Original article

Evaluation of luteinizing hormone regulation of maturation and apoptosis, expression of LHR and FSHR in cumulus-oocyte complexes in Lanzhou fat-tailed sheep

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Abstract

The present study aimed to assess LH effects on *in vitro* maturation (IVM) and apoptosis and also to explore the gene expressions of LHR and FSHR in cumulus-oocyte complexes (COCs) of the sheep. COCs were *in vitro* matured 24h in the IVM medium supplemented with varying concentrations of LH (0, 5, 10, 20 and 30 μg/mL). They were allocated into LH-1 (control group), LH-2, LH-3, LH-4 and LH-5 groups, respectively. FSH (10 IU/mL) addition was as a positive control (FSH group). COCs apoptosis was assessed by TUNEL. The qPCR and Western blotting were utilized to detect mRNA and protein expressions of FSHR and LHR, respectively. The results showed maturation rates of oocytes improved as LH concentration increased from 0 to 10 μg/mL (IU/mL), reaching a peak value of 44.3% in the LH-3 group. Maturation rate of LH-5 group was lower than that of LH-3 and FSH-treated groups. The lowest apoptosis rate was found in LH-3 group. The germinal vesicle break down (GVBD) rates of LH-2, LH-3 and LH-4 groups were also increased in comparison with that found in LH-1 group (control group). GVBD rate of LH-5 was lower than that in LH-3 group. The germinal vesicle (GV) rates in LH-3 and LH-4 groups were lower than those in LH-1 and LH-5 groups ($p<0.05$, or $p<0.01$). The lowest GV rate was found in LH-3 group. GV rates in LH-2, LH-4 and LH-5 groups were higher than that in FSH group ($p<0.05$). At hours 20, 22 and 24 after oocytes IVM, caspase-3 concentrations in four LH-treated groups were decreased in comparison with that in LH-1 group. At 24h, caspase-3 concentrations of LH-2 and LH-3 groups were lower than that in LH-1 group ($p<0.05$). Expression levels of FSHR and LHR mRNAs rose when LH concentrations in IVM medium increased. The greatest expressions of FSHR and LHR mRNAs were found in LH-5 and LH-3 groups ($p<0.01$) in comparison with those in the control group (LH-1). Meanwhile, FSHR mRNA expressions in LH-2, LH-3 and LH-4 groups were lower than that in FSH group ($p<0.01$ or $p<0.05$). Expression levels of FSHR proteins revealed no significant differences among all groups. Expression levels in LHR proteins were increased. LHR protein level in LH-2 group was higher than that in LH-1 group. In conclusion, LH treatment could promote the maturation rate and GVBD rate. LH reduced apoptosis rate, GV rate of sheep oocytes, and caspase-3 concentrations in IVM medium fluids and additionally enhanced expressions of FSHR and LHR mRNAs of sheep COCs.

Key words: *In vitro* maturation, apoptosis, luteinizing hormone, cumulus oocyte complexes, Caspase-3, receptor

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Introduction

In vitro maturation (IVM) of oocytes is conducted by supplementing the medium with exogenous hormones to induce oocytes expansion, nuclear maturation (Xiao et al. 2014), and to improve follicular development of sheep (Lee et al. 2007). Oocyte IVM is often conducted in the media supplemented with gonadotropins such as follicle-stimulating hormone (FSH) and luteinizing hormone (LH) to induce expansion and nuclear maturation of cumulus-oocyte complexes (COCs) (Xiao et al. 2014). LH treatment could increase the rate of germinal vesicle break down (GVBD), the cleavage and blastocyst rates of ovine oocytes as compared to the control group after being cultured *in vitro* for 24h. Therefore, LH may play a role in the delay of GVBD occurrence, and prolongs the cytoplasmic maturation (Li et al. 2011). Initial studies recorded that LH affects on oocyte IVM in the sheep (de Frutos et al. 2013). For instance, addition of 10 μg/mL LH increased the IVM rate of ovine oocytes (Rebecca et al. 2012). The optimal concentration of FSH and LH in IVM medium was 5 μg/mL and 50 IU/mL, respectively, for oocytes IVM of yak. However, a high concentration of LH (100 IU/ml) in the IVM medium decreased the subsequent cleavage rate of yak oocytes (Xiao et al. 2014). Currently, the information about LH effects on IVM of sheep oocytes is very limited. It is undetermined whether supplementation of LH into the IVM medium impacts maturation and apoptosis of oocytes in Lanzhou fat-tailed sheep oocytes (de Frutos et al. 2013, Wei et al. 2016a). Up to date, it is undetermined which dose of LH in the IVM medium is the optimum concentration for sheep oocytes IVM. Meanwhile, it remains unclear whether LH supplementation into the IVM medium influences IVM rate, apoptosis incidence and expression levels of FSHR and LHR genes of sheep oocytes (de Frutos et al. 2013).

For LH to act, its specific receptors (LHR) must be expressed by the cumulus cells (Calder et al. 2003, Xiao et al. 2014). Our previous investigation confirmed that LHR and FSHR are expressed in ovaries of the sheep (Wei et al. 2013). Another report in cattle also indicated that FSHR and its mRNA were expressed in cumulus cells and granulosa cells, but not mRNA of LHR. However, no information is available on LHR and FSHR expressions in sheep oocytes (de Frutos et al. 2013, Nor-Azlina, et al. 2014). Nowadays, some data have been reported about LH effects on expression levels of LHR and FSHR mRNAs and proteins in COCs of human and animals (Nor-Azlina et al. 2014).

Apoptosis is characterized by specific structural changes. The aspartate-specific cysteine protease 3 (caspase-3) is the most critical apoptosis protease in the downstream of caspases cascade (Earnshaw et al. 1999). FSH down-regulates caspase-3 mRNA levels in the granulosa cells of dominant follicles (Li et al. 2013). However, there is scarce information regarding LH treatment affecting the activity of the caspase family of sheep oocytes (Cao et al. 2014).

Lanzhou fat-tailed sheep are unique sheep breeds in Lanzhou areas (latitude N 35°34' to N 38°10', longitude E 102°30' to E 104°37') of China. The breed is characterized by a big tail, fast development, full fat and delicious mutton. However, the lambing percentage is only about 117% of Lanzhou fat-tailed sheep, and fecundity is poor (Zhang 2010). Therefore, improving reproductive performance of Lanzhou fat-tailed sheep to raise the population quantity has become an urgent work. So far, there has been little information about oocytes IVM in this species. In this investigation, the effects of different concentrations of LH on IVM and apoptosis of Lanzhou fat-tailed sheep oocytes were studied. Additionally, expression levels of FHSR and LHR mRNAs and proteins were determined. The present investigation aimed to improve the ovine fecundity by assessing LH effects on IVM and apoptosis, gene expressions of LHR and FSHR in COCs of Lanzhou fat-tailed sheep.

Materials and Methods

Preparation of maturation medium

Basal culture medium (bMM) consisted of 9.5 g Medium 199 powder (Sigma, St. Louis, MO, USA), 2.2 g NaHCO₃, 25 mg sodium pyruvate, 4.8 g 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 NaHCO₃, 10% fetal calf serum (v/v; HyClone, Logan, UT, USA), and 5% fetal bovine serum (FBS; Minhai Company, Lanzhou, China).

Collections of sheep ovaries

Ovaries were collected between April and July 2015 from 530 immature and noncyclic Lanzhou fat-tailed sheep (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) supplemented with 100 000 IU penicillin, 100 mg streptomycin and 250 mg amphotericin B per liter,

and maintained at 30-35ºC within 3 h after collection (Paola et al. 2005; de Frutos et al. 2013). All subsequent procedures were conducted at a constant temperature of 37ºC. 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 covering the ovary were removed using sterile scissors. The ovaries were placed on a petri dish, to which 2 mL pre-equilibrated extraction fluid [phosphate-buffered saline (PBS) containing 3 mg/mL bovine serum albumin, incubated at 38ºC overnight] was added, while they were gently held in place using sterile ophthalmic tweezers. Follicles (sizes of 3-5 mm in diameter) on the ovarian surface were then scratched with a scalpel blade. Cumulus-oocyte complexes (COCs) were collected from ovarian follicles by gently cutting follicles with a scalpel in DPBS on a petri dish. 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). Only COCs with at least 3 complete layers of cumulus cells and finely granulated homogeneous ooplasm were selected as suitable for IVM and classified into four grades (A, B, C and D) according to their cumulus cell layers (Nandi et al. 2002, Tripathi et al. 2016). A total of 1828 oocytes were used for subsequent experiments.

IVM of Oocytes

A micro-well culture system was utilized in this experiment. IVM of sheep oocytes was performed in accordance with earlier described procedure (Wei et al. 2016b). Intact COCs were collected in IVM medium. 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 LH (Ningbo Sansheng Hormone Factory, Ningbo, China) covered with 300 µL mineral oil. Additions of 0, 5, 10, 20, or 30 µg/mL LH were assigned to LH-1 (control group), LH-2, LH-3, LH-4 and LH-5 groups, respectively. FSH (10 IU/mL) treatment was con-

sidered as a positive control (FSH group). COCs were then left to complete their maturation at 38.5ºC in an atmosphere of 5.0% carbon dioxide and 90%-95% relative humidity for 24h. IVM fluids were collected at 24h.

Evaluation of maturation status

To assess the rate of meiosis at the end of IVM, oocytes were separated in groups according to LH treatment. Following IVM, oocytes were denuded by 0.3% hyaluronidase following three PBS rinses. The harvested oocytes were observed under an inverted microscope (Leica, Japan), and those displaying an intact first polar body were identified as matured oocytes. Mature denuded oocytes were then fixed and subjected to Giemsa staining to determine their progression to metaphase II. Oocytes were classified as: immature (germinal vesicle, GV) and germinal vesicle breakdown stage, GVBD), intermediate (metaphase I and anaphase I), and matured (telophase I and metaphase II) (Wei et al. 2016b). Experiments were replicated at least 3 times.

Apoptosis detection of COCs

Morphological analysis of COC apoptosis COCs having undergone IVM were mounted and observed under a microscope to identify apoptosis cells according to morphological criteria (Yang and Rajamahendran 2000). The cells with nuclei containing condensed chromatin that had either in large compact granular masses, or a single regularly shape and pyknotic appearance, or multiple fragments, or formation of apoptotic bodies were considered as apoptosis.

Detection of COCs apoptosis by TUNEL To estimate rates of apoptosis, COCs were analyzed according to treatment group using a kit of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), following the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, COCs were fixed in 4% PBS-buffered paraformaldehyde for 20 min at 23-25ºC, 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 37.5ºC. COCs were placed in 30-mL drops of TUNEL solution and incubated in the dark for 1 h at 37.5ºC. For the negative control group, the TUNEL reagent was omitted. Apoptosis rate was reported as the number of labeled cells expressed as a percentage of the total cell number.

Caspase-3 detection of IVM medium fluids

Caspase-3 concentrations were detected using caspase-3 detection kit for mice (ELISA). The concentrations were measured strictly according to the manufacturer's instructions (Northern biotechnology institute, Beijing). Optical density (OD) of each hole was read with MK-3 type enzyme-label instrument (Rayto, USA) at 450 nm wavelength. The samples were measured in triplicate. The sensitivity was 0.01 ng/mL, and the minimum limit of detection was 0.03 ng/mL. The intra- and inter-assay CV was lower than 8%. The correlation coefficient of the standard curve was 0.9995 (Wei et al. 2012).

Real time fluorescence quantitative RT-PCR (qRT-PCR)

Primer designs In order to access the effect of addition of LH in IVM media on FSHR and LHR mRNAs expression in COCs after IVM, the specific primers of LHR (GenBank accession number: L36329.1) and FSHR (GenBank accession number: NM-001009289.1) were designed using Beacon Designer 7.0 software (Premier Biosoft International, Palo Alto, CA USA) and Primer-BLAST on NCBI. Sheep GAPDH gene (GenBank accession number: HM-043737.1) was chosen 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 assessed using the melting curve analysis. Thus, only those concentrations of primers which showed dimmer-free reactions were used for the final analysis. Primers and probes were synthesized by Beijing AoKeDingSheng Biotechnology Co. Ltd. China. The sequences of the primers used in the qPCR were as follows: LHR, forward 5'-CCTGAAGAAGATGCACGATGACGCC-3' and reverse, 5'- ACCCATTCCCTGTCTGCCAGT CT-3'; FSHR, forward 5'- TCTTTGCTTTTGCAGT TG CC-3'and reverse, 5'- GCACAAGGAG-GGACATAACATAG-3'; GAPDH, forward, 5'-CTTCAACAGCGACACTCACTCT-3'and reverse, 5'- CCACCACCCTGTTGCTGTA-3'.

Total RNA extraction Total RNA was extracted from cumulus cells after IVM (during which, cells were exposed to IVM medium containing different LH concentrations) using TRIzol reagent (Invitrogen, Beijing, China) according to the manufacturer's instructions, before being reverse transcribed (Wei et al. 2013, 2016a). Reverse transcription of total RNA was performed by a commercial kit (Takara, China).

The qRT-PCR detection Expression levels of LHR and FSHR mRNAs were detected using qRT-PCR of Bole T100 gradient PCR instrument (Bio-rad, USA) (Wei et al. 2013, 2014). 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 95ºC, 40 cycles of amplification were performed on the basis of the following thermo cycling profiles: denaturation for 30 s at 95ºC, annealing for 20 s at 60ºC and extension for 20 s at 72ºC. The relative amount of each mRNA was determined by the $2^{-\Delta\Delta CT}$ comparative method and normalized to an endogenous reference gene (GAPDH). Each experiment was replicated three times with 30 COCs for each replicate.

Western blotting of LHR and FSHR proteins

To evaluate LHR and FSHR expression levels in sheep COCs during IVM, western blotting was conducted following our previous report (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 LHR and FSHR (Sigma, diluted 1:200), and rabbit anti-sheep β-actin (1:1000) polyclonal antibodies at 4ºC 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 Quantity One software (Bio-Rad, Hercules, CA, USA). Relative protein expression was calculated as the ratio of the gray value of the target band (LHR and FSHR) to that of the β-actin band. Samples were run in triplicate. The negative control was not incubated with the primary antibodies.
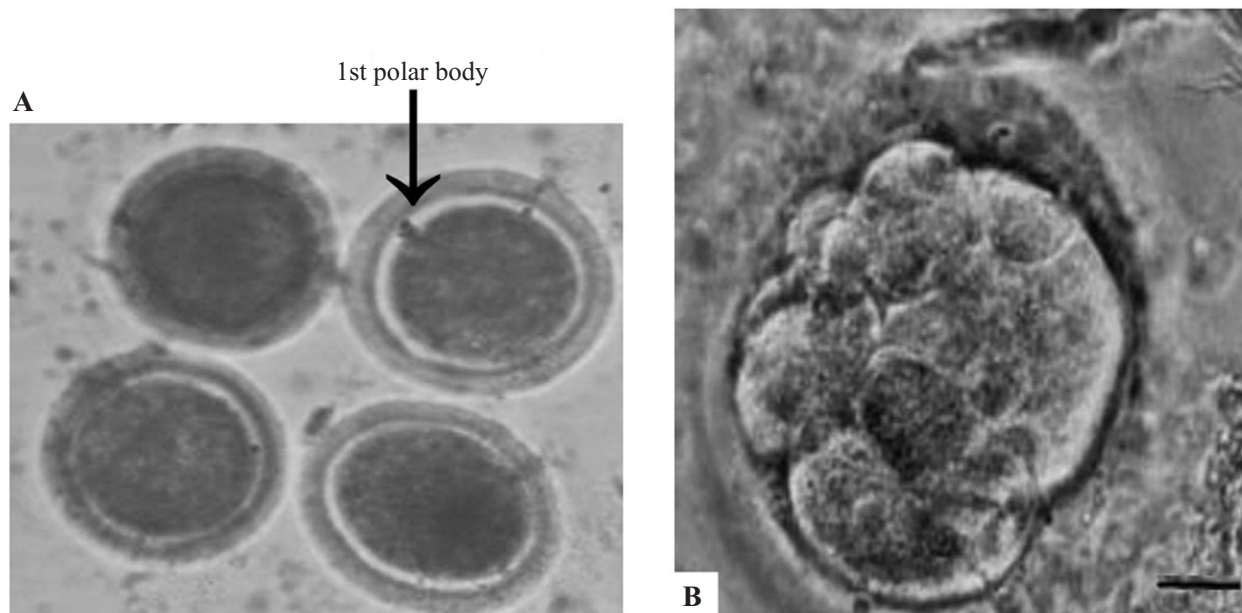
Fig. 1. The extrusion of the first polar body (A) and multiple nuclear fragment of oocytes (B). (Scale bar=25 μm)

Table 1. Effects of different LH doses on *in vitro* maturation of oocytes in sheep.

Hormones	Group	Doses	Cultured oocytes	Matured oocytes	Maturation rate %	Apoptotic rate %	Apoptosis rate %
	LH-1	0	298	105	35.2	84	28.1
	LH-2	5	306	117	38.2[#b]	72	23.5
LH (μg/mL)	LH-3	10	291	129	44.3[*a]	59	20.3*
	LH-4	20	296	113	38.2[#b]	65	21.9
	LH-5	30	286	95	33.2[a#]	75	26.2[b#]
FSH (IU/mL)	FSH	10	351	170	48.5	73	20.8

Note: * $p<0.05$ when compared to LH-1 group (blank control); ** $p<0.01$ when compared to LH-1 group (blank control). # $p<0.05$ when compared to FSH group (normal control). Different superscript letters indicate a significant difference between LH-treated groups ($p<0.05$).

Data statistical analyses

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 (Scheffe 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

IVM evaluations of sheep COCs

Based on the morphological observation of oocytes under microscope, the extrusion of the first polar body was observed (Fig. 1A). Apoptosis morphological features were evident in cumulus masses of COCs at 24h of IVM. Apoptosis features included the shrinkage or occurrence of cytoplasmic condensation, membrane blebbing and multiple nuclear fragments of COCs (Fig. 1B) or apoptosis body formation. These revealed that the Lanzhou fat-tailed sheep COCs went through the shrinkage and DNA fragmentation.
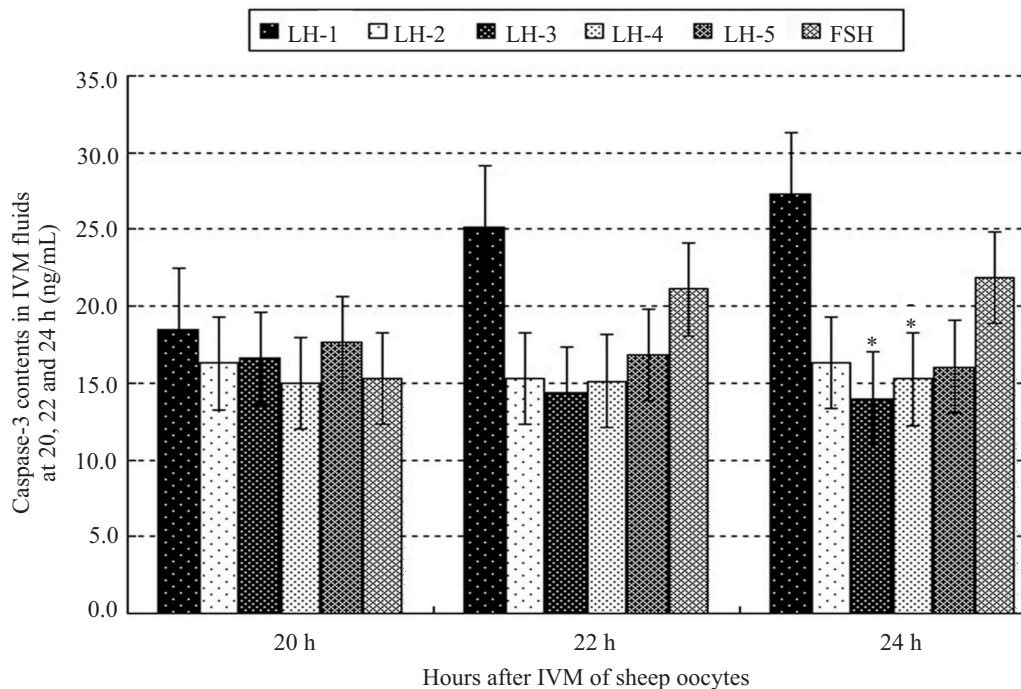
Fig. 2. Changes in caspase-3 concentrations in IVM medium fluids (ng/mL)

IVM rates of Sheep COCs

As shown in Table 1, maturation rates of sheep COCs gradually improved as LH concentration increased from 0 (LH-1 group) to 10 µg/mL (LH-3 group), reaching a peak value of 44.3%, then maturation rates were declined. Maturation rates in LH-2, LH-4 and LH-5 groups were lower than that in FSH group (positive control). Furthermore, maturation rate in LH-5 group was lower than that in LH-3 group. Our results demonstrated that 10 µg/mL LH addition into the IVM medium was optimal LH concentration for IVM of sheep COCs.

TUNEL assays and apoptosis rate

The incidence of apoptosis was demonstrated by TUNEL assay in COCs. Apoptosis rates of COCs varied in an opposition tendency to maturation rates (Table 1). Apoptosis rates were gradually reduced after LH doses increased from 0 to 10 µg/mL. The minimum apoptosis rate was calculated in LH-3 group which was significantly lower than that in control (LH-1) group (20.3% vs 28.1%).

GV and GVBD rates of oocytes

Data in Table 2 show that GVBD rates of LH-2, LH-3 and LH-4 groups were also increased in comparison with that in LH-1 group (control group). GVBD rate of LH-5 was lower than that in LH-3 group. GV rates of LH-3 and LH-4 groups were lower than that in LH-1 and LH-5 groups (p<0.05, or p<0.01). The lowest GV rate was found in LH-3 group. GV rates of LH-2, LH-4 and LH-5 groups were higher than that in FSH group (p<0.05). The results demonstrated that addition of LH into the IVM medium could increase the GVBD rate and decrease GV rate of oocytes.

Caspase-3 concentrations of IVM medium fluids

Caspase-3 concentrations of IVM medium fluids were detected. As shown in Fig. 2, at hours 20, 22 and 24 after IVM, caspase-3 concentrations in four LH-treated groups were decreased in comparison with that in LH-1 group. At 24h, caspase-3 concentrations of LH-2 and LH-3 groups were lower than that in LH-1 group (p<0.05). There was no significant difference between LH-treated groups and FSH-treated group. The results indicated that LH treatment could reduce caspase-3 concentrations of IVM medium fluids.

Expressions of FSHR and LHR mRNAs in cumulus-oocyte complexes (COCs)

FSHR and LHR mRNAs were detected in sheep COCs after 24h of IVM (Fig. 3). The expression levels

Table 2. GV and GVBD rates of ovine oocytes after IVM (mean ± SEM).

Group	Oocytes	GVBD rate %	GV rate %
LH-1	298	39.6 ± 3.9	23.5 ± 2.5
LH-2	306	41.2 ± 3.7	18.0 ± 2.1[#]
LH-3	291	43.0 ± 4.3[*b]	12.1 ± 1.9[**c]
LH-4	296	40.4 ± 3.7	17.6 ± 2.8[*b#]
LH-5	286	38.1 ± 3.2[a]	26.9 ± 3.3[a##]
FSH	351	42.5 ± 4.3	11.5 ± 1.1

Note: GV-germinal vesicle, GVBD – germinal vesicle break down. * $p<0.05$ when compared to LH-1 group (blank control); ** $p<0.01$ when compared to LH-1 group (blank control). # $p<0.05$ when compared to FSH group (normal control); ## $p<0.01$ when compared to FSH group (normal control). The different superscript letters indicate a significant difference between LH-treated groups. The adjacent superscript (such as ab, bc) indicate the difference was significant ($p<0.05$), while interval superscript (such as ac) the difference was highly significant ($p<0.01$).

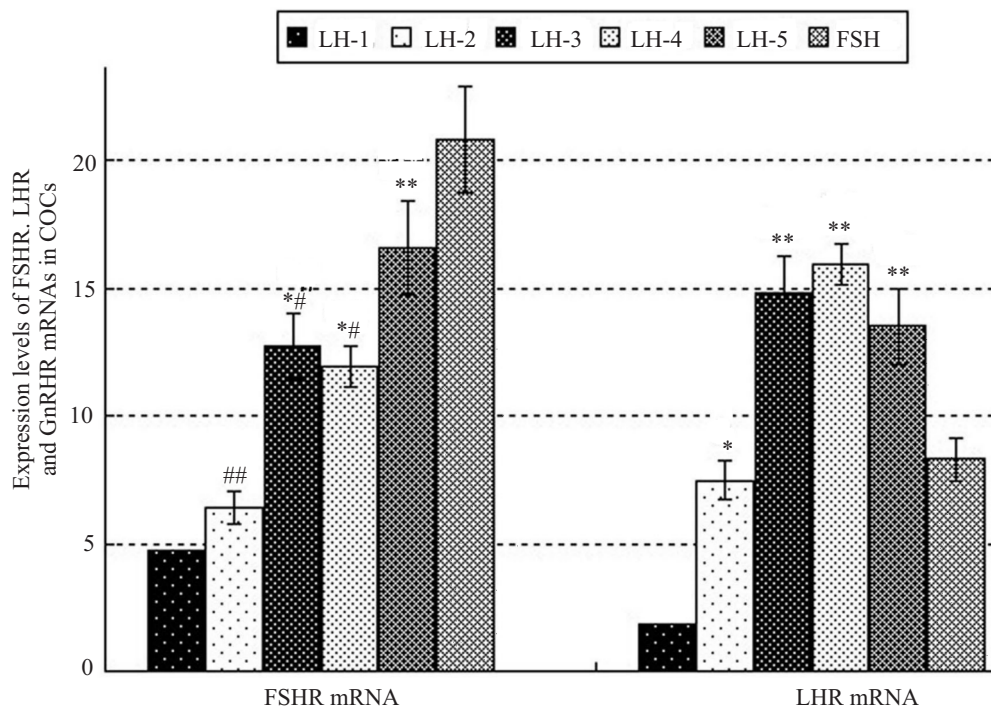Fig. 3. Expression of FSHR and LHR mRNAs. *$p<0.05$ and **$p<0.01$ compared to the blank control group (LH-1). # $p<0.05$ when compared to FSH group (positive control); ## $p<0.01$ when compared to FSH group (positive control). Expression levels of FSHR and LHR mRNAs were enhanced after LH was added to the IVM medium. The greatest increment of FSHR and LHR mRNAs was detected in LH-5 and LH-4 groups, respectively as compared those in the control group (LH-1).

of FSHR and LHR mRNAs rose when LH concentrations in IVM medium increased. The greatest expressions of FSHR and LHR mRNAs were found in LH-5 and LH-3 groups ($p<0.01$) in comparison with those in control group (LH-1). Meanwhile, FSHR mRNA expressions of LH-2, LH-3 and LH-4 groups were lower than that in FSH group ($p<0.01$ or $p<0.05$). Our findings confirmed that LH treatment could enhance expressions of FSHR and LHR mRNAs of sheep COCs.

Western blotting results of FSHR and LHR proteins

Expression levels of FSHR and LHR proteins were assessed by Western blotting in sheep COCs (Fig. 4). Expression levels of FSHR proteins revealed no significant differences among all the groups. Expression levels of LHR proteins were increased. LHR protein level of LH-2 was greater than that in LH-1 group. The findings demonstrated that supple-
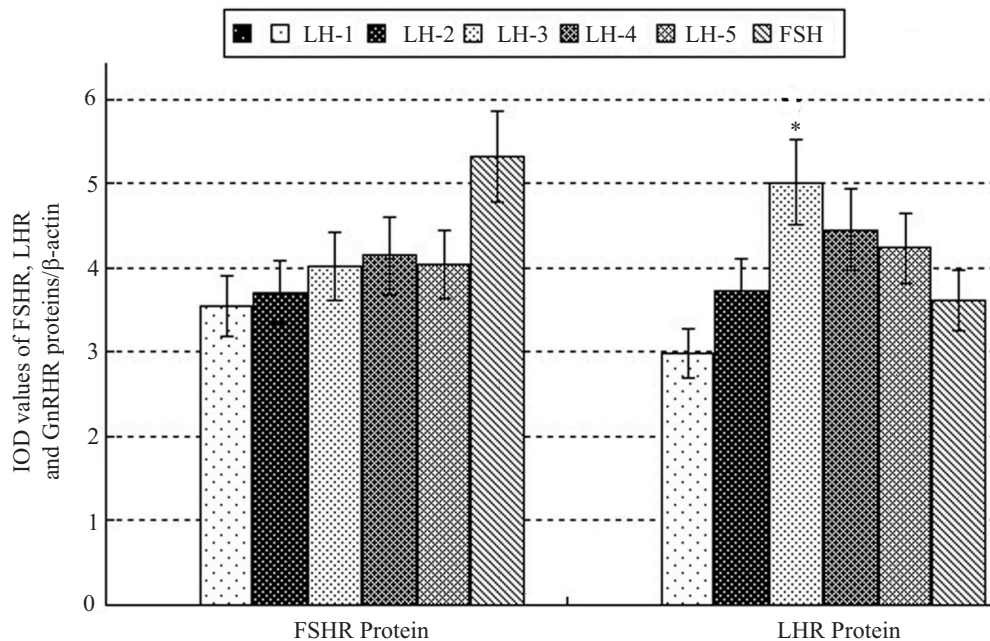
Fig. 4. Expression of FSHR and LHR proteins IOD = integrated optical density. *p<0.05 when compared to the control group (LH-1 group). Expression level of LHR proteins in LH-2 group was increased in comparison to that in LH-1 group.

ment of LH could enhance expressions of LHR proteins.

Discussion

IVM of oocytes

The meiotic cell cycle of mammalian oocytes starts during embryogenesis and then pauses until luteinizing hormone (LH) acts on the granulosa cells of the follicles (Shuhaibar et al. 2016). A series of physiological changes including maturation of the nucleus and cytoplasm happens during oocyte IVM. The extrusion of the first polar body is a crucial sign of oocytes IVM. LH promoted the cytoplasm maturation of porcine oocytes (Li et al. 2011). Addition of 5-10 μg/mL LH has been demonstrated to increase the IVM rate of ovine oocytes (Rebecca et al. 2012, de Frutos et al. 2013). Nevertheless, 20 μg/mL LH inhibited oocyte maturation. Up to date, it is undetermined which dose of LH in IVM medium is optimum for IVM of Lanzhou fat-tailed sheep oocytes although the initial studies indicated that the addition of 1.5 μg/mL recombinant bovine LH (rbLH) affected oocytes IVM in sheep (de Frutos et al. 2013).

In the present study, different doses of LH (0, 5, 10, 20 and 30 μg/mL) were added into the IVM medium to determine the LH effects on maturation and apoptosis of sheep COCs. These results showed that the maturation rates of sheep COCs gradually improved as LH concentration increased from 0 to 10 μg/mL and then declined. Maturation rates of LH-2, LH-4 and LH-5 groups group were lower than that in 10IU/mL FSH treatment group.

Apoptosis rates of sheep COCs were gradually reduced after LH doses increased from 0 to 10 μg/mL which showed a minimum apoptosis rate. After IVM, the extrusion of the first polar body occurred, and obvious apoptosis morphological features were observed in COCs. These indicated that sheep COCs went through oocyte shrinkage and DNA fragmentation. Our results confirmed that addition of 10 μg/mL LH into the IVM medium was optimal LH concentration for IVM of sheep COCs. These results were consistent with earlier reported findings (Rebecca et al. 2012, de Frutos et al. 2013, Lettcia et al. 2016). However, FSH (10IU/mL) was more efficient than LH in promoting IVM of sheep oocytes. The actual mechanism needs to be thoroughly investigated.

LH effects on GV and GVBD rates of sheep oocytes

Many hormones can be used to promote oocyte IVM, although different hormones exert divergent effects in this respect (Yang et al. 2015). LH inhibited GVBD of oocyte in its initial stage during IVM and declined GVBD rate, because LH could prolong the initiation of GVBD for sheep oocytes

(Li et al. 2011). Currently, few studies reveal the LH effects on GV and GVBD rates of sheep oocytes (Yang et al. 2015). In the present work, GVBD rates in LH-2, LH-3 and LH-4 groups were increased in comparison with that in LH-1 group (control group). GV rates of LH-3 and LH-4 groups were lower than that in LH-1 and LH-5 groups. The lowest GV rate was found in LH-3 group. The results demonstrated that addition of LH into the IVM medium could increase the GVBD rates and decrease GV rates of oocytes. These findings were in agreement with earlier data (Xiao et al. 2014, Adeldust et al. 2015). However, the molecular mechanism needs to be thoroughly studied.

Detection of caspase-3 levels in IVM medium

Apoptosis of COCs can be a key marker for oocyte developmental quality (Nor-Azlina et al. 2014). Apoptosis is characterized by specific structural changes. COCs apoptosis affected follicular quality and ovarian function, because of the presence of molecules that regulate the apoptosis mechanism in the maternal mRNA stored in the oocytes (Metcalfe et al. 2004). Although multiple genes are involved in apoptosis of oocytes, the key mediators are aspartate specific cysteine proteases (Caspases). Caspase-3 is the most critical apoptosis protease in the downstream of caspases cascade (Earnshaw et al. 1999). However, there is scarce information about LH treatment affecting the activity of the caspase family of Lanzhou fat-tailed sheep oocytes (Nor-Azlina et al. 2014).

In this experimental study, caspase-3 concentrations of IVM medium fluids were detected so as to determine the effects of LH addition into the IVM medium on apoptosis of oocytes. At 20, 22 and 24h of oocytes IVM, caspase-3 concentrations in four LH-treated groups were decreased in comparison with that in LH-1 group (control group). At 24h, caspase-3 levels of LH-2 and LH-3 groups were lower than that in LH-1 group. The results indicated that LH addition into the IVM medium could reduce caspase-3 production of sheep oocytes. The results were similar to the findings described in the initial report (Cao et al. 2014). The action mechanism has to be thoroughly explored in other animals.

Expression of FSHR and LHR mRNAs and proteins in COCs

In order for LH and FSH to exert their effects, the specific receptors must be present on follicle cells, especially granulosa cells (Tisdall et al. 1995). Cumulus cells have large numbers of FSHRs, but few or no LHRs (Xiao et al. 2014). Our previous studies demonstrated that FSHR and LHR were expressed in the sheep ovaries and follicles (Wei et al. 2012, 2014). In the present study, LHR and FSHR mRNAs were also detected in sheep COCs so as to investigate influences of LH supplementation into the IVM medium on expression levels of LHR and FSHR mRNAs and protein in sheep COCs. The results showed that expression levels of FSHR and LHR mRNAs in four LH-treated groups were higher than that in LH-1 (control group). The greatest levels of FSHR and LHR mRNAs were found in LH-5 and LH-3 groups in comparison with those in LH-1. Expression levels of LHR proteins were increased. LHR protein level of LH-2 was higher than that in LH-1 group. There were no significant differences in FSHR protein expression between LH-treated groups. These findings demonstrated that supplementation of LH into the IVM medium could increase the levels of FSHR mRNAs, and enhance the levels of LHR mRNAs and proteins of sheep oocytes. LH had more efficacy than FSH to improve expression of LHR. The outcomes were consistent with previously reported (Hashim et al. 2013, Sullivan et al. 2013, Xiao et al. 2014).

In conclusion, LH addition into IVM medium can promote the maturation rates and GVBD rates, and enhances expressions of FSHR and LHR mRNAs of sheep COCs. Additionally, LH attenuates apoptosis rate and GV rate, furthermore declines caspase-3 level of IVM medium fluids. The present data provide a better understanding of the involvement of LH in the regulation of the oocytes IVM in Lanzhou fat-tailed sheep.

Acknowledgements

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