FSH and eCG impact follicles development and expression of ovarian FSHR and caspase-9 in mice

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Summary

The study aimed to investigate the effects of FSH and eCG on the ovarian and follicular development, expression levels of FSHR and caspase-9 of ovaries *in vivo*. One hundred and five prepuberty mice were allocated into FSH-1, FSH-2, FSH-3, eCG-1, eCG-2, eCG-3 groups and control group (CG). Mice in FSH-1, FSH-2 and FSH-3 were intramuscularly injected with 5, 10 and 20 IU FSH twice (on day 0 and 4), respectively. Mice in eCG-1, eCG-2 and eCG-3 were intraperitoneally injected with 10, 20 and 40 IU eCG on day 0 and 4. Mice in the CG were injected with 0.5 ml normal saline on day 0 and 4. Left and right ovaries of each mouse were dissected aseptically on days 7, 14 and 21, respectively. The results showed that on days 14 and 21 the ovarian sizes and follicle numbers of FSH-3 and eCG-3 groups were greater than CG ($P<0.05$). FSHR mRNA of FSH-2 and eCG-1 were higher than CG on days 14 and 21 ($P<0.05$). FSHR proteins of FSH-3 were higher than CG on days 14 and 21 ($P<0.05$). Caspase-9 mRNA in FSH and eCG groups was less than CG. There were positive correlations between follicle numbers and FSH and eCG doses. FSHR protein expressions had positive correlations between ovarian weights and sizes of ovary and follicle numbers ($r=0.971$, $P<0.05$) in FSH-treated mice. Serum FSH concentrations of FSH-2, FSH-3, eCG-2 and eCG-3 groups were greater than that of CG. In conclusion, eCG and FSH promoted the ovarian development, follicle genesis, FSH secretion, FSHR mRNA and protein expressions in ovaries of mice. FSH and eCG inhibited the expression of ovarian caspase-9 mRNA.

Key words: Caspase-9, Equine chorionic gonadotrophin, Follicle stimulating hormone receptor, Gene expression, Ovarian development

Introduction

The ovarian structures and functions undergo continuous change during the reproductive life span (Zeleznik, 2004). Dramatic changes occur in the follicle following ovulation that involve the transformation of granulosa cells to lutein cells, resulting in the formation of corpus luteum within the ovary. Follicle stimulating hormone (FSH) can promote proliferation and differentiation of preantral follicles (Miro and Hillier, 1996), and thus induce follicular growth and maturation of ovarian follicles, resulting in the generation of mature eggs and the production of estrogens (Hunzicker-Dunn and Maizels, 2006).

FSH control of ovarian and follicular development rests on a network of intrafollicular paracrine interactions (Hillier, 2001) and paracrine mechanisms (Erickson *et al*., 2001). The experiments demonstrated that the ovarian response to FSH stimulation depends on the FSHR genotype (Maritza *et al*., 2000; Ali *et al*., 2012). However, the exogenous FSH could only induce the follicle recruitment in early vitellogenic females (Susan and Roy, 2000). Therefore, the roles of FSH in the development of primordial follicles are controversial (Roy and Albee, 2000). The actual effects and mechanisms of FSH on ovarian and follicular develop-ments during the different stages are still unclear (Wei *et al*., 2013a, b).

Equine chorionic gonadotrophin (eCG, previously known as pregnant mare serum gonadotrophin, PMSG) is widely used for the superovulation of mammalians due to its efficacies of FSH and LH (Zhang *et al*., 2007). Injection of 4 IU eCG could not increase the development of primordial and primary follicles in mice 21 days old (Hu *et al*., 2005), but 4-5 week-old rats are more sensitive to eCG superovulation, giving a maximum number of oocytes at the dose of 20 IU eCG (Zhang *et al*., 2007). However, there were no significant effects in the primary follicle and antral follicle amounts (El-Nefiawy and Nagwa, 2011). Therefore, the exact effects of eCG on ovarian and follicular developments remain unknown (Seekallu *et al*., 2010; Alejandro *et al*., 2012).

The caspases are highly conserved cysteine protease that play an important role in the process of apoptosis. Caspase-9 is one of the initial factors of cell apoptosis which are mainly involved in the mitochondrial pathway. Cytochrome *c* binds Apaf-1 (apoptotic protease-activating factor-1), which then binds to an initiator caspase-9. The formation of this complex is named apoptosome, results in the activation of caspase-9 (Ene *et al*., 2013). The activated caspases-9 further activates the caspases-3 and caspases-6, triggering a cascade reaction

of caspase families (McStay *et al*., 2008). FSH down-regulates caspase-9 and caspase-3 mRNA levels in the granulose cells of dominant follicles. As a result, FSH prevented atresia in dominant follicles (Li *et al*., 2013). However, there has been little information regarding FSH administration affecting caspase-9 activity. The mechanism of how FSH inhibits mitochondrial apoptosis is still unclear (Cao *et al*., 2014). Thus, investigation of the action of FSH on mitochondrial apoptosis could aid in the understanding of cell apoptosis and follicular atresia.

The present study aimed to evaluate the effects of FSH and eCG administration at different doses on the ovary development and follicle apoptosis of mice, and to investigate the efficacy on expressions of FSHR and caspase-9 mRNAs, as well as level of FSHR proteins in the mice ovaries, in order to provide scientific bases for improving reproductive capacity of animals.

Materials and Methods

Animals and experimental designs

The experiments were performed in a total of 105 Kunming mice (*Mus musculus*), 30 days old and body weight of 26.31 ± 2.53 g, purchased from Experiment Animal Center, Lanzhou University [License No. SCXK (Gansu) 2005-0007]. All mice were randomly assigned into three groups: FSH group, eCG and control group (CG). Each experimental group was randomly divided into three groups, marked as FSH-1, FSH-2, FSH-3, eCG-1, eCG-2 and eCG-3 (n=15), respectively. For FSH group, referring to the early report of Zhou *et al*. (2013), mice in FSH-1, FSH-2 and FSH-3 were intramuscularly injected with 5, 10 and 20 IU FSH (Ningbo Sansheng Pharmaceutical Co. Ltd., Ningbo, Zhejiang, China) twice (on day 0 and 4), respectively. Mice in eCG-1, eCG-2 and eCG-3 groups were intramuscularly injected with 10, 20 and 40 IU eCG (Ningbo Sansheng Pharmaceutical Co. Ltd., Ningbo, Zhejiang, China) twice (on day 0 and 4), respectively. Mice in the CG were injected with 0.5 ml normal saline twice (on day 0 and 4). Injections of mice were performed in the morning (at 8 to 9 AM).

All mice were raised in group and kept in mice cages equipped with automatic water dispensers under the same conditions in the room maintained at 22-24°C and 30% to 50% relative humidity. The light cycle in the room provided 12 h light/day. Mice received a commercial diet (Lanzhou Taihua Feed Co. Ltd., China), according to the physiological condition. Water was provided *ad libitum*. The experiment was started after a 10-day adaptation period, when mice were 40 days of age. The body weight of each mouse was weighed on an electronic scale once a day. All procedures referring to animal treatment were approved by the Experiment Animal Care and Use Committee of Gansu province, the People's Republic of China.

Sample collection and measurements

Five mice from each group were anesthetized by

intramuscular injection of 0.1 mg/kg xylazine, they were sacrificed by cervical dislocation on days 7, 14 and 21, respectively. Left and right ovaries of each mouse were dissected aseptically and immediately weighed on an electronic scale. The values of the ovarian weights and ovarian sizes were measured. The ovarian widths and lengths of the left and right ovaries were also measured with a Vernier caliper, respectively. The average value was calculated from the left and right ovaries for each mouse. The antral follicle and mature follicle numbers on the ovaries were examined under the inverted micros-cope (Hu *et al*., 2005).

Blood samples were taken aseptically using vacutainers (Zhejiang Gongdong Medical Technology Co. Ltd., Zhejiang, China) from cervical veins on days 7, 14 and 21 after the first FSH injection, respectively. The samples were allowed to coagulate during 2 h at room temperature, then centrifuged (3000 × g, 20 min), the serum was stored at -20°C until analysis.

Real time fluorescence quantitative PCR (qPCR)
Primer design

The primers of FSHR (GenBank accession number: NM_013523.3), caspase-9 (GenBank accession number BC056447.1) and glyceraldeyhyde-3-phosphate dehydrogenase (GAPDH, GenBank accession number: NM_008084.2) were designed using Primer Premier 5.0 software. GAPDH was used as an internal control. TaqMan probes were labeled at the 5′ end with the fluorescent reporter FAM and at the 3′ end with the fluorescent quencher BHQ1. 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 dimer-free reactions were used for the final analysis. Primers and probes were synthesized by Takara Bio, Dalian, China (Table 1).

RNA extraction and cDNA synthesis

About 10 mg ovary in each mouse was used for total RNA extraction using the TRIzol reagent (Invitrogen Distributer, Beijing, China), according to the manu-facturer's instructions, as described previously (Wei *et al*., 2013b). Briefly, total RNA was treated with the gDNA wipeout buffer, supplied with the QuantiTect reverse transcription kit (Qiangen, Beijing, China) to remove traces of genomic DNA contamination. Assessment of RNA quality was performed using a 1.2% agarose gel containing ethidium bromide (EB) and photographed with the Bio-BEST 140E imaging system (SIM Company, USA). RNA samples were quantified using a Nanodrop spectrophotometer (Zhiyan Company, Shanghai, China). The absorbance ratios of 260/280 nm in all samples were more than 1.9, indicating the high RNA purity. cDNA was synthesized with the superscript™ III first-strand synthesis system for RT-PCR (Invitrogen, Beijing, China), according to the manufacturer's instructions. The resulting single stranded cDNA products were quantified using a

Table 1: Primers of FHSR and GAPDH mRNAs for qPCR

Gene	Primer	Sequence (5´-3´)	Tm	bp
FSH-R	Forward	AGGTACAGCTCTGCCATGCT	60.04	171
	Reverse	GTACGAGGAGGGCCATAACA	59.96	
Caspase-9	Forward	GCCCTTGCCTCTGAGTAGTG	60.01	168
	Reverse	CCAACCAAATGAAGCCAAGT	59.97	
GAPDH	Forward	ACCCAGAAGACTGTGGATGG	59.96	172
	Reverse	CACATTGGGGGTAGGAACAC	60.09	

FSHR gene (GenBank accession number NM_013523.3), caspase-9 mRNA (GenBank accession number BC056447.1), and GAPDH (GenBank accession number NM_008084.2)

Nanodrop spectrophotometer (Zhiyan Company, Shanghai, China), and then diluted 50-fold with deionized water prior to their use as templates for the qPCR reactions.

Fluorescence quantitative RT-PCR (qPCR)

Expression levels of FSHR mRNA were determined using real time fluorescence quantitative polymerase chain reaction (qPCR) with a SLAN thermocycler (Hongshi, Shanghai, China) (Wei *et al*., 2013b). Each 25 µL reaction in a 96-well plate comprised 4 µL of 50 × diluted cDNA template, 1 µL of each primer pair at 10 µM, 0.25 µL probe at 10 µM and 12.5 µL of 10 × TaqMan Universal PCR Master Mix that contains DNA polymerase, buffer, dNTP and SYBR® Green II (Promega). PCR amplification included an initial denaturation at initial denaturation at 95°C for 1.5 min, 35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 20 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 5 min. FSHR mRNA expression levels were recorded as the threshold cycle (C_T) values that corresponded to the number of cycles at which the fluorescence signal could be detected above the threshold value. GAPDH was used as an endogenous control. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative values of mRNA abundances, compared to the CG. The samples were run triplicate.

Western blotting analysis of FSHR protein in ovaries

To evaluate the FSHR expression of ovaries in protein level after FSH and eCG administration, the Western blotting was performed (Wei *et al*., 2013b). Briefly, the ovary samples were lysed in lysis buffer (0.5% Nonidet P [NP] 40, 10 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 1 mmol/L ethylene diamine tetra-acetic acid [EDTA], 1 mmol/L sodium orthovanadate (Na_3VO_4) containing protease inhibitor (1 mmol/L phenylmethylsulfonyl fluoride [PMSF]). Proteins were loaded on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to polyvinylidene fluoride (PVDF) membranes and blocked in 5% non-fat milk in 10 mmol/L Tris, pH 7.5, 100 mmol/L NaCl, 0.1% (w/v) Tween 20 for 2 h. Rabbit anti-sheep GnRHR, FSHR and LHR polyclonal antibodies (Sigma, 1:200) and b-actin polyclonal antibody (1:1000) were diluted and incubated at 4°C overnight, followed by 1 h incubation with the appropriate secondary antibody

(1:2000). Anti-b-actin mouse monoclonal antibody was diluted in 1:10000 for sample loading control. The blots were further developed using a chemiluminiscence reagent (SuperSignal West Pico, Rockford, IL, USA). The integral optical density (IOD) of the scanned band images was done by using Quantity One Software (Bio-Rad Company, Hercules, CA, USA). The relative contents of GnRHR, FSHR and LHR proteins were presented as the ratio between gray values of GnRHR, FSHR and LHR divided by that of β-actin. A negative control was performed without primary antibody. The experiments were run in triplicate.

Detection of serum FSH levels

Serum FSH concentrations were determined using commercially available ELISA FSH detection kit for mice, respectively, according to the manufacturer's instructions (CAT. No: F10843, Shanghai Yanjing Biotechnology Co. Ltd., Shanghai, China). The analytical specificity was 100% for FSH and LH. The analytical sensitivities of FSH and LH were 0.03 IU/L and 0.02 ng/ml. The inter-assay CV was less than 4%. The correlation coefficient of the standard curve was 0.9995.

Statistical analysis

Statistical analysis was done using SPSS v. 18.0 (SPSS Inc. Chicago, IL, USA). For each group, the averages of all parameters described above were calculated based on the data of 5 mice in each group, respectively. Data are presented as means±SEM. After a square root transformation of the data, all variables complied with the assumptions for one-way ANOVA. Pearson's model was utilized to analyze the correlations between ovarian parameters, serum hormone levels and FSHR expression respectively. When significant differences were identified, supplementary Tukey's post-hoc tests were performed to investigate pair wise differences. The p-value equal to or lower than 0.05 was considered to be significant difference (P<0.05 and P<0.01).

Results

Ovarian weights and sizes of mice

During the entire experiment, ovarian weights of six experimental groups were higher than that of the CG (Table 2). Ovarian weights of eCG group were higher

than that of FSH group, with a maximum increment of eCG-3 group. On days 7, 14 and 21, ovarian weight of eCG-3 group was greater than that of CG (P<0.05). Meanwhile, on day 7 ovarian weight of eCG-2 was also significantly increased in comparison to CG (P<0.05). This demonstrated that eCG or FSH promoted the growth of mice ovarian structures. ECG had higher effects.

Table 2: Ovarian weights of mice

Group	7d	14d	21d
CG	0.015±0.003	0.017±0.004	0.021±0.004
FSH-1	0.029±0.004*	0.023±0.002	0.025±0.002
FSH-2	0.026±0.003	0.019±0.003	0.025±0.002
FSH-3	0.017±0.001	0.019±0.002	0.028±0.003
eCG-1	0.029±0.005*	0.027±0.003	0.026±0.003
eCG-2	0.023±0.004*	0.032±0.003*	0.036±0.003*
eCG-3	0.038±0.006**	0.034±0.002*	0.044±0.003**

* P<0.05 and ** P<0.01 when compared to CG

Based on measurements of the ovarian diameters using the Vernier caliper, the ovarian sizes of all experimental groups were larger than that of CG during the experiment with a maximum ovarian size of FSH-3 group (Table 3). However, there was no significant difference when compared to CG. The findings indicated that FSH and eCG treatments slightly enhanced the ovarian development of mice.

Follicle numbers on the ovaries of mice

The antral follicle and mature follicle numbers on the ovaries were examined under the inverted microscope as described above. As listed in Table 3, the follicle numbers of FSH and eCG groups were larger than that of CG on day 14 and 21. The follicle number of FSH-3 group was more than that of the remaining groups and CG on day 14 and 21 (P<0.05 or P<0.01). Additionally, the follicle numbers of eCG-3 group significantly increased when compared to CG (P<0.05). This also demonstrated eCG and FSH can promote the follicle genesis and development.

FSHR mRNA and protein expression levels in ovaries of mice

As shown in Fig. 1, FSHR gene mRNA expression levels of FSH-2 and eCG-1 groups were significantly greater than that of CG on days 14 and 21. FSHR mRNA levels in the remaining groups slightly exceeded CG.

This illustrated the FSH and eCG treatments may improve the FSHR mRNA expression in the ovaries.

The Western blotting analysis indicated that the expression levels of FSHR proteins in ovaries of FSH and eCG were slightly increased compared to CG (Fig. 2). FSHR protein levels of FSH-3 group were significantly higher than CG on days 14 and 21 (P<0.05).

Expression levels of caspase-9 mRNA in the ovaries of mice

It can be seen in Fig. 3 that the expression levels of
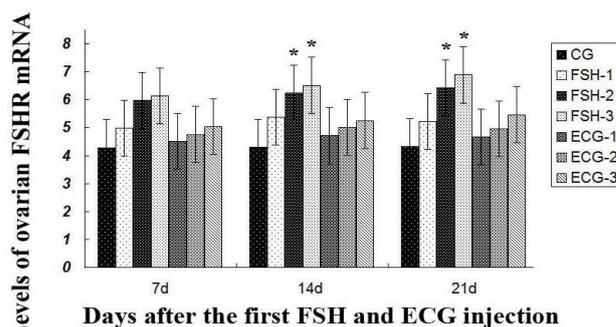

Fig. 1: The expression levels of FSHR mRNA in the ovaries. FSHR gene mRNA expression levels of FSH-2 were significantly greater than that of CG on days 14 and 21. * The difference was significant (P<0.05) when compared to CG, and ** The difference was highly significant (P<0.01) when compared to CG
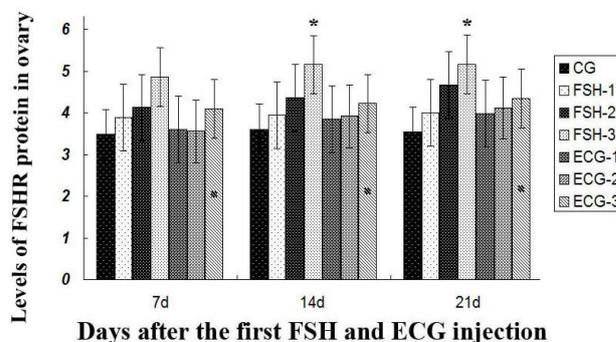

Fig. 2: The expressions of FSHR protein in the ovaries. FSHR protein levels of FSH-3 group were significantly higher than CG on days 14 and 21 (P<0.05). * The difference was significant (P<0.05) when compared to CG, and ** The difference was highly significant (P<0.01) when compared to CG

Table 3: Ovarian sizes and follicle numbers of mice

Group	Ovarian sizes (μm)			Follicle numbers	
	7d	14d	21d	14d	21d
CG	0.267 ± 0.018	0.290 ± 0.020	0.290 ± 0.018	3.3 ± 0.67	15.6 ± 1.28
FSH-1	0.291 ± 0.074	0.316 ± 0.080	0.316 ± 0.080	4.55 ± 1.33	17.9 ± 2.44
FSH-2	0.307 ± 0.066	0.334 ± 0.072	0.343 ± 0.072	5.33 ± 1.56**	22.66 ± 4.67*
FSH-3	0.324 ± 0.071	0.352 ± 0.081	0.352 ± 0.081	9.22 ± 2.78**	29.27 ± 4.44**
eCG-1	0.313 ± 0.087	0.340 ± 0.062	0.348 ± 0.052	4.52 ± 1.67	16.12 ± 3.25
eCG-2	0.321 ± 0.062	0.341 ± 0.021*	0.361 ± 0.052*	4.98 ± 1.8	20.13 ± 3.86
eCG-3	0.308 ± 0.069	0.335 ± 0.021	0.369 ± 0.042*	7.12 ± 2.1*	25.21 ± 4.33*

* P<0.05 and ** P<0.01 when compared to CG

ovarian caspase-9 mRNA of FSH and eCG groups were less than that of CG during the whole experiment, with a minimum of FSH-3 group. It demonstrated that administration of FSH and eCG could inhibit caspase-9 mRNA expression, which probably decreased the atresia and apoptosis of follicles.

Serum FSH concentrations of mice

As show in Fig. 4, serum FSH concentrations in six experimental groups rose along with the increase of FSH and eCG doses. On day 7, FSH concentrations of FSH-2 and FSH-3 groups significantly increased in comparison with CG ($P<0.05$). On day 21, FSH concentrations of eCG-2 and eCG-3 groups were greater than that of CG ($P<0.01$).

The findings demonstrated that eCG and FSH treatments could promote the synthesis and secretion of FSH of mice, and also enhance its serum concentrations.

Correlations between ovarian parameters and protein expression

As shown in Table 4, Pearson's correlation analyses indicated that there was a significant positive correlation between follicle numbers and the administrated dose of FSH and eCG ($r=0.995$, $r=0.981$, $P<0.01$). In FSH group, FSH doses had significant positive correlations with ovarian size and FSHR protein expression levels ($r=0.966$, $r=0.977$, respectively; $P<0.05$) on day 21. FSHR protein expressions had positive correlations between the FSH dose, ovarian weights, follicle numbers, ovarian sizes and FSHR mRNA ($P<0.05$ or $P<0.01$). FSH administration could enhance FSHR mRNA and protein expressions in the ovaries of mice.

In eCG group, eCG administration doses had significant positive correlations with the ovarian weight, follicle numbers, FSHR mRNA and protein expression levels on day 21 ($P<0.05$ or $P<0.01$). FSHR protein expression also had a positive correlation with FSHR mRNA expression ($P<0.05$ or $P<0.01$). This demonstrated that the FSHR protein expression was in relation to FSHR mRNA expression levels. FSH and eCG administration could enhance FSHR mRNA and protein expressions in the ovaries of mice.
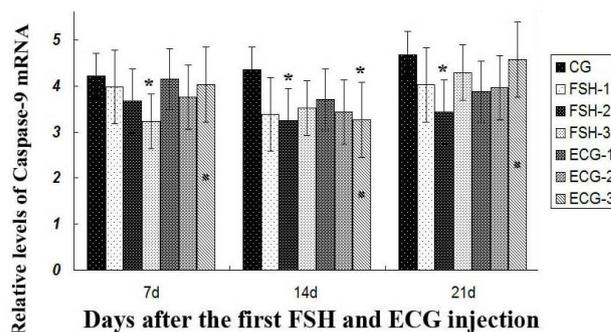

Fig. 3: Relative expression levels of caspase-9 in the ovaries. The expression levels of ovarian caspase-9 of FSH and eCG groups were less than that of CG with a minimum of FSH-3 group during the entire experiment ($P<0.05$). * The difference was significant ($P<0.05$) when compared to CG
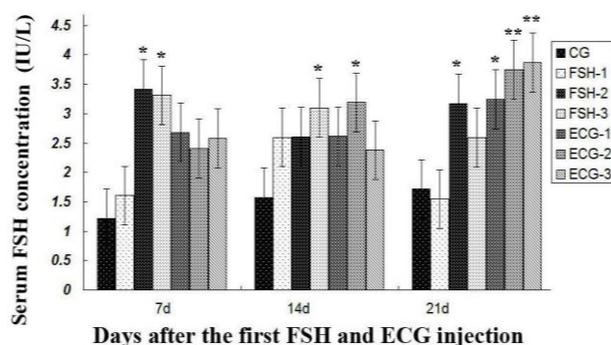

Fig. 4: Serum FSH concentrations. On day 7, FSH concentrations of FSH-2 and FSH-3 groups were higher than that of CG ($P<0.05$). On day 21, eCG-2 and eCG-3 groups were greater than CG ($P<0.01$). * The difference was significant ($P<0.05$) when compared to CG, and ** The difference was highly significant ($P<0.01$) when compared to CG

Discussion

FSH is known to regulate the transzonal connection between the oocytes and the surrounding granulosa cells, as well as modulate the development of the primary follicles to preantral and antral stages (Thomas and Vanderhyden, 2003; Menon et al., 2007). eCG could

Table 4: Pearson's correlation analyses of ovarian parameters of mice on day 21

Group	Parameter	Dose	Weight	Number	Size	mRNA	Protein
FSH group	Weight	0.922					
	Number	0.995**	0.882				
	Size	0.966*	0.972*	0.947			
	mRNA	0.943	0.929	0.933	0.987*		
	Protein	0.977*	0.930	0.971*	0.990*	0.992**	
	Caspase-9	-0.258	-0.482	-0.220	-0.490	-0.557	-0.448
eCG group	Weight	0.952*					
	Number	0.981*	0.922				
	Size	0.491	0.257	0.405			
	mRNA	0.997**	0.937	0.968*	0.551		
	Protein	0.937	0.848	0.864	0.730	0.960*	
	Caspase-9	0.082	0.232	0.226	-0.767	0.008	-0.269

* Indicates there was a significant correlation ($P<0.05$), and ** Indicates there was a high significant correlation ($P<0.01$)

restore the retarded follicular and oocyte nucleolar growth rates of mice ovaries. However, the effects of eCG vary with the doses, ages and seasons. There were no significant effects in the primary follicle and antral follicle amounts (El-Nefiawy and Nagwa, 2011).

In this study, 105 Kunming mice are studied. The follicle numbers (including the antral follicle and mature follicles) of FSH and eCG groups were more than that of CG. Follicle numbers of FSH treatment group were more than that of eCG group. The ovarian weights and ovarian sizes of FSH-treated and eCG-treated mice were greater than CG. However, ovarian weights of eCG group were higher than that of FSH group with a maximum of eCG-3 group. This demonstrated FSH and eCG treatments could improve the ovarian development of mice. FSH had a greater efficacy in promoting the follicle development in comparison with eCG since FSH can promote proliferation and differentiation of preantral follicles, and thus induce follicular growth and maturation of ovarian follicles (Hunzicker-Dunn and Maizels, 2006).

ECG possesses a dual activity of FSH and LH. It can obviously improve the follicular development and ovulation (David *et al*., 2009). Currently, there is little information on whether eCG has more remarkable effects on ovarian growth and follicular development than FSH in the same animal. In this study, the relatively high doses of eCG were administered in referring to the early reports (Zhou, 1995; Zhang *et al*., 2007). Our results were consistent with previous reports (Hu *et al*., 2005). The exact mechanisms of eCG on ovarian and follicular developments remain unknown (Seekallu *et al*., 2010; Alejandro *et al*., 2012).

FSH inhibits the granulosa cell apoptosis and can rescue granulosa cells from atresia of mouse (Layman and McDonough, 2000; Zhang *et al*., 2011) and apoptosis (Asahara *et al*., 2003). This was confirmed in our experiment. The results demonstrated that the administration of FSH and ECG inhibited caspase-9 mRNA expression, which possibly decreased the atresia and apoptosis of follicles.

In the present experiments, FSHR mRNA and protein expression levels of FSH and eCG groups were greater than that of CG on days 14 and 21. This illustrated the FSH and eCG treatments may improve the FSHR mRNA expression in the ovaries. The findings were similar to our previous reports in ewes, which indicated that Alarelin active immunization enhanced expressions of FSHR and LHR proteins in ovaries (Wei *et al*., 2013b). The ovarian response to FSH stimulation depends on the FSHR genotype (Maritza *et al*., 2000; Ali *et al*., 2012). The FSHR genotypes were not determined in this study. The effect needs to be further explored with researches in the future.

FSH and LH are synthesized and stored in gonadotrophin cells under the regulation of multiple mechanisms (Crawford *et al*., 2009). It is reported that FSH and LH levels reached peak levels after 96 h of injection of urinary gonadotropin (human menopausal gonadotropin, HMG), and showed obvious changes in the reproductive hormones in rabbits. In this study, serum FSH concentrations in eCG and FSH mice increased following the cloprostenol and eCG injections. However, FSH concentrations in eCG mice were higher than that in CLO and CG mice on day 21, with a maximum increment in eCG-3 group. The findings are similar to an early report (Wei *et al*., 2013b).

There is little information about the correlation between FSH and eCG treatments and ovarian development indexes as well as FSHR expression levels (Yuan *et al*., 2008; El-Nefiawy and Nagwa, 2011). Pearson's correlation analyses in the present study demonstrated that there was a significant positive correlation between follicle numbers and the administrated dose of FSH and eCG. FSHR protein expressions had positive correlations with FSH dose, ovarian weights, follicle numbers, ovarian sizes and FSHR mRNA. ECG administration had significant positive correlations with ovarian weight, follicle numbers, FSHR mRNA and protein expression levels on day 21. FSH and eCG administrations could enhance FSHR mRNA and protein expressions in the mouse ovaries. So far, similar reports have not been published (Roberta *et al*., 2012; Ene *et al*., 2013). Thus, the actual statistical correlations between these parameters need further research. Our results laid a novel thought and method for studying quantitatively the effects of eCG and FSH on the reproductive functions in animals.

FSH and eCG treatments could promote the ovarian development, follicle genesis, FSHR mRNA and protein expression levels in the ovaries of mice. FSH and eCG could also enhance FSH serum concentrations, and inhibit the ovarian caspase-9 mRNA expression. Administration of doses of FSH and eCG had significant positive correlations to weights and sizes of ovaries, follicle numbers as well as expression of FSHR mRNA and protein. These are very important to improving the reproductive capacity of animals.

Acknowledgements

Conflict of interest

All authors have no financial or personal relationship with organizations or people that could influence or bias the study.

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