Endometrial prostaglandin F2α *in vitro* production and its modulation regarding dominant follicle position in cattle

Produção in vitro de PGF F2α endometrial e sua modulação referente ao folículo dominante em bovinos

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Abstract

Prostaglandin F2α (PGF2α) determines luteolysis in cattle, and the ability to manipulate its endogenous synthesis is indispensible for large-scale animal breeding. Estradiol (E_2) and progesterone (P_4) modulate several molecular pathways in endometrial cells, including the synthesis of PGF2α; however, its specific mechanisms are still not totally known. This study investigated the production *in vitro* and possible modulation of endometrial PGF2α due to a local effect of endogenous E_2 in the ipsilateral uterine horn (UH) containing the dominant follicle (DF) or from P_4 in ipsilateral horn containing the corpus luteum (CL). The PGF2α stimulators oxytocin (OT) and phorbol 12,13-dibutyrate (PDBu) were incubated with endometrial explants, and PGF2α content was measured. For that, cycling cows were synchronized, the development of DF and CL was examined by ultrasonography and on the seventh day of the estrous cycle, endometrial explants were collected and cultured in medium supplemented with 10^{-6} M PDBu or 10^{-6} M OT or non-supplemented. Media samples were collected immediately after treatment and 60 min later. Radioimmunoassay showed that the PGF2α content of the UH ipsilateral to the DF was 49% less than that of the contralateral UH (8.22 ± 0.95 vs. 12.24 ± 0.95 pg/mL/mg tissue, respectively; $P < 0.01$). However, the PGF2α levels did not differ between the UHs as a function of the CL position (9.46 ± 0.95 vs. 11 ± 0.95 pg/mL/mg; $P > 0.05$). The cellular stimulators promoted an increase in PGF2α synthesis ($P < 0.02$), and the effects differed among the animals ($P < 0.04$). The PGF2a production was higher in the explants treated with PDBu rather than OT (13.68 ± 1.16 vs. 10.01 ± 1.16 pg/mL/mg tissue, respectively; $P < 0.05$). In conclusion, PGF2α synthesis is modulated by the presence of the DF (local E_2) but not the CL (local P_4), and both PDBu and OT stimulated PGF2a synthesis.

Keywords: Cattle. Luteolysis. PGF2α synthesis. Reproductive physiology.

Resumo

A prostaglandina F2α (PGF2α) determina a luteólise em bovinos. A capacidade de manipular sua síntese endógena é indispensável para a produção animal em grande escala. O estradiol (E_2) e a progesterona (P_4) modulam diversas vias moleculares das células endometriais, incluindo a síntese de PGF2α; no entanto, pouco se sabe sobre seus mecanismos específicos. Este trabalho investigou a produção *in vitro* e a possível modulação da PGF2α endometrial devido a um efeito local do E_2 endógeno no corno uterino ipsilateral ao folículo dominante (FD) ou da P_4 no corno ipsilateral ao corpo lúteo (CL). Os estimuladores de PGF2α oxitocina (OT) e 12,23-dibutirato de forbol (PDBu) foram incubados com explantes endometriais, e o conteúdo de PGF2α foi mensurado. Para tal, vacas cíclicas foram sincronizadas, o desenvolvimento de FD e CL foi examinado por ultrassonografia, e no 17º dia do ciclo estral os explantes endometriais foram coletados e cultivados em meio ou suplementados com PDBu 10^{-6}M ou 10^{-6}M OT. As amostras de meio foram coletadas imediatamente após o tratamento e sessenta minutos depois. O radioimunoensaio mostrou que o conteúdo de PGF2α do corno ipsilateral ao FD foi 49% menor que o do corno contralateral (8,22 ± 0,95 vs. 12,24 ± 0,95 pg/mL/mg de tecido, respectivamente, $P < 0{,}01$). No entanto, os níveis de PGF2α não diferiram entre os cornos em função da posição do CL (9,46 ± 0,95 versus 11 ± 0,95 pg/mL/mg; $P > 0{,}05$). Os estimuladores celulares promoveram um aumento na síntese de PGF2α ($P < 0{,}02$), e os efeitos diferiram entre os animais ($P < 0{,}04$). A produção de PGF2α foi maior nos explantes tratados com PDBu em comparação à OT (13,68 ± 1,16 versus 10,01 ± 1,16 pg/mL/mg de tecido, respectivamente, $P < 0{,}05$). A conclusão obtida foi que a síntese de PGF2α é: modulada pela presença do FD (E_2 local), mas não do CL (P_4 local); e estimulada por PDBu e OT.

Palavras-chave: Bovino. Luteólise. Síntese de PGF2α. Fisiologia reprodutiva.

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Introduction

In cattle, the reproductive cycle is determined by a well-coordinated set of physiological events. Luteolysis, one of these events, is characterized by the functional and morphological regression of the corpus luteum (CL) resulting in the decline in the plasma concentrations of progesterone (P_4), favoring the growth of the dominant follicle (D_F) and an increase in the serum concentrations of estradiol (E_2); hence, initiating an acute release of luteinizing hormone (LH) and ovulation. Prostaglandin F2α (PGF2α) is known to be the main luteolytic factor in cows, and it has been reported that its action on the CL is probably mediated by other factors such as immune and endothelial cells and pericytes. In this species, the occurrence of five to eight pulsatile releases of PGF2α within an interval of two to three days determines luteolysis between days fifteen and nineteen of the estrous cycle (MCCRACKEN et al., 1999; MEIDAN et al., 1999; SKARZYNSKI; OKUDA, 2010).

The endometrial synthesis of PGF2α results from a complex cascade of highly coordinated events involving endocrine factors such as estradiol (E_2), oxytocin (OT) and luteinizing hormone (LH). Molecularly, the arachidonic acid (AA) stored in the phospholipid membranes is the main primary precursor of prostaglandins (DIAZ et al., 2002). In the classic model, oxytocin (OT), acting through its receptor on the endometrial cell membrane, activates a guanosine nucleotide-binding protein (G protein), which promotes the activation of phospholipase C (PLC) that cleaves phosphatidylinositol triphosphate into inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 binds to receptors on the endoplasmic reticulum, promoting an increase in the cytoplasmic calcium concentration. DAG activates protein kinase C (PKC), a serine/threonine kinase that is dependent on calcium for activation. Activated PKC phosphorylates phospholipase A_2 (PLA2). The IP3-induced increase in cytosolic calcium stimulates the calcium-dependent PLA_2 activity, and PLA_2 preferentially cleaves the sn-2 position of phosphatidylcholine, releasing AA. The free AA is then converted into PGG_2 by the cyclooxygenase (COX, prostaglandin G/H synthase) enzyme. PGG_2 is converted into PGH_2 by a peroxidase and, finally, converted into PGF2α by PGF synthase (WLODAWER et al., 1976; CLARK et al., 1991; BURNS et al., 1997; GIJÓN; LESLIE, 1999).

Another PGF2α stimulator is the phorbol ester PDBu (phorbol 12, 13 dibutyrate [PDBu]). Phorbol esters stimulate PKC activity, activates COX-2 gene expression and PGF2α secretion via the mitogen-activated protein kinase (MAPK) pathway (THATCHER et al., 2001). The abilities of PDBu and OT to stimulate PGF2α synthesis in bovine endometrial tissue endometrial have been reported by Danet-Desnoyers et al. (1994); Mann (2001); Guzeloglu et al. (2004) and Rodriguez-Sallaberry et al. (2006).

The role of endogenous estradiol in this context is not fully understood. It was reported that E_2 is important for PGF2α synthesis during bovine luteolysis and, therefore, for female reproductive physiology (KARSCH et al., 1970; IRELAND et al., 1984; VILLA-GODOY et al., 1985; HUGHES et al., 1987; ZHANG et al., 1991; SALFEN et al., 1999), and it is also known that it exerts several other physiological roles in the organism (BERKANE et al., 2017; COOKE et al., 2017; ZENDEDEL et al., 2017). In cattle, E_2 administration between days 13 and 18 of the estrous cycle stimulates the production of 13,14-dihydro-15-keto-prostaglandin F2α (PGFM), the main PGF2α metabolite (LEMON, 1975; BARTOL et al., 1981; KNICKERBOCKER et al., 1986; THATCHER et al., 1986; LARSON et al., 1991). Bertan (2004) and collaborators compared the PGF2α synthesis capacity after E_2 administration on days 15, 17 and 19 of the estrous cycle and observed that the magnitude of the E_2 response progressively increased during this period.

It is assumed that the triggering of PGF2α synthesis by E_2 is dependent on a previous and current priming of the endometrium by several factors throughout the estrous cycle. With respect to the roles of E_2 on PGF2α synthesis, several studies have reported that E_2 induces an increase in the numbers of endometrial OT receptors; hence, increasing the capacity of the endometrium to respond to OT (BEARD; LAMMING, 1994; SPENCER et al., 1995; BERIO et al., 2017).

Progesterone is produced in large amounts by the CL, and endometrial priming by P_4 is important for PGF2α production (VALLET et al., 1990; ZHANG et al., 1991; PINEDA; DOOLEY, 2003). It was reported that in the absence of previous priming by progesterone, PGF2α synthesis is inhibited (SILVIA; RAW, 1993; LAMMING; MANN, 1995; RAW et al., 1995). Bogacki et al. (2002) observed that P_4 has an inhibitory effect on the production and/or activation of OT receptors and on the binding of OT to its receptor.

Although roles for E_2 and P_4 in PGF2α synthesis have been illustrated, the mechanism of action of these hormones on luteolysis is still unclear. It is known that E_2 and P_4 are present in the systemic circulation and that their concentrations vary throughout the estrus cycle, consistent with their modulation of cellular mechanisms. However, it is also possible that local modulation of PGF2α synthesis by E_2 and P_4 may occur, where the uterine horn ipsilateral to the DF would be influenced by locally produced E_2 and the uterine horn ipsilateral to CL would be influenced by the local P4 concentration.

The present work investigated whether endometrial explants primed with endogenous E_2 or P_4 can produce PGF2α *in vitro* when stimulated with OT or PDBu, and whether the response would be affected by the dominant follicle position (ipsi or contralateral to the endometrial explants). These results contribute to a better understanding of bovine reproductive physiology and provide new insights on bovine endocrine modulation, an essential feature for an optimized animal breeding program.

Methods

Non-lactating, non-pregnant and cycling crossbred cows (*Bos taurus* x *Bos indicus*, n = 5) were maintained in grass (*Brachiaria decumbens* var. marandu) paddocks supplemented with minerals and water *ad libitum*.

Cows presenting DF and responsive CL received 150 µg D-cloprostenol IM (N.C. Preloban® – Intervet) to induce estrus. Heat behavior was observed every 12 h for the subsequent five days. D0 of the estrus cycle was considered the day when estrus was observed. On D6, ultrasonographic examinations (Aloka SSD500, 7.5 MHz linear transductor) were performed, and females presenting with a DF > 7.5 mm received 0.15 mg of a synthetic GnRH analog IM (Fertagyl®) to induce DF ovulation, emergence of a new follicular wave and occurrence of three-wave cycles. DFs from the second follicular wave were observed ultrasonographically on days 14, 15 and 16 of the estrus cycle. On the 17th day of the estrous cycle, the uterine horns were collected from the cows at the slaughterhouse, placed in ice and immediately brought to the laboratory (approximately 15 min). The uterine horns were dissected, endometrial explants from both horns collected, and fragments of 80 – 100 mg of intercaruncular tissue incubated in borosilicate tubes containing 0.5 mL Krebs-Hensleit bicarbonate medium (KHB; 118 mM NaCl, 4.7 mM KCl, 2.56 mM CaCl2, 1.13 mM MgCl2, 25 mM NaHCO3, 1.15 mM NaH2PO4, 5.55 mM glucose, 20 mM Hepes, 0.013 mM phenol red; pH 7.4). The fragments were incubated for 1 h at 37°C and 40 rpm, washed twice with 0.5 mL KHB and again incubated for 1 h and washed. The samples were then incubated in 1 mL with the *in vitro* treatments: KHB (control), KHB supplemented with 10^{-6} M OT (Sigma Chemicals O-6379 reconstituted in acetic acid 5%, final concentration of 0.25 g/mL) or KHB supplemented with 10^{-6} M PDBu (Sigma Chemicals P-1269; reconstituted in ethanol at 1 mg/mL). The treatments were conducted in quadruplicate. The concentrations of both stimulators were based on previous reports (BURNS et al., 1997; ARNOLD et al., 2000). Aliquots (250 µL) of the culture medium were removed immediately after treatment administration (time 0) and again after 60 min of incubation. The PGF2α concentrations of these samples were analyzed using a radioimmunoassay as previously described (DANET-DESNOYERS et al., 1994) and average PGF2α production between time 0 and 60 was analyzed (DIF 60).

The PGF2α concentrations at times 0 and 60 were adjusted to pg/mL/mg of endometrial tissue. The intra-assay coefficients of variation for the radioimmunoassay for reference samples containing low (250 pg/mL), medium (1,000 pg/mL) and high concentrations (3,500 pg/mL) were 4.15%, 17.52% and 21.69%, respectively. The inter-assay coefficients of variation for the low, medium and high concentration reference samples were 1.98%, 18.16% and 26.77%, respectively.

Statistical analysis

Preliminary analysis demonstrated that data was not adequate regarding analysis of normality of residues (Shapiro-Wilk test, P < 0,01) and variance homogeneity (F test, P < 0,01). The data was converted to square roots and submitted to variance analysis. The difference between the PGF2α concentrations at times 0 and 60 (DIF 60) was the dependent variable, and the independent variables were: animal, uterine horn (ipsi- or contralateral to DF or CL), *in vitro* treatments and interactions. The data were analyzed by ANOVA (proc GLM) (SAS INSTITUTE, 1988) and are presented as untransformed LSmeans ± SEM. The means of the treatments were compared by specific contrasts.

Results

A local effect of the presence of the DF was observed when endometrial PGF2α content was compared between the uterine horns independent of the *in vitro* stimulation (Figure 1). Overall, the PGF2α concentration in the UH contralateral to the DF was 49% higher than that of the ipsilateral UH (12.24 ± 0.95 vs. 8.22 ± 0.95 pg/mL/mg of tissue, respectively; $P < 0.01$). However, the endometrial PGF2α levels were not different between the UHs as a function of the position of the CL (Figure 2; 9.46 ± 0.95 vs. 11 ± 0.95 pg/mL/mg of tissue; $P > 0.05$).

When both uterine horns were considered, higher PGF2α concentrations were observed when the explants were treated with PDBu (13.09 ± 1.16 pg/mL/mg of tissue) than when they were treated with OT (10.02 ± 1.16 pg/mL/mg of tissue) or left untreated (7.59 ± 1.16 pg/mL/mg of tissue; $P < 0.05$) independent of the DF or CL position (Figures 3 and 4).

Under the conditions of the present investigation, large variations in PGF2α production among individuals were observed, resulting in an animal effect ($P < 0.05$). An interaction between animal and i*n vitro* treatment was observed ($P < 0.05$). Two of the animals did not respond to either OT or PDBu, whereas three responded to both OT (minimum of 4.1% and maximum of 60.7%) and PDBu (minimum of 37.3% and maximum of 111.8%, data not shown). However, no interaction between animal and uterine horn was observed. Regardless of the magnitude of the PGF2α production, a higher concentration in the UH contralateral to the DF was consistently observed.

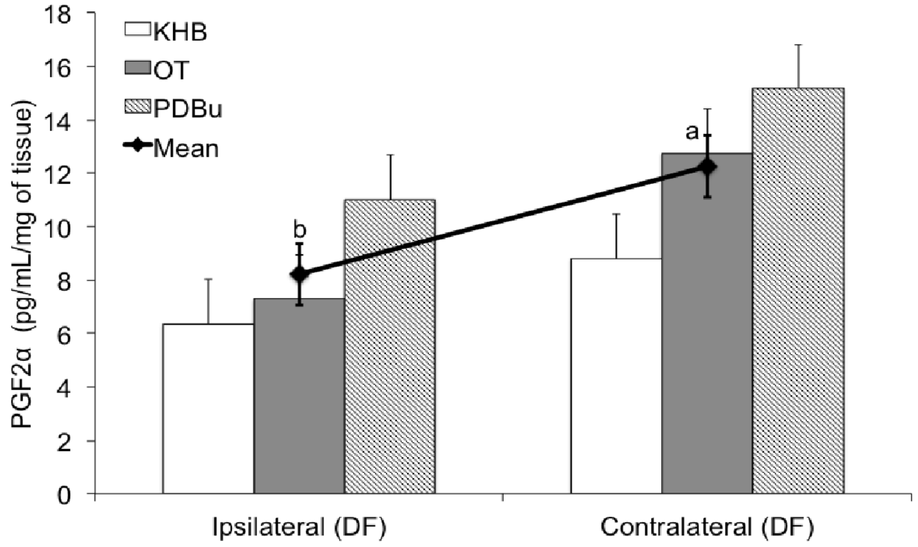

Figure 1 – Effect of DF position (ipsi- or contralateral uterine horns) on average PGF2α production of endometrial explants, treated or not (KHB), with OT 10^{-6}M or PDBu 10^{-6}M o (DIF 60; LSmeans + SEM). a, b: Different letters represent statistical difference

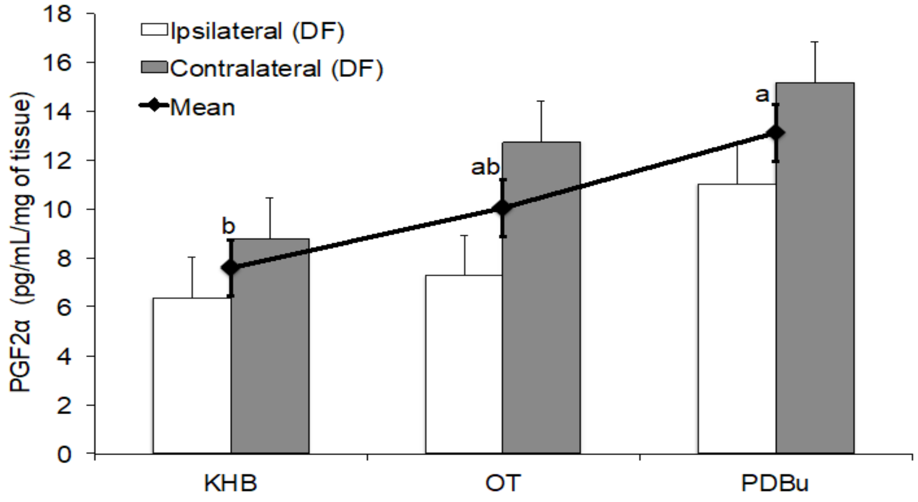

Figure 2 – Effect of *in vitro* treatments on endometrial explants from ipsi- or contralateral uterine horn to the DF, treated or not (KHB), with OT 10^{-6}M or PDBu 10^{-6}M on average PGF2α production (DIF 60; LSmeans + SEM). a, b: Different letters represent statistical difference

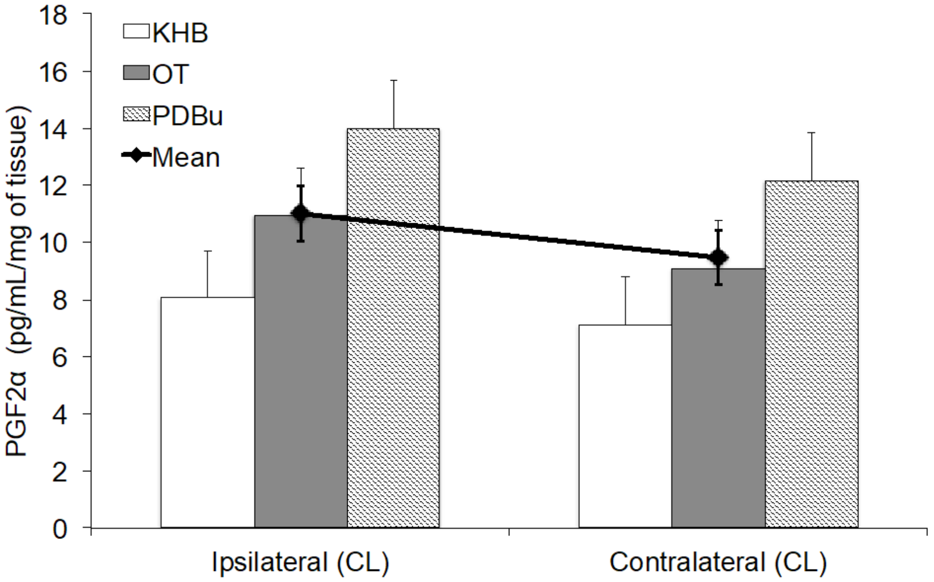

Figure 3 – Effect of CL position (ipsi- or contralateral uterine horns) on average PGF2α production of endometrial explants, treated or not (KHB), with OT 10^{-6}M or PDBu 10^{-6}M o (DIF 60; LSmeans + SEM)

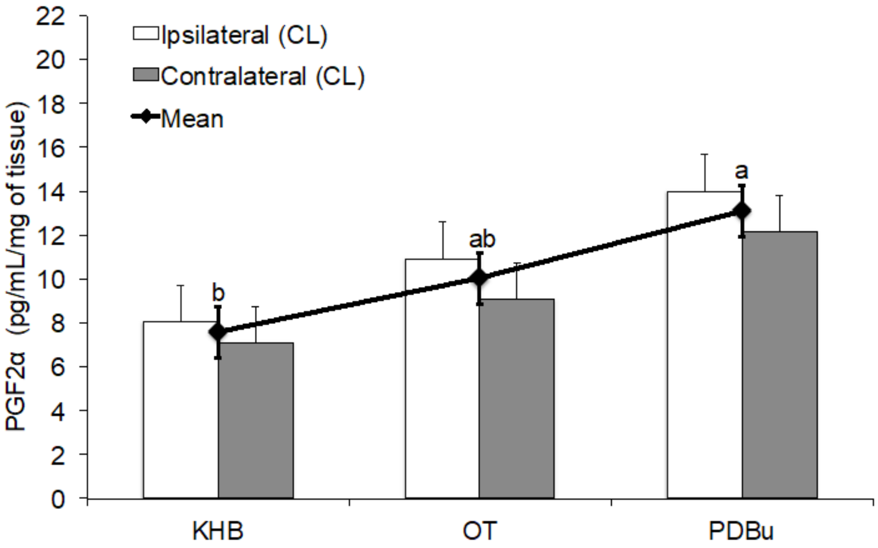

Figure 4 – Effect of *in vitro* treatments on endometrial explants from ipsi- or contralateral uterine horn to the CL, treated or not (KHB), with OT 10^{-6}M or PDBu 10^{-6}M on average PGF2α production (DIF 60; LSmeans + SEM)

Discussion

It has been widely reported that the E_2 is produced by the ovarian follicle due to the interaction between the theca cells and the granulosa cells and, after deviation, the DF presents higher steroidogenic capacity, resulting in greater concentrations of E_2 in the follicular fluid (XU et al., 1995; BEG et al., 2001). The uterine and ovarian vascularization system of the bovine exhibits a particular anatomical feature, an entanglement between these vessels that leads to speculation regarding a possible local role of E_2 from the DF affecting the ipsilateral uterine horn. This vein is closely associated with the ovarian artery, permitting transport of PGF2α from the uterine vein into the ovarian artery by the classic mechanism of counterflow (MAPLETOFT; GINTHER, 1975).

Herein it can be speculated that E_2 synthesized by the DF was, via this counterflow mechanism, transferred from uterine vein to the artery, providing E2 in higher concentrations to the uterine horn ipsilateral to the ovary bearing the DF. Similarly, P_4 produced by the CL would be provided in higher concentrations to the uterine horn ipsilateral to the CL. It is known that both E_2 and P_4 are involved in PGF2α synthesis and are produced in DF and CL, respectively. It is therefore plausible to infer a possible local action of these hormones that would promote endometrial PGF2α synthesis in the uterine horn ipsilateral to these structures.

In the present investigation, greater levels of PGF2α synthesis in the UH contralateral to the DF were observed, indicating a local modulation of the E_2 after PGF2α production stimulation. Although the exact mechanisms of action of estradiol on PGF2α synthesis are not yet known, a few facts about estradiol are already well established. It is known that estradiol action is mediated by intracellular receptors, and two predominant types of receptors have been characterized: α and β estrogen receptors, and the first has been shown as the most important subtype for the regulation of uterine physiological processes (KUIPER et al., 1996; COUSE; KORACH, 1999; WALTHER et al., 1999; MURAMATSU; INOUE, 2000). Typically, estradiol exerts its functions by binding to its receptors, which act as transcription factors (MANGELSDORF et al., 1995) and, therefore, stimulate gene transcription and the synthesis of new proteins (MANGELSDORF et al., 1995; HO; LIAO, 2002).

Indeed, it is known that PGF2α production is dependent on protein synthesis (BINELLI et al., 2000) and, therefore, the presence of different concentrations of E_2 in each uterine horn, as reported by Ireland et al. (1994), is probably highly involved with quantitative and qualitative differences on protein synthesis between horns and, as a result, in PGF2α production.

Bertan (2004) and collaborators suggested that the role of E_2 in triggering PGF2α synthesis is dependent on previous progressive endometrial priming with several factors throughout estrous cycle. It was speculated here that during the period in which the PGF2α synthesis was higher in the uterine horn contralateral to the DF, lower concentrations of the factors responsible for such previous priming are probably found in the UH ipsilateral to the DF. Lower synthesis capacity of PGF2α production may be beneficial to the future pregnant uterine horn. However, further studies are needed to unravel the mechanisms in which the UH bearing the DF present lower levels of PGF2α when stimulated and focusing on blood flow and cell-to-cell communication features through extracellular vesicles might explain such modulation. Indeed, extracellular vesicles such as exosomes or microvesicles were already described in body fluid such as seminal plasma, uterine fluid and even follicular fluid (BURNS et al., 2014; TANNETTA et al., 2014; BRESSAN et al., 2015). These vesicles contain bioactive molecules such as RNAs, miRNAs and proteins that are able to mediate physiological processes. For instance, miRNAs involved in the regulation of estradiol and progesterone concentrations were already reported in exosomes present in the follicular fluid in humans (SANG et al., 2013).

PGF2α acts in the developing CL as a local regulator to enhance progesterone secretion directly and indirectly by stimulating angiogenic factors VEGF and FGF2, probably explaining why the developing CL does not acquire luteolytic capacity until several days following ovulation (MIYAMOTO et al., 2010). Therefore, a possible and yet not completely understood endocrine mechanism of communication between UHs could explain the similarity observed in PGF2α synthesis in both UHs regardless of the CL position herein when D17 of the estrous cycle was studied. Nevertheless, both UHs showed capacity to respond to PGF2α production when stimulated *in vitro*.

Interestingly, greater stimulation of PGF2α synthesis in explants treated with PDBu and not OT was observed when compared to control. It has been demonstrated that oxytocin-stimulated PGF2α secretion is associated with its binding to receptors, activity of PKC and the gene expression of enzymes involved in PGF2α synthesis, e.g., PLA2, COX-2, and PGFS and that the endometrial sensitivity to oxytocin varies during the estrous cycle, presenting high responsiveness during the period of luteal regression until the early luteal stage of the next estrous cycle (SILVIA et al., 1991; MIRANDO et al., 1993; OKUDA et al., 2002).

These characteristics may explain the results obtained herein, as well as some previous results that did not observe a stimulatory effect of OT in endometrial explants retrieved from D17 cows (SKARZYNSKI et al., 1999; BERTAN, 2004).

It has been reported that PGF2α produced by the endometrial cells is transported through the uterine vein by the prostaglandin transporter (PGT), a 12-transmembrane solute carrier organic anion transporter protein, and activation of ERK1/2 pathways and interactions between ERK1/2 and PGT protein appear to be PGT-mediated efflux transport function (LEE et al., 2013).

Thatcher et al. (2001) showed that treatment of endometrial (BEND) cells with the MEK-1 inhibitor, PD98059 completely abolished PDBu-induced secretion of PGFα, showing that the PKC-Rafl/MEK-l/ERK1/2 pathway was required for PDBu induction of COX-2 activation and PGFα, secretion in this model, probably due to activation of this pathway through PKC-mediated phosphorylation of Raf-1 and subsequent activation of MEK-1 and ERK1/2 (PRU et al., 2001; THATCHER et al., 2001). In summary, different specific mechanisms of action of both OT and PDBu, allied with the temporal variation of endometrium responsiveness to OT probably lead to different *in vitro* results of PGF2α after stimulation in this study.

In the present investigation, PGF2α production varied greatly among the animals. Similar effects were also observed by Arnold et al. (2000) that used a protocol like the present one. An interaction between the animal and the treatment was also observed, where the magnitude of the response to the stimulators differed among animals. These observations suggested that the stimulatory action of both cell stimulators might be linked to the presence or absence of a set of proteins that comprise the PGF2α-generating cascade. It is possible that the magnitude of the response of the endometrial cells may be related to the concentrations of these proteins, thus generating responses with a wide range of magnitude.

In conclusion, it was demonstrated herein that the production of PGF2α in both uterine horns is similar regardless of the location of the CL. However, estradiol produced by the DF may have a local effect by inhibiting PGF2α synthesis. Furthermore, exogenous OT and PDBu were able to stimulate PGF2α synthesis in the endometrial explants, with PDBu treatment resulting in a greater stimulation when compared to control.

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