Original Paper

FSHR and LHR Expression and Signaling as Well as Maturation and Apoptosis of Cumulus-Oocyte Complexes Following Treatment with FSH Receptor Binding Inhibitor in Sheep

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Key Words

Cumulus-oocyte complexes • *In vitro* maturation • Apoptosis • Follicle stimulating hormone • FSH receptor binding inhibitor • Receptors

Abstract

Background/Aims: Currently, it remains unknown whether FSH receptor binding inhibitor (FRBI) influences follicular development and reproduction functions in humans and animals. The present study aimed to investigate FRBI effects on *in vitro* maturation (IVM) and apoptosis of cumulus-oocyte complexes (COCs) of sheep, to determine the effect of FRBI on mRNA and protein levels of FSHR and LHR in COCs, and to elucidate the signal pathway of FRBI effects.

Methods: COCs were *in vitro* cultured for 24h in the IVM media supplemented with varying concentrations of FRBI (0, 10, 20, 30 and 40 µg/mL) and FSH (10 IU/mL). The harvested COCs were observed under an inverted microscope and maturation rates of COCs were determined. Real time RT-PCR and Western blotting were utilized to detect mRNA and protein levels of FSHR and LHR. The concentrations of FSH, LH and caspase-3 were determined using especial ELISA kits for sheep, respectively. **Results:** Maturation rates of COCs decreased gradually as FRBI concentrations increased from 0 to 40 µg/mL, reaching a bottom value of 23.76% of the FRBI-4 group. The maximal apoptosis rate was detected in the FRBI-4 group. IP3 contents of FRBI-3 and FRBI-4 groups were reduced as compared to control group (CG) and FSH groups ($P < 0.05$). Levels of FSHR protein of FRBI-3 and FRBI-4 groups as well as LHR protein of FRBI-4 group were significantly less than that of CG and FSH group. FSH contents of four FRBI treatment groups were gradually decreased along with the supplementation doses of FRBI.

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Caspase-3 contents of FRBI groups were reduced with a maximum reduction of the FRBI-2 group. **Conclusion:** Our results revealed supplement of FRBI into IVM media could dose-dependently decrease the maturation rate and increase apoptosis rate of sheep COCs. A lower dose of FRBI treatment slightly promoted IP3 production, but a higher dose of FRBI reduced IP3 production. FRBI suppressed the mRNA and protein expression levels of FSHR and LHR in sheep COCs. Our study will help to therapy effectively ovarian diseases, improve ovarian and follicular functions, and further to promote fertility of humans and animals.

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Introduction

Ovarian follicular development is a continuous process under the influence of follicle stimulating hormone (FSH) and luteinizing hormone (LH) [1]. FSH activates multiple signaling pathways to regulate genes necessary for follicular maturation [2]. FSH acts via cognate FSH receptor (FSHR) existed on the granulosa cells of ovary follicles [3]. FSHR is an important mediator in human and animals' reproduction [4]. FSH-FSHR interaction activates a cascade of intracellular signaling events leading to the production of steroidogenesis that are involved in modulating the proliferation and differentiation of ovarian granulosa cells [5].

Follicular fluids contain a number of factors acting as inhibitors of FSH-FSHR interaction [6]. One non-steroidal low molecular weight factor, FSH receptor binding inhibitor (FRBI), was identified from the follicular fluids of sheep and human [7, 8]. This FRBI, as an FSH antagonist, blocks the binding of hFSH to rat granulosa cells. *In vivo* administration of FRBI caused the suppression of ovulation and induced follicular atresia in mice [7], further impaired fertility of marmosets at doses of 100µg/day and 300µg/day [9].

After rat granulosa cells were treated with FRBI in the presence or absence of FSH, progesterone (P) secretion was inhibited in a dose-dependent manner [10]. FRBI blocked the binding of FSH to FSHR, and altered FSH action at the receptor level [3, 9]. Currently, little information has been recorded about FRBI effects on follicular development and reproduction functions in human and animals [2].

FRBI can initiate the primary signaling cascades via the production of the cAMP and inositol trisphosphate (IP3), thereby regulating steroidogenesis. These signaling cascades adjusted by FSH and LH in the granulosa cells play a crucial role in follicular development and ovulation [11]. However, the exact mechanism of FRBI actions remains still unclear and the regulatory signaling pathways are undetermined [2, 12, 13]. IP3 is an important intracellular second messenger that participates in signal transduction pathways in diverse cell types. Binding of IP3 to its receptor (IP3R) induces Ca²⁺ release from intracellular stores and enables the initiation of intracellular calcium ion-dependent signaling. Up to date, whether FRBI treatment of COCs influences this signal transduction remains unknown [14].

The present work was conducted to investigate the effects of FSH receptor binding inhibitor (FRBI) on IVM of sheep COCs, and to understand the FRBI mechanism of inhibiting the interaction of FSH to FSHR in COCs and granulosa cells, additionally to elucidate the signal pathway of FRBI actions in sheep COCs.

Materials and Methods

Preparation of FSH receptor binding inhibitor (FRBI)

FRBI peptide of 99.9% purity was synthesized by Nanjing Peptide Biotech Co. Ltd., Nanjing, China (CAS: 163973-98-6) and was characterized before using it for experimental work. The homogeneity of FRBI peptide was checked by analytical RP-HPLC. The amino acid composition and the peptide content were evaluated by amino acid hydrolysis of FRBI peptide. FRBI activity was determined by the radio receptor assay as reported earlier [9].

To prepare FRBI solution (1.0mg/mL), 100mg FRBI was dissolved in 10% demethyl sulfoxide (DMSO), the sterile normal saline was added into the above solution to a total volume of 100mL, the concentration of FRBI was 1000µg/mL. The prepared FRBI solution was kept at -20°C.

Preparation of basal maturation media (bMM)

Basal maturation media (bMM) were prepared as our previous methods [15]. The bMM was consisted of 9.5 g Medium 199 powders (Sigma, St. Louis, MO, USA), 2.2 g NaHCO₃, 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 media were filtered through a 0.22-µm membrane and stored at 4°C. *In vitro* maturation (IVM) media comprised the bMM supplemented with 0.68 mM L-glutamine, 2.1 g NaHCO₃ and 5% fetal bovine serum (FBS; Minhai Company, Lanzhou, China). All experiments were performed in sterile conditions.

Collections of sheep ovaries

Ovaries were collected between May to July of 2016 from 509 immature and noncyclic ewes (6-7 months old) immediately after their slaughter at the local abattoirs [15], and then transported to the laboratory in 30-35°C Dulbecco's phosphate-buffered saline (DPBS) (Sigma Co. Ltd, Beijing, China) 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 in China.

Collection of COCs

After the extraneous tissues and fat on the ovary surfaces were removed using sterile scissors, the ovaries were placed in a petri dish, to which 2 mL pre-equilibrated extraction fluid [phosphate-buffered saline (PBS) containing 3 mg/mL bovine serum albumin, incubated at 38°C overnight] was added, while they were gently holden using sterile ophthalmic tweezers. Subsequently, the follicles on ovarian surface were scratched from a total of 1006 ovaries with a scalpel blade. Cumulus-oocyte complexes (COCs) were recovered from antral follicles (3.0-5.0mm in diameter) by gently cutting follicles with a scalpel on the 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). Only COCs with at least three complete cumulus cell layers were considered suitable for IVM [16]. COCs taken from all animals were collected together in one instrument tray. A total of 1296 COCs were suitable for subsequent experiments.

IVM of sheep COCs

IVM of sheep COCs was performed referring to the early methods [15, 17]. A microwell culture system was used in this experiment. Collected COCs were rinsed three times with extraction fluid, and pre-equilibrated for 3 h before IVM culture. At least 30 COCs were randomly taken from the instrument tray and placed in one culture well (Nunc Inc., Naperville, IL, USA) containing 600 µL maturation media covered with 300 µL mineral oil. In order to evaluate the influence of FRBI on IVM of sheep COCs, the basal maturation media were supplemented with different doses of FRBI-8 at 0, 10, 20, 30 or 40µg/mL and 10 IU/mL FSH, respectively. They were allocated to control group (CG), FRBI-1, FRBI-2, FRBI-3, FRBI-4 and FSH groups, respectively (Table 1). COCs were then left to complete their maturation at 38.5°C in an atmosphere of 5.0% carbon dioxide in humidified air continuously for 24h. The development and maturation of oocytes were observed at 20, 22 and 24 h, respectively. IVM medium fluids were also collected at 20, 22 and 24h respectively. The supernatant was separated and stored at -20°C for analysis.

Table 1. Protocol of IVM of sheep COCs, FSH: Follicle stimulating hormone; FRBI: Follicle stimulating hormone binding, Inhibitor; IVM: *in vitro* maturation

Group	COCs	Addition doses of FSH or FRBI into the IVM media
CG	203	Neither FRBI nor FSH was added into the IVM media, as a blank control.
FSH	355	10 IU/mL of FSH was added into the IVM media, as a positive control.
FRBI-1	172	10 µg/mL of FRBI was added into the IVM media.
FRBI-2	183	20 µg/mL of FRBI was added into the IVM media.
FRBI-3	181	30 µg/mL of FRBI was added into the IVM media.
FRBI-4	202	40 µg/mL of FRBI was added into the IVM media.

IVM evaluations and apoptosis detection of COCs

After IVM, COCs were denuded by using 0.3% hyaluronidase digestion following three PBS rinses. Mature denuded COCs were then fixed and subjected to Giemsa staining to determine their progression to metaphase II. The harvested COCs were observed under an inverted fluorescence microscope (Leica, Japan), and those displaying an intact first polar body were identified as matured COCs. Experiments were executed in triplicate [18].

To detect apoptosis of COCs, COCs was analyzed according to treatment group using a TUNEL kit in accordance with the manufacturer's instructions (Roche Molecular Biochemicals, Mannheim, Germany). Briefly, COCs was fixed at 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, TUNEL reagent was omitted. Apoptosis rate was reported as the number of labeled cells expressed as a percentage of the total cell numbers.

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

FSHR and LHR mRNAs were determined using real time reverse-transcription polymerase chain reaction (qRT-PCR) and cloning techniques.

Primer design The primers specific for FSHR (GenBank accession number: NM-001009289.1) and LHR (GenBank accession number: L36329.1) were designed with Beacon Designer 7.0 software (Premier Biosoft International, Palo Alto, CA, USA) according to manufacturer's guidelines and Primer-BLAST at NCBI. Ovine GAPDH gene (GenBank accession number: HM-043737.1) was selected as the reference gene for normalizing expression levels of target genes [15, 18]. The sequences of the primers used in the qPCR were as follows: FSHR, forward 5'-TCTTTGCTTTTGCAGTTGCC-3' and reverse, 5'-GCACAAGGAGGGACATAACATAG-3'; LHR, forward 5'-CCTGAAGAAGATGCACGATGACGCC-3' and reverse, 5'-ACCCATTCCCTGTCTGCCAGTCT-3'; GAPDH, forward, 5'-CTTCAACAGCGACACTCACTCT-3' and reverse, 5'-CCACCACCCTGTTGCTGTA-3'. Primers were synthesized by Beijing AoKeDingSheng Biotechnology Co. Ltd., China. The concentrations of the primers (100 nM, 200 nM, 300 nM and 500 nM) were evaluated, and formation of primer-dimers was evaluated using the melting curve analysis. Thus, only those concentrations of primers which showed dimmer-free reactions were used for the further analysis.

Total RNA extraction. After IVM under different FRBI concentrations in IVM media, total RNA of 30 COCs was extracted using the TRIzol reagent (Invitrogen, Beijing, China), according to the manufacturer's instructions, then reverse transcribed [15, 19]. The extraction was replicated three times with 30–40 COCs.

qRT-PCR detection of mRNAs. The expression level of ERβ mRNA was determined using qRT-PCR, respectively [19, 20]. The relative amount of each mRNA was determined by the $2^{-\Delta\Delta CT}$ method and normalized to an endogenous reference gene of GAPDH. Each sample was executed in triplicate, and each experiment was replicated three times with 30 COCs for each replicate.

Western blotting of FSHR and LHR proteins in COCs

To evaluate the effects of FBRI treatment on expression levels of FSHR and LHR proteins in COCs of sheep, Western blotting was implemented according to our previous report [18, 19]. The integral optical density (IOD) of the scanned band images was obtained by using Quantity One software (Bio-Rad Company, Hercules, CA, USA). The relative levels of FSHR and LHR proteins were presented as the ratio between the gray values of FSHR and LHR divided by β-actin value. A negative control was performed without primary antibody. Assays were performed in triplicate.

Detection of caspase-3 contents

To access the effects of FRBI treatment on the apoptosis relative factor, caspase-3, the contents of caspase-3 in the IVM medium fluids were determined using an especial caspase-3 ELISA kit for sheep according to the manufacturer's instructions (Cusabio Biotech Co., Ltd. Wuhan, P.R. China), respectively. Analytical sensitivities were 0.10–0.40 ng/mL. The correlation coefficient of the standard curve was more than 0.9960. All samples were tested in triplicate in the same assay. The detailed operation steps were presented in our initial research [19].

Measurement of FSH and LH concentrations

Concentrations of FSH and LH in the IVM medium fluids were also determined using an especial ELISA kit of FSH and LH for sheep. The detailed operation steps were presented in our initial research [19]. Analytical sensitivities were 0.10-0.40 ng/mL for FSH and LH. The intra- and inter-experimental coefficients of variation were lower than 2.71% and 6.72%. The correlation coefficient of the standard curve was more than 0.9960. All samples were tested in triplicate in the same assay.

Inositol triphosphate (IP3) assay in IVM medium fluids

In order to determine the FRBI regulatory effects on inositol trisphosphate (IP3) production of sheep COCs and further to elucidate the signal pathway of FRBI action, IP3 produced by the COCs in response to FRBI supplement into the IVM media was detected with inositol triphosphate ELISA kit for sheep according to the manufacturer's instructions (Xinyu Biotech Co., Ltd., Shanghai, China).

Statistical data analyses

Statistical analysis was done using SPSS (version 21.0; Inc. Chicago, IL, USA). Data was presented as means $\pm$ SEM. All variables of five groups complied with the assumptions for a one-way analysis of variance (ANOVA). When significant differences were identified, supplementary Tukey's post hoc tests were performed to investigate pairwise differences. $P < 0.05$ was considered to be significant.

Results

In vitro maturation of sheep COCs

In order to evaluate FRBI influence on IVM and apoptosis of COCs, COCs were *in vitro* cultured for 24 h applying a microwell method. As shown in Table 2, maturation rate of sheep COCs of FSH group was greater than that of CG ($P < 0.05$). However, maturation rates of four FRBI-treated groups gradually declined as FRBI concentrations increased from 0 (control group) to 40 μ g/mL (FRBI-4 group), reaching a bottom value of 23.76% of the FRBI-4 group. Maturation rates of FRBI-2, FRBI-3 and FRBI-4 groups were significantly less than that of the FSH group ($P < 0.05$ or $P < 0.01$).

The incidence of apoptosis in COCs was determined by TUNEL assay, and the findings are presented in Table 2. Apoptosis rates of COCs demonstrated a trend opposite to that observed in maturation rates of COCs. Apoptosis rates were gradually increased as FRBI dose increased from 0 to 40 μ g/mL. The highest apoptosis rate was detected in the FRBI-4 group (36.14%) as compared to CG and FSH group ($P < 0.05$ or $P < 0.01$). Our results revealed supplement of FRBI into the IVM media could dose-dependently reduce the maturation rate and enhance the apoptosis rate of sheep COCs.

Expression of FSHR and LHR mRNAs of sheep COCs

To evaluate the effect of FRBI on expression and mRNA levels of FSHR and LHR of COCs, qRT-PCR was implemented. These transcripts were detected in sheep COCs after IVM, and their levels were augmented as the concentration of FRBI in the IVM medium was increased (Fig. 1). The expression levels of FSHR and LHR mRNAs of FSH group were enhanced as compared to CG ($P < 0.05$). However, the expression levels of FSHR and LHR mRNAs in four FRBI-treated groups were gradually declined along with supplementing doses of FRBI into IVM medium, with the least value of the FRBI-4 group ($P < 0.05$).

Table 2. Effects of FRBI on *in vitro* maturation of sheep COCs, FRBI- Follicle stimulating hormone receptor binding Inhibitor.

* $P < 0.05$ as compared to CG (blank control).
$P < 0.05$ and ## $P < 0.01$ as compared to FSH group

Group	Doses	Cultured COCs	Matured COCs	Maturation rate %	Apoptosis COCs	Apoptosis rate %
CG	0	203	66	32.51	59	29.06
FSH	10IU/mL	355	150	42.25 ^a	72	20.28 ^a
FRBI-1	10ug/mL	172	63	36.63	45	26.16
FRBI-2	20ug/mL	183	62	33.88 [#]	54	29.51 [#]
FRBI-3	30ug/mL	181	54	29.83 [#]	61	33.7 [#]
FRBI-4	40ug/mL	202	48	23.76 ^{##}	73	36.14 ^{##}

Fig. 1. Expression levels of FSHR and LHR mRNAs. In four FRBI-treated groups were gradually reduced as of FRBI doses rose, with the least value of the FRBI-4 group. * $P < 0.05$ as compared to control group (CG); # $P < 0.05$ and ## $P < 0.01$ as compared to FSH group.

Fig. 2. Expression levels of FSHR and LHR proteins. Western blotting analysis showed that FSHR and LHR proteins were expressed in sheep COCs with β -actin as a reference gene. Levels of FSHR protein of FRBI-3 and FRBI-4 groups as well as LHR protein of FRBI-4 group were significantly less than that of CG and FSH group. * $P < 0.05$ as compared to control group (CG); # $P < 0.05$ and ## $P < 0.01$ as compared to FSH group.

Expression levels of FSHR and LHR proteins of sheep COCs

Expression of FSHR and LHR proteins of sheep COCs was identified by Western blotting (Fig. 2). As shown in Fig. 2, levels of FSHR and LHR proteins of FSH group were slightly increased as compared to CG. But, in FRBI-treated groups, the expression levels of these three receptors were gradually decreased along with the increase of adding FRBI doses. Levels of FSHR protein of FRBI-3 and FRBI-4 groups as well as LHR protein of FRBI-4 group were significantly less than that of CG and FSH group ($P < 0.05$ or $P < 0.01$).

Content of caspase-3 in IVM medium

As shown in Fig. 3, caspase-3 contents of FSH- and FRBI-treated groups were reduced as compared to CG with a maximum reduction of the FRBI-4 group ($P < 0.05$). There was no significant difference between four FRBI groups. These findings revealed that FRBI treatment decreased the caspase-3 production of sheep COCs.

Concentrations of FSH and LH

FSH concentration of FSH-treated group was increased in comparison with CG (Fig. 3). FSH contents of four FRBI treatment groups were dose-dependently decreased along with the increase of adding doses of FRBI into the IVM medium, leading to a significant reduction of the FRBI-4 group in comparison with CG ($P < 0.05$) and FSH groups ($P < 0.01$). LH concentrations had no significant difference between all groups (Fig. 3). The outcomes demonstrated that FRBI treatment could suppress FSH production of sheep COCs.

Effect of FRBI on inositol triphosphate (IP3) production

After IVM of COCs for 20h to 24h, IP3 production was detected (Fig. 4). IP3 content of FSH group was higher than that of CG with a maximum increment at 24h. IP3 contents of FRBI-1 and FRBI-2 groups were greater than that of CG at 22h and 24 h, but there was no

Fig. 3. Concentrations of FSH, LH and caspase-3 in the IVM medium (ng/mL or pg/mL). FRBI treatment decreased the production of FSH and caspase-3 of sheep COCs. FSH concentration of FRBI-4 group was significantly less than that of CG. * $P < 0.05$ as compared to control group (CG); # $P < 0.05$ and ## $P < 0.01$ as compared to FSH group.

Fig. 4. Effect of FRBI on IP3 contents in IVM medium. At 22 and 24h of COCs IVM, IP3 contents of FRBI-1 and FRBI-2 group were higher than that of CG. But, IP3 contents of FRBI-3 and FRBI-4 were less than that of CG and FSH group. * $P < 0.05$ as compared to control group (CG); # $P < 0.05$ and ## $P < 0.01$ as compared to FSH group.

significant difference. IP3 contents of FRBI-3 and FRBI-4 groups were less than that of CG and FSH groups at 22h and 24h ($P < 0.05$). The results demonstrated that supplementing FRBI of 10 $\mu\text{g/mL}$ and 20 $\mu\text{g/mL}$ into IVM medium could slightly promote IP3 production of sheep COCs. However, higher doses of FRBI (from 30 to 40 $\mu\text{g/mL}$) could reduce IP3 production.

Discussion

In vitro maturation (IVM) of COCs is often conducted with media supplemented with gonadotropins. The extrusion of the first polar body is a principal sign of oocyte IVM and nucleus maturation. The previous studies have confirmed that addition of 10 $\mu\text{g/mL}$ FSH and 10 $\mu\text{g/mL}$ LH has been shown to increase the IVM rate of ovine oocytes [5]. Supplementation in IVM media with 1.5 $\mu\text{g/mL}$ rbFSH yielded a high degree of cumulus expansion of sheep oocytes [17]. These results indicate that FSH induced sheep oocyte maturation [17]. However, FSH-receptor binding inhibitor (FRBI), as an FSH antagonist, influenced the efficacy of FSH by blocking the binding of FSH to FSHR in the presence of FSH [8, 10]. *In vivo* administration of FRBI resulted in the suppression of ovulation and induced follicular atresia and apoptosis of mice [7, 21] and further impaired the proliferation of granulosa cells. Therefore, FRBI interfered with FSH-FSHR interaction leading to a blockage of FSH action [3]. Currently, little information has been recorded about FRBI effects on follicular development and reproduction functions in human and animals [2, 13]. In the present study, different doses of FRBI were added into the IVM medium for sheep COCs so as to assess FRBI effects on IVM and apoptosis of sheep COCs. Our results showed that maturation rates of COCs were decreased when adding FRBI concentration increased from 0 to 40 $\mu\text{g/mL}$. Apoptosis rates were increased as FRBI doses rose. Apoptosis level was the highest in the

group FRBI-4 group. These results indicated supplement of FRBI into the IVM medium could dose-dependently reduce the maturation rate and increase apoptosis rate of sheep COCs. Our findings were in agreement with an earlier report [21-23].

Signaling molecules in the regulation of the apoptosis are distributed among cells in the COCs [22]. One key signaling cascade regulated by FSH-FSHR interaction is the production of second messenger molecules (cAMP) and IP3. The cAMP and IP3 further result in the activation of the downstream cascade of protein kinases [24]. A previous study reported that FSH treatment increased cAMP production of rat granulosa cells, but FRBI treatment (500 and 1000 µg) declined cAMP production. In addition, FRBI treatment increased IP3 production as compared to the FSH-untreated granulosa cells in rats [23]. The present study indicated that a low dose of FRBI (10 µg/mL and 20 µg/mL) slightly promoted IP3 contents of sheep COCs. However, higher doses of FRBI (from 30 to 40 µg/mL) declined IP3 production. The findings revealed that a higher dose of FRBI suppressed IP3 production of sheep COCs because FRBI interfered at the receptor level with FSH-FSHR complex, and blocked IP3 and cAMP response [23]. Our results were partially a disagreement with an early report [23]. The difference was probably owing to FRBI doses and different animal species (rats and sheep). However, the actual effects of FRBI on IP3 production of COCs still need to be further investigated.

Treatment of granulosa cells with FRBI inhibited FSH action and progesterone secretion, and then hampered the growth and differentiation of granulosa cells [9, 23]. FSH concentrations in IVM medium were increased after IVM medium was added with different doses of FSH or LH [18].

Caspases play important roles in the process of early apoptosis. Caspase-3 is a critical apoptosis protease in the downstream of the caspase cascade. Its activity reflects early apoptosis. In the present study, FSH concentrations of FRBI-treated groups were gradually declined along with adding doses of FRBI. Caspase-3 contents of FSH- and FRBI-treated groups were reduced as compared to CG with a maximum reduction of the FRBI-2 group. However, TUNEL assay demonstrated that supplement of FRBI into the IVM medium could dose-dependently promote apoptosis rate of sheep COCs. Namely, FRBI could enhance the late apoptosis of sheep COCs. Our findings were consistent with the previous document [23, 25]. These were probably because the animal species and sampling stages were different. The samples were collected in the pre-puberty of sheep. However, the action mechanism has to be thoroughly explored in the future.

FSHR and LHR are important mediators in human and animal reproduction [26]. FSHR is expressed in the pituitary gland and ovaries of sheep [20]. LHR initiates a signal cascade. FSHR induces aromatase release in the ovaries, which influence the developmental ability of oocytes [4]. Addition of FSH into IVM media could enhance the expression of *FSHR* proteins in COCs, resulting in an increase of the maturation rate of COCs [15, 18]. However, supplementation of FSH to IVM media had no obvious effect on expression levels of *LHR* proteins. In the current study, the mRNA and protein levels of FSHR and LHR of COCs were gradually declined along with supplementing doses of FRBI into the IVM medium as compared to CG, with the least value of the FRBI-4 group. FRBI treatment could dose-dependently decrease the expression of FSHR and LHR mRNAs and proteins in sheep COCs. These findings demonstrated that FRBI treatment could decrease the mRNA and protein expression levels of FSHR and LHR in sheep COCs. Our outcomes were similar to early reports [27, 28]. These findings need to be further verified in the future.

In summary, our findings revealed supplement of FRBI into IVM medium could dose-dependently reduce the maturation rate and increase apoptosis rate of sheep COCs. Lower dose of FRBI treatment slightly promoted IP3 production, but a higher dose of FRBI reduced IP3 production. FRBI suppressed the mRNA and protein expression of FSHR and LHR mRNAs in sheep COCs. Therefore, FRBI inhibited the production of FSH and caspase-3. Our study provides a scientific basis for thoroughly elucidating the mechanism of FRBI as an antagonist of FSHR. These will be beneficial to therapy effectively ovarian diseases, improve ovarian and follicular functions, and further to promote fertility of human and animals.

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Disclosure Statement

All authors declare that they have no Disclosure Statement.

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