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ANIMAL REPRODUCTION

Role of fibroblast growth factor 18 in regulating the cascade of pre-ovulatory events in cattle

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ABSTRACT: Suboptimal LH surges can lead to delayed or absent ovulation during reproductive management programs in cattle. Several signaling pathways may enhance the LH signal, potentially including growth factors. In the present study, we determined whether Fibroblast growth factor 18 (FGF18), which mimics some actions of the LH surge on granulosa cells, may potentiate the action of a suboptimal dose of LH using an established bovine granulosa cell (GC) culture system. Addition of recombinant FGF18 increased the concentration of progesterone (P4) in the culture medium. A combination of LH and FGF18 in the culture medium increased the abundance of mRNA encoding EREG, EGR1 and EGR3, proteins that are early modulators of the LH-induced pre-ovulatory cascade. This study demonstrated that FGF18 may be a co-factor in the regulation of the pre-ovulatory cascade in bovine ovarian granulosa cells.
Key words: FGF18, ovulation, granulosa cells.

Função do fator de crescimento fibroblástico 18 na regulação da cascata de eventos pré-ovulatórios em bovinos

RESUMO: Os picos de LH abaixo do ideal podem bloquear ou retardar a ovulação durante programas de manejo reprodutivo em bovinos. Várias vias de sinalização podem aumentar o sinal de LH, incluindo em especial fatores de crescimento. No presente estudo, determinamos se o fator de crescimento de fibroblastos 18 (FGF18), que estimula algumas ações do pico de LH nas células da granulosa, pode potencializar a ação de uma dose sub fisiológica de LH em um sistema de cultivo de células da granulosa bovina (GC). A adição de FGF18 recombinante aumentou a concentração de progesterona (P4) no meio de cultura. A combinação de LH e FGF18 no meio de cultura aumentou a abundância de mRNA para EREG, EGR1 e EGR3, proteínas que são moduladoras precoces da cascata pré-ovulatória induzida por LH. Este estudo demonstrou que o FGF18 pode ser um cofator na regulação da cascata pré-ovulatória em células da granulosa bovina.
Palavras-chave: FGF18, ovulação, células da granulosa.

INTRODUCTION

Sub-optimal fertility in cattle can cause considerable economical losses, at least part of which may be caused by failure to ovulate. For example, 10% of synchronized dairy cows exhibited delayed ovulation and suboptimal LH surges (BLOCH et al., 2006). The rupture of the follicle wall and release of the oocyte-cumulus complex involve a cascade of signaling events initiated by the LH surge. The initial stage of the ovulatory trigger is the release of epidermal growth factor (EGF)-like factors amphiregulin (AREG) and epiregulin (EREG) from the surface of mural granulosa cells (RICHARDS et al., 2002; SEKIGUCHI et al., 2004; PANIGONE et al., 2008); which then act in a paracrine manner to stimulate the EGF receptor on cumulus cells (PARK et al., 2004; ANDRIC & ASCOLI, 2008). A key element of follicle rupture is the production of prostaglandins by granulosa cells (ANDRIC & ASCOLI, 2008) by the enzyme, prostaglandin-endoperoxide synthase 2 (PTGS2) (DOS SANTOS et al., 2022). Prostaglandins act, at least in part, by stimulating the expression of proteases in the follicle wall, including plasminogen activators (PA) (PORTELA et al., 2015; RICHARDS et al., 2002; SARTORI et al., 2001), and EGF stimulates PA activity and inhibits abundance of PA inhibitors such as SERPINE2 (CAO et al., 2006).

The LH-induced pre-ovulatory cascade can be regulated by a number of additional pathways including angiotensin (PORTELA et al., 2011) and the Hippo pathway (SUN & DIAZ, 2019; DOS

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SANTOS et al., 2022). The activation of the Hippo pathway is controlled by intracellular effectors called YAP/TAZ; when YAP1 is phosphorylated, this protein is retained in the cytoplasm and does not induce gene transcription, whereas when the pathway is inactive, nonphosphorylated YAP translocates into the nucleus where it stimulated gene transcription and cell survival. The LH surge promotes the inactivation of YAP1 through the MAPK3/1 pathway in pre-ovulatory follicles (JI et al., 2017), and the Hippo pathway acts through expression of EGF receptor mRNA and protein, modulates *EREG* and *PTGS2* expression (DOS SANTOS et al., 2022) and results in the cessation of granulosa cell proliferation.

Another intraovarian factor that may modulate granulosa responsive to LH is FGF18. This growth factor is produced mainly by the endothelial cells of the ovary (ESTIENNE et al., 2022) and, like the LH surge, inhibits granulosa cell proliferation and decreases SERPINE2 protein abundance and estradiol production (PORTELA et al., 2010). We; therefore, hypothesized that FGF18 is involved as a co-factor involved in the LH-induced pre-ovulatory cascade and may potentiate LH signaling. The objectives of the present study were to verify the role of FGF18 associated with LH in the cellular regulation of pre-ovulatory events in cattle.

MATERIALS AND METHODS

Cell culture

Cells were cultured in a system that permits LH-responsive expression of mRNA encoding PTGS2 and EGF-like ligands (PORTELA et al., 2011). Ovaries were obtained from adult bovine females at different stages of the estrous cycle from a local slaughterhouse and transported to the laboratory in phosphate-buffered saline (PBS) at 35°C containing penicillin (100 IU/ml), streptomycin (100 µg/ml), and fungizone (1 µg/ml). Granulosa cells were aspirated from large follicles (≥ 10 mm in diameter) and washed three times in a culture medium (DMEM-F12) by centrifugation at 900 x *g* for 10 min. Cell viability was estimated using 0.4% trypan blue. Cells were seeded into 24-well culture plates (Sarstedt®) at a density of 1 x 10^6 viable cells per well in 1 ml DMEM-F12 supplemented with sodium bicarbonate (10 mM), sodium selenite (4 ng/ml), BSA (0.1 %), penicillin (100 IU/ml), streptomycin (100 g/ml), bovine transferrin (2. 5 g/ml), non-essential amino acids (1.1 mM), androstenedione (10^{-7} M), FSH (1 ng/ml), insulin (10 ng/ml), and 2 % fetal bovine serum (FBS).

Cell cultures were maintained at 37°C in 5% CO2 for 24 h. Afterward, the culture medium was replaced (100%) with DMEM-F12 without FBS or supplementation for 18 h before experimental treatments described below. For each treatment, 6 wells were used for RNA extraction and progesterone quantification.

Experimental design

To determine the effect of FGF18 on progesterone secretion, cells were treated with LH (10 ng/ml), with FGF18 (10 ng/ml) (PeproTech – FUNPEC, Ribeirão Preto, SP, Brazil) or with LH (10 ng/ml) plus FGF18 (10 ng/ml); controls received vehicle (PBS) alone. Medium and cells were recovered after 6, 12 and 24 h culture; medium was frozen before P4 assay and total cell protein was extracted to express P4 relative to cell number.

To determine the effect of FGF18 on the gene expression, cells were cultured with LH (10 ng/ml), with FGF18 (10 ng/ml) or with LH (10 ng/ml) plus FGF18 (10 ng/ml). Cells were recovered at 6, 12 and 24 h of treatment and RNA extracted to evaluate abundance of mRNA of the LH-responsive genes *AREG, EREG, EGR1, EGR3,* and *PTGS2,* and the Hippo pathway target genes ankyrin repeat domain contamination protein 1 (*ANKRD1*), connective tissue growth factor (*CTGF*), YAP1, baculoviral IAP repeat-containing 5 (BIRC5; also known as survivin), and cysteine-rich angiogenic inducer 61 (*CYR61*).

The doses of FGF18 and LH were chosen as they have been shown to be the minimal effective doses previous studies with granulosa cells (PORTELA et al., 2010; PORTELA et al., 2015), and a lower dose of LH was selected to mimic 'sub optimal' LH surges oberved in animals with delayed ovulation (BLOCH et al., 2006). All experiments were performed on three independent biological replicates.

Nucleic acid extraction and reverse transcriptase reaction

Total RNA was extracted using a PureLink RNA Mini Kit-based protocol according to the manufacturer's instructions (Invitrogen) and quantified by measuring UV absorbance at 260 nm using NanoDrop technology. DNase I treatment was performed after purification (referring to the PureLink™ RNA Mini Kit manual) to ensure highly pure RNA without genomic DNA contamination. RNA (200 ng) was subjected to reverse transcription by adding RNase-free water, 5x Mix iScript

(containing primer and dNTPs), and iScript enzyme for a total volume of 20 µl.

The reaction was terminated by incubation at 25°C for 5 min, 46°C for 20 min, 95°C for 1 min, and 4°C for 10 min. Negative controls were run with water instead of RNA to verify the absence of genomic RNA.

Polymerase chain reaction (PCR)

All genes were measured by real-time PCR in a thermocycler (Bio-Rad, Hercules, CA, USA) using GoTaq® Master Mix (Promega Corporation, Madison, USA) as a reagent. For these experiments, common thermal cycling parameters were used (3 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C) to amplify each transcript. A dissociation curve analysis was performed for each transcript to verify that no extraneous products were present. The samples were processed in duplicate and expressed relative to histone H2AFZ as a constitutive gene. A standard curve was generated to determine the efficiency of the primers using 2-fold serial dilutions of the samples and to measure the genes according to the standard curve results. The primers for the genes used in this study are listed in table 1. Negative controls were run water replacing cDNA.

Progesterone quantification

Progesterone concentrations were determined using an ELISA kit (Cayman Chemical Company, Ann Arbor, MI, USA) as described by PORTELA et al. (2011). Progesterone was measured in duplicate, as previously described, with mean intra- and inter-assay coefficients of variation of 7.2% and 18%, respectively. The sensitivity of the assay was 4 pg/tube, which is equivalent to 20 ng/mg protein.

Statistical analysis

Statistical analysis were performed using the JMP software (SAS Institute, Cary, NC). Data were transformed to logarithms if they were not normally distributed (Shapiro Wilk test). Gene expression (mRNA) and steroid concentration results were compared using an analysis of variance (PROC GLM; General Linear Model Procedure). Differences between means were tested with Fisher protected least significant difference test or by Dunnett test for specific comparisons with controls. Main effects of treatments, time and time-treatment interaction were tested, with culture replicate as a random effect in the model. Data are expressed as least squares means and means considered different at $P < 0.05$.

RESULTS

Treatment of granulosa cells with FGF18 significantly increased the concentration of P4 in medium ($P < 0.05$) at 6 h, but not at 12 h and 24 h of culture. Neither LH alone nor LH plus FGF18 altered P4 secretion (Figure 1).

Treatment with LH or with FGF18 alone did not significantly alter *EGR1* or *EGR3* mRNA abundance, however the combination of LH and FGF18 significantly increased abundance of mRNA encoding both EGR1 and EGR3 (Figure 2).

Addition of LH stimulated *EREG* mRNA abundance after 6 and 12 h of treatment, but not at 24 h, whereas the addition of FGF18 alone increased *EREG* mRNA abundance at 12 h.

Coculture with LH plus FGF18 did not further increase the effect of LH at 6 h. Neither LH nor FGF18, alone or in combination, significantly affected *PTGS2* or *AREG* mRNA abundance (Figure 2).

Table 1 - Gene and primers sequence used.

Gene symbol	----------Forward primer----------	----------Reverse primer----------
H2AFZ	GAGGAGCTGAACAAGCTGTTG	TTGTGGTGGCTCTCAGTCTTC
EGR1	TCCCCTGTTCACAATGGTTT	TGGGAGAAAAGGTGGTTGTC
EGR3	GGAGCAAATGAAATGTTGGTG	AGGAAAACCTATGGGGAATG
AREG	CTTTCGTCTCTGCCATGACCTT	CGTTCTTCAGCGACACCTTCA
EREG	ACTGCACAGCATTAGTTCAAACTGA	TGTCCATGCAAACAGTAGCCATT
CTGF	AGCTGAGCGAGTTGTGTACC	TCCGAAAATGTAGGGGGCAC
CYR61	GGCTCCCCGTTTTGGAATG	TCATTGGTAACGCGTGTGGA
BIRC5	CTGAGAACGAGCCCGACTTG	ATGTTCTTCTATAGGGTCGTCATCT
ANKRD1	ATCAGTGCGCGGGATAAGTT	GGGAGTATCTCCTTCCCGGT
YAP1	TCCTTTGAGATCCCTGACGATG	TGACGTTCATCTGGGAGAGC
COX-2	CCTGTGTTCCACCAGGAGAT	CCCTGGCTAGTGCTTCAGAC

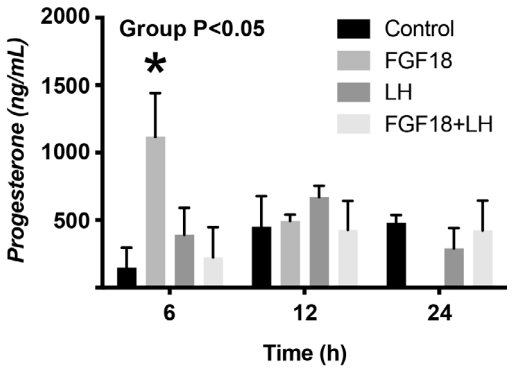

Figure 1 - Effects of FGF18 and LH on the secretion of progesterone (P4) from bovine granulose cells. Cells were cultured for 18 h in serum-free medium without supplementation and collected at 6, 12, and 24 h after adding FGF18 and LH (10 ng/ml) to treatment groups. Data are presented as means ± SEM of three independent replicates. The asterisk denote statistical differences between the groups by time ($^{*}P < 0.05$).

None of the treatments altered abundance of mRNA encoding ANKRD1, CTGF, CYR61 or YAP1, whereas treatment with LH or FGF18 alone, but not combined, caused a significant ($P < 0.05$) reduction in *BIRC5* mRNA abundance at 24 h of treatment (Figure 3).

DISCUSSION

The objectives of the present study were to determine whether FGF18 acts on pre-ovulatory granulosa cells and whether it enhances elements of the LH-induced pre-ovulatory cascade. The results suggest that FGF18 may acutely stimulate progesterone secretion and may cooperate with LH to enhance the expression of key upstream genes including *EGR1*, *EGR3* and *EREG*. These data add another layer to the complex regulation of the LH-induced ovulatory process.

Delayed or failed follicle rupture could be caused by suboptimal LH surges, and the present data suggest that treatment of granulosa cells with FGF18 could enhance some typical LH-dependent responses including progesterone secretion, expression of the upstream transcription factors EGR1 and EGR3 and the growth factor EREG. It is known that granulosa cell progesterone secretion increases in response to the LH surge and that progesterone is essential for ovulation (LIPNER & GREEP, 1971; SNYDER et al., 1984; ROBKER et al., 2000); therefore, the increase in progesterone secretion by FGF18 could enhance ovulatory or post-ovulatory events. This result is; however, in constrast to a study showing that FGF18 decreased granulosa cell progesterone secretion (PORTELA et al., 2010), but the two studies used a different culture system; that of PORTELA et al., (2010) used a serum-free non-luteinizing model with cells from small follicles that are not responsive to LH, whereas the current study used cells from large follicles that are responsive to LH.

Another positive effect of FGF18 was observed for abundance of *EGR1*, *EGR3* and *EREG* mRNA when added in combination with LH. High (ovulatory) doses of LH increased the expression of EREG in vivo and in vitro in cattle (PORTELA et al., 2011; SAYASITH et al., 2013), and LH (GnRH) stimulated EGR1 mRNA in the rat ovary (ESPEY et al., 2000) and EGR1 protein in the bovine ovary (DA ROSA et al., 2016), suggesting that FGF18 could enhance the ability of LH, especially at lower doses, to initiate the pre-ovulatory cascade.

These data are consistent with the ability of FGF18 to increase *EGR1* mRNA abundance in FSH-stimulated granulosa cells (JIANG et al. 2013).

The role of EGR3 in ovulation has not been reported, and FGF18 did not increase *EGR3* mRNA abundance in granulosa cells from small follicles (HAN et al., 2017); therefore, the increase in EGR3 mRNA levels in LH-stimulated 'pre-ovulatory' granulosa cells is a novel observation. Overexpression of EGR1 increased both *EREG* and *EGR3* mRNA abundance in granulosa cells of small follicles, suggesting that the increase in *EREG* and *EGR3* mRNA abundance by LH plus FGF18 treatment in the present study may be downstream of an increase in *EGR1* mRNA production.

Despite the above increases in upstream mRNA abundance, there was no effect on the abundance of mRNA encoding PTGS2, the enzyme responsible for prostaglandin synthesis and a major target of LH signaling in the pre-ovulatory cascade. This is likely due to the suboptimal levels of LH, and also a limitation of this culture system in which *PTGS2* mRNA abundance is not consistently increased by LH (PORTELA et al., 2011).

Additional signaling pathways have been implicated in the ovulatory cascade, including the Hippo pathway. Inhibition of YAP-TEAD transcriptional activity reduced EGR1 and EREG mRNA abundance in EGF-treated granulosa cells using the same culture system as employed herein, and reduced ovulation in

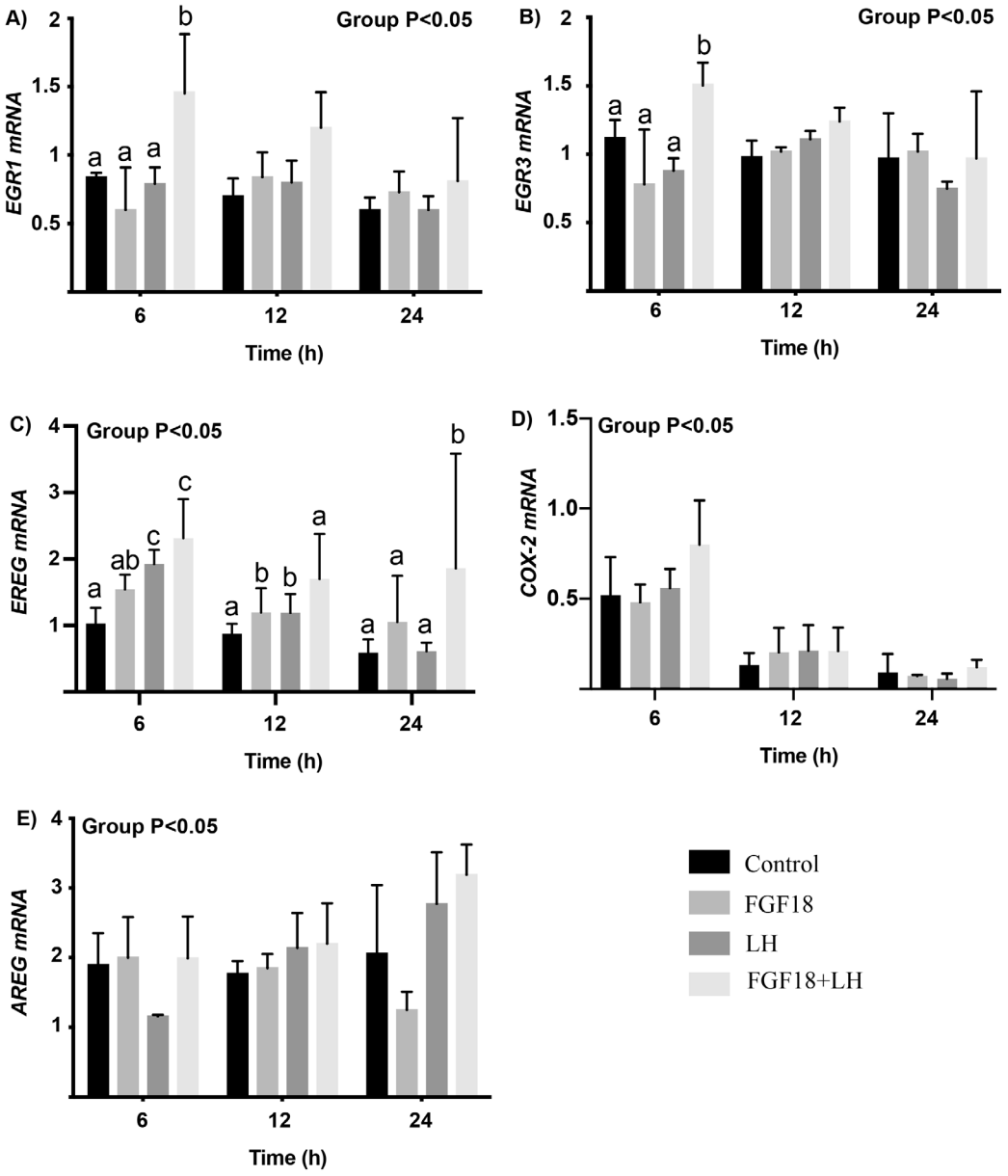

Figure 2 - Effects of FGF18 and LH on the expression of genes critical for ovulation. Cells from large follicles (≥10 mm) were cultured for 18 h in serum-free medium without supplementation and collected at 6, 12, and 24 h after adding FGF18 and LH (10 ng/ml) to treatment groups. mRNA abundance was measured using real-time PCR. Data are presented as means ± SEM of three independent replicates. Different letters show statistically significant differences between all treatment means by time within this experiment (P < 0.05).

vivo in cattle (DOS SANTOS et al., 2022), as well as typical Hippo target genes *CCN1* (also known as CYR61) and *CCN2* (also known as CTGF). However, in the present study, abundance of neither *CCN1* nor *CCN2* were altered by suboptimal LH concentrations or the combination of LH and FGF18.

CONCLUSION

We concluded that FGF18 was able to potentiate the action of low doses of LH in a model of pre-ovulatory bovine granulosa cells, specifically by stimulating progesterone secretion and abundance of

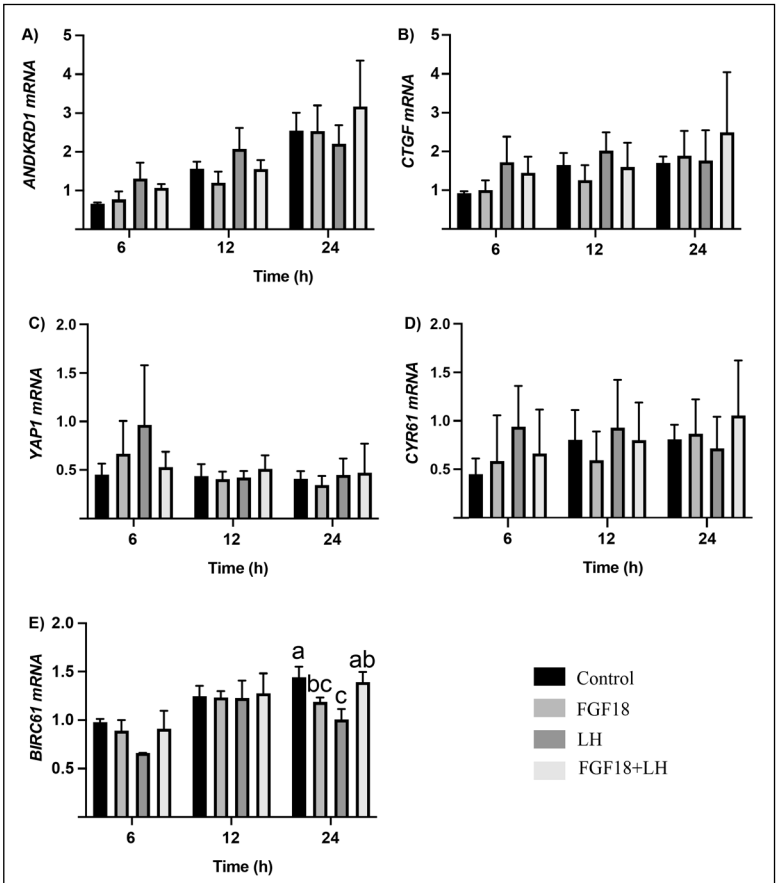

Figure 3 - Effects of FGF18 and LH on the expression of the Hippo pathway target genes during the pre-ovulatory cascade. Cells from large follicles (≥10 mm) were cultured for 18 h in serum-free medium without supplementation and collected at 6, 12, and 24 h after adding FGF18 and LH (10 ng/ml) to treatment groups. mRNA abundance was measured using real-time PCR. Data are presented as means ± SEM of three independent replicates. Different letters show statistically significant differences between all treatment means by time within this experiment ($P < 0.05$).

mRNA of the upstream pre-ovulatory genes EGR1, EGR3, and EREG. If validated in in-vivo studies, this may lead to strategies to increase ovulation efficiency in bovine reproductive management programs.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHORS' CONTRIBUTIONS

All authors contributed equally to the conception and writing of the manuscript. All authors critically revised the manuscript and approved the final version.

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