Kisspeptin-10 modulates hormone secretion and proliferation of follicular granulosa cells by upregulating expressions of the kiss-1r gene

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ABSTRACT

The present study aimed to determine the subcellular localization of KISS-1 protein, and also to investigate the effects of Kisspeptin-10 (KP-10) on the proliferation viability and hormone secretion of follicular granulosa cells (FGCs), and its molecular mechanisms. FGCs were cultured in vitro in DMEM media supplied with the different doses of KP-10 (0,10,100 and 1000 nmol/L) which were allocated into a control group (CG), experiment group 1 (EP-1), experiment group 2 (EP-2) and experiment group 3 (EP-3), respectively, CG, EP-1, EP-2 and EP-3. Subcellular localization of FSHR and KISS-1 proteins in FGCs were assayed using immunofluorescence assay. A Cell Counting Kit 8 (CCK8), real time quantitative PCR (qPCR), and western blotting were used to detect the proliferation activities and expression levels of genes and proteins in the FGCs. Elisa kits were applied to measure the contents of estrogen (E2) and progesterone (P4). Immunofluorescence assay showed that KISS-1 protein was positively expressed in sheep FGCs and mainly distributed in the cytoplasm. At 48h and 72h following KP-10 treatment, the cell viability of the FGCs in groups EP-2 and EP-3 were significantly enhanced compared to CG ($P<0.05$ or $P<0.01$). The estrogen (E2) and progesterone (P4) contents of group EP-2 were increased in comparison with CG ($P<0.01$). Additionally, expression levels of Kiss-1R, StAR, 3β-HSD and CYP11 mRNAs in group EP-2 were remarkably increased compared to CG ($P<0.05$ or $P<0.01$) with increments of 65.9%, 30.7%, 73.4% and 71.4%, respectively. In conclusion, KISS-1 proteins were located mainly in the cytoplasm of FGCs. KP-10 could enhance FGC vitality, and promote progesterone and estrogen secretion, which is possibly achieved by upregulation of expression levels of Kiss-1R, StAR, 3-β-HSD and CYP11 genes. KP-10 significantly upregulated mRNA and protein levels of Bcl-2 and PI3K ($P<0.01$), and downregulated expressions of mRNA and protein levels of Bcl-2 and PI3K ($P<0.05$). Our outcomes provide a solid basis for searching after novel improvements of reproduction of animals.

Key words: kisspeptin; follicular granulosa cells; estrogen and progesterone; proliferation

Introduction

Follicular atresia is often caused by apoptosis of follicular granulosa cells (FGCs) (LEE et al., 1999). Therefore, FGC apoptosis is a key factor in mammalian follicular cell atresia and provides nutrition for promoting oocyte proliferation (KE et al., 2019). FGCs can also promote ovarian synthesis

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of estrogen and progesterone (ARMSTRONG et al., 2002). The apoptosis of FGCs is influenced by many factors, such as Bax, Bcl-2, Caspase-3, PI3K, and AKT (CHOI et al., 2004; MAMEDE et al., 2017). However, the exact functional role of these apoptosis factors in sheep follicular atresia and cell apoptosis still remains poorly understood (LEE et al., 2021; GREENHILL, 2023).

Kisspeptin, a KISS-1 gene-encoded peptide in the hypothalamus, was first found in the placentas of women. KISS-1 (a complementary DNA, cDNA) was first discovered from malignant melanoma cells by LEE at al. (1996). KISS-1 is known as a novel tumor metastasis suppressor gene, encoding a 145-amino acid peptide which is subsequently cleaved to 54 amino acids in length, which is truncated further into smaller kisspeptin fragments of KP-14, KP-13 and KP-10 (PAPAOICONOMOU et al., 2014; SONG and ZHAO, 2015). It has been proved that kisspeptin is distributed and expressed in the pituitary glands, ovaries (CASTELLANO et al., 2006), testes (AINARA et al., 2020; GIBULA-TARLOWSKA et al., 2019), and the arcuate nucleus of the hypothalamus (OHTAKI et al., 2001; STATHAKI et al., 2019). Additionally, KiSS-1 receptor (KiSS-1R) is massively expressed in the placenta, pituitary gland, pancreas, brain, and spinal cord (KOTANI et al., 2001; MUIR et al., 2001). So far, numerous studies have been performed to explore the physiological roles of kisspeptins on reproduction endocrine in mammals. Early studies demonstrated that kisspeptin serves as a potent stimulator of GnRH secretion (GOODMAN et al., 2022). However, the actual effects and molecular mechanisms still remain undetermined (YOSHIHISA and TSUKAMURA, 2023).

KP-10 directly affected GnRH neurons by binding to GPR54 (KISS-1R) (OZCAN et al., 2011). The administration of KP-10 increased viability of primary granulosa cells (LIU et al., 2017) and enlarged the size of oocytes (BYRI et al., 2017) and increased plasma levels of LH (XIAO et al., 2012). Kisspeptin participates in the mechanism through stimulation of hormone production (QIN et al., 2020). Therefore, Kisspeptins play a key role in regulating the physiological functions of reproduction (KASUM et al., 2017). An initial study reported that the expression level of kisspeptin increased continuously from the early follicle to pre-ovulation, leading to the production of the LH surge and ovulation (LATIF and RAFIQUE, 2015; ZHAI et al., 2017). Intravenous injection of 5μg/kg KP-10 increased LH and FSH contents in bovine blood, inducing LH surge and ovulation (ZHAI et al., 2017).

Kisspeptin promoted the proliferation of cumulus granulosa cells, inhibited cell apoptosis, enhanced oocyte vitality, and promoted follicle maturation (OWENS et al., 2017), while high doses had inhibitory effects on oocyte maturation (SAADELDIN et al., 2012). KP-10 increases the maturation rate of COCs in sheep cultured in vitro. 10 μg/mL KP-10 had the best effects. However, the precise molecular mechanisms of KP-10 remain unknown (BYRI et al., 2017).

On the basis of the above-mentioned documents, we hypothesized that kisspeptin-10 may effect the secretion of estrogen and progesterone, and improve the proliferation activities of follicular granulosa cells (FGCs), probably by regulating the associated key genes and proteins of FGCs. Therefore, the present study will thoroughly explore how kisspeptin-10 modulates the secretion of estrogen (E2) and progesterone (P4) and proliferation activities, and regulates expression levels of Kiss-1R, StAR, 3-β-HSD, and CYP11 genes, as well as expression levels of Bax, Bcl-2, PI3K, and AKT proteins so as to develop novel reproduction increasing and proliferation methods and improve the fecundity of animals (JIANG et al., 2023).

Materials and methods

Collection of follicular granulosa cells and in vitro culture. A total of 378 ovaries were aseptically collected from 206 pre-pubertal sheep (aged 4-5 months, small-tailed han sheep) in a local slaughterhouse, placed in DPBS at 37°C with 100 IU/mL penicillin and 100 μg/mL streptomycin, and immediately brought to the laboratory in a heated box. Each ovary was punctured with a syringe needle to harvest the 3-5 mm follicles. Follicular cell fluids were collected and centrifuged at 1000 g for 8 min. Follicular granulosa cells (FGCs) were

harvested. Then, FGCs (1×10^5 cells/well) were cultured in a 4-well plate (Nunc, Roskilde, Denmark) using EMDM/F12 (Dulbecco's modified Eagle's medium; Thermo Fisher Scientific, Inc., Waltham, MA, USA), with 10% FBS (newborn bovine serum; Gibco, Grand Island, NY, USA) and a 1% penicillin-streptomycin solution added (Gibco; Thermo Fisher Scientific, Inc.), and maintained in a humidified atmosphere containing 5% CO_2 at 37.0°C (YONG et al., 2022). At 24h, 48h and 72h after culture, FGCs were collected for assay using the Cell Counting Kit-8 (CCK-8). Culture media were partially changed every 48 h. The spent medium was stored at -20°C prior to assay. All experimental procedures involving animal care, were approved by the Institutional Animal Care and Use Committee of Northwest Minzu University (NWMU 2018-0089).

KP-10 treatment of FGCs. Kispeptin-10 (Freeze-dried powder, 048-56, purity of 99.9%) was synthesized by Beijing Phoenix biotech Co., Ltd. FGCs were cultured in vitro in DMEM/F12 (1:1) media (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in which different doses of KP-10 (0, 10, 100 and 1000 nmol/L) were supplied. These treatments were allocated into the Control Group (CG), Experiment Group 1 (EP-1), Experiment Group 2 (EP-2) and Experiment Group 3 (EP-3), respectively.

Subcellular localization of FSHR and KISS-1 proteins. Immunofluorescence technology is used for subcellular localization of FSHR and KISS-1 proteins, as referred to in previous reports (BARBERIS et al., 2021; FU et al., 2017). Briefly, 3×10^5 FGCs per well were inoculated in a 6-well plate. 500 μL 0.5% Triton-X-100was added to each well for transparenting for 20 min. Then 200 μL of primary antibody (anti-FSHR Rabbit pAb, ab113421, Abcam, Shanghai, China, 1: 500; anti-KISS-1 Rabbit pAb, ab275874, Abcam, Shanghai, China, 1: 1000), 1: 200 PBS and 200μL of fluorescein labeled secondary antibody (FITC labeled goat anti-rabbit IgG) were supplemented for incubation for 1 hour, respectively. Finally, 200 μL of DAPI (4', 6-diamidino-2-phenylindole, Sigma-Aldrich, CAS:28718-90-3) was added into each well for 30 min. The images were acquired under a fluorescent microscope (BX-53; Olympus, Tokyo, Japan) and examined using a 40 × objective lens.

Proliferation viability assays of FGCs. Cell proliferation activity was assessed using the Cell Counting Kit-8 assay (CCK-8; Dojindo, Kumamoto Japan) according to the manufacturer's instructions. After 3 h of incubation, the absorbance was read at 450 nm using an Elx800 Reader (Bio-Tek Instruments Inc., USA). This assay was conducted in triplicate. According to the calculation formula for the CCK-8 kit, the proliferation activities of FGCs were calculated for each group.

*Apoptosis assays***.** FGCs were harvested after 24 h, 48 h and 72 h of culture. The apoptosis effect was measured using the Annexin V-FITC Apoptosis Detection Kit (KeyGEN, Nanjing, China). Briefly, FGCs were cultured in 6-well trays and transfected with plasmids for 48 h. Then, 500 μL of 1X Annexin V buffer was added to gently resuspend the cells, and 5 μL of Annexin V-FITC and 5 μL of propidium iodide staining solution were added and mixed. Flow cytometry was performed after incubation for 15 min at room temperature. Apoptosis cells were quantified by flow cytometry according to the manufacturer's instructions. The data were analyzed using FlowJo (TreeStar, Inc., Ashland, OR).

Quantitative real-time PCR (qRT-PCR) of key genes

*Primer design***.** For qPCR, the specific primers were chosen in well-conserved regions of KISS-1R(NM-001318077.1), StAR(NM-001009243.1), β-SD(NM-001135932.1), YP11(NM-001093789.1), PI3K(XM-004016920.4), AKT(XM-027956972.2), Bax(XM-027978594.2), Bcl-2(XM-027960877.2), Caspase-3(XM-015104559.3) and GAPDH(NM-001190390.1) (Table 1). The primers were synthesized by Beijing Aoke Dingsheng Biological Company.

RNA extraction and cDNA synthesis. FGCs were cultured for 72 h, and washed twice with DPBS. Total RNA was extracted by lysing granulosa cells in TRIzol reagent (Invitrogen; Carlsbad, CA, USA), according to the manufacturer's instructions. The RNA concentration was determined by absorbance at 260 nm using UV-2600i, UV-2700i ultraviolet spectrophotometer (Daojin Co. Ltd, Shanghai, China). 1 μg of total RNA was reverse transcribed

Table 1. Primer sequences

Primers	Primer sequence (5'-3')	Product size/bp
PI3K	F: AAGAACAATGCCAAACCCAGGAGC	174
	R: CCTGCTTCTTCAAGTCTTCTTCCAACC	
AKT	F: CACAAGCGAGGGGAGTACATC	129
	R: GAAGTTGTTGAGGGGCGACTC	
Bax	F: GCCCTTTTGCTTCAGGGTTT	121
	R: TCAGACACTCGCTCAGCTTC	
Bcl-2	F: TTGGGAAGTTTTCAGAGCAGC	141
	R: ACCTCCTCCGTGATGTGGTA	
Caspase-3	F: CTGCAACGTTGTGGCTGAAC	122
	R: AGTCCACTGATTTGCTTCCGT	
StAR	F: CAGCAGAAGGGTGTCATCAGAG	154
	R: GCGAGAGGACCTGATTGATGA	
3β-HSD	F: GCAGGAAATCCGGGTGCTA	127
	R: CCTCTTCAGGCACTGCTCATC	
KISS-1R	F: CTTAATGTTCCTGCTGTGCTGC	112
	R: CGAGACCTGCTGGATGTAGTTGA	
CYP11	F: GTTTCGCTTTGCCTTTGAGTC	120
	R: ACAGTTCTGGAGGGAGGTTGA	
GAPDH	F: TGGCATCGTGGAGGGACTTA	266
	R: CATCATACTTGGCAGGTTTCTCC	
TNC	F: GCTACGGAGGTTCAGTCGGAATC	146
	R: GTCAGGCTGTAAGAGGTGGTGTC	
THBS1	F: GTGCCTGACAAGAAGTTCCAAGAC	156
	R: GGAGACCACGCTGAAGACCTG	
Bax	F: GCCCTTTTGCTTCAGGGTTT	121
	R:TCAGACACTCGCTCAGCTTC	
Bcl-2	F:TTGGGAAGTTTTCAGAGCAGC	141

into cDNA using the PrimeScript™ RT reagent kit with gDNA Eraser (Takara; Dalian, China) according to the manufacturer's instructions.

*Real time quantitative PCR (qPCR)***.** Expression levels of the gene mRNAs described above were determined using qPCR, as referred to in the previous report by our team (BAI et al., 2020). The qPCR was performed with a SYBR Premix Ex Taq™ II kit (Takara; Dalian, China) utilizing the CFX96 real-time PCR system (Bio-Rad; Hercules, CA, USA). The reaction was incubated for pre-denaturation at 95°C for 10 min, 40 cycles of denaturation at 95°C

for 30 s and annealing/ elongation at 60°C for 55 s. Fluorescence signals were collected during the annealing step. GAPDH was employed as the endogenous control for these genes. The relative amount of each mRNA was determined by the $2^{-\Delta\Delta(Ct)}$ method. All the reactions were performed in triplicate.

Western blotting of Bcl-2, Bax, PI3K and AKT proteins in FGCs. To determine the expression levels of Bcl-2, Bax, PI3K and AKT proteins, total protein was extracted from the FGCs using a Total Protein Extraction Kit (Applygen Technologies, Beijing, China) according to the manufacturer's instructions. Protein concentrations were determined with a BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Haumen, China). Western blotting assay was carried out according to our previous studies (LAI et al., 2018; YANG et al., 2019). Quantity One software was utilized to analyze the integral optical density (IOD) of the scanned band image and the relative level of each protein.

The primary antibodies were as follows: anti-Bcl-2 antibody (EPR17509, 1:1000; ab182858, Abcam, Cambridge, UK), anti-Bax antibody (ERP18283, 1:1000; ab2733, Abcam), anti-PI3K antibody (EPR25156-60, 1:1000; ab302958, abcom), anti-pan-AKT antibody (1:1000; ab8805, Abcam) and β-actin (1:1500; SC-47778, Santa Cruz). Goat anti-rabbit IgG (H+L) secondary antibody (31460, 1:5000, Thermo Pierce) was used.

Detection of estrogen and progesterone concentrations. Concentrations of estrogen (E2) and progesterone (P4) in the media fluid were determined using special ELISA kits of E2 and P4 for sheep, following the operation manual (Cusabio Biotech Co., Ltd., Wuhan, China), respectively. Detection limits were 0.02ng/mL for E2 and 0.01ng/mL for P4. The inter- and intra- experimental variation coefficients were less than 6% and 5%. All samples were tested in triplicate. The detailed methods were reported in our earlier studies (WEI et al., 2013; WEI et al., 2017).

Data statistics and Pearson correlation analysis. Each experiment was performed in triplicate, and the data were analysed by SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). Data are presented as means ± SEM. Statistical differences between groups of different KP-10 doses were determined by one-way analysis of variance (ANOVA). Tukey's test was used to estimate the significance of the results. The relationships between KP-10 doses and other indexes were statistically assessed with Pearson

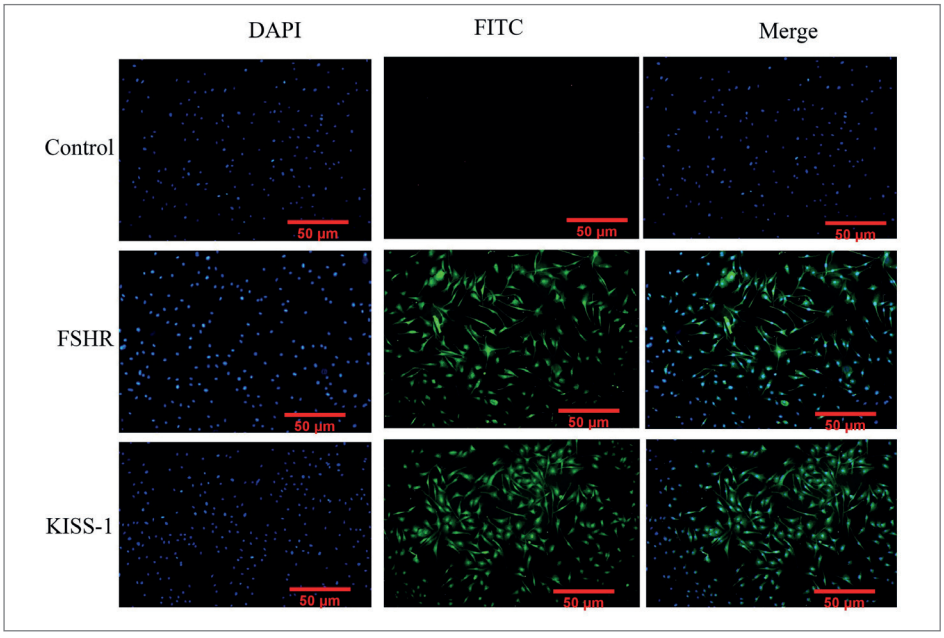

Fig 1. Immunofluorescence staining results of FSHR and KISS-1 proteins in FGCs (40 ×)

Note: DAPI: DAPI stains nucleus (blue fluorescence zone); FITC: FSHR and KISS-1 are immune positive cells (green fluorescent zone); Merge: the nucleus and positive area.

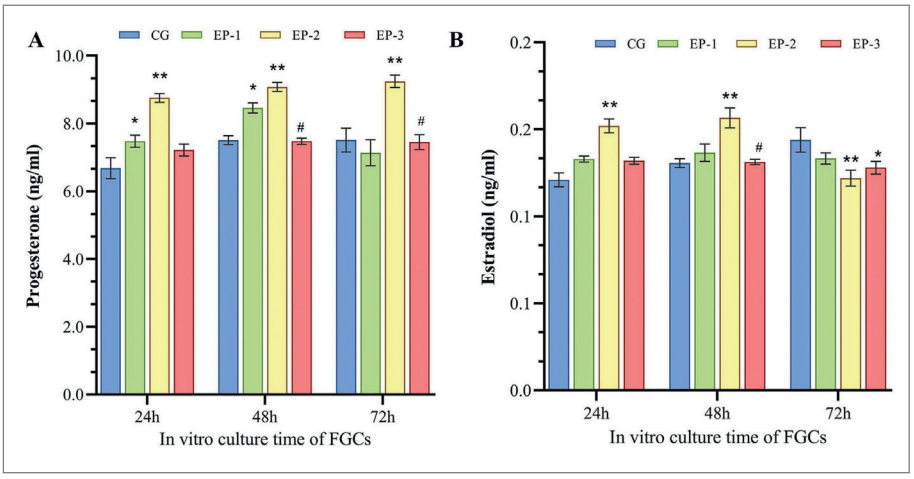

Fig. 2. Effect of KP-10 on estrogen and progesterone secretion in FGCs

A: Progesterone (ng/ml); B: Estrogen (ng/ml); * P<0.05; ** P<0.01, compared to the CG group; # P<0.05, compared to EP-2 group

correlation analysis in SPSS. P<0.05 was considered to be significant.

Results

Subcellular localization of FSHR and KISS-1 proteins in FGCs. Immunofluorescence detection results showed that the nucleus of FGCs appeared as blue fluorescence after DAPI staining. FSHR and KISS-1 displayed clear green fluorescence in the cytoplasm of the FITC staining positive FGCs. KISS-1 protein was positively expressed in ovine FGCs, mainly distributed in the cytoplasm (Fig. 1). These results revealed that both FSHR and KISS-1 existed and expressed in FGCs with very high purity.

KP-10 promote secretion of progesterone (P4) and estrogen (E2) in FGCs. ELISA assay results indicated that the P4 concentrations in the EP groups was significantly increased when compared to CG (Fig. 2). At 24h, 48h and 72 h after KP-10 treatment, P4 concentration in group EP-2 was significantly higher than that in CG (P<0.01). Meanwhile, P4 levels in group EP-2 were higher than in EP-3 (P<0.05) at 48h and 72h. E2 concentration in group EP-2 were significantly higher than in the CG at 24h and 48h (P<0.01). E2 contents of EP-2 were greater than that of EP-3 at 48h (P<0.05), but obviously decreased at 72h (P<0.01).

KP-10 improved expression levels of KISS-1R, StAR, 3 β-HSD and CYP11A1 mRNAs. The expression levels of KISS-1R, StAR, 3 β-HSD and CYP11A1 mRNAs were detected with qPCR in sheep FGCs treated with KP-10 at different times. The results indicated that expression levels of KISS-1R, StAR, 3 β-HSD and CYP11A1 mRNAs in group EP-2 were significantly higher than those in CG and group EP-1 at 48h and 72h (P<0.01) (Fig. 3).

KP-10 affected the proliferation activity of Sheep FGCs. CCK8 test results showed that the cell viability of FGCs gradually increased after KP-10 treatment compared to CG (Fig. 4). The largest increment was seen in group EP-2. The proliferation activity of FGCs in EP-2 (P<0.01) and EP-3 (P<0.05) were significantly higher that in CG at 48h and 72h. This indicated that 100 nmol/L of KP-10 (EP-2 group) enhances the proliferation activity of sheep FGCs.

KP-10 impacted apoptosis of FGCs. The apoptosis rate assay indicated that KP-10 decreased the apoptosis rates of sheep FGCs. Following KP-10 treatment, the apoptosis rates of the three experiment groups were decreased in comparison with the CG (Fig. 4). The apoptosis rates of group EP-2 were significantly lower than the CG at 24h (P<0.05), 48h and 72h (P<0.01). Furthermore, the apoptosis rates

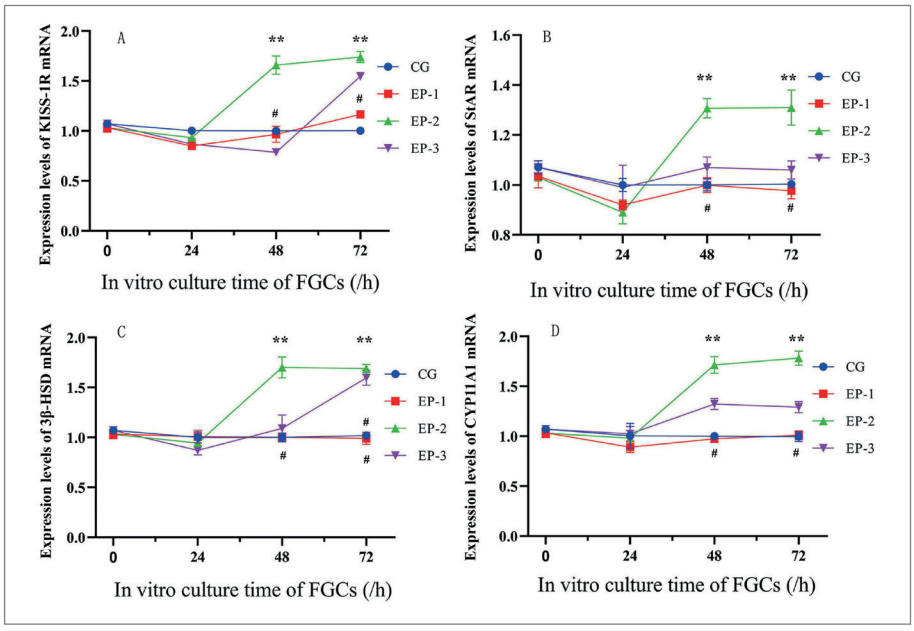

Fig. 3. Effects of KP-10 on genes expressions at different times. Expression levels of KISS-1R, StAR, 3 β-HSD and CYP11A1 mRNAs in EP-2 group were significantly higher than those in CG and EP-1 group at 48h and 72h. KP-10 treatment could enhance levels of KISS-1R, StAR, 3 β-HSD and CYP11A1 genes in FGCs

* P<0.05; ** P<0.01, compared to the CG group; # P<0.05, compared to EP-2 group

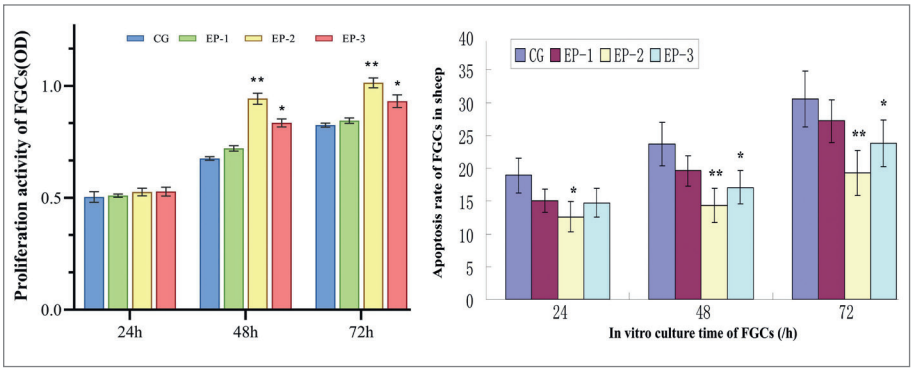

Fig. 4. Effects of KP-10 on proliferation activity and apoptosis rate of FGCs at the different times

* P<0.05, ** P<0.01, compared to the CG

of group EP-3 were also lower than the CG at 48h and 72h (P<0.05).

KP-10 regulated expressions of Bax, Bcl-2, caspase-3, PI3K and AKT mRNAs in FGCs. The qPCR results showed that expression levels of Bax mRNAs in the KP-10 treatment groups were decreased with the increase of KP-10 dosage (Fig. 5), and Bax mRNA levels of EP-3 groups were lower than in the CG at 48h and 72h (P<0.01). In addition, the expression levels of caspase-3 in group EP-2 were lower than the CG at 48h (P<0.05) and 72h (P<0.01). However, expression levels of Bcl-2 in EP-2 (P<0.05) and EP-3 (P<0.01) were significantly higher than in the CG at 48h and 72h. Furthermore, expression levels of PI3K mRNA of group EP-3 were higher than in the CG at 48h (P<0.01) and 72h (P<0.05). At 48h, the

level of AKT mRNA of group EP-2 was higher than in the CG (P<0.05). These outcomes demonstrated that KP-10 may obviously attenuate expression levels of Bax and caspase-3 mRNAs, and heighten levels of Bcl-2, PI3K and AKT genes related to the cell proliferation and apoptosis of sheep FGCs, thereby enhancing FGC cell viability.

KP-10 influenced expression levels Bax, Bcl-2, PI3K and AKT proteins. The expression levels of Bax and AKT proteins in KP-10 treatment groups were significantly lower than those in the CG (P<0.01) (Fig. 6). However, expression levels of PI3K protein in group EP-3 at 72h and Bcl-2 levels in group EP-2 at 48h were significantly higher than in the CG (P<0.05).

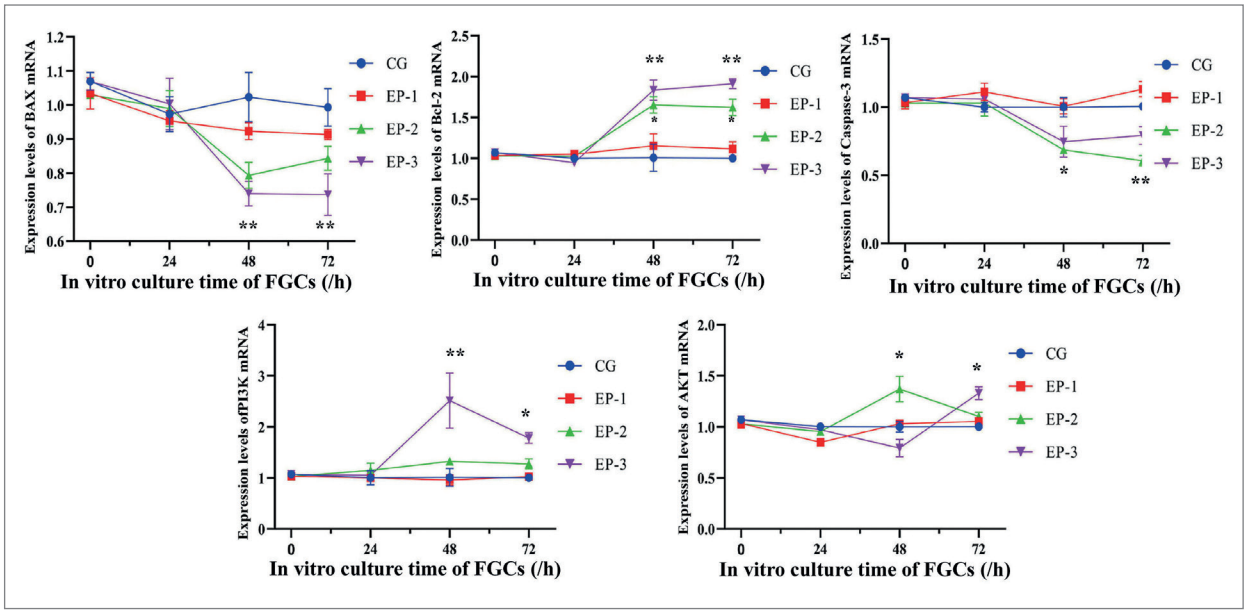

Fig. 5. Effects of KP-10 on expression levels of the associated genes in FGCs
* P<0.05, ** P<0.01, compared to the CG

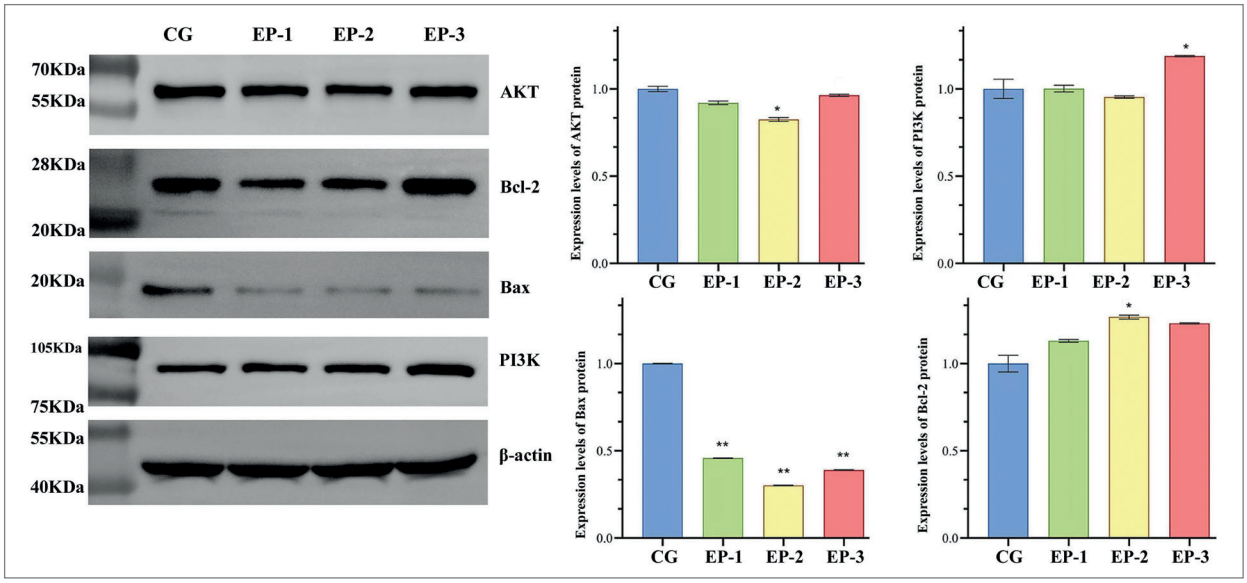

Fig. 6. Effects of KP-10 on expression levels of Bax, Bcl-2, PI3K and AKT proteins in FGCs
* P<0.05, ** P<0.01, compared to the CG group

Correlation analyses among indexes. Pearson analysis indicated that the KP-10 doses were positively correlated to cell activities, and expression levels of Bcl-2 and PI3K mRNAs and proteins in FGCs (Table 2). However, the KP-10 dose was negatively correlated to the BAX protein. The proliferation activities of FGCs were also positively correlated to levels of Bcl-2 mRNA and protein. Meanwhile they were negatively correlated to expressions of Bax mRNA and protein.

Table 2. Pearson correlation analysis among KP-10 doses and other indexes

Items	Dose	Cells activities	PI3K mRNA	Bax mRNA	Bcl-2 mRNA	AKT mRNA	PI3K protein	Bcl-2 protein	Bax protein
Cells activities	0.73**								
PI3K mRNA	0.81**	0.35							
Bax mRNA	0.84**	-0.70*	-0.67*						
Bcl-2 mRNA	0.93**	0.78**	0.74**	-0.74**					
AKT mRNA	-0.22	0.43	-0.57	0.07	-0.10				
PI3K protein	0.62*	-0.01	0.81**	-0.51	0.46	-0.81**			
Bcl-2 protein	0.88**	0.89**	0.51	-0.84**	0.81**	0.21	0.27		
Bax protein	-0.81**	-0.78**	-0.40	0.71**	-0.73**	-0.19	-0.17	-0.93**	
AKT protein	-0.34	-0.80**	0.14	0.36	-0.41	-0.77**	0.50	-0.69*	0.73**

Note: * P<0.05, ** P<0.01.

Discussion

Granulose cells (GCs) are the main functional cells of the ovary, playing a crucial role in determining whether follicles continue to develop or undergo atresia. GCs mainly synthesize and release estrogen and progesterone. Previous studies showed that Kisspeptin is an important marker for neuroendocrine regulation during the reproductive process (BHALAKIYA et al., 2019; JIANG et al., 2023). Kisspeptin may be a key molecule in the maturation and regulation of female reproductive organs (GARCIA-ORTEGA et al., 2016).

Recently Kisspeptins have been shown to be essential to reproductive functions in mammals. The distribution of KP-10 immunoreactive cells has been determined in the ovine hypothalamus. The pattern of distribution of these KP-10 immunoreactivity cells in the hypothalamus suggests the role of kisspeptin in the estrogen-dependent regulation of GnRH and LH secretion in ewes. Therefore, Kisspeptin is considered the most effective inducer of the secretion, pulses, and surges of gonadotropin and GnRH (WEN et al., 2016).

An early study showed that the KISS-1 gene was expressed in the FGCs of rats, chickens, cows, pigs, sheep (HU et al., 2018). KP-10 significantly enhanced the vitality of FGCs in chicken before ovulation, and promoted progesterone secretion in a dose-dependent manner (XIAO et al., 2011). The crucial mechanism was upregulation of expression levels of 3 β-HSD, P450, and StAR mRNA (NAVARRO et al., 2011). Previous investigations found that the KISS-1 protein was expressed in the cytoplasm of sheep FGCs, and a certain concentration of KP-10 can dose-dependently increase the secretion of E2 and P4 in FGCs within a certain concentration range. However, after 72h of treatment with 100 and 1000 nmol/L KP-10, E2 secretion was significantly inhibited.

Kisspeptin and its receptor (KISS-1R) staining was detected in primordial, preantral and antral follicles throughout all reproductive stages (OBERHAUS

et al., 2015). Kisspeptin/kisspeptin receptor (Kiss-Kissr) system has a correlation with the regulation of reproduction (WANG et al., 2017). The immunofluorescence findings in our study demonstrated KISS-1 was expressed positively in FGCs, mainly distributed in the cytoplasm. These outcomes agreed with earlier reports (CIELESH et al., 2017).

KP-10 directly stimulates GnRH neurons to secrete GnRH (DHILLO, 2019). The intravenous injection of 45 mg human or murine KP-10 in heifers increased plasma LH concentrations, accompanied by a small or non-existent elevation of FSH level (JAYASENA et al., 2014). In this study, the concentrations of estrogen (E2) and progesterone (P4) following KP-10 treatment were determined using a special ELISA kit. P4 concentration in the EP group was significantly increased when compared to the CG. At 24h, 48h and 72h, P4 concentration in group EP-2 was significantly higher than in CG and group EP-3. E2 concentrations in group EP-2 were significantly increased. The findings revealed that KP-10 could significantly enhance the secretion of progesterone and estrogen in FGCs. Our findings lay a foundation for a thorough understanding of the effects and mechanism of KP-10 on reproduction secretions (MARRAUDINO et al., 2018).

Another study indicated that when yak FGCs were treated with 1000 nmol/L KP-10 for 12 hours, their cell viability was significantly inhibited). However, over time, the cell viability of FGCs was significantly improved compared to the control group, and progesterone secretion was dose-dependent on the concentration of KP-10 (MISHRA et al., 2019; XIN et al., 2019).

KP-10 treatment could enhance the proliferation activity of sheep FGCs, where the largest increment was seen in group EP-2, and is beneficial for exploiting follicular and ovarian functions (ZHENG et al., 2023). Our results are consistent with a previous study (BAO et al., 2021). Additionally, the experimental results indicated that KP-10 secretion of progesterone and estrogen concentrations in the EP groups was significantly increased at 48h and 72h. This demonstrated that KP-10 could promote the secretion of progesterone and estrogen in sheep FGCs. Our outcomes are in agreement with initial reports (ROMERO-RUIZ et al., 2019; XIAO et al., 2011).

In this study, we detected the expression levels of KISS-1R, StAR, 3 β-HSD and CYP11A1 genes associated with hormone secretion in FGCs. The expression levels of these four genes in group EP-2 were heightened at 48h and 72h compared to the CG and group EP-1. The qPCR results revealed that expression levels in group EP-2 were significantly higher than those in the CG at 48h and 72h. The increases in the relative expression levels of 3 β-HSD, StAR and CYP11A1 may promote estrogen and progesterone secretion in GCs. Therefore, KP-10 treatment could increase hormone secretion, probably by accelerating the expressions of KISS-1R, StAR, 3 β-HSD and CYP11A1 genes. Our results are consistent with a previous study (LIU et al., 2018). Moreover, we found that 100 nmol/L of KP-10 could enhance levels of KISS-1R, StAR, 3 β-HSD and CYP11A1 genes in FGCs. KP-10 regulated secretion of estrogen and progesterone, probably by increasing the expressions of these genes. These findings indicated that FSHR immunofluorescence assay can be applied to identify the KISS-1 protein efficiently in FGCs. Therefore, we believed that these proteins jointly mediated the apoptosis and promoted hormonogenesis of the ovaries and FGCs. The outcomes provided the experimental basis for exploring more deeply the genes that are involved in the steroidogenesis of granulosa cells in animals and humans, and revealing the underlying molecular mechanisms.

Cell apoptosis is programmed cell death, controlled by related genes which play an important role. Bcl-2 and Bax proteins are mutually antagonistic proteins in regulating apoptosis. Increasing levels of Bcl-2 and Bax proteins could inhibit or promote the formation of Bax/Bcl-2 heterodimer, which activates the expression of downstream Caspase family proteins, and promotes the release of apoptosis factors. The results in the present study revealed that expression levels of Bax and AKT proteins in the KP-10 treatment groups were reduced. Levels of Bax proteins in groups EP-2 and EP-3 were significantly lower than in the CG. However, expression levels of both Bcl-2 levels in group EP-2 and PI3K protein in group EP-3 significantly increased. Therefore, KP-

10 may significantly upregulate or downregulate the expression levels of Bcl-2, Bax, PI3K and AKT proteins in FGCs. This is consistent with the results of the qPCR assay. Kisspeptin-10 increased hormone secretion, modulated proliferation activity, and inhibited apoptosis of FGCs, which was probably through activation of the PI3K/AKT signal pathway.

KP-10 significantly enhanced the cell viability of FGCs by upregulating the expression of Bcl-2 and downregulating the expression of Bax and Caspase-3. KP-10 could improve the proliferation activity of FGCs in chicken and yaks. We found that the cell viability of FGCs gradually increased after KP-10 treatment. The largest increment was found in the EP-2 group. The results demonstrated that KP-10 can upgrade the proliferation activity of sheep FGCs, which is beneficial for the development of follicular and ovarian functions.

Conclusions

KISS-1 proteins were predominately distributed and expressed in the cytoplasm of FGCs. KP-10 (100 nmol/L) could enhance proliferation activity of FGCs by decreasing apoptosis rates via upregulation of the mRNA and protein levels of Bcl-2 and PI3K, and downregulation of mRNA and protein levels of Bcl-2 and PI3K of sheep FGCs. Additionally, KP-10 could promote the secretion of progesterone (P4) and estrogen (E2), probably by strengthening the expressions of KISS-1R, StAR, 3 β-HSD and CYP11A1 gene. 100 nmol/L KP-10 was the optimum dose. These data establish the scientific experimental basis for thorough investigation of the regulation effects and molecular mechanism, as well as the signaling pathways of KP-10, in relation to reproductive function and improvement of the fecundity of animals and humans using Kisspeptin (Greenhill, 2023; JIANG et al., 2023).

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Author contributions

Dr. Pei Mengyuan and Miss Xu Linglong carried out the cell culture and Western assay. Miss Gao Enyu did premier design and the statistical analysis of data. Dr. Yuan Zhaofang detected receptor gene expression. Professor and Dr. Wei Suocheng designed the experiments and wrote the manuscript. All authors interpreted the data, critically revised the manuscript for important intellectual content, and approved the final version.

Availability of data and material

All data are available upon reasonable requests.

Declaration of competing interest

The authors declare no conflict of interest.

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SAŽETAK

Cilj istraživanja bio je odrediti supcelularni smještaj proteina KISS-1 i istražiti učinak kispeptina 10 (KP-10) na umnažanje, održivost i izlučivanje hormona folikularnih granuloza-stanica (FGC). Također, istraženi su i molekularni mehanizmi koji prate navedene procese. FGC-e su kultivirane *in vitro* u mediju DMEM obogaćenom različitim dozama KP-10 (0, 10, 100 i 1000 nmol/L) te su prema tome razvrstani u kontrolnu skupinu (CG), pokusnu skupinu 1 (EP-1), pokusnu skupinu 2 (EP-2) i pokusnu skupinu 3 (EP-3). Supcelularni smještaj proteina FSHR i KISS-1 u FGC-ama analiziran je imunofluorescencijskim testom. Upotrijebljen je set za brojenje stanica 8 (CCK8), PCR u stvarnom vremenu (qPCR) i *western blotting* kako bi se ustanovila proliferacijska aktivnost i ekspresija gena i proteina u FGC-ama. ELISA kompleti primijenjeni su za mjerenje sadržaja estrogena (E2) i progesterona (P4). Imunofluorescencijski je test pokazao da je protein KISS-1 pozitivno eksprimiran u FGC-ama ovaca i većinom se izlučuje u citoplazmu. Nakon 48 sati i 72 sata tretmana s KP-10 održivost FGC-a u skupinama EP-2 i EP-3 znakovito je poboljšana u odnosu na kontrolnu skupinu ($P<0,05$ odnosno $P<0,01$). Sadržaj estrogena (E2) i progesterona (P4) u skupini EP-2 povećan je u usporedbi s kontrolnom skupinom ($P<0,01$). Osim toga, razine izražaja gena Kiss-1R, StAR, 3β-HSD i CYP11 mRNA u skupini EP-2 znatno su povećane u usporedbi s kontrolnom skupinom ($P<0,05$ odnosno $P<0,01$) s porastom od 65,9% za Kiss-1R, 30,7% za StAR, 73,4% za 3β-HSD i 71,4% za CYP11. Zaključeno je da su proteini KISS-1 bili smješteni većinom u citoplazmi FGC-a. KP-10 može povećati održivost FGC-a i izlučivanje progesterona i estrogena, što se može postići povećanom izražajnošću gena Kiss-1R, StAR, 3-β-HSD i CYP11. KP-10 znakovito je povećao razine mRNA i proteina u Bcl-2 i PI3K ($P<0,01$) te smanjio izražajnost mRNA i razine proteina u Bcl-2 i PI3K ($P<0,05$). Navedeni rezultati pružaju dobru osnovu za daljnja istraživanja poboljšanja reprodukcije životinja.

Ključne riječi: kispeptin; folikularne granuloza-stanice; estrogen; progesteron; proliferacija