Effect of superstimulation on the expression of microRNAs and genes involved in steroidogenesis and ovulation in Nelore cows

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ARTICLE INFO

ABSTRACT

To better understand the impact of ovarian superstimulation on bovine follicular microenvironment, Nelore cows (*Bos taurus indicus*) were subjected to ovarian superstimulation with follicle stimulating hormone (FSH, n = 10; P-36 protocol) or FSH combined with eCG (n = 10; P-36/eCG protocol). Follicular fluid was analyzed for cholesterol concentration. Granulosa cells were analyzed by RT-qPCR to assess the expression of genes involved in steroidogenic and ovulatory and expression of microRNAs involved in final follicular development and luteinizing hormone/choriogonadotropin receptor *(LHCGR)* expression. Plasma concentration of estradiol was also measured. Follicular fluid from the P-36 group showed higher concentration of cholesterol than that of control (non-superstimulated) cows. Plasma concentration of estradiol was higher in the P-36/eCG group. Abundance of *STAR* and *FSHR* mRNAs were lower in granulosa cells from the P-36/eCG group. In contrast, *LHCGR* mRNA abundance was higher in superstimulated granulosa cells from the P-36 group and showed a pattern opposite to that of miR-222 expression. Ovarian superstimulation did not affect the expression of other markers (mmu-miR-202-5p, has-miR-873, has-miR-144, and their target genes, *CREB, TGFBR2,* and *ATG7*) of antral follicle development. However, the mRNA expression of VEGF pathway components was modulated by P-36 treatment. Taken together, these results demonstrate that superstimulatory protocols modify steroidogenic capacity, increase plasma estradiol, and regulate the abundance of VEGF system, *LHCGR* mRNA and suppress the expression of miR-222 in bovine granulosa cells.

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1. Introduction

Follicular growth and development is an endocrine-dependent process, involving gonadotropic hormones (follicle stimulating hormone [FSH] and luteinizing hormone [LH]) and steroid hormones (estradiol and progesterone) [1,2]. Ovarian follicle development depends on the coordinated action of FSH and LH (the well-known two-cell, two-gonadotropin model) via estradiol synthesis. Follicle development is characterized by the proliferation and functional differentiation of granulosa cells, which is essential for the final development and ovulation [3,4]. The expression of gonadotropic receptors (luteinizing hormone receptor [LHCGR]) and steroidogenic enzymes (CYP19A1 and STAR) is a characteristic of this differentiation, in addition to the secretion of high levels of estradiol [3,4]. Androgens are synthetized by theca cells in response to LH and are transported to granulosa cells, where they are aromatized to estrogens under the influence of FSH [5–7].

Numerous factors other than hormones are involved in follicular development such as vascular endothelial growth factor (VEGF), known to be a potent mitogen of endothelial cells [8] and a stimulator of vascular permeability [9]. VEGF acts through its receptors (FLT-1 and FLK-1) that are expressed in granulosa cells. VEGF expression changes throughout follicle development, with higher expression in dominant follicles [10]. The VEGF system functions in the growth of capillaries around follicles [11,12] and in the proliferation of the granulosa cells in ovaries in bovines [13].

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Another class of molecules that influences follicular development is microRNAs (miRNAs) [14]. MiRNAs are 18–24 nucleotide-long, small non-coding RNAs involved in post-transcriptional control of gene expression [15]. Recent studies demonstrated the different regulatory roles of specific miRNAs in the ovarian follicles [16–22], including their involvement in bovine steroidogenesis [17,22,23]. Among the several miRNAs, miR-222 was detected in ovine [24] and bovine [25] fetal ovaries. LHCGR was indicated as a possible target for its action [25].

Other miRNAs are involved in antral follicle development. Recently, Donadeu et al. [17], suggested that miR-202, miR-873, and miR-144 could be used together as markers of steroidogenic potential and antral follicle development. According to Sontakke et al. [23], the miRNAs miR-144, miR-202, and miR-873 are expressed in healthy dominant follicles and participate in signaling pathways involved in follicular cell proliferation, steroidogenesis and oocyte maturation, through modulation of predicted target genes, *TGFBR2, SPRED1,* and *ATG7.*

Improved understanding of follicular development led to the development of superstimulatory treatments capable of increasing the number of competent oocytes that can produce embryos [26]. Several superstimulation protocols have been developed, including the P-36 protocol and its variation, the P-36/eCG protocol. Both protocols use FSH to stimulate follicular growth. It is believed that on the last day of treatment, follicular granulosa cells exhibit LHCGR [27]. Therefore, the last two doses of FSH are replaced by eCG doses in the P-36/eCG protocol. Recent data in Nelore cows demonstrated the impact of superstimulatory protocols on the oviductal transcript profile [28], lipid profile of follicular fluid [29], and the expression of genes involved in LHCGR signaling in granulosa cells [30]. However, the effects of P-36 and P-36/eCG protocols on follicular development need further investigation. The aim of the present study was to evaluate the effects of ovarian superstimulation on the expression of miRNAs and their target genes involved in steroidogenesis and the ovulatory capacity of granulosa cells from Nelore cows.

2. Material and methods

2.1. Ovarian superstimulation

A scheme of the experimental design from the study was presented in Fig. 1. This study was conducted on a farm located in Santa Cruz do Rio Pardo (São Paulo, Brazil, latitude 22° 53′ 56″, longitude 49° 37′ 57″, altitude 467 m). The cattle were maintained in a pasture (*Brachiaria brizantha*), with *ad libitum* access to water and a mineral supplement. Non-lactating, multiparous, 5–7-year-old Nelore cows, with body condition scores ranging from 2.0 to 3.5, were submitted to ovarian superstimulatory protocol, P-36 (FSH, n = 10) or P-36/eCG (FSH + eCG, n = 10). Control group (n = 10) did not receive either treatment. The experiments were approved by the local *Ethics Committee on Animal Use* from the Institute of Bioscience (University of São Paulo State UNESP, Botucatu, São Paulo, Brazil; protocol number: 379/2012).

For ovarian superstimulation, as described by Santos et al. [29], cows at random stages of estrous cycle received progesterone-releasing vaginal inserts (1.0 g, PRIMER ®, Tecnopec, São Paulo, SP, Brazil) and estradiol benzoate (2.5 mg, i.m., Estrogin ®, Farmavet, São Paulo, SP, Brazil) on day 0.

The P-36 protocol was performed by administering pFSH (Folltropin-V®, Bioniche Animal Health, Belleville, ON, Canada), twice daily (AM & PM) from days 5–8, in decreasing doses of 40% (day 5), 30% (day 6), 20% (day 7) and 10% (day 8) of the total amount used (200 mg). All the animals were given, twice on day 7 (7 a.m. and 7 p.m.), 150 mg of d-cloprostenol (Prolise®, Tecnopec, São Paulo, SP,

Brazil) intramuscularly. Progesterone-releasing vaginal inserts were removed at 7 p.m. on day 8 and the cows were sacrificed on day 9 at 7 a.m. For P-36/eCG treatment, the final two doses of FSH were replaced by two doses of eCG (total doses = 400 IU, i.m., Novormon®, Syntex, Buenos Aires, Argentina).

2.2. Follicular fluid and granulosa cell recovery

The follicles were detected by ovarian ultrasonography performed 12 h before slaughter. Ovaries were collected and transported to the laboratory in saline solution (0.9%), at 4 °C and evaluated for the presence of *corpora lutea* or previous ovulations. The average diameter of each follicle was derived from two measurements, approximately perpendicular to each other, using a caliper. Follicular fluid and granulosa cells were recovered from the only dominant follicle of control cows and one of the three largest follicles from P-36 and P-36/eCG groups. The granulosa cells recovered from each follicle was determined as its respectively sample. The diameter of follicles in all the cows ranged from 11 to 14 mm. The total number of superstimulated follicles was around 20 follicles and did not differ between the P-36 and P-36/eCG groups.

Subsequently, the antral cavity was flushed repeatedly with cold saline and granulosa cells were pelleted by centrifugation at 1200g for 1 min. The pooled granulosa cells from each follicle were placed in Trizol (Invitrogen, São Paulo, SP, Brazil) and homogenized using Precellys 24 Tissue homogenizer (Bertin Technologies®, Montigny Le Bretonneux, France) by 3 cycles of 30 s each.

2.3. Intrafollicular concentration of cholesterol

The follicular fluid from each follicle was used to quantify intrafollicular concentration of cholesterol (n = 10 follicles/group). For the analysis, it was use the kit from Laborlab® (São Paulo, SP, Brazil), according to manufacturer's protocol. Cholesterol was released by the enzymatic hydrolysis of esters by a lipase and was subjected to the action of cholesterol oxidase. The hydrogen peroxide released by the reaction between cholesterol and molecular oxygen oxidizes the phenol and 4-aminophenazone in the reagent, resulting in the formation of quinone-imine (red color). Color intensity was determined spectrophotometrically at 505 nm using ULTROSPEC 2000 (Pharmacia Biotech, Cambridge, England).

2.4. Estradiol assay

Blood samples were collected from the jugular vein on day 9, to quantify the plasma concentration of estradiol using a solid phase competitive immunoassay (Immulite 1000; Siemens® Flanders, New Jersey, USA). For analysis, 300 μL of samples from control group (n = 10), P-36 (n = 10) and P-36/eCG (n = 10) and reagents (liquid phase - alkaline phosphatase conjugated with estradiol) were incubated together with specific anti-body coated spheres (solid phase) for 60 min. The spheres were washed repeatedly to eliminate unconjugated sample. Chemiluminescent substrate was added to generate the signal. The intensity of the signal is proportional to the amount of bound enzyme. The technique is reliable in the concentration range of 20 pg/mL to 2000 pg/mL and sensibility of 15 pg/mL.

2.5. RNA extraction and target gene expression

Total RNA was extracted from the granulosa cell samples according to the manufacturer's protocol from Trizol Reagent (Invitrogen, São Paulo, SP, Brazil) and stored at -80 °C. Possibility of cross-contamination of granulosa cells samples due to passage of
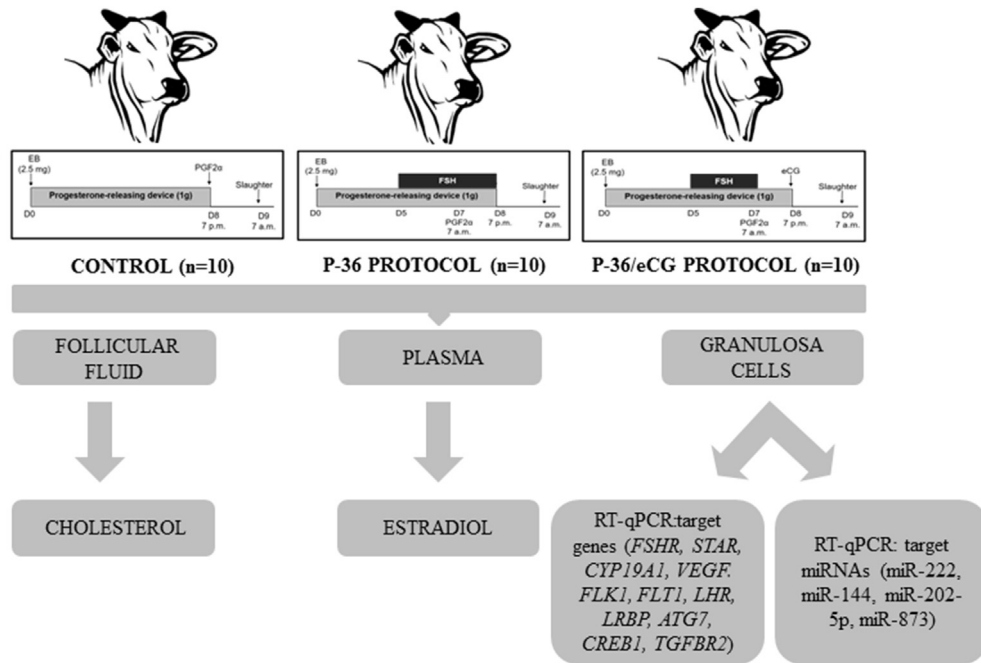
Fig. 1. Experimental design to investigate the effects of ovarian superstimulation in Nelore cows. It was examined the intrafollicular levels of cholesterol, concentration of estradiol in plasma and the expression of target genes and microRNAs in granulosa cells from cows subjected to superstimulatory treatments, P-36 or P-36/eCG, or control cows (n = 10 cows/group).

the needle through the theca cells layers was eliminated by testing for mRNA encoding 17α-hydroxylase (*CYP17*), by PCR as described by Buratini *et al.* [31]. The *CYP17* expression is analyzed because its expression is only expect in theca cells. In the present experiment, none granulosa cells sample was contaminated by theca cells once CYP17 was not detected in our samples.

The total RNA (1 μg) from each of the samples was incubated with DNAse (1 U/μg; Invitrogen, São Paulo, SP, Brazil) and then reverse transcribed, using High Capacity Kit (Applied Biosystems, São Paulo, SP, Brazil) containing random primers, according to the manufacturer's protocol.

The mRNA expression of each of the 11 target genes (*FSHR, STAR, CYP19A1, VEGF, FLK1, FLT1, LHR, LRBP, TGFBR2, ATG7* and *CREB1*) was analyzed by RT-qPCR (Table 1) using Power Sybr® Green PCR Master Mix system (Applied Biosystems, São Paulo, SP, Brazil) and QuantStudio™ 7 Flex. The total reaction volume of 25 μL contained 1 μL sample and 24 μL probe plus primers. The cycling conditions were: initial denaturation at 95 °C for 10 min followed by 40 cycles at 95 °C for 10 s, and annealing and extension for 1 min (temperature of this step of the cycle varied between genes).

Reactions were optimized for maximum amplification efficiency for each gene. The specificity of each PCR product was determined by melting curve analysis. The amplicon size was determined using electrophoresis on 1.5% agarose gels. Each sample was analyzed in duplicate and all the experiments included negative controls.

To choose the most stable reference gene for detailed analyses of granulosa cells, peptidylprolyl isomerase A (*PPIA*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and histone H2AFZ (*H2AFZ*) amplification profiles were compared using the geNorm applet for Microsoft Excel [32]. The most stable housekeeping gene for granulosa cells was *PPIA*. The ΔΔCt method with efficiency correction was used to calculate relative expression values (target genes/*PPIA*) for each target gene, using one control sample as calibrator [33].

2.6. MiRNA expression

MiRNA was isolated from 40 μg total RNA using *mir*Vana™ miRNA isolation kit (Life Technologies®, São Paulo, SP, Brazil) according to the manufacturer's instructions and stored at -80 °C. Target miRNAs (bta-miR-222, mmu-miR-202-5p, hsa-miR-873, has-miR-144; Table 2), were reverse transcribed using TaqMan®

Table 1
Primers used in RT-qPCR.

Gene	Forward	Reverse	Final concentration (mM)	Temp. annealing (°C)
FSHR	5′AGCCCCTTGTCACAACTCTATGTC 3′	5′GTTCCTCACCGTGAGGTAGATGT 3′	300	60
STAR	5′CCCAGCAGAAGGGTGTCATC 3′	5′TGCGAGAGGACCTGGTTGAT 3′	400	62
CYP19A1	5′CTGAAGCAACAGGAGTCCTAAATGTACA 3′	5′AATGAGGGGCCCAATTCCCAGA 3′	400	62
VEGF	5′CCCAGATGAGATTGAGTTCATTTT 3′	5′ACCGCCTCGGCTTGTCAC3′	300	60
FLK1	5′TGGCCCAACAATCAGAGCAG 3′	5′GAACGGAGCCCATGTCAGTG 3′	300	60
FLT1	5′GAAGGACGGGATGAGGATGC 3′	5′ATGGCCGTTGAGCGGAATGGA 3′	300	60
LHR	5′GCATCCACAAGCTTCCAGATGTTACGA 3′	5′GGGAAATCAGCGTTGTCCCATTGA 3′	300	60
LRBP	5′TGTTGTCAGAAGTCCTGCTGGTGT 3′	5′TAAGTTGAGGCCCACTCTCCCATT 3′	300	56
TGFBR2	5′GAAGTCCTGCTGGAGCAACT 3′	5′AGGCAATCTTGGGGTCATGG 3′	300	60
ATG7	5′GGCCTCTCCAGATTGCAGTT 3′	5′GCTTCGTCTAGCCGGTACTC 3′	300	60
CREB1	5′AATCCTAGTGCCCAGCAACC 3′	5′CGCTGTGCGAATCTGGTATG 3′	300	60

Table 2
Details of miRNA assays used in RT-qPCR.

MiRNA	Mature miRNA Sequence	Code	MirBase accession number
Has-miR-222	AGCUACAUCUGGCUACUGGGU	002276	MIMAT0000279
Has-miR-144	UACAGUAUAGAUGAUGUACU	197375_mat	MIMAT0000436
Mmu-miR-202-5p	UUCCUAUGCAUAUACUUCUUU	002579	MIMAT0004546
Has-miR-873	GCAGGAACUUGUGAGUCUCCU	002356	MIMAT0004953
Has-miR-191	CAACGGAAUCCCAAAAGCAGCUG	002299	MIMAT0000440
RNU43	GAACTTATTGACGGGCGGACAGAAACTGTGTGCTGATTGTCACGTTCTGATT	001095	

Reverse Transcription Reagents (Applied Biosystems, São Paulo, SP, Brazil) following manufacturer's protocols. qPCR analyses were performed using TaqMan® Universal PCR Master Mix (Applied Biosystems, São Paulo, SP, Brazil) and QuantStudio™ 7 Flex. First, it was prepared a mix containing TaqMan® Universal PCR Master Mix, deionized and purified water and specific TaqMan™ MicroRNA Assays to the target miRNA. The mix was distributed in wells for each samples and its respective duplicate. Final, the sample was added in wells. The total reaction volume of 20 μL contained 1,33 μL sample and 18,67 μL probe plus primers and cycling conditions were: enzyme activation at 95 °C for 10 min, followed by 40 cycles of denaturation (95 °C for 15 s) and annealing/extension (60 °C for 60 s). All samples were run in duplicates.

To quantify the relative abundance of target miRNAs, we used the geometric mean of the expression of RNU43 and has-miR-191 as reference. The ΔΔCt method with efficiency correction was used to calculate relative expression values (target genes/RNU43_has-miR-191 geometric mean) for each miRNA, using one control sample as calibrator [33].

2.7. Statistical analysis

Data were transformed to logarithmic values, if not normally distributed. ANOVA was used to determine the effect of ovarian superstimulation on the concentration of estradiol and cholesterol, as well as the relative abundance of miRNAs and target gene expression. The differences between the means were determined with Tukey-Kramer test. Analyses were performed using JMP software (SAS Institute Cary, NC). Data are presented as means ± SEM. Differences were considered significant when $p \leq .05$.

3. Results

Cholesterol level in follicular fluid from the P-36 group was higher compared with that in fluid from control cows ($p = .0176$; Fig. 2A). The plasma estradiol concentration was higher in the P-36/eCG group than in the P-36 or control group ($p = .0016$; Fig. 2B).

Among the genes involved in steroidogenesis, the mRNA abundance of FSHR was higher in the control cows than in cows subjected to superstimulation ($p = .0126$, Fig. 2C). Furthermore, the abundance of STAR mRNA was lower in the P-36/eCG group than in the control group ($p = .0017$; Fig. 2D). The abundance of CYP19A1 mRNA was unaffected by ovarian superstimulation (Fig. 2E). Level of VEGF mRNA was higher in the P-36 group than in the control group ($p = .0501$; Fig. 2F). Conversely, mRNA abundance of FLT1 and FLK1 were lower ($p = .0072$ and $p = .003$ respectively; Fig. 2G and 2H) in the P-36 group compared to control group.

LHCGR mRNA abundance were higher in the P-36 group than in the control group ($p = .003$; Fig. 3A. The mRNA abundance of LRBP was not affected by ovarian superstimulation (Fig. 3B), but the miR-222 abundance was higher in granulosa cells from the control group than in cells from the P-36 group (Fig. 3C).

Ovarian superstimulation did not affect the expression of genes involved in follicle development (ATG7, CREB1 and TGFBR2) and their predicted miRNA regulators (has-miR-144, hsa-miR-873 and mmu-miR-202-5p; Fig. 4).

4. Discussion

In the present work, we demonstrated the effects of ovarian superstimulation on the steroidogenic capacity as well as on the mRNA and miRNAs profiles in granulosa cells from Nelore cows subjected to the superstimulatory protocol P-36 or P-36/eCG. Our results showed that the intrafollicular concentration of cholesterol and the plasma concentration of estradiol increased after superstimulation. The superstimulation also positively affected the regulation of genes involved in steroidogenic and ovulatory bovine granulosa cells. Finally, we demonstrated that LHCGR was upregulated in a pattern that is opposite to that of miR-222 expression in granulosa cells.

Cholesterol is the substrate for androstenedione production in theca cells. Androstenedione is, in turn, the substrate for the FSH-mediated biosynthesis of estradiol in granulosa cells [7]. Estradiol has a fundamental role in granulosa cell differentiation and follicular maturation [34]. The concentration of circulating estradiol is a key factor in the physiological cascade involved in stimulating estrous behavior and inducing the gonadotropin surge [35]. In the present study, we observed higher levels of estradiol in the plasma of cows submitted to ovarian superstimulation with FSH in combination with eCG. Similar results were obtained in a study on buffaloes, where eCG treatment increased the concentration of estradiol in plasma [36]. Studies reported similar findings for bovine heifers treated with FSH or human menopausal gonadotropin [37]. In that study, the authors demonstrated that plasma concentration of estradiol and progesterone were increased by human menopausal gonadotropin treatment. Our results are also consistent with those of the studies in sheep, which showed that individual follicles from eCG-stimulated animals secrete more estradiol in culture than those from non-treated sheep, suggesting that the increased steroidogenic capacity of individual follicles contributes to the increase in plasma steroid concentration [38]. Despite the higher concentration of plasma estradiol in cows subjected to P-36/eCG protocol, there was no difference in the abundance of aromatase mRNA. An increase in CYP19A1 is expected in recruited follicles and after the selection, there is an increase abundance of mRNA steroidogenic enzymes. It has been hypothesized that dominant follicle is selected because its acquire LHCGR on granulosa cells and the estradiol synthesis allows in response to LH as well as FSH [8]. Besides that Ferrara and Davis-Smith [8], also described a decrease in aromatase abundance in granulosa cells from follicles after the deviation.

Cholesterol is transported by steroidogenic acute regulatory protein (STAR) to the inner mitochondrial membrane and this transport is considered a rate-limiting step in steroidogenesis [39]. Cholesterol is then converted to pregnenolone by another enzyme, the P450 cholesterol side chain cleavage complex [5,40]. Cholesterol is responsible for progesterone synthesis in granulosa cells.
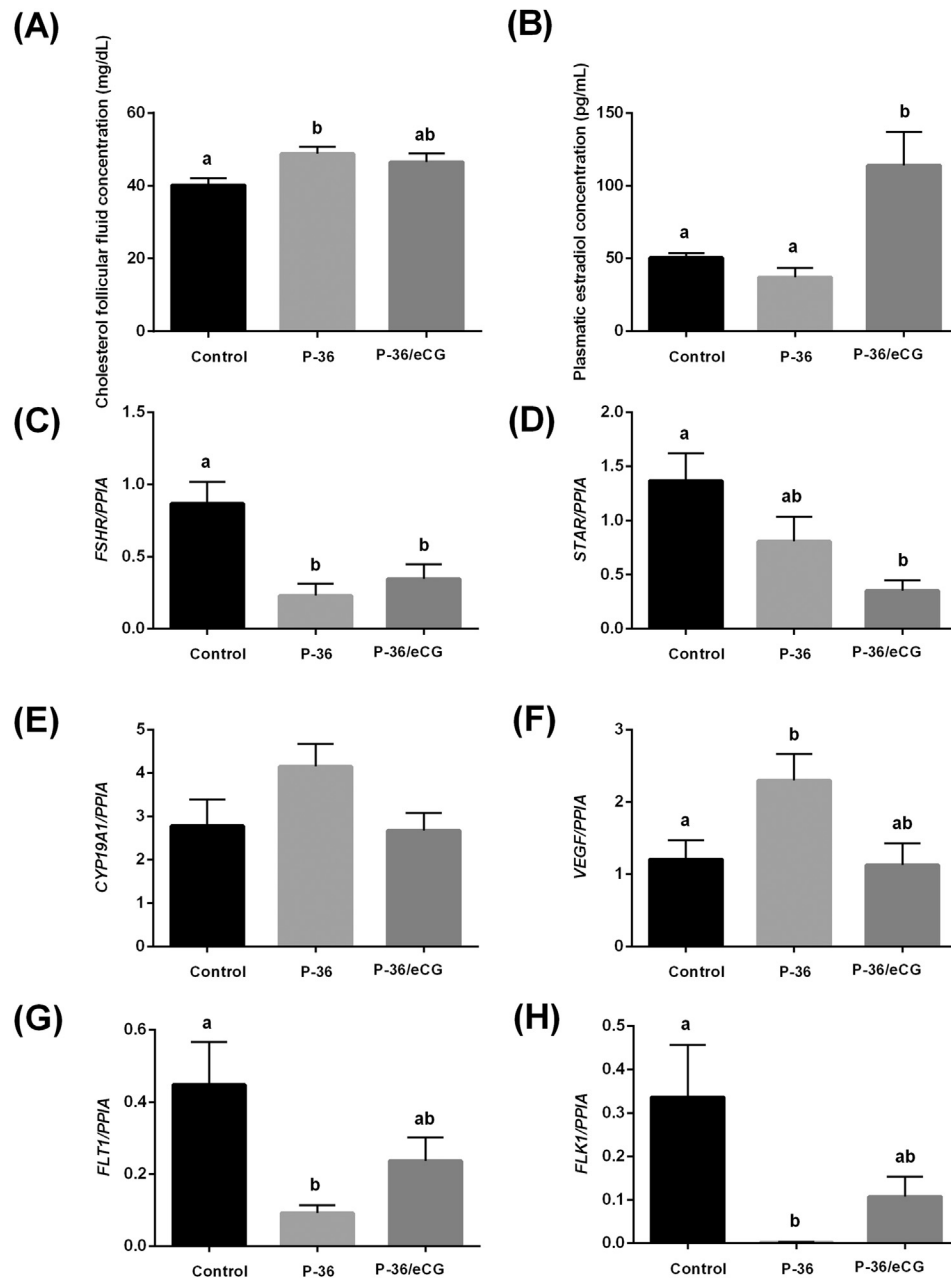
Fig. 2. Effects of ovarian superstimulation (P-36 or P-36/eCG) on levels of intrafollicular cholesterol (A) and plasma estradiol (B; mean ± SEM) and relative abundance (mean ± SEM) of *FSHR* (C), *STAR* (D), *CYP19A1* (E), *VEGF* (F), *FLT1* (G) and *FLK1* (H) mRNAs in granulosa cells. Messenger RNA abundance was measured by real-time PCR. The expression values are relative to a calibrator sample and were calculated by the ΔΔCt method with efficiency correction. Data are from 10 follicles/experimental group and presented as mean ± SEM. Bars with different letters ("a" and "b") are significantly different ($p < .05$).

Recently, Walsh *et al.* [39] found a significant increase in the expression of *STAR* in granulosa cells during follicle differentiation in cows. However, in the present work, cows receiving P-36/eCG showed lower abundance of *STAR* mRNA, compared to control group. In fact, these results are compatible with the behavior of late pre-ovulatory follicles obtained from cows treated with gonadotropin-releasing hormone (GnRH) and delayed LH surge, showing that superstimulatory treatment could be stimulating the dominant to preovulatory transition of follicles in these animals and perhaps modifying the ovulatory capacity in these cows.

We observed that granulosa cells from cows subjected to superstimulation exhibited lower abundance of *FSHR* mRNA compared to those from control cows. This decrease seems to be a result of the negative feedback from FSH, and is in agreement with results from several studies [41—44], in which FSH treatment of granulosa cells led to the down-regulation of *FSHR*. The lower expression of *FSHR* mRNA in granulosa cells from superstimulated groups is also in agreement with the findings of Nimz *et al.* [45], who showed that *FSHR* is down-regulated in late preovulatory follicles obtained from cows treated with GnRH.

In another approach, we demonstrated that ovarian superstimulation using FSH alone increased the expression of *LHCGR* mRNA. The role of FSH in the recruitment of follicular wave and sustaining the follicular growth before granulosa cells acquire LH receptor, is well known [1,2]. In granulosa cells, the transcriptional regulation of the *LHCGR* gene is FSH-dependent and is crucial for
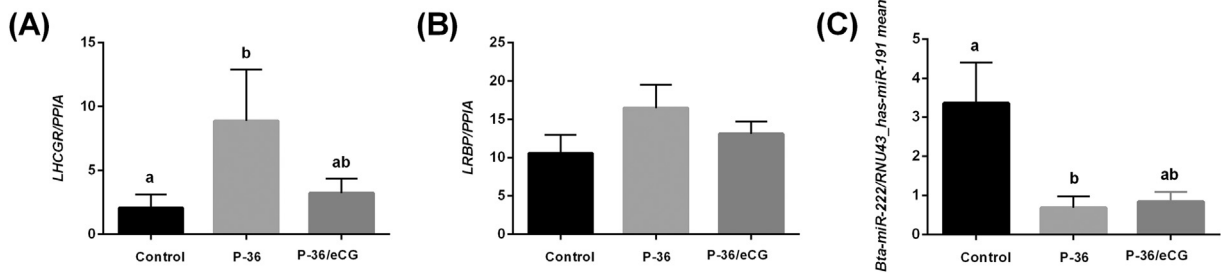
Fig. 3. Effects of ovarian superstimulation (P-36 or P-36/eCG) on the relative abundance (mean ± SEM) of *LHCGR* (A), *LRBP* (B) mRNAs and bta-miR-222 (C) in granulosa cells. mRNA and miRNA abundance were measured by real-time PCR and were calculated using the ΔΔCt method with efficiency correction. The expression values are relative to that of the calibrator sample, *PPIA* (mRNA) and relative genes media (RNU43 and miR-191; miRNA). Data are from 10 follicles/experimental group and presented as mean ± SEM. Bars with different letters ("a" and "b") are significantly different ($p < .05$).

the differentiation of this cell type [46]. Our *in vivo* results are consistent with those of previous studies where the *LHCGR* transcripts were increased in bovine granulosa cells cultured in the presence of FSH [47]. It has been suggested that ovulatory capacity is linked to the acquisition of LHCGR by granulosa cells [48–51]. Therefore, the present data suggest that the up-regulation of mRNA

encoding LHCGR could be correlated with the improvement of ovulatory capacity in superstimulated cows.

LH plays a key role in controlling the physiological processes in the ovary, such as the development of antral follicles and ovulation [48]. *LHCGR* was up-regulated in granulosa cells from cows of P-36 group, but no such effect was demonstrated in granulosa cells from
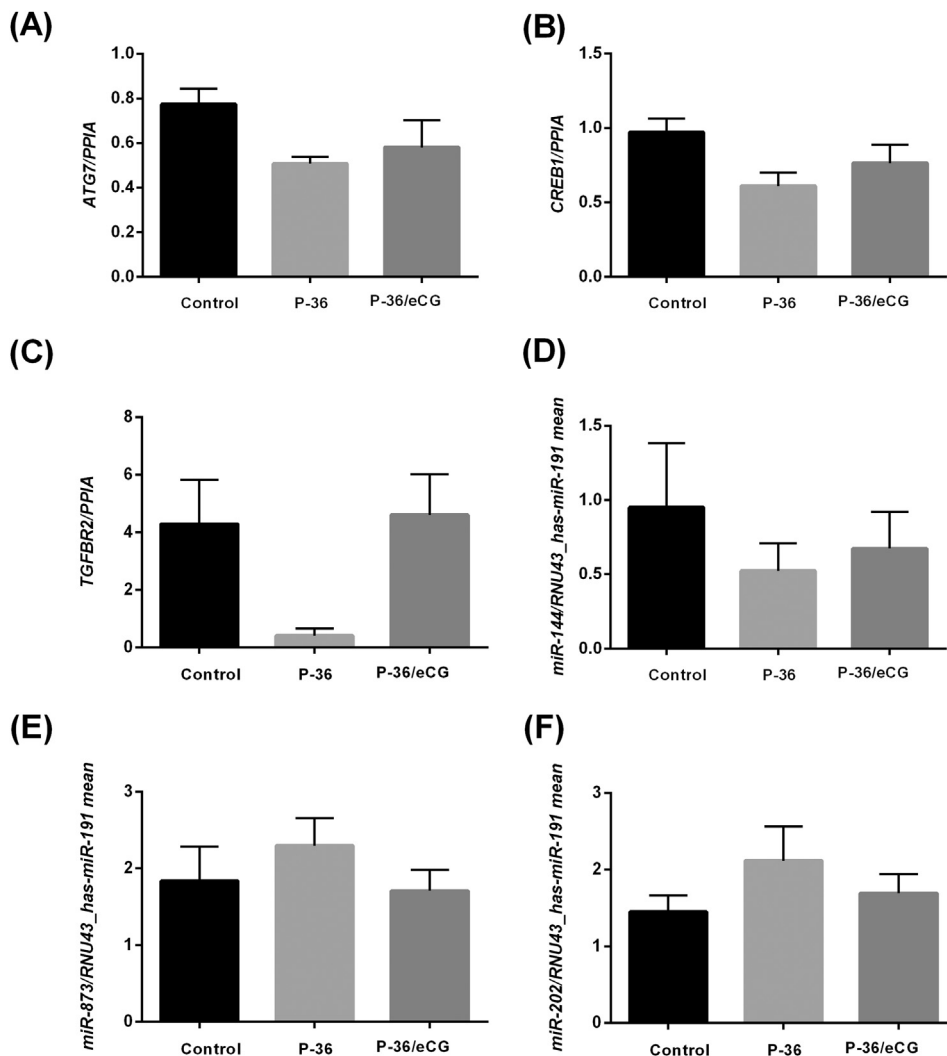

Fig. 4. Relative abundance (mean ± SEM) of *ATG7* (A), *CREB1* (B), *TGFBR2* (C) mRNA and the miRNAs has-miR-144 (D), hsa-miR-873 (E) and mmu-miR-202-5p (F) in bovine granulosa cells. Messenger RNA and miRNA abundance were measured by real-time PCR and expression values calculated by the ΔΔCt method with efficiency correction, are relative to that of a calibrator sample. Data are from 10 follicles/experimental group and presented as mean ± SEM.

cows subjected to FSH/eCG superstimulatory protocol. One effect to be considered is the inherent LH activity of eCG. eCG interacts quite well with ruminant FSH and LH receptors [52]. It is known that high concentrations of hCG or LH may lead to lower concentrations of the LH receptor on the cell surface, with concomitant decrease of all alternative *LHCGR* transcripts [53]. Thus, the LH activity of eCG could explain the absence of *LHCGR* up-regulation in granulosa cells from cows in the P-36/eCG group. eCG seems to be effective in increasing the abundance of mRNA of proteins involved in Gs-protein/adenylyl cyclase/cAMP/protein kinase A (PKA) system and phospholipase Cβ/IP3 pathways [30], which reinforce the role of eCG in the regulation of granulosa cell differentiation and its participation in the control of intracellular pathways involved in ovulation.

LRBP and some miRNAs are among the possible factors involved in LHCGR regulation in granulosa cells. LRBP is an mRNA binding protein that binds to the LHCGR coding region and represses its expression [54]. The negative correlation between *LRBP* expression and *LHCGR* mRNA regulation, at the follicular deviation stage in cattle, was also reported by Ereno et al. [55]. The authors suggest that the lower abundance of *LRBP* mRNA in dominant follicles is consistent with the involvement of LHCGR/LRBP system during follicle selection, to ensure the expression of *LHCGR* mRNA and the acquisition of ovulatory capacity. However, we did not observe any effect of ovarian superstimulation on the abundance of *LRBP* mRNA, suggesting that *LHCGR* in granulosa cells could not be regulated by this protein in response to superstimulation, as described for follicle deviation.

Post transcriptional regulation of *LHCGR* by miRNA in ovary has been reported in several species [21,56—58]. There are reports of miRNA expression and their specific roles in bovine ovary [59], including that of miR-222, which was reported by Hossain et al. [25] as a possible regulator of LHCGR expression. Besides that, Salilew-Wondim et al. [60] demonstrated the expression of miR-222 in theca and granulosa cells of bovine antral follicles with lower expression in granulosa cells from bovine dominant follicles. Corroborating the possible role of miR-222 in the development of antral follicles, recently study demonstrated higher abundance of miR-222 expression in theca and granulosa cells from atretic follicles [61]. Similarly, in our study, miR-222 expression was lower in granulosa cells from superstimulated cows, compared to that in control animals and was inversely proportional to the abundance of *LHCGR* mRNA. In addition, confirming the role of miR-222 in granulosa cell differentiation, possibly controlling the mRNA expression of LHCGR, superstimulatory protocols failed to regulate the mRNA abundance of *LHCGR* in theca cells (unpublished data). Taken together, these data suggest that the up-regulation of *LHCGR* in superstimulated granulosa cells could be supported by the down-regulation of miR-222 and that lower levels of miR-222 might be required to improve follicular health in bovine ovary and antral follicle development.

According to Gebremedhn et al. [62], the miRNAs miR-202 and miR-873 were up regulated in dominant follicular granulosa cells unlike in subordinate follicles, showing a possible involvement of miRNAs in follicular development. Additionally, Donadeu et al. [17] showed that miR-202 and miR-873 could be used in combination, as markers for steroidogenic capacity. Besides that, Sontakke et al. [23] demonstrated that the combination of miR-144, miR-202 and miR-873 may play a key role in the final maturation of the dominant follicle in cattle through their predicted target genes (*TGFBR2, ATG7* and *CREB1*), which are involved in multiple signaling pathways related to follicular cell proliferation, steroidogenesis, and prevention of premature luteinization. In the present study, the absence of any effect on these miRNAs used as steroidogenic markers and their predicted targets suggests that despite affecting specific pathways to promote the final maturation of preovulatory follicles (e.g. steroidogenic and ovulatory capacity), super-stimulatory treatments did not alter the abundance of biomarkers predicted to be essential for final maturation, indicating that follicles subjected to superstimulatory protocols could reach the ovulation stage.

Besides that, Sontakke et al. [23] found a linear correlation between the expression of *CYP19A1* and the expression of miR-144, miR-202 and miR-873 in dominant follicles. We found a similar correlation in the present study, since neither the abundance of miRNAs nor the expression of *CYP19A1* were affected by super-stimulation. Recently, Donadeu et al. [17] proposed that the abundance of miR-202 and miR-873 in combination with the expression of *CYP19A1* and *LHCGR* could be used to determine the steroidogenic capacity and health status of follicles. Thus, our results suggest that theses superstimulatory protocols were able to preserve the health status of the follicles.

Our findings also demonstrated that FSH stimulation decreased the expression of *FLT1* and *FLK1* and increased the expression of *VEGF*. VEGF is an important factor that promotes follicular development [63]. According to Ortega Serrano et al. [64], healthy follicles contain a vascular network that carries oxygen molecules, gonadotropins, and nutrients required by the follicles during the period of rapid cell proliferation and higher levels of estradiol synthesis. VEGF components are also associated with the selection of dominant follicles [65]. *FLT1* and *FLK1* mRNA are down-regulated in healthy follicles and are overexpressed in subordinate follicles [65]. Taken together, these data suggest that ovarian super-stimulation using FSH could improve antral follicle development through the up-regulation of VEGF and down-regulation of its cognate receptors.

5. Conclusion

Collectively, our data showed that superstimulatory treatments seemed to induce molecular changes in granulosa cells and modify the steroidogenic capacity through the transcriptional regulation of steroidogenic enzymes and FSH receptor. Moreover, the FSH seems to be a key hormone that increases the abundance of *LHCGR* mRNA in granulosa cells and, in combination with eCG or alone, is seems to be able to suppress the expression of miR-222.

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