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PHYSIOLOGY

Effects of luteinizing hormone and follicle stimulating hormone on the developmental competence of porcine preantral follicle oocytes grown in vitro

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Abstract

Background There has been controversy over the role of FSH in the regulation of preantral follicle development. LH is a survival and differentiation factor that increases oocyte maturation in FSH-supplemented cultures of mouse preantral follicles. However, little information exists on the action of LH and FSH in the developmental competence of porcine preantral follicle oocytes in vitro.

Materials and methods Porcine preantral follicles were cultured for 3 days in the presence or absence of FSH or LH. Oocytes from these follicles were then matured, fertilized in vitro, and embryos were cultured. Estradiol secretion and histological analysis of cultured follicles were also carried out.

Results FSH or combined LH and FSH significantly enhanced follicular growth compared to LH alone or the controls. Combined LH and FSH treatment of preantral follicles significantly increased the percentage (59±5%) of oocytes competent to undergo cleavage to the two-cell stage after fertilization. A significant effect was seen on oocyte competence to develop from the two-cell to the blastocyst stage (30±6%) compared to FSH alone treatment (45±7 and 14±5%, respectively). The amount of estradiol on days 2 and 3 of culture was significantly higher in follicles cultured with FSH (48.75±17, 70.5±14 pg/ml) or combined LH and FSH (63.25±16, 72.5±12 pg/ml) than that cultured with the untreated controls (16±10, 5.66±4 pg/ml).

Conclusions The results indicated that FSH is essential for the in vitro growth of porcine preantral follicles, estradiol secretion, and for oocytes to acquire competence to resume meiosis and undergo fertilization and embryonic development. LH with FSH treatment of porcine preantral follicles can improve the quality of oocytes by promoting growth and a higher frequency of embryonic development.

Keywords Developmental competence · FSH · Growth · LH · Porcine preantral follicles

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Introduction

Development of the ovarian follicle is a dynamic process involving important morphological and functional changes in thecal and granulosa cells (proliferation, steroidogenesis and gonadotropin sensitivity), and in oocytes (cytoplasmic and nuclear maturation). The gonadotropins follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are heterodimeric glycoproteins produced within the adenohypophysis that, in the female, act primarily at the level of the ovarian follicle. The actions of gonadotropins are targeted to ovarian somatic cells through specific cell-surface receptors. These receptors, namely the LH receptor (LHR) and the FSH receptor (FSHR), are members of the G protein-coupled receptor superfamily.

In sheep, FSHR mRNA can be observed in granulosa cells of early preantral follicles with one or two cell layers,

and its gene expression continues throughout folliculogenesis [1]. FSH is an essential survival hormone for the prevention of the programmed demise of early antral follicles in rodents [2–4]. Moreover, FSH plays an important role in the final differentiation of granulosa cells in antral and preovulatory follicles, to allow the biosynthesis of estrogens and to prepare the preovulatory follicles for ovulation [5]. However, there has been controversy over the role of FSH in the regulation of preantral follicle development.

In vivo, preantral follicles are considered to be gonadotropin-independent because animal or human preantral follicles can develop to the antral stage in conditions with minimal circulating gonadotropins [6–8], but several studies have suggested that development of early follicles is under the influence of gonadotropin. In vitro, although several studies have demonstrated an important role for FSH in preantral follicle growth [9–12], other studies have indicated that treatment with FSH does not enhance preantral follicle growth [13, 14].

LH receptors are expressed on theca cells from preantral and antral follicles, and on granulosa cells from large antral follicles [15–18]. LH may play multiple roles throughout follicular development, but most studies have focused on the action in late-stage follicles and during the periovulatory period. In contrast, the action of LH in preantral follicle development has received less attention. Cortvrindt et al. [19, 20] have suggested that LH is a survival and differentiation factor that increases oocyte maturation in FSH-supplemented cultures of mouse preantral follicles, and LH has a stage-limited effect on mouse preantral follicle development in vitro. However, little information exists on the action of LH and FSH in the developmental competence of porcine preantral follicle oocytes in vitro.

The objective of this study was to evaluate the effects of FSH and/or LH on in vitro growth of porcine preantral follicles, estradiol secretion, antrum formation, oocyte maturation, and subsequent embryonic development.

Materials and methods

Animal and tissue collection

Ovaries were collected from prepubertal gilts at a local abattoir and transported to our laboratory in Dulbecco's phosphate-buffered saline (DPBS; Gibco 11500-030, Grand Island, NY, USA) supplemented with 3 mg/ml bovine serum albumin (BSA; A-8022, fraction V, Sigma, St. Louis, MO, USA) maintained at 30–37°C. Serum was collected from prepubertal gilts and stored in aliquots at −20°C until they were used.

Preantral follicles and in vitro culture

The preantral follicles were collected and cultured as previously described [21, 22]. The ovaries were cut into small pieces (1–3 mm) and preantral follicles were isolated mechanically using watchmaker's forceps in DPBS with 3 mg/ml BSA.

Preantral follicles 296±8 μm in diameter were collected into four-well multidishes (Nunclon; Nunc, IL, USA) containing the collecting medium NCSU23 supplemented with 3 mg/ml BSA. The follicles were transferred from the collecting medium into the culture medium that consisted of NCSU23 supplemented with 3.5 μg/ml insulin (I5523, Sigma), 10 μg/ml transferrin (T5391, Sigma), 100 μg/ml L-ascorbic acid (A4544, Sigma), and 7.5% porcine serum. Depending on the experiment, culture medium was supplemented with 1.5 ng/ml ovine FSH (OFSH-20, 4,453 IU/mg; National Hormone and Pituitary Program of National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), Torrance, CA, USA), and/or ovine LH (OLH-26; National Hormone and Pituitary Program of NIDDK) at various concentrations. The follicles were randomly distributed to different experimental groups and cultured for 3 days in 24-well cell culture cluster plates (3524; Costar, Corning, NY, USA), with three follicles per well in 280 μl culture medium. The culture was carried out at 38.5°C in 5% CO_2 in air. Culture medium was changed every day with freshly prepared medium. The diameters of follicles were measured using a stereomicroscope with an ocular scale at a magnification of ×50.

Histological investigation of follicles

Histological investigation of follicles was completed using a protocol described by Wu et al. [20]. In brief, cultured follicles were fixed in 3% paraformaldehyde and 2.5% glutaraldehyde in phosphate buffer. After washing, the fixed follicles were dehydrated in increasing concentration of ethanol. The follicles were pre-infiltrated in 1:1 mixture of 100% ethanol and Technovit 7100 together with Hardener I (Heraeus Kulzer, Germany). Infiltration was then carried out by placing the follicles into Technovit 7100 with Hardener I. For embedding, the follicles were transferred to Beem capsules in Technovit 7100 with Hardener I and II. Serial 1-μm sections were cut through the follicles, and the sections were placed on glass slides and stained with methylene blue solution.

Measurement of estradiol

Estradiol in culture media was measured using an ELISA method (Serono Diagnostics, Woking, Surrey). The inter-

and intra-assay coefficients of variation were ≤5% and the sensitivity was <20 pmol/ml.

In vitro maturation of oocyte–cumulus complexes (OCCs)

Maturation of OCCs was evaluated as described by Wu et al. [21] After the culture was completed, the follicles were opened using two needles and the OCCs were flushed into DPBS supplemented with 3 mg/ml BSA. After washing three times in NCSU23 medium supplemented with 0.23 mM pyruvate and 10% porcine serum, the OCCs were cultured for 48 h in the same medium, which was further supplemented with 0.12 μg/ml OFSH, 2.5 μg/ml OLH, 20 ng/ml epidermal growth factor (EGF), 50 μg/ml L-ascorbic acid and 10–20 antral follicular shell pieces.

In vitro fertilization (IVF) and embryo culture

After maturation, oocytes were washed three times with IVF medium consisting of modified Tris-buffered medium, 2 mM caffeine and 2 mg/ml BSA (A7888, Sigma). The oocytes were then transferred to 50 μl IVF medium that was covered with warm mineral oil in a 35×10-mm^2 tissue culture dish (Corning, Corning, NY, USA). The dishes were kept in a CO_2 incubator for about 30 min until spermatozoa were added for fertilization.

Porcine sperm (SGI, Cambridge, MA, USA) was prepared as described previously [21, 23]. The prepared sperm suspension (50 μl) was added to 50 μl oocyte-containing medium (final concentration of 5×10^5 cells/ml). The oocytes were incubated with spermatozoa for 5–6 h at 38.5°C in an atmosphere of 5% CO_2 in air. After insemination, the oocytes were washed three times in embryo culture medium (NCSU23 containing 3 mg/ml BSA and 0.5% (*v*/*v*) minimum essential medium amino acids), and cultured in 500 μl embryo culture medium in a 60×15 mm center-well organ culture dish (Becton Dickinson, Falcon, NJ, USA) until examination. At 48 and 168 h after IVF, cleavage rate and blastocyst formation were evaluated under a stereomicroscope. Fertilization rate was calculated by adding the cleaved embryos and one-cell oocytes that had been penetrated by spermatozoa. One-cell oocytes and blastocytes were fixed in 25% (*v*/*v*) acetic acid in ethanol, stained with 1% (*w*/*v*) orcein in 45% (*v*/*v*) acetic acid, and examined under a phase-contrast microscope.

Results

Role of FSH and/or LH in porcine follicular growth

Without FSH or LH (0 control), follicles grew slowly to a final size of 357±5 μm, which was significantly smaller than that of follicles cultured with FSH (471±7 μm) (Table 1). Antrum formation was not observed in these control cultures. With LH alone, follicles reached a final size of 366±7–378±8 μm but did not develop to the antral stage. The three different amounts of LH (15, 30 or 60 ng/ml) did not produce significantly different final sizes of follicles. FSH significantly enhanced follicular growth compared to LH alone or the controls. Combined LH and FSH produced rapid growth of follicles with a mean final diameter of 479±10–481±9 μm. However, the final follicle sizes were not significantly larger than those achieved with FSH alone.

Table 1 Effect of FSH, LH or LH with FSH on development of porcine preantral follicles in vitro

Variable concentration (ng/ml)	Follicle diameter (mean+SEM, μm)		Antrum formation (%)
	Start size (day 0)[a]	End size (day 3)	
0 (Control)	296±8	357±5*	0
FSH 1.5	297±9	471±7	89±2
LH 15	297±6	366±7	0
30	297±8	377±6	0
60	296±8	378±8	0
FSH 1.5+LH			
15	296±7	479±10	86±4
30	298±6	485±11	90±2
60	297±7	481±9	87±3

[a] The diameters between conditions were not different on day 0.
*$p<0.05$ as compared with other conditions.

Effect of FSH or combination of LH and FSH on developmental competence of oocytes from preantral follicles

After culture of preantral follicles with FSH or LH (30 ng/ml) and FSH, the capacity of the oocytes to mature, fertilize and develop into embryos was evaluated. Oocytes from preantral follicles cultured with FSH or LH and FSH reached the metaphase II stage in nearly the same proportion (52±8 and 53±6%, respectively) (Table 2). For fertilization, no significant difference was observed between FSH alone and LH and FSH treatment. Surprisingly, combined LH and FSH treatment of preantral follicles significantly increased the percentage (59±5%) of oocytes competent to undergo cleavage to the two-cell stage after fertilization, the most profound, a significant effect was seen on oocyte competence to develop from the two-cell to the blastocyst stage (30±6%), compared to FSH alone treatment (45±7 and 14±5%, respectively).

Table 2 Maturation, fertilization and embryonic development of oocytes from porcine preantral follicles under the different conditions

Conditions (ng/ml)	Maturation (%)	Fertilization (%)	2 cells (%)	Blastocyst (%)
FSH 1.5	52±8*	54±6*	45±7**	14±5**
FSH 1.5+LH				
15	51±7	55±7	51±6	23±6
30	53±6	57±5	59±5	30±6
60	52±6	55±6	56±5	27±5

*$p>0.05$ as compared with other conditions.
**$p<0.05$ as compared with other conditions.

Role of FSH or combination of LH and FSH in induction of estradiol secretion by follicles

Estradiol secretion by follicles was assessed in cultures with FSH, combined LH (30 ng/ml) and FSH, or in untreated controls. Without FSH or LH (30 ng/ml) supplementation, follicles secreted progressively lower amounts of estradiol. In contrast, follicles cultured with FSH or LH and FSH supplementation, released progressively larger amounts of estradiol. Moreover, the amount of estradiol on days 2 and 3 of culture was significantly higher in follicles cultured with FSH (48.75±17, 70.5±14 pg/ml) or LH and FSH (63.25±16, 72.5±12 pg/ml) than that in with the untreated controls (16±10, 5.66±4 pg/ml). However, there was no significant difference in estradiol secretion between follicle culture with FSH and combined LH and FSH (Fig. 1).

Histological observations of cultured follicles

Histological examination of sections of follicles cultured for 3 days with three different conditions (control, FSH, and combined LH and FSH) showed that oocytes were at the germinal vesicle (GV) stage and the cytoplasm had a homogeneous structure. In the controls, a few granulosa cells had proliferated and no antrum formation was observed (Fig. 2a). With FSH, oocytes were healthy and surrounded by one or two layers of granulosa cells (cumulus cells). Granulosa cells had proliferated, and normal organization and antrum formation was seen (Fig. 2b). With combination of LH and FSH, oocytes were surrounded by one layer of granulosa cells (cumulus cells). Granulosa cells had proliferated and showed normal organization and antrum formation (Fig. 2c).

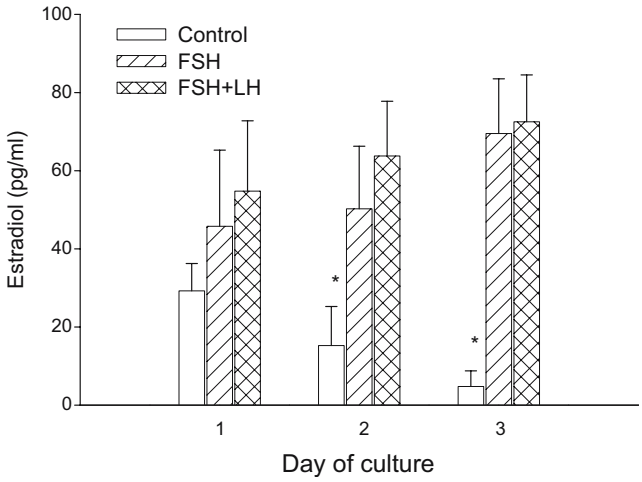

Fig. 1 Comparison of the effects of medium containing FSH, LH and FSH, or no gonadotropins (control) on production of estradiol by follicles in vitro. *Indicates $P<0.05$ as compared with other conditions

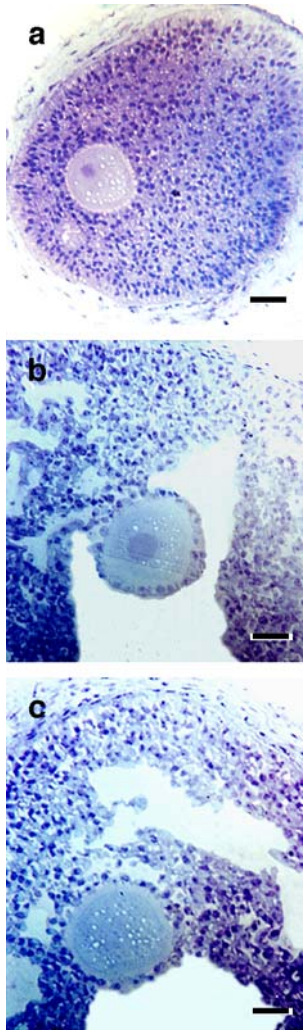

Fig. 2 Examples of porcine follicles fixed after 3 days of culture under different conditions. **a** Controls (no gonadotropins); **b** FSH; **c** Combined FSH and LH (30 ng/ml). *Bar* 50 mm

Discussion

Processes occurring during follicular oocyte development establish the foundation for embryogenesis. Transcripts essential for early embryo development are produced, and stored in dormant from throughout oocyte growth, and are activated and translated during meiotic maturation and late

embryo development. Oocyte growth and development occur in an ovarian follicular environment involving highly coordinated proliferation and differentiation of theca and granulosa cells. This coordination can be offset by exogenous gonadotropins or other factors.

This study tested the hypothesis that FSH or combined LH and FSH treatment of porcine preantral follicles improves the quality of oocytes by promoting growth and higher frequency of embryonic developmental competence. The results supported this hypothesis. When FSH was added to the culture medium, preantral follicles grew rapidly to the antral stage, half of their oocytes matured, and subsequently developed to the blastocyst stage after IVF. In contrast, without FSH, preantral follicles failed to grow to the antral stage and none became mature oocytes. These results support previous studies [9, 10, 21, 24–27] suggesting that FSH plays an important role in the development of preantral follicles in vitro. FSH promotes preantral follicle growth by inducing granulosa cell proliferation and differentiation, because granulosa cell growth accounts for the majority of this follicle expansion [28]. Moreover, FSH may indirectly drive the theca to make the androgen substrate that is converted to estrogen by the granulosa cell aromatase. FSH and secreted estrogen coordinate to induce antral formation in cultured preantral follicles. In contrast, it is considered that FSH may not be a required element for in vitro follicular development [13, 14]. Recently, Eppig et al. [29] have reported that FSH treatment of cultured oocyte–granulosa cell complexes does not significantly affect oocyte growth, oocyte competence to resume meiosis or undergo fertilization, and preimplantation development. Furthermore, treatment of the complexes with both FSH and insulin produces a highly deleterious effect on competence to undergo development from the two-cell to blastocyst stage.

As compared with FSH treatment alone, combined LH and FSH treatment of preantral follicles significantly increased the percentage of their oocytes competent to undergo cleavage to the two-cell stage after IVF. Moreover, there was a significant effect on oocyte competence to develop from the two-cell to the blastocyst stage. LH acts synergistically with FSH in promoting follicular development and function. An example of this synergic action is estrogen production via the two-cell, two-gonadotropin model, where LH drives the theca to make the androgen substrate that is converted to estrogen by granulosa cell aromatase, under the influence of FSH. Steroids might be important regulators of the essential oocyte cytoplasmic changes required for normal fertilization [30]. This synergic action is also supported by previous work [31] showing that bovine embryonic development to the blastocyst stage is significantly higher when oocytes mature with FSH combined with a high concentration of LH, rather than FSH or LH alone.

In this study, combined LH and FSH produced rapid growth of follicles and resulted in a final follicle size that was significantly larger than that achieved with LH alone. Recent immunohistochemical studies have demonstrated that LHRs and FSHRs are also expressed in cumulus cells during follicular development, suggesting that LH and FSH might interfere during the oocytes' entire growth phase [17, 32–35]. In this study, histological observations revealed different effects of combined LH and FSH on granulose cell differentiation. The number of cells forming the cumulus mass in LH and FSH combination cultures was lower than that with FSH alone. This result is consistent with previous work from Cortvrindt et al. [19], suggesting more pronounced differentiation of the granulosa cells in the presence of LH. However, the exact mechanisms by which gonadotropins influence embryonic development are unknown. FSH and LH have previously been shown to influence the protein synthetic capacity of oocytes [36]. Recent work from Anderiesz et al. [31] has suggested that gonadotropins may improve oocyte viability by influencing oocyte protein content, stimulating synthesis in oocytes. This protein content might be essential for embryonic development. Additionally, combination of LH and FSH may be able to improve embryonic development by modulating the oocyte's nutritional or appropriate steroid environment, or other effects which are concurrent with the LH and FSH-induced effects on follicle differentiation. However, these were not directly investigated. The result of the present study showed that combined LH and FSH significantly increased embryo developmental competence. This indicates that combination of LH and FSH can improve cytoplasmic maturation of porcine oocytes required for further development.

In summary, this study shows that FSH is essential for in vitro growth of porcine preantral follicles, antral formation, estradiol secretion, and for oocytes acquiring competence to resume meiosis and undergo fertilization and embryonic development. Perhaps for first time, combined LH and FSH treatment of porcine preantral follicles can improve the quality of oocytes by promoting growth and a higher frequency of embryonic developmental competence.

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