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Gene expression and Doppler flowmetry of the reproductive tract in Nelore (*Bos indicus*) cows synchronized with estradiol and equine chorionic gonadotrophin

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Abstract: Forty Nelore cows were submitted to timed artificial insemination (TAI) protocol and randomly assigned to groups according to estradiol (E2) ester (cypionate or benzoate, i.e. EC or EB) and equine chorionic gonadotrophin treatment (eCG or No-eCG) in a factorial 2x2 design. On a random day of the estrous cycle (Day 0), cows received a P4 intravaginal device plus 2 mg im injection of EB. At the time of P4 device removal (Day 8), cows were treated with 0.150 mg im of D-cloprostenol (PGF) and randomly divided to receive or not 400 UI im of eCG, as well as randomly assigned to receive 1 mg of EC immediately (D8), or 1 mg of BE, 24 h after it (D9) as ovulatory stimuli. Therefore, the groups formed were EC, EC+eCG, EB and EB+eCG. Were evaluated the effects of treatments on VEGF, OXTR, and PGR gene expression, E2/P4 concentrations, and Doppler-flowmetry of the reproductive tract of the investigated models. Ovulation rate was higher in animals subjected to the EC + eCG treatment ($p < 0.05$). Follicular vascularization was not influenced by the ovulation inducer ($p > 0.05$), although it was influenced by the eCG treatment ($p < 0.05$). EB enabled higher vascularized area rate in CL ($P = 0.0192$). VEGF and PGR genes were more expressed in animals treated with EB and eCG. On the other hand, OXTR expression was less evident in eCG-treated cows. EC has led to higher E2:P4 ratio in follicular fluid. This outcome indicated higher follicular estrogenic activity at D10, which justified the higher ovulation rate, when EC was associated with eCG ($p < 0.05$). Finally, the results of the present study corroborate the hypothesis that the different E2 esters and eCG differentially regulate vascularization, steroidogenesis, and utero-ovarian gene expression in Bos indicus cows submitted to TAI protocols.

Key words: Timed artificial insemination, estradiol ester, gonadotrophin, genes, ultrasoun

1. Introduction

Reproductive efficiency is the trait that mostly influences commercial cattle herds' productivity and profitability. The programmed use of reproductive hormones, and of their analogues in timed artificial insemination (TAI) protocols is the main strategy adopted to increase the service rate of cows in reproduction processes. This procedure allows synchronizing both the ovulation and insemination of all females available for reproduction. However, mean fertilization rates at the first artificial insemination procedure have historically reached 50% [1,2], and it indicates that a significant number of inseminated females failed to get pregnant.

Different strategies have been suggested to help improving the effectiveness of synchronization techniques applied to TAI. Among them, one finds the use of equine chorionic gonadotropin (eCG) to stimulate preovulatory follicle growth, increase progesterone (P4) corpus luteum (CL) production, and improve pregnancy rates [3–5]. In addition, estradiol benzoate (EB) is administered at the early protocol application stage in order to induce atresia in developing follicles, and it leads to the emergence of new follicular wave [6]. More recently, estradiol cypionate (EC) has been widely used at intravaginal device removal time in order to induce LH peak between 45 and 49 hours, as well as ovulation between 67 and 70 h after treatment [7].

The use of these exogenous hormones in TAI protocols leads to differential gene expression in bovine endometrium [8]. Genomic effects of steroid hormones, estradiol (E2), and P4 on the endometrium are mainly mediated by their

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connection to their receptors [9,10]. Among the genes of importance in this context, one finds VEGF (vascular endothelial growth factor), OXTR (oxytocin receptor), and PGR (progesterone receptor), which are associated with angiogenesis, follicular and uterine development [11,12], as well as with uterine receptivity for embryonic implantation [13–15].

Changes in P4 serum concentrations at early luteal stage can lead to temporal variations in the composition and number of transcripts in bovine endometrium [16,17]. E2 increases the expression of its own receptors, as well as of PGR and OXTR in the endometrium [9,18,19].

In addition, exogenous hormones are capable of modulating the blood vascular pattern in animals' reproductive tract [20,21]. The uterus of female cattle, as well as of other species, undergoes physiological changes throughout the estrous cycle, and each cycle stage is directly linked to uterine vascular perfusion [22,23]. These changes are regulated by steroidal hormones and by a network of complex interactions between local growth factors secreted by the uterine epithelium and stroma [23–25].

E2 is a steroidal hormone known for its vasodilatory effect [26,27], whereas P4 increases uterine receptivity to the embryo through the induction of endometrial angiogenesis [28]. Some studies have emphasized the influence of eCG administration on CL formation and vascularization, as well as on P4 production and increased uterine blood flow, both in embryo recipients and donors [29–31].

Thus, gene expression and blood vascular pattern in the reproductive tract can be regulated by exogenous hormones used in TAI protocols. Therefore, the aim of the current study was to analyze the influence of hormonal protocols on hemodynamics and gene expression, which are directly linked to follicles' ovulatory capacity, CL development, gonadal steroidogenesis, and associated endometrial responses.

2. Materials and methods

2.1 Study site and animals

The current study was carried out in a commercial farm in Barra do Corda County, Maranhão State, Brazil (latitude 5°30`21" South and longitude 45°14`34" West). Animal models were kept in an extensive breeding system comprising Mombasa grass pasture (*Megathyrsus maximus*), mineral supplementation, and water ad libitum. The experiment comprised 40 multiparous lactating Nelore (*Bos indicus*) cows, at mean age 48.0 ± 0.0 months, in anestrus, with postpartum period ranging from 35 to 60 days, and body condition score (BCS) of 3.0 ± 0.0, based on Machado et al. [32].

Experimental procedures were approved by the Ethics Committee on Animal Research of Federal University of Maranhão (CEUA-UFMA), under Protocol N. 23115.014522/2020-37.

2.2 Experimental design

Nelore cows were submitted to timed artificial insemination (TAI) protocols and randomly assigned to groups according to E2 ester (EC or EB) and eCG treatment (eCG or No-eCG) in a factorial 2 x 2 arrangement (Figure 1). The experiment followed a complete randomized block design (RCBD). For grouping the experimental units into homogeneous groups or blocks based on similar ovulatory potential, the cows were stratified according to the dominant follicle diameter at P4 device removal time (D8; day 18 of protocol application) and allocated to 4 treatments with 10 cows each.

2.3 Hormonal protocol

At the start of the study (designated Day 0), all cows were given a intravaginal P4 device (1 g of P4; Sincrogest®, Ourofino, São Paulo, Brazil) and 2 mg of EB im (EB; Estrogin, Farmavet, São Paulo, Brazil). On Day 8, P4 devices were removed, and cows received 0.150 mg of D-cloprostenol im (PGF; Prolise, Arsa, Buenos Aires, Argentina). At this time (D8), the cows were randomly divided to receive or not 400 UI of eCG im (SincroeCG, Ourofino, São Paulo, Brazil), as well as randomly assigned to receive 1 mg of EC (ECP, Zoetis Saúde Animal, São Paulo, Brazil) immediately (D8), or 1 mg of BE, 24 h after it (D9) (Figure 1). Therefore, the groups formed were EC (n=10), EC + eCG (n=10), EB (n=10) and EB + eCG (n=10).

2.4 Ultrasound evaluations

Animals were subjected to 5 ultrasound evaluations at protocol application days 0, 8, 9, 10, and 18 (Figure 1); these evaluations were performed with the aid of an ultrasound device (Mindray, Model Z5Vet, Digital Ultrasonic Diagnostic Imaging System) equipped with 7.5 MHz transrectal linear multifrequency transducer.

The examination was initially performed in bidimensional mode (B-mode; grayscale) in order to identify the uterine-ovarian structures and measure the diameters of the dominant follicle (DF) and the corpus luteum (CL). Next, Color-Doppler mode was activated to determine the blood perfusion area in the structures; images were recorded for further analysis (Figure 2).

One-minute-long real-time continuous cross-section scanning of uterine horns and ovaries was recorded in order to show the largest blood perfusion area possible with a better quality image (adapted from Ferreira et al. [33]). The vascular perfusion area (in color) proportional to the total area of uterus and ovaries (0 to 100%) was subjectively assessed by six appraisers. The maximum and

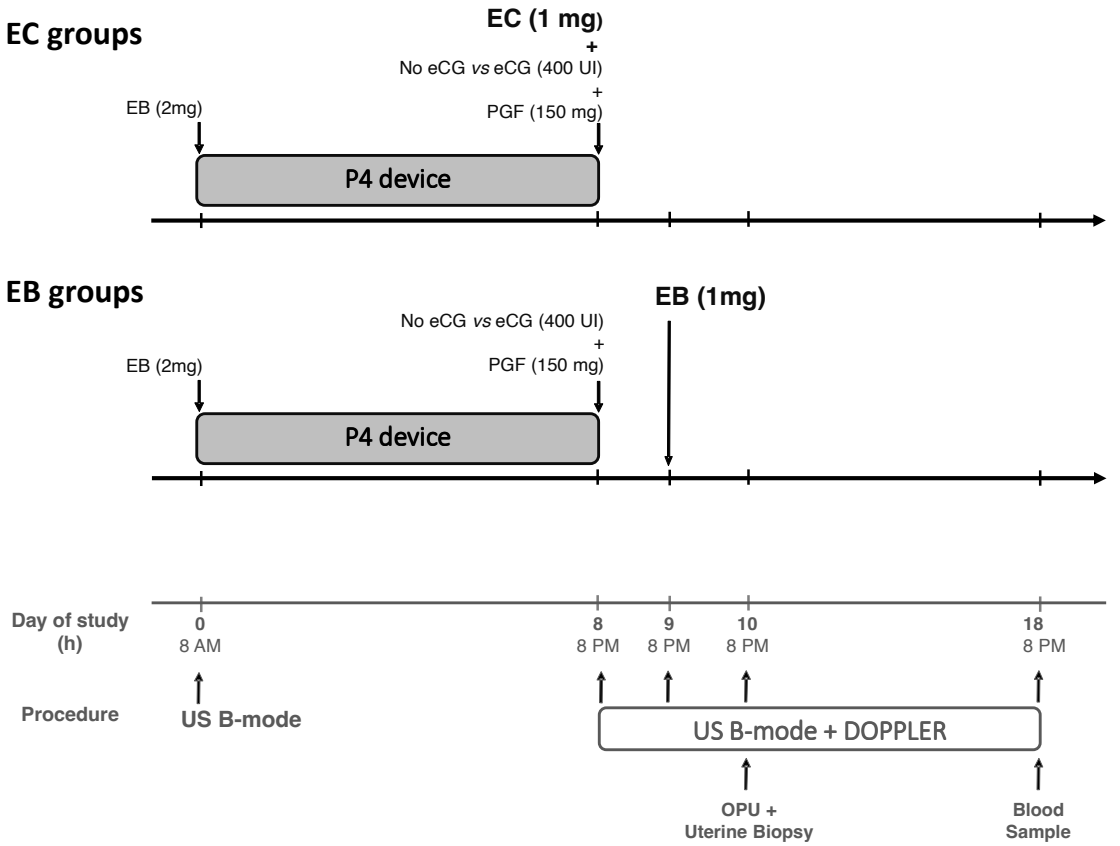

Figure 1. Schematic view of TAI protocol and proceedings following ovulation synchronization in Nelore (*Bos indicus*) cows subjected to an P4/E2-based timed artificial insemination (TAI) protocol using EC or EB as ovulatory stimuli and its association, or not, with eCG.[†], [†] TAI protocol. On a random day of the estrous cycle (Day 0), cows received a P4 intravaginal device plus 2 mg im injection of EB. At the time of P4 device removal (Day 8) cows were treated with 0.150 mg im of D-cloprostenol (PGF) and randomly divided to receive or not 400 UI im of eCG; as well as randomly assigned to receive 1 mg of EC immediately (D8), or 1 mg of BE, 24 h after it (D9). US B-mode = ultrasound examination in bidimensional mode (grey-scale); DOPPLER = ultrasound examination in Color-Doppler mode; EC = estradiol cypionate; EB = estradiol benzoate; OPU = ovum pickup / ultrasound-guided aspiration of follicular fluid in vivo; P4 device = Sincrogest

minimum scores were discarded, whereas the median scores were used. Objective evaluations were performed based on the intensity of the colored images (in number of pixels), as previously described for mares and heifers [34–36]. The number of colored pixels in the analyzed images was counted in image editing software (Adobe Photoshop CS5) in order to measure the extension of the uterine horn, ovary, follicular wall, and corpus luteum vascularization at pixel scale [37] (Figure 2).

2.5 Follicular fluid recovery in vivo

Dominant follicle aspiration was carried out at the end of the synchronization protocol (D10) - which corresponded to TAI time – to enable follicular fluid recovery in vivo, which was guided by ultrasound equipped with a guiding device for the Ovum Pickup 10-gauge needle. Follicular content was recovered through aspiration, based on the application of manual negative pressure, via Teflon circuit (Wtavet, Brazil) - 2 mm in internal diameter and 80 cm in length - connecting the needle directly to a 10 mL syringe.

2.6 Uterine biopsy

Uterine endometrium sample was collected at follicular aspiration time (D10). It was removed from the region of the middle curvature of the uterine horn ipsilateral to the ovulatory follicle, with the aid of Yomann forceps, through transcervical access. Next, collected fragments were stored in RNALater (Ambion, Austin, USA) at –20 °C, until mRNA extraction, reverse transcription, and real-time PCR at the laboratory.

2.7 RNA extraction and cDNA synthesis

Total mRNA was extracted by using the RNeasy Micro Kit (Qiagen GmbH, Hilden, Germany), according to the manufacturer's specifications, and treated with Dnase. Total RNA in 1 µL of the sample was quantified through spectrophotometry with the aid of ND-100

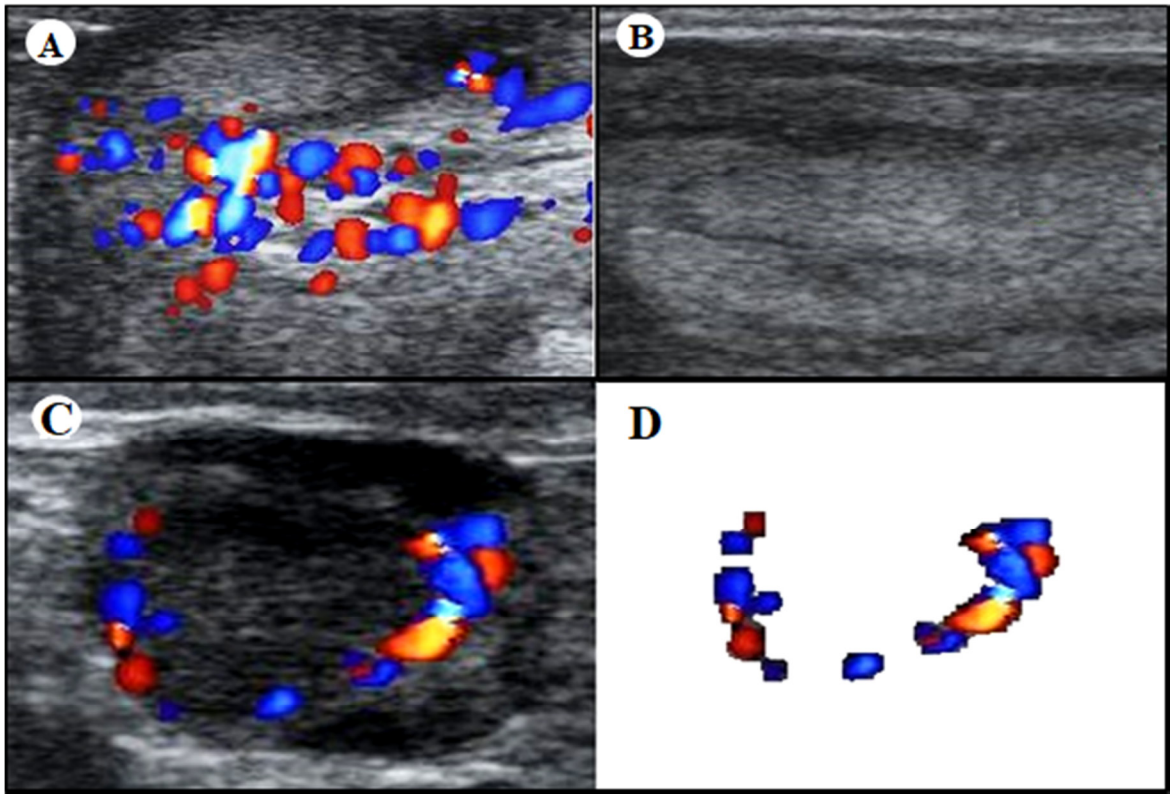

Figure 2. Uterine ultrasonography showing blood flow in Color Doppler mode (A) and the same structure in B-mode (B). The image of the CL in Color Doppler mode (C) and the same image edited by Adobe Photoshop CS5 for color pixels count (D).

spectrophotometer (NanoDrop, Wilmington, USA). The mRNA was retrotranscribed into cDNA by using the Super Script III First-Strand Synthesis System commercial kit for RT-PCR (Invitrogen, Carlsbad, CA, USA), for the final volume reaction of 20 µL. In addition, 3.0 µL of DEPC water, 1.0 µL of Random Hexamer (50 µg / µL), 1.0 µL of Anneling Buffer, 2.0 µL of RNase OUTTM Enzime Mix, 10.0 µL of 2x First were used for cDNA synthesis -Strand Reaction Mix. The following parameters were used for cDNA synthesis: 65 °C for 5 min, incubation in ice for 1 min, 25 °C for 10 min, and 50 °C for 50 min. Subsequently, cDNA was subjected to conventional PCR to assess whether the amount of RNA was enough or not. Samples were subjected to electrophoresis in 2% agarose gel, right after PCR, in order to confirm fragment size.

2.8 Quantitative real-time RT-PCR (RT-qPCR)

RT-qPCR was performed based on 2.5 µL of cDNA, 10 µM of primer pair specific to each gene, and 12.5 µL of Platinum SYBR Green qPCR SuperMix (Platinum SYBR Green qPCR SuperMix - with ROX, Invitrogen) at final volume of 20 µL per reaction. The following parameters were used for RT-qPCR: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 s and 60 °C for 1 min; RT-qPCR was conducted in ABI 7500 thermocycler (Applied Biosystems). Data were analyzed based on the comparative Ct method [38]. Ct values were normalized based on GAPDH expression. The sequence of used primers is described in Table 1.

2.9. E2 and P4 concentrations in follicular fluid and blood plasma

Blood samples were collected after each ultrasound evaluation in order to determine serum concentrations of ovarian steroid hormones such as P4 and E2. In order to do so, animals' caudal vein was punctured, and blood was directly collected in 10 mL Vacutainer vacuum tubes, without anticoagulant (Becton-Dickinson & Company, USA), at room temperature. Each sample was centrifuged (3000 g for 10 min; Excelsa Baby Centrifuge, Fanem, Brazil) right after collection; the serum was separated from the sample, labeled, and stored in sterile tubes at –20 °C. Samples were diluted in PBS to adapt to the standard curve of the quantification kit to enable determining the steroid hormone (P4 and E2) concentrations in follicular fluid. The dilution was performed due to high E2 (dilution ratio 1:100) and P4 (dilution ratio 1:50) concentrations in follicular fluid.

Table 1. Sequence of genes and primers used in RT-qPCR.

Gene	Tissue / associated function	Primers (5'-3')	NCBI sequence of reference
GAPDH	Endogenous Gene of reference	5'- GAGCTTGACAAAGTGGTCGTTGAG-3' 3'-CCAACGTGTCTGTTGTGGATCTGA-5'	NM_001034034.1
PGR	Endometrial gene	5'-GCCGCAGGTCTACCAGCCCTA-3' 3'-GTTATGCTGTCCTTCCATTGCCCTT-5'	NM_001205356.1
VEGF	Endometrial gene	5'-GCCATGGAGCGCTTTGG-3' 3'-CCACAGTCAGCAATGGTGATCT-5'	NM_174216
OTR	Endometrial gene	5'-AAGATGACCTTCATCGTCGTG-3' 3'-CGTGAAGAGCATGTAGATCCAG-5'	NM_174134.2

Analyses were based on the immunoenzymatic assay technique (ELISA); they used commercial kits to determine progesterone and estradiol in solid phase (Progesterone ELISA and 17beta-Estradiol ELISA kit, IBL International).

2.10 Statistical analysis

Data analysis was performed in the Statistical Analysis System for Windows SAS software (version 9.4. SAS Inst. Inc., Cary, NY, USA). The normality of residues for each variable was analyzed through Shapiro–Wilk test (SAS PROC UNIVARIATE application). Whenever necessary, data were transformed (logarithm in base 10 - Log10 X) to meet the assumptions of the analysis of variance. Continuous dependent variables (follicular diameter and pixels) were subjected to ANOVA and expressed as mean and standard error of the mean (mean ± SEM), whereas means between treatments were compared by Duncan test (PROC GLM from SAS). Ovulation rate was featured at D18, based on the identification of corpus luteum formed after the luteinization of the dominant follicle aspirated at D10 [39], and analyzed through Fisher's exact test (GraphPad Instat) [40]. Hormonal concentrations, follicular vascularization area, and mRNA expression for PGR, VEGF, and OXTR genes have shown non-normal distribution; the model was adjusted for Poisson distribution, and they were analyzed through Poisson regression (PROC GLIMMIX from SAS), based on Wang et al. [41] and Svensson et al. [42].

The association between variables was investigated based on the Principal Component Analysis (PCA) method in Statistica 7.1 software [43]. According to the aforementioned method, the two produced axes graphically represent the strongest data pattern, i.e. it explains the important role played by the two main components in total data variation. The significance level to reject H0 (null hypothesis) was 5%, i.e. a significance level lower than 0.05 indicated the effect of the classificatory variables and of the interactions between them.

3. Results

Dominant follicle and corpus luteum diameter did not differ among treatments ($p > 0.05$). Ovulation rate was significantly higher in animals subjected to the EC + eCG treatment than in those subjected to EC ($p < 0.05$). Groups treated with EB did not show the effect of eCG on ovulation rate, whereas groups treated with EC and E + eCG have shown intermediate ovulation rate ($p > 0.05$). Overall, there was no interaction between inducers and eCG (Inducer x eCG) in DF and CL diameters or in ovulation rate (Table 2).

There was no significant difference in uterine vascularization area at D8 among treatments ($p > 0.05$). However, there was an effect of both EC and eCG administration, which significantly increased uterine vascularization, from D8 to D9 ($P = 0.0006$) (Table 3).

The ovulation inducer did not have an effect on DF wall vascularization; however, eCG administration presented a significant interaction with E2 ester, as it increased DF vascularization only on D8 for EC and only on D9 for EB (E2 x eCG interaction = 0.0019 on D8 and = 0.0058 on D9) (Table 3). This finding has shown that the best vascular response of the inducer in the current study depended on its association with eCG and on its administration time (D8 for EC and D9 for EB).

Cows treated with EB recorded a higher vascularized area rate both in CL ($P = 0.0192$) and in the uterus ($p = 0.0315$), in the diestrus subsequent to protocol application (D18). On the other hand, there was no effect or interaction of EB with eCG ($p > 0.05$) (Table 3).

Animals treated with EC recorded higher E2:P4 ratio (Figure 3) in the follicular fluid collected at protocol application D10 (dominant preovulatory follicle). Surprisingly, this association was lower in groups treated with eCG. On the other hand, eCG had a positive effect on P4 concentration increase in the subsequent diestrus (D18), particularly when it was associated with EC. Serum estradiol concentrations at D18 were similar to each other,

Table 2. Effects of estradiol esters (EB and EC) as ovulatory stimuli and its association, or not, with eCG, on the ovarian follicular response in Nelore (Bos indicus) cows evaluated by ultrasound at days 8, 9, 10, and 18 of a P4/E2-based timed artificial insemination (TAI) protocol. †

Variable	EB		EC		*P-value*		
	eCG	No eCG	eCG	No eCG	*E2*	*eCG*	*E2 x eCG*
n	**10**	**10**	**10**	**10**	-	-	-
DF diammeter at D8 (mm)	10.2 ± 0.8	10.3 ± 0.8	9.7 ± 0.6	9.9 ± 0.8	*0.5273*	*0.8571*	*0.9521*
DF diammeter at D9 (mm)	11.5 ± 0.7	10.8 ± 0.8	11.7 ± 0.6	10.7 ± 0.9	*0.9071*	*0.2990*	*0.8914*
DF diammeter at D10 (mm)	12.1 ± 0.7	12.2 ± 0.8	13.3 ± 0.6	11.8 ± 0.8	*0.6058*	*0.4059*	*0.3047*
CL diammeter at D18 (mm)	17.4 ± 1.3	17.0 ± 1.4	15.5 ± 2.9	15.2 ± 3.1	*0.3290*	*0.3956*	*0.3936*
Ovulation rate*	70% (7/10)^{ab}	80% (8/10)^{ab}	100% (10/10)^{a}	50% (5/10)^{b}	*0.1000*	*0.2700*	---

† TAI protocol. On a random day of the estrous cycle (Day 0), cows received a P4 intravaginal device plus 2 mg im injection of EB. At the time of P4 device removal (Day 8) cows were treated with 0.150 mg im of D-cloprostenol (PGF) and randomly divided to receive or not 400 UI im of eCG; as well as randomly assigned to receive 1 mg of EC immediately (D8), or 1 mg of BE, 24 hours after it (D9).
* Ovulation rate; featured by the identification of corpus luteum at D18; analyzed through Fisher's exact test.
^{ab} Means followed by different superscripts on the same line significantly differ from each other, based on the Duncan test, at 5% significance level.
EB = estradiol benzoate; EC = estradiol cypionate; eCG = equine chorionic gonadotropin; DF = dominant follicle

Table 3. Effects of estradiol esters (EB and EC) as ovulatory stimuli and its association, or not, with eCG, on the uterovarian vascular response in Nelore (Bos indicus) cows evaluated by Color Doppler ultrasonography at days 8, 9, 10, and 18 of a P4/E2-based timed artificial insemination (TAI) protocol. †

Variables	EB		EC		*P-value*		
	eCG	No eCG	eCG	No eCG	E2	eCG	E2 x eCG
n	**10**	**10**	**10**	**10**	-	-	-
Uterine vascularization area at D8 (%)	9.8 ± 0.9	11.9 ± 1.0	12.3 ± 1.1	11.9 ± 1.0	0.2334	0.3958	0.2334
Uterine vascularization area at D9 (%)	18.0.9 ± 1.3^{a}	10.7 ± 1.3^{b}	19.7 ± 3.9^{a}	18.2 ± 3.3^{a}	0.0006	0.0008	0.0117
Uterine vascularization area at D10 (%)	16.3 ± 1.3^{ab}	19.9 ± 1.5^{a}	17.6 ± 1.0^{ab}	15.5 ± 1.2^{b}	0.2714	0.6443	0.0420
Uterine vascularization area at D18 (%)	15.8 ± 1.2^{a}	12.4 ± 1.2^{ab}	11.4 ± 1.1^{b}	11.5 ± 1.1^{b}	0.0315	0.2120	0.1800
DF vascularization area at D8 (%)	12.1 ± 1.1^{b}	11.6 ± 1.0^{bb}	16.9 ± 1.3^{a}	8.7 ± 0.9^{c}	0.8035	0.0005	0.0019
DF vascularization area at D9 (%)	15.5 ± 1.2^{a}	10.7 ± 1.0^{b}	10.9 ± 1.0^{b}	12.8 ± 1.1^{ab}	0.3461	0.2540	0.0058
DF vascularization area at D10 (%)	15.0 ± 1.2^{a}	10.3 ± 1.0^{b}	11.2 ± 1.1^{b}	10.9 ± 1.0^{b}	0.2231	0.0384	0.0740
CL vascularization area at D18 (%)	40.0 ± 2.5^{a}	43.5 ± 2.3^{a}	32.5 ± 1.8^{b}	38.7 ± 3.1^{ab}	0.0192	0.0549	0.4816

† TAI protocol. On a random day of the estrous cycle (Day 0), cows received a P4 intravaginal device plus 2 mg im injection of EB. At the time of P4 device removal (Day 8) cows were treated with 0.150 mg im of D-cloprostenol (PGF) and randomly divided to receive or not 400 UI im of eCG; as well as randomly assigned to receive 1 mg of EC immediately (D8), or 1 mg of BE, 24 hours after it (D9).
^{ab} Means followed by different superscripts on the same line significantly differ from each other, based on the Duncan test. at 5% significance level. EB = estradiol benzoate; EC = estradiol cypionate; eCG = equine chorionic gonadotropin; DF = dominant follicle.

regardless of the adopted treatment ($p > 0.05$) (Figures 3 and 4).

Overall, EB has induced greater endometrial expression of the investigated genes ($p < 0.0001$; Figure 5). E2 esters and eCG had isolated effect on VEGF-system gene expression, which was significantly higher in animals treated with EB ($p < 0.0001$), eCG ($p < 0.0140$) and its association (EB + eCG; $p < 0.05$) (Figures 5 and 6). There was also high OXTR gene expression in the group treated with EB ($p < 0.0001$), although its expression was

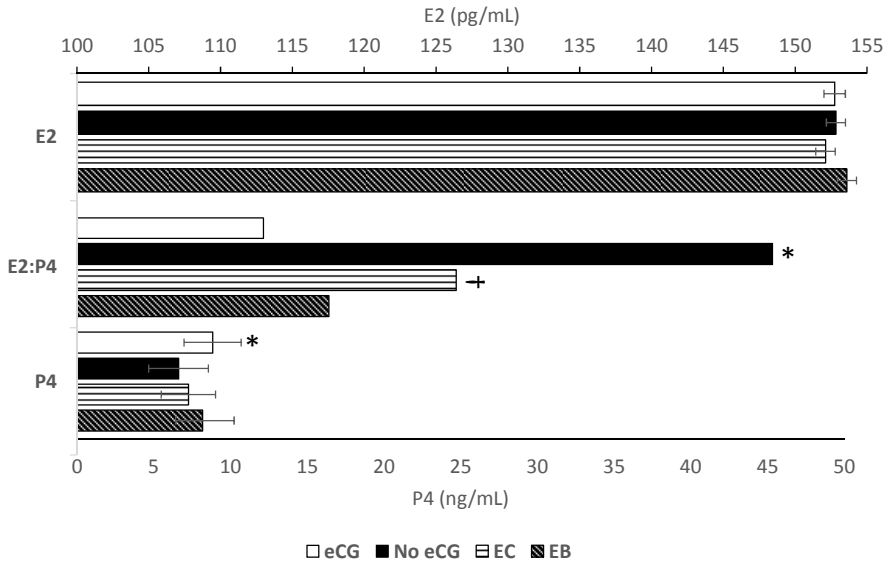

Figure 3. Main effects of estradiol esters (EB and EC) as ovulatory stimuli and eCG administration, on the P4 and E2 concentrations in blood serum (D18) and on E2:P4 ratio in follicular fluid (D10) of Nelore (Bos indicus) subject to an P4/E2-based timed artificial insemination (TAI) protocol. [a] TAI protocol. On a random day of the estrous cycle (Day 0), cows received a P4 intravaginal device plus 2 mg im injection of EB. At the time of P4 device removal (Day 8) cows were treated with 0.150 mg im of D-cloprostenol (PGF) and randomly divided to receive or not 400 UI im of eCG; as well as randomly assigned to receive 1 mg of EC immediately (D8), or 1 mg of BE, 24 h after it (D9). Values are expressed as mean ± SEM. * Significant difference between eCG vs No eCG ($p < 0.05$). † Significant difference between EB vs EC ($p < 0.05$).

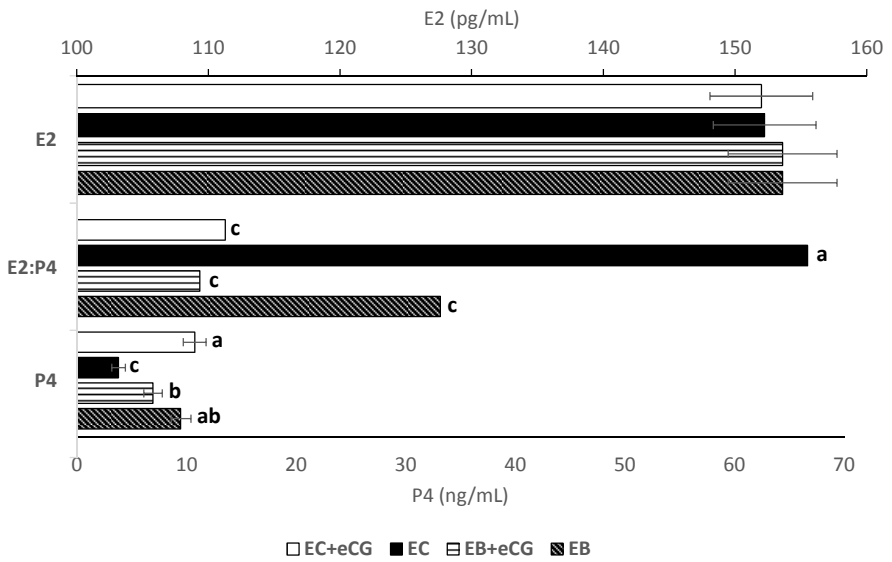

Figure 4. Effects of treatments with estradiol esters (EB and EC) as ovulatory stimuli and its association, or not, with eCG, on the P4 and E2 concentrations in blood serum (D18) and on E2:P4 ratio in follicular fluid (D10) of Nelore (Bos indicus) subject to an P4/E2-based timed artificial insemination (TAI) protocol. [†, ††]TAI protocol. On a random day of the estrous cycle (Day 0), cows received a P4 intravaginal device plus 2 mg im injection of EB. At the time of P4 device removal (Day 8) cows were treated with 0.150 mg im of D-cloprostenol (PGF) and randomly divided to receive or not 400 UI im of eCG; as well as randomly assigned to receive 1 mg of EC immediately (D8), or 1 mg of BE, 24 h after it (D9). Values are expressed as mean ± SEM. [abc]Different letters indicate significant difference between treatments ($p < 0.05$).

less evident in animals treated with eCG. The CE + eCG group has shown the lowest OXTR expression among treatments (Figures 5 and 6). PGR gene expression was also significantly higher in groups treated with BE ($p < 0.0001$) and the use of eCG had a positive influence on the expression of this gene, regardless of the inducer (p

< 0.0001). This finding has shown that increased PGR expression depends on the use of eCG in association with inducers (p = 0.0002) (Figures 5 and 6).

The association between variables analyzed through Mode-B ultrasonography, uterine-ovarian Color-Doppler, ovarian steroids serum concentration, and uterine gene expression was investigated through Principal Component Analysis (PCA) (Figure 7). Results have shown that the two main components, altogether, explained 40.21% of total data variation. Ovulation rate (OVU), OXTR, and PGR were the most representative variables in Component 1, since they presented longer-length vectors that were closer to the Component 1 axis. DF vascularization (DFV) and follicular E2 concentration (E2) were the variables that mostly contributed to Component 2; they were highly related to each other, but they were not related to OXTR and PGR. VEGF expression and uterus vascularization (UTV) have shown strong association with each other, since they formed acute angles between their respective vectors, as shown in Figure 7.

4. Discussion

Although the current study did not find a difference in follicular diameter among experimental groups, the use of inducers in association with eCG has influenced the higher ovulation rate observed for the CE + eCG group

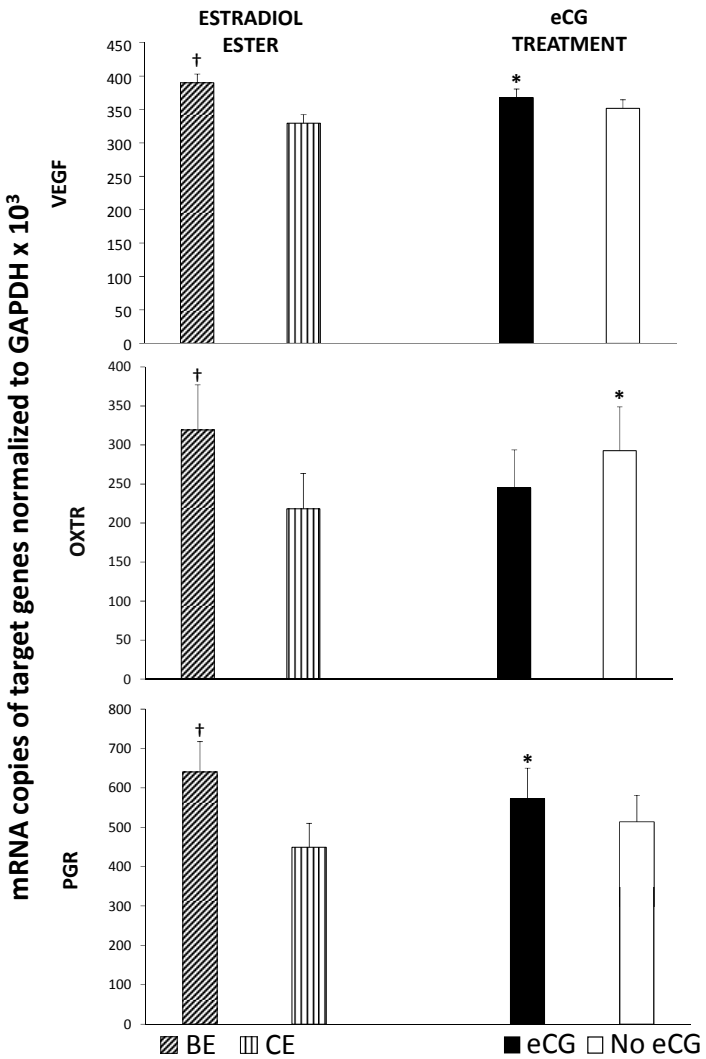

Figure 5. Main effects of estradiol esters and eCG administration on the endometrial expression of VEGF, OXTR and PGR at day ten (D10) of protocol application to Nelore cows synchronized to TAI. Values are expressed as mean ± SEM. * Significant difference between eCG vs No eCG ($p < 0.05$). † Significant difference between EB vs EC ($p < 0.05$).

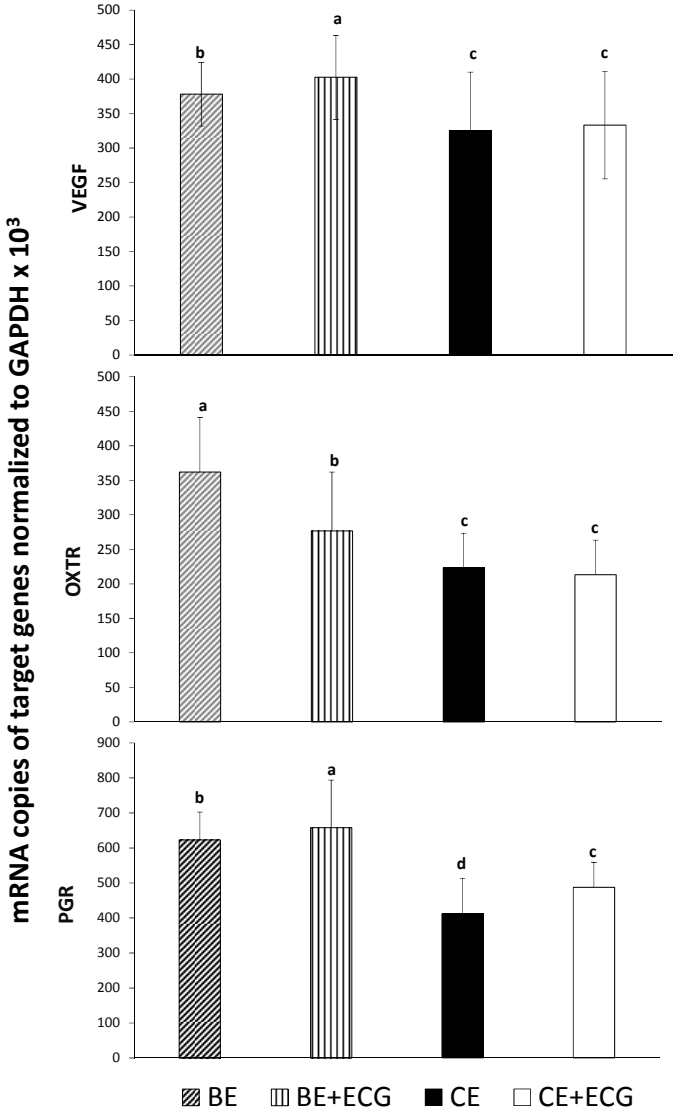

Figure 6. Effects of treatments with BE, BE + eCG, CE or CE + eCG on the endometrial expression of VEGF, OXTR and PGR at day ten (D10) of protocol application to Nelore cows synchronized to TAI. Values are expressed as mean ± SEM. [abc]Different letters indicate significant difference between treatments ($p < 0.05$).

(100%). This outcome was already expected, since many other studies have shown that eCG leads to increased fertilization rate because it makes the LH preovulatory peak more consistent, as well as increases dominant follicles' responsiveness to ovulation inducers and increases ovulation rate, mainly in anestrous animals [30, 44–52]. In addition, according to Sá Filho et al. [53], EC accounts for increasing the incidence of estrus and for better pregnancy rates after AI than EB.

These results have shown that, despite the divergence of information about the synchronization capacity of ovulations induced by EC administration at P4 device removal time, the use of this ovulation inducer resulted in fertilization rates similar to, or better than, those recorded for BE, in insemination procedures performed 48h [54,55], between 52 and 56 h [56] and between 54 and 58h [57] after P4 removal. This outcome was mainly observed when EC was used in association with eCG, which has enhanced synchronization and luteinization, besides stimulating follicular development [58].

Thus, EC administration at the P4 device removal day (three management protocol; Torres-Júnior et al. [59]) has been widely used to replace BE as ovulation inducer, without compromising indices recorded for progesterone

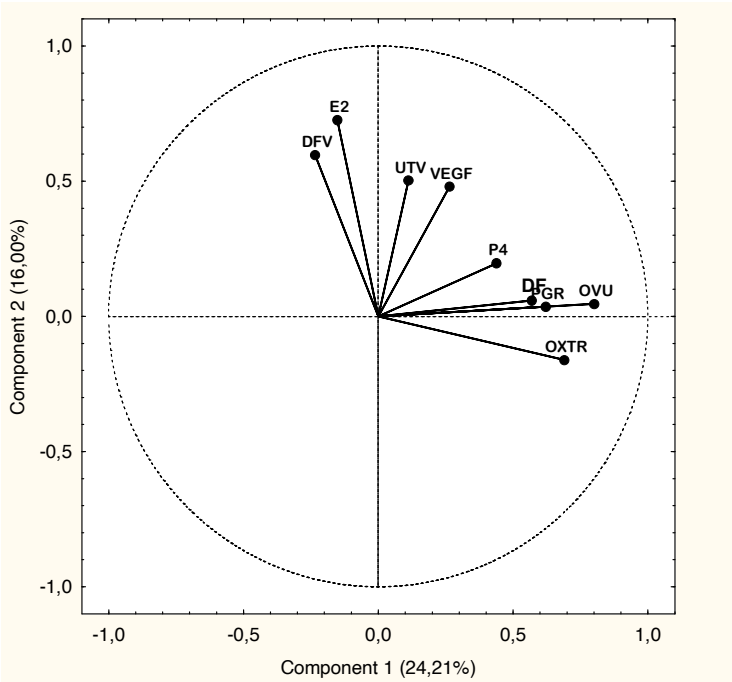

Figure 7. Biplot of principal component analysis (PCA) applied to the association between variables, based on B-mode and Doppler ultrasonography, sex steroid concentrations in follicular fluid and uterine gene expression in Nelore cows (*Bos indicus*) subjected to synchronization protocol with different estradiol esters and eCG. OVU = ovulation rate; DF = dominant folicle diammeter; DFV = dominant follicle vascularization; UTV = uterine vascularization; E2 = estradiol concentrations in follicular fluid; P4 = progesterone concentrations in follicular fluid; VEGF = endometrial expression of vascular endothelial growth factor gene; OXTR = endometrial expression of oxytocin receptor gene; PGR = endometrial expression of progesterone receptor gene.

plus eCG-based protocols. Thus, EC is an alternative used to simplify synchronization programs, since it enables reducing the number of times animals should be managed without affecting synchronization treatment effectiveness, mainly in large herds [56,57,60,61].

Endometrial blood vascularization is an important factor in uterine receptivity to the fetus [62]. The vascularized uterine area rate (%) observed in the current study has changed among treatments, from 24 h after P4 device removal onwards (D9). It may have happened due to decreased P4 concentration after P4-device withdrawal and to animals' sequential exposure to E2, which have led to vasodilation and, consequently, to increased uterine vascular flow [63]. Another recent study has shown that dairy cows subjected to 10 mg of intramuscular EB application during the postpartum period, as well as to color Doppler ultrasound assessment, have shown increased blood flow volume and speed, as well as uterine artery diameter [64].

The action of eCG in increasing uterine blood flow was also notable in the current study, mainly when it was associated with EB. The use of gonadotropins such as FSH, hCG, or eCG in TAI protocols has increased follicular estradiol concentrations [65]; estradiol worked as an important vasodilator in the uterine artery [26,66]. In addition, other studies have positively correlated increased uterine blood flow to estrogen levels during estrus in cows [20,21,67]. Honnens et al. [31] observed increased blood flow volume and decreased uterine artery pulsatility index in a study conducted with cows subjected to superovulation treatment with gonadotropins.

Animals treated with one of the estradiol esters, either with benzoate or cypionate, did not show changes in the blood vascular pattern of DF, when they were not subjected to concomitant eCG application. However, each ovulation inducer, used in association with eCG, has induced vascular flow increase in DF, at its respective administration day (EC at D8 and EB at D9). The group treated with EB presented a permanent vascularization increase from D9 to D10; this outcome was likely influenced by increased intra-follicular E2 concentrations in this group in comparison to the one treated with EC. It may have happened in response to the increased pharmacological bioavailability of EB. Vynckier et al. [68] have observed a fast increase in E2 level in the blood plasma of Holstein cows treated with 10 mg of EB; the maximum E2 level was recorded between 1 and 23 h after EB application. On the other hand, these very same authors have observed later peak plasma E2 concentrations,

after EC administration. A similar outcome was reported by Sales et al. [7], who observed LH peak approximately 30 h after EC administration and area under the curve twice as persistent in comparison to that of EB (8.6 ± 0.2 vs 16.5 ± 1.0; $p < 0.05$) [7].

Blood vascularization at follicle margin throughout proestrus and estrus is quite discreet; it presents noticeably increased blood flow approximately 6 hours before the beginning of the LH peak [69]. Interestingly, Ginther [35] has shown that color Doppler does not detect blood flow signs in follicles that do not ovulate, even if they have preovulatory diameter. This outcome indicates the potential of the Color-Doppler tool to assess blood flow in dominant follicles in TAI protocols, as long as vascular effects inherent to the administration time, dose, and estradiol ester used to induce ovulation are also taken into consideration at test interpretation time [20]. Ovulatory-follicle wall collapses during ovulation. In addition, angiogenic and mitogenic factors such as insulin-like growth factor, fibroblast growth factor, and endothelial vascular growth factor (VEGF) under LH influence lead to corpus luteum growth and vascularization [70]. Groups treated with EB have formed more vascularized CLs in the diestrus subsequent to protocol application (D18). It may have happened due to higher VEGF gene expression in this group.

A positive correlation between CL vascularization and P4 production was previously described by Arruda [71] and Ginther et al. [72]. However, the use of eCG in the current study has increased P4 concentrations, but it did not influence CL vascularization. On the other hand, the EB-treated group presented a larger number of vascularized CLs, but it did not present increased P4 concentrations. In addition, it is important emphasizing that, despite the different CL vascularization patterns observed among estradiol esters, its diameter remained unchanged.

According to Bollwein et al. [23], there was no association between CL diameter and changes in blood flow. This outcome has suggested that CL neovascularization process was not associated with its dimension. Blood flow has increased in parallel with increased circulating progesterone concentration at the early CL development stage; thus, vascularization was associated with the potential to produce progesterone [73].

As in CL, uterine vascularization at D18 (diestrus) was significantly higher in the EB group; therefore, it did not follow the expected negative correlation pattern often observed between both. The presence of CL influences uterine resistance (IR) and pulsatility (IP) rates, which are often higher in animals without CL, thus indicating lesser blood perfusion [35,74]. Similar results were observed by Rawy et al. [64] in study conducted with dairy cows subjected to 10 mg of intramuscular EB application during the postpartum period. Animals were evaluated with the aid of color Doppler ultrasound, which showed increased blood flow volume and speed, as well as uterine artery diameter.

According to Hervé et al. [75], the variability in VEGF system expression is associated with functional changes in animals' uterus and ovaries. Among the factors accounting for increasing VEGF expression, one finds ovarian steroid hormones [76] and prostaglandins [77]. VEGF expression in the diestrus subsequent to protocol application in the current study was significantly higher in animals treated with EB, and it may also explain the greater uterine vascularization observed at the same time. Therefore, the vascular effects of P4 resulted from its stimulatory action in VEGF production [78–81]. VEGF is highly mitogenic to endothelial cells [82]. This protein accounts for inducing endothelial cell proliferation, maturation, and migration, as well as for inhibiting apoptosis. VEGF regulates angiogenesis, blood vessel permeability [83], as well as vascular development under normal [84,85] and pathological conditions [86].

In addition, the BE + eCG group presented higher VEGF expression, which enabled inferring that the effects of the stimulation treatment with eCG have changed gene expression in the uterus in the short-term and, consequently, they modulated the blood perfusion pattern. VEGF expression can also be regulated by ovarian steroid hormones [76], prostaglandins [77], FSH [87], hCG [88], or by eCG itself [89], although eCG did not influence VEGF expression in the group treated with EC.

The EB group has also recorded higher OXTR gene expression, despite the fact that it presented a lower E2:P4 ratio than that of animals treated with EC. However, the EB group recorded higher absolute E2 concentration in follicular fluid than that of EC (1,637.50 ± 14.3 vs 1,500.00 ± 12.3 pg/mL; $p < 0.05$). Periovulatory E2 variations, as well as their interrelation with P4, are known to stimulate endometrial contractions mediated by increased oxytocin bonds to their receptor [9,90,91].

The PGR gene was more expressed when eCG was used in the protocols. This finding was again in compliance with the higher P4 concentration found in both follicular fluid (D10) and blood serum (D18). According to Souza et al. [92], eCG promotes increase in the number of luteal cells, which account for approximately 80% of P4 synthesis and give it greater volume and steroidogenic capacity. Accordingly, Baruselli et al. [93] have indicated eCG use as a potential tool to improve plasma P4 concentrations in estrous cycles subsequent to TAI protocol applications.

These data have shown eCG effectiveness in increasing ovulation rates, as well as CL and circulating P4 volume in diestrus subsequent to TAI application - these conditions are essential to embryonic formation and survival [30, 44-52].

Finally, the results of the present study corroborate the hypothesis that the different estradiol esters and eCG adminstration differentially regulate vascularization, steroidogenesis, and utero-ovarian gene expression in Bos indicus cows submitted to TAI protocols.

Conflict of interest

The authors declare no conflict of interest.

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