

# Effects of PI3K and FSH on steroidogenesis, viability and embryo development of the cumulus–oocyte complex after *in vitro* culture

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## Summary

The purpose of this study was to evaluate the effects of FSH and PI3K on the nuclear maturation, viability, steroidogenesis and embryo development of bovine cumulus–oocyte complexes (COCs). Oocyte maturation was achieved with MIV B, MIV B+100  $\mu$ M LY294002, MIV B+10 ng/mL follicle stimulating hormone (FSH), or MIV B+10 ng/mL FSH+100  $\mu$ M LY294002 treatments for 22–24 h. After the cultured COCs were denuded, oocytes were separated into those that extruded polar bodies (mature) and those that did not, and real-time polymerase chain reaction (PCR) for BAX, BCL2, LHR, FSHR, CYP11A1, CYP19A1 and HSD17B1 genes was performed. The culture medium was collected to determine the levels of 17 $\beta$ -estradiol (E2) and progesterone (P4). The trypan blue test was used to study COC viability, and embryo development was evaluated. FSH increased nuclear maturation and PI3K blocked the maturation but did not influence oocyte viability. BAX and BCL2 expression levels in the cumulus cells were only affected by FSH, and the BAX levels decreased after treatment with LY294002. FSH increased the levels of E2 and P4, however inhibition of PI3K decreased E2 levels. MIV B enhanced levels of LHR, FSHR, CYP11A1, CYP19A1 and HSD17B1, whereas LY294002 inhibited the expression levels of all genes. MIV B+FSH decreased the expression levels of all genes except CYP11A1. LY294002 did not demonstrate any effects in the presence of FSH. Embryo development was significantly decreased when the MIV B+FSH medium was used. In conclusion, FSH controls the steroidogenesis, viability and gene expression in COCs. PI3K plays essential roles in nuclear maturation, steroidogenesis and embryo development.

Keywords: COC, Embryo development, FSH, PI3K, Steroidogenesis, Viability

## Introduction

Ovarian follicles are the fundamental units of the reproductive cycle and are responsible for gametogenesis and steroidogenesis. Oocytes are attached to and are surrounded by granulosa cells, which form the cumulus–oocyte complex (COC), and by theca cells (Morgan *et al.*, 2015; Zhang & Liu, 2015). The maturation process of the oocyte includes both the nuclear (meiosis) and cytoplasmic maturation processes (reorganization of organelles and cytoskeleton, transcription of RNAs, and translation of proteins related to embryonic development) (Ferreira *et al.*, 2009). The *in vitro* viability of COCs is essential for

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embryo development and is affected by the pubertal status of the female (Pawlak *et al.*, 2015) and FSH levels, which maintain the expression of *BAX* and *BCL2* genes in the granulosa cells (Mani *et al.*, 2010).

Steroidogenesis is one of the most important and well known factors involved in oocyte maturation both *in vivo* and *in vitro*. The intrafollicular concentrations of progesterone and 17 $\beta$ -estradiol are important to the oocyte's competence in generating embryos after *in vitro* fertilization (Aardema *et al.*, 2013) and affect the expression of zona pellucida proteins (Kempisty *et al.*, 2012). Supplementing the COC culture medium with 17 $\beta$ -estradiol and progesterone improves the rate of embryos that develop to the morula stage, compared with medium containing only LH and FSH (Zheng *et al.*, 2003). Higher *in vitro* concentrations of estradiol increased chromosomal aberrations of oocytes and diminished embryo development, whereas the addition of FSH reversed both effects (Beker *et al.*, 2002).

In addition, 17 $\beta$ -estradiol influences the viability and oxidative stress levels of cultured neurons by suppressing Bax and stimulating Bcl-2 expression levels (Li *et al.*, 2013a; Li *et al.*, 2013b). Phosphatidylinositol 3-kinase (PI3K) is essential for the viability effects of 17 $\beta$ -estradiol, and similarly, steroidogenesis is influenced by the levels of PI3K and the gonadotropins LH and FSH.

PI3K family proteins, which were discovered in 1980 (Liu *et al.*, 2009), are kinases that phosphorylate 3'-hydroxyl inositol groups found in membrane lipid molecules known as phosphatidylinositols to generate phosphatidylinositol 3,4,5-trisphosphate (PIP<sub>3</sub>) and other phosphatidylinositol phosphate molecules. The latter components recruit AKT/PKB to activate intracellular cascades that control growth, proliferation, survival/viability, metabolism, intracellular trafficking, and differentiation of cells (Hawkins *et al.*, 2006; Engelman *et al.*, 2006; Yuan & Cantley, 2008; Liu *et al.*, 2009; Vanhaesebroeck *et al.*, 2010).

PI3K proteins are classified into three different classes according to phosphorylation substrates and regions of sequence homology as follows (Hawkins *et al.*, 2006; Engelman *et al.*, 2006): classes I (A and B), II and III; however, the last two classes are not completely understood. Class I is related to cell surface receptors; class II seems to be related to the internalization of receptors, cellular migration, glucose metabolism, exocytosis, apoptosis; and class III controls the intracellular transport of vesicles in the Golgi apparatus, cellular growth and autophagy (Engelman *et al.*, 2006; Vanhaesebroeck *et al.*, 2010).

Class I proteins are clearly related to intracellular signalling, and the subtype IA proteins are activated by tyrosine kinase receptors and use p85 as the regulatory subunit, whereas subtype IB proteins are activated by G-protein-coupled receptors and do not use p85

(Vanhaesebroeck *et al.*, 2010). The catalytic subunit can be p110 $\alpha$ , p110 $\beta$ , p110 $\gamma$  or p110 $\delta$  and promotes the activation of complex intracellular signalling using proteins including AKT/PKB, FOXO, PDK1, mTOR, MAPK and others (Engelman *et al.*, 2006; Vanhaesebroeck *et al.*, 2010; Zheng *et al.*, 2012).

PI3K proteins participate in the activation and survival of primordial follicles and in the recruitment of follicles in ovarian cycles (Zheng *et al.*, 2012). FSH activates cAMP/PKA and PI3K, resulting in the increase of aromatase (*CYP19*) expression (Stocco, 2008). Aromatase converts androgens into estradiol, enhances the effects of FSH on COCs and progresses ovarian follicle development to a preovulatory stage (Stocco, 2008).

The effects of PI3K have been mostly evaluated in granulosa cells but not in bovine COCs. The aim of the present study was to analyze the *in vitro* effects of PI3K and its relationship to the effects of FSH on COC maturation, viability, steroidogenesis and embryo development. An inhibitor of PI3K, LY294002, was used to understand the intracellular effects of PI3K *in vitro*. It is noteworthy that oocytes were completely viable after culture, based on trypan blue tests, and viability, steroidogenesis and embryo development were significantly affected by PI3K.

## Materials and methods

Bovine ovaries were obtained from a local slaughterhouse (Distrito Federal, Brazil) and transported to the laboratory in sterile saline solution (0.9% NaCl, w/v) at 35–37°C. The ovaries were rinsed twice in saline solution to remove the blood and were maintained at 35–37°C.

Follicles (1 to 8 mm in diameter) were aspirated with 21-gauge needles to obtain immature COCs. Ovarian follicles with clear fluid and well vascularized appearance were selected. The COCs were suspended in phosphate-buffered solution (PBS) (Invitrogen, Brazil) and BSA (Sigma, USA) under sterile conditions, and those characterized by compact cumulus cells and evenly granulated cytoplasm were selected (Viana *et al.*, 2004).

After selection, COCs were cultured in defined, patented, serum free, basic maturation *in vitro* medium (MIVB) [applicant: Fundação Universidade de Brasilia]. MIVB is composed of a diluent alpha-MEM medium (Invitrogen-GIBCO, Brazil), HEPES (Sigma, USA), sodium bicarbonate, polyvinyl alcohol (PVA), non-essential amino acids, transferrin (Invitrogen-GIBCO, Brazil), 10<sup>-7</sup> M androstenedione, and antibiotics, penicillin and streptomycin, supplemented with or without FSH 10 ng/mL (Sigma, USA). The inhibitor LY294002 (Sigma, USA) was used to determine the role of PI3K.

## Experimental design

The experimental groups were MIVB with no FSH (0FSH), MIVB with no FSH supplemented with 10  $\mu$ M or 100  $\mu$ M of LY294002 (0FSH 10LY and 0FSH 100LY, respectively), MIVB with FSH 10 ng/ml (10FSH) and MIVB with FSH 10 ng/ml and 10  $\mu$ M or 100  $\mu$ M of LY294002 (10FSH 10LY and 10FSH 100LY, respectively). The doses of LY294002 were based on a study conducted by Hoshino *et al.* (2004). The group with the 10  $\mu$ M dose was excluded from PCR assays and polar body extrusion rate determination because it did not demonstrate any effects.

All experiments procedures after *in vitro* maturation was based on the idea of comparing oocytes that matured *in vitro* with those ones that did not mature (extrude polar body) in terms of viability and gene expression.

### *In vitro* maturation of COCs and the viability test

COCs (35–40 COCs/well) were cultured in 400  $\mu$ l of medium for 22–25 h at 38.5°C, 5% CO<sub>2</sub>, and 95% humidity. The trypan blue exclusion test was performed to determine oocyte viability before and after culture, and no non-viable oocyte was found. Viability of COCs was also evaluated by embryo development *in vitro*.

The maturation rates were determined by the polar body extrusions after *in vitro* culture and were expressed as the average of percentages of matured oocytes per experiment.

### *In vitro* fertilization and embryo culture

After culture, the COCs were fertilized according to the methods described by Mota *et al.* (2015), and embryo culture was performed using SOF medium as described by Gulart (2015).

### Hormone level measurement

The activity of steroidogenesis was assessed by the measurement of hormones in the medium after culture. The culture medium of each well was collected and frozen at –20°C until hormone level analysis. Chemiluminescence was performed to measure 17 $\beta$ -estradiol (E2) and progesterone (P4) concentrations. The concentrations of both hormones were expressed as ng/ml, and the values were used to determine the E2/P4 ratios for each sample.

### Isolation of cumulus cells and oocytes (polar body extrusion) after culture

After culture, the COCs were mechanically denuded and oocytes were isolated from cumulus cells. Isolated cumulus cells were centrifuged twice in PBS for 5 min

at 6000 rpm. The oocytes and the pelleted cumulus cells were resuspended in PBS and TRIzol Plus (Life Technologies, Brazil) and stored at –80°C until RNA extraction.

The oocytes were divided into two categories: oocytes that matured and extruded polar bodies (progressed to metaphase II) and immature ones that did not extrude polar bodies [germinal vesicle/germinal vesicle breakdown (GV/GVBD), metaphase, anaphase or telophase I]. The rate of polar body extrusion was correlated to 17 $\beta$ -estradiol (E2) and progesterone (P4) concentrations in the medium.

### RNA extraction from cumulus cells

The TRIzol Plus (Life Technologies, Brazil) protocol was used for total RNA extraction. The isolated RNA was resuspended in RNase and pyrogen free water (Invitrogen) and stored at –80°C. RNA concentration and quality were measured using the NanoDrop 2000/2000c (Thermo Fisher Scientific) and 2100 Bioanalyzer (Agilent Technologies). The samples that presented NanoDrop A<sub>260</sub>/A<sub>280</sub> ratios between 1.8 to 2.0 were used to synthesize cDNA samples.

### cDNA synthesis and real-time PCR

The QuantiTect Reverse Transcription (Qiagen, USA) kit was used to perform cDNA synthesis based on the manufacturer's instructions, and the resulting samples were stored at –20°C.

Real-time PCR reactions were performed using the SYBR® Green Master Mix (Applied Biosystem, USA) with 50–100 ng/ $\mu$ l of cDNA, RNase and pyrogen free water (Invitrogen), and primer mixes that contained 100 nM forward and 100 nM reverse primers in the final volume of PCR reaction. The primers (Table 1) were designed based on the GenBank code and using the IDTSci Tools (Integrated DNA Technologies, USA), which included BLAST analysis. The real-time PCR conditions were previously standardized to guarantee high efficiency.

The cycling conditions included the following steps: 95°C for 20 min, 45–50 cycles of 95°C for 3 s, and 60°C for 30 s, as described for the Fast SYBR® Green Master Mix. The melting curves confirmed that a single specific product was generated for each target gene, and no-template controls showed the absence of contaminants.

The relative quantification was calculated using the  $\Delta\Delta$ CT method, and cyclophilin A (CYC) was chosen as the reference gene. The mean and standard deviation (SD) of CTs were 22.9 ( $\pm$ 2.07, SD) for cumulus cells, and 32.68 ( $\pm$ 2.86, SD) for oocytes. The calibrator group was the immature cumulus cells obtained from non-cultured COCs (GV). Three or four biological samples from each experimental group, and three or four

**Table 1** Sequences and temperature melting of target bovine gene primers

Gene	Primers sequence		T <sub>m</sub>	GenBank code
CYC	F: TGGCAAGTCCATCTATGGCGAGAA	R: ATCCAACCACTCAGTCTTGGCAGT	77.39	NM_178320
FSHR	F: AGAAGAAAGCAGGTGGATGG	R: GTTAGAGCAGTGACAGAGTCCG	79.78	NM_174061
LHR	F: TGAAAGCACAGCAAGGAGAC	R: GGAGTGTCTTGGGTAAGCAG	72.17	U20504
CYP11A1	F: GCTCCAGAGGCAATAAAGAAC	R: GATGTCCCCTACAAACTTCCG	79.48	NM_176644
CYP19A1	F: CTTGGGCTATGTGGACGTG	R: CCCCTGGATGTTAACCACG	69.05	U18447
HSD17B1	F: CCGTCCCAGAGCTTCAAAG	R: GAATCTGCATCCCTCACGTC	83.79	NM_001102365
BAX	F: GTTGTCGCCCTTTTCTACTTTG	R: AAGGAAGTCCAATGTCCAGC	79.81	NM_173894
BCL2	F: CCCTGT TTGATTTCTCCTGGCTGT	R: TGGGCTTCACTTATGGCCAG ATA	80.81	NM_001166486

FSHR: FSH receptor; LHR: LH receptor; CYP11A1: cytochrome P450, family 11-A1; CYP19A1: aromatase; HSD17B1: hydroxysteroid dehydrogenase 17 $\beta$  1; CYC: cyclophilin A (peptidylprolyl isomerase A); BAX: BCL2 associated X; BCL-2: B cell CLL/lymphoma-2. T<sub>m</sub>: melting temperature (°C).

replicates per biological sample, were analysed using PCR reactions.

A biological sample contained 15–25 oocytes or cumulus cells from 25 oocytes (each oocyte contains approximately 10<sup>5</sup> cumulus cells) (Calado *et al.*, 2005). The COC pool was cultured for 24 h and after denudation, were grouped into oocytes that either extruded polar bodies and those did not. The cumulus cells were not separated based on the oocyte maturation state to avoid degeneration.

### Statistical analysis

The chi-squared test was performed to analyze oocyte maturation (polar body extrusion). Two-way analysis of variance (ANOVA), followed by one-way ANOVA and the Bonferroni post hoc test were performed to analyze hormone measurements and E2/P4 ratios. Student's *t*-test was performed to determine the significant differences in the hormone measurements and E2/P4 ratios in the culture medium between the groups. The Kruskal–Wallis followed by Student–Newman–Keuls tests were performed to determine the differences in real-time PCR analysis among the groups.

The correlation between 17 $\beta$ -estradiol or progesterone and the nuclear maturation was evaluated by simple linear regression and Pearson's correlation. The differences were considered significant when the *P*-value was < 0.05.

## Results

Medium supplemented with FSH significantly increased polar body extrusion compared with all groups, and PI3K inhibition blocked polar body extrusion of COCs cultured in 10FSH medium (Fig. 1). Interestingly, no degenerated COCs or oocytes were observed after culture. Only a few cumulus cells of

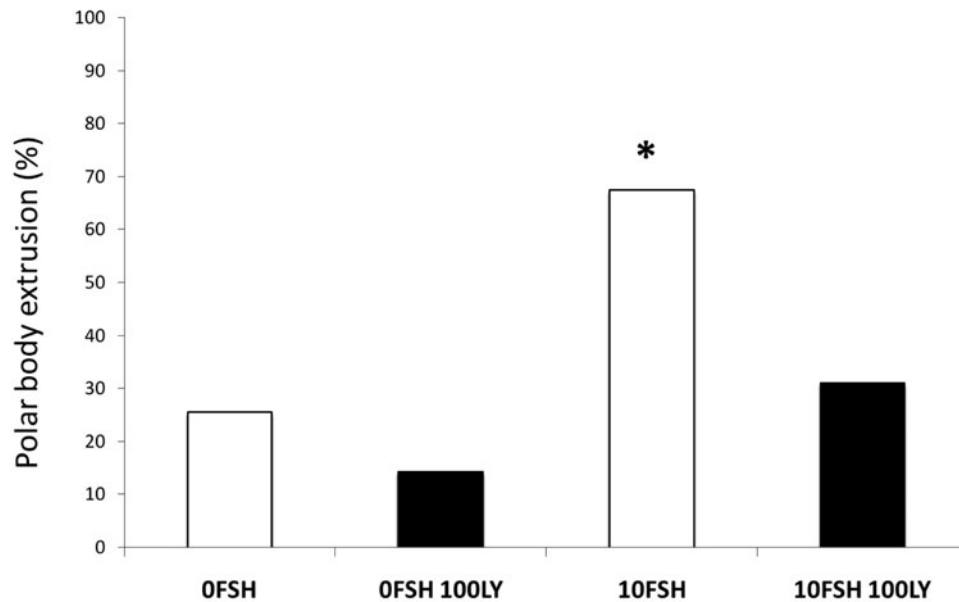
one COC were degenerated and detached after culture (Figure S1). BAX and BCL2 gene expression levels were also tested in the cumulus cells and oocytes from cultured COCs and were differentially regulated based on the cell type (Figs 2 and 3).

In cumulus cells, both BCL2 and BAX expression levels were upregulated by FSH, however PI3K affected only BAX expression levels (Fig. 2). The oocytes expressed lower levels of BCL2 with the exception of immature oocytes cultured in the 0FSH 100LY medium (Fig. 3). Oocytes also expressed significantly lower levels of BCL2 mRNA compared with cumulus cells (data not shown; *P* < 0.05).

The BAX expression levels of oocytes (Fig. 3) are similar to those of cumulus cells (Fig. 2; *P* > 0.05); however, higher levels of mRNA were observed in both matured and immature oocytes after 24 h of culture (Fig. 3; *P* > 0.05).

In terms of steroidogenesis, FSH supplementation in the medium significantly enhanced the concentrations of E2 and P4; however, it is noteworthy that 0FSH medium was also able to sustain steroidogenesis (Table 2) in the presence of androstenedione. PI3K inhibition (100  $\mu$ M of LY294002) significantly decreased E2 production and E2/P4 ratios in the 0FSH and 10FSH groups, whereas PI3K inhibition enhanced P4 production only in the 0FSH medium (Table 2). Progesterone did not demonstrate any relationship with polar body extrusion ( $R^2 = 0.009$ , *P* = 0.73); however, 17 $\beta$ -estradiol showed a significant correlation to nuclear maturation and polar body extrusion ( $R^2 = 0.564$ , *P* = 0.0020). The results demonstrate the essential role of PI3K in the steroidogenesis of COCs *in vitro*.

Based on the activity of steroidogenic pathways as evaluated by E2 and P4 concentrations, the gene expression levels were evaluated after culture. The culture medium, absent of FSH (0FSH), enhanced the gene expression levels of LHR (Fig. 4A), FSHR (Fig. 4B), CYP11A1 (Fig. 5A) and CYP19A1 (Fig. 5B), but did not change the expression of HSD17B1, compared with the



**Figure 1** Effects of FSH and PI3K inhibitor, LY294002, on polar body extrusion of oocytes isolated from bovine COCs that were matured *in vitro*. Oocytes per treatment 0FSH: 51 oocytes; 0FSH 100LY: 42 oocytes; 10FSH: 44 oocytes; 10FSH 100LY: 73 oocytes. The result was obtained from three independent experiments (replicates) of IVM. \*Statistical difference from all groups (chi-squared test,  $P < 0.05$ ). Values are expressed as averages of the maturation percentages of each experiment.

10FSH group. In contrast, the 10FSH group presented lower levels of *LHR*, *FSHR*, *CYP19A1* and *HSD17B1* expression levels (Figs 4 and 5).

PI3K inhibition decreased the expression of *LHR* (Fig. 4A), *FSHR* (Fig. 4B), *CYP11A1* (Fig. 5A), *CYP19A1* (Fig. 5B) and *HSD17B1* (Fig. 5C) when 0FSH medium was used. The FSH action was not affected by PI3K inhibition after 24 h of culture (Figs 4 and 5).

The *in vitro* rates of embryo development were similar to those described by Mota *et al.* (2015), and no differences were observed between 0FSH and 0FSH 100LY groups (Fig. 6). 10FSH was able to increase embryo cleavage but not blastocyst development, and 10FSH 100LY decreased the cleavage and blastocyst production *in vitro* (Fig. 6). These results indicate the relevance of FSH to embryo development and show that PI3K alters FSH action in embryo development.

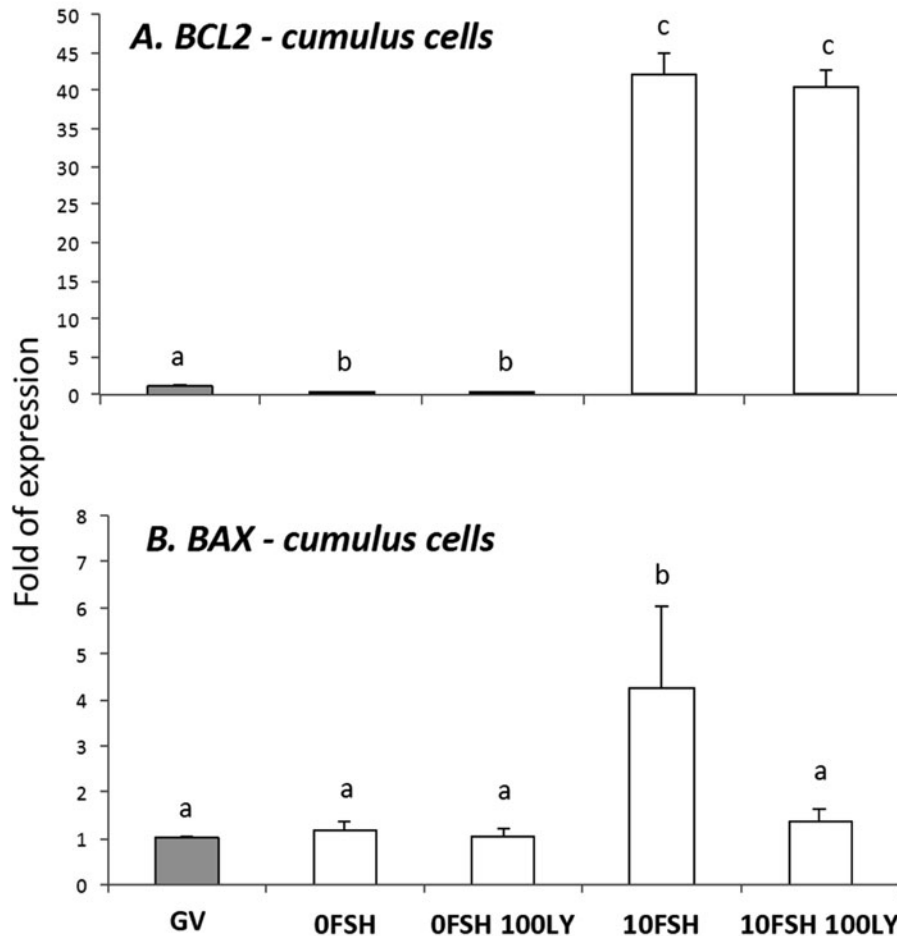
## Discussion

PI3K influenced nuclear maturation and blocks FSH effects in polar body extrusion, and PI3K inhibition did not degenerate oocytes after culture, despite changes in the gene expression levels of *BAX* and *BCL2* in oocytes and cumulus cells. Steroidogenesis, mostly 17 $\beta$ -estradiol, was also influenced by PI3K; it was not so clear, however, how the interactions between FSH and

PI3K influenced gene expression. Similarly, estrogen was correlated to polar body extrusion, as well as PI3K and FSH. Interestingly, FSH increased embryo cleavage and the inhibition of PI3K severely decreased blastocyst development *in vitro*. To our knowledge, this is the first paper that describes the relevance of PI3K to bovine COCs *in vitro*.

Oocyte maturation corresponds to nuclear (polar body extrusion) and cytoplasmic maturation, which includes the production of RNAs and proteins for embryo development (Ferreira *et al.*, 2009). Polar body extrusion is strongly influenced by FSH (Mota *et al.*, 2015) and PI3K. The F-actin in oocytes is dependent on PtdIns(3,4,5)P<sub>3</sub>, which is produced by proteins such as PI3K. The inhibition of PtdIns production modifies the migration of the meiotic fuse to the peripheric oolemma (Zheng *et al.*, 2013) and could potentially explain the inhibition of polar body extrusion by LY294002 even in the presence of FSH in the medium.

Despite the nuclear maturation results, viability was not influenced by PI3K *in vitro* as shown by the trypan blue test. The PI3K/AKT proteins control apoptosis, and LY294002 increased the number of dead cumulus cells from porcine COCs cultured with FSH (Shimada *et al.*, 2003). In addition, *BAX* and *BCL2* levels of the oocytes were not strongly modulated by PI3K, and the cumulus cells were very responsive to FSH and PI3K. As previously described, the *BAX* and *BCL2* levels of the granulosa cells are controlled and stimulated



**Figure 2** BAX and BCL2 gene expression levels in cumulus cells isolated from COCs after being cultured in a medium with or without FSH and PI3K inhibitor, LY294002. <sup>a,b,c</sup>Different letters indicate significant statistical differences among groups ( $P < 0.05$ ).

by FSH, and only BAX levels respond to PI3K (Mani *et al.*, 2010); this is similar to the results presented here.

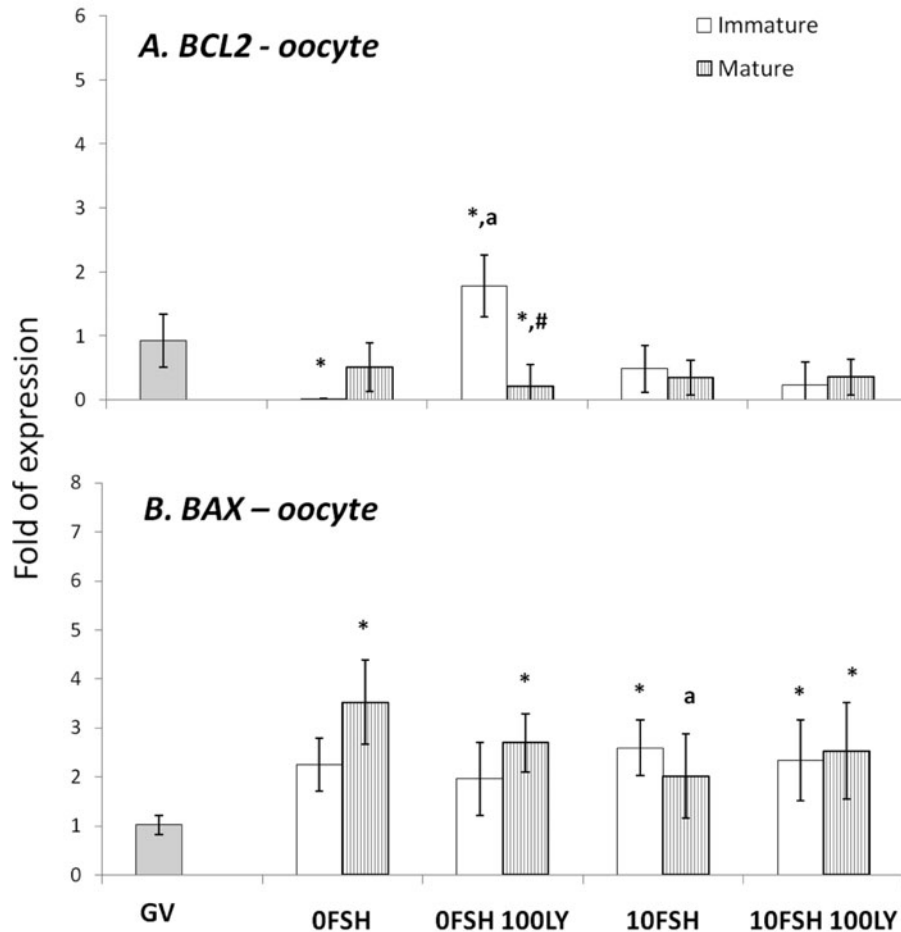
The viability of cultured cells is clearly influenced by  $17\beta$ -estradiol (Li *et al.*, 2013a, b). Higher concentrations of  $17\beta$ -estradiol in the COC culture medium ( $10^{-4}$  mol/l) significantly increased oocyte diameter, nuclear maturation and cumulus expansion in porcine oocytes (Kubo *et al.*, 2015); however, higher rates of chromosomal aberrations were identified in bovine oocytes (Beker *et al.*, 2002). Progesterone and estradiol also influenced the expression of zona pellucida glycoproteins in canine oocytes (Kempisty *et al.*, 2012), demonstrating the relevance of progesterone and estradiol to the fertilization process.

The 0FSH medium was able to sustain steroidogenesis based on androstenedione precursor and, as expected, the addition of FSH enhanced its ability. The patented MIV B medium is different from MIV C due to its lack of hormones as shown in a previous analysis (Vasconcelos *et al.*, 2013). MIV C was described

as a pro-estrogenic medium in a study in which the wall sections of bovine ovarian follicles were cultured *in vitro* with medium containing no FSH and supplemented with androstenedione and other hormones (Vasconcelos *et al.*, 2013). The MIV B medium is also estrogenic and sustains COCs *in vitro* even when FSH is absent (E2:P4 ratio is approximately 1.0).

FSH is a well known hormone related to follicle steroidogenesis (Gutiérrez *et al.*, 1997; Silva *et al.*, 2006; Zheng *et al.*, 2008), and cumulus cells are considered more progesteronic than mural granulosa cells when exposed to higher concentrations of FSH (Armstrong *et al.*, 1996). Supplementation with 10 ng/ml of FSH was able to maintain higher levels of  $17\beta$ -estradiol compared with progesterone (Zheng *et al.*, 2008). MIV B+FSH sustained the E2:P4 ratio at approximately 1.0, similar to the 0FSH medium; however, the absolute levels of both steroid hormones were higher in the FSH medium as expected.

The mRNA expression levels were differently regulated based on the presence or absence of FSH.



**Figure 3** BAX and BCL2 gene expression levels in mature and immature oocytes isolated from COCs after being cultured in a medium with or without FSH and the PI3K inhibitor, LY294002. \*Indicates significantly different from GV ( $P < 0.05$ ). <sup>a</sup>Indicates significantly different from 0FSH group during the same cell status ( $P < 0.05$ ). #Indicates significantly difference between immature and mature oocytes in the same experimental treatment.

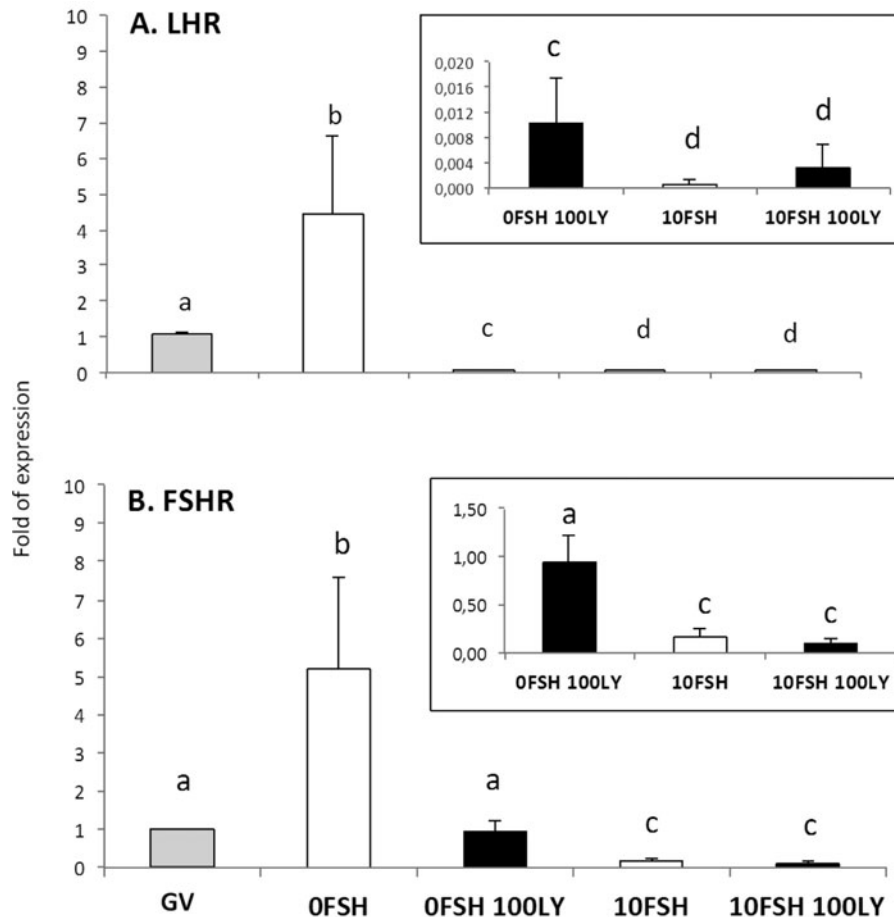
**Table 2** PI3K and FSH effects in  $17\beta$ -estradiol and progesterone concentrations and E2/P4 ratio of *in vitro* matured COCs

	0 FSH	10 FSH	Significance
17 $\beta$ -Estradiol (ng/ml)			
0 LY294002	7.26 $\pm$ 2.47 <sup>a</sup>	16.40 $\pm$ 6.10 <sup>a</sup>	$P = 0.008$
10 $\mu$ M LY294002	5.02 $\pm$ 2.60 <sup>a</sup>	14.26 $\pm$ 3.67 <sup>a</sup>	$P = 0.023$
100 $\mu$ M LY294002	2.33 $\pm$ 0.44 <sup>b</sup>	5.33 $\pm$ 2.60 <sup>b</sup>	$P = 0.009$
Progesterone (ng/ml)			
0 LY294002	8.91 $\pm$ 4.18 <sup>a</sup>	27.06 $\pm$ 10.72 <sup>a</sup>	$P = 0.003$
10 $\mu$ M LY294002	16.13 $\pm$ 1.50 <sup>a</sup>	37.50 $\pm$ 8.53 <sup>a</sup>	$P = 0.012$
100 $\mu$ M LY294002	17.68 $\pm$ 6.41 <sup>b</sup>	38.04 $\pm$ 11.26 <sup>a</sup>	$P = 0.003$
17 $\beta$ -Estradiol/progesterone ratio (E2/P4 ratio)			
0 LY294002	1.23 $\pm$ 0.95 <sup>a</sup>	0.75 $\pm$ 0.68 <sup>a</sup>	$P > 0.05$
10 $\mu$ M LY294002	0.32 $\pm$ 0.17 <sup>a</sup>	0.40 $\pm$ 0.14 <sup>a</sup>	$P > 0.05$
100 $\mu$ M LY294002	0.13 $\pm$ 0.02 <sup>b</sup>	0.13 $\pm$ 0.06 <sup>b</sup>	$P > 0.05$

Significance expressed in the table is between 0FSH and 10FSH groups (Student's *t*-test).

<sup>a,b</sup>Letters indicates statistical difference between 0  $\mu$ M, 10  $\mu$ M and 100  $\mu$ M LY294002 [one-way analysis of variance (ANOVA),  $P < 0.05$ ].

Two-way ANOVA indicates statistical difference between the profile of 0FSH and 10FSH in each dose of inhibitor in  $17\beta$ -estradiol ( $P = 0.0007$ ) and progesterone ( $P < 0.0001$ ), but not in E2/P4 ratio ( $P > 0.05$ ).



**Figure 4** The roles of FSH and PI3K inhibition in the gene expression of the receptors of LH (*LHR*) and FSH (*FSHR*) in cumulus cells from bovine COCs cultured *in vitro*. <sup>a,b,c,d</sup>Different letters indicate significant statistical differences among groups ( $P < 0.05$ ).

Exposure to FSH for 24 h decreased the expression levels of steroidogenic enzymes (with exception of *CYP11A1*) and gonadotropin receptors, despite the enhancement of steroidogenic activity, which was demonstrated by E2 and P4 concentrations. Culture medium that did not contain FSH sustained higher levels of *LHR*, *FSHR*, *CYP11A1*, *CYP19A1* and *HSD17B1* expression.

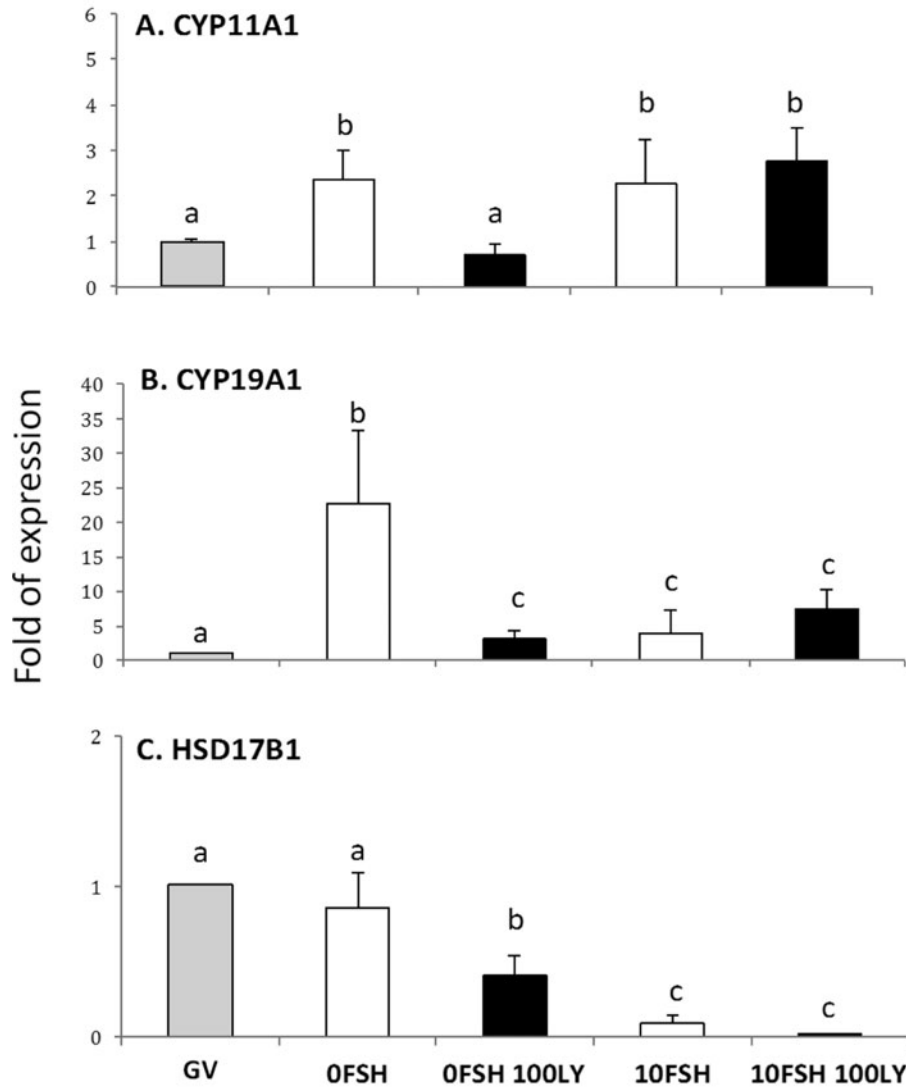
Steroid production depends on gonadotropins and enzymes, and previous studies have demonstrated that FSH controls the expression levels of *CYP11A1*, *CYP19A1*, *HSD17B1*, *LHR* and *FSHR* in COCs and granulosa cells (Silva & Price, 2002; Calder *et al.*, 2003; Sahmi *et al.*, 2006). However, the gene expression levels of gonadotropins and enzymes are not directly related to hormone production. The lower levels of expression can be related to the downregulation of FSH receptors after 24 h of exposure to hormones (Houde *et al.*, 1994) or the mRNA stability during culture (Sahmi *et al.*, 2006; Payne, 2015). Cultured COCs showed decreased expression levels of the *FSH* receptor, whereas the *LH*

receptor levels increased (Calder *et al.*, 2003; Salhab *et al.*, 2011). The levels of *CYP11A1* and *CYP19A1* decreased during culture (Salhab *et al.*, 2011), and *HSD17B1* expression in COCs has not been fully explored. In bovine granulosa cells, mRNA stability was observed for 3 h for *CYP19A1* mRNA and for 12 h for *CYP11A1* and *HSD17B1* mRNAs (Sahmi *et al.*, 2006).

In terms of intracellular signalling, PI3K has been extensively investigated in cancer cells, and its mutations are frequently identified in cancer cell lines and somatic cells (Yuan and Cantley, 2008; Liu *et al.*, 2009), making this protein a pharmacological target (Liu *et al.*, 2009, 2011). PI3K is one of the most relevant proteins that controls steroidogenesis (Moore *et al.*, 2001; Shimada *et al.*, 2003; Yu *et al.*, 2005; Su *et al.*, 2006; Ebeling *et al.*, 2011) and has defined actions in the activation and survival of primordial follicles, and in the recruitment of follicles (Zheng *et al.*, 2012).

PI3K controls steroidogenesis, an effect that has been observed by using 100  $\mu$ M of its inhibitor, LY294002, a dose used by Hoshino *et al.* (2004). The steroid





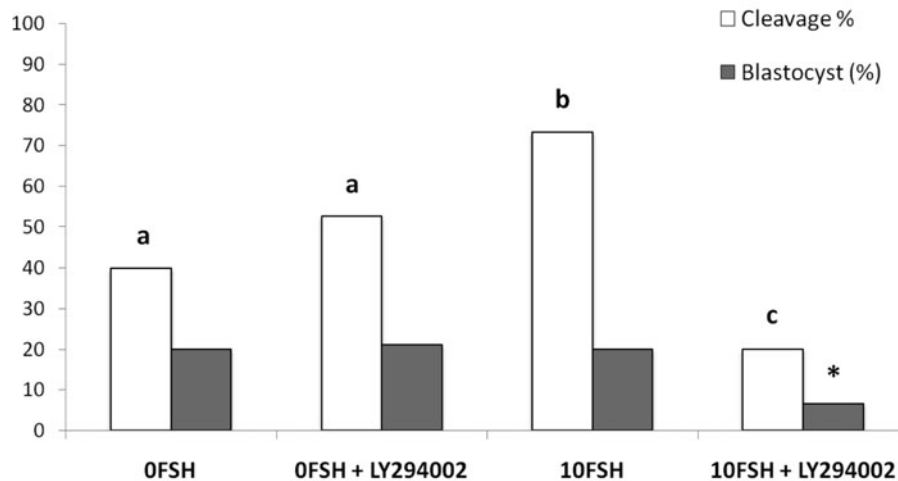
**Figure 5** The roles of FSH and PI3K inhibition in the gene expression of the receptors of *CYP11A1*, *CYP19A1* (aromatase) and *HSD17B1* in cumulus cells from bovine COCs cultured *in vitro*. <sup>a,b,c</sup>Different letters indicate significant statistical differences among groups ( $P < 0.05$ ).

concentrations in the culture medium after 24 h of culture indicate the relevance of PI3K, which decreases  $17\beta$ -estradiol and the E2:P4 ratio in the presence or absence of FSH. In the absence of FSH, progesterone production was significantly enhanced after PI3K inhibition. LY294002 caused a significant decrease in *LHR*, *FSHR*, *CYP11A1*, *CYP19A1* and *HSD17B1* expression levels in 0FSH medium but demonstrated no effects in 10FSH.

Previous results have indicated that, in the presence of FSH, progesterone production is stimulated by LY294002 in cumulus cells (Shimada *et al.*, 2003) and MAPK (protein controlled by PI3K) is inhibited, which indicates that progesterone levels decrease and estradiol levels increase in cumulus and granulosa cells (Moore *et al.*, 2001; Yu *et al.*, 2005; Su *et al.*,

2006; Ebeling *et al.*, 2011). Our data demonstrated that steroidogenesis is dependent on, but not exclusively so, PI3K action because steroidogenesis occurs even in the presence of LY294002.

Previous results have shown that PI3K or MAPK inhibition, in the presence of FSH, resulted in differing effects on *CYP11A1* and *CYP19A1* gene expression levels in COCs and granulosa cells (Moore *et al.*, 2001; Su *et al.*, 2006; Ebeling *et al.*, 2011), and no studies have reported the effects on *FSHR*, *LHR* and *HSD17B1* gene expression levels. The lack of additional information about the relevance of PI3K to steroidogenic gene expression in COCs cultured *in vitro* makes the current study an important contribution to the understanding of oocyte maturation. It is noteworthy that steroidogenesis inhibitors (AGT) reduce the production of estradiol



**Figure 6** Cleavage and blastocyst rates of COCs after culture in the presence or absence of FSH and the PI3K inhibitor, LY294002. There were 168 oocytes, and 28 blastocyst embryos developed *in vitro*. The number of oocytes per replicate was 41 to 45 COCs, and the experiment was repeated three times. <sup>a,b,c</sup>Different letters indicate significant statistical differences among groups ( $P < 0.05$ ). \*Indicates significantly decreased compared with all groups.

and progesterone and, as expected, impair the *in vitro* maturation of COCs. Supplementation of both hormones did not recover maturation rates, which indicates that other relevant factors are involved (Wang *et al.*, 2006).

In terms of *in vitro* embryo development, COCs cultured in the 0FSH medium developed to the blastocyst stage independently of the presence of LY294002, in accordance with the observations of Mota *et al* (2015). The 10FSH medium increased embryo cleavage but not blastocyst development, and COCs matured *in vitro* with LY294002 decreased both parameters. The inhibition of PI3K blocked the essential effects of FSH in oocyte competence, including embryo development *in vitro*, despite the expression levels of *BAX* and *BCL2*. The inability to extrude polar bodies can be associated with low-quality embryos, lower *in vitro* development and genomic aberrations (Somfai *et al.*, 2005). The concentration of  $17\beta$ -estradiol in the culture medium was also lower in the group 10FSH 100LY, indicating some possible correlation.

In conclusion, the relevance of FSH and PI3K to COC maturation is essential as observed by polar body extrusions, steroidogenesis and embryo development *in vitro*, although the gene expression levels were not directly related to oocyte competence and blastocyst rates.

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## Supplementary material

To view supplementary material for this article, please visit <https://doi.org/10.1017/S0967199417000703>

## Conflicts of interest

The authors claim that there are no conflicts of interest.

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## Supporting information

Additional Supporting information is available online at the publisher's website.

**Figure S1** Viability test of COC after culture in the presence or absence of FSH and the PI3K inhibitor, LY294002.

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