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Effects of Prostaglandins E2 and F2α on the in vitro maturation of bovine oocytes

Sarah A.D. Rodrigues[a], Thais P. Pontelo[b], Nayara R. Kussano[c], Taynan S. Kawamoto[d], Ligiane O. Leme[e], Felippe M.C. Caixeta[a], Luiz F.M. Pfeifer[f], Mauricio M. Franco[d,g], Margot A.N. Dode[a,g,*]

[a] University of Brasília, Animal Science, Brasilia, DF 70910-900, Brazil
[b] Federal University of Lavras, Veterinary Science, Lavras, MG 32700-000, Brazil
[c] University of Brasília, Animal Biology, Brasilia, DF 70910-900, Brazil
[d] Federal University Uberlândia, Animal Science, Uberlândia, MG 38400-902, Brazil
[e] Federal University of Espírito Santo, Animal Science, Vitória, ES 29075-073, Brazil
[f] Embrapa Rondônia, Porto Velho, RO 76815-800, Brazil
[g] Embrapa Genetic Resources and Biotechnology, Brasília, DF 70770-917, Brazil

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ABSTRACT

We aimed to elucidate the effects of PGE2 and PGF2α on the in vitro maturation (IVM) of bovine oocytes. First, cumulus-oocyte complexes were matured in the media supplemented with or without PGE2, PGF2α, or PGE2 plus PGF2α for the final 24, 12, or 6 h of culture. Then, the cumulus-oocyte complexes were matured in the absence or presence of a PG endoperoxide synthase 2 (PTGS2) enzyme inhibitor (NS398) supplemented with PGE2, PGF2α, or PGE2 plus PGF2α. Finally, the expression of genes associated with PGs activity in cumulus cells (PTGS2, PG E-synthase-1 [PTGES1], and aldo-keto reductase 1 [AKR1B1]) or oocytes (receptors for PGE2 [PTGER2] and PGF2α [PTGFR]) of different competencies was quantified. Supplementation of the IVM medium with PGs did not improve in vitro embryo production or embryo quality ($P > 0.05$). During maturation, the relative abundance of PTGS2 transcripts increased ($P < 0.05$) only in the less-competent group, whereas those of PTGES1 increased in the less-competent and in the more-competent groups. Conversely, AKR1B1 expression decreased only in the less-competent group ($P < 0.05$). Receptors for the PGE2 and PGF2α genes were very low or undetectable in oocytes. In conclusion, PGE2 and PGF2α are not recommended for media supplementation during maturation because they have no effect on embryo development. Although genes related to PGs activity are differentially expressed in cumulus cells of cumulus-oocyte complexes of different competence during maturation, the expression of PGE2 and PGF2α receptor genes was either not detectable or was detected at low levels in oocytes.

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1. Introduction

For in vitro embryo production (IVP) and other assisted reproduction techniques in domestic animals, oocytes are removed prematurely from the follicular environment and then a heterogeneous population of oocytes with different levels of competence is used. Such a difference in oocyte competence leads to a lower rate of embryo production compared with that obtained with in vivo matured oocytes [1]. This suggests that to increase IVP efficiency, a better understanding of the mechanisms involved in the maturation process is necessary so that the oocytes can be provided with adequate conditions for complete maturation.

* Corresponding author. Tel.: 055 61 3448 4700x4692; fax: 055 61 3340 3624.
E-mail address: margot.dode@embrapa.br (M.A.N. Dode).

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One alternative to improve maturation conditions is to mimic the in vivo environment in which oocyte maturation occurs physiologically. Research in this area has focused on the addition of substances that are normally present in follicular fluid or are necessary for the final preparation of the follicle for ovulation. Various substances have already been tested in the medium during in vitro maturation (IVM), such as growth factors [2–4], antioxidants [5,6], and hormones [7,8]. However, the results have been inconsistent regarding the compounds that should be added during maturation to obtain better results in IVP.

In the follicle environment, the preovulatory peak of LH stimulates maturation, which is completed just before ovulation [9]. During this period, in which maturation occurs and the follicle rupture approaches, the intrafollicular concentration of PGE2 and PGF2α increases in several species [10–13]. This suggests that these PGs may play a role in ovulation and oocyte maturation processes.

Prostaglandins are products of the metabolism of arachidonic acid, synthesized from the sequential actions of the PG endoperoxide synthase 2 (PTGS2) enzyme and specific terminal synthases, including PG E synthase (PTGES1–S3) and PG F synthase (PTGFS). Although the role of PTGES in the synthesis of PGE2 has been well characterized [14–18], little is known about the specific enzyme responsible for the production of PGF2α. Despite the lack of information, the main PTGFS reported in the literature belong to the aldo-keto reductase 1C (AKR1C) and 1B (AKR1B1) family [19,20].

Previous studies have reported that the presence of PGE2 in the oocyte microenvironment stimulatescumulus cell (CC) expansion [21,22], cell cycle progression, and initial embryonic development [23], as well as promoting the regulation of apoptosis [24]. In fact, the presence of PGE2 receptors on the oocyte and the COC has also been demonstrated, indicating the possible direct action of PGE2 in the oocyte [22,23]. Although PGF2α is often related to the ovulatory process in ruminants [25], few studies have been carried out to identify the role of PGF2α during COC maturation. However, Barreta et al [26] demonstrated that PGF2α added during IVM induces the resumption of meiosis in the same way as PGE2, suggesting a possible role for this PG in IVM.

Based on these considerations, we hypothesized that the inclusion of PGE2 and PGF2α in the IVM culture improves oocyte maturation and embryo production. We evaluated the addition of both PGs at various time points during IVM and used a specific PTGS2 inhibitor, NS398, to elucidate the effect of PGs in maturation. We also attempted to determine whether the competence of the oocyte could affect the expression profile of genes encoding the enzymes involved in PGs synthesis and activity.

2. Material and methods

Unless otherwise indicated, chemicals were purchased from Sigma (St. Louis, MO).

2.1. Recovery and selection of cumulus-oocyte complexes

Ovaries from crossbred females (*Bos indicus* × *Bos taurus*) were collected from local abattoirs immediately after slaughter, and transported in saline solution (0.9% NaCl) supplemented with antibiotics (100 mg/mL streptomycin and 100 IU/mL G penicillin) at temperatures of 35°C to 36°C. Cumulus-oocyte complexes were aspirated from follicles of 3 to 8 mm diameter using an 18-gauge needle attached to a 10 mL syringe. Only the COCs presenting homogenous cytoplasm and at least 3 layers of CCs were used.

To obtain COCs with differing degrees of competence, we used the follicle size model previously described [27–29]. Briefly, follicles were dissected from the ovarian cortex, measured using a graduated ocular (eyepiece micrometer OSM-4 Olympus, Tokyo, Japan), and morphologically classified. Follicles measuring 1 to 2.9 mm and 6 to 8 mm in diameter were selected, and the oocytes were used to represent less and the more competent oocytes, respectively. The COCs were released from the follicles by rupturing the follicle wall. Only those with at least 3 layers of CCs and a homogeneous cytoplasm were used for further analysis.

2.2. In vitro maturation

After selection, COCs were washed and transferred to droplets of the maturation medium. In the experiments with oocytes with differing degrees of competence, the COCs were transferred to 50 μL (≤10 oocytes) or 100 μL (≥11 oocytes) droplets of IVM medium and covered with mineral oil. In the other experiments, groups of up to 50 COCs were transferred to a 5-well culture dish (WTA, Cravinhos-SP, Brazil) containing 500 μL of the IVM medium. The IVM medium consisted of TCM-199 with Earl's salts supplemented with 0.4% fatty acid–free BSA, 0.075 mg/mL amikacin, 1 μM pyruvate, 1 μM cysteamine, and 0.01 UI/mL FSH. The culture was performed for 24 h at 39°C and 5% CO_2 in air.

To evaluate cumulus expansion, each COC was measured using the Motic Image Plus 2.0 program (Motic China Group Co, Ltd, Xia-men, China) before and after maturation. Cumulus cell expansion during IVM was determined by the difference between the mean area of all COCs from each treatment before and after IVM.

For gene expression analyses, oocytes and CCs were collected after 0 and 24 h of maturation and stored in RNAlater (Ambion, Life Technologies, Carlsbad, CA) at −80°C until RNA extraction. To remove CCs, the COCs were transferred to a drop of 50 μL saline in PBS, where they were stripped by successive pipetting until complete removal of the CCs. After being completely denuded, the oocytes were washed 3 times in PBS without magnesium and calcium and individually transferred (2 μL) to a 0.2 mL tube. Then, twice the volume of RNAlater was added to the tube. The 50 μL drop of PBS containing the CCs removed from COCs was washed by centrifuging twice for 2 min at 700 × *g*. The supernatant was removed and RNAlater was added to the pellet.

2.3. Embryo production

After maturation, groups of up to 50 COCs were transferred to 200 μL drops of the fertilization medium, which consisted of Tyrode's albumin lactate pyruvate supplemented with penicillamine (2 mM), hypotaurine (1 mM), epinephrine (250 mM), and heparin, 10 mg/mL

[30]. Frozen semen from a bull of proven fertility was used for all treatments and replicates; this bull has been used for several years as a reference bull for IVP in our laboratory. Motile spermatozoa were obtained using the Percoll (GE Healthcare, Piscataway, NJ) gradient method in microtubes [31] and were added to the fertilization drop at a final concentration of 1×10^6 spermatozoa/mL. Spermatozoa and oocytes were coincubated for 18 h at 39°C with 5% CO_2 in air. The day of in vitro insemination was considered day 0. Eighteen hours after insemination, the presumptive zygotes were washed and transferred to 200 μL drops of synthetic oviduct fluid medium with amino acids, citrate, and inositol [32] supplemented with 0.4% BSA-FAF and 0.5 μg/mL of ITS (insulin 10 mg/L, transferrin 5.5 mg/L, selenium 5 μg/L) and incubated at 39°C in an atmosphere of 5% CO_2 in air. Embryos were evaluated on day 2 for cleavage and on days 6 and 7 for blastocyst rates.

2.4. Total cell count and apoptosis rate

To determine the total number of cells and percentage of apoptotic cells, D7 expanded blastocysts were submitted to TUNEL analysis and H33342 treatment using the Click-iT TUNEL Alexa Fluor Thermo Fisher Kit (Waltham, MA). Expanded blastocysts were washed in PBS supplemented with polyvinyl pyrrolidone (PVP; 1 mg/mL) and then fixed in 3.7% paraformaldehyde for 15 min. After washing in 1 mg/mL PVP, the blastocysts were incubated in 0.5% Triton-X for 20 min. Finally, the embryos were exposed to the TUNEL and Alexa Fluor 488 enzymatic mix for 1 h at 37°C and later to H33342 for 10 min. Finally, the embryos were washed with PBS-PVP, mounted on glass slides, and observed under a fluorescence microscope, using 495/519 nm excitation wavelength filters for Alexa Fluor 488 and 350/461 nm excitation for H33342. For each blastocyst, the total number of individual cells (blue nuclei, H33342) and the total number of apoptotic cells (green nuclei, TUNEL) were determined.

2.5. Real-time quantitative PCR

To analyze gene expression, the relative abundance of transcripts for 5 genes associated with PGs activity was analyzed. PTGS2, PTGES1, and AKR1B1 were quantified in CCs using RT-qPCR, whereas the receptors for PGE2 (PTGER2) and PGF2α (PTGFR) were quantified in oocytes. The primer sequences, amplicon size, primer efficiency, and primer concentration are listed in Table 1.

Total RNA was isolated from 4 pools of CCs obtained from 17 COCs and 3 pools of 6 or 10 oocytes for each treatment using the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA was used for cDNA synthesis, which was performed using the Super Script III First-Strand cDNA Synthesis kit (Invitrogen Carlsbad, CA), following the manufacturer's recommendations in a final volume of 30 μL. Complementary deoxyribonucleic acid was synthesized in a thermocycler at 65°C for 5 min and 50°C for 50 min, followed by inactivation of the enzyme at 85°C for 5 min. Quantitative polymerase chain reaction was performed using the Fast SYBR Green Master Mix kit (Applied Biosystems, Foster City, CA). Each biological sample was analyzed in triplicate, and the specificity of each PCR product was determined by melting curve analysis and determination of the amplicon size in agarose gel. Reactions were carried out in a final volume of 25 μL using a cDNA template corresponding to 0.88 CCs or 0.66 oocytes per reaction, according to each experiment. The conditions of the qPCR were 95°C for 5 min, followed by 50 cycles of denaturation at 95°C for 10 s, and annealing and extension at 60°C for 30 s.

To determine which housekeeping gene to use for data normalization, 3 constitutive genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, and peptidylprolyl isomerase A, were analyzed using the GeNorm program [33]. The analysis revealed GAPDH to be the most stable gene for use in subsequent experiments with CCs and PPIA for use in experiment with oocytes. The relative expression of each gene was calculated using the DDCt method with efficiency correction by the Pfaffl method [34].

The expressions of genes not detected in oocytes were assessed in different tissues (corpus luteum [CL]: 35 ng/reaction; endometrium [End]: 35 ng/reaction; placenta [Plac]: 50 ng/reaction; CCs [CC1: 0.8 CC/reaction and CC2: 1.6 CC/reaction]) and in different concentrations of cDNA from oocytes (0.6 oocyte/reaction: 1.2 oocytes/reaction and

Table 1
Information of specific primers used for amplification of gene fragment analysis by real-time PCR.

Gene	Primer sequence	Amplicon size (bp)	Primer concentration (nM)	Primers efficiency	GenBank n°/reference
GAPDH	F: GGC GTG AAC CAC GAG AAG TATAA R: CCC TCC ACG ATG CCA AAG T	118	300	94.54	NM_001034034.2
PTGS2	F: GAG GAA CTT ACA GGA GAG AAG R: CGG GAG AGC ATA TAG GAT TAC	193	250	105.3	NM_174445.2
PTGES1	F: TGG TCA TCA AAA TGT ACG TGG T R: AGT AGA CAA AGC CCA GGA ACA G	201	200	103.62	NM174443
AKR1B1	F: GTC TCC AAC TTC AAC CAT CTC R: CTA TAG GCA GTC ACC ACA ATG	158	300	109.23	NM_001012519.1
PTGER2	F: GCC ACT CTG CTG CTG TTG C R: CCA TGG ATA CCC TTT CCG CT	151	200	92.59	Nuttinck et al, 2011 [23]
PTGFR	F: CAT CCA GCT TCT GGG TAT AAT G R: GCGTCTGGTACACACATATAG	220	300	87.43	NM_181025.3

Abbreviations: F, primer forward; R, primer reverse; bp, base pairs.

2.4 oocytes/reaction). Those PCR products were run on gels to confirm their presence and identity based on the expected size, corroborating the Tm and CT data.

2.6. Experimental design

2.6.1. Experiment 1: effects of PGE2 and PGF2α during IVM of bovine oocytes on embryo production

This experiment aimed to evaluate whether the addition of PGs during IVM of bovine oocytes would improve embryo production. Initially, the COCs obtained by follicular aspiration (3–8 mm diameter) were selected and distributed into 4 groups: (1) CTL, control; (2) PGE2, the maturation medium was supplemented with 1 μM of PGE2; (3) PGF2α, the maturation medium was supplemented with 1 μM of PGF2α; and (4) PGE2 + PGF2α, the maturation medium was supplemented with 1 μM of PGE2 and 1 μM of PGF2α. The cleavage rate on D2 and the blastocyst rates on D6 and D7 were evaluated.

Then, we aimed to determine whether the addition of PGs at different time points during IVM would affect subsequent embryo production. Owing to the number of treatments, it was not possible to supplement the media with PGE2 and PGF2α simultaneously; therefore, 2 separate experiments were carried out. For PGE2, COCs obtained by follicular aspiration (3–8 mm in diameter) were selected and distributed into 4 treatment groups: (1) CTL; (2) PGE2 during the 24 h of IVM; (3) PGE2 during the last 12 h of IVM; and (4) PGE2 during the last 6 h of IVM. In this sequence, the PGF2α effect was evaluated in a similar manner as PGE2, using the same treatments; the difference was that instead of PGE2, the PGF2α was added during the 24 h, the last 12 h, and the last 6 h of IVM. Cleavage on D2 and blastocyst rate on D6 and D7 were evaluated. Concentrations of PGE2 and PGF2α used in all the experiments were based on those reported by Barreta et al [26].

2.6.2. Experiment 2: effects of PTGS2 inhibition during maturation on cumulus expansion and embryo production

To ensure that the presence of PGs does not affect oocyte IVM, we selected a PTGS2 inhibitor, which would prevent the synthesis of both PGE2 and PGF2α. The COCs were matured in the presence of a specific inhibitor of PTGS2 (NS398), and the medium was supplemented with either PGE2 or PGF2α. The COCs obtained by follicular aspiration (3–8 mm in diameter) were selected and distributed into 5 groups: (1) CTL; (2) NS398, the maturation medium was supplemented with 10 μM of NS398; (3) NS398 + PGE2, the maturation medium was supplemented with 10 μM of NS398 and 1 μM of PGE2; (4) NS398 + PGF2α, the maturation medium was supplemented with 10 μM of NS398 and 1 μM of PGF2α, and (5) NS398 + PGE2+ PGF2α, the maturation medium was supplemented with 10 μM of NS398, 1 μM of PGE2, and 1 μM of PGF2α. Expansion of the CCs during maturation and the cleavage and blastocyst rates were evaluated for each group. The total number of cells and percentage of cells with DNA fragmentation was determined by the TUNEL test in expanded D7 blastocysts. The concentrations of NS398 used were according to Marei et al [17].

2.6.3. Experiment 3: expression of genes involved in the activity of PGE2 and PGF2α during maturation in CCs and oocytes with different levels of competence

In this experiment, we aimed to evaluate whether the expression of genes related to PGs activity varies during IVM. Considering that the in vivo expression of PTGS2, which is essential for PGs synthesis, depends on follicle size, we aimed to determine the association between gene expression and the oocyte competence. The expression of PTGS2 (PGE2 and PGF2α synthesis), PTGES1 (PGE2 specific synthase), and AKR1B1 (PGF2α specific synthase) were evaluated in CCs, and that of PTGER2 (PGE2 specific receptor) and PTGFR (PGF2α specific receptor) were evaluated in oocytes from COCs of different categories. The COCs used were obtained from follicles of 6 to 8 mm diameter and from follicles of 1 to 2.9 mm in diameter, which were previously described [27–29] as more competent (COM) and less competent (INC), respectively.

3. Statistical analyses

Data on embryo development were analyzed by the chi-square test ($P < 0.05$), total cell number by ANOVA, and DNA fragmentation (TUNEL) by the Kruskal–Wallis test. Cumulus cell expansion and gene expression were compared between treatments by ANOVA and Tukey's test if the data were normally distributed, whereas data not normally distributed were evaluated using the Kruskal–Wallis test. Differences were considered statistically significant at the 95% confidence level ($P < 0.05$). All analyses were performed using Prophet Statistics software, version 5.0 (BBN Systems and Technologies, Cambridge, MA; 1996).

4. Results

4.1. Experiment 1: effect of PGE2 and PGF2α during IVM of bovine oocytes on embryo production

When COCs were cultured in the maturation media supplemented with PGE2, PGF2α, or PGE2 and PGF2α, no differences ($P > 0.05$) in the cleavage rate were observed, except for the PGE2 group, which presented a lower ($P < 0.05$) rate than the CTL group. However, the blastocyst rates

Table 2
Embryo development of bovine oocytes submitted to in vitro maturation for 24 h in the absence (CTL) or in the presence of PGE2, PGF2α, or PGE2 associated to PGF2α.

Treatments	No total	D2	D6	D7
		Cleavage N° (%)	Blastocysts N° (%)	Blastocysts N° (%)
CTL	97	79 (81.4)[a]	7 (7.2)	22 (22.6)
PGE2	81	51 (62.9)[b]	6 (7.4)	23 (28.3)
PGF2α	68	52 (76.4)[ab]	7 (10.2)	21 (30.8)
PGE2 + PGF2α	104	77 (74.0)[ab]	11 (10.5)	30 (28.8)

Abbreviations: CTL, control; D2, day 2 of in vitro embryo development; D6, day 6 of in vitro embryo development; D7, day 7 of in vitro embryo development.
[a,b]Different letters in the same column indicate significant differences ($P < 0.05$). Data analyzed by the chi-square test ($P > 0.05$).

Table 3
Embryo development of bovine oocytes submitted to in vitro maturation for 24, 12, or the last 6 hours in the absence (CTL) or in the presence of the PGE2.

Treatments	N° total	D2	D6	D7
		Cleavage N° (%)	Blastocysts N° (%)	Blastocysts N° (%)
CTL	231	202 (87.4)^{ab}	25 (10.8)^{ab}	73 (31.6)
PGE2 24 h	211	187 (88.6)^{a}	24 (11.4)^{a}	62 (29.4)
PGE2 12 h	223	183 (86.5)^{b}	19 (8.5)^{ab}	58 (26.0)
PGE2 6 h	214	189 (88.3)^{ab}	13 (6.1)^{b}	59 (27.6)

Abbreviations: CTL, control; D2, day 2 of in vitro embryo development; D6, day 6 of in vitro embryo development; D7, day 7 of in vitro embryo development.
^{a,b}Different letters in the same column indicate significant differences ($P < 0.05$). Data analyzed by the chi-square test ($P > 0.05$).

at D6 and D7 were similar ($P > 0.05$) for all treatment groups (Table 2). Similarly, the addition of PGE2 or PGF2α at different time points during IVM did not alter embryo development (Tables 3 and 4).

4.2. Experiment 2: effects of PTGS2 inhibition during maturation on cumulus expansion and embryo production and quality

To ensure that PGs did not affect maturation, COCs were matured in the absence and presence of a PG synthesis inhibitor, with or without supplementation with PGE2, PGF2α, or PGE2+ PGF2α. The results showed that the presence of PGE2 or PGF2α did not affect ($P > 0.05$) CC expansion (Fig. 1).

Embryonic development data are presented in Table 5. The cleavage rate was similar ($P > 0.05$) for all groups, regardless of the treatment used. On D6, oocytes treated with PGF2α + NS398 presented a lower rate of blastocyst formation than oocytes from NS398 or CTL groups ($P < 0.05$). On D7, the CTL group presented a higher blastocyst rate ($P < 0.05$) compared with other groups. Thus, PGs affected embryonic development, and only the group in which NS398 alone was added presented blastocyst production similar to that in the CTL group. However, no difference was observed between groups regarding the total number of cells and the percentage of apoptotic cells (Table 6).

Table 4
Embryo development of bovine oocytes submitted to in vitro maturation for 24, 12, or the last 6 hours in the absence (CTL) or in the presence of the PGF2α.

Treatments	N° total	D2	D6	D7
		Cleavage N° (%)	Blastocysts N° (%)	Blastocysts N° (%)
CTL	220	184 (83.6)	9 (4.1)	57 (25.9)
PGF2α 24 h	227	198 (87.2)	11 (4.8)	59 (26.0)
PGF2α 12 h	243	204 (84.0)	11 (4.5)	50 (20.6)
PGF2α 6 h	232	180 (77.6)	15 (6.5)	47 (20.3)

Abbreviations: CTL, control; D2, day 2 of in vitro embryo development; D6, day 6 of in vitro embryo development; D7, day 7 of in vitro embryo development.
Data analyzed by the chi-square test ($P > 0.05$).

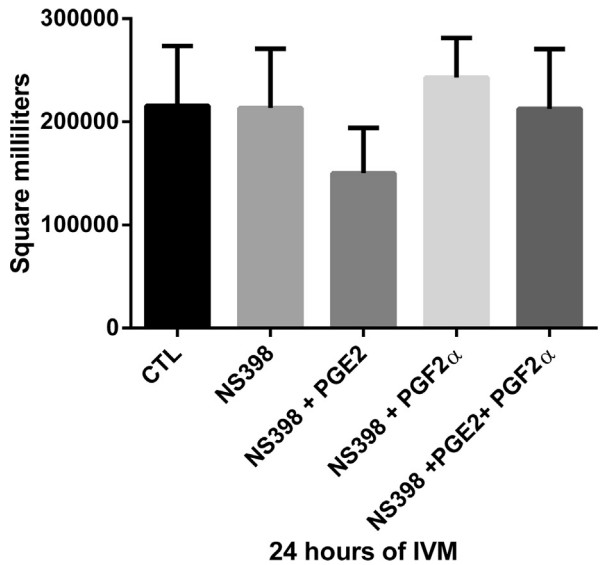

Fig. 1. Expansion area of the cumulus-oocyte complexes matured for 24 h in the absence (CTL) or presence of NS398, supplemented with PGE2, PGF2α or PGE2 and PGF2α. The area was obtained from the difference between the mean area of all COCs from each treatment before and after in vitro maturation. CTL, control; COCs, cumulus-oocyte complexes.

4.3. Experiment 3: expression of genes involved in the activity of PGE2 and PGF2α during maturation in CCs and oocytes with different competence

To determine the expression of genes involved in the activity of PGE2 and PGF2α, and to evaluate whether the expression profile varies during maturation according to the competence of the COCs, 3 genes (PTGS2, PTGES1, and AKR1B1) were quantified in CCs and 2 genes (PTGER2 and PTGFS) in oocytes. The results showed that the expression of PTGS2 increased ($P < 0.05$) in the INC group during maturation (Fig. 2A) and that of PTGES1 increased in the INC and COM groups (Fig. 2B). Regarding the AKR1B1 gene, the expression decreased in the INC group ($P < 0.05$) during maturation (Fig. 2C).

When the expression of genes in CCs was compared among groups of COCs at the same time of maturation, no differences were found at 0 and 24 h of IVM, except for the PTGS2 gene, which was expressed at lower levels in the COM group ($P < 0.05$) compared with the INC group at 24 h (Fig. 2A).

Expression of the PTGER2 and PTGFR genes was quantified in immature and mature oocytes from the 2 groups of COCs. The results revealed no expression of those genes in any of the oocytes categories. To confirm that the genes were not expressed in the oocytes, their expression was also quantified in different bovine tissues, collected at abattoirs, which were used as positive CTLs (CCs, CL, End, and bovine Plac). As shown in Table 7 and Figure 3, CT values associated with PGFR expression could not be determined, even when template cDNA corresponding to 2.4 oocytes/reaction was used. By contrast, the CT values for the remaining tissues were between 27 and 32. Similarly, expression of the PTGER2 gene was very low or

Table 5
Embryo development of bovine oocytes submitted to maturation for 24 h in the absence (CTL) or in the presence of the inhibitor of the enzyme PG endoperoxide synthase 2 (NS398) supplemented with PGE2, PGF2α, or PGE2 and PGF2α.

Treatments	N° total	D2	D6	D7
		Cleavage N° (%)	Blastocysts N° (%)	Blastocysts N° (%)
CTL	254	207 (81.4)	20 (7.9)[a]	88 (34.6)[a]
NS398	241	186 (77.1)	22 (9.1)[a]	72 (29.8)[ac]
NS398 + PGE2	236	186 (78.8)	16 (6.8) [ab]	60 (25.4)[bc]
NS398 + PGF2α	215	163 (75.8)	8 (3.7)[b]	44 (20.4)[b]
NS398 + PGE2 + PGF2α	231	186 (80.5)	18 (7.7)[ab]	61 (26.4)[bc]

Abbreviations: CTL, control; D2, day 2 of in vitro embryo development; D6, day 6 of in vitro embryo development; D7, day 7 of in vitro embryo development.
[a,b,c]Different letters in the same column indicate significant differences ($P < 0.05$). Data were analyzed by the chi-square test ($P > 0.05$).

undetectable in all oocyte samples, whereas CT values varied from 29 to 34 in other tissues (Table 7). The PCR product was placed on the gel to confirm its presence and was identified based on the expected size, corroborating the Tm and CT data. This gene was expressed in all tissues tested, but was not detected with any concentration of oocytes (Fig. 3).

5. Discussion

During the maturation of the COC and the preparation of follicles for ovulation, concentrations of PGE2 and PGF2α increase in the follicular fluid of several species [11,12,35,36], suggesting that those substances may play an important role in the in vivo maturation process. Therefore, in an attempt to elucidate the effect of PGs E2 and F2α during the IVM of bovine COCs, we evaluated the ability of oocytes matured in vitro in the presence or absence of both PGs, and in the presence of an inhibitor of PG synthesis, to attain normal embryo development. In addition, we quantified the expression of genes associated with the activity of these PGs in CCs or oocytes before and after maturation to better understand the effects or PGs during IVP.

Table 6
Total number of cells, and number and percentage of apoptotic cells in expanded blastocysts, at day 7 of development, originating from cumulus-oocyte complexes in the absence (CTL) or in the presence of the inhibitor (NS398) of the enzyme PTGS2 supplemented with PGE2, PGF2α, or PGE2 and PGF2α.

Treatments	N° total BX	N° cells ±SD	N° apoptotic Cells ±SD (%)
CTL	33	177.8 ± 39.3	7.1 ± 2.8 (4.0)
NS398	30	188.9 ± 46.5	8.8 ± 4.5 (4.6)
NS398 + PGE2	33	185.2 ± 30.6	7.9 ± 3.3 (4.3)
NS398 + PGF2α	29	185.0 ± 42.2	7.6 ± 4.1 (4.1)
NS398 + PGE2+ PGF2α	31	183.7 ± 39.6	8.8 ± 3.5 (4.8)

Abbreviation: CTL, control.
Data were analyzed by ANOVA (number of cells) and the Kruskal–Wallis test (number and percentage of apoptotic cells).

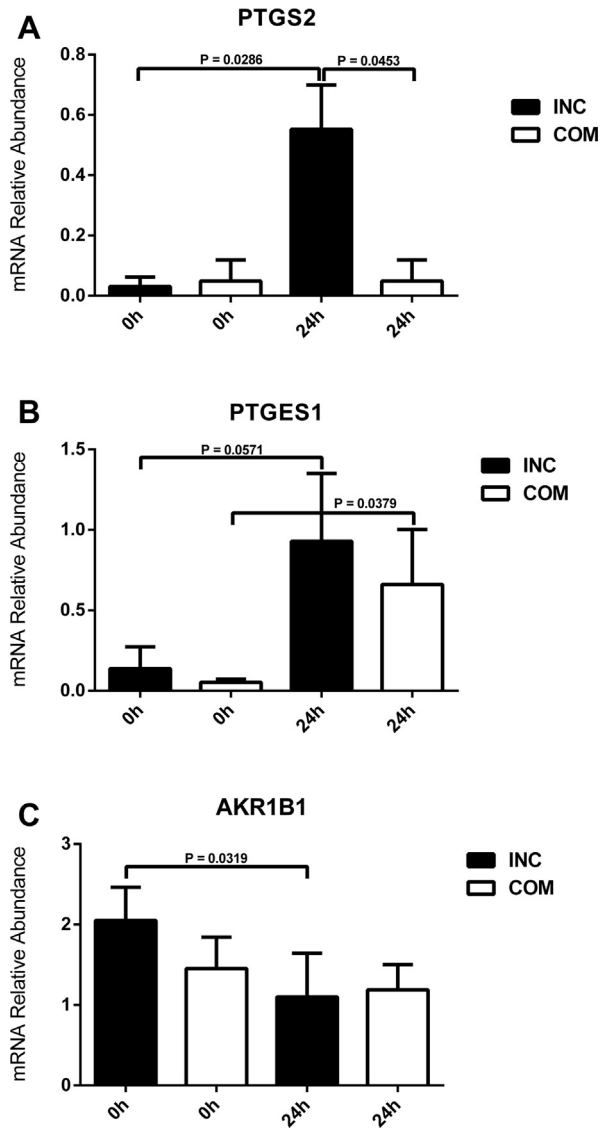

Fig. 2. Levels of transcripts of the (A) PG endoperoxide synthase 2 (PTGS2), (B) PG E synthase 1 (PTGES1), and (C) aldo-keto reductase 1B (AKR1B1) genes, quantified by RT-qPCR in bovine cumulus cells of different competencies, follicles derived from 1.0 to 2.9 mm (incompetent group; INC) and 6.0–8.0 mm (competent group; COM), before (0 h) and after (24 h) in vitro maturation. Data (mean ± SD) were normalized by the GAPDH gene.

The first experiment aimed to confirm whether PGs can be used as additives to improve embryo development of bovine oocytes. First, the maturation medium was supplemented with PGE2, PGF2α, or PGE2 and PGF2α. The results showed that supplementation of the IVM medium with PGs do not promote additional effects in the in vitro production of embryos. Similar results were reported by Marei et al (2014) and Nuttinck et al (2017) after the addition of different concentrations of PGE2 to the IVM medium. However, PGF2α was not added to the IVM medium in either of those studies.

Considering that under physiological conditions, PGE2 and PGF2α secretion is highest at the end of the maturation

Table 7
Threshold cycle and melting temperature values for cDNA amplification of glyceraldehyde-3-phosphate dehydrogenase, PGE2-specific receptor and PGF2α-specific receptor genes, in duplicate, run using samples from corpus luteum, endometrium, cotyledon, oocyte (Ov1: 0.6 oocyte/reaction, Ov2: 1.2 oocytes/reaction, and Ov3: 2.4 oocytes/reaction), and cumulus cells (CC1: 0.8 CC/reaction and CC2: 1.6 CC/reaction).

Sample name	Biological replicate	GAPDH		PTGER2		PTGFR	
		Ct	TM	Ct	TM	Ct	TM
Negative control		46.19	72.25	40.99	73.61	46.83	72.42
Corpus luteum	1	21.53	79.35	30.77	82.89	27.18	74.45
Corpus luteum	2	21.53	79.52	30.74	82.89	27.16	74.28
Endometrium	1	20.23	79.68	29.24	83.06	31.47	74.28
Endometrium	2	20.21	79.68	29.09	83.06	30.80	74.45
Cotyledon	1	18.64	79.68	33.54	81.71	29.80	74.28
Cotyledon	2	18.69	79.68	34.80	81.54	29.46	74.45
CC 1	1	19.37	79.85	33.97	81.88	32.33	74.45
CC 1	2	19.44	79.85	34.33	82.22	31.88	74.62
CC 2	1	18.08	79.68	32.55	82.05	30.89	74.62
CC 2	2	18.12	79.68	32.86	82.05	30.77	74.62
Ov. 1	1	27.88	79.52	39.97	72.25	Undetermined	67.53
Ov. 1	2	28.08	79.52	Undetermined	72.09	Undetermined	73.61
Ov. 2	1	26.75	79.52	36.87	78.00	Undetermined	68.37
Ov. 2	2	26.69	79.52	40.46	73.61	Undetermined	73.10
Ov. 3	1	21.80	79.85	37.06	72.09	Undetermined	66.34
Ov. 3	2	21.80	79.85	42.41	71.58	Undetermined	67.70

Abbreviations: CTL, control; Ct, threshold cycle; TM, melting temperature.

period [37], we questioned whether the lack of effect observed on the presence of PGs throughout the 24 h could be due to an excessive exposure to PGs during maturation. Then, we also evaluated the addition of these PGs at different times during IVM (final 24, 12, or 6 h of culture). However, no differences in cleavage and blastocyst rates were also detected at any supplementation times. These data suggested that neither PGE2 nor PGF2α are suitable additives for enhancing the embryonic development of bovine oocytes.

Taking into account our results, in which PGs have no effect during maturation and the information that PGs are synthesized during maturation both in vivo and in vitro [18,23,36,38–41], one could think that the PGs synthesized in culture is sufficient to cause their effects. If this is true, it is not necessary to supplement the media with PGs. To test that, we used a specific inhibitor of PTGS2 during IVM. First, the results showed that neither the addition of an inhibitor nor supplementation of NS398 with PGs altered the levels of CC expansion. Similar results were reported by Marei et al (2014) with the same inhibitor (NS398), or with siRNA-PTGS2 (PTGS2-specific interference RNA), or NS398 and siRNA-PTGS2. However, others have reported a beneficial effect of PGE2 on expansion after addition to the IVM medium [21,22]; however, the effect was only significant when COCs were cultured in the presence of serum or in the absence of hormones [22,42]. Serum was not used in the present study, and the levels of expansion in the treated groups were similar to those in the CTL group; therefore, it is possible that the presence of FSH induced the expansion, and that PGE2 and PGF2α had no effect under the conditions used in this study.

Similar to CC expansion, supplementation of the maturation medium with the inhibitor and PGs did not affect the cleavage rate. However, the addition of PGE2 and PGF2α to the NS398-treated medium affected embryo development, resulting in lower rates of D7 blastocysts in all PG-treated groups compared with the CTL group. Notably, no changes in embryo quality were observed. Results indicate that PGs synthesis is not essential for IVM and that the addition of PGE2 and PGF2α to the medium treated with NS398 may negatively affect embryo production. These

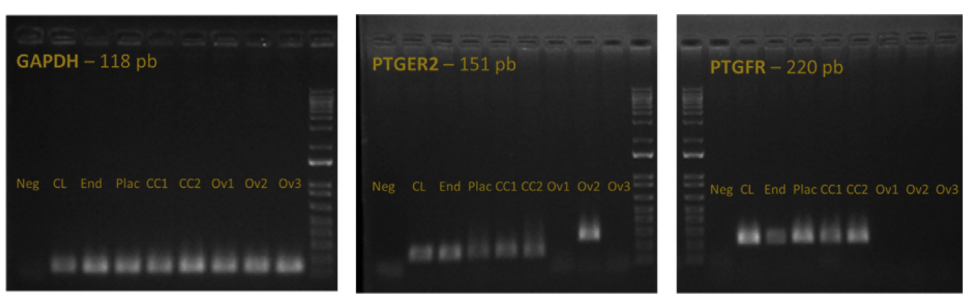

Fig. 3. Validation of PGE2-specific receptor (PTGER2) and PGF2α-specific receptor (PTGFR) gene expression in different bovine tissues (oocyte–Ov1: 0.6 oocyte/reaction, Ov2: 1.2 oocytes/reaction and Ov3: 2.4 oocytes/reaction; cumulus cells–CC1: 0.8 CC/reaction and CC2: 1.6 CC/reaction; corpus luteum–CL: 35 ng/reaction; endometrium–End: 35 ng/reaction; and placenta–Plac: 50 ng/reaction). GAPDH gene was used as the endogenous control. Electrophoresis of 2% agarose gels showing specific amplicons for GAPDH (118 bp), PTGER2 (151 bp), and PTGFR (220 bp); the DNA ladder used is 1 kb plus (Invitrogen).

results are not consistent with those reported in the literature, as recent studies using bovine COCs have reported that the addition of PGE2 during IVM and IVF treated with NS398 restored the negative effects on the blastocyst rate in D6, the number of blastomeres in D6 and D7 embryos [23], and on the average length of D14 embryos [24]. Conversely, it is well established that the synthesis and activity of those substances derived from the metabolism of arachidonic acid depend on the microenvironment, and the correct distribution of enzymes and receptors in different tissues and cells [39,43,44]. Thus, differences between study findings may be due to methodological differences, including the types and condition of medium, as well as the type of oocytes used. For example, in the study of Nuttinck et al, 2011 [23] and 2017 [24], PGE2 was added to the IVM and IVF media and serum was used. In our study, PGs were added only to the IVM medium, and BSA was used instead of serum. Also, pools of oocytes from a donor of different breeds (mainly *Bos indicus* breeds), raised under different conditions were used. Therefore, the results did not support the hypothesis that inclusion of PGE2 and PGF2α in the IVM culture will improve oocyte maturation and embryo production. Therefore, the role of PGE2 and PGF2α during the IVM of bovine oocytes remains unclear.

Considering that the expression of PTGS2 in vivo, which is essential for PGs synthesis, depends on follicle size [10], we wanted to determine if the effect of PGs varied according to the level of competence of the oocytes. Therefore, we evaluated the expression profiles of genes encoding the enzymes involved in PGs synthesis and activity in CCs and oocytes with different degrees of competence. Follicle size was used to obtain COCs with different competencies; this model is well established in our laboratory [27–29]. Therefore, large follicles were used to obtain competent COCs, and smaller follicles were used to obtain less-competent COCs. The results showed that the relative abundance of the PTGS2 gene increased after 24 h of IVM in the incompetent group, and remained unchanged in the other group. Previous studies have reported a variable expression pattern for this gene because PTGS2 expression levels increased [18,22,36,42,45,46] or were not altered at the end of maturation [17,22].

Prostaglandin endoperoxide synthase 2 is an enzyme responsible for the conversion of arachidonic acid to PGH2, a common precursor of all PGs. In vivo, the gonadotrophic peak induces PTGS2 expression in granulosa cells of the preovulatory follicle [10–12,35]. However, expression under physiological conditions is dependent on the follicle size and the moment of induction because it only occurs in follicles that reach a minimum diameter of 8 mm in cattle [10]. By contrast, in vitro COCs aspirated from 3 to 6 mm follicles express PTGS2 shortly after aspiration and during IVM [42]. The levels of PTGS2 during IVM have been shown to increase at 6 h, reaching maximum concentrations and then decreasing at 12 [17], 18, or 24 h [22]. In the present study, the expression of PTGS2 increased after 24 h of culture in the incompetent group only. Therefore, it is possible that the induction of PTGS2 in the CCs of this group was delayed, whereas in the competent groups, the highest expression may have occurred during the first hours of maturation before subsequently decreasing, as demonstrated in the literature [17,22]. These results, therefore, confirm that the level of PTGS2 induction may be related to follicular size [10,17,18,36,42,47,48].

In addition to PTGS2, the relative abundance of PTGES1 also increased during IVM in this study. The increased expression occurred in the CCs of incompetent oocytes and also in the competent group, suggesting a possible role of this enzyme during maturation. PTGS2 and PTGES1 are inducible enzymes associated with the intracellular membrane and are responsible for the synthesis of PGE2 in several tissues [16,36,49]. In COCs, Nuttinck et al [18] reported that the induction of PTGES1 expression occurs in parallel with the induction of PTGS2. The study by Marei et al [17] showed that expression of both of these genes was induced during IVM, and that after 24 h, there was an increase only in the relative abundance of the PTGES1 gene. Therefore, the induction of PTGS2 and PTGES1 activity appears to occur sequentially throughout IVM. An increase in PTGS2 observed in the competent group may have occurred early during IVM, which could have triggered the increase in PTGES1 observed at 24 h of maturation. In the incompetent group, the induction of PTGS2 may have occurred later; therefore, at 24 h of IVM, PTGS2 expression remained high, which would have induced PTGES1 transcription.

Although PTGS2 expression in CCs has also been associated with increased PGF2α concentration in the follicular fluid and maturation media [11,12,17,18,35,36,38,41], few studies have investigated the specific synthase enzyme responsible for the conversion of PGH2 to PGF2α in the COC. As the PTGFS involved in PGF2α synthesis in the follicle remains unknown, we selected the AKR1B1 gene because it is considered to be the main enzyme responsible for PGF2α synthesis in the bovine and human End [19,50]. Our results showed that AKR1B1 expression decreased in the CCs of the incompetent group after 24 h of maturation, whereas expression remained unchanged in the other group. Considering that this is the first report of AKR1B1 in bovine COCs of different competencies, its characterization is important. According to Pépin et al [19], the enzyme AKR1B1 is constitutively expressed at moderate levels in the bovine End, and the production of PGF2α in this tissue results from the induced expression of PTGS2. Based on those reports and the result of the present study, we can hypothesize that the AKR1B1 gene is also constitutively expressed in the CCs. Thus, in the competent group, the levels of AKR1B1 remained unchanged during maturation. In the incompetent group, there may have been a decrease in mRNA levels because of its translation and subsequent degradation. Therefore, it is possible that those CCs had to use that RNA and were unable to respond to the stimulus to replenish the mRNA stock.

When the expression of each gene at a given time (0 or 24 h) was compared between groups, PTGS2 was the only differentially expressed gene and was expressed at lower levels in the competent group at 24 h than in the incompetent groups. Although we did not compare the expression of PTGS2 and PTGES1 at different time points of maturation, our results led us to suppose that the greatest expression of PTGS2 in the competent group may have occurred in the first hours of maturation, which could be triggering PTGES1 activity. Although abundance of mRNA

for PTGS2, PTGES1, and AKR1B1 showed a differential profile during maturation according to the competence of the COCs, the expression was similar between those COCs when they were analyzed at a specific time point during maturation. These results are consistent with the literature regarding the possible use of PTGS2 as a marker of competence [36,47,48,51].

Prostaglandin E2 acts through 4 receptors, PTGER1–PTGER4, and PGF2α functions through the PTGFR; the characteristics, physiological effects, and mechanisms of action of each subtype differ [52]. Bovine CCs express all PTGER isoforms, except for PTGER1, whereas only PTGER2 is expressed on the oocyte [23,42]. Conversely, no study has quantified the expression of PTGFR in this cell. In the present study, we evaluated the expression of specific PGE2 (PTGER2) and PGF2α (PTGFR) receptors in oocytes of different competencies. Our results showed that PGE2 was expressed at low levels, and that PGF2α receptor expression was undetectable in oocytes, regardless of the competence level. The mRNA for both receptors was detected in CCs, CL, End, and bovine Plac, but not in oocytes. Although PTGER2 expression has been reported in oocytes [23,42], Calder et al [42] showed that the composition of the maturation medium has a marked effect on the relative abundance of PTGER2 mRNA. In addition, numerous reports have shown that the transcriptional levels, as well as the rates of development and embryonic morphology, are affected by protein supplementation [30,53]. Thus, in the present study, the use of BSA may have influenced the expression of the receptors, unlike other studies that used serum or another protein source.

The results of our study indicate that during IVM, PGE2 and PGF2α have no positive effect on bovine embryo development; therefore, supplementation with these agents during IVM/IVF is not recommended. This study also reported the expression of AKR1B1 in bovine COCs of different competencies for the first time. Although genes involved in the activity of these PGs are differentially expressed in CCs of COCs of different competence during maturation, low or no expression of PGs receptor mRNA was detected in oocytes.

CRediT authorship contribution statement

Sarah A.D. Rodrigues: Conceptualization, Data curation, Formal analysis, Investigation, Validation, Visualization, Writing - original draft. **Thais P. Pontelo:** Validation, Formal analysis. **Nayara R. Kussano:** Validation, Formal analysis. **Taynan S. Kawamoto:** Validation, Formal analysis. **Ligiane O. Leme:** Formal analysis, Supervision, Validation, Writing - review & editing. **Felippe M.C. Caixeta:** Formal analysis. **Luiz F.M. Pfeifer:** Conceptualization, Methodology. **Mauricio M. Franco:** Methodology, Formal analysis, Writing - review & editing. **Margot A.N. Dode:** Conceptualization, Funding acquisition, Supervision, Methodology, Project administration, Formal analysis, Writing - review & editing.

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The authors declare no conflicts of interest.

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