BEATRIZ DE OLIVEIRA CARDOSO

Requirement of follicular estradiol for the onset of luteolysis in *Bos indicus* cows supplemented with progesterone at early diestrus

Pirassununga

2017

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Dissertation submitted to the Postgraduate Program in Animal Reproduction of the School of Veterinary Medicine and Animal Sciences of the University of São Paulo to obtain the Master's degree in Sciences.

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Prof. Mario Binelli, Ph.D.

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UNIVERSIDADE DE SÃO PAULO

FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "Cytobrush: uma ferramenta para avaliação molecular do endométrio bovino", protocolada sob o CEUA nº 5816230217, sob a responsabilidade de **Mario Binelli** *e equipe; Beatriz de Oliveira Cardoso* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 06/04/2017.

We certify that the proposal "Cytobrush: a tool for molecular evaluation of bovine endometrium ", utilizing 10 Bovines (10 females), protocol number CEUA 5816230217, under the responsibility of **Mario Binelli** *and team; Beatriz de Oliveira Cardoso* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 04/06/2017.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 02/2017 a 03/2017 Área: Reprodução Animal

Origem:	Prefeitura do Campus da USP de Pirassununga						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	3 a 13 anos	N:	10
Linhagem:	Nelore			Peso:	500 a 700 kg		

Resumo: Em bovinos, a compreensão molecular da biologia uterina in vivo requer um exame do útero por meio de técnicas invasivas, tais como a biópsia. Coletas de amostras repetidamente podem causar inflamação, danos físicos e afetar a fertilidade. Portanto, a validação de técnicas de amostragem menos traumáticas, repetíveis e que permitem a coleta de amostras representativas e confiáveis é necessária. Neste contexto, esse estudo pretende: (1) comparar os aspectos estrutural e funcional de amostras endometriais coletadas por biópsia transcervical e por cytobrush e (2) caracterizar o perfil de expressão de genes envolvidos no mecanismo de luteólise no endométrio bovino utilizando a técnica de cytobrush. Para tanto, o cio de cinco vacas Nelore será sincronizado utilizando-se um protocolo a base de progesterona e estradiol. O dia da ovulação será detectado por ultrassonografia. Dez dias após a ovulação, amostras endometriais serão coletadas do corpo uterino por meio de escova citológica adaptada à extremidade de um aplicador convencional de inseminação artificial e, posteriormente, utilizando-se um aparato de biópsia. As amostras serão submersas em reagente Trizol e armazenadas a -80 °C até a extração do RNA. A abundância de transcritos característicos de epitélio (KRT18), estroma (VIM), células imunes (CD3D) e células endoteliais (FLT1) e transcritos envolvidos na função uterina durante o ciclo estral (PGR, PTGES, AKR1C4) será medida por RT-qPCR e comparada entre as técnicas de coleta. Posteriormente, alo cio de cinco vacas Nelore foi sincronizado e o dia da ovulação (D0) foi estimado por ultrassonografia. Nos dias 10, 13, 16 e 19 amostras endometriais foram coletadas utilizando-se o método cytobrush. A abundância dos transcritos OXTR, ESR1 e PGR2 foi medida por qPCR.

Local do experimento: Instalações do Departamento de Reprodução Animal - CBRA - FMVZ - USP Campus de Pirassununga

São Paulo, 13 de julho de 2017

Profa. Dra. Denise Tabacchi Fantoni
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Roseli da Costa Gomes
Secretaria Executiva da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

CERTIFICADO

Certificamos que a proposta intitulada "Requerimento do estradiol na antecipação da luteólise em vacas Bos indicus (Nelore) suplementadas com progesterona no início do diestro ", protocolada sob o CEUA nº 8717061115, sob a responsabilidade de **Mario Binelli** - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 02/03/2016.

We certify that the proposal "Estradiol role on progesterone-induced CL regression in beef cows", utilizing 75 Bovines (75 females), protocol number CEUA 8717061115, under the responsibility of **Mario Binelli** - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 03/02/2016.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 01/2016 a 03/2016 Área: Reprodução Animal

Origem:	Prefeitura do Campus da USP de Pirassununga						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	a	N:	75
Linhagem:	Nelore			Peso:	a		

Resumo: A suplementação com progesterona (P4) no início do diestro pode ser uma estratégia para aumentar as taxas de concepção em vacas de corte. No entanto, observou-se a ocorrência de antecipação da regressão funcional e estrutural do corpo lúteo (CL; i.e., luteólise precoce) e consequente perda gestacional em uma parcela dos animais submetidos a este tratamento. Supõe-se que a redução da eficácia dessa estratégia pode ser devida à ocorrência da luteólise precoce. Entende-se que este processo tem pelo menos dois componentes, não excludentes, a serem estudados: um componente uterino que está associado à liberação precoce de pulsos luteolíticos de PGF2? endometrial e um componente luteínico que está associado à formação de um CL sub-funcional. Considerando que o estradiol (E2) proveniente dos folículos ovarianos durante a fase luteal apresenta um papel central na indução da liberação de PGF2? e no desencadeamento do processo de lise do CL, o objetivo deste trabalho é investigar o requerimento do E2 para a ocorrência da luteólise precoce verificada em bovinos de corte suplementados com P4 no diestro inicial. Para tanto, vacas Bos indicus (Nelore) multíparas e ciclando terão as ovulações sincronizadas e serão distribuídas aleatoriamente em arranjo fatorial 2 x 2, a fim de receberem tratamento com placebo ou P4 injetável de longa ação no início do diestro, e ainda, serem submetidas ou não à ablação folicular por meio de OPU ao longo da fase luteal. O crescimento folicular e o desenvolvimento e vascularização luteínica serão avaliados por ultrassonografia, ao passo que as concentrações plasmáticas de P4 e E2 serão avaliadas por radioimunoensaio. Amostras endometriais serão coletadas por escovas citológicas e amostras luteínicas por biópsia. A abundância de transcritos será medida por qPCR. Será verificada a dinâmica das alterações temporais na abundância de transcritos de genes que (1) controlam a liberação de PGF2? no endométrio e (2) de genes associados ao desenvolvimento, funcionamento, e resposta à PGF2? do CL, ao longo do diestro.

Local do experimento:

São Paulo, 13 de julho de 2017

Profa. Dra. Denise Tabacchi Fantoni
Presidente da Comissão de Ética no Uso de Animais

Roseli da Costa Gomes
Secretaria Executiva da Comissão de Ética no Uso de Animais

EVALUATION FORM

Author: CARDOSO, Beatriz de Oliveira

Title: **Requirement of follicular estradiol for the onset of luteolysis in *Bos indicus* cows supplemented with progesterone at early diestrus**

Dissertation submitted to the Postgraduate Program in Animal Reproduction of the School of Veterinary Medicine and Animal Sciences of the University of São Paulo to obtain the Master's degree in Sciences.

Date: ___/___/_____

Committee Members

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

Prof. _____

Institution: _____ Decision: _____

DEDICATION

I dedicate this dissertation to my dad, Jair Cardoso. The one who taught me the value of knowledge, of studying, and of character. The one who always said that his inheritance would not be money, but rather the study and honesty. Certainly, this is true.

All my love to you.

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My deep thanks to all those mentioned here and those I did not mention but who directly or indirectly helped me to get here.

“The important thing is not to stop questioning. Curiosity has its own reason for existing.”

Albert Einstein

RESUMO

CARDOSO, B. O. **Requerimento do estradiol folicular para o momento da luteólise em vacas *Bos indicus* suplementadas com progesterona no início do diestro.** [Requirement of follicular estradiol for the onset of luteolysis in *Bos indicus* cows supplemented with progesterone at early diestrus]. 2017. 81 f. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, 2017.

Em vacas de corte, a suplementação com progesterona injetável de longa ação (iP4) no diestro inicial tem efeitos paradoxais, uma vez que tanto aumenta a fertilidade a campo quanto induz a antecipação da luteólise em uma parte dos animais tratados. Considerando que o estradiol (E2) proveniente dos folículos ovarianos durante a fase luteal desempenha um papel central na indução da liberação de PGF2α uterina e regressão luteal, testou-se a hipótese central de que a ausência de folículos pós-desvio previne a antecipação do momento da luteólise e prolonga o ciclo estral em vacas *Bos indicus* suplementadas com iP4. Esta dissertação consiste em dois estudos. Um primeiro estudo foi realizado para validar a utilização de escovas citológicas (cytobrush) como uma ferramenta para coletar amostras sequenciais do endométrio para caracterizar as mudanças temporais no transcriptoma ao longo do ciclo estral. Concluiu-se que a técnica de cytobrush foi confirmada como um método confiável, repetível, e seguro para o estudo da biologia uterina bovina *in vivo*. No segundo estudo, vacas Nelore cíclicas foram sincronizadas e distribuídas em um arranjo fatorial 2 x 2 para receber tratamento com placebo ou 300 mg de iP4 três dias após a ovulação (D0), e serem submetidos ou não à aspiração folicular (AF) durante a fase lútea. Amostras endometriais foram coletadas no D15, D17 e D19 usando a técnica de cytobrush, e a abundância de transcritos foi medida por qPCR. A iP4 diminuiu o tamanho do maior folículo no D6 e a área do CL entre D8 e D10 comparado aos grupos placebo. Vacas submetidas à AF tiveram um ciclo mais longo, porém o momento da luteólise foi semelhante ao das vacas não aspiradas. O tratamento com iP4 e AF tendeu a adiantar o momento da regressão lútea estrutural (≤ D16). Além disso, nos momentos precedentes à luteólise (D11 ao D16), vacas desse grupo detectadas em luteólise antes

ou no D16 apresentaram um CL menor em comparação com vacas do mesmo grupo em luteólise após o D16. Adicionalmente, vacas tratadas com iP4 com luteólise precoce apresentaram um menor CL ao longo da fase lútea. Não houve diferenças significativas na abundância de transcritos endometriais relacionados com a programação (PGR, ESR1) ou execução (OXTR, PTGS2) da liberação de PGF2α. Em resumo, a suplementação com iP4 no início do diestro afetou o crescimento do CL mas não modulou os transcritos endometriais associados com a luteólise, sugerindo que o componente uterino pode não estar envolvido neste processo. Conclui-se que CLs comprometidos pela exposição à iP4 durante a luteogênese são mais susceptíveis à regredir antecipadamente, e isto pode ser exacerbado por baixas concentrações de E2 resultantes da AF. Em conclusão, rejeitamos a hipótese de que a redução do E2 folicular previne a antecipação da luteólise induzida pela iP4 em vacas *Bos indicus*.

Palavras-chave: Útero. Corpo lúteo. Cytobrush. Aspiração folicular. Bovinos.

ABSTRACT

CARDOSO, B. O. **Requirement of follicular estradiol for the onset of luteolysis in *Bos indicus* cows supplemented with progesterone at early diestrus.** [Requerimento do estradiol folicular para o momento da luteólise em vacas *Bos indicus* suplementadas com progesterona no início do diestro]. 2017. 81 p. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, 2017.

In beef cows, long-acting injectable progesterone (iP4) supplementation in the early diestrus has paradoxical effects, as it both increases fertility in the field as well as induces anticipation of luteolysis in part of the treated animals. Considering that estradiol (E2) from the ovarian follicles during the luteal phase plays a central role in the induction of uterine PGF2α release and luteal regression, here we tested the central hypothesis that the absence of post-deviation follicles (i.e., a low E2 tone) delays the early onset of luteolysis and extends the estrous cycle in *Bos indicus* cows supplemented with iP4. This dissertation consists of two studies. A first study was carried out to validate the use of cytologic brushes (cytobrush) as a tool to collect sequential samples of endometrium to characterize temporal changes in the transcriptome along the estrous cycle. It was concluded that the cytobrush technique was confirmed as a reliable, repeatable, and safe method for studying bovine uterine biology *in vivo*. In the second study, cyclic Nelore cows were synchronized and distributed in a 2 x 2 factorial arrangement to receive treatment with placebo or 300 mg iP4 three days after ovulation (D0), and to be subjected or not to follicular aspiration (FA) during the luteal phase. Endometrial samples were collected on D15, D17 and D19 using the cytobrush technique, and abundance of transcripts was measured by qPCR. The iP4 decreased the size of the largest follicle on D6 and the area of the CL between D8 and 10 compared to the placebo groups. Cows submitted to FA had a longer cycle but timing of luteolysis was similar to non-FA cows. Treatment with iP4 and submitted to FA tended to advance the timing of structural luteal regression (≤ D16). Furthermore, in the moments preceding luteolysis (D11 to D16), cows from this group that underwent luteolysis before or at D16 presented a smaller CL compared with cows of the same group that underwent

luteolysis after D16. In addition, cows treated with iP4 that presented early luteolysis had a smaller CL across the luteal phase. There were no significant differences in the abundance of endometrial transcripts related to programming (PGR, ESR1) or execution (OXTR, PTGS2) of PGF2α release. In summary, supplementation of P4 at early diestrus affected CL growth but did not modulate endometrial transcripts associated with luteolysis, suggesting that the uterine component might not be involved in this process. It is concluded that CLs impaired by exposure to iP4 during luteogenesis are most likely to undergo early luteal regression, and this may be exacerbated by low E2 concentrations resulting from FA. In conclusion, we rejected the hypothesis that reduced follicular E2 could prevent iP4-induced early onset of luteolysis in *Bos indicus* cows.

Keywords: Uterus. Corpus luteum. Cytobrush. Follicular aspiration. Bovine.

LIST OF FIGURES

LIST OF TABLES

SUMMARY

1 INTRODUCTION

Worldwide attention has focused on Brazilian beef industry due to the “green” type of production systems and the potential for expansion. Brazil is the second largest beef producer, accounting for 15.4% of the total meat production of the ten largest producer countries in the world (USDA, 2017). This reflects directly on the country’s economy, since agribusiness accounts for 23% of the Brazilian gross domestic product (ABMRA, 2017). Although the Brazilian beef industry is on the rise, reproductive efficiency is generally low and deserves attention. For example, Brazil has around 80.6 million females of reproductive age and produces only 55.2 million calves per year (ANUALPEC, 2015). In addition, calving interval in Brazil is in average 16.3 months (ANUALPEC, 2015), still far from what is considered the optimal reproductive efficiency (BARUSELLI et al., 2016).

At the herd level, one component of reproductive efficiency is the number of pregnant cows/number of inseminated cows (P/AI). According to Vanroose et al. (2000), 20% to 40% of conception failures in beef herds are due to embryonic mortality, which most occur between the second and third weeks of gestation (DUNNE; DISKIN; SREENAN, 2000; DISKIN et al., 2016). During this period, the intra-uterine microenvironment to which the embryo is exposed after fertilization plays a crucial role for gestational success. An inadequate environment limits conceptus development and production of interferon-tau (IFNT). Insufficient IFNT signaling is permissive to the secretion of endometrial prostaglandin F2alpha (PGF2α) that induces corpus luteum (CL) regression and interrupts gestation (MANN; LAMMING, 2001).

The biochemical composition of the uterine microenvironment is under the control of ovarian steroids, such as progesterone (P4). Indeed, the probability of conception is lower in heifers (DISKIN; MORRIS, 2008; PERES et al., 2009) and cows (STRONGE et al., 2005; PERES et al., 2009) that have reduced plasma concentration of P4 during post-insemination diestrus. In contrast, greater concentrations of P4 in the first week

after ovulation favor embryonic development, production of IFNT and maintenance of pregnancy (CARTER et al., 2008; O'HARA et al., 2014). Considering the benefits of P4 in this crucial period, a logical strategy to minimize embryonic losses and increase conception rates in beef herds would be through exogenous P4 supplementation after fertilization. Several studies were conducted using this strategy, such as daily injections of intramuscular P4 (GINTHER, 1970; GARRETT et al., 1988b), single administration of a long acting P4 (PUGLIESI et al., 2014b, 2016), or by an insertion of an intra-vaginal P4-releasing device (NASCIMENTO et al., 2013; MONTEIRO JR et al., 2014; O'HARA et al., 2014). However, the fertility outcome of this technology is inconsistent and ranges from positive to negative, depending on the study (MANN; LAMMING, 1999; YAN et al., 2016).

One main explanation for negative effects of P4 supplementation on fertility is the occurrence of early luteolysis (i.e., before day 16 after estrus) in a proportion of animals, as reported previously (GINTHER, 1970; GARRETT et al., 1988a; PUGLIESI et al., 2014b). Indeed, CL regression before maternal recognition of pregnancy causes pregnancy loss. Nonetheless, the causes of early luteolysis are unknown and deserve critical investigation. There are at least two main targets for research in this area, the CL and the endometrium.

Regarding the CL, the preovulatory luteinizing hormone (LH) surge triggers the luteinization of follicular cells. LH induces differentiation of granulosa and thecal cells into large and small luteal cells, angiogenesis, and steroidogenesis (NISWENDER et al., 2000). Accordingly, LH is the main luteotropic hormone in cattle and it is required for normal luteal development and function (NISWENDER; NETT, 1988). Additionally, P4 exerts a negative feedback on tonic GnRH-induced LH secretion (IRELAND; ROCHE, 1982; BATTISTA et al., 1984; GIORDANO et al., 2012). Thus, it is possible that exogenous P4 supplementation during early luteal phase inhibits the proper LH support, compromising the CL. In fact, Ginther (1970) found that treatment with a luteotropic agent (hCG) prevented the shortening cycle effect of exogenous P4 given on day 1 to 4 after estrous. Consistently, supplementation with intravaginal P4 devices (1.55 g) from days 3 to 5 post estrus along with administration of a eCG dose (750 IU) at day 3

resulted in fewer shorted cycles compared to the P4 supplemented group without eCG (O'HARA et al., 2016). Furthermore, in a recent a study, Parr et al. (2017) highlighted the vulnerability of the early developing CL to exogenous P4 supplementation in beef heifers. These are strong evidences to support the hypothesis that supplementation with P4 at early diestrus compromises luteal development, resulting in a subfunctional CL likely to regress early (BURKE et al., 1994; O'HARA et al., 2016; PARR et al., 2017).

Regarding the endometrium, the release of luteolytic pulses that cause CL regression is timed precisely by an intricate sequence of molecular events. A mechanism for the control of episodic pulsatile release of PGF2α from the uterus was proposed for ewes (reviewed by SILVIA et al., 1991) and it is generally accept for other ruminants. Specifically, during early to mid diestrus, P4 acting in its own receptors inhibits the expression of estradiol (E2) and oxytocin (OT) receptors (ESR and OXTR, respectively) in the endometrium (VALLET; LAMMING; BATTEN, 1990; MCCRACKEN; CUSTER; LAMSA, 1999). After continuous exposure of the uterus to P4, this steroid gradually downregulates its own receptors (MILGROM; THI; ATGER, 1973; LAMMING; MANN, 1995). The downregulation of P4 receptors (PGR) allows the rise of ESR, which in the presence of high concentrations of follicular E2 stimulates the increase of OXTR in the endometrium (MCCRACKEN; SCHRAMM; OKULICZ, 1984; BEARD; LAMMING, 1994; GOFF, 2004). Binding of OT to its receptors triggers a chain of events that results in PGF2α synthesis, and consequently, luteolysis (MCCRACKEN; SCHRAMM; OKULICZ, 1984; MCCRACKEN et al., 1995; SPENCER; BAZER, 1995). Moreover, effects of E2 are much more pronounced after exposure to P4, and its role inducing uterine PGF2α release may be through the stimulation of uterine responsiveness to OT (SILVIA et al., 1991). In this context, it is evident that E2 plays a central role in the activation of the luteolytic mechanism (SILVIA et al., 1991; SALFEN et al., 1999; BINELLI et al., 2001).

Treatment with supplemental P4 at early diestrus advances endometrial gene expression (FORDE et al., 2009) and may lead to an early regulation of endometrial factors involved in the control of the luteolytic mechanism (PUGLIESI et al., 2014b). Interestingly, treatment with exogenous P4 on days 1 to 10 post-estrus did not reduce

cycle length in heifers after ipsilateral removal of uterine horn, as it did in intact heifers (WOODY; GINTHER, 1968), indicating the involvement of the endometrium in such event. This is consistent with the findings of Garrett et al. (1988), in which P4-treated cows exhibited earlier release of PGF2α from the endometrium compared to controls. These authors suggested that P4 supplementation might lead to an early maturation of the uterus that stimulates an advance release of luteolytic pulses, and thus, CL regression. In this regard, it can be hypothesized that supplemental P4 advances the molecular mechanisms that trigger the release of PGF2α pulses, and consequently, the onset of luteolysis.

Here we attempted to distinguish between the CL and the endometrium as targets to the supplemental P4 in the process of early luteolysis. Because of the central role played by E2 triggering endometrial mechanisms of PGF2α release, **the central hypothesis was that the absence of post-deviation follicles delays the early onset of luteolysis and extends the estrous cycle in *Bos indicus* cows supplemented with P4.** Indeed, if onset of luteolysis depends on E2-induced synthesis of OXTR, and supplemental P4 advances this process, under a lower E2 tone the P4 effects should be inhibited.

The aims of this study were: (1) to verify whether the ablation of post-deviation follicles prevents the earlier onset of luteolysis in cows supplemented with injectable P4 and (2) to evaluate the temporal changes in the abundance of transcripts that control the release of endometrial PGF2α in cows supplemented with P4 in the presence and absence of post-deviation follicles. To meet these aims, cows were randomized on a 2 x 2 factorial arrangement in order to receive long-acting injectable P4 or placebo treatment three days after ovulation and to undergo follicular or sham follicular aspiration throughout the luteal phase. The moment of luteal structural regression was determined and sequential endometrial samples were taken in order to study the molecular regulation of this event. Endometrial samples were taken using a cytologic brush (cytobrush). In the present dissertation, this technique was validated and shown to provide samples of adequate quality for transcript analyses. Because sampling could be performed frequently in the same animals, we used the cytobrush technique, for the first

time, to gain molecular understanding of uterine events at specific time-points.

This dissertation was divided in two chapters. Chapter 1 describes the validation of a novel approach to collect uterine samples from cows throughout the estrous cycle and it was published in Reproduction in Domestic Animals (Cardoso et al., 2017; Attachment A). Chapter 2 comprises the investigation of the involvement of E2 on the timing of onset of luteolysis in cows treated with P4 at early diestrus. This last chapter was organized in a journal manuscript format. At the end of this dissertation there are final considerations that summarize the research results and discuss the perspectives.

In general, it is expected that a better understanding of the key players controlling the timing of luteolysis in *Bos indicus* cattle will enable the development of strategies to reverse the negative effects and maximize de benefits of P4 supplementation at early diestrus.

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2 CHAPTER 1: CYTOBRUSH: A TOOL FOR SEQUENTIAL EVALUATION OF GENE EXPRESSION IN BOVINE ENDOMETRIUM[1]

2.1 INTRODUCTION

In cattle, to understand the cyclic cellular and molecular changes of uterine function, it is often necessary to probe the uterus throughout the estrous cycle or early pregnancy. Most studies in the literature used *post mortem* samples of the endometrium, collected in specific target days after estrus (BAUERSACHS et al., 2005; PALMA-VERA; SHARBATI; EINSPANIER, 2015). Nevertheless, with this method it is impossible to characterize the endometrial transcriptome in the same animal along the estrous cycle. Alternatively, *in vivo* endometrial samples may be obtained by uterine biopsies; however, multiple biopsies in the same animal result in a local inflammatory process that may affect endometrial function, and impair the subsequent fertility (ZAAYER; VAN DER HORST, 1986). Collecting endometrial cytological samples using a cytobrush technique is less traumatic than using biopsy apparatuses. To the best of our knowledge, there is no study that used the cytobrush technique repeatedly, in the same animal, to evaluate molecular changes that occur throughout the estrous cycle. We aimed: 1) to compare molecular aspects of endometrial samples collected by transcervical biopsy and cytobrush; and 2) to characterize the abundance of transcripts involved in the PGF2α synthesis associated with luteolysis in beef cattle, using the cytobrush technique.

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[1] This study was submitted to the journal Reproduction in Domestic Animals as a Short Communication (Cardoso et al., 2017. Cytobrush: a tool for sequential evaluation of gene expression in bovine endometrium. Reproduction in Domestic Animals. 2017. DOI: 10.1111/rda.13037) and was accepted for its publication on June of 2017. The proof correction of the manuscript is in the Attachment A.

2.2 MATERIALS AND METHODS

Animal procedures were approved by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science of the University of São Paulo (CEUA-FMVZ/USP nº 5816230217). Non-lactating, multiparous Nelore (*Bos indicus*) cows containing no gross reproductive anomalies were kept in grazing conditions and water *ad libitum*.

2.2.1 Experiment designs

Experiment 1: Characterization of samples obtained by biopsy or cytobrush techniques

Ovulations of five Nelore cows were synchronized using an estradiol/progesterone based TAI protocol. Ten days after ovulation, cows were submitted to collection of endometrial samples by a cytobrush technique followed by biopsy. The RNA was extracted and abundance of specific transcript markers of epithelium (cytokeratin; KRT18), stroma (vimentin; VIM), immune cells (T-cell receptor T3 delta; CD3D), endothelial cells (vascular endothelial growth factor receptor 1; FLT1) and myometrium (myostatin; MSTN), and transcripts involved in uterine function during the estrous cycle (progesterone receptor; PGR, prostaglandin E synthase; PTGES, aldo-keto reductase family 1 member C4; AKR1C4) was measured by qPCR.

Experiment 2: Characterization of genes involved in luteolytic mechanisms

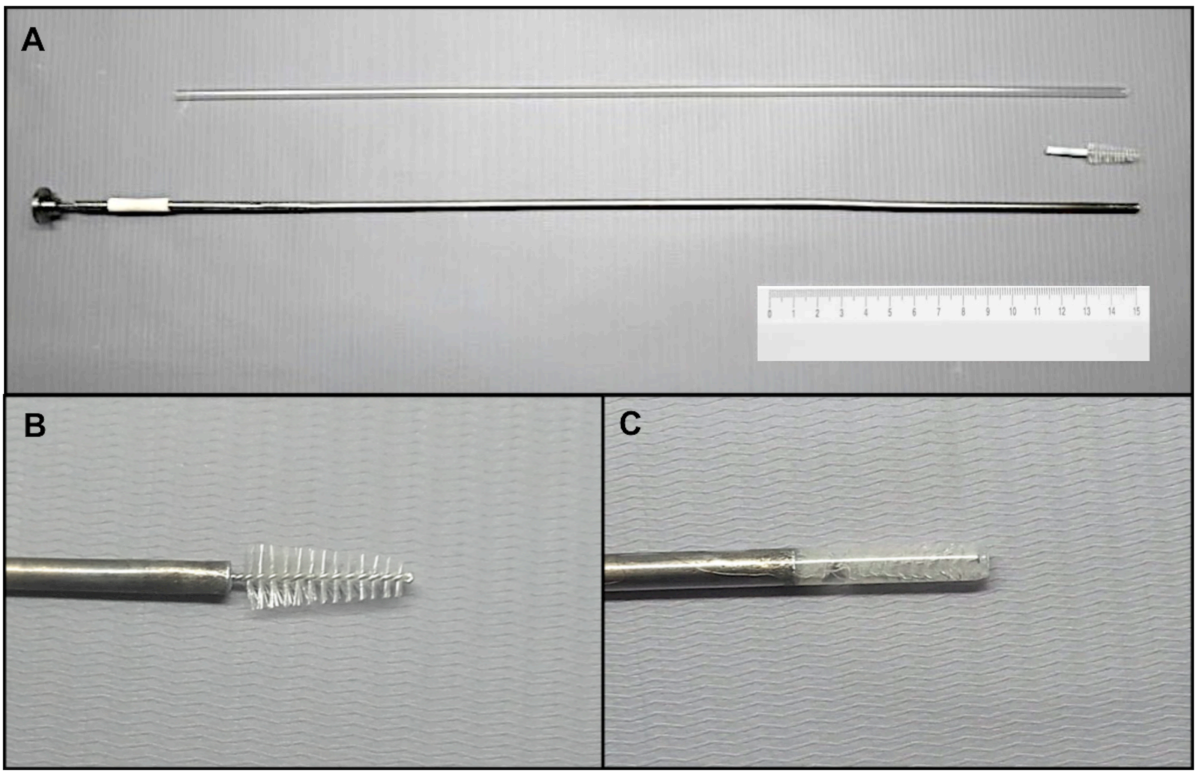
Ovulations of ten Nelore cows were synchronized as mentioned. On days 10, 13, 16 and 19 after ovulation, endometrial cytobrush samples were collected from each animal. Each day, luteolysis was verified by ultrasonography as described previously (PUGLIESI et al., 2014a). Five of those ten cows were randomly selected

for molecular analysis and transcript abundance was determined for oxytocin receptor (OXTR), estrogen receptor-1 (ESR1) and progesterone receptor-2 (PGR2) by qPCR.

2.2.2 Cytobrush and biopsy procedures

After epidural anesthesia (60 mg, lidocaine), a cytological brush (Disposable cytology sampling brush 8”; Viamed Ltd, West Yorkshire, UK) was coupled to the tip of a conventional AI gun, covered by a disposable AI sheath and protected by a sanitary sheath (Figure 1). Apparatus was inserted via cervix and cytobrush rotated to collect cells from uterine body. The cytobrush was uncoupled from the apparatus and placed in a 2 mL cryo-tube filled with 1 mL of Trizol® reagent (Life Technologies, California, USA) and stored at −80 ºC for later processing.

Figure 1 - Images of the apparatus used in the cytobrush technique



Source: (CARDOSO, B.O., 2017)
Legend: Image A illustrates the entire apparatus with the uncovered cytological brush and the disposable AI sheath. Image B illustrates the uncovered cytological brush before insertion of the disposable AI sheath and the sanitary sheath. Image C illustrates the cytological brush covered by a disposable AI sheath after sample collection.

Immediately afterwards, a biopsy sample (~50mg) was obtained from uterine body of each cow using an Eppendorfer uterine biopsy forceps as reported previously (PUGLIESI et al., 2014c) and stored at −80 ºC.

2.2.3 RNA extraction, synthesis of cDNA and qPCR

After RNA extraction by Trizol® reagent, cDNA was synthesized from 500 ng total RNA (High Capacity cDNA Synthesis kit; Life Technologies, SP, Brazil). Transcripts abundance was measured using SYBR Green qPCR (Step One Plus Real Time System; Life Tech) (MESQUITA et al., 2014; Table 1).

2.2.4 Statistical analyses

In Experiment 1, fixed effect of technique was analyzed by one-way ANOVA and in Experiment 2, transcript abundance on different days was analyzed by repeated-measures ANOVA (PROC MIXED from SAS software).

Table 1 - Primer sequences of target and reference genes analysed using qPCR

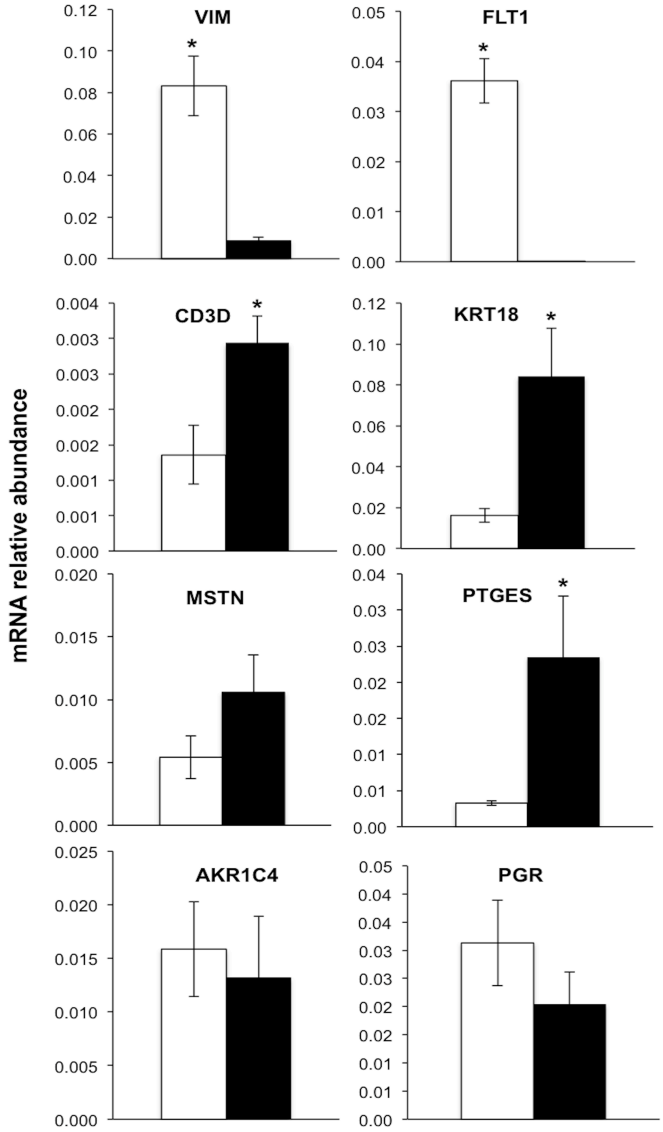
Gene symbol	Gene name	Forward primer	GenBank ID	Amplicon size
KRT18	Cytokeratin 18	F: GGAACACCTGGAGAAGAAG R: AGCAAGACGGGCATTATC	NM_001192095.1	168
VIM	Vimentin	F: TCAGGAGCGCAAGATAGA R: CAAGGCGTCTTCGGTAAA	NM_173969.3	146
CD3D	T-cell Receptor T3 Delta Chain	F: TCCAGGACCCAAGAGGAATG R: GGAGCACAGTGGCAATGATG	NM_001034033.2|	116
FLT1	Fms Related Tyrosine Kinase 1	F: CCGAAAACTGAAAAGGTCGTCTT R: ACTTCATCCGGGTCCATGATAA	NM_001191132.2	168
MYST	Myostatin	F: GCACCTAAAAGATATAAGGCCAATTACT R: GGTTTGCTTGGTGCACAAGA	NM_001001525.3	100
AKR1C4	Aldo-keto reductase family 1, member C4	F: TCCTGTCCTGGGATTTGGAACCTT R: ATCGGCAATCTTGCTTCGAATGGC	NM_181027.2	166
PTGES	Prostaglandin E synthase	F: GCTGCGGAAGAAGGCTTTTGCC R: GGGCTCTGAGGCAGCGTTCC	NM_174443.2	101
PGR	Progesterone receptor	F: GCCGCAGGTCTACCAGCCCTA R: GTTATGCTGTCCTTCCATTGCCCTT	NM_001205356.1	199
ACTB	Actin Beta	F: GGATGAGGCTCAGAGCAAGAGA R: TCGTCCCAGTTGGTGACGAT	NM_173979.3	78
PGR2	Progesterone Receptor 2	F: CTACCCGCCCTATCTCAACTA R: TTGTGCTGCCCTTCCATTT	NM_000926.4	189
ESR1	Estrogen Receptor 1	F: CAGGCACATGAGCAACAAAG R: TCCAGCAGCAGGTCGTAGAG	XM_002690343.1	82
OXTR	Oxitocin Receptor	F: AAGATGACCTTCATCGTCGTG R: CGTGAAGAGCATGTAGATCCAG	NM_174134.2	177
RPS18	Ribosomal Protein S 18	F: GTGGTGTTGAGGAAAGCAGACA R: TGATCACACGTTCCACCTCATC	NM_001033614.2	100

2.3 RESULTS

Experiment 1

Abundance of VIM and FLT1 was 9.2 and 275 fold greater in biopsy samples, respectively ($P<0.05$; Figure 2). Abundance of KRT18, CD3D and PTGES was 5.2, 2.2 and 7.2 folds greater in cytobrush samples, respectively ($P<0.05$). There was no difference in the relative expression levels of MSTN, AKR1C4 or PGR between the two probing methods ($P>0.05$).

Figure 2 - Abundance of VIM, FLT1, CD3D, KRT18, MSTN, PTGES, AKR1C4 and PGR transcripts normalized to ACTB in uterine samples (n=5/group) collected by biopsy (open bars) and cytobrush technique (filled bars) 10 days after ovulation; mean ± SEM



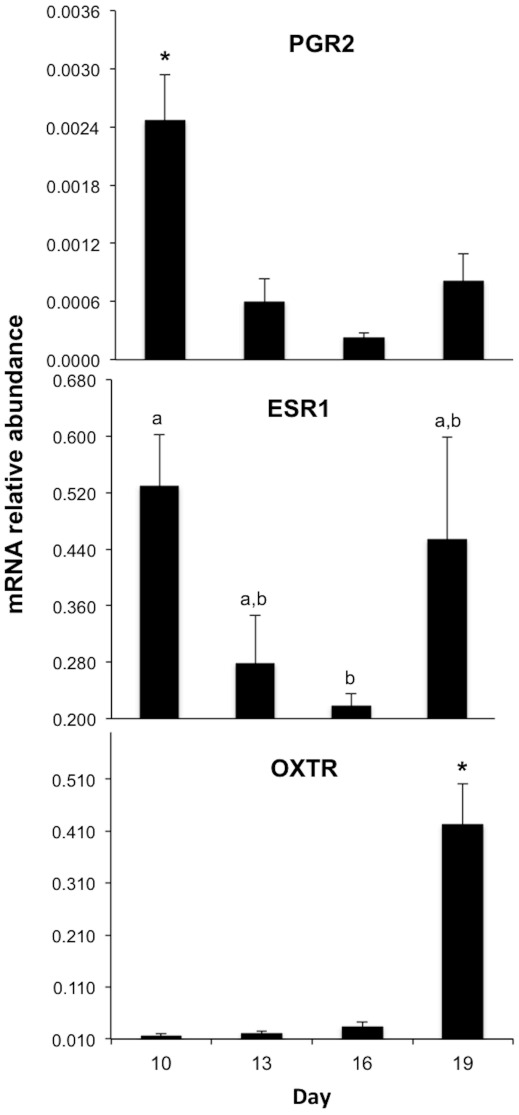
Source: (CARDOSO, B.O., 2017)
Legend: An asterisk (*) means P ≤ 0.05

Experiment 2

There was an effect of day on abundance of all transcripts (Figure 3). Levels of ESR1 mRNA tended to be greater on days 10 and 19 (P=0.10). The abundance of

PGR2 mRNA was maximum on day 10, gradually decreased and increased again on day 19 (P<0.05). There was a gradual increase in abundance of OXTR over time that reached a peak on day 19. Structural luteolysis was between days 16 and 19 (day 18 ± 0.63).

Figure 3 - Abundance of PGR2, ESR1 and OXTR transcripts normalized to RPS18 in uterine samples (n=5) collected by cytobrush technique during estrous cycle; mean ± SEM



Source: (CARDOSO, B.O., 2017)
Legend: An asterisk (*) means P ≤ 0.05 and a,b: 0.05 < P > 0.10

2.4 DISCUSSION

The present study demonstrated that the cytobrush technique can be successful used for sequential evaluation of endometrial gene expression in cattle. The cellular composition of biopsy samples were different from that obtained by cytobrush. The former contained a greater portion of endometrial stroma (VIM) and vascular cell (FLT1) markers than cytobrush samples (JOHNSON et al., 1999). Because of the more superficial sampling compared to biopsies, cytobrush samples were enriched in endometrial epithelial cells (KTR18) and immune cells (CD3D) markers (LI et al., 2016). It is unlike that either probing technique reached the myometrial layer, because abundance of MSTN was similar between groups. Detection of sex-steroid modulated endometrial markers; PGR, AKR1C4 and PTGES in both sample types confirmed the adequacy of both techniques for the study of events relevant for reproduction. In summary, biopsy samples are enriched in stromal and endothelial cells, while cytobrush samples are enriched in epithelial and immune cells, which direct their use for different purposes.

In Experiment 2, temporal changes of transcripts involved in the luteolytic mechanism in cytobrush samples were consistent with a previous report (OKUMU et al., 2010). Specifically, PGR2 expression decreased gradually from the middle to end of cycle, associated with an increase of ESR1 expression, followed by a marked increase of OXTR transcription. As an additional observation, seven out of 10 cows in Experiment 2 became pregnant after a single artificial insemination at the subsequent estrus. This indicated that cytobrush technique did not interfere with the immediate fertility, which reinforces the safety to use this technique.

In conclusion, repeated bovine endometrial sampling using the cytobrush method is safe and provides representative samples for the study of the superficial endometrium *in vivo*. Nonetheless, this technique is not adequate for the study of deeper layers of the endometrium.

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3 CHAPTER 2: REQUIREMENT OF FOLLICULAR ESTRADIOL FOR THE ONSET OF LUTEOLYSIS IN *BOS INDICUS* COWS SUPPLEMENTED WITH PROGESTERONE AT EARLY DIESTRUS

3.1 INTRODUCTION

In cattle, most pregnancy losses occur between fertilization and maternal recognition of pregnancy, around day 16 after estrus (DUNNE; DISKIN; SREENAN, 2000; DISKIN; MORRIS, 2008). At this moment, the elongated conceptus relies solely on the uterine luminal secretome to provide molecular support for its development. A key regulator of uterine secretory function is P4. Indeed, P4 plays a key role in gestational success. Low concentrations of P4 have been associated with lower conception rate in heifers (DISKIN; MORRIS, 2008) and cows (STARBUCK, 1999; STRONGE et al., 2005). In contrast, an increase in P4 concentration post ovulation is associated with a greater probability of the cow to become pregnant (DEMETRIO et al., 2007; PERES et al., 2009). Early diestrus increases in P4 concentrations lead to changes in the abundance of endometrial transcripts (FORDE et al., 2009) that contribute to conceptus development (CARTER et al., 2008), production of interferon-tau, and consequently, the maintenance of pregnancy (MANN; LAMMING, 1999; O'HARA et al., 2014).

Over the past few decades, a number of strategies to supplement P4 after ovulation have been proposed as means to reduce embryonic losses and increase conception rates in cattle operations (GINTHER, 1970; GARRETT et al., 1988b; BELTMAN et al., 2009; O'HARA et al., 2014; PUGLIESI et al., 2016). However, the fertility outcomes of P4 supplementation are remarkably variable and inconsistent across experiments (reviewed by YAN et al., 2016). A common finding in studies that supplement P4 at the beginning of diestrus is the advanced onset of luteolysis (WOODY; FIRST; POPE, 1967; GINTHER, 1970; VAN CLEEFF et al., 1996; PUGLIESI et al., 2014b). Such event certainly contributes to the failure of maternal recognition and leads to embryo loss, compromising the benefit of such strategy. Although the paradoxal effects of P4 supplementation early in the estrous cycle on

fertility is well recognized (O'HARA et al., 2014) the physiological mechanisms involved in the P4-induced early luteal regression are not well characterized. The overarching hypothesis is that P4 supplementation during early diestrus advances the molecular mechanisms associated with the release of endometrial PGF2α thereby causing early onset of luteolysis.

In ruminants, the uterine luteolytic mechanism that occurs spontaneously at each estrous cycle requires sequential actions of P4, E2, and OT through their respective receptors (SPENCER; BAZER, 1995; MCCRACKEN; CUSTER; LAMSA, 1999). Luteal P4 progressively downregulates its own receptor (PGR) in the uterus to reach nadir concentrations around the mid-luteal phase (MILGROM; THI; ATGER, 1973). This allows the expression of endometrial estrogen receptors (ESR), which in turn are activated by follicular E2 that subsequently induces expression of OT receptor (OXTR; SILVIA et al., 1991). Binding of OT to its endometrial receptors triggers PGF2α release (MCCRACKEN; CUSTER; LAMSA, 1999). Thus, circulating E2 concentrations at late diestrus play a central role in the timing of the onset of luteolysis (SILVIA et al., 1991; SALFEN et al., 1999). It is well known that the main source of E2 is the post-deviation ovarian follicle. Thus, a scenario of increased sensitivity of the endometrium to E2 along with the presence of dominant follicles is likely to be associated with the initiation of PGF2α pulses and luteolysis. Indeed, follicle cauterization (VILLA-GODOY et al., 1985), follicle aspiration (ARAUJO et al., 2009) or *in vivo* treatment with steroid-free follicular fluid (i.e., rich in inhibin; SALFEN et al., 1999) all delayed the onset of luteolysis. Conversely, E2 administration stimulated PGFM release and advanced luteolysis (THATCHER et al., 1986; ARAUJO et al., 2009).

Considering that ultrasound-guided follicular aspiration is a reliable alternative method to decrease plasma concentrations of E2 (TOHEI et al., 2001), here we tested the hypothesis that in the absence of post-deviation follicles (i.e., follicles with increasing E2 synthesis) the early onset of luteolysis stimulated by P4 supplementation is prevented. The specific objectives of this study were to: (1) verify whether consecutives follicle aspiration delays the onset of luteolysis in cows supplemented with P4, and (2) measure the temporal changes in the abundance of endometrial transcripts associated with the control of PGF2α release in response to P4 supplementation in the presence or absence of post-deviation follicles.

3.2 MATERIAL AND METHODS

3.2.1 Local and animals

The experiment was carried out at Pirassununga campus of the University of São Paulo, Brazil. All animal procedures described herein were approved by the Ethics and Animal Handling Committee of the School of Veterinary Medicine and Animal Science of the University of São Paulo (CEUA–FMVZ/USP nº 8717061115).

Thirty-five nonlactating nulliparous (n=11) and multiparous (n=24) Nelore (*Bos indicus*) cows presenting no gross reproductive abnormalities with an average body condition score between 3-3.5 (1=emaciated, 5=obese) were maintained in grazing conditions and fed with sugar cane and concentrate to fulfill their maintenance requirements. Cows had access to a mineral supplement and water *ad libitum*.

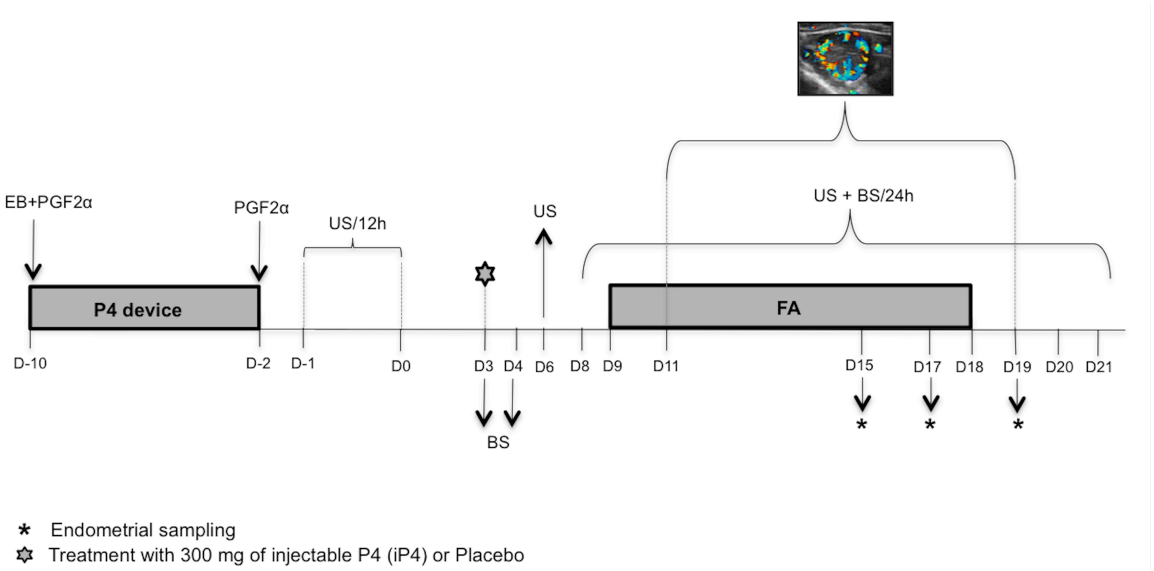
3.2.2 Reproductive management and experimental design

To synchronize ovulations, on D−10 animals received an intravaginal P4-releasing device (1g; Sincrogest®, Ourofino Saúde Animal, Cravinhos, São Paulo, Brazil) along with intra-muscular (im) injections of estradiol benzoate (EB; 2 mg; Sincrodiol®, Ourofino Saúde Animal), and PGF2α (500 µg of sodium cloprostenol; Sincrocio®, Ourofino Saúde Animal). On D−2, the P4-releasing device was removed, animals received an im administration of PGF2α (500 µg of sodium cloprostenol; Sincrocio®, Ourofino Saúde Animal), and a heat detector patch (Estrotect®, Rockway, Inc. Spring Valley, WI, USA). From D−1, ovaries were examined twice daily by ultrasonography until detection of ovulation The day of ovulation detection was defined as experimental day 0 (D0) and only cows that ovulated between 48 and 72 hours from the removal of P4-device were kept in the study (n=30). Three days after ovulation (D3), animals were assigned to one of four experimental groups: I)

intra-muscular (im) administration of placebo solution on D3 and sham follicular aspiration (FA) daily from D9 to D18 (−iP4−FA, n=6), II) im administration of placebo solution on D3 and FA daily from D9 to D18 (-iP4+FA, n=6), III) im administration of long action injectable P4 (iP4; 300 mg; Sincrogest®, Ourofino Saúde Animal) on D3 and sham FA daily from D9 to D18 (+iP4−FA group, n=9), and IV) im administration of iP4 (300 mg; Sincrogest®, Ourofino Saúde Animal) on D3 and FA daily from D9 to D18 (+iP4+FA group, n=9; Figure 4). Due to clinical condition and a cystic CL, two cows were removed from the experiment, one from +iP4+FA and the other from the +iP4−FA group. Ultrasound scanning (US) was performed on D6, and daily from day D8 until detection of subsequent ovulation. The US assessment using color Doppler was performed from D11 until the day after structural luteolysis detection. Endometrial samples were collected using a cytological brush on D15, D17 and D19. Blood samples were collected on D3 and D4, and then, daily from D8 until two days after the detection of structural luteolysis (Figure 4).

The dose and timing of iP4 injection were chosen based on our previous work. Pugliesi et al. (2014) reported that 57% of cows receiving an identical treatment advanced the timing of luteolysis (i.e., detection of functional luteolysis before D15) compared to 0% in cows not supplemented with P4. Thus, such treatment was expected to be effective in inducing early onset of luteolysis at least in 50% of the animals in the P4 treated groups in this experiment. The FA period was chosen because in a previous study, Araujo et al. (2009) successfully postponed the onset of luteolysis in *Bos taurus* heifers using FA technique performed in a similar way from D9 to D17 after ovulation.

Figure 4 - Experimental design



Source: (CARDOSO, B.O., 2017)
Legend: The estruses of 35 Nelore cows were synchronized using an E2/P4 based protocol. All cows received an intravaginal P4-device (1g; Sincrogest®, Ourofino), an injection of 2 mg of EB (Sincrodiol®, Ourofino), and 500 µl im of PGF2α (Sincrocio, Ourofino). Eight days later, the P4-device was removed and 500 µl of PGF2α (Sincrocio, Ourofino) was im administrated. Twenty-four hours from this day, the ovaries of all cows were scanned every 12h to determine the day of ovulation (D0). Three days after ovulation (D3), cows were allocated to one of four experimental groups (see text for details): −iP4−FA (n=6), −iP4+FA (n=6), +iP4−FA (n=8) or +iP4+FA (n=8). Endometrial samples were collected using a cytological brush on days 15, 17, and 19 after ovulation. Blood samples (BS) were taken on D3, D4 and daily from D8 until two days after the detection of structural luteolysis. Ultrasound scanning (US) of both ovaries was performed on D6 and every 24h from D8 until detection of the subsequent ovulation.

3.2.3 Ultrasound examinations and follicular aspiration

A B-mode (gray-scale) and color Doppler ultrasound instrument (M5vet, Mindray, China) equipped with a 7.5-MHz linear transducer was used for the ovarian scanning. Cows had their ovaries scanned twice daily from 24 h to 96 h after the P4-device removal to detect the ovulation. The day of ovulation was defined by the disappearance of the dominant follicle seen in the previous scan and confirmed 12 h later in the next examination. On D6, the mean diameter of the largest follicle present in the ovary was measured. The follicular diameter was considered the average between measurements of the largest perpendicular axes. Follicular dynamic was

monitored from D8 until the subsequent ovulation. The evaluation of CL development and regression were performed from D8 until the following day that structural luteolysis was determined. The CL area was determined using the B-mode and the tracing function of ultrasound. For CL with an anechoic fluid-filled cavity, the area of the cavity was subtracted from the total area. Evaluation of the percentage of CL area with signals of blood perfusion (CLBP) was performed on D8 and daily from D11 until the next day after structural luteolysis detection (Figure 4), as described previously by Pugliesi et al. (2014a). Accordingly, a scale from 0 to 100% with 5 interval points was used to determine the CLBP. All scans were performed at a constant setting (6.5 MHz, Gain: 62 and Pulse Repeated Frequency: 5.3 kHz) and a velocity setting of 5.4 cm/s. In each CL exam, the maximal area and the blood perfusion of the CL were evaluated to detect structural luteolysis, which was defined as the day when there was a 25% reduction in the largest CL area measured between D8 and D10 and 50% of the CLBP measured on D8. All ultrasound evaluations were performed by a single operator throughout the experiment period.

Additionally, ultrasound evaluations were performed every day from D9 after ovulation until D18 in order to map and measure follicles present in the ovaries, which were subjected daily to FA using an *ovum* pick up (OPU) procedure (Figure 4). For this purpose, animals were restrained in a chute and received epidural anesthesia before each aspiration section (Lidovet®, Bravet, Rio de Janeiro, Brazil) to facilitate ovarian manipulations per rectum. All antral follicles identified by ultrasonography were aspirated from each ovary using a 5 MHz convex array transducer (DP 2200vet, Mindray, China) housed in a plastic vaginal probe with a stainless steel needle 20 G (0.9 x 50 mm; Terumo® Europe NV, Belgium) connected to an aspiration equipment and a vacuum system (100 mmHg; V-MAR 5000, Cook Australia, Queensland, Australia). An 1.1 mm inner diameter by a 120 cm length circuit (Watanabe Tecnologia Aplicada, WTA, Ltda, Cravinhos, São Pualo, Brazil) were attached to this equipment for follicular aspirate recovery in a 50 mL conical tube containing 15 mL of saline solution and 5,000 IU/mL sodium heparin (Hemofol®, Cristália, Itapira, São Paulo, Brazil). The recovered content was discarded after each aspiration section. Animals allocated to the −iP4−FA and +iP4−FA groups were subjected to sham FA conducted daily from D9 to D18. Specifically, after epidural anesthesia and by the aid of rectal manipulation, the aspiration probe was introduced

into the vagina and the vaginal fornix was perforated. All FA procedures were performed by a single experienced operator.

3.2.4 Blood sampling and hormone assay

Blood samples were collected on D3 and D4, and then, every 24 hours from D8 until two days after the detection of structural luteolysis (Figure 4). Blood samples were collected by jugular venipuncture using heparinized Vacutainer tubes (Vacuette, Greiner bio-one, São Paulo, Brazil), which were kept on ice. Samples were centrifuged at 4ºC and 1,500 x g for 15 min and plasma was stored at −20ºC for further analysis of plasma P4 concentrations by radioimmunoassay.

3.2.5 Endometrial samples collection and processing

Endometrial samples were collected on days 15, 17 and 19 after ovulation (Figure 4), for evaluation of transcripts abundance by quantitative polymerase chain reaction analysis (qPCR). The samples were collected using a cytological brush as described by Cardoso et al. (2017). Briefly, before collection, epidural anesthesia was performed using 3 mL of lidocaine (60 mg; Lidovet®, Bravet). Thereafter, a previously autoclaved cytological brush (Disposable cytology sampling brush 8”; Viamed Ltd, West Yorkshire, UK) was coupled to the tip of a conventional AI gun, covered by a disposable AI sheath and protected by a sanitary sheath. The apparatus was inserted via the cervix into the uterine body. The cytobrush was exposed and rotated twice in a clockwise direction in contact to the endometrial surface to collect endometrial cells. After retraction to the sanitary sheath, the apparatus was removed from the animal, the cytobrush tip was uncoupled from the apparatus and transferred to a 2 mL cryo-tube containing 1 mL of Trizol® reagent (Life Technologies, California, USA). Tube was immersed in liquid nitrogen and transported to the laboratory to be stored at −80 ºC for later processing. Unfortunately, individual conformation of the cervix prevented access to the uterine lumen for sampling in certain occasions. This fact reduced the

number of samples collected for molecular analyses from each group, most specially the −iP4+FA group (n = 1, 2, 2), that was completely removed from the analyses. Therefore, the remaining samples for each group were −iP4−FA (n = 4, 4, 4) +iP4−FA (n = 4, 4, 4), +iP4+FA (n = 5, 5, 5), for days 15, 17, and 19 respectively.

Samples were thawed and vortexed to release the cells of the cytobrush bristles. The cytobrush was discarded and the RNA extraction was performed using the Trizol® reagent as per manufacturer's instruction. Briefly, 266 µl of chloroform was added per 1 mL of Trizol reagent in a 1.5 mL RNA/DNA-free microcentrifuge tube. Samples were vortexed for 15 seconds, incubated at room temperature for 5 minutes, and then centrifuged at 12,000 x g for 15 min at 4 ºC. The aqueous phase was carefully transferred to a fresh tube and 333 µl of isopropanol was added. Samples were incubated for 10 minutes at room temperature and stored at −80 ºC overnight. Total RNA was precipitated by centrifugation at 15,000 x g for 8 min at 4°C, and the supernatant was discarded. The remaining pellet was washed twice with 600 µl of 75% ethanol. The final pellet was dried for 5 minutes at room temperature and then dissolved in 40 µl of diethylprocarbonate-treated water. Then, samples were heated to 55 ºC for 15 min and manually homogenized. The RNA concentration was measured using a spectrophotometer (NanoDrop; Thermo Scientific, Wilmington, MA, USA). Before reverse transcription (RT), 500 ng total RNA was treated with DNAse Amplification Grade (Invitrogen™, LifeTechnologies) according to the manufacturer's instructions. cDNA was synthesized using a High Capacity cDNA Reverse Transcript Kit (Applied Biosystems™, Life Technologies) as per manufacturer's instructions. RNA samples were incubated at 25 ºC for 10 min and then at 37 ºC for 2h, followed by incubation at 85 ºC for 5 min for enzyme inactivation. The cDNA was stored at −20 ºC for molecular analysis.

3.2.6 Quantitative polymerase chain reaction

Quantification of endometrial transcript abundance was performed by quantitative polymerase chain reaction analysis (qPCR) using the StepOne Plus apparatus (Applied Biosystems Real-Time PCR System; Life Technologies). Transcript abundance of receptors involved in luteolysis (progesterone receptor, PGR; estrogen receptor 1, ESR1; oxytocin receptor, OXTR) and an enzyme involved in PGF2α synthesis (prostaglandin-endoperoxidase synthase 2, PTGS2) was determined. The qPCR cycle condition were as follows: an initial step at 95 ºC for 10 minutes, 40 cycles of 15 seconds at 95 ºC and at 60 ºC for 1 minute, followed by a melt curve analysis obtained by stepwise increases in the temperature from 60 to 95 ºC.

Primers for the selected transcripts were designed using the Primer Express 3.0 based on GenBank Ref-Seq mRNA sequences of target genes. A pool of cDNA samples was used to obtain a standard curve of five progressive dilutions in duplicates of each primer pair. Primers validation consisted of meeting the following criteria: 1) amplification efficiency of the standard curve between 90 and 110%, 2) a single peak in the dissociation curve, and 3) absence of amplification of the negative control (diethyl pyrocarbonate treated water replacing template cDNA on the qPCR reaction). Identity of qPCR products was confirmed by electrophoresis and sequencing. Details of primers are provided in Table 2. Reactions for comparing transcript abundance among treatments were conducted in triplicates, on 96-well plates in a final volume of 20 µl using 10 µL of Power SYBR Green qPCR Master Mix (Life Technologies). Determination of qPCR efficiency and Cq (quantification cycle) values per sample was performed with LinRegPCR software (V2014.2; http://linregpcr.nl/). A set of normalizing genes was chosen using GeNorm software. The effect of group, day, and group by day interaction on the abundance of five endogenous genes (RPS18, GAPDH, β-actin, Cyclophilin and RPL15) was tested alone and in combination. In this regard, RPS18 alone was the only endogenous gene not affected by day, group and its interaction, and thus, it was considered to be the best endogenous control to be used. Quantification was obtained after normalization of the target gene expression values (Cq values) by the endogenous

control gene expression using the equation described by Pfaffl (2001). Relative transcript abundance was calculated as a ratio of the abundances of the target gene and the endogenous control gene and given as an arbitrary unit (AU).

Table 2 - Gene name, primers forward (F) and reverse (R) sequences, representative identification number, and amplicon size of genes analyzed using qPCR

Gene symbol	Gene name	Forward primer	GenBank ID	Amplicon size
PGR	Progesterone receptor	F: GCCGCAGGTCTACCAGCCCTA R: GTTATGCTGTCCTTCCATTGCCCTT	NM_001205356.1	199
ESR1	Estrogen receptor 1	F: CAGGCACATGAGCAACAAAG R: TCCAGCAGCAGGTCGTAGAG	XM_002690343.1	82
OXTR	Oxytocin receptor	F: AAGATGACCTTCATCGTCGTG R: CGTGAAGAGCATGTAGATCCAG	NM_174134.2	177
PTGS2	Prostaglandin endoperoxide synthase 2	F: CCAGAGCTCTTCCTCCTGTG R: GGCAAAGAATGCAAACATCA	NM_1744445.2	161
RPS18	Ribosomal Protein S 18	F: GTGGTGTTGAGGAAAGCAGACA R: TGATCACACGTTCCACCTCATC	NM_001033614.2	100

3.2.7 Statistical analyzes

Data from the CL area, CLBP, and P4 concentrations measured along across time were analyzed as repeated measures using the MIXED procedure of SAS version 9.3. The model was set up as a 2 x 2 factorial arrangement of treatments (factor 1: iP4 and factor 2: FA), day, and all possible interactions, as fixed effects. The random effect of animal nested within the interaction between iP4 and FA was used as an error term for the effect of iP4, FA and their interaction. The type of variance–covariance structure used was chosen based on the smallest magnitude of the corrected Akaike's information criterion (AICC) obtained for several structures tested. The residual and influencing diagnostics outputs from the MIXED procedure were checked for the assumption of normality of the data. Further, studentized residual error outputs were also checked for normality by the Shapiro–Wilk test using the UNIVARIATE procedure. A cow from the +iP4+FA group that had the latest timing of luteolysis skewed the residual error variances of most variables and was characterized as an outlier and removed from analyzes, leaving only 7 animals in this group. A secondary set of analyzes were ran, considering in the model the effect of iP4 treatment (+iP4 and −iP4), the retrospectively accessed timing of luteolysis (TL;

before or at day 16, and after day 16), day and their interactions. For analyses of abundance of transcripts (PGR, ESR1, OXTR, PTGS2), the effect of group (–iP4−FA, +iP4−FA, and +iP4+FA) was considered in the model of repeated measures. Because of the technical constraints associated with the −iP4+FA group (described previously), the iP4 x FA factorial structure was not maintained.

The discrete variables (day of structural and functional luteolysis, interval between luteolysis and ovulation, cycle length, and diameter of the largest follicle on the day of luteolysis) were analyzed considering the 2 x 2 factorial arrangement as fixed effect in the PROC MIXED. The variable follicle diameter on D6 was analyzed considering only the iP4 treatment (+iP4 and –iP4), as fixed effect since FA procedures started only on D9.

The Kenward-Roger adjustment for denominator's degree of freedom has been incorporated into all PROC MIXED models. Effects were determined by F-test using Type III sums of squares. The least significant difference test (LSD; i.e., the DIFF option of the LSMEANS statement) was used for the comparison among the means. When interactions were significant, the slice command was incorporated to the SAS program to screen which of the factors or in which days treatment effect occurred. On D16, the effect of group on the frequency of luteolysis was analyzed by the exact Fisher’s test, using the FREQ procedure of SAS.

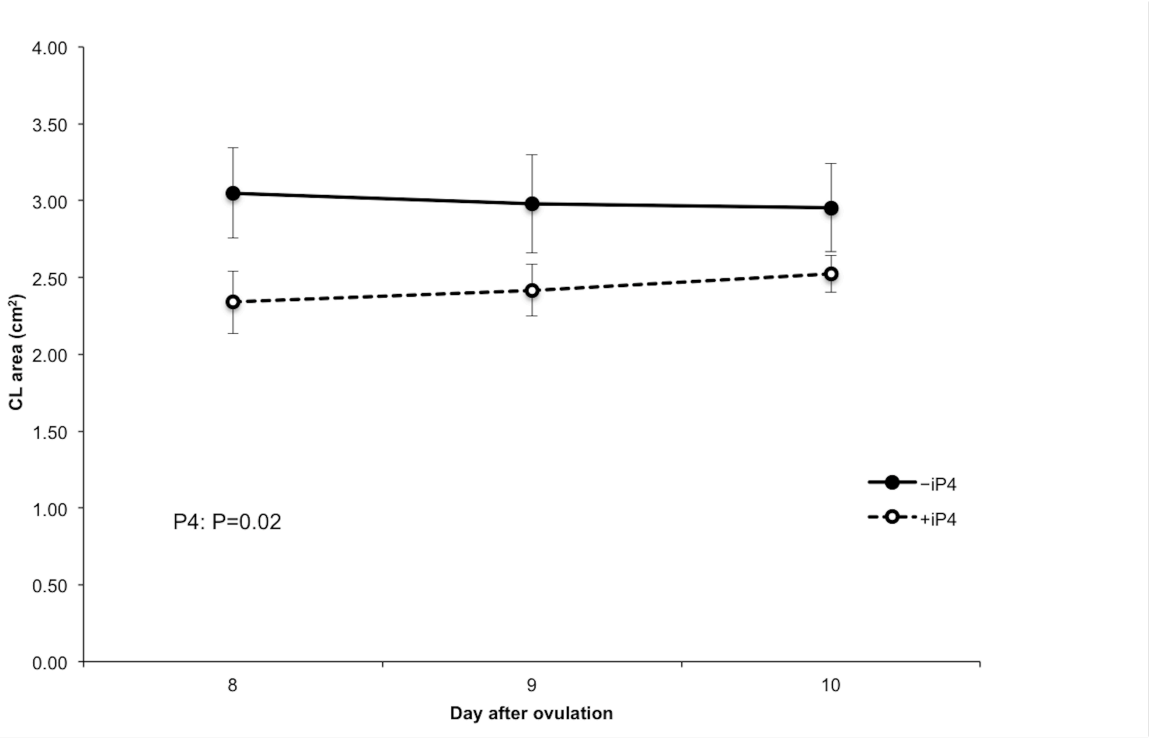
Data were presented as MEAN ± SEM (standard error of the mean). In all cases $P \leq 0.05$ indicated that the difference was significant, and $0.05 > P \leq 0.1$ indicated a tendency for the difference to be significant.

3.3 RESULTS

In our experimental design, the iP4 was administered on D3 post ovulation with the expectation that it would stimulate endometrial mechanisms that would lead to early onset of luteolysis in *Bos indicus* cows. Furthermore, animals were subjected to daily FA from D9 to D18 aiming to delay the onset of luteolysis in iP4 treated cows.

Diameter of the first-wave dominant follicle on D6 was smaller for cows treated with iP4 (11.8±0.5 vs. 9.64±0.4 mm; P<0.05). Between D8 and D10, CL area was smaller in cows treated with iP4 (P<0.05; Figure 5).

Figure 5 - CL area on D8, D9, and D10 of cows treated with placebo (−iP4; n=12) or iP4 (+iP4; n=15) three days after ovulation; mean ± SEM



Source: (CARDOSO, B.O., 2017).

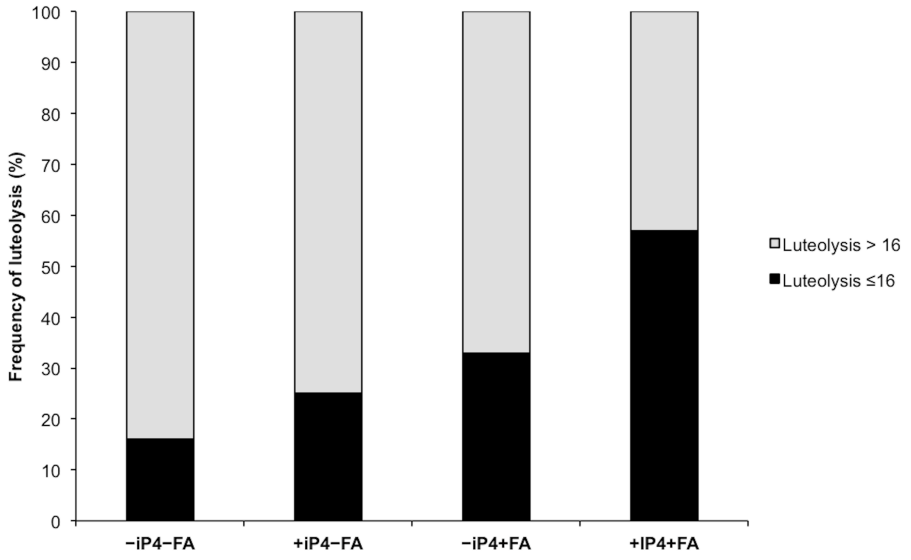
Cows subjected to FA presented a smaller diameter of the largest follicle on the day of structural luteolysis (P<0.05; Table 3). Consequently, the FA increased the length of the estrous cycle about 3 days and the interval between luteolysis and ovulation in approximately 2.5 days (P<0.05; Table 3). Surprisingly, contrary to anticipation, in animals treated with iP4, FA tended to hasten the timing of luteal structural regression (iP4 vs. FA interaction; P<0.06; Table 3). Consistently, frequency of luteolysis at or before D16 was numerically greater in +iP4+FA group (Figure 6).

Table 3 - Variables associated with luteolysis in cows treated with placebo or iP4 (−/+iP4) and submitted or not to FA (+/−FA)

Variable	Mean ± SEM				P value		
	−iP4−FA (n=6)	+iP4−FA (n=8)	−iP4+FA (n=6)	+iP4+FA (n=7)	iP4	FA	iP4*FA
Structural luteolysis[1] (days)	17.2±0.7^{xy}	18.2±0.6^{x}	18.0±0.7^{x}	16.4±0.7^{y}	0.73	0.48	0.06
Interval between luteolysis and ovulation (days)	3.6±1.0	5.3±1.0	6.3±1.0	7.4±1.0	0.18	0.03	0.74
Cycle length (days)	21.2±0.9	23±0.8	24.7±0.8	25.2±0.9	0.19	0.01	0.47
Diameter of the largest follicle on the day of luteolysis (mm)	11.34±0.8	10.16±0.6	3.89±0.7	1.74±0.6	0.15	<.0001	0.66

Legend: Different subscripts represent different means (P<0.10).
[1]: Structural luteolysis was defined as the day when a 25% reduction in the largest CL area measured between D8 and D10 and 50% of the CL blood perfusion measured on D8 was observed.

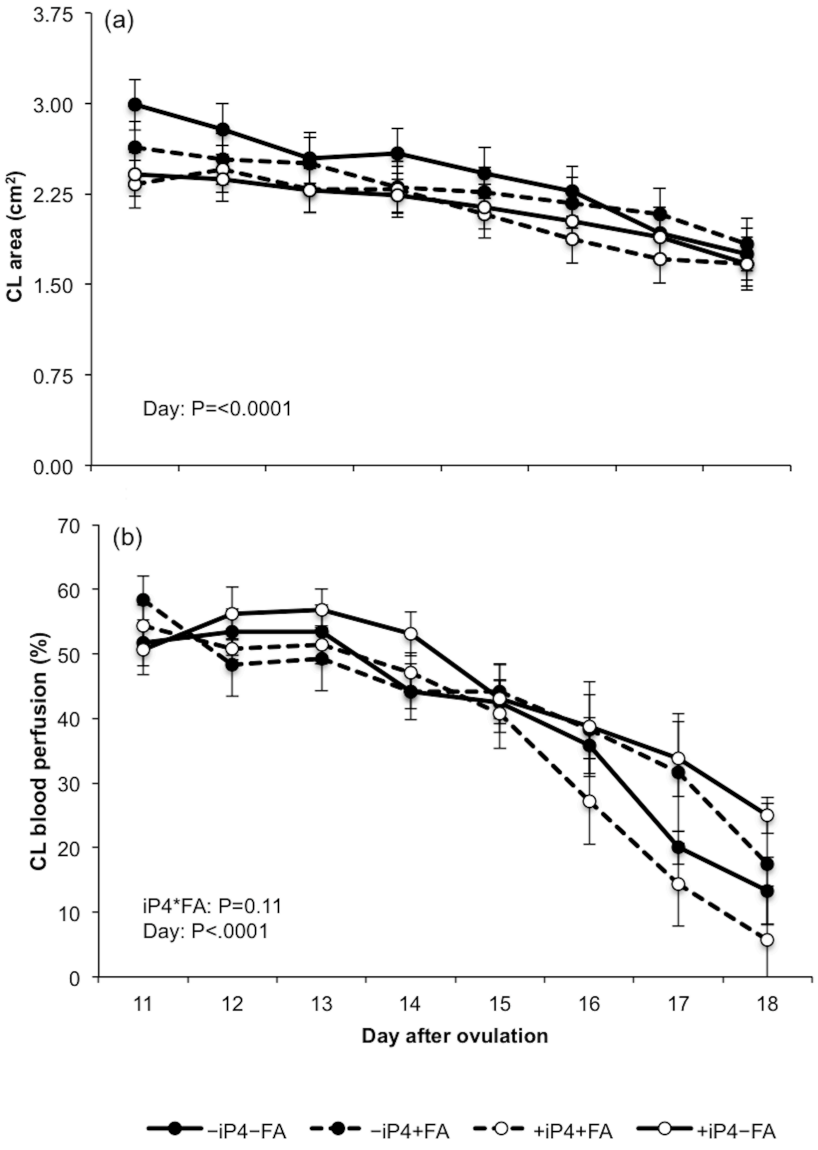
Figure 6 - Frequency of cows treated with placebo or iP4 (−/+iP4) and submitted or not to FA (+/−FA), according to the day of luteolysis (before or on D16 or after D16)



Source: (CARDOSO, B.O., 2017).
Legend: Data analyzed by Fisher's exact test (P>0.1).

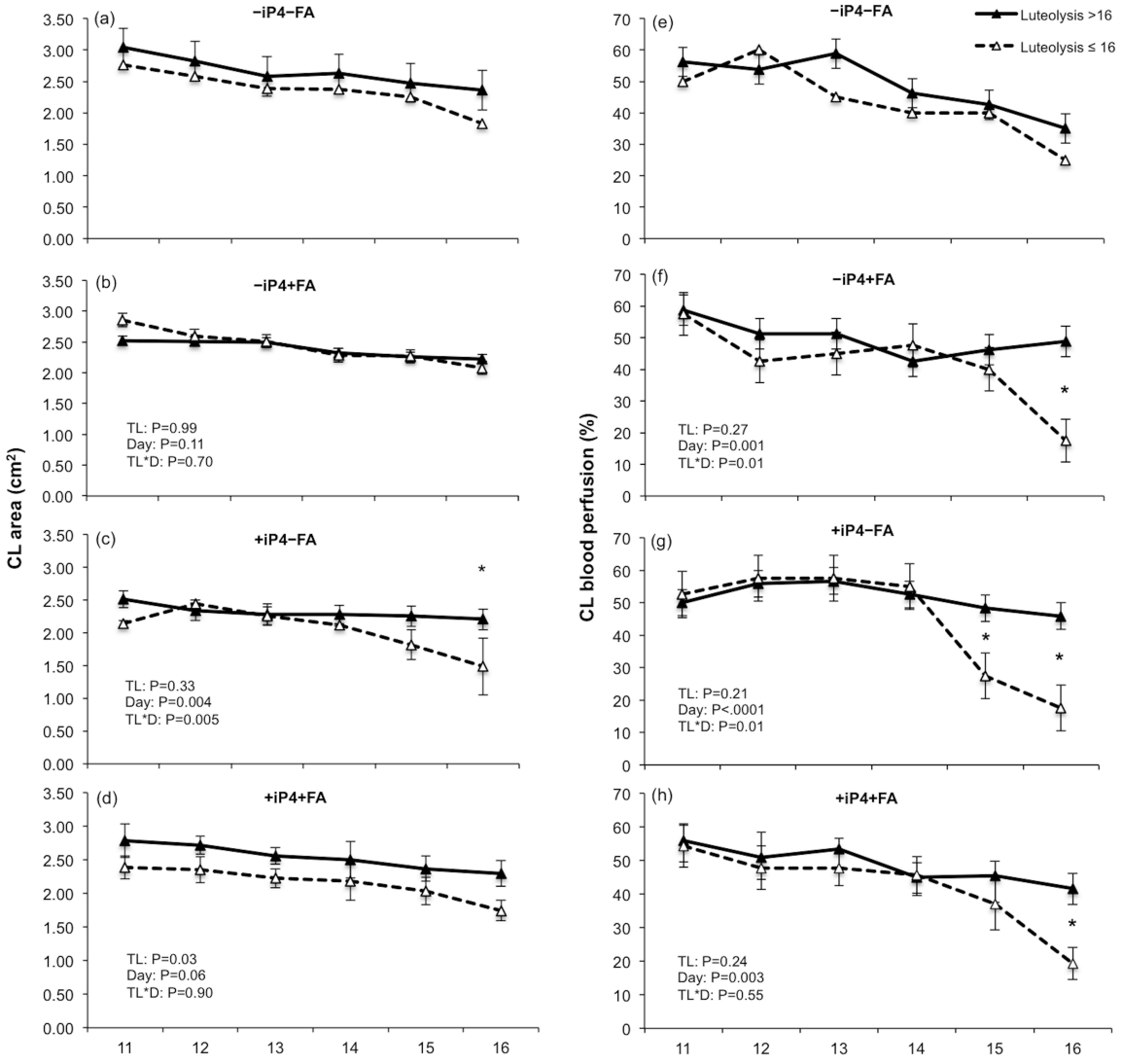
CL area decreased over time (day effect; P<0.01; Figure 7 a) as expected, but size was not affected by treatments. Between D16 and D18, consistent to the timing of luteal structural regression, the CLBP tended to decrease earlier in iP4-treated cows subjected to FA compared to cows treated with iP4 and subjected to sham aspirations (iP4 vs. FA interaction; P=0.1; Figure 7 b). To better understand the effect of treatments on CL regression, we analyzed CL size and CLBP between D11 and D16 within particular groups of animals that were sub-divided retrospectively according to the day of structural luteolysis, i.e., before or at D16 (early luteolysis) or after D16. In the +iP4+FA group alone, CL area was less in cows that regressed the CL at or before D16 (effect of timing of luteolysis [TL]; P<0.05), indicating that cows that underwent earlier luteal regression already had a CL approximately 1,18 cm^2 smaller since D11 (Figure 8 d). However, only a day effect was noted for CLBP in this group (P<0.05; Figure 8 h). Further, there was an interaction of timing of luteolysis by day for CL area in the +iP4−FA group on D16, where cows presenting luteolysis before or at D16 had a smaller CL at this day (Figure 8 c). The iP4 vs. FA interaction was also noted in this group for CLBP on D15 and D16, whereas this interaction was only observed on D16 in the −iP4+FA group (Figure 8 f, g). The −iP4−FA group had a single cow that underwent early luteolysis, thus, no statistics were conducted in that group and means are shown for illustrative purposes.

Figure 7 - CL area (panel a) and CLBP (panel b) from D11 to D18 after ovulation from cows treated with placebo or iP4 (−/+iP4) and submitted or not to FA (+/−FA); mean ± SEM



Source: (CARDOSO, B.O., 2017).

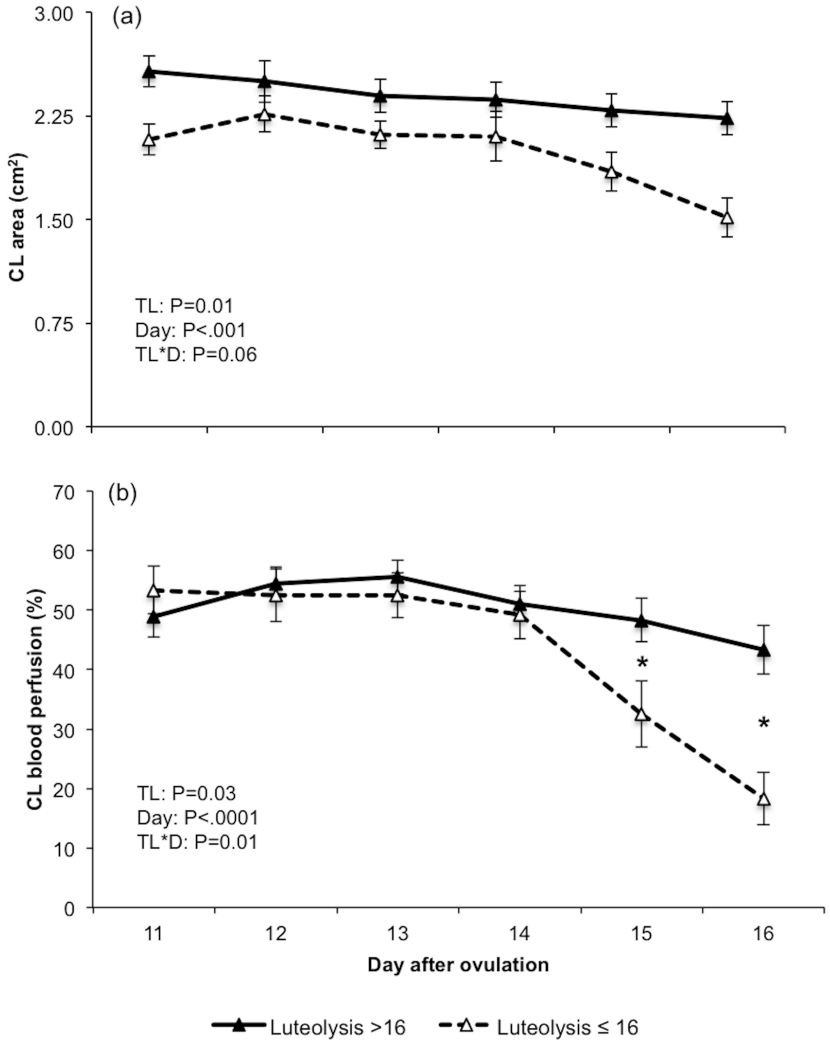
Figure 8 - CL area (left panels) and CLBP (right panels) from D11 to D16 after ovulation from cows treated with placebo or iP4 (−/+iP4) and submitted or not to FA (+/−FA), that were sub-grouped according to the day of luteolysis (before or on D16; or after D16); mean ± SEM



Source: (CARDOSO, B.O., 2017).
Legend: An asterisk (*) means P ≤ 0.05.

Cows treated with iP4 that presented early luteolysis had a smaller CL over-time (effect of timing of luteolysis; P<0.05; Figure 9 a) and a lower CLBP on D15 and D16 (effect of timing of luteolysis by day; P<0.05; Figure 9 b) compared to those detected in luteolysis after D16. In summary, there are sub-populations of animals that vary on their susceptibility to iP4-induced early luteolysis. Such susceptibility is associated with CLs that are less developed throughout the luteal phase.

Figure 9 - CL area (panel a) and CLBP (panel b) from D11 to D16 after ovulation only from cows treated with iP4 that were grouped according to the day of luteolysis (before or on D16; n=9; or after D16; n=18); mean ± SEM

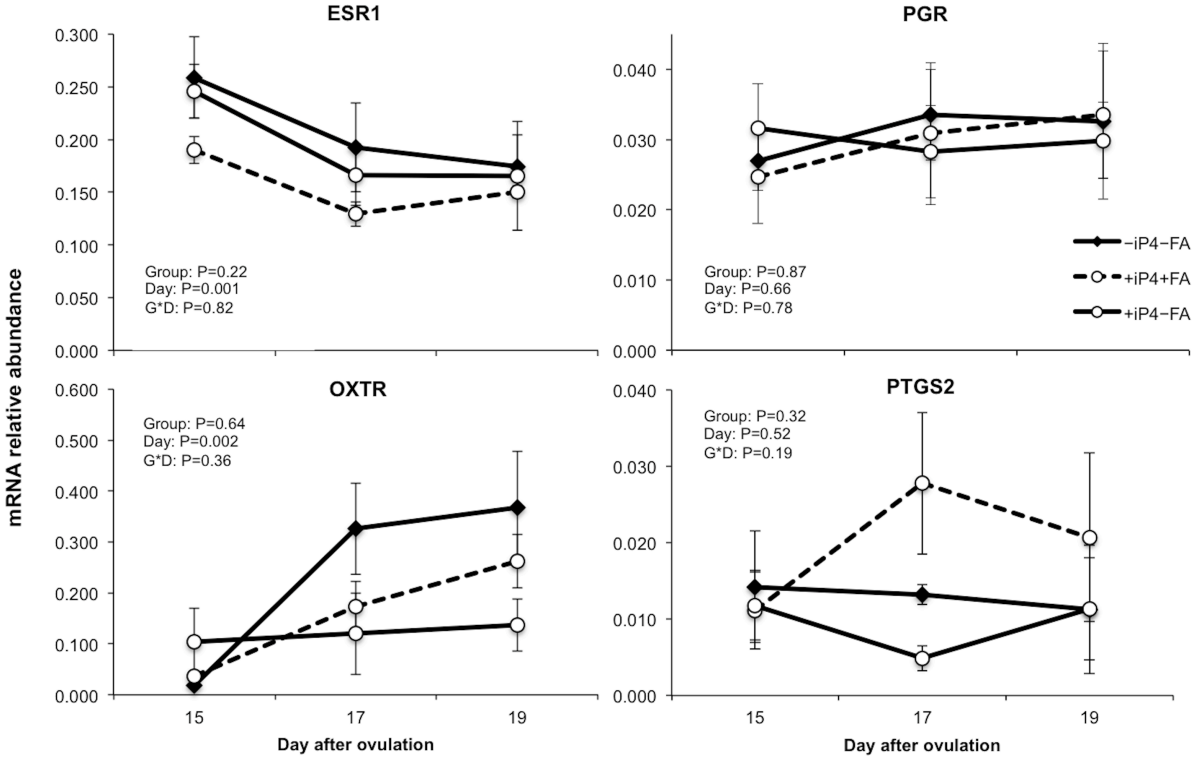


Source: (CARDOSO, B.O., 2017).
Legend: TL means timing of luteolysis. An asterisk (*) means P ≤ 0.05.

Within each of the −iP4−FA, +iP4−FA, +iP4+FA groups, there were respectively 1/6, 2/8 and 4/7 cows that presented luteolysis at or before D16. There was no main effect of group nor the effect of group by day interaction on the abundance of any of the endometrial transcripts measured (P>0.1; Figure 10). However, an effect of day for ESR1 and OXTR mRNA abundance was observed (P<0.01). Specifically, abundance of ESR1 transcripts decreased while that of OXTR

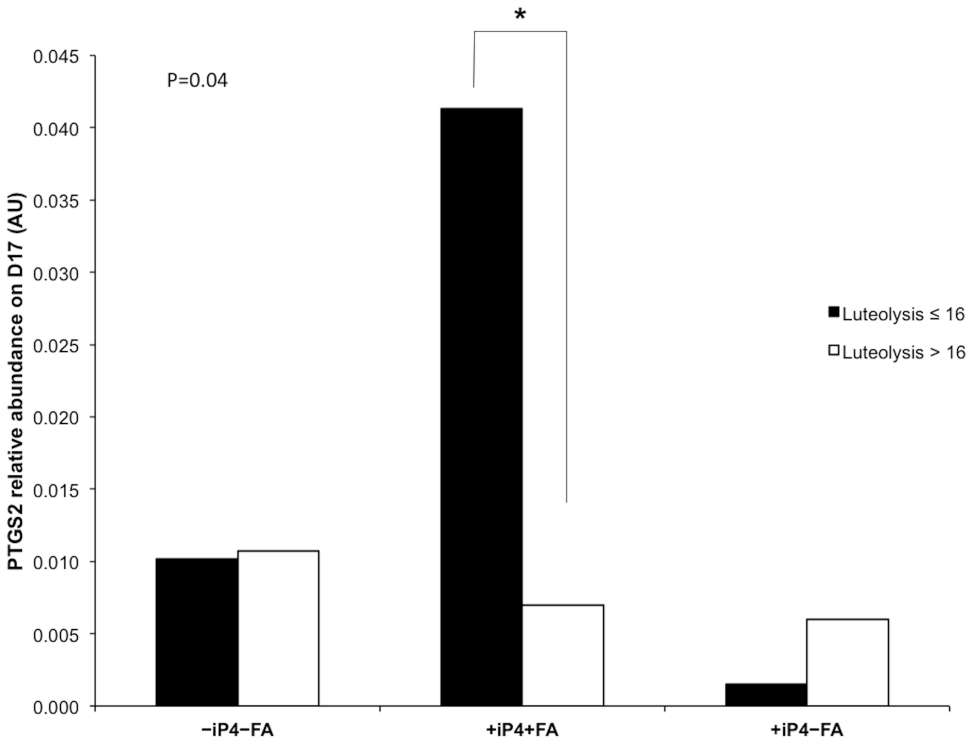
increased between D15 and D19. No effect of group, day or their interaction was detected for the abundance of PGR and PTGS2 mRNA over-time. A retrospective analysis of the abundance of PTGS2 on D17 revealed that in the group +iP4+FA, this transcript was upregulated in the endometrium of cows that underwent early luteolysis (P<0.05; Figure 11).

Figure 10 - Abundance of ESR1, PGR, OXTR, and PTGS2 transcripts normalized to the abundance of RPS18 in endometrial samples from cows of the –iP4−FA (n=4), +iP4−FA (n=4), and +iP4+FA groups (n=5); mean ± SEM



Source: (CARDOSO, B.O., 2017).

Figure 11 - Abundance of PTGS2 transcript on D17 after ovulation normalized to the abundance of RPS18 in endometrial samples from cows of the –iP4−FA, +iP4+FA, and +iP4−FA groups, that were sub-divided according to the day of luteolysis (before or on D16; n=1, 3, 1; or after D16; n=3, 2, 3; respectively)



Source: (CARDOSO, B.O., 2017).
Legend: An asterisk (*) means P ≤ 0.05.

3.4 DISCUSSION

Progesterone supplementation after ovulation has been used as an alternative to reduce embryo mortality and improve the efficiency of reproductive programs in beef cattle (MANN; LAMMING, 2001; PUGLIESI et al., 2016). However, such strategy has a paradoxical effect. At the same time that supplemental P4 favors conceptus development (CARTER et al., 2008), it can stimulate an earlier onset of luteolysis (GARRETT et al., 1988a; O'HARA et al., 2014; PUGLIESI et al., 2014b). Physiologic explanations for the causes of such deleterious effects are unknown, but may include at least three, non-exclusive, components: an uterine component that is associated with the advanced release of luteolytic pulses of endometrial PGF2α, a luteal component that is associated with the direct actions of P4 in the newly formed

CL that renders it subfunctional, and another luteal component that results from the effects of the supplemental P4 in the hypothalamic-pituitary axis to inhibit GnRH pulses, and thus, LH pulses required for adequate luteogenesis. Endometrial release of PGF2α pulses that occurs in natural estrous cycles requires E2 (MCCRACKEN; SCHRAMM; OKULICZ, 1984; SILVIA et al., 1991; SALFEN et al., 1999; BINELLI et al., 2001). In the present dissertation, we attempted to determine whether the endometrial component was involved in the iP4-induced early onset of luteolysis by frequently aspirating antral follicles as a strategy to decrease circulating E2 concentrations. Contrary to our expectations, there was no iP4-induced early luteolysis and there was no FA effect on the length of the luteal phase. However, combination of both iP4 and FA caused CL failure earlier than in the other groups. Such effect was independent from alterations in the abundance of endometrial transcripts associated with the release of PGF2α pulses. Furthermore, cows that underwent early luteolysis had lower CL size during the luteal phase. Collectively, these findings support the notion that iP4 affects mainly the CL to induce early luteolysis.

Supplementation of P4 at early diestrus affects CL growth and function. It is well known that LH is required for follicle growth and luteogenesis in cows. Previous studies have shown a negative association between plasma P4 concentration and LH pulse frequency (BERGFELD et al., 1996; FIKE et al., 2004; GIORDANO et al., 2012). Although serum LH concentration and pulse frequency were not evaluated in the present study, it is likely that in cows treated with iP4, the bolus P4 at the beginning of luteal phase reduced LH pulsatility, resulting in a smaller size first-wave dominant follicle (DF1) and CL on days 8 to 10 after ovulation. Likewise, Burke et al. (1994) reported that greater concentrations of P4 during metaestrus and early diestrus affected negatively CL diameter and growth of the DF1. Consistent with such detrimental effects of P4 on CL development, iP4-treated cows presenting luteolysis on day 16 or before had a smaller-size CL throughout the luteal phase compared to those that underwent luteolysis after D16, suggesting a deleterious action of P4 on the luteal structure. Accordingly, other studies reported a reduction in CL function in response to P4 supplementation from days 1 to 4 or days 3 to 5 post-estrus (GARRETT et al., 1988a; O'HARA et al., 2014). In these studies as well as the present study, decreased luteal function may lead to premature luteolysis. This

possibility is based on the fact that the administration of luteotrophic agents, such as hCG or eCG associated with P4 supplementation, can rescue CL function and luteal lifespan (GINTHER, 1970; O'HARA et al., 2016). Accordingly, Farin, Nett and Niswender (1990) observed a reduction in the CL weight and P4 concentrations on Day 12 of estrous cycle of hypophysectomized ewes, whereas those receiving LH replacement from Day 5 to 12 maintained luteal weight and P4 concentrations similar to intact ewes. They concluded that the reduced CL weight was due to a decrease in the size of both small and large luteal cells, highlighting the importance of LH release from the pituitary for normal luteal development. Therefore, based on evidences showed here and by others, we propose that the CL component is critically involved in the early onset of luteolysis in cows supplemented with P4 at early diestrus.

Supplementation of P4 at early diestrus did not modulate the abundance of endometrial transcripts related to programing (PGR, ESR1) or execution (OXTR, PTGS2) of PGF2α release. Based on other reports, we expected that iP4 alone would cause an earlier onset of luteolysis at least on a proportion of treated animals (WOODY; FIRST; POPE, 1967; GINTHER, 1970; O'HARA et al., 2014; PUGLIESI et al., 2014b). Furthermore, we hypothesized that such an effect would be through actions on the PGF2α–synthesizing machinery in the endometrium. Indeed, P4 induces changes in the endometrial transcriptome (FORDE et al., 2009). Garrett et al. (1988) postulated that exogenous P4 given at early diestrus stimulates earlier maturation of the uterus, resulting in an early release of PGF2α from the endometrium. Accordingly, it would be expected an advancement in temporal changes in the abundance of endometrial transcripts that controls endometrial PGF2α release. It is generally accepted that during mid to late luteal phase, the endometrial release of luteolytic pulses is dependent on a well-coordinated action of P4, E2 and OT through their respective uterine receptors in sheep (SILVIA et al., 1991; SPENCER; BAZER, 1995) and cows (LAMMING; MANN, 1995; MCCRACKEN; CUSTER; LAMSA, 1999; ROBINSON et al., 2001). Specifically, towards luteolysis, PGR and ESR1 would be expected to be downregulated and OXTR and PTGS2 upregulated in the endometrium. This pattern was observed partially in the present experiment, but was not influenced by iP4. Different authors highlight the requirement of uterine OXTR upregulation for triggering luteolysis in ruminants (MCCRACKEN; SCHRAMM; OKULICZ, 1984; JENNER; PARKINSON;

LAMMING, 1991; SPENCER; BAZER, 1995). However, there may be differences among ruminant species. For example, Mann and Lamming (2006) showed that OXTR were undetectable well before the start of luteolysis in cows. Despite of an increase of receptor concentration prior to the first detection of PGF2α release, peak concentrations were reached only after luteolysis had been completed. In an elegant study, Robinson et al. (2001) collected biopsy samples from cyclic cows over three estrous cycles and demonstrated that the upregulation of OXTR in the luminal epithelium around day 16 was not related to preceding changes in the endometrial expression of either ESR1 or PGR. In addition, other authors have suggested that OT play a more facilitating than a mandatory role in luteolysis in cattle (MEYER; MITTERMEIER; SCHAMS, 1988; HANSEL; BLAIR, 1996; KOTWICA et al., 1997). Collectively, while the sequential role of the key players triggering luteolysis is well established in ewes, it still remains uncertain what is the precise temporal relationship between acquisition of ESR1 and OXTR and the onset of luteolysis in cattle. The present data showed no clear indication of molecular changes associated with the control of the onset of luteolysis in cows supplemented with iP4. This suggests that the uterine component might not be involved in this process.

Follicle aspiration did not affect timing of the onset of luteolysis nor did it regulate the transcriptional machinery associated with PGF2α release. In the present study we attempted to inhibit the E2 tone to the endometrium by aspirating antral follicles daily. The motivation was that follicular E2 was hypothesized to play a stimulatory role to the onset of luteoysis. Thus, removing antral follicles was expected to delay the onset of luteolysis, as shown previously (FOGWELL et al., 1985; VILLA-GODOY et al., 1985; ARAUJO et al., 2009). We used ultrasound-guided follicular aspiration that was shown as a reliable method to decrease plasma concentrations of E2 (TOHEI et al., 2001; ARAUJO et al., 2009). The follicle aspiration regime adopted in the present experiment was effective, given that antral follicles never reached the deviation size (i.e., 6-7 mm; GIMENES et al., 2008) before being aspirated (data not shown). This is important because the steroidogenic capacity of ovarian follicles markedly increases after deviation (KULICK et al., 1999; GINTHER et al., 2000). Furthermore, length of the estrous cycle was significantly longer in the FA groups. However, there was no evidence that follicular E2 was required for the occurrence of luteolysis in *Bos indicus* cattle. Similarly, Bisinotto et al. (2012) demonstrated that

daily follicular aspiration of *Bos indicus* and crossbred cows from day 13 to 25 failed to extend luteal lifespan. Furthermore, in Holstein heifers of three follicular waves, emergence of the third wave occurred hours before the beginning of a decrease in P4 concentrations (GINTHER et al., 2014), indicating that apparently elevated E2 concentrations were not required to initiate luteolysis. Although some studies using models to remove E2 during peri-luteolysis period successfully extended the luteal phase (FOGWELL et al., 1985; VILLA-GODOY et al., 1985; ARAUJO et al., 2009), they could not prevent later luteal regression, suggesting that E2 might be permissive but not an absolute requirement for luteolysis in cows (ARAUJO et al., 2009). In summary, our results showed that luteolysis in the FA groups occurred even in the presence of follicles that were smaller than the size of deviation (3.89±0.7 and 1.74±0.6 mm for the –iP4+FA and +iP4+FA groups, respectively; Table 3).

Combination of supplemental P4 and FA stimulated early CL regression. This result was unexpected and contradicted our hypothesis. This finding could not be explained by changes in the abundance of endometrial transcripts involved in PGF2α release, because they were not affected by either treatment or the combination. One possible explanation is that a CL that has already been compromised due to P4 supplementation at the beginning of the diestrus, may be affected negatively further under low concentrations of E2. Effects of E2 to possibly favor the CL are at least two-fold: a paracrine/autocrine action in the CL during the luteal phase (BERISHA; PFAFFL; SCHAMS, 2002) and a central action in the hypothalamic-hypophyseal axis to stimulate LH release. Regarding a local effect of E2 in the CL, according to Shibaya et al. (2007), ESR1 protein level is increased in luteal tissues at the early and mid-luteal stages followed by a dramatic decrease at the regressed luteal stage, which can result in progression of structural luteolysis in cattle. These authors suggested that E2 probably acts differently via the different receptors (ESR1 and ESR2) in the bovine CL. While E2 binding to ESR2 would be important during CL regression, binding to ESR1 would play an important role in the CL maintenance. In this sense, we speculate that E2 may have a luteotrophic action in the CL, thus, the reduction in E2 concentrations due to FA may have negatively affected the CL and contributed to its earlier demise. Regarding a central effect of E2, studies over the past 40 years have demonstrated that E2 has a positive feedback on LH pituitary release (HOBSON; HANSEL, 1972; BECK; CONVEY, 1977; FORREST;

KALTENBACH; DUNN, 1981; BRAUN; SCHAMS; LEIDL, 1986). Evidences show that P4 and E2 modulate LH release through indirect mechanisms that regulate hypothalamic GnRH secretion or by a direct stimulation or suppression of the pituitary response (SCHOENEMANN et al., 1985; RISPOLI; NETT, 2005; STEVENSON; PULLEY, 2016). For example, Rudolf and Kadokawa, (2013) cultured anterior pituitary cells for 3 days and treated them with increasing concentrations of E2. They showed that greater concentrations of E2 increased GnRH-stimulated LH secretion. However, an interesting finding was that lower concentrations of E2 significantly suppressed the secretion of LH. These authors concluded that this inhibitory effect might be partly due to contribution of G-protein coupled receptor 30 (GPR30), which is an E2 receptor located on the plasma membrane of the anterior pituitary, and that the low E2 suppression of LH secretion may have another undefined non-genomic pathway in the bovine anterior pituitary. It was demonstrated that low concentrations of E2 exerts a negative feedback on hypothalamic-GnRH secretion in the ewe (EVANS et al., 1994). Additionally, Hoffmann et al. (1974) provided evidence for the need for LH luteotrophic support for CL maintenance in cattle, demonstrating that treatment with LH antiserum during the mid luteal-phase inhibited CL function. Although ultrasound-guided follicular aspiration was evidently effective in the present study, such technique may not provide complete removal of circulating E2 and low E2 level are maintained in circulation, as previously reported by Araujo et al. (2009). Collectively, we suggest that low concentrations of E2 that result from follicular aspiration could suppress LH secretion, which combined with the negative effect of iP4 on luteogenesis may have further impaired CL function of cows from +iP4+FA group, significantly reducing the luteal lifespan in this group.

In summary, the present study provided further evidence to the fact that elevated follicular E2 is not absolutely required for the onset of luteolysis in *Bos indicus* cows. In addition, P4 supplementation compromises CL development probably by decreasing LH pulsatile release. Accordingly, our experimental results did not provide plausible signs that the uterine component could be involved in the early onset of luteolysis in cows supplemented with P4 at early diestrus. We concluded that impaired CLs are most likely to undergo early luteal regression. In addition, it was shown for the first time that P4 supplementation together with reduced E2 concentration after FA during the mid-luteal phase hastened the

luteolysis onset in *Bos indicus* cows. We speculate that inadequate luteotrophic support both during CL development and maintenance has led to severe luteal impairment, culminating in its earlier regression. Further studies will be required to elucidate whether the interruption of luteotrophic support after FA was due to a reduced direct action of E2 on its own receptor in the CL, reduced E2 indirect regulation on the hypothalamic-hypophyseal axis, or both.

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4 FINAL CONSIDERATIONS

It has been shown that P4 supplementation at initial diestrus causes an early maturation of the uterus. Specifically, it advances endometrial PGF2α release, culminating in premature luteal regression. Considering the important role of E2 in triggering the pulsatile release of PGF2α, here we studied the requirement of follicular E2 for the onset of luteolysis in *Bos indicus* cows supplemented with P4 at early diestrus. In order to gain molecular insights on possible uterine mechanisms involved in such event, we used, for the first time, the cytobrush technique at specific time-points during the luteolytic period.

The present study presented a new approach for sequential molecular evaluation of the endometrial epithelial lining from cows *in vivo*, along the estrous cycle. Even though the cytobrush technique requires specific training and equipment, the samples can be obtained in a practical and safe manner. However, this technique is not adequate for the study of deeper layers of the endometrium, directing its use to events comprised in the endometrial luminal epithelial cells. Furthermore, results of the present study suggested that the cytobrush technique does not interfere with the immediate fertility of animals. Thus, the cytobrush maybe an useful tool for identification of possible marker genes for fertility in the field. A major limitation of the use of this technique is that the cervix must have an adequate conformation to facilitate the insertion of the apparatus through it. This may be challenging when performed during diestrus.

In addition, the present data showed no clear indication of molecular changes associated with the control of the onset of luteolysis in cows supplemented with iP4. This suggests that the uterine component might not be critically involved in this process, as we anticipated. Instead, we speculate that corpora lutea affected by exposure to iP4 during luteogenesis are most likely to undergo early luteal regression. Furthermore, this process may be exacerbated by low E2 concentrations resulting from FA. Collectively, these results lead me to **reject the central hypothesis of this dissertation** and to conclude that reduced follicular E2 cannot prevent iP4-induced early onset of luteolysis in *Bos indicus* cows.

Finally, the mechanism by which P4 advances the release of PGF2α in cows is not clearly understood yet. Although it still necessary to study the precise temporal relationship between acquisition of endometrial ESR1 and OXTR for the onset of luteolysis in cattle, the results of this dissertation indicated that E2 is not absolutely required for the occurrence of this event in *Bos indicus* cows. Further studies are necessary to determine the exact mechanism by which supplemental P4 produces its effects, and also, the precise temporal molecular events involved in triggering luteolysis in cows.

ATTACHMENT

ATTACHMENT A – Proof correction of the manuscript accepted in the journal Reproduction in Domestic Animals

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SHORT COMMUNICATION

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Cytobrush: A tool for sequential evaluation of gene expression in bovine endometrium

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Contents

Aims were to (i) compare specific transcript abundance between endometrial samples collected by transcervical biopsy and cytobrush and (ii) measure the abundance of endometrial transcripts involved in PGF2a synthesis in samples collected by cytobrush. In Experiment 1, endometrial samples were taken transcervically by cytobrush and biopsy 10 days after ovulation. Compared to biopsy samples, abundance of transcripts for MSTN, AKR1C4 and PGR was similar, VIM, FLT1 and PTGES was lower ($p < .05$) and KRT18 and CD3D was greater in cytobrush samples ($p < .05$). Thus, there was an enrichment of epithelial and immune cells in the cytobrush samples. In Experiment 2, endometrial samples were collected by cytobrush on days 10, 13, 16 and 19 after ovulation. Abundance of PGR2 mRNA was maximum on day 10 then decreased ($p < .05$). Abundance of ESR1 decreased gradually from day 10 to day 16 then increased again on day 19. The greatest abundance of OXTR was noted on day 19. The sequential alterations in abundance of these transcripts are consistent with the release of PGF2a associated with luteolysis. In summary, cytobrush sampling provides representative, physiologically relevant samples of the luminal epithelium in cattle.

1 | INTRODUCTION

In cattle, to understand the cyclic cellular and molecular changes of uterine function, it is often necessary to probe the uterus throughout the oestrous cycle or early pregnancy. Most studies in the literature used *post-mortem* samples of the endometrium, collected in specific target days after oestrus (Bauersachs et al., 2005; Palma-Vera, Sharbati, & Einspanier, 2015). Nevertheless, with this method, it is impossible to characterize the endometrial transcriptome in the same animal along the oestrous cycle. Alternatively, in vivo endometrial samples may be obtained by uterine biopsies; however, multiple biopsies in the same animal result in a local inflammatory process that may affect endometrial function, and impair the subsequent fertility (Zaayer & van der Horst, 1986). Collecting endometrial cytological samples using a cytobrush technique is less traumatic than using biopsy apparatuses. To the best of our knowledge, there is no study that used the cytobrush technique repeatedly, in the same animal, to evaluate molecular changes that occur throughout the oestrous cycle. We aimed (i) to compare molecular aspects of endometrial samples collected by transcervical biopsy and cytobrush and (ii) to characterize the abundance of transcripts involved in the PGF2a synthesis associated with luteolysis in beef cattle, using the cytobrush technique.

2 | MATERIALS AND METHODS

Animal welfare guidelines and handling procedures recommended by the São Paulo State (Brazil) law number 11.977 were strictly followed.

Non-lactating, multiparous Nelore (*Bos indicus*) cows containing no gross reproductive anomalies were kept in grazing conditions and water ad libitum.

3 | EXPERIMENT DESIGNS

3.1 | Experiment 1: characterization of samples obtained by biopsy or cytobrush techniques

Ovulations of five Nelore cows were synchronized using an oestradiol/progesterone-based TAI protocol. Ten days after ovulation,

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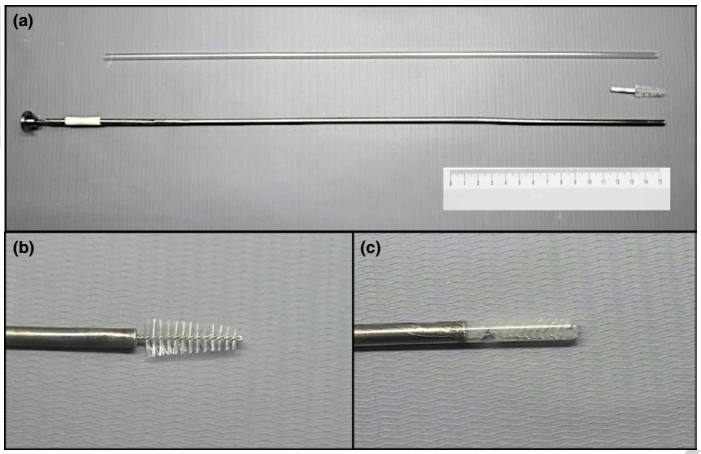

FIGURE 1 Images of the apparatus used in the cytobrush technique. Image (a) illustrates the entire apparatus with the uncovered cytological brush and disposable AI sheath. Image (b) illustrates the uncovered cytological brush before insertion of the disposable AI sheath and the sanitary sheath. Image (c) illustrates the cytological brush covered by a disposable AI sheath after sample collection

cows were submitted to collection of endometrial samples by a cytobrush technique followed by biopsy. The RNA was extracted, and abundance of specific transcript markers of epithelium (cytokeratin; KRT18), stroma (vimentin; VIM), immune cells (T-cell receptor T3 delta; CD3D), endothelial cells (vascular endothelial growth factor receptor 1; FLT1) and myometrium (myostatin; MSTN), and transcripts involved in uterine function during the oestrous cycle (progesterone receptor; PGR, prostaglandin E synthase; PTGES, aldo-keto reductase family 1 member C4; AKR1C4) was measured by qPCR.

3.2 | Experiment 2: characterization of genes involved in luteolytic mechanisms

Ovulations of ten Nelore cows were synchronized as mentioned. On days 10, 13, 16 and 19 after ovulation, endometrial cytobrush samples were collected from each animal. Each day, luteolysis was verified by ultrasonography as described previously (Pugliesi, Miagawa et al., 2014). Five of those ten cows were randomly selected for molecular analysis, and transcript abundance was determined for oxytocin receptor (OXTR), oestrogen receptor-1 (ESR1) and progesterone receptor-2 (PGR2) by qPCR.

3.3 | Cytobrush and biopsy procedures

After epidural anaesthesia (60 mg, lidocaine), a cytological brush (Disposable cytology sampling brush 8"; Viamed Ltd, West Yorkshire, UK) was coupled to the tip of a conventional AI gun, covered by a disposable AI sheath and protected by a sanitary sheath (Figure 1). Apparatus was inserted via cervix and cytobrush rotated to collect cells from uterine body. The cytobrush was uncoupled from the apparatus and placed in a 2-ml cryo-tube filled with 1 ml of Trizol® reagent (Life Technologies, California, USA) and stored at −80°C for later processing.

Immediately afterwards, a biopsy sample (~50 mg) was obtained from uterine body of each cow using an Eppendorfer uterine biopsy forceps as reported previously (Pugliesi, Scolari et al., 2014) and stored at −80°C.

3.4 | RNA extraction, synthesis of cDNA and qPCR

After RNA extraction by Trizol® reagent, cDNA was synthesized from 500 ng total RNA (High Capacity cDNA Synthesis kit; Life Technologies, SP, Brazil). Transcripts abundance was measured using SYBR Green qPCR (Step One Plus Real Time System; Life Tech; Mesquita et al., 2014; Table S1).

3.5 | Statistical analyses

In Experiment 1, fixed effect of technique was analysed by one-way ANOVA, and in Experiment 2, transcript abundance on different days was analysed by repeated-measures ANOVA (PROC MIXED from SAS software).

4 | RESULTS

4.1 | Experiment 1

Abundance of VIM and FLT1 was 9.2- and 275-fold greater in biopsy samples, respectively ($p < .05$; Figure 2). Abundance of KRT18, CD3D and PTGES was 5.2-, 2.2- and 7.2-fold greater in cytobrush samples, respectively ($p < .05$). There was no difference in the relative expression levels of MSTN, AKR1C4 or PGR between the two probing methods ($p > .05$).

4.2 | Experiment 2

There was an effect of day on abundance of all transcripts (Figure 3). Levels of ESR1 mRNA tended to be greater on days

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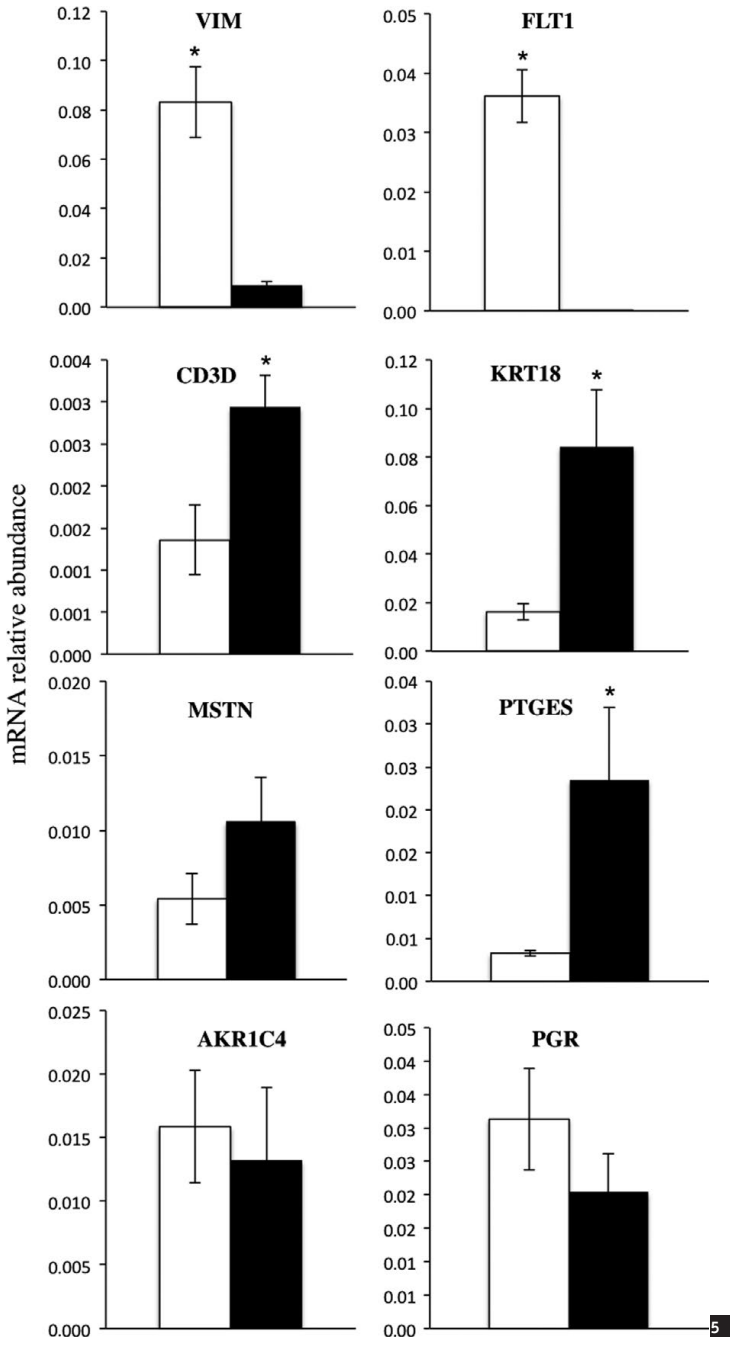

FIGURE 2 Abundance of VIM, FLT1, CD3D, KRT18, MSTN, PTGES, AKR1C4 and PGR transcripts normalized to ACTB in uterine samples (n = 5/group) collected by biopsy (open bars) and cytobrush technique (filled bars) 10 days after ovulation (Mean ± *SEM*)

10 and 19 (p = .10). The abundance of PGR2 mRNA was maximum on day 10, gradually decreased and increased again on day 19 (p < .05). There was a gradual increase in abundance of OXTR over time that reached a peak on day 19. Structural luteolysis was between days 16 and 19 (day 18 ± 0.63).

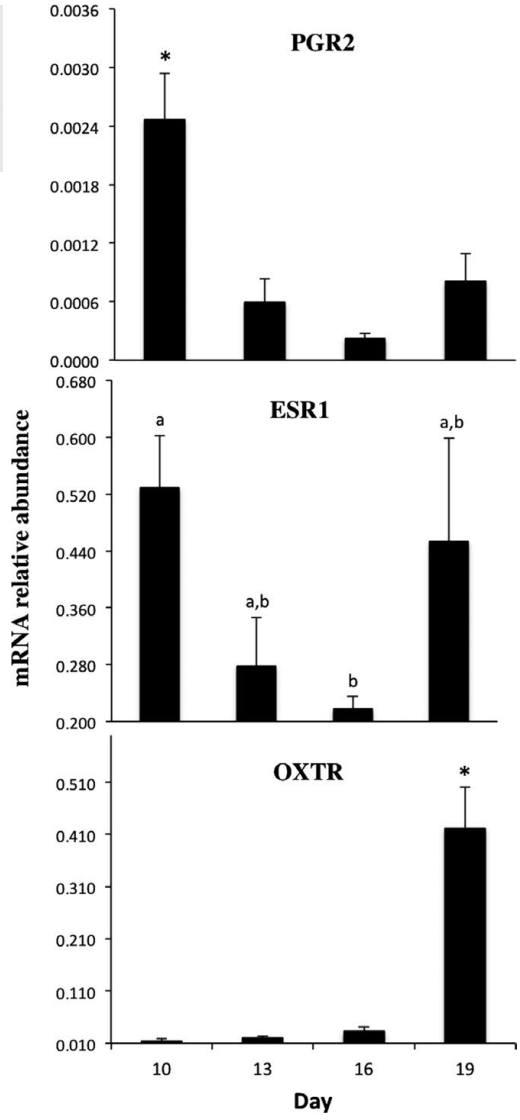

FIGURE 3 Abundance of PGR2, ESR1 and OXTR transcripts normalized to RPS18 in uterine samples (n = 5) collected by cytobrush technique during oestrous cycle. An asterisk (*) means $p \leq .05$ and a, b: $.05 < p > .10$ (Mean ± *SEM*)

5 | DISCUSSION

The present study demonstrated that the cytobrush technique can be successful used for sequential evaluation of endometrial gene expression in cattle. The cellular composition of biopsy samples was different from that obtained by cytobrush. The former contained a greater portion of endometrial stroma (VIM) and vascular cell (FLT1) markers than cytobrush samples (Johnson, Austin, Collins, Murdoch, & Hansen, 1999). Because of the more superficial sampling compared to biopsies, cytobrush samples were enriched in endometrial epithelial cells (KTR18) and immune cells (CD3D) markers (Li et al., 2016). It is unlike that either probing technique reached the myometrial layer, because abundance of MSTN was similar between groups. Detection of sex-steroid modulated endometrial markers, PGR, AKR1C4 and PTGES, in both sample types confirmed the adequacy of both techniques for the study of events relevant for reproduction. In summary, biopsy samples are enriched in stromal and endothelial cells, while cytobrush samples are enriched in epithelial and immune cells, which directs their use for different purposes.

In Experiment 2, temporal changes of transcripts involved in the luteolytic mechanism in cytobrush samples were consistent with a previous report (Okumu et al., 2010). Specifically, PGR2 expression decreased gradually from the middle to end of cycle, associated with an increase of ESR1 expression, followed by a marked increase of OXTR transcription. As an additional observation, 7 of 10 cows in Experiment 2 became pregnant after a single artificial insemination at the subsequent oestrus. This indicated that cytobrush technique did not interfere with the immediate fertility, which reinforces the safety to use this technique.

In conclusion, repeated bovine endometrial sampling using the cytobrush method is safe and provides representative samples for the study of the superficial endometrium in vivo. Nonetheless, this technique is not adequate for the study of deeper layers of the endometrium.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

Cardoso was responsible for sample collection, processing, RNA isolation and cDNA synthesis, gene expression analyses, results interpretation and writing the manuscript. Oliveira was responsible for sample collection of Experiment 2, RNA isolation and worked on experimental design and interpretation. Pugliesi performed samples collection of Experiment 1, statistical analysis of both experiments and helped with results interpretation and revision of the manuscript. Batista was responsible for revising and editing the manuscript. Binelli worked on the experimental design, interpretation of results and manuscript revisions and editing.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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