IGOR GARCIA MOTTA

Impacts of estradiol on $PGF_{2\alpha}$ release and corpus luteum function during early pregnant in beef heifers

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CERTIFICADO

Certificamos que a proposta intitulada "Impactos do uso do estradiol durante o período de reconhecimento materno da gestação em novilhas Bos indicus e Bos taurus", protocolada sob o CEUA nº 6550291019 (ID 007333), sob a responsabilidade de **Guilherme Pugliesi** *e equipe; Igor Garcia Motta* - 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 Universidade de São Paulo (CEUA/FMVZ) na reunião de 17/12/2019.

We certify that the proposal "Impacts of estradiol use during maternal recognition period of pregnancy in Bos indicus and Bos taurus heifers", utilizing 100 Bovines (100 females), protocol number CEUA 6550291019 (ID 007333), under the responsibility of **Guilherme Pugliesi** *and team; Igor Garcia Motta* - 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 12/17/2019.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 03/2020 a 12/2022 Área: Reprodução Animal

Origem:	Prefeitura do Campus da USP de Pirassununga			
Espécie:	Bovinos	sexo: Fêmeas	idade: 24 a 36 meses	Quantidade: 50
Linhagem:	Nelore		Peso: 300 a 400 kg	
Origem:	Animais de proprietários			
Espécie:	Bovinos	sexo: Fêmeas	idade: 24 a 36 meses	Quantidade: 50
Linhagem:	Aberdeen Angus		Peso: 300 a 400 kg	

São Paulo, 30 de agosto de 2023

Prof. Dr. Marcelo Bahia Labruna
Coordenador da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

Profa. Dra. Camilla Mota Mendes
Vice-Coordenadora da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia
Universidade de São Paulo

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Author: MOTTA, Igor Garcia

Impacts of estradiol on PGF2α release and corpus luteum function during early pregnant in beef heifers

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Date: _____/_____/_____

Committee Members

Prof.Dr._____

Institution:_____Decision: _____

Prof.Dr._____

Institution:_____Decision:_____

Prof.Dr._____

Institution:_____Decision:_____

Prof.Dr._____

Institution:_____Decision:_____

Prof.Dr._____

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I dedicate this thesis to the most important people in my life,
My son Arthur and my wife Mayuki,
My parents, José Luiz, and Solange,
My brothers Rodrigo, Diego, and Vitor,
My uncle José Fernando and my nephew Pietro.

RESUMO

MOTTA, I. G. **Impactos do estradiol na secreção de PGF2α e na função do corpo lúteo durante o início da gestação em novilhas de corte.** 81p. Tese (Doutorado em Ciências e Ciências Veterinárias) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2023.

No primeiro estudo, foram estudados os efeitos do 17β-estradiol (E2) ou do benzoato de estradiol (BE) na secreção de $PGF_{2\alpha}$ em novilhas de corte, gestantes e não gestantes. Foram utilizadas 32 novilhas da raça Nelore, com 16 a 18 meses de idade, pesando em média 314 ± 31 kg e escore de condição corporal (ECC) entre 3 a 4. No dia 14 após a inseminação artificial em tempo fixo (IATF) as novilhas receberam um dispositivo intravaginal de 1g de P4, novo (Sincrogest®, Ourofino Saúde Animal e foram distribuídas em três grupos: Controle (C, sem tratamento adicional); E2 (1 mg E2 + 9 mg P4; Betaproginn®, Boehringer Ingelheim); ou BE (1 mg; Sincrodiol®, Ourofino). Amostras de sangue foram colhidas de hora em hora, durante 8 horas após o tratamento para mensurar as concentrações plasmáticas do metabolito de $PGF_{2\alpha}$ (PGFM). O dispositivo de P4 foi removido no D22 e o diagnóstico de gestação realizado no D28. A proporção de novilhas gestantes não diferiu entre controle, E2 e BE. A concentração média de PGFM durante as 9 horas de colheita foi maior no grupo E2 (55,3 ± 10,2) do que no grupo BE (37,2 ± 4,7) e grupo controle (25,1 ± 1,1). Além disso, a luteólise ocorreu mais cedo no grupo E2 do que no grupo BE. Nas novilhas gestantes, 1 mg de BE, 14 dias após a IATF não induziu a liberação de $PGF_{2\alpha}$, e apenas o número de pulsos proeminentes de PGFM e a concentração máxima foram superiores ao grupo controle. No estudo 2, Experimento 1, foram avaliados os efeitos de diferentes doses de BE, 13 dias após a ovulação, sobre a liberação de $PGF_{2\alpha}$ e o tamanho e função do corpo lúteo em novilhas Nelore gestantes e não gestantes. Foram utilizados 69 ciclos de 47 novilhas da raça Nelore, com idade entre 18 e 24 meses, pesando em média 390 ± 35 kg e ECC entre 3 e 4. O estro foi sincronizado e as novilhas foram subdivididas aleatoriamente em não inseminadas ou inseminadas. Treze dias após a ovulação, foram distribuídas aleatoriamente em três grupos: 0, 1 ou 2 mg de BE. Amostras de sangue foram colhidas no D13, na hora 0 (H0) (antes do tratamento) e de hora em hora da H3 a H12 para avaliar a PGFM. A ultrassonografia Doppler foi realizada diariamente do

D13 ao D19 e o diagnóstico de gestação foi realizada no D28. Independentemente da dose, a luteólise foi antecipada nas novilhas não inseminadas tratadas com BE em relação ao grupo controle (16,3 ± 0,2 vs.17,3 ± 0,6 dias). A proporção de novilhas gestantes foi menor nos grupos tratados com 1 mg (50%; 8/16) e 2 mg (29,2%; 7/24) quando comparado ao grupo controle (90%; 9/10). A concentração média de PGFM durante as 10 horas de colheita foi maior nos grupos tratados com 1 (46,3 ± 3,6 pg/mL) e 2 mg de BE (46,9 ± 3,3 pg/mL) do que no grupo controle (28,0 ± 1,2 pg/mL) independentemente do status gestacional. No Experimento 2, foram avaliados os efeitos de 0 e 1 mg de BE, 13 dias após a ovulação sobre os componentes da cascata endometrial da síntese de $PGF_{2\alpha}$ em novilhas de corte, não inseminadas e gestantes. Foram utilizados 60 ciclos de 40 novilhas da raça Nelore, com idade entre de 24 e 30 meses, pesando em média 437 ± 45 kg e ECC entre 3 e 4. Treze dias após a ovulação, as novilhas foram distribuídas aleatoriamente em dois grupos: 0 ou 1 mg de BE. Três horas após os tratamentos, foram realizadas citologias do endométrio e amostras foram posteriormente avaliadas por qPCR. A abundância de OXTR e PGR foi afetada pelo tratamento com BE, sendo maior nas novilhas tratadas com 1 mg de BE. A abundância de ESR2 foi menor nas novilhas gestantes, independentemente do tratamento com BE. A abundância das principais enzimas envolvidas na liberação de $PGF_{2\alpha}$ não foi afetada pelo tratamento com BE. O *status* gestacional não teve influência na expressão gênica de IL1-β, mas, as novilhas tratadas com 1 mg de BE tiveram uma menor expressão em comparação com as novilhas do grupo controle. Em conclusão, o tratamento com 1 ou 2 mg BE, 13 dias após a ovulação em novilhas de corte induz a secreção de $PGF_{2\alpha}$ e antecipa a luteólise em um dia nas novilhas não gestantes.

Palavras-chave: luteólise; estrógeno; PGFM; ressincronização

ABSTRACT

MOTTA, I. G. **Impacts of estradiol on PGF2α release and corpus luteum function during early pregnant in beef heifers.** 81p. Tese (Doutorado em Ciências e Ciências Veterinárias) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2023.

In the first study, the effects of 17β-estradiol (E2) or estradiol benzoate (EB) on $PGF_{2\alpha}$ release were studied in bred-non-pregnant and pregnant beef heifers. Thirty-two Nelore heifers, aged between 16 and 18 months, weighing 314 ± 31 kg, and body condition score (BCS) between 3 and 4 were used. The females received an intravaginal P4 device (1g Sincrogest®, Ourofino Saúde Animal) 14 days after timed artificial insemination (TAI) and were randomly assigned in three groups: Control (C, P4 device only); E2 (1 mg E2 + 9 mg P4; Betaproginn®, Boehringer Ingelheim); or EB (1 mg; Sincrodiol®, Ourofino). Blood samples were collected hourly for 8 hours after treatment to measure plasma concentrations of a $PGF_{2\alpha}$ metabolite (PGFM). The P4 device was removed on D22 and pregnancy was diagnosed on D28. The proportion of pregnant heifers did not differ between the control (7/12), E2 (5/10) and EB (5/10) groups. The average PGFM concentration during the 9 hours was greater in E2 group (55.3 ± 10.2) than EB (37.2 ± 4.7) and the control group (25.1 ± 1.1). In addition, luteolysis occurred earlier in the E2 group than in the EB group. Also, in pregnant heifers, 1 mg EB 14 days after TAI did not induce $PGF_{2\alpha}$ release, and only the number of prominent PGFM pulses and maximum concentration were greater than controls. In study 2, Experiment 1, was evaluated the effects of different doses of EB at 13 days after ovulation on the release of $PGF_{2\alpha}$ and the size and function of the corpus luteum in pregnant and non-pregnant heifers. Forty-seven Nelore heifers, aged between 18 and 24 months, weighing 390 ± 35 kg, and body condition score (BCS) between 3 and 4 were used. Estrus was synchronized and heifers were randomly subdivided into non-inseminated or inseminated. Thirteen days after ovulation (D13), the heifers were randomly assigned to three groups to receive 0, 1 or 2 mg of EB. On D13, blood samples were taken at hour 0 (H0) (before treatment) and hourly from H3 to H12 to measure the PGFM. Doppler ultrasonography was performed daily from D13 to D19. Pregnancy was diagnosed on D28. Regardless of the dose, luteolysis was earlier in non-inseminated heifers treated with EB than in the

control group (16.3 ± 0.2 vs. 17.3 ± 0.6 days). The proportion of pregnant heifers was lower in the groups treated with 1 mg (50%; 8/16) and 2 mg (29.2%; 7/24) when compared to the control group (90%; 9/10). The average concentration of PGFM during the 10 hours of collection was greater in EB groups treated with 1 mg (46.3 ± 3.6 pg/mL) and 2 mg of BE (46.9 ± 3.3 pg/mL) than in the control group (28.0 ± 1.2 pg/mL) regardless of gestational status. In Experiment 2, the effects of 0 and 1 mg of EB, 13 days after ovulation on the components of the endometrial cascade of $PGF_{2\alpha}$ synthesis in non-inseminated and in pregnant beef heifers were evaluated. Forty Nelore heifers, aged between 24 and 30 months, weighing 437 ± 45 kg, and body condition score (BCS) between 3 and 4 were used. Thirteen days after ovulation, the heifers were randomly assigned to two groups: 0 or 1 mg of EB. Three hours after the treatments, endometrial cytology was performed and samples were subsequently evaluated by qPCR. The abundance of OXTR and PGR was affected by EB treatment, being greater in heifers treated with 1 mg of EB. ESR2 abundance was lower in pregnant heifers, regardless of EB treatment. The abundance of the main enzymes involved in $PGF_{2\alpha}$ release was not affected by EB treatment. Pregnancy status had no influence on IL1-β gene expression, but heifers treated with 1 mg EB had a lower expression compared to heifers in the control group. In conclusion, treatment with 1 or 2 mg EB 13 days after ovulation in beef heifers induces $PGF_{2\alpha}$ release and anticipates luteolysis by one day in non-pregnant heifers.

Keywords: luteolysis; estrogen;; PGFM; resynchronization

Sumário

1. GENERAL INTRODUCTION

In 2021, the Brazilian cattle herd reached an effective record of approximately 196.4 million heads (ABIEC, 2022). Brazil stands out in the production of cattle due to the largest commercial herd and for being the leader of meat exporters. In 2022, Brazil produced 10.3 million equivalent tons of carcass, representing 17.3% of world production, which placed the country in second place in the world's meat production (USDA, 2023). The United States, on the other hand, produced 12.8 million equivalent tons of carcass, 21.5% of the 59.3 million tons produced worldwide, and lead the world ranking in meat production (USDA, 2023) with a herd 2.1 times smaller than Brazil (94.5 million head) (ABIEC, 2022).

Reproductive efficiency is essential to increase productivity in beef and dairy herds. A strategy to improve reproductive effectiveness is use of fixed-time artificial insemination (TAI) (BARUSELLI et al., 2019). This tool enables to control of the estrous cycle and ovulation through pharmacological manipulation, that being so, the females are inseminated at pre-determined times, without the need for estrus observation (SA FILHO et al., 2013). Moreover, the TAI is extremely important as it induce cyclicity in anestrous females (BARUSELLI et al., 2004), and provides an increase in reproductive efficiency (BARUSELLI et al., 2017). In addition, it is possible to use semen from genetically superior bulls, accelerating genetic gain and resulting in more productive calves that generate a greater economic return for meat and milk producers (BARUSELLI et al., 2019).

There are basically two major types of protocols for TAI: (1) Ovsynch, based in the administration of gonadotropin-releasing hormone (GnRH), and (2) based on the use of estradiol (E2)-based compounds associated with progesterone (P4) releasing devices (BÓ et al., 2018). Regardless of hormonal combinations, the protocols aim to: (1) synchronize the emergence of a new follicular wave, through ovulation of the dominant follicle after treatment with GnRH or through inhibition of gonadotropins after treatment with E2 compounds associated with P4 (CONSENTINI et al., 2021); (2) control the luteal phase, maintaining high circulating concentrations of P4 during the development of the new preovulatory follicular wave to prevent ovulation and consequently reduce P4 concentrations at the end of the protocol for ovulation to occur (MADUREIRA et al., 2020). Finally, it aims to promote the ovulation of a follicle with

appropriate size and age synchronously (3) through the application of GnRH or E2 esters (CONSENTINI et al., 2021).

The preference for one of these protocols by professionals depends on several factors but is limited to the availability and permission of the use of hormones in a given country (BÓ et al., 2016). Protocols based on E2/P4 are commonly used in South American herds. In these places, there is the availability of E2 ester products, and they are efficient in both heifers and cows in postpartum anestrus (BÓ et al., 2018). While GnRH-based protocols tend to be more used in North America, Europe, and New Zealand, where E2 use is forbidden in cattle (BÓ et al., 2016).

To achieve better genetic and production gains, reproductive strategies should focus on concentrating results at the beginning of the breeding season (BARUSELLI et al., 2017). Therefore, it is necessary to improve service rates at the beginning of the breeding season and to identify non-pregnant females prematurely for a second chance of conception at an early stage as well. (BÓ et al., 2016; BARUSELLI et al., 2017; BARUSELLI et al., 2019). For this goal, one interesting reproductive strategy is the resynchronization of ovulation. Such biotechnique enables the increase of calves born from artificial insemination; eliminates the need for estrus observation, enables a decrease in the number of bulls on the farm, enables a new chance of conception in females returning to anestrus and increases the pregnancy rate at the end of the season (STEVENSON et a., 2003; BÓ et al., 2016; CREPALDI et al., 2017).

The resynchronization programs can start only in non-pregnant females after a pregnancy diagnosis, or in all females without knowing the pregnancy status (MARQUES et al., 2015; SA FILHO et al., 2014; MOTTA et al., 2020).

One recent alternative is the association of an early resynchronization with the use of color-Doppler ultrasonography to diagnose non-pregnant females with accuracy of 91% in beef cows from 20 to 22 days after insemination (PUGLIESI et al., 2014; DALMASO DE MELO et al., 2020). This approach enabled the development of a new resynchronization strategy, in which the protocol starts between 12 to 14 days after TAI in beef cattle (PUGLIESI et al., 2019; JUNIOR et al., 2021). However, this period precedes the maternal recognition of pregnancy in cattle (BINELLI et al., 2001) and treatments with E2 or its esters at this period are controversial because of the E2 role in $PGF_{2\alpha}$ secretion by the uterus (ARAUJO et al., 2009; PUGLIESI et al., 2011). In non-inseminated heifers, E2 administration 12 to 14 days after ovulation caused an increase in pulsatile secretion of $PGF_{2\alpha}$, which is characterized by an increase in the

concentrations of $PGF_{2\alpha}$ metabolite, 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) between 6 to 7 hours after E2 administration (ARAUJO et al., 2009; GINTHER et al., 2010a; PUGLIESI et al., 2011). In inseminated animals, administration of 1.5 or 2 mg estradiol benzoate (EB) 13 or 14 days after insemination, respectively, in dairy and beef cows, induced luteolysis and impacted the establishment of pregnancy (VIEIRA et al., 2014; SILVA et al., 2022). On the other hand, the administration of 1 mg E2 or 1 mg EB on day 14 after TAI in beef heifers did not compromise the pre-existing pregnancy (MOTTA et al., 2020). Therefore, the threshold between toxicity and effectiveness is very close and a better understanding is needed.

A series of coordinated molecular events are required, to lead the luteolytic cascade induced by the pulsatile release of $PGF_{2\alpha}$. In fact, the pulsatile synthesis and release of $PGF_{2\alpha}$ to the circulation, ultimately occurs, after upregulation of oxytocin receptors (OXTR) (SPENCER and BAZER, 1995; FLEMING et al., 2006). Recent studies have been performed to understand the response of an exogenous administration of E2 in the expression of E2 receptors alpha (ERα) and beta (ERβ) and OXTR in the endometrium of pregnant and non-pregnant cows. Results from Oliveira, et al. (2022), using non-inseminated cows indicate that animals that received an intravenous infusion of E2 on day 15, after estrus detection, showed an upregulation of OXTR in the endometrium four hours after treatment when compared with cows that received a placebo treatment. Interestingly, in the same study, no differences in gene expression of OXTR were observed between the groups, seven hours after treatment. Nonetheless, the effect of E2 injection on the gene expression of ESR1, ESR2, and OXTR in the endometrium of pregnant cows is still unclear.

Considering the controversial results of E2 treatment during early pregnancy, it is necessary to better understand the effects of different E2 doses on $PGF_{2\alpha}$ secretion, as well as evaluate them in inseminated females. Therefore, in the present thesis, we aimed to evaluate for the first time the effects of E2 doses on PGFM concentrations, corpus luteum fuction, uterine gene expression, and pregnancy maintenance in inseminated Nelore heifers. The results obtained in these studies will be reported as two manuscripts and presented in two separate chapters. The first chapter refers to the first study that was already published in the journal Domestic Animal Endocrinology in 2021 (MOTTA et al., 2021). The second chapter refers to the second study and contemplates two experiments. In the first experiment, the heifers were divided into inseminated and non-inseminated and 13 days after ovulation they were divided into

three treatments: 0, 1 and 2mg of EB, to assess CL characteristics, P4 and E2 concentration and $PGF_{2\alpha}$ release. In the second experiment, the heifers were divided in the same way, but 13 days after ovulation they were divided into two treatments: 0 and 1 mg of EB, to evaluate the components of the endometrial cascade of PGF synthesis.

1.1 OBJECTIVES

The aim of the first study was to evaluate the effects of the administration of 1 mg E2 or 1 mg EB on PGFM concentrations in beef heifers subjected to resynchronization 14 days after TAI. In the second study, the aim was: (1) to evaluate the PGFM concentrations after administration of 1 *vs*. 2 mg of EB, 13 days after ovulation in pregnant and non-inseminated beef heifers; (2) to assess the effects of applying 1 mg *vs.* 2 mg EB in corpus luteum (CL) blood perfusion and size, and circulating P4 concentrations in pregnant and non-inseminated beef heifers; (3) to identified in the endometrial cells collected by Cytobrush from the body of the uterus the expression of transcripts for ESR1, ESR2, OXTR, P4 receptor (PGR), and the enzymes related to PGF2α synthesis, , induced by EB administration 13 days after ovulation in pregnant and non-inseminated beef heifers.

2. LITERATURE REVIEW

2.1 PHYSIOLOGICAL BASES OF ESTROUS CYCLE AND EARLY PREGNANCY IN CATTLE

2.1.1 Estrous Cycle

Bovine females are polyestrous animals, which means, that photoperiod had no influence on the estrous cycle occurrence in cows, in contrast to horses, ewes, or buffalo females, classified as seasonal species. The estrous cycle is referred to as, the interval between the two following estrus phases, induced by cyclical reproductive events mediated by the ovarian activity, characterized by alternating periods of non-receptivity to receptivity (estrus), ultimately enabling mating, used by female animals, to achieve greater chances to the establishment of pregnancy (FORDE et al., 2011). Bovine follicular development occurs in a pattern of waves of follicle development, reaching 2 to 4 waves, each estrous cycle, according to breed or category. Each follicular wave is divided into four phases: emergence; deviation; dominance; ovulation or atresia of the dominant follicle (VIANA et al., 2000; WOLFENSON et al., 2004).

The neuroendocrine control of the estrous cycle is regulated, through a coordinated system of positive and negative feedback, by the following hormones, gonadotrophin-releasing hormone (GnRH), produced by the hypothalamus, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), from the anterior pituitary, and hormones produced by ovaries, P4, E2 and inhibin, and from the uterus, prostaglandin F-$_{2\alpha}$ ($PGF_{2\alpha}$) (ROCHE, 1996). Initiated by the release of GnRH, a decapeptide produced by the hypothalamus, and regulates the secretion of FSH and LH, gonadotrophins, produced by the anterior pituitary (BABA et al., 1971; SCHALLY et al., 1971). In mammals, the onset of estrous cycles starts after puberty; however, in heifers, it usually occurs around 6 to 12 months of age, even more, Nelore heifers, reach puberty later than *Bos taurus* heifers (BÓ et al., 2007). Generally, cattle present regular intervals between estrus behavior, approximately every 21 days, ranging from 17 to 24 days, according to the breed, age, and other physiological factors (FORDE et al., 2011).

The bovine estrous cycle consists of two distinct phases, the Follicular phase, which, persists during 4 – 6 days of the estrous cycle, is characterized by the absence

of a functional CL in the ovary, being that ovarian follicles are the main structure of the ovary, then, at this stage of the cycle the female is under the stimulation of E2, also referred as the estrogenic phase. On the other side, the Luteal phase, also called as progesteronic phase, length from 14 to 18 days, and is characterized by the CL formation and establishment, and leads, the female under the stimulation of P4 (NEBEL et al., 2004).

Nonetheless, the follicular phase is divided into two different stages, proestrus, and estrus, the proestrus is initiated by the CL regression and ovarian follicle growth, consequently, a decrease of P4, and an increase of E2 production, produced by the granulosa cells, in response of rapid proliferation of the pre-ovulatory dominant follicle (DF), extends from 2 to 4 days (FERREIRA, 2010; FORDE et al, 2011). The estrus stage is characterized by the presence of a preovulatory follicle in the ovary, leading to the peak of E2, concurrent with a lower P4 concentration, promotes a GnRH surge, inducing a surge in FSH and LH, then, allows to display of estrus behavior, which heifers/cows are sexually receptive and will stand to be mounted (FRANDSON et al., 2003; BARUSELLI at al., 2007). Length from 10 to 18 hours, however, is usually shorter in *Bos indicus* females when compared to *Bos taurus* beef cattle (12.9 ± 2.9 hours vs. 16.3 ± 4.8 hours, for Nelore and Angus, respectively) (MIZUTA, 2003). Insights from Wiltbank et al., (2006), related, a negative correlation between milk production and length of estrus, due to the greater hepatic metabolization of steroid hormones (E2 and P4), so, high-producing dairy cows, had a shorter estrus stage.

Whereas metaestrus and diestrus, are present in the luteal phase. Metestrus starts after estrus and lasts 3 to 5 days. The main event during this stage, is the ovulation, about 10 to 15 hours after the end of estrus, in response to LH surge, and the CL undergoing to development, then, an increase in P4 concentrations is observed (SENGER, 2003).

In mammals, CL is a temporary endocrine gland that produces P4 for the establishment and maintenance of pregnancy (SCHAMS and BERISHA, 2004). CL development occurs rapidly within 2-3 days after ovulation, derived by active angiogenesis and vascularization. Hormonal, vascular, and immune events trigger the development of CL (NISWENDER ET AL., 2000; PATE and LANDIS KEYES, 2001). Production of basic fibroblast growth factor (FGF2) and vascular endothelial growth factor (VEGF) A, in the bovine CL, induce neovascularization and proliferation of granulosa cells and endothelial cells, these factors are usually related to cell growth,

differentiation, transformation, and angiogenesis, thus, blood vessels and endothelial cells within the CL have crucial roles in luteal function (GOSPODAROWICZ et al. 1985; ROBINSON et al. 2008).

Finally, diestrus is the period when the CL is completely formed and producing P4, produced mainly by large luteal cells, classified as steroidogenic cells, produce and secrete a large amount of P4 during CL presence, playing a crucial role in pregnancy establishment and maintenance (GIRSH et al., 1995; MIYAMOTO and SHIRASUNA 2009). If pregnancy does not occur, the CL regresses through the release of $PGF_{2\alpha}$, ending the diestrus phase (FORDE et al., 2011). Therefore, luteolysis in cattle is essential for the initiation of a new estrous cycle, and it is a process that involves the functional and structural regression of CL, induced by the endometrial $PGF_{2\alpha}$, the main luteolytic agent (KINDAHL et al., 1976; MCCRACKEN et al., 1984; GINTHER at al., 1989).

2.1.2 Luteolysis and its blockage by conceptus signaling

The steroid hormones contribute to the release of endometrial $PGF_{2\alpha}$ (GINTHER et al., 2010b). Therefore, it has been established that direct communication between the uterus and the ovary is necessary for uterine $PGF_{2\alpha}$ to reach the CL and promote luteolysis. It was speculated that this communication occurred through vascular contact between the uterine vein and the ovarian artery (GINTHER, 1976). The mechanism behind the pulsatile release of $PGF_{2\alpha}$ is regulated by orderly events. Primally P4-induced downregulation of P4 receptors, thus, circulating E2, stimulates expression of ERα and ERβ receptors in the endometrium, followed by upregulation of transcription of OXTR, simulating the activity of phospholipase C, Protein Kinase C (PKC) and phospholipase A2 (PLA2) (SPENCER and BAZZER, 1995; FLEMING et al., 2006; TITHOF et al., 2007). First, diacylglycerol or phospholipid are converted into arachidonic acid (AA) by PLA2, being converted into prostaglandin H_2 (PGH_2) by cyclooxygenase 2 (PTGS2) (ASSELIN et al., 1997). Finally, PGH_2 was converted into $PGF_{2\alpha}$, by aldo-keto reductase family 1 member B1 (AKR1B1) and AKR1C4 (FORTIER et al., 2008).

However, some species have a very well-elucidated mechanism to interrupt the luteolytic cascade for successful pregnancy. In ruminants, the maternal recognition of pregnancy is regulated by interferon-tau (IFN-t), the main molecule responsible for

preventing luteolysis (SPENCER et al., 2007; ANTONIAZZI et al., 2013). The IFN-t is a cytokine produced by the trophectoderm of the embryo, initiated as early as the seventh day of gestation (SPONCHIADO et al., 2017; RASHID et al., 2018). Secretion begins at the early blastocyst stage, and increases during embryo elongation, from 13 to 15 days of pregnancy, and achieves a peak around the 20th day of pregnancy (FORDE and LONERGAN, 2017). This cytokine acts by preventing the pulsatile release of $PGF_{2\alpha}$, through the inhibition of OXTR and ERα and Erβ in the endometrium, which is essential to induce $PGF_{2\alpha}$ pulse (SPENCER et al., 1995; SAĞSÖZ et al., 2011).

The IFN-t can stimulate the gene expression of some targets, in peripheral blood immune cells, and other tissues, also referred to as, interferon-stimulated genes (ISGs), where, gene expression of ISGs, has been widely used, as a method for early diagnosis of pregnancy in cattle (PUGLIESI et al., 2014; YOSHINO et al., 2017; DALMASO DE MELO et al., 2020). In an oppositive effect of $PGF_{2\alpha}$, prostaglandin E_2 (PGE_2) is a metabolite of PGH_2, produced in endometrial bovine cells, by the prostaglandin E synthase (PTGES), which exerts a crucial role in bovine CL maintenance, acting as a luteotrophic or antiluteolytic agent. During pregnancy, when PGE_2 is secreted by the conceptus, induces an immunosuppressive response to protect the semi-allogenic conceptus, from maternal immune system rejection (LOW and HANSEN 1988; PRATT et al., 1995).

Beltman et al. (2010) and Binelli et al. (2015) observed that cows with a viable embryo at 6-7 days of pregnancy have an upregulation of *PGTES* in the uterus. Interestingly, there is evidence that IFN-t can regulate the ratio of PGE_2:$PGF_{2\alpha}$ production, where, endometrial cells treated with 20 ug/ml of IFN-t, had 27-fold stimulation of PGE_2, compared to the control group (PARENT et al., 2002). Those results suggest that IFN-t and PGE_2 work together to reach pregnancy establishment.

The effect of E2 in the luteolytic cascade can be evidenced by determining the circulating concentrations of the $PGF_{2\alpha}$ metabolite, 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) (KNICKERBOCKER et al., 1986; GINTHER et al., 2010a, table 1). After the action of $PGF_{2\alpha}$, it is metabolized into PGFM, which can be detected in peripheral blood. In addition, E2 stimulates the expression of mRNA for OXTR in endometrial cells, which binds the oxytocin molecules and enables the activation of $PGF_{2\alpha}$ biosynthesis (SIVAL et al., 1991; MANN et al., 2001;2006). Insights from Pugliesi et al. (2011) indicate that there is an increase in the prominence of PGFM pulses due to

increased doses of E2, suggesting that E2 has a crucial role in the production of $PGF_{2\alpha}$, in accordance with several reports that E2 is directly involved in $PGF_{2\alpha}$ release (SILVA et al., 1991; DIAZ et al., 2002). Indeed, Araujo et al (2009) showed a delay in the luteal regression following a subsequent reduction in circulating E2 concentrations after follicular ablation. Both, intravenous and intramuscular administration of E2 stimulated the release of $PGF_{2\alpha}$ in cattle, inducing the luteolytic cascade, which was evidenced by the increase in PGFM. Thus, PGFM concentration increases about 6-7 hours, in non-inseminated heifers, following administration of 1 mg of E2 or EB, from day 12 to 14 after ovulation (SOUZA et al., 2005; ARAUJO et al., 2009; GINTHER et al., 2010a; PUGLIESI et al., 2011). Interestingly, distinct moments in relation to the increase in plasma concentration of PGFM, after the application of E2 or EB, (4-6 *vs* 5-6, respectively) are reported (MOTTA et al., 2021). This difference could be related to the esterification of EB that reduces the amount of E2, where EB is converted to about 72% of E2, inducing a different circulating E2 profile among the treatments.

Previous studies have also shown the effects of E2 on the characteristics of PGFM pulses (Table 1). There are reports indicating that animals treated with E2 had a greater number of prominent pulses (peak of >100 pg/mL) compared to the control group, according to the type of E2 used (E2 vs EB), regardless of gestational status (MOTTA et al, 2021). Moreover, treatment with E2 generated a greater concentration of PGFM in the peak, possessing a dose-dependent effect, being greater in animals treated with E2 than EB (GINTHER et al., 2010a; PUGLIESI et al., 2011; MOTTA et al, 2021), however, these effects were not observed in pregnant heifers (MOTTA et al., 2021). Treatment with E2 (0.05 and 0.1 mg) on day 16 after ovulation resulted in a lower prominence of the PGFM pulse when compared to treatment on day 15 after ovulation (PUGLIESI et a., 2012).

Oliveira et al., (2022), in a study conducted with *Bos indicus* beef cows, showed that cows that received an intravenous injection, on day 15 after estrus, containing 3 mg of E2, diluted in 6 mL of 50% ethanol, had a greater plasma PGFM concentration, earlier luteal regression, and an upregulation of OXTR in the endometrium, four hours after treatment, compared those of were submitted to placebo treatment.

Table 1. Previous studies that evaluated characteristics of PGFM pulses (Mean ± SEM) during diestrus, showing the experimental unit, reproductive status, time of treatment application and the corresponding treatments used in each experiment.

Reference	Experimental unit	Reproductive Status	Momento of injection	Treatments	No. of PGFM pulses[x]	No. of prominent PGFM pulses[x]	Plasma PGFM pulse concentration at peak (pg/mL)	Area under pulse curve (pg/mL x h)
GINTHER et al., 2010a	Holstein heifers	Cycling	day 14 after ovulation	Vehicle	1.0 ± 0.3	not evaluated	162.7 ± 32.5^{b}	63.4 ± 9.9^{b}
				1 mg E2	1.5 ± 0.2	not evaluated	307 ± 43.5^{a} *	132.0 ± 16.2^{a} **
PUGLIESI et al., 2011	Holstein heifers	Cycling	day 14 after ovulation	Vehicle	not evaluated	not evaluated	53.1 ±6.8^{b}	83.5 ± 3.7^{b}
				0.01 mg E2	not evaluated	not evaluated	44.5 ±15.0^{b}	56.9 ± 26.4^{b}
				0.05 mg E2	not evaluated	not evaluated	124.0 ± 34.8^{b}	286.9 ± 105.5^{ab}
				0.1 mg E2	not evaluated	not evaluated	231.8 ± 43.5^{a} **	635.9 ± 165.0^{a} **
PUGLIESI et al., 2012	Holstein heifers	Cycling	day 15 after ovulation	Vehicle	not evaluated	not evaluated	106.8 ± 44.1^{b}	165.3 ± 87.2^{b}
				FAV	not evaluated	not evaluated	63.1 ± 2.90^{b}	85.2 ± 15.3^{b}
				FAE -0.05 mg E2	not evaluated	not evaluated	274.0 ± 37.4^{ac}	655.7 ± 117.3^{ac}
				FAE -0.1 mg E2	not evaluated	not evaluated	274.0 ± 37.4^{ac} *	692.0 ± 133.5^{ac} *
			day 16 after ovulation	Vehicle	not evaluated	not evaluated	128.7 ± 60.3	226.8 ± 118.1
				FAV	not evaluated	not evaluated	89.4 ± 35.0	129.2 ± 68.1
				FAE -0.05 mg E2	not evaluated	not evaluated	174.2 ± 35.6^{d}	281.4 ± 50.0^{d}
				FAE -0.1 mg E2	not evaluated	not evaluated	95.1 ± 44.4^{d} *	105.3 ± 38.8^{d} *
MOTTA et al., 2021	Nelore heifers	Non-pregnant	day 14 after TAI	Control	1.2 ± 0.2	0.0 ± 0.0^{b}	26.1 ± 3.0 ^{b}	22.7 ± 2.3^{b}
				1 mg E2 + 9 mg P4	1.2 ± 0.2	0.8 ± 0.4^{a}	123.7 ± 22.6^{a}	150.8 ± 47.5^{a}
				1 mg EB	1.2 ± 0.4	0.2 ± 0.2 ^{ab} #	87.6 ± 43.3^{ab} *	114.5 ± 70.2^{ab} *
	Nelore heifers	Pregnant	day 14 after TAI	Control	0.7 ± 0.3	0.0 ± 0.0 ^{b}	38.0 ± 6.9	39.1 ± 5.3
				1 mg E2 + 9 mg P4	1.2 ± 0.2	0.6 ± 0.2 ^{a}	113.0 ± 35.8	173.1 ± 59.9
				1 mg EB	0.4 ± 0.2	0.0 ± 0.0 ^{b} **	39.6 ± 3.4	41.7 ± 8.1

[ab] Means within a column and within the same work with different superscripts are different ($P < 0.05$).
[cd] Means within a column and end point with different superscripts are different between days ($P < 0.05$).
[x] Prominent PGFM pulse (peak of >100 pg/mL).
#: indicates $0.05 < p \geq 0.10$
*: indicates $p \leq 0.05$
**: indicates $p \leq 0.01$
***: indicates $p \leq 0.001$
FA = follicle ablation; V = vehicle; E = estradiol

It is well known that E2 presents a dose-dependent effect in inducing luteolysis; however, it is still unclear if the exogenous application of E2 can alter ESR and OXTR expression in the endometrium as well as E2-stimulated PGFM release during early

pregnancy in a dose-dependent manner, which could compromise CL function and pregnancy establishment.

2.1.3. Pharmacological control of the estrous cycle in bovine females

Improving reproductive performance is one of the most important goals to increase economic gain in beef and dairy herds. Several strategies have been developed to improve reproductive efficiency, such as the use of fixed-time artificial insemination (TAI) (BARUSELLI et al., 2019). In contrast to artificial insemination after estrus observation, TAI protocols minimize or eliminate estrus observation, where, cows are inseminated at a pre-determined day, due to, control of the estrous cycle and ovulation, using hormonal administration, which promotes numerous advantages for beef and dairy production (SA FILHO et al., 2013). In South American herds, the use E2 ester products are not forbidden, thus, most TAI protocols are based on E2/P4, and, those protocols had been demonstrated, as a good tool for heifers and cows in postpartum anestrus (BÓ et al., 2016; 2018). Moreover, resynchronization raises the chances of the cows becoming pregnant after the pregnancy check. Usually, conventional resynchronization occurs after a confirmatory pregnancy check, which occurs mainly about 30 days after insemination, using B-mode ultrasonography, reaching an interval between insemination, around 40 days, being later than 19 days after the expected return of estrus, which generally happens 20 to 21 days after breed (MARQUES at al.,2015).

A variety of methods to predict early gestation have been widely studied among researchers. Among them, the use of color Doppler ultrasonography, which is a real-time and non-invasive method of early pregnancy predictor, based on the subjective evaluation of luteal blood perfusion, and can reach an accuracy higher than 90% in beef cattle (PUGLIESI et al., 2014; MELO et al., 2020). The applied use of color Doppler ultrasonography provided a super early resynchronization, initiated at 12 to 14 days after the first TAI (PENTEADO et al 2016; PUGLIESI et al., 2019), even though the pregnancy status is unknown, reducing the interval among first and second insemination in 18 days (from 40 to 22 days) (BARUSSELI et al., 2017; PUGLIESI et al., 2019). However, the timing of the initiation of super early resynchronization coincides with the moment of maternal recognition of gestation, assigned as a critical

period for successful pregnancy (BINELLI, et al., 2001). Due to fact, a treatment with EB, which is widely used in the hormonal protocols in South America, could induce an embryonic loss, since E2 is involved in the synthesis of $PGF_{2\alpha}$ (THACHER et al., 1986; ARAUJO et al., 2009). Therefore, an exogenous injection of E2 can stimulate luteolysis in cattle, in a dose-dependent manner (PUGLIESI et al., 2011).

Several researchers are interested in studying, different strategies of super early resynchronization, using different associations of hormones, doses, and time of initiation, in order to not harm the pregnancy. In a recent study, performed by our group, using beef heifers, was observed that treatment with 1 mg EB or 1 mg E2 +9 mg P4 at 14 days post-TAI, did not affect the pregnancy rate (MOTTA et al., 2020). The administration of 1.5 mg of E2 and 50 mg of P4, 13 days after TAI in beef heifers, did not impair the pregnancy rate (COLAZO et al., 2006). Palhão et al. (2020) and Silva et al. (2022) have shown that administration of 1 mg EB associated with a P4-device in primiparous and multiparous beef cows 13 and 14 days after TAI had no impact on the pregnancy rate at the first TAI. In agreement with those results, administration of 1 mg EB, 13 days after first TAI, had no effect on pregnancy in beef (Stevenson et al., 2003) and dairy cows (EL-ZARKOUNY et al., 2004). In contrast, previous results have reported, an expressive reduction in the P/AI at first service of 34.5% (19/55) to 5.4% (3/55), in non-lactating Nelore beef cows, treated with 5 mg of E2, at day 12 after insemination (MACHADO et al., 2008). Also, reports from Cutaia et al. (2002) and Vieira et al. (2014), demonstrated that, doses of 1 or 1.5 mg EB, administrated at day 13 after insemination, promotes luteolysis, inducing pregnancy loss, in beef heifers and lactating dairy cows, respectively.

Therefore, it is essential to thoroughly assess whether the application of E2 is promoting the release of $PGF_{2\alpha}$ in pregnant bovine females.

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3. CHAPTER 1 - EFFECTS OF ESTRADIOL TREATMENTS ON $PGF_{2\alpha}$ RELEASE IN BEEF HEIFERS SUBMITTED TO ESTROUS RESYNCHRONIZATION 14 DAYS AFTER TIMED-AI

I.G. Motta[a], C.C. Rocha[ab] D.Z. Bisinotto[a], G.D. Melo[c], G.A.A. Júnior[a], T. K. Nishimura[a], A.M.G. Diaza[b], T. Castro[d], O. J. Ginther[d], G. Pugliesi[a]

[a] Department of Animal Reproduction, School of Veterinary Medicine and Animal Science, University of São Paulo, Pirassununga, São Paulo, Brazil

[b] North Florida Research and Education Center, University of Florida, FL, USA

[c] Department of Animal Science - Texas A&M University of Texas, TX, USA

[d] Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, MDN, Wisconsin

Adapted from: I.G.Motta, C.C.Rocha, D.Z.Bisinotto, G.D.Melo, G.A.A.Júnior, T.K.Nishimura, A.M.G.Diaza, T.Castro, O.J.Ginther, G.Pugliesi. Effects of estradiol treatments on $PGF_{2\alpha}$ release in beef heifers submitted to estrous resynchronization 14 days after timed-AI. Domestic Animal Endocrinology. 2021; 106625.

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3.1. ABSTRACT

The effects of 17β-estradiol (E2) or estradiol benzoate (EB) on $PGF_{2\alpha}$ release were studied in bred-non-pregnant and pregnant Nelore beef heifers. The day of timed artificial insemination (TAI) was designated day 0 (D0), and a single treatment was given on D14. All heifers also received an intravaginal P4 device on D14, and were randomly assigned to three groups: Control (C, P4 device only, n = 12); E2 (1 mg E2 + 9 mg P4, n = 10); or EB (1 mg, n = 10). Blood samples were collected hourly for 8 hours after treatment (Hours 0–8) to measure plasma concentrations (pg/mL) of a $PGF_{2\alpha}$ metabolite (PGFM). The P4 device was removed on D22 and pregnancy was diagnosed on D28. Pregnancy rate was not different among groups (C, n = 7/12; E2, n = 5/10; EB, n = 5/10). More ($P < 0.05$) heifers had a CV-identified prominent PGFM pulse (peak of > 100 pg/mL) in E2 group (6/10) than in EB (1/10) and C (0/12) groups. Hourly concentration of PGFM for Hours 0 to 8 showed significant effects of group and hour and an interaction of group by hour but did not show an interaction of group or hour with pregnancy status. In preliminary post-hoc analyses, PGFM concentrations during Hours 0 to 8 and pulse characteristics were analyzed within each pregnancy status. For the non-pregnant heifers, a group-by-hour interaction was detected tentatively indicating an increase ($P < 0.005$) in PGFM concentrations in E2 group from Hours 4 to 6 and in EB group at Hours 5 and 6. Maximum PGFM concentration during Hours 0 to 8 did not differ ($P > 0.1$) between E2 (124 ± 23) and EB (110 ± 30) groups, but was greater ($P < 0.05$) in each group than in C (32 ± 3). Furthermore, PGFM concentrations of pulses at the peak, amplitude, and area under pulse curve (pg/mL/h) were greater ($P < 0.05$) in E2 group than in C group whereas the EB group did not differ ($P > 0.1$) from the other groups. For pregnant heifers, no effects of group, hour, or their interaction were detected in PGFM concentrations during the hourly sessions, except that maximum PGFM concentration was greater ($P < 0.05$) in E2 than in EB and C groups. In addition, the number of prominent pulses was greater in E2 group than in Control or EB groups. In conclusion, PGFM increased earlier and in greater concentration combined for bred-non-pregnant and pregnant heifers treated 14 days after TAI with 1 mg E2 plus 9 mg P4 than with 1 mg EB. Tentatively, a positive effect for each of E2 and EB on PGFM concentrations was attenuated in pregnant heifers.

3.2 INTRODUCTION

Timed artificial insemination (TAI) commonly uses a program to synchronize ovulation in heifers and cows [1]. Resynchronization provides an opportunity for a second round of insemination and may increase the reproductive efficiency of beef and dairy cattle [1]. With the recent advent of methods for prediction of pregnancy status at 20 to 22 days after TAI using color Doppler ultrasonography [2,3], a novel strategy for resynchronization of ovulation starting 12 to 14 days after the first TAI was developed [4,5].

An intravaginal P4 device and E2 or EB are commonly used for recruitment of a new follicle wave in TAI programs [1]; however, it is not known if the administration of E2 or EB as early as 14 after TAI would cause pregnancy loss as E2 is involved in pulsatile $PGF_{2\alpha}$ release from the uterus [6,7]. In non-inseminated heifers the effects of estrogens on $PGF_{2\alpha}$ release are indicated by increased concentrations of the $PGF_{2\alpha}$ metabolite, 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) between 6 to 7 hours after treatments with 1 mg E2 or EB on days 12 to 14 post-ovulation [6,7,8,9]. The greater esterification in the EB molecule results in a reduced peak and longer circulating E2 profile compared to E2 treatment [10]. Thus, EB may also induce the $PGF_{2\alpha}$ release differently from E2. A harmful effect on pregnancy establishment was not detected when a 1 mg E2 or EB was administered to heifers 14 days after TAI [5], but the effects of E2 or EB on $PGF_{2\alpha}$ release and CL function of pregnant heifers is not known.

Therefore, we studied the effects of 1 mg E2 combined with 9 mg P4 or 1 mg EB on PGFM concentrations in beef heifers submitted to a resynchronization 14 days after TAI.

3.3 MATERIAL AND METHODS

The experiment was approved by the ethics committee of the School of Veterinary Medicine and Animal Science of the University of São Paulo and was conducted at the campus of Pirassununga – SP of University of São Paulo.

3.3.1 Heifer and management

Thirty-two Nelore (Bos taurus indicus) cycling heifers, aged between 16 and 18 months, weighing 314 ± 31 kg, and body condition score of 3 or 4 (BCS; on a scale of 1 to 5; 1 [emaciated] and 5 [obese]) were used [11]. Heifers were maintained on a *Brachiaria decumbens* pasture with ad libitum access to water and a mineral supplement during the experimental period.

3.3.2 Reproductive management, experimental design, and treatments

Heifers had their estrus synchronized and managed as previously described [5]. Briefly, heifers were submitted to an E2/P4 based protocol for TAI (D0). On D14 three experimental groups were used as shown (Fig. 1). All heifers received a new P4 intravaginal device (1 g, Sincrogest, Ourofino Saúde Animal, Brazil). Heifers in the control group (n = 12; C) did not receive further treatment. Heifers of E2 group (n = 10; E2) received 1 mg E2 + 9 mg P4 (0.36 mL, i.m., Betaproginn, Boehringer Ingelheim, Brazil). Heifers in the EB group (n = 10, EB) received 1 mg EB (1 mL; i.m., Sincrodiol, Ourofino Saúde Animal). The addition of 9 mg P4 in the E2 group occurred as there is no other commercial E2 formulae for cattle available in Brazil, but an influence of this additional P4 on $PGF_{2\alpha}$ release is not expected considering the insertion of a P4 device in all groups. On D22, an early detection of non-pregnant heifers was performed based on luteolysis detection by color-Doppler ultrasonography as previously described in beef cows [2,3] and devices were removed, but a second TAI was not performed. Confirmatory pregnancy diagnosis was done on D28 based on detection of an embryo with heartbeat.

On D14, blood samples were collected hourly for 9 hours (Hours 0–8) from the jugular vein for determination of plasma PGFM and P4 concentrations. Samples were harvested using a 10 mL vacuum tube containing heparin (BD Vacutainer, São Paulo, SP, Brazil). The samples were centrifuged at 3600 x g for 15 minutes at 4°C and the plasma was stored at –20°C for subsequent assay.

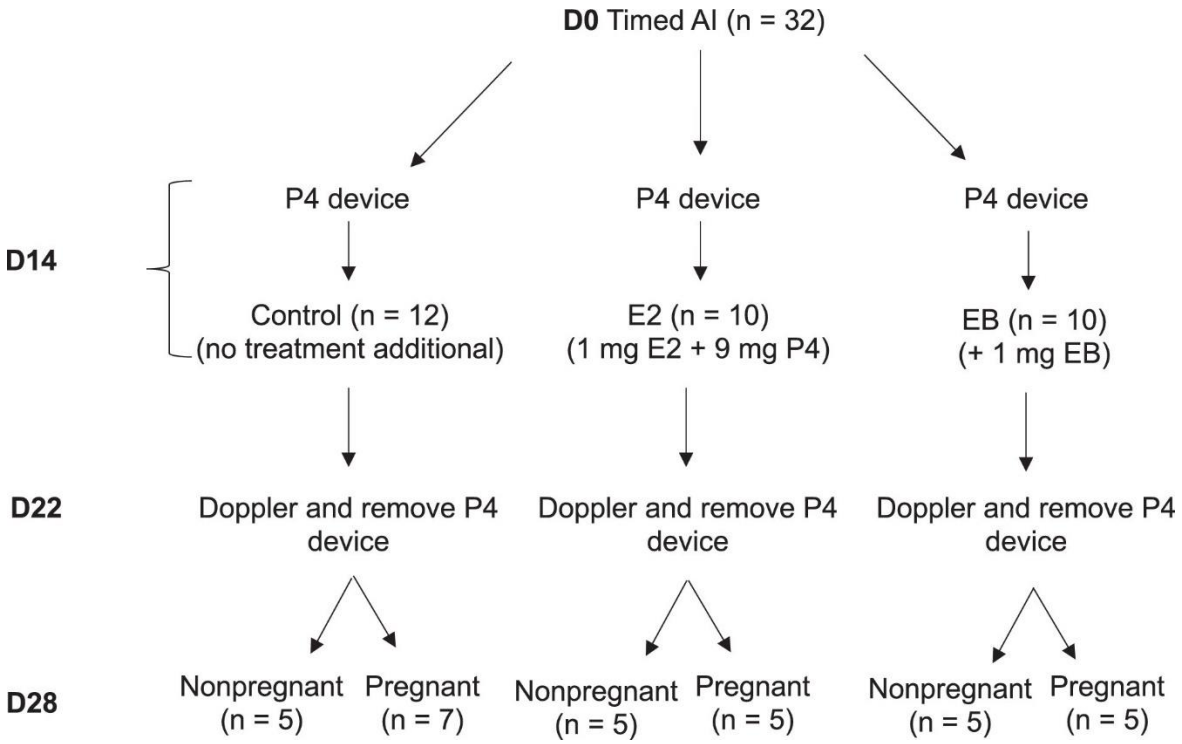

Fig. 1. Experimental protocol. Heifers (n = 32) were synchronized to TAI (D0). On D14 three experimental groups were used. All heifers received a new P4 intravaginal device. Heifers from the control group (n = 12) did not receive further treatment. Heifers in E2 group (n = 10) received or 1 mg E2 + 9 mg P4 and heifers of EB group (n = 10) received 1 mg EB. On D22, diagnosis of pregnancy was performed by color-Doppler ultrasonography and devices were removed. Confirmatory pregnancy diagnosis was done on D28. Pregnancy was confirmed on D28 in the indicated number of heifers.

3.3.3 Hormone assays

The plasma samples were assayed for PGFM by an ELISA that was developed and validated for use with bovine plasma [12]. Plasma P4 concentration was assayed with a solid-phase RIA kit containing antibody-coated tubes and I125-labeled P4 (Coat-A-Count Progesterone; Diagnostic Products Corporation, Los Angeles, CA, USA) as described [12]. The intra- and interassay CVs and sensitivity for PGFM were 5.4%, 6.7% and 1.6 pg/mL, respectively. The intra-assay CV and sensitivity for P4 were 4.2% and 0.02 ng/mL, respectively.

3.3.4 Identification of PGFM pulses

Pulses of PGFM were identified using the coefficient of variance (CV) method as described [9,12]. Pulses with a PGFM peak concentration greater than 100

pg/mL were considered prominent pulses as described [9,12]. The average PGFM concentration for a 9 hours session and the PGFM concentration at the peak of the most prominent pulse during a session were compared among groups. The pulse characteristics were nadir 1, peak, nadir 2, and the amplitude (difference between peak and nadir 1). Area under the pulse curve (AUC) was also considered. The concentration of PGFM from –2 hour to 2 hour (0 hour = pulse peak) of the PGFM pulse with the greatest peak per 9 hours session was compared among the three groups.

3.3.5 Statistical analyses

The data were submitted to two statistical analyses using SAS (version 9.2, SAS Institute Inc., Cary, NC, USA). Firstly, the experiment was analyzed as a 2 × 3 factorial (two pregnancy statuses and three treatment groups). Data were examined for normality with the use of the Shapiro-Wilk test. All the continuous data for PGFM and P4 were analyzed for the main effect of group, hour, pregnancy status, and their interactions. The SAS PROC MIXED procedure was used with a REPEATED statement to account for autocorrelation between sequential measurements. When there was no interaction that involved pregnancy status, a post-hoc analysis was done separately for non-pregnant and pregnant heifers on a preliminary or tentative basis for potential consideration in future studies. The LSD or Duncan test was used when comparisons between hours were made within a group or among more than two means or for discrete data of PGFM pulse characteristics. A Fisher's exact test was used for comparison of the frequency of PGFM pulses among groups. A probability of $P \leq 0.05$ indicated that a difference was significant and $0.05 < P \leq 0.1$ indicated approached significance. Data are presented as the mean ± SEM.

3.4 RESULTS

3.4.1 PGFM concentrations during the 9-hour sessions

The proportion of pregnant heifers did not differ ($P > 0.1$) among the control, E2 and EB groups (Fig. 1). In the combined bred-non-pregnant and pregnant heifers, the mean concentrations of PGFM in 9 hour sessions of hourly sampling showed main effects of treatment group and hour and an interaction of group by hour (Fig. 2a). The PGFM concentrations (pg/mL) over the 9 hour sessions were greater in the E2 group (55.3 ± 10.2) than in the EB (37.2 ± 4.7) and control (25.1 ± 1.1) groups. The interaction reflected an increase in PGFM concentrations between Hours 4 to 6 in the E2 group and between Hours 5 and 6 in the EB group, compared with no increase in the control group. The PGFM concentrations were greater in the E2 group than in the EB group at Hours 5, 6, and 7 and greater than in the control group at Hours 5, 6, 7, and 8. Concentrations were greater in the EB group than in the control group at Hours 7 and 8.

An interaction of treatment group-by-pregnancy status (non-pregnant or pregnant) was not detected for PGFM concentrations during the 9 hour sessions (not shown). When the data were analyzed separately for each pregnancy status on a preliminary or tentative basis, the effects of group, hour, and interaction occurred only in the non-pregnant heifers (Fig. 2b). The interaction reflected an increase in PGFM concentrations between Hours 4 to 6 in the E2 group and between Hours 5 and 6 in the EB group compared with no increases in control group. The group effect represented greater PGFM concentrations in the E2 group (59.4 ± 11.7) than in the EB (45.0 ± 8.6) and control (21.3 ± 1.2) groups. Concentrations were greater in the E2 group than in the EB group at Hour 6 and greater than in the control group at Hours 4 to 8. Concentrations were greater in EB group than in the control group at Hours 7 and 8. For pregnant heifers, no effect on PGFM concentrations during the 9 hour sessions was found (Fig. 2c). Tentatively, the PGFM concentration was greater in the E2 group than in the control group at Hours 1 and 7.

When the PGFM concentrations were compared tentatively between the pregnant and non-pregnant heifers in each treatment group, no effect of pregnancy status occurred on PGFM concentrations for any group.

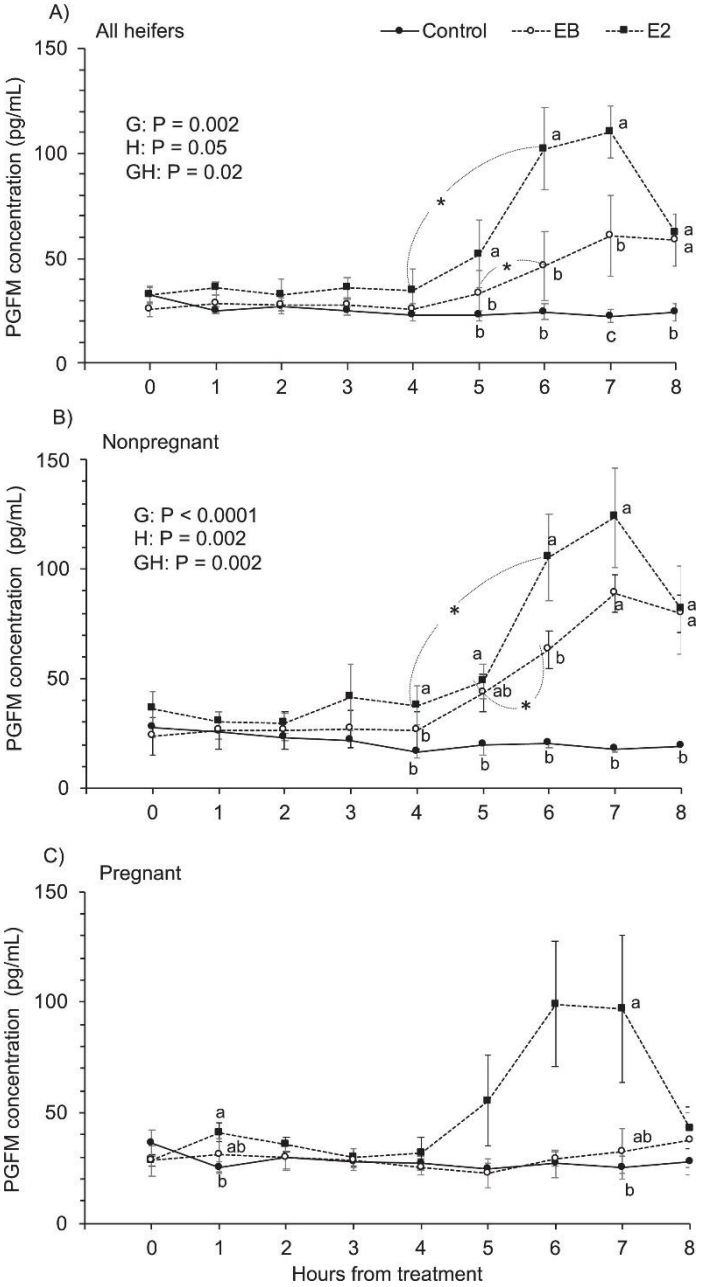

Fig. 2. Plasma PGFM concentration combined for the two pregnancy statuses (a) and in nonpregnant (b) and pregnant (c) Nelore heifers receiving an intravaginal P4 device combined with no additional treatment (control), E2 (1 mg E2 + 9 mg P4) or EB (1 mg estradiol benzoate) on day 14 after TAI. Main effects of treatment group (G), hour (H) and the interaction (GH) that were significant are shown. Different lowercase letters indicate hours of a difference ($P < 0.05$) in PGFM concentration among groups. A dotted line with an asterisk between the days represents an interaction ($P < 0.05$).

3.4.2 Identified PGFM pulses

Regarding the characteristics of PGFM pulses (Table 1), a group effect occurred for 8 of 10 end points. A status effect (pregnant or non-pregnant) or a group by status interaction were not found for any end point. The frequency of heifers with an identified

PGFM pulse was greater in the E2 group than in the EB group, and the control did not differ from either group. The number of all (prominent and non-prominent) PGFM pulses per heifer did not differ among treatment groups. The frequency of heifers with a prominent PGFM pulse (> 100 pg/mL) and the number of prominent PGFM pulses were greater in E2 group than in the EB and control groups. The PGFM concentration at the peak and nadir 2 was greater in the E2 group than in the control group whereas concentration in the EB group was intermediate and did not differ from the other groups. The amplitude was greater in the E2 group than in the EB and control groups. The maximum concentration did not differ between E2 and EB groups, and each was greater than in the control group. The AUC was greater in the E2 group than in the control group and in the EB group was intermediate and not different from the other two groups.

Pregnancy status tended to affect (P = 0.06) number of all PGFM pulses per heifer; number was greater in non-pregnant heifers (1.2 ± 0.14 pulses) than in pregnant heifers (0.76 ± 0.16).

Table 1. Mean ± SEM characteristics of PGFM pulses during hourly sampling combined for non-pregnant and pregnant Nelore heifers that received an intravaginal P4 device and no additional treatment (control), E2 (1 mg E2 + 9 mg P4), or EB (1 mg estradiol benzoate) on day 14 after TAI.

	Control	**E2**	**EB**	**Probability**
No. of heifers	12	10	10	
Heifers with PGFM pulses (%)	75%[ab] (n= 9/12)	100%[a] (n= 10/10)	60%[b] (n= 6/10)	0.01
Heifers with a prominent PGFM pulses (%)[a]	0%[b] (n=0/12)	60%[a] (n= 6/10)	10%[b] (n= 1/10)	0.001
No. of all PGFM pulses	0.9 ± 0.2	1.2 ± 0.1	0.8 ± 0.2	0.34
No. of prominent PGFM pulse[a]	0.0 ± 0.0[b]	0.7 ± 0.2[a]	0.1 ± 0.1[b]	0.001
PGFM pulse concentration (pg/mL)				
Nadir 1[b]	19.0 ± 2.5	27.6 ± 4.1	30.0 ± 3.7	0.11
Peak	31.4 ± 3.8[b]	118.4 ± 20.0[a]	71.6 ± 29.2[ab]	0.01
Nadir 2[b]	16.7 ± 3.1[b]	62.2 ± 12.3[a]	37.0 ± 13.3[ab]	0.008
Amplitude[c]	12.4 ± 2.0[b]	90.8 ± 18.7[a]	41.6 ± 27.5[b]	0.01
Maximum concentration	37.2 ± 3.5[b]	119.5 ± 19.5[a]	79.5 ± 18.0[a]	0.01
Area under pulse curve (pg/mL x h)	29.9 ± 3.8[b]	161.9 ± 36.2[a]	90.2 ± 47.0[ab]	0.02

ab Means within a row without a common superscript are different (P < 0.05).
[a] Prominent PGFM pulse (peak of >100 pg/mL).
[b] Nadir 1 and 2 are the nadirs at the beginning and the end of a PGFM pulse, respectively.
[c] Amplitude is the difference in concentration between Nadir 1 and peak

The group-by-status interaction was not significant for any PGFM pulse characteristic. A post-hoc analyses for non-pregnant heifers alone and pregnant heifers alone on a preliminary basis tentatively indicated some differences between groups for each pregnancy status (Table 2). For non-pregnant heifers, the number of prominent PGFM pulses approached significance (P = 0.09) among treatment groups; the number was greater in the E2 group than in the control group whereas in the EB group, the number was intermediate and did not differ from the other groups. The PGFM concentrations at the peak, nadir 2, amplitude and AUC were greater for heifers in the E2 group than in the control group, whereas concentration in the EB group was intermediate and did not differ from the other groups. The maximum concentration did not differ between E2 and EB groups and was greater than in the control group. For pregnant heifers, the number of prominent PGFM pulses and maximum concentration per 9 hour was greater tentatively in the E2 group than in the control and EB groups. The other end points in pregnant heifers were not different and did not approach a difference among groups.

Table 2. Mean ± SEM characteristics of PGFM pulses during hourly sampling in each of nonpregnant and pregnant Nelore heifers receiving an intravaginal P4 device combined with no additional treatment (control), E2 (1 mg E2 + 9 mg P4) or EB (1 mg estradiol benzoate) on day 14 after TAI.

End point	Control	E2	EB	Probability
Non-pregnant				
No. of heifers	5	5	5	
No. of all PGFM pulses	1.2 ± 0.2	1.2 ± 0.2	1.2 ± 0.4	0.50
No. of prominent PGFM pulses[a]	0.0 ± 0.0[b]	0.8 ± 0.4[a]	0.2 ± 0.2[ab]	0.09
PGFM pulse concentration (pg/mL)				
Nadir 1[b]	16.4 ± 3.4	30.8 ± 7.3	26.8 ± 4.9	0.15
Peak	26.1 ± 3.0[b]	123.7 ± 22.6[a]	87.6 ± 43.3[ab]	0.03
Nadir 2[b]	14.0 ± 2.7[b]	81.4 ± 20.3[a]	46.1 ± 18.6[ab]	0.02
Amplitude[c]	9.6 ± 1.1[b]	92.9 ± 23.8[a]	60.8 ± 39.0[ab]	0.07
Maximum concentration	31.8 ± 3.0[b]	123.7 ± 22.6[a]	110.2 ± 29.7[a]	0.02
Area under pulse curve (pg/mL x h)	22.7 ± 2.3[b]	150.8 ± 47.5[a]	114.5 ± 70.2[ab]	0.02
Pregnant				
No. of heifers	7	5	5	
No. of PGFM pulses	0.7 ± 0.3	1.2 ± 0.2	0.4 ± 0.2	0.20
No. of prominent PGFM pulses[a]	0.0 ± 0.0[b]	0.6 ± 0.2[a]	0.0 ± 0.0[b]	0.007
PGFM pulse concentration (pg/mL)				
Nadir 1[b]	22.1 ± 3.3	24.4 ± 4.1	36.4 ± 0.5	0.15
Peak	38.0 ± 6.9	113.0 ± 35.8	39.6 ± 3.4	0.16
Nadir 2[b]	20.1 ± 6.2	43.0 ± 9.4	18.8 ± 7.9	0.14
Amplitude[c]	15.9 ± 3.8	88.6 ± 31.7	3.20 ± 2.9	0.10
Maximum concentration	41.1 ± 5.3[b]	115.3 ± 34.4[a]	48.8 ± 10.1[b]	0.02
Area under pulse curve (pg/mL x h)	39.1 ± 5.3	173.1 ± 59.9	41.7 ± 8.1	0.25

ab Means within a row without a common superscript are different (P < 0.05).
[a] Prominent PGFM pulse (peak of >100 pg/mL).
[b] Nadir 1 and 2 are the nadirs at the beginning and the end of a PGFM pulse, respectively.
[c] Amplitude is the difference in concentration between Nadir 1 and peak.

For PGFM concentrations from –2 to 2 hour of the PGFM pulse/heifer, group and hour effects were detected in non-pregnant heifers (Fig. 3a). In non-pregnant heifers, the overall PGFM concentrations during –2 to 2 hour of the PGFM pulse was greater ($P < 0.05$) in the E2 group than in the EB group and the EB group was greater ($P < 0.05$) than in the control group. In non-pregnant heifers (Fig. 3a) and pregnant heifers (Fig. 3b), there was an hour effect representing a greater ($P < 0.05$) concentration at the peak of pulses, as expected from centralizing PGFM concentrations to the peak.

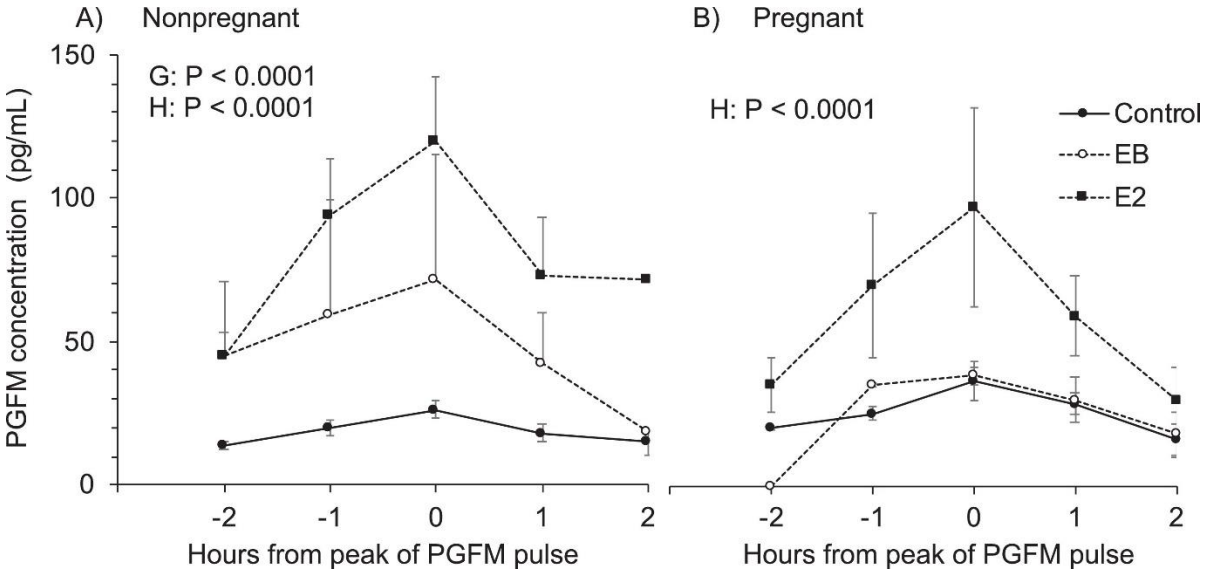

Fig. 3. Plasma PGFM concentration for PGFM pulses from -2 to 2 hour in nonpregnant (a) and pregnant (b) Nelore heifers receiving an intravaginal P4 device combined with no additional treatment (control), E2 (1 mg E2 + 9 mg P4) or EB (1 mg estradiol benzoate) on day 14 after TAI. Main effects of treatment group (G), hour (H) and the interaction (GH) that were significant are shown.

3.4.3 P4 concentrations during the 9-hours session

For P4 concentrations, only an hour effect occurred in each of non-pregnant and pregnant groups (Fig. 4a, b). The hour effect reflected an increase in P4 concentrations between Hours 0 and 1. There were no significant differences between non-pregnant and pregnant heifers.

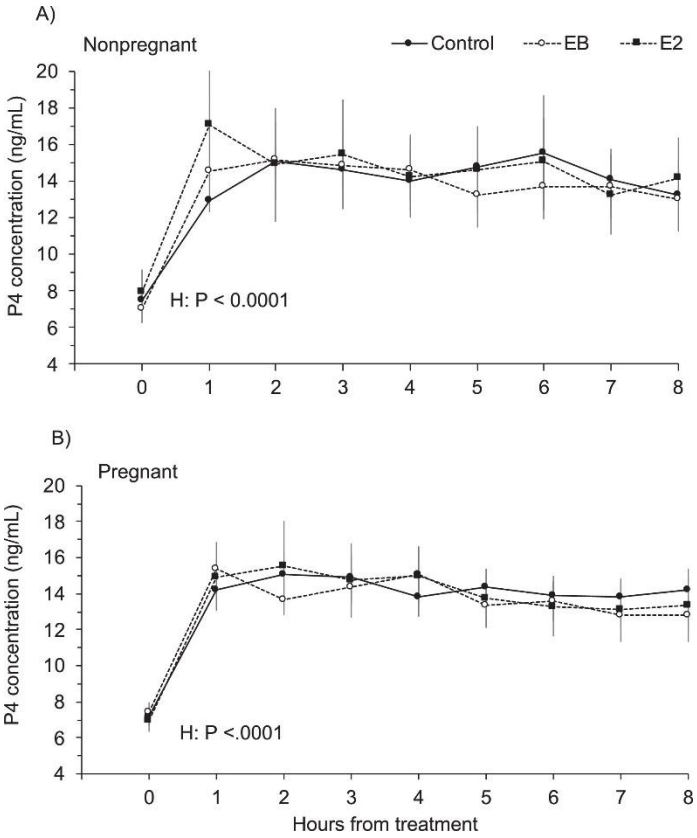

Fig. 4. Plasma P4 concentration in nonpregnant (a) and pregnant (b) Nelore heifers receiving an intravaginal P4 device combined with no additional treatment (control), E2 (1 mg E2 + 9 mg P4) or EB (1 mg estradiol benzoate) on day 14 after TAI. Main effects of treatment group (G), hour (H) and the interaction (GH) that were significant are shown.

4 DISCUSSION

The recent development in resynchronization protocols starting between 12 to 14 days after the first TAI and prediction of pregnancy status by detecting luteolysis 8 days later [2,3] allows a reduced interbreeding interval [13,14]. The hormonal treatments begin near the period of maternal pregnancy recognition [15] and must not jeopardize the existing pregnancy. Considering that the E2 has substantial relevance in the luteolytic process and may compromise pregnancy establishment, the effects of E2 or EB treatment during early pregnancy on $PGF_{2\alpha}$ release were studied for the first time in the present experiment.

The $PGF_{2\alpha}$ release was stimulated in E2-treated heifers regardless of pregnancy status, but only a slight stimulus occurred in EB-treated heifers. The esterification of EB reduces the amount of E2 (1 mg EB = 0.72 mg E2) and induces a distinctive circulating E2 profile in cows treated with EB compared to E2 [10]. This at

least partly accounts for the reduced effect of 1 mg EB vs 1 mg E2 on PGFM concentrations. Nonbred dairy cows treated with 0.5 mg E2 had greater E2 maximal concentrations when compared to 0.5 mg EB and a shorter interval from treatment to maximal concentrations (4 vs 16 hours) and from treatment to return to nadir concentrations [10]. Moreover, the PGFM concentrations peaked 6 to 7 hours after 0.05 and 0.1 mg E2 treatment [9] or at 8 hours after 1 mg EB treatment [6] in nonbred dairy heifers. The present results of a lower peak and later occurrence of PGFM pulses from EB than E2 treatment are consistent with the reported findings in nonbred heifers [6,7]. The 9 hour harvest windows in the present study likely were inadequate for detection of PGFM pulses in EB-treated heifers. Also, a controlled group with nonbred heifers was not used to evaluate the effect of pregnancy status; thus, occurrence of pregnancy loss before day 22 post-TAI may have increased the variation in the PGFM response in non-pregnant heifers.

The E2 stimulus on $PGF_{2\alpha}$ release was independent of pregnancy status in the primary analyses of the present study. However, the preliminary analyses of PGFM response tentatively indicated that $PGF_{2\alpha}$ release occurred predominantly in non-pregnant heifers. This was indicated in non-pregnant heifers by the greater PGFM concentrations during the 9 hours windows and more CV-identified PGFM pulses in the E2-treated group compared to the control and EB-treated heifers. The only two PGFM end points that differed in the pregnant heifers were the greater number of prominent PGFM pulses and greater maximum PGFM concentration during the 9 hour window in E2-treated group than in EB and control groups. Although the pregnancy rate was not affected in heifers receiving 1 mg E2 or EB in the present or previous studies [5,21], this is the first report of an E2-induced $PGF_{2\alpha}$ release in pregnant heifers. This is an interesting result as in early pregnancy, interferon-tau (IFN-t) from the elongating embryo blocks the luteolytic process [16,17]. The IFN-t directly silences transcription of the ERα and indirectly the E2-induced expression of oxytocin receptors in uterine luminal and superficial glandular epithelia negates the endometrial pulsatile release of $PGF_{2\alpha}$ [18,19]. In the present study, pregnant heifers in the E2 group tentatively had greater number of prominent pulses, which are expected to decrease in P4 concentrations [9,12]; For complete luteolysis, two or three pulses of $PGF_{2\alpha}$ occur over a period of 24 hours [20]. Instead, the plasma P4 concentration increased in the present study as expected after the insertion of the P4 device. The P4 device prevented the determination of the effects on CL function and P4 secretion; however,

evaluating the ultrasonography CL characteristics, a previous study from our group [5] indicated that the luteolysis is anticipated in E2 and EB-treated heifers.

5 CONCLUSIONS

In conclusion, $PGF_{2\alpha}$ release was more stimulated and occurred earlier in bred-non-pregnant and pregnant heifers submitted to estrous resynchronization with an intravaginal P4 device combined with 1 mg E2 + 9 mg P4 than with 1 mg EB. When analyzed separately for each pregnancy status on a preliminary basis, $PGF_{2\alpha}$ release was greater in non-pregnant heifers treated with E2. In pregnant heifers, 1 mg EB 14 days after TAI did not induce $PGF_{2\alpha}$ release, and only the number of prominent PGFM pulses and maximum concentration were greater for E2 treatment than for controls. Further studies using greater doses of estrogens and without P4 with the E2 treatment are indicated for a better understanding of the dose-dependent estrogen effects on $PGF_{2\alpha}$ release, luteolysis, and pregnancy maintenance.

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6. EFFECTS OF ESTRADIOL ON $PGF_{2\alpha}$ SYNTHESIS AND CORPUS LUTEUM FUNCTION DURING EARLY PREGNANCY IN BEEF HEIFERS

6.1 INTRODUCTION

The use of timed artificial insemination (TAI) enables an increase in profitability in beef and dairy herds (BARUSELLI et al., 2019). Resynchronization provides an opportunity for a second round of insemination and increase the reproductive efficiency in beef and dairy cattle (BO et al., 2016; BARUSELLI et al., 2017). With the recent methods for prediction of pregnancy status at 20 to 22 days after TAI using color Doppler ultrasonography (SIQUEIRA et al., 2013; PUGLIESI et al., 2014a; DALMASO DE MELO et al., 2020), a novel strategy for resynchronization of ovulation starting 12 to 14 days after the first TAI was developed (PUGLIESI et al., 2019; MOTTA et al., 2020).

In South America, an association of intravaginal progesterone (P4) devices and estradiol benzoate (EB) administration is commonly used for the recruitment of a new follicular wave in TAI protocols (BO et al., 2016). Though, the use of estradiol (E2) and its esters during the first weeks after TAI would cause pregnancy loss (VIEIRA et al., 2014) as E2 is involved in pulsatile $PGF_{2\alpha}$ release from the uterus (ARAUJO et al., 2009; PUGLIESI et al., 2011). In non-inseminated heifers the effect of estrogens on $PGF_{2\alpha}$ release is indicated by increased concentrations of the $PGF_{2\alpha}$ metabolite, 13,14-dihydro-15-keto- $PGF_{2\alpha}$ (PGFM) between 6 to 7 hours after treatments with 1 mg E2 or EB on days 12 to 14 post-ovulation (ARAUJO et al., 2009; GINTHER et al., 2010a; PUGLIESI et al., 2011). It has also been shown that this stimulus is dose dependent. A harmful effect on pregnancy establishment was not detected when a 1 mg E2 or EB was administered to heifers 14 days after TAI (MOTTA et al., 2020); however, the application of 1mg E2 induced prominent pulses of PGFM (> 100 pg/mL at the peak of PGFM pulse) in pregnant heifers (MOTTA et al., 2021). The administration of 1 mg EB associated with a P4-device in primiparous and multiparous beef cows 13 days after TAI had no impact on the pregnancy rate at the first TAI (PALHÃO et al., 2020); however, 1.5 mg EB on 13 days after TAI in dairy cattle (VIEIRA et al., 2014) and 2 mg EB on 14 days after TAI in suckled Nelore cows (SILVA et al., 2022) impaired the pregnancy rate of the first TAI.

Several researchers have strongly focused on improving the understanding of the effect of exogenous E2 on $PGF_{2\alpha}$ synthesis as well as the enzymes involved in this process. Oliveira et al. (2022), demonstrated that the intravenous injection of 3 mg of E2 on day 15 after estrus in non-pregnant animals, induced a greater abundance of *OXTR,* but a temporary reduction of enzymes involved in the $PGF_{2\alpha}$ synthesis cascades, such as *AKR1B1*, and *AKR1C4*. Similarly, Feltrin et al. (2020), using non-pregnant Nelore heifers, reported that the treatment with 1mg 17β-E2 on day 15 after estrus, induced a downregulation for the transcripts *PLA2G4* and *AKR1C4* at 90 and 180 min after administration. Although the E2 effects on $PGF_{2\alpha}$ synthesis were determined in cycling animals, the effects in pregnant animals, which would have the endometrium under the effect of IFN-t, are still unclear. It is likely that the IFN-t directly silences transcription of the ERα and the expression of OXTR in uterine luminal and superficial glandular epithelia negates the endometrial pulsatile release of $PGF_{2\alpha}$ (SPENCER et al., 1996; SPENCER et al., 2007; BAZER et al., 2017).

Therefore, further studies are needed to better understand the endocrine and molecular mechanisms related to $PGF_{2\alpha}$ synthesis in inseminated females. Recently, our research group studied the effects of administering 1 mg EB and 1 mg E2 on PGFM concentrations in pregnant beef heifers (MOTTA et al., 2021; Chapter 1]. However, as heifers were submitted to a TAI protocol and were resynchronized, the insertion of P4 and the presence of animals that could have experience IFN-t stimulus in the non-pregnant group may influence the uterine expression of enzymes involved in the $PGF_{2\alpha}$ synthesis. Therefore, to better understand the endocrine and molecular mechanisms without the exogenous P4 influence from an insertion of a P4 device, we proposed in the herein study a more physiological model, in which pregnant and cycling heifers will receive EB treatments after spontaneous ovulations.

We aimed to evaluate the effects of different EB doses (0, 1, and 2 mg), 13 days after ovulation on: 1) the characteristics of the CL, $PGF_{2\alpha}$ release, and induction of luteolysis in beef heifers; and 2) the expression of E2 receptors and the main enzymes involved in $PGF_{2\alpha}$ synthesis. It was hypothesized that: 1) the conceptus presence reduces the E2-stimulated pulsatile release of $PGF_{2\alpha}$; 2) the release of $PGF_{2\alpha}$ pulses and induction of luteolysis in pregnant heifers occurs in E2 dose-dependent manner; 3) the transcription of E2 (ESR1 and ESR2) and oxytocin (OXTR) receptors is up-regulated by E2 only in non-pregnant heifers.

6.2 MATERIAL AND METHODS

Experiments were submitted and approved by the ethics committee of the School of Veterinary Medicine and Animal Science of the University of São Paulo institution, under the protocol number 6550291019.

6.2.1 Heifers management and experimental design

6.2.1.1 Experiment 1

Experiment 1 was carried out from June to October 2020 at the Department of Animal Reproduction of the School of Veterinary Medicine and Animal Science of the University of São Paulo (FMVZ-USP, campus of Pirassununga – SP). The experiment was carried out to evaluate whether doses of 1 or 2 mg EB when given 13 days after ovulation in pregnant (inseminated) and non-inseminated beef heifers affect the CL blood perfusion and circulating PGFM and P4 concentration.

In this experiment, 69 cycles of 47 Nelore (*Bos taurus indicus*) heifers, aging between 18 and 24 months, weighing on average 390 ± 35 kg, and body condition score of 3 and 4 (BCS; on a scale of 1-5; 1 [emaciated] and 5 [obese]) (AYRES et al., 2009) were used. Heifers were maintained on a *Brachiaria decumbens* pasture with *ad libitum* access to water and a mineral supplement during the experimental period and received supplementation with corn silage and concentrate as established by the NRC 2016.

The experiment was conducted in three replicates. In both replicates the heifers had estrus synchronized with two i.m administrations of 0.53 mg of sodium cloprostenol (2 mL, i.m., Sincrocio, Ourofino Saúde Animal, Brazil) in 12 days of interval. At the time of the last administration, estrus detection patches (Boviflag, ABS Pecplan, Uberaba, MG, Brazil) were applied halfway between the hip and tail head to determine the occurrence of mounting behavior associated with estrus. In addition, after the second application, estrus was observed for one hour every 12 hours. Heifers that did not respond to estrus synchronization were excluded. Heifers that demonstrated estrus were randomly signed into two groups (non-inseminated, n = 19;

or inseminated n = 50; Fig. 1). Ultrasonography evaluations were performed daily to determine the ovulation. The day of ovulation (D0) was defined as the daily ultrasonography evaluation where the disappearance of a dominant follicle was identified. Thirteen days after ovulation (D13), heifers were randomly signed to one vehicle-treated (EB-0) and two EB groups with 1 and 2 mg (EB-1 and EB-2; Fig. 2). Heifers received a single intramuscular treatment in a total volume of 2 mL, with 0, 1, or 2 mg of EB (Sincrodiol, Ourofino Saúde Animal, Brazil). Considering the possible impairment of EB treatment on pregnancy establishment, more inseminated heifers were included in the EB-1 (n=16) and EB-2 (n=24) treatment groups than in the EB-0 group (n=10). Ultrasonography evaluations were performed daily from D13 to D19. Confirmatory pregnancy diagnosis was done on D28 based on the detection of an embryo with heartbeats.

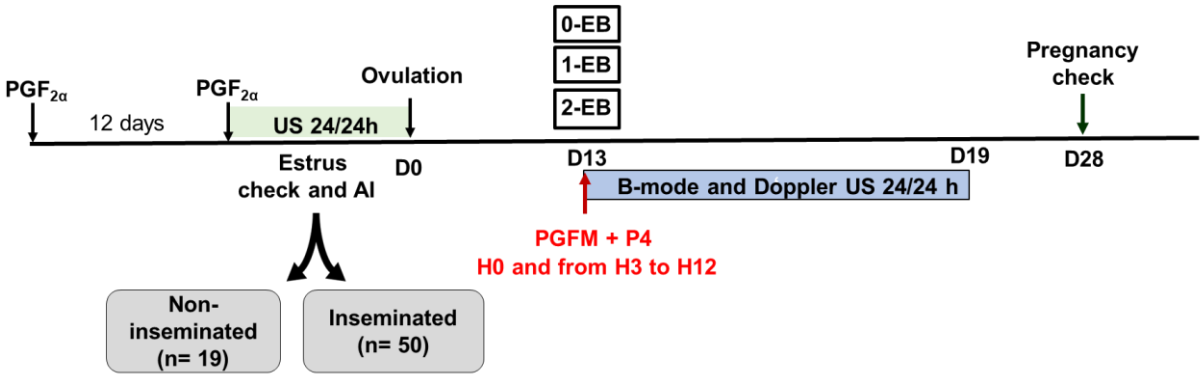

Fig 1. Heifers had estrus synchronized with two administrations of 0.53 mg of sodium cloprostenol in 12 day of interval. Estrus detection patches were applied to determine the occurrence of mounting behavior associated with estrus. In addition, estrus was observed every 12 hours. Heifers that demonstrated estrus were randomly subdivided into non-inseminated or inseminated. Daily ultrasonography exams were performed to determine ovulation. Ovulation was defined (D0). On D13 three experimental groups were used. Heifers were randomly signed to one vehicle-treated (EB-0) and two estradiol benzoate (EB) treated groups with 1 and 2 mg (EB-1 and EB-2). On D13, blood samples were collected at hour 0 (H0) which was before the treatment, and thereafter hourly from H3 to H12 for determination of PGFM and P4 concentrations. Ultrasonography evaluations were performed, and blood samples were collected daily from D13 to D19. A confirmatory pregnancy diagnosis was done on D28.

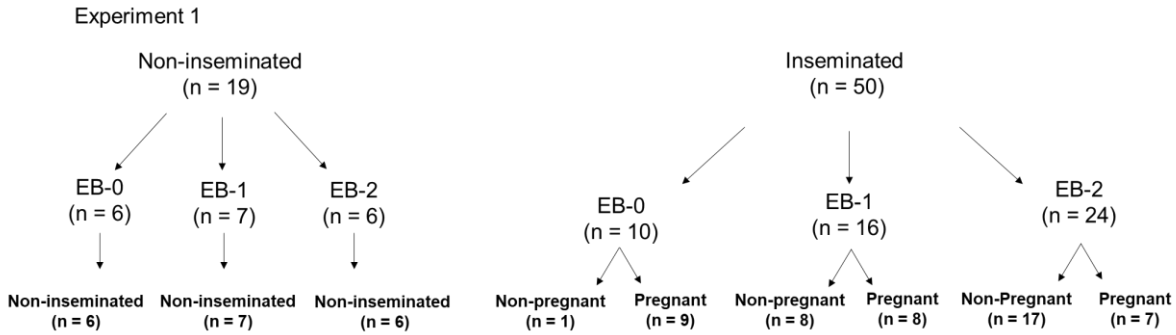

Fig 2. Heifers had estrus synchronized with two administrations of 0.53 mg of sodium cloprostenol in 12 days of interval. Heifers were randomly subdivided into two groups (non-inseminated, n = 19; or inseminated n = 50). Thirteen days after ovulation three experimental groups were used. Heifers were randomly assigned to one vehicle-treated group (EB-0) and two estradiol benzoate (EB) treated groups with 1 or 2 mg (EB-1 and EB-2). A pregnancy diagnosis was done on D28.

On D13, blood samples were collected from the jugular vein just before the treatment at Hour 0 (H0) and thereafter hourly from H3 to H12 for determination of plasma PGFM and P4 concentrations and in a subgroup of pregnant heifers (n= 6/group) to measure the concentrations of E2. Also, blood samples were collected daily from D13 to D19 for determination of plasma P4 concentrations. Samples were harvested using a 10 mL vacuum tube containing heparin (BD Vacutainer, São Paulo, SP, Brazil). The samples were centrifuged at 3600 x g for 15 minutes at 4°C and the plasma was stored at –20°C for subsequent assay.

Ultrasonographic exams were performed daily from D13 to D19 after ovulation by a single operator to monitor luteal dynamics. Exams were done using a duplex B-mode and pulse-wave color-Doppler ultrasound instrument (MyLab Delta, Esaote Healthcare, Italy) and a linear transducer in B (setting: RES-A, gain 50%, P 74 mm, X/M, PRS 1) and color-Doppler modes (setting: gain 61%, PRF 730Hz, frequency 6.3 MHz, WF 4, PRS 3 and PRC M/2).

The CL area was determined through the tracing function in the B-mode. When the CL presented a cavity, the area of the cavity was subtracted from the total CL area, as previously described by Rocha et al. (2019). The animals were submitted to evaluations of the blood perfusion of CL with color-Doppler ultrasonography. The proportion of the CL area with colored signals indicating blood perfusion was estimated in relation to the total CL area and indicated as a percentage (GINTHER et al., 2007). The day of structural luteolysis was defined by a retrospective evaluation as the day when the CL presented for the first-time blood perfusion ≤ 25% (PUGLIESI et al., 2014). The confirmatory diagnosis of pregnancy was performed with mode B

ultrasonography, 28 days after the first TAI, by detection of an embryo with heartbeats.

6.2.1.2 Experiment 2

The objective of this study was to evaluate the effects of systemic administration of EB on the components of the endometrial cascade of $PGF_{2\alpha}$ synthesis in beef heifers 13 days after ovulation. To test this hypothesis, the effects of administration of 1 mg EB, 13 days after ovulation were evaluated on: (1) the area and blood perfusion of the CL; and (2) relative quantification of the abundance of transcripts of genes related to $PGF_{2\alpha}$ synthesis.

For these, 60 cycles of 40 Nelore (*Bos taurus indicus*) heifers, aging between 24 and 30 months, weighing on average 437 ± 45 kg, and body condition score of 3 and 4 (BCS; on a scale of 1-5; 1 [emaciated] and 5 [obese]) (AYRES et al., 2009). Heifers were maintained on a *Brachiaria decumbens* pasture with *ad libitum* access to water and a mineral supplement during the experimental period.

The experiment was conducted in four replicates. As done in Experiment 1, in all replicates the heifers were submitted to an estrus synchronization protocol but ovulation was not induced. The protocol consisted of the insertion of an intravaginal P4-releasing device (Progestar, Biogénesis Bagó, Brazil) along with i.m. administration of 2 mg EB (2 mL, i.m., Bioestrogen, Biogénesis Bagó, Brazil) and 0.150 mg of sodium D-Cloprostenol (2mL, i.m., Croniben, Biogénesis Bagó, Brazil). After six days, 0.150 mg of sodium D-Cloprostenol was given. After 48 hours, the P4 devices have been removed and a new dose of 0.150 mg of sodium D-cloprostenol was given. In addition, the heifers received an estrous detection patch (Boviflag, ABS Pecplan, Uberaba, MG, Brazil) which was applied halfway between the hip and tail head to determine the occurrence of mounting behavior associated with estrus. Estrous behavior was observed for one hour every 12 hours for five days after the $PGF_{2\alpha}$ analog treatment. All Heifers were randomly signed into non-inseminated (n = 21) or inseminated (n = 39) groups. Ultrasonography evaluations were performed daily, every 12 hours to determine the ovulation. Ovulation was defined (D0) as the disappearance of a follicle identified as a dominant follicle during the preceding examination (Fig. 3). Thirteen days after ovulation (D13), heifers were randomly signed to one vehicle-treated (EB-0; n = 10 non-inseminated and 18 inseminated; Fig. 4) and one EB-treated group with

1 mg EB (EB-1; n = 11 non-inseminated and 21 inseminated; Fig. 4). The treatment time was considered as Hour 0 (H0). Heifers received a single intramuscular treatment in a total volume of 1 mL, with 0 or 1 mg of EB (Sincrodiol, Ourofino Saúde Animal, Brazil). Ultrasonography evaluations were performed daily from D13 to D19. Exams were done as in Experiment 1. Confirmatory pregnancy diagnosis was done on D28 based on the detection of an embryo with heartbeats.

Three hours after vehicle or EB treatments (H3), heifers were submitted to epidural anesthesia using 0.06 g of lidocaine hydrochloride and immediately after to a cytological sampling of endometrium using a cytobrush procedure in the body of the uterus according to Cardoso et al. (2017). After sampling, each 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 initially in liquid nitrogen (–196C°) and later at –80C° for later processing.

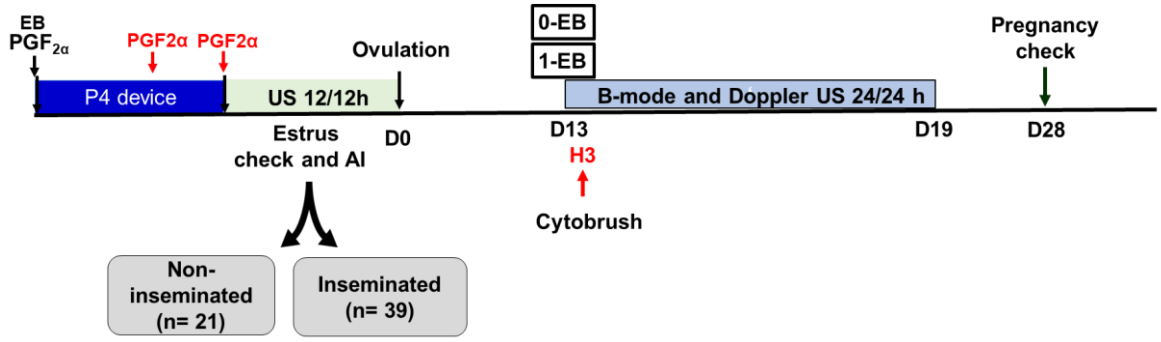

Fig 3. Heifers were submitted to an estrus synchronization P4/E2-based protocol but ovulation was not induced. The $PGF_{2\alpha}$ analog was given at the beginning of the protocol and after 6 days. After 48 hours, the P4 devices have been removed and a new dose of $PGF_{2\alpha}$ analog was given. Estrus detection patches were applied to determine the occurrence of mounting behavior associated with estrus. In addition, estrus was observed every 12 hours for five days. Heifers that demonstrated estrus were randomly subdivided into non-inseminated or inseminated. Daily ultrasonography exams were performed to determine ovulation. Ovulation was defined (D0). On D13, all heifers were randomly signed into non-inseminated (n = 21) or inseminated (n = 39) and between two experimental groups: with 0 or 1 mg of EB. (0-EB and 1-EB). On D13, the treatment was given at hour 0 (H0), four hours later (H4) the heifers were subjected to endometrial cytology (cytobrush) and the samples were subsequently subjected to qPCR. Ultrasonography evaluations were performed daily from D13 to D19. A confirmatory pregnancy diagnosis was done on D28.

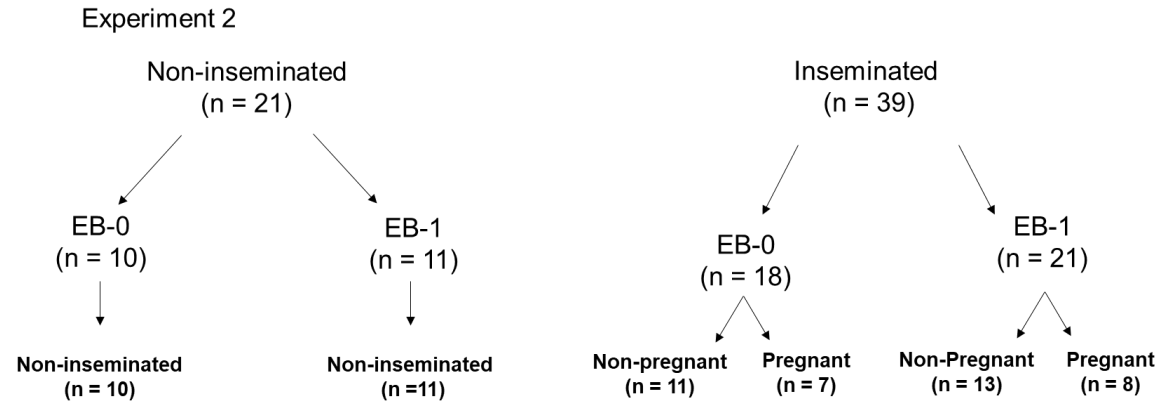

Fig 4. Heifers were randomly subdivided into two groups (non-inseminated, n = 21; or inseminated n = 39). Thirteen days after ovulation, heifers were randomly assigned to one vehicle-treated group (EB-0) and one estradiol benzoate (EB) treated group with 1 mg (EB-1). A pregnancy diagnosis was done on D28.

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6.2.2. Hormone assays

For Experiment 1, plasma P4 concentration was assayed with a RIA commercial kit (CT Progesterone, MP Biomedicals LCC, Solon, OH, USA). Plasma PGFM concentration was assayed with an in-house ELISA, as described elsewhere (GINTHER et al., 2007). Because of the limited availability of the ELISA kit, plasma E2 concentration was assayed only at two moments in a subgroup of pregnant heifers (n= 6/group) using a competitive ELISA (DetectX® Serum Estradiol Enzyme Immunoassay Kit, Abor Assays, Ann Arbor, USA). The samples collected at Hours 5 and 6, and Hours 11 and 12 were combined as 2 mL of plasma was needed for the protocol of steroid extraction before running the ELISA.

The intra- and inter-assay CV and sensitivity for P4 were 4.3%, 3.45%, and 0.025 ng/mL, respectively. The intra- and inter-assay CVs and sensitivity for PGFM were 6.6%, 10.3%, and 2.8 pg/mL, respectively. The intra-assay CV and sensitivity for E2 were 4,4%, and 11.9 pg/mL, respectively.

6.2.3. Identification of PGFM pulses

In Experiment 1, the pulses of PGFM were identified using the coefficient of variance (CV) method as described (GINTHER et al., 2010a; GINTHER et al., 2010b). Pulses with a PGFM peak concentration greater than 100 pg/mL were considered

prominent pulses as previously described (GINTHER et al., 2010a; GINTHER et al., 2010b). The average PGFM concentration for the 11 samples/session and the PGFM concentrations of the most prominent pulse during a session were compared among groups. The pulse characteristics that were compared were nadir 1, peak, nadir 2, and amplitude (difference between peak and nadir 1).

6.2.4 Evaluation of transcripts

In Experiment 2, the abundance of transcripts from *ISG15, RSAD2, ESR1*, *ESR2*, *OXTR, PGR, AKR1C4, PTGS2, PLA2G4 and IL1-β* was evaluated in a subgroup of non-inseminated heifers (EB-0, n= 7 and EB-1, n = 7); and in pregnant heifers (EB-0, n = 9 and EB-1, n = 8).

The RNA extraction was performed with Trizol reagent (Life Technologies, Frederick, USA) according to the manufacturer's instructions. We measured the concentration of total RNA in the extracts with a NanoVue ™ Plus, Spectrophotometer (GE Healthcare, UK) at 260 nm absorbance, and the purity of total RNA was estimated based on the ratio of absorbance at 260 and 280 nm. Samples with a 260/280 ratio ranging from 1.7 to 2.0 were used for transcript abundance analyses. After the extraction, the cDNA synthesis was performed with “High-Capacity cDNA Reverse Transcription kit (Life Technologies), according to the manufacturer's instructions, and the cDNA of each sample was stored at −20 °C until qPCR analysis. For the reaction of qPCR was used the Step One Plus™ Real Time System thermocycler (Applied Biosystems®, Life Technologies Corporation) with programming of the following temperature cycles: 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. After amplification, the plate remained at 95°C for 15 minutes, 60°C for 1 minute and again 95°C for 15 minutes, to obtain the dissociation curve (“Melt Curve”). Specific primers for each selected gene (Table 1) were designed using Primer Quest (IDT; http://www.idtdna.com/Primerquest/Home/Index) or obtained from previous reports: Krishnaswamy et al. (2009); Mesquita et al. (2014); Gonela et al. (2015); Oliveira et al. (2015); Batista et al., (2019); Rocha et al., (2020); and Oliveira et al., (2022).

Table 1. Forward (F) and reverse (R) primers sequences of target and reference genes analyzed in the 2, using qPCR.

Target	Number	Foward primer sequence (5'-3')	Reverse primer sequence (5'-3')
ISG15 [A]	NM_174366	GGTATCCGAGCTGAAGCAGTT	ACCTCCCTGCTGTCAAGGT
RSAD2 [A]	NM_001045941.1	TGGTTCCAGAAGTACGGTGAA	ACCACGGCCAATAAGGACAT
ESR1 [B]	NM_001001443.1	CAGGCACATGAGCAACAAAG	TCCAGCAGCAGGTCGTAGAG
ESR2 [C]	NM_174051.3	GTAGAGAGCCGCCATGAATAC	CAATGGATGGCTAAAGGAGAGA
OXTR [D]	NM_174134.2	AAGATGACCTTCATCGTCGTG	CGTGAAGAGCATGTAGATCCAG
PGR [E]	NM_011205356.1	GCCGCAGGTCTACCAGCCCTA	GTTATGCTGTCCTTCCATTGCCCTT
AKR1C4 [F]	NM_181027.2	TCCTGTCCTGGGATTTGGAACCTT	ATCGGCAATCTTGCTTCGAATGGC
PTGS2 [F]	NM_174445.2	CCAGAGCTCTTCCTCCTGTG	GGCAAAGAATGCAAACATCA
PLA2G4 [G]	NM_001075864.1	GATGCCGCTGTCGCAGATCCTG	TGTGAGGCCACTGTCCACCACAT
IL1-β [A]	NM_174093.1	AATCGAAGAAAGGCCCGTCT	ATATCCTGGCCACCTCGAAA
GAPDH [B]	NM_001034034.2	GCCATCAATGACCCCTTCAT	TGCCGTGGGTGGAATCA
PPIA [C]	NM_178320.2	GCCATGGAGCGCTTTGG	CCACAGTCAGCAATGGTGATCT

Adapted from Oliveira et al. (2022). Superscript letters in target: [A] Rocha et al. (2020); [B] Mesquita et al. (2014); [C] Gonela et al. (2015); [D] Krishnaswamy et al. (2009); [E] Batista et al. (2019); [F] Oliveira et al. (2015); [G] PrimerQuest;

6.2.5. Statistical analyses

For each experiment, statistical analyses were performed using SAS (version 9.2, SAS Institute Inc., Cary, NC, USA) considering each heifer as an experimental unit. Continuous data were examined for normality with the use of the Shapiro-Wilk test and for homogeneity using Levene's test. Experiment 1 was analyzed as a 2 × 3 factorial (two pregnancy status [pregnant *vs.* non-inseminated] and three treatment groups); whereas Experiment 2 was analyzed as a 2 × 2 factorial (two pregnancy statuses and two treatment groups). All the continuous data for CL (area, blood perfusion, and day of structural luteolysis) were analyzed for the main effect of group, time (days), status, and their interactions. The PGFM and P4 were analyzed for the main effect of group, hour, pregnancy status, and their interactions. The PROC MIXED procedure was used with a REPEATED statement to account for autocorrelation between sequential measurements. For PGFM concentrations, Hour 0 Was used as covariate, and concentrations were evaluated from Hours 3 to 12. The abundance of the transcripts was analyzed considering only the main effects of the group (EB-0 or EB-1), pregnancy status (non-inseminated or pregnant), and their interaction. The LSD test was used to compare time within a group or among more than two means or for discrete data of CL. The LSD test was used to compare between hours within a group or among more than two means or for discrete data of P4 concentration and PGFM pulse characteristics. A Fisher's exact was used for the comparison of the frequency

of PGFM pulses among groups. The pregnancy rate was analyzed by Fisher's exact test. For both experiments, a probability of $P \leq 0.05$ indicated that a difference was significant and $0.05 < P \leq 0.1$ indicated approached significance. Data are presented as the mean ± SEM.

6.3 RESULTS

6.3.1 Experiment 1

6.3.1.1 Ovarian and pregnancy outcomes

No significant ($P>0.1$) interaction between the treatment group and pregnancy status (non-inseminated *vs.* pregnant) was detected for the diameter of dominant follicle at the time of artificial insemination. The diameter (mm) of dominant follicle at the time of artificial insemination and the day of structural luteolysis did not differ ($P>0.05$) among the EB-0, EB-1, and EB-2 groups (Table 2). However, for no-inseminated heifers, when the EB-1 and EB-2 groups were combined, the day of structural luteolysis was earlier than in the EB-0 group (16.3 ± 0.2 *vs.*17.3 ± 0.6 days, $P = 0.04$). The proportion of pregnant heifers was greater ($P < 0.05$) in the EB-0 group than in the EB-1 and EB-2 groups (Table 2).

Table 2. Dominant follicle diameter at time of artificial insemination, structural luteolysis, pregnancy rate (%) at day 28 of heifers non-inseminated and inseminated on the EB-0, EB-1 and EB-2 groups, treated on day 13 after ovulation.

End point	EB-0	EB-1	EB-2	Probability
Non-inseminated				
No. of heifers	6	7	6	
Dominant follicle at AI (mm)	12.7 ± 0.4	12.4 ± 0.4	13 ± 0.8	0.78
Luteolysis (days)	17.2 ± 0.7	16.6 ± 0.3	16 ± 0.6	0.19
Inseminated				
No. of heifers	10	16	24	
Dominant follicle at AI (mm)	12.7 ± 0.4	12.5 ± 0.4	12.4 ± 0.3	0.78

End point	EB-0	EB-1	EB-2	Probability
Luteolysis (days)*	-	16.4 ± 0.2	16.3 ± 0.3	0.19
Pregnancy rate on D28	90%[A] (n = 9/10)	50% [B] (n = 8/16)	29.2% [B] (n = 7/24)	0.004

A B Different upper-case letters on the same row indicate a difference ($P < 0.05$) between groups by Fisher's exact test.

* The day of luteolysis in inseminated heifers was compared only between the EB-1 and EB-2 groups, because in the EB-0 group luteolysis was detected only in one heifer that did not become pregnant.

When the data of the CL area was analyzed over D13 to D19, a significant triple interaction among the treatment group, time, and pregnancy status was not detected, but a significant interaction between time and pregnancy status was observed (Fig. 5A). The CL area was maintained in pregnant heifers; whereas, it decreased progressively from D14 to D18 in non-inseminated heifers. Regardless of pregnancy status, there was also an interaction between the treatment group and time for the CL area (Fig. 5B). In the EB-0 group, the CL area did not change over time, but in the EB-1 group, the CL area decreased from D14 to D15 and from D17 to D18; whereas in EB-2, the CL area decreased from D15 to D18.

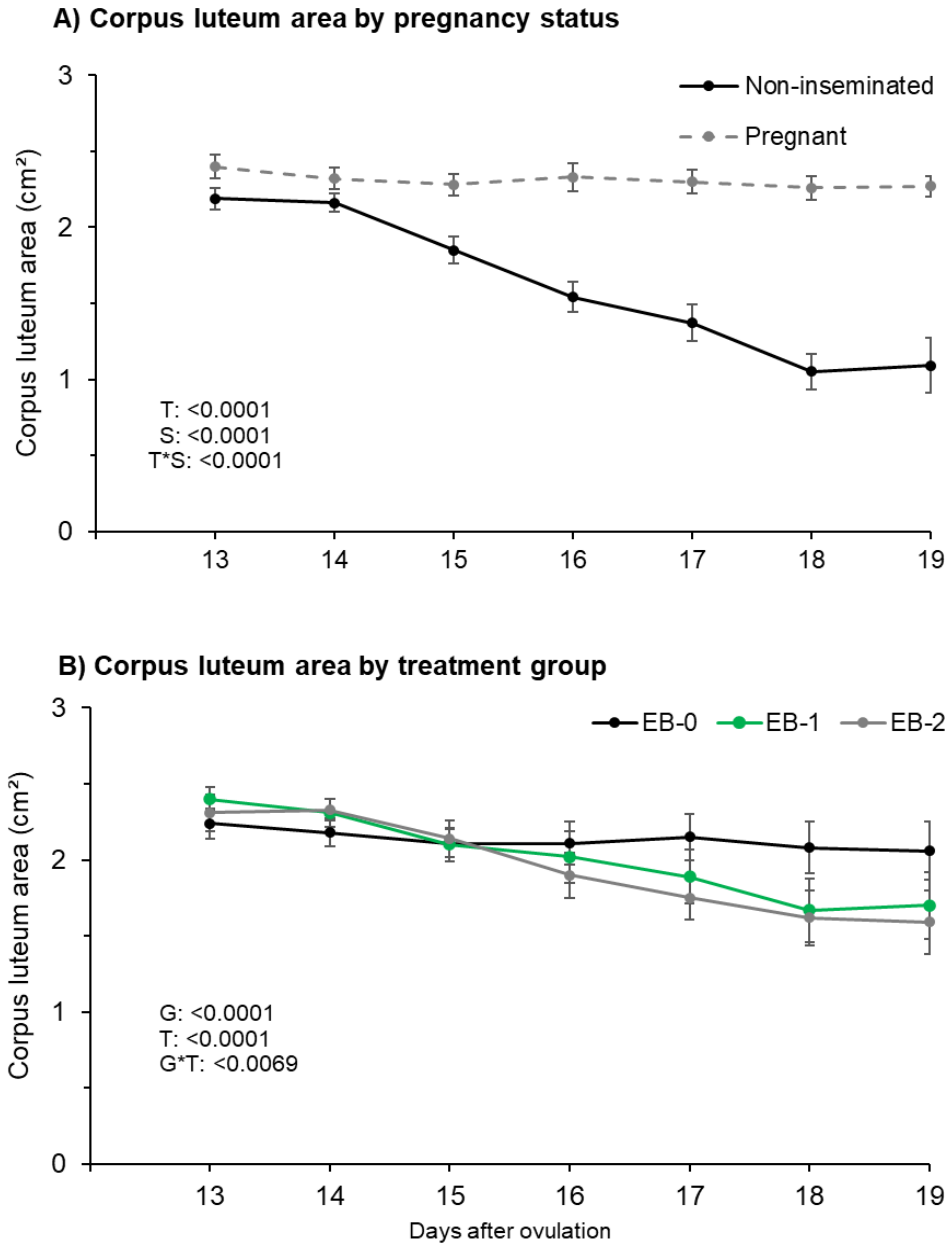

Fig 5. Painel A and B: mean ± SEM CL area (cm^2) from 13 to 19 days after ovulation in Nelore heifers receiving 0, 1 or 2 mg of estradiol benzoate (EB) 13 days after ovulation. Panel A: non-inseminated and pregnant, regardless the treatment group, and Panel B: EB-0, EB-1 and EB-2 groups, regardless of pregnancy status. Main effects of time (T), pregnancy status (S) and the interaction (TS) that were significant are shown. Main effects of treatment group (G), time (T) and the interaction (GT) that were significant are shown.

When the data of CL blood perfusion was analyzed over D13 to D19 there was a significant triple interaction (P = 0.03) between treatment group, time, and status. In non-inseminated heifers, the CL blood perfusion decreased slower in the EB-0 group than in the EB-1 and EB-2 groups (Fig. 6A). In pregnant heifers, the CL blood perfusion in the EB-0 group increased from D15 to D18; whereas, in the EB-1 group the CL blood perfusion increased from D13 to D14, and in the EB-2 group, increased from D15 to D16 (Fig. 6B).

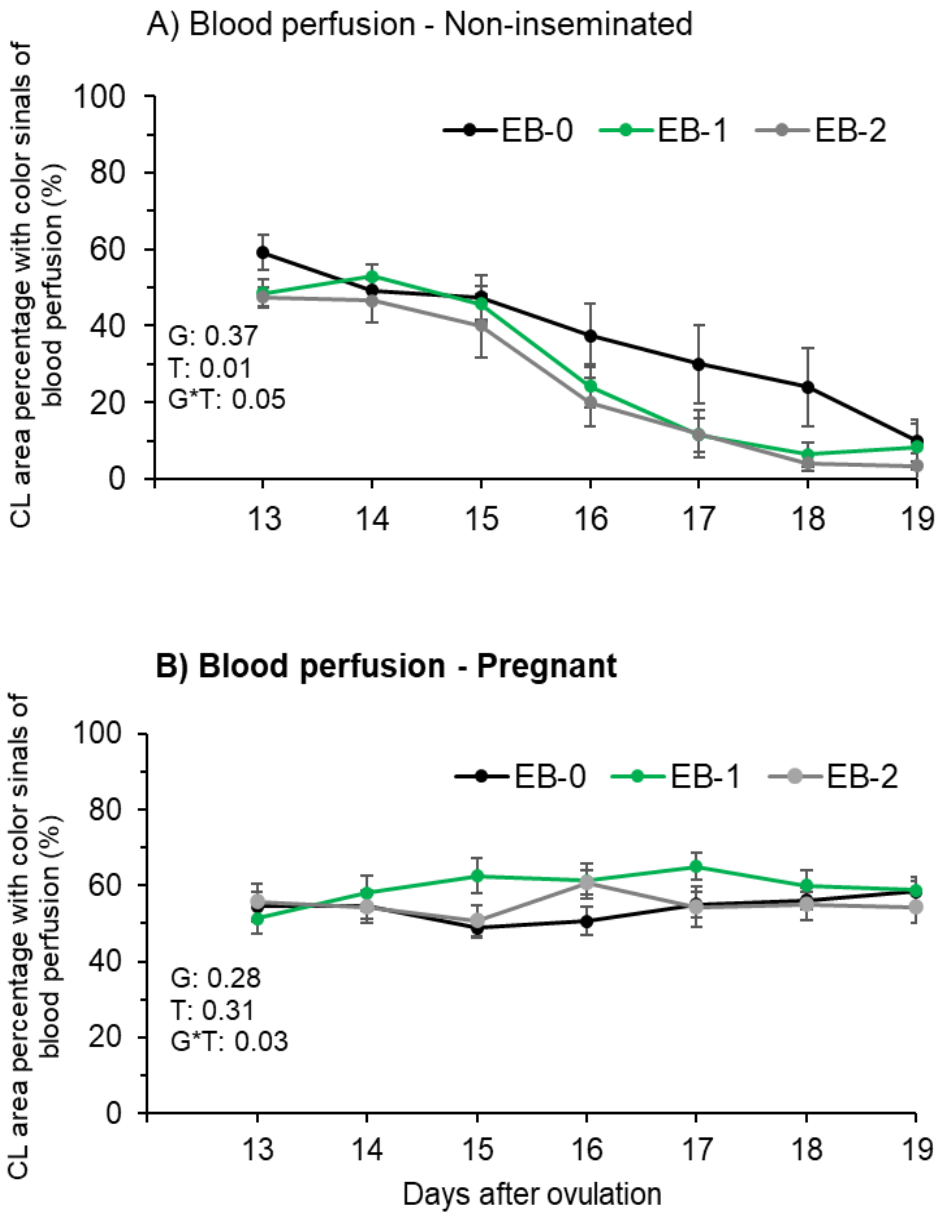

Fig 6. Mean ± SEM proportion of luteal tissue with color signals of blood from D13 to D19 of EB-0, EB-1 and EB-2 group in non-inseminated (A) and pregnant (B) heifers. Main effects of treatment group (G), time (T) and the interaction (GT) that were significant are shown.

6.3.1.2 Daily plasma P4 concentrations

When the data of the P4 concentrations was analyzed over D13 to D19, a significant triple interaction among treatment group, time, and pregnancy status was not detected, but a significant interaction between pregnancy status and time ($P<0.000$; Fig. 7A), and an approached significant interaction between treatment group and time ($P = 0.06$; Fig.7B) were observed. In non-inseminated heifers, the P4

concentration decreased progressively from D14 to D19; whereas in pregnant heifers, the P4 concentration did not change from D13 to D19. Regardless of the pregnancy status, the P4 concentration decreased from D14 to D18 in the EB-0 group. In the EB-1 group, the P4 concentration decreased from D13 to D15 and again from D15 to D17; whereas in the EB-2 group, the decrease was from D14 to D16.

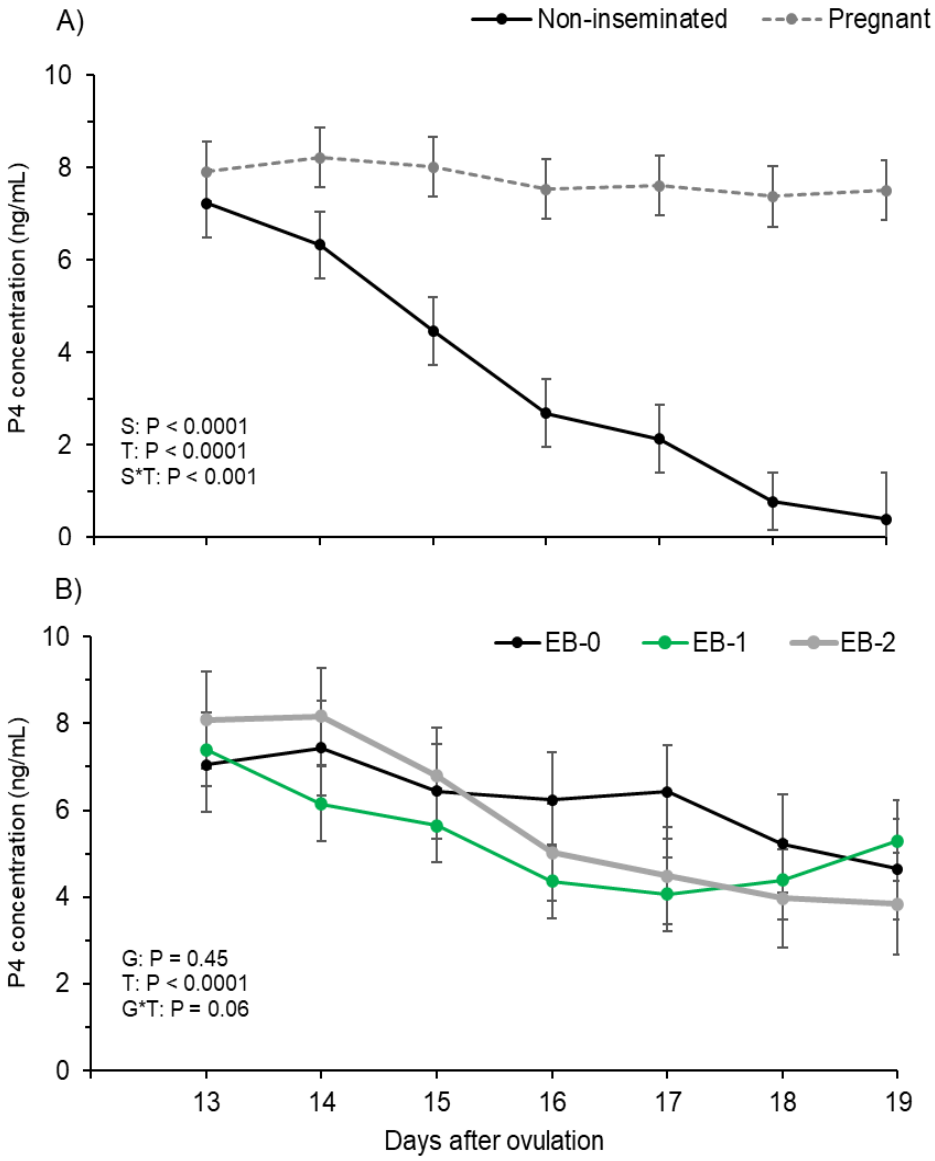

Fig 7. Mean ± SEM P4 concentrations from 13 to 19 days after ovulation in Nelore heifers receiving 0, 1 or 2mg of EB 13 days after ovulation. Painel A: non-inseminated and pregnant, regardless the treatment group and Painel B: EB-0, EB-1, and EB-2 groups, regardless of pregnancy status. Main effects of time (T), pregnancy status (S) and the interaction (TS) that were significant are shown. Main effects of treatment group (G), time (T) and the interaction (GT) that were significant are shown.

6.3.1.3 Plasma PGFM and E2 concentrations

A significant triple interaction among treatment group, time, and pregnancy status was not detected. The average PGFM concentrations over H3 to H12 did not differ between the EB-1 and EB-2 groups. Regardless of the EB treatment group, an approached significant interaction of pregnancy status by time ($P<0.06$) indicated that PGFM concentrations increased from Hour 5 to Hour 6 in non-inseminated and from Hour 7 to Hour 8 in pregnant heifers (Fig. 8A). The averaged PGFM concentrations over H3 to 12 were greater ($P<0.002$) in the EB-1 (46.3 ± 3.6 pg/mL) and EB-2 (46.9 ± 3.3 pg/mL) groups than in the control group (EB-0; 28.0 ± 1.2 pg/mL), regardless the pregnancy status (Fig 8B). When the data of the E2 concentrations was analyzed in pregnant heifers at Hours 5/6 and Hours 11/12, a significant interaction between the treatment group and time was not detected ($P=0.38$). Although a significant effect of time was not detected ($P=0.67$), E2 concentrations were affected by the treatment group ($P<0.0001$). The E2 concentration was greater in the EB-2 group (38.18 ± 3.7 pg/mL) than in the EB-1 group (26.04 ± 4.9 pg/mL), which was greater than in the EB-0 group (2.53 ± 0.4 pg/mL).

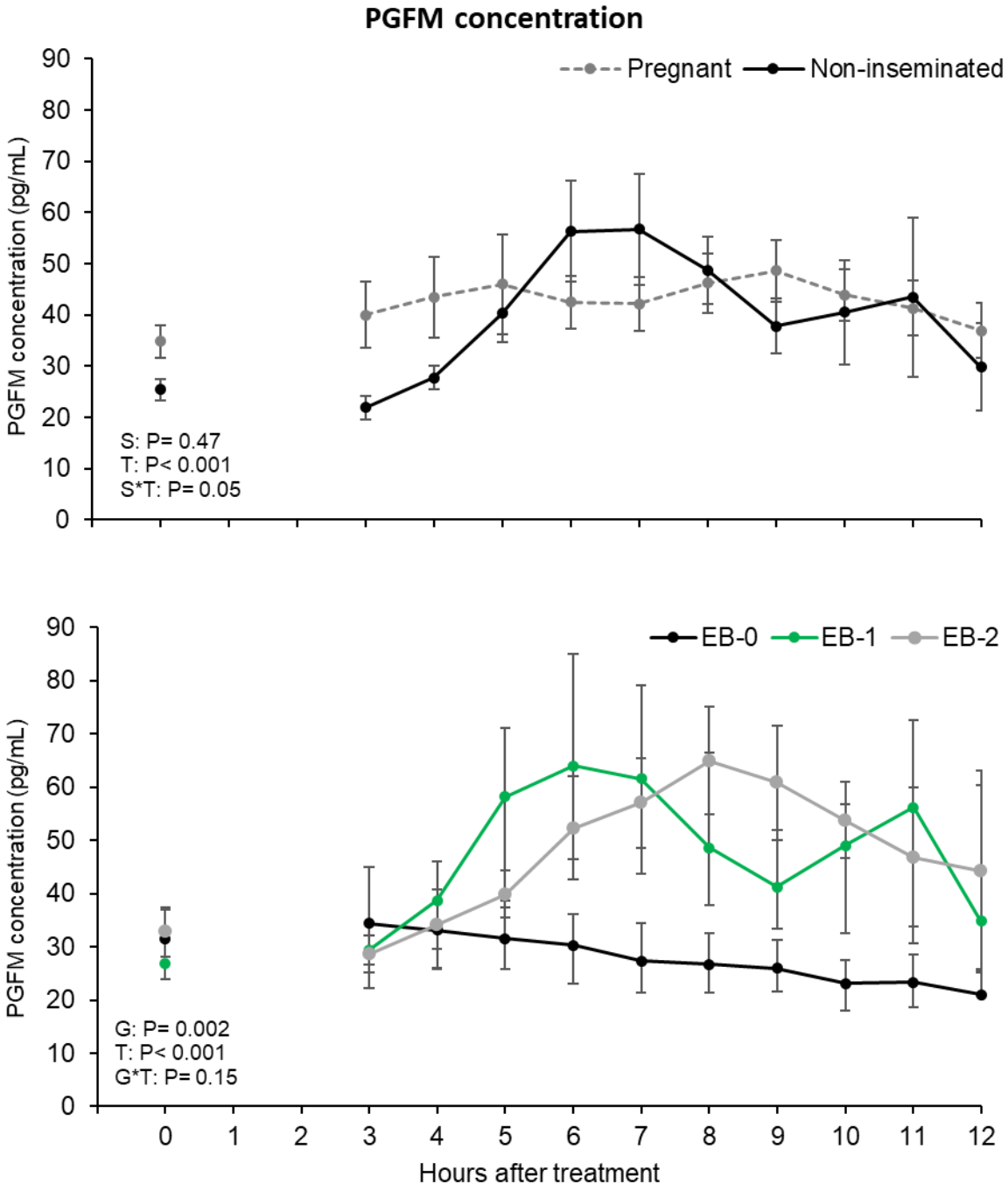

Fig. 8 Mean ± SEM for plasma PGFM concentrations from Hour 0 until Hour 12 in Nelore heifers receiving 0, 1, or 2mg of EB. Painel A: non-inseminated and pregnant, regardless of the treatment group, and Painel B: EB-0, EB-1, and EB-2 groups, regardless of pregnancy status. The main effects of time (T), pregnancy status (S), and the interaction (TS) that were significant are shown. The main effects of the treatment group (G), time (T), and the interaction (GT) that were significant are shown.

6.3.1.4 Identified PGFM pulses

Regarding the characteristics of PGFM pulses (Table 3), a significant or approached significant group effect occurred for 5 of 8 end points. A significant

pregnancy status effect (non-inseminated or pregnant) or a group by status interaction were not found for any end point, but the PGFM concentration at nadir 1 tended (P=0.08) to be greater in pregnant heifers than non-inseminated, regardless of treatment groups. The number of all (prominent and non-prominent) PGFM pulses per heifer did not differ between treatment groups and between pregnancy status (Table 3). The number of prominent PGFM pulses was greater in the EB-2 group than in the EB-0 group, whereas the number of prominent PGFM pulses in the EB-1 group was intermediate and did not differ from the other groups. The PGFM concentration at the peak, the amplitude, maximum concentration, and AUC of PGFM did not differ between the EB-1 and EB-2 groups, but they were greater in both groups than in the EB-0 group. The PGFM concentration at the nadir 2 did not differ between treatment groups and between pregnancy status.

Table 3. Mean ± SEM characteristics of PGFM pulses during hourly sampling in Nelore heifers receiving 0, 1 or 2mg of EB, 13 days after ovulation.

End point	**EB-0**	**EB-1**	**EB-2**	**Group**
No. of cycles	13	13	13	
No. of all PGFM pulse	1.4 ± 0.2	1.5 ± 0.2	1.3 ± 0.3	0.67
No. of prominent PGFM pulse[x]	0.0 ± 0.0[b]	0.4 ± 0.3[ab]	0.5 ± 0.2[a]	**0.07**
PGFM pulse concentration (pg/mL)				
Nadir 1[y]	20.6 ± 4.4	25.2 ± 5.9	31.4 ± 5.8	0.34
Peak	35.2 ± 8.3[B]	107.0 ± 35.8 [A]	90.4 ± 15.0[A]	**0.003**
Nadir 2[y]	14.6 ± 3.5	33.4 ± 9.0	28.9 ± 7.3	0.12
Amplitude[z]	20.6 ± 8.1[b]	73.6 ± 26.3[a]	61.5 ± 19.0[a]	**0.08**
Maximum concentration	43.4 ± 9.4[B]	102.0 ± 30.9[A]	91.9 ± 13.2[A]	**0.0003**
Area under pulse curve (pg/mL x h)	43.5 ± 18.7[b]	201.0 ± 71.9[a]	184.2 ± 59.4[a]	**0.06**

[AB] Means within a row without a common superscript are different (P < 0.05).
[ab] Means within a row without a common superscript are different (P < 0.1).
[x] Prominent PGFM pulse (peak of >100 pg/mL).

[y] Nadir 1 and 2 are the nadirs at the beginning and the end of a PGFM pulse, respectively.

[z] Amplitude is the difference in concentration between Nadir 1 and peak

For PGFM concentrations from –2 to 2 hour of the PGFM pulse/heifer, treatment group (P= 0.003) and hour effects were detected (P<0.0001). The overall PGFM concentrations during –2 to 2 hour of the PGFM pulse was greater in EB-1 (69.2 ± 8.00) and EB-2 (62.9 ± 4.35) groups, than in the EB-0 group (24.8 ± 2.21), however did not differ between EB-1 and EB-2 groups. There was an hour effect representing

a greater ($P < 0.05$) concentration at the peak of pulses, as expected from centralizing PGFM concentrations to the peak.

Although the group-by-status interaction was not significant for any PGFM pulse characteristic, post-hoc analyses for non-inseminated heifers alone and pregnant heifers alone on a preliminary basis tentatively indicated some differences between groups for each pregnancy status (Table 4). For non-inseminated heifers, the PGFM concentrations at the peak, amplitude, and AUC were greater for heifers in the EB-1 and EB-2 groups than in the EB-0 group. Also, the maximum concentration observed within Hours 3 to 12 was greater in the EB-1 and EB-2 groups than in the EB-0 group. For pregnant heifers, the PGFM concentration at Nadir 2 was greater in the heifers from the EB-2 group than in the EB-0 and EB-1 groups. Also, PGFM concentration at the peak of a PGFM pulse, and maximum concentration within Hours 3 to 12 tended to differ among the groups (Table 4). Both end points were greater ($P<0.05$) in the EB-2 group than in the EB-0, whereas the EB-1 group did not differ from the other groups. The other end points in pregnant heifers were not different and did not approach a difference among groups.

Table 4. Mean ± SEM characteristics of PGFM pulses during hourly sampling in each of non-inseminated and pregnant Nelore heifers receiving 0, 1 or 2mg of EB, 13 days after ovulation.

End point	EB0	EB1	EB2	Probability
Non-pregnant				
No. of heifers	6	6	6	
No. of all PGFM pulses	1.50 ±0.28	1.33 ±0.21	1.00 ±0.31	0.45
No. of prominent PGFM pulses[x]	0	0.66 ±0.33	0.40 ±0.24	0.20
PGFM pulse concentration (pg/mL)				
Nadir 1[y]	20.40 ±6.0	23.96 ±6.28	21.16 ±5.83	0.93
Peak	32.12 ±8.13^{B}	123.44 ±36.93^{A}	93.37 ±18.33^{A}	**0.02**
Nadir 2[y]	15.90 ±3.87	27.99 ±9.09	17.18 ±5.21	0.63
Amplitude[z]	16.22 ±4.63^{B}	95.44 ±30.80^{A}	76.19 ±22.91^{A}	**0.02**
Maximum concentration	32.25 ±4.56^{B}	123.44 ±36.93^{A}	94.49 ±18.16^{A}	**0.002**
Area under pulse curve (pg/mL x h)	36.43 ±8.73^{B}	250.84 ±69.64^{A}	244.23 ±72.37^{A}	**0.007**
Pregnant				
No. of heifers	7	7	7	
No. of PGFM pulses	1.33 ±0.33	1.60 ±0.24	1.50 ±0.22	0.80
No. of prominent PGFM pulses[x]	0	0.20 ±0.20	0.66 ±0.21	0.10
PGFM pulse concentration (pg/mL)				
Nadir 1[y]	20.70 ±2.83^{B}	26.31 ±5.50^{B}	41.65 ±5.78^{A}	**0.04**
Peak	38.21 ±8.51^{b}	90.47 ±34.74^{ab}	87.34 ±11.59^{a}	**0.09**
Nadir 2[y]	13.21 ±3.08	38.71 ±8.81	40.64 ±9.44	0.19
Amplitude[z]	25.00 ±11.54	51.75 ±29.64	46.70 ±15.18	0.82
Maximum concentration	54.50 ±14.31^{b}	80.56 ±24.80^{ab}	89.22 ±8.13^{a}	**0.07**
Area under pulse curve (pg/mL x h)	50.51 ±28.69	151.05 ±74.20	124.16 ±46.41	0.39

^{AB} Means within a row without a common superscript are different (P < 0.05).
^{ab} Means within a row without a common superscript are different (P < 0.1).
[x] Prominent PGFM pulse (peak of >100 pg/mL).
[y] Nadir 1 and 2 are the nadirs at the beginning and the end of a PGFM pulse, respectively.
[z] Amplitude is the difference in concentration between Nadir 1 and peak.

6.3.2 Experiment 2

6.3.2.1 Ovarian outcomes

No significant (P>0.1) interaction between the treatment group and pregnancy status was detected for to diameter of the dominant follicle at the time of artificial insemination. The diameter (mm) of the dominant follicle at the time of artificial insemination did not differ (P>0.1) between non-inseminated and inseminated heifers

and between the EB-0 and EB-1 groups. The day of structural luteolysis did not differ (P>0.1) between non-inseminated and inseminated non-pregnant heifers. However, was anticipated (P = 0.007) in the EB-1 group than in the EB-0 group (16.6 ± 0.20 vs. 17.1 ± 1.08 days), regardless of pregnancy status. The proportion of pregnant heifers did not differ (P=1.00) between EB-0 and EB-1 groups (Table 4).

When the data of the CL area were analyzed over D13 to D19 there was no significant triple interaction among treatment group, time, and pregnancy status for CL area. However, regardless of pregnancy status, there was an interaction between the treatment group and time for CL area (Fig. 9). In both groups, the CL area decreased from D15 to D16, but the reduction was more pronounced in the EB-1 than in the EB-0 group, which resulted in smaller CL area from D16 to D18 in the EB-1 group. Regardless of the treatment group and time effects, there was an effect of pregnancy status (P=0.001) on the CL area. In non-inseminated heifers, the CL area (cm^2) was smaller than pregnant heifers (2.09 ± 0.12 *vs.* 2.33 ± 0.11).

Table 4. Dominant follicle diameter at the time of artificial insemination, structural luteolysis, pregnancy rate (%) at day 28 of heifers non-inseminated and inseminated on the EB-0, EB-1 groups, treated on day 13 after ovulation.

End point	EB-0	EB-1	Probability
Non-inseminated			
No. of heifers	10	11	
Dominant follicle at AI (mm)	12.6 ± 0.5	12.8 ± 0.6	0.67
Luteolysis (days)	18.2 ± 0.6	16.6 ± 0.3	0.47
Inseminated			
No. of heifers	18	21	
Dominant follicle at AI (mm)	12.7 ± 0.5	12.8 ± 0.3	0.67
Luteolysis (days)	15.8 ± 2.3	16.6 ± 0.3	0.47
Pregnancy rate on D28	36.8% (n = 7/18)	38.1% (n = 8/21)	1.00

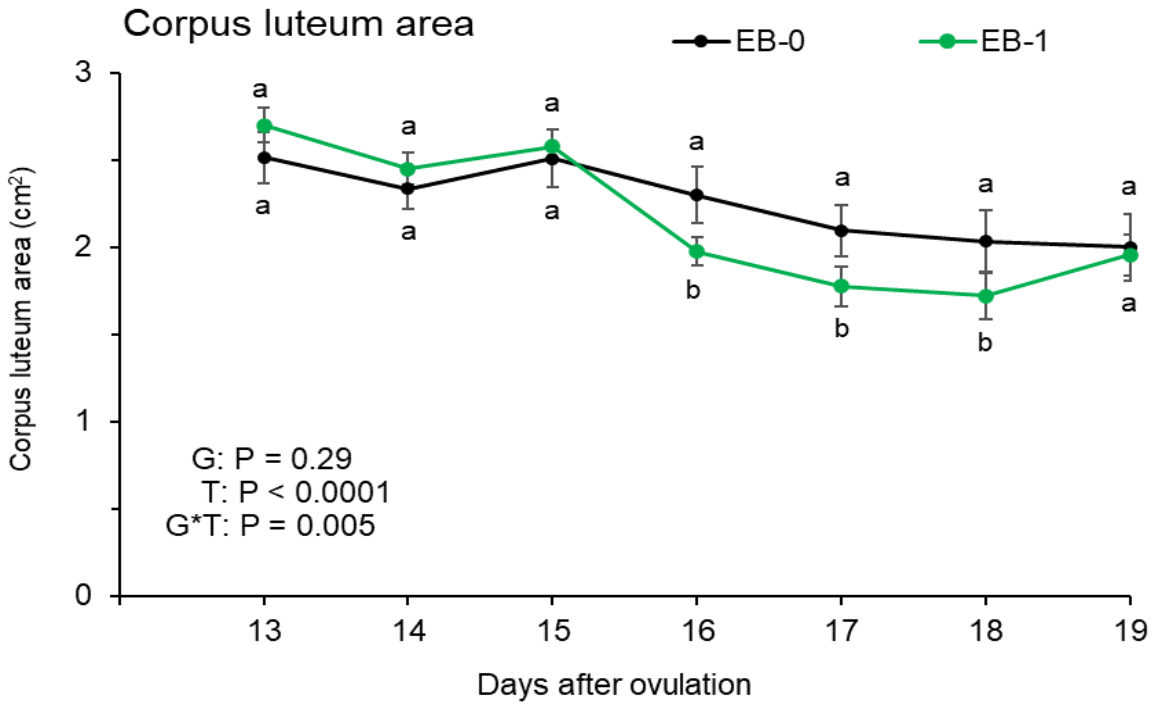

Fig 9. Mean ± SEM corpus luteum area (cm2) from 13 to 19 days after ovulation in Nelore heifers, regardless pregnancy status that receiving 0 or 1 mg of estradiol benzoate (EB) 13 days after ovulation.

Similarly to the CL area, when the data of CL blood perfusion were analyzed over D13 to D19 there was no significant triple interaction among treatment group, time, and pregnancy status for CL blood perfusion. However, there was a significant

(P = 0.05) interaction between treatment group and time and a tendency for significant interaction (P = 0.09) between status and time. In EB-0 group, the CL blood perfusion decreased from D17 to D18 and from D18 to D19; whereas in EB-1 group, the CL blood perfusion decreased from D15 to D18 (Fig. 10A). This resulted in a smaller CL area from D16 to D19 in the EB-1 group than in the EB-0 group. In non-inseminated heifers the CL blood perfusion decreased from D15 to D16 and continued to decrease until D18; whereas in inseminated pregnant heifers, the CL blood perfusion decreased from D16 to D17 and continued to decrease until D18 (Fig. 10B).

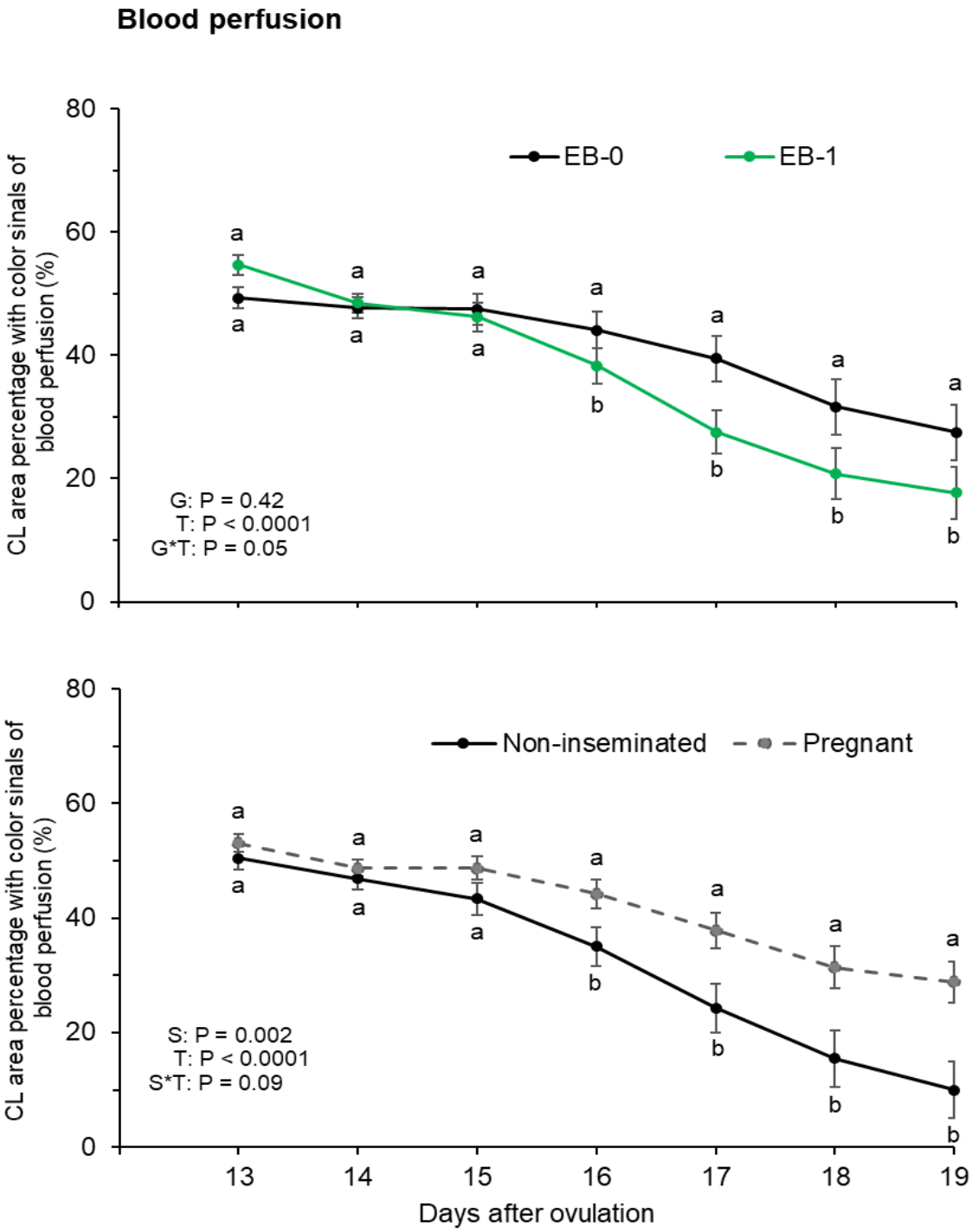

Fig 10. Mean ± SEM proportion of luteal tissue with color signals of blood perfusion from D13 to D19 of EB-0 and EB-1 group (A) and in non-inseminated and pregnant heifers (B). Main effects of treatment group (G); time (T), pregnancy status (S) and the interaction (GT and TS) that were significant are shown.

6.3.2.2 Endometrial gene expression

A significant interaction of pregnancy status by treatment group was not detected for any of the transcripts analyzed.

For the abundance of *ISG15* and *RSAD2* transcripts, no significant (P>0.1) main effect of pregnancy status and treatment group was detected (Fig. 11).

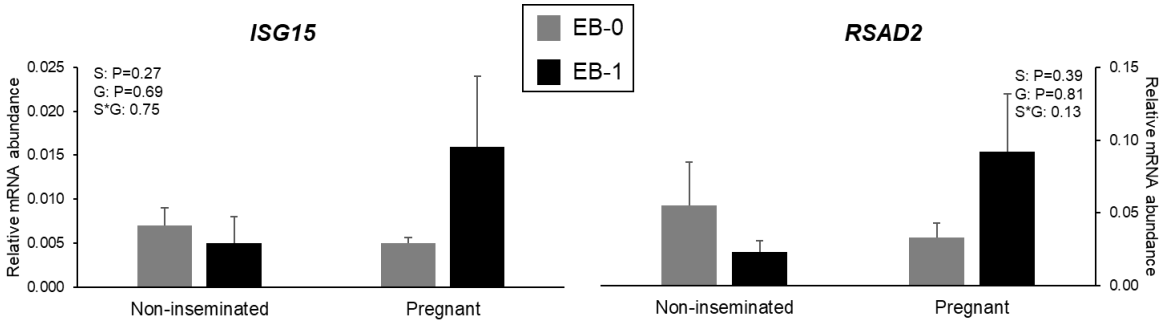

Fig 11. Mean ± standard error of the mean (SEM) of the relative mRNA abundance of ISG15 and RSAD2, in Nelore heifers, at D13 after ovulation, in animals which received no treatment, EB-0 (n = 9 non-inseminated and 7 pregnant), and from heifers, which received a treatment with1 mg of estradiol, EB-1 (n = 7 non-inseminated and 8 pregnant).

For *PGR, and OXTR,* (Fig. 12), no significant effect of pregnancy status was observed, but a significant effect of the treatment group was detected. The abundance of these transcripts was, respectively, 2.3, and 7.0-fold greater in the EB-1 group than in the EB-0 group, regardless of the pregnancy status. For *ESR1,* no significant (P>0.1) main effect of pregnancy status and treatment group was detected (Fig. 12). For the gene expression of *ESR2,* a significant effect of pregnancy status was observed (P=0.04). The expression was 9.5-fold greater in non-inseminated heifers compared to pregnant heifers.

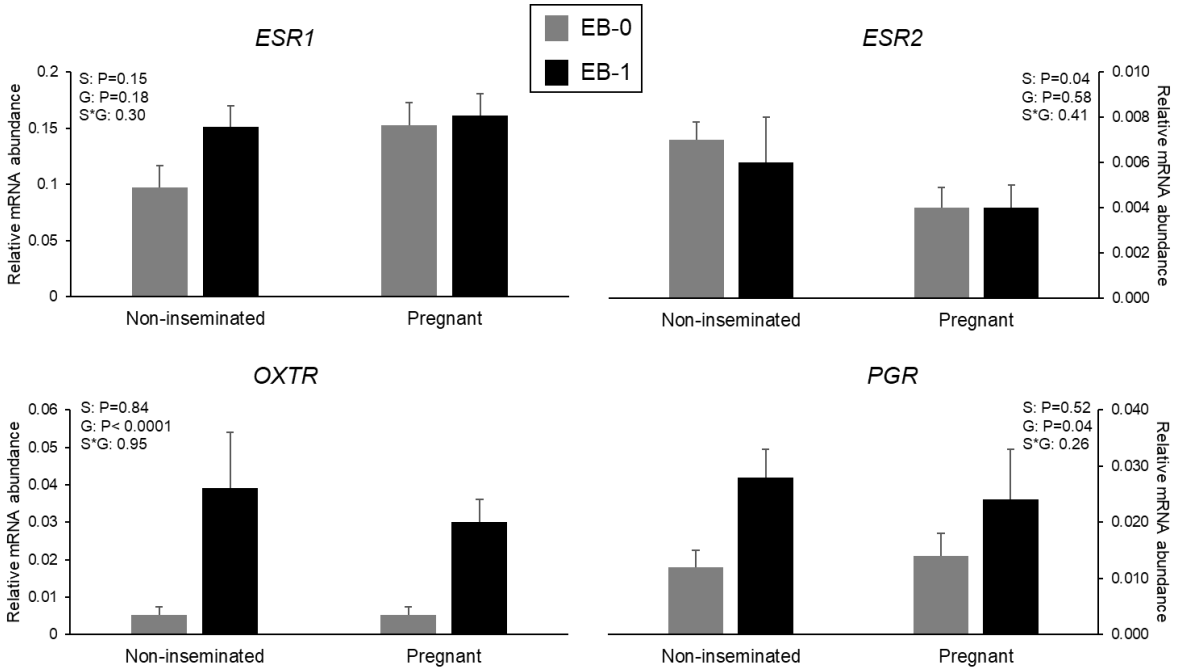

Fig 12. Mean ± standard error of the mean (SEM) of the relative mRNA abundance of *ESR1, ERS2, OXTR,* and *PGR*, in Nelore heifers, at D13 after ovulation, in animals which received no treatment, EB-0 (n = 9 non-inseminated and 7 pregnant), and from heifers, which received a treatment with1 mg of estradiol, EB-1 (n = 7 non-inseminated and 8 pregnant).

For the specific enzymes related to $PGF_{2\alpha}$ synthesis (Fig. 13), the abundance of *AKR1C4*, *PTGS2,* and *PLA2G4* was not affected by pregnancy status or treatment group (P>0.1). For *IL1-β*, a pro-inflammatory cytokine, the transcript abundance was 2.9-fold greater (P=0.005) in the EB-0 group than in the EB-1 group, regardless of pregnancy status.

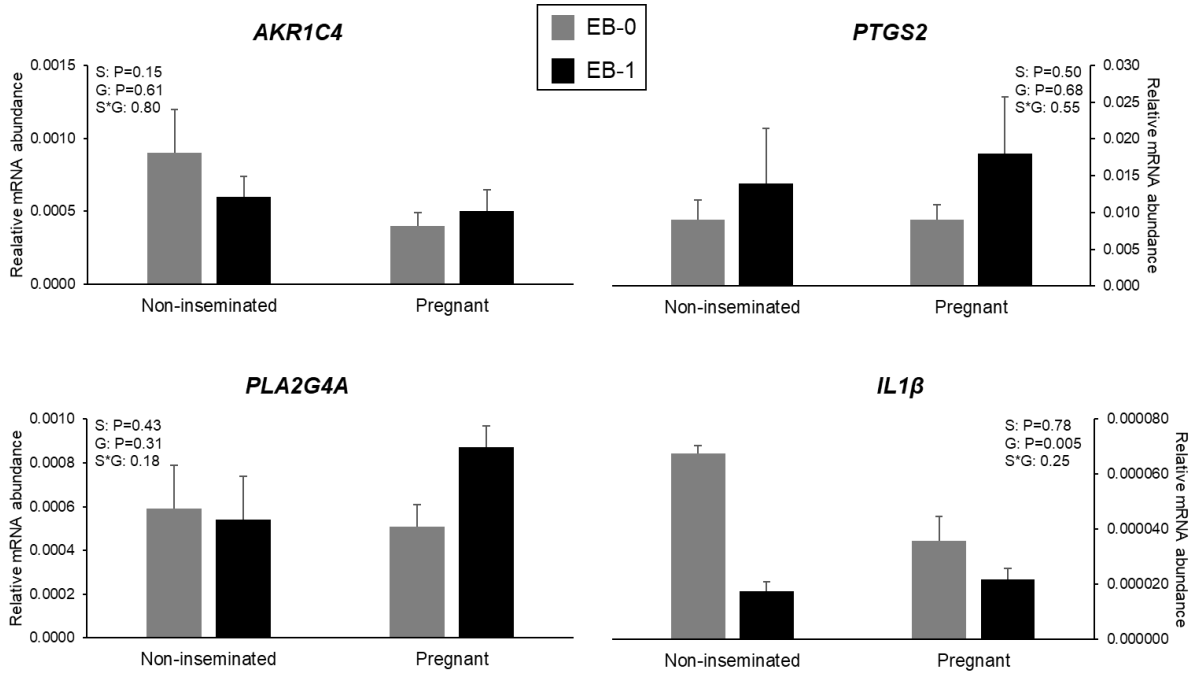

Fig 13. Mean ± standard error of the mean (SEM) of the relative mRNA abundance of enzymes related in the $PGF_{2\alpha}$ release (*AKR1C4, PTGS2 and PLA2G4*) and pro inflammatory cytokine (*IL1-β*), in Nelore heifers, at D13 after ovulation, in animals which received no treatment, EB-0 (n = 9 non-inseminated and 7 pregnant), and from heifers, which received a treatment with1 mg of estradiol, EB-1 (n = 7 non-inseminated and 8 pregnant).

7 DISCUSSION

The use of E2 esters treatment before the critical period for maternal recognition of pregnancy has increased due to the recent use of resynchronization protocols for TAI starting at 12 to 14 days after the first TAI in beef cattle, mainly in South America (PUGLIESI et al., 2023). However, a better understanding of the molecular mechanisms related to E2-induced $PGF_{2\alpha}$ synthesis in inseminated females is needed to comprehend how the $PGF_{2\alpha}$ synthesis and CL function are affected by E2 treatments in pregnant and non-pregnant animals. In the herein study, the effects of different EB doses (0, 1, and 2 mg), 13 days after ovulation in inseminated and non-inseminated *Bos indicus* heifers were investigated for the first time and indicated that doses of 1 or 2 mg EB induce $PGF_{2\alpha}$ synthesis, regardless of the pregnancy status, anticipating the luteolysis in non-pregnant animals. In addition, the 1 mg EB treatment up-regulated the transcription of the key endometrial receptors for $PGF_{2\alpha}$ synthesis within 3 hours, regardless of the pregnancy status.

In non-pregnant animals, the effectiveness of E2 on induction of $PGF_{2\alpha}$ synthesis one to several days before expected luteolysis has been previously demonstrated in cycling (non-inseminated) *Bos taurus* and *Bos indicus* cattle (ARAUJO et a., 2009; GINTHER et al., 2010; PUGLIESI et al., 2011; OLIVEIRA et al., 2022). The E2 administration 12 to 14 days after ovulation causes an increase in pulsatile secretion of $PGF_{2\alpha}$, which is characterized by a pulse of PGFM, reaching a peak between 6 to 7 hours after E2 administration (ARAUJO et al., 2009; GINTHER et al., 2010; PUGLIESI et al., 2011). Previous studies have shown that a dose-dependent stimulus of E2 on $PGF_{2\alpha}$ synthesis is observed (PUGLIESI et al., 2011). The current study differs in that an E2 ester, EB, was used instead of the E2 treatment. The effectiveness of EB treatment on stimulation of $PGF_{2\alpha}$ synthesis was already demonstrated by enhanced plasma PGFM concentrations after treatment with 1mg EB on day 13 after ovulation in *Bos taurus* heifers (ARAÚJO et al., 2009). However, differences in $PGF_{2\alpha}$ release after EB or E2 treatment have been recently reported by our group (MOTTA et al., 2021) and indicated that PGFM increased earlier and in greater concentration in heifers treated 14 days after TAI with 1 mg E2 plus 9 mg P4 than with 1 mg EB. The novel results in the present study indicate that a dose-dependent effect on PGFM concentrations is not likely to occur using 1 or 2 mg EB on day 13 post-ovulation in non-inseminated *Bos indicus* heifers*.* The averaged PGFM concentrations and PGFM pulse characteristics (peak, amplitude, concentrations during PGFM pulse) were similar between heifers receiving 1 or 2 mg EB and greater than the vehicle-treated animals. This equivalent effect is probably occurring as both doses result in an effective circulating E2 concentration for maximal $PGF_{2\alpha}$ synthesis. In this regard, 1 and 2 mg EB represents 0.72 and 1.44 mg of E2, which are doses much greater than the minimal dose of E2 reported (0.05 mg) for $PGF_{2\alpha}$ synthesis on day 14 post-ovulation (PUGLIESI et al., 2011). In addition, although greater E2 concentrations were observed in heifers treated with 2 mg EB compared to 1 mg until Hours 11/12, both resulted in Supraphysiological levels for late diestrus or during functional luteolysis (ARAUJO et al., 2009). In the present study, non-pregnant heifers (non-inseminated) treated with EB (1 or 2 mg) had anticipation of luteolysis in about one day compared to those not receiving EB. Also, the CL blood perfusion and area decreased more rapidly in EB-treated heifers regardless of the dose, reinforcing that 1mg and 2 mg EB have similar effectiveness in inducing the luteolytic mechanisms in non-pregnant animals.

Hypothesis 1 that the conceptus presence reduces the E2-stimulated pulsatile release of $PGF_{2\alpha}$ was not supported. The PGFM concentrations increased and a PGFM pulse was induced after EB treatments, independently of the pregnancy status (pregnant or non-inseminated) or EB dose. Thus, this novel result indicates that even with the presence of viable conceptus releasing IFN-t, uterine machinery for $PGF_{2\alpha}$ synthesis was not modulated to prevent the stimulus of enhanced E2 in the bloodstream. However, the preliminary analyses of PGFM response in each pregnancy status tentatively indicated that $PGF_{2\alpha}$ release induced by 1 mg EB is less pronounced in pregnant heifers. Significant increases in PGFM pulse characteristics in pregnant heifers were only observed in the EB-2 group compared to the EB-0 group. Still, no difference was observed between the 1 and 2 mg EB on PGFM concentrations and PGFM pulse characteristics in pregnant heifers, which is not in agreement with our Hypothesis 2 that the release of $PGF_{2\alpha}$ pulses and induction of luteolysis in pregnant heifers occurs in E2 dose-dependent manner. Furthermore, the E2-induced $PGF_{2\alpha}$ synthesis in inseminated heifers can be associated with the reduction in pregnancy rate in EB-treated heifers. Although not statistically significant, the negative impact of EB doses on pregnancy maintenance was apparently stronger in heifers receiving 2 mg compared to 1 mg of EB in Experiment 1, as the pregnancy rate was about 71% greater in heifers treated with 1 mg (29.2% *vs.* 50%). On the other hand, this negative effect on pregnancy establishment was not confirmed in Experiment 2 as the treatment with 1 mg EB did not impact the pregnancy rate. Previous reports have also shown that E2 can affect pregnancy maintenance (CUTAIA et al., 2002; VIEIRA et al., 2014; SILVA et al., 2022), but this impact may depend on the E2 or EB dose used. In suckled Nelore cows submitted to resynchronization, 2mg EB reduced the pregnancy rates and increased pregnancy loss compared to 1 mg EB on day 14 after TAI. These controversial results also point to several factors that could be affecting pregnancy establishment such as the age and weight of the heifers, which were different in the present experiments. Heifers from Experiment 2 were older and heavier. In addition, the pregnancy rate of heifers in the control group of experiment 1 was well above the average for this type of animal and this makes interpretation even more difficult.

The similar PGFM profile and PGFM pulse characteristics in pregnant and non-inseminated heifers are not in agreement with the expected in pregnant and non-bred animals at early gestation. Pinaffi et al. (2018) reported that although the frequency of the PGFM pulses did not differ between nonbred and pregnant heifers, the mean

PGFM concentration was lower in pregnant heifers. One possible explanation for this difference between studies may have occurred because of the timing of the sampling. In the previous study, the PGFM were compared between nonbred and pregnant heifers on days 16, 17, and 18 post ovulation; while in our study, the PGFM were compared 13 days after ovulation. An abundant increase in INF-t secretion starts 14 days of pregnancy and reaches the highest concentration on days 19 to 21 (ROBERTS et al., 2008). Therefore, it seems that before the peak of conceptus releasing IFN-t, high circulating E2 concentrations induce the uterine machinery to release a prominent $PGF_{2\alpha}$ pulse (>100 pg/mL). Also, although the EB treatment reduced the pregnancy rates in Experiment 1, it may not result in sequential prominent $PGF_{2\alpha}$ pulses capable of inducing luteolysis and pregnancy loss in all inseminated heifers. In this regard, a minimum of four sequential prominent $PGF_{2\alpha}$ are reported to be needed for complete luteolysis induction in *Bos taurus* heifers (GINTHER et al., 2010).

The uterus is a fundamental organ for the control of the estrous cycle and the establishment of pregnancy. The conceptus-derived pregnancy recognition signal in cattle, IFN-t, significantly modifies the transcriptome of the endometrium (SPENCER et al., 2007; ANTONIAZZI et al., 2013). In pregnant heifers and cows, the reduction in uterine production of $PGF_{2\alpha}$ by inhibiting OXTR and ERα (MEYER et al., 1995) is reported as one of the several mechanisms for blocking luteolysis (BAZER; THATCHER, 2017; SPENCER; HANSEN, 2015). However, one huge limitation in evaluating the local maternal response in the uterine environment is that a validated *in vivo* model for evaluation along the pregnancy recognition period in females with a further successful pregnancy has not been described. Most published studies used post-mortem samples of the endometrium, collected in specific target days during early pregnancy (SPENCER et al., 2013; SPONCHIADO et a., 2017; SPONCHIADO et a., 2019). Also, *in vivo* endometrial samples have been collected by uterine biopsies and uterine flushing at day 6 post-TAI in studies of our group (PUGLIESI et al., 2014b) without severely affecting pregnancy maintenance. Thus, to our knowledge, this is the first study to probe the superficial portion of the endometrium during an ongoing pregnancy during the elongation phase of conceptus. A negative impact of the uterine sampling on pregnancy establishment cannot discarded as the pregnancy rates in the EB-0 group were about one-third in Experiment 2 compared to Experiment 1, and only about 39% of heifers were detected as pregnant. However, the cytobrush procedure

on 13 post-ovulation allowed an effective sampling to investigate the endometrial gene expression in an ongoing pregnancy in cattle.

Therefore, using the cytobrush procedure we were able in the present study to test Hypothesis 3 - that transcription of *ESR1*, *ESR2*, and *OXTR* is up-regulated by E2 only in non-pregnant heifers. Our hypothesis was based on the assumption that the conceptus reduces the E2-stimulated pulsatile release of $PGF_{2\alpha}$ through the modulation of uterine receptors and enzymes related to $PGF_{2\alpha}$ synthesis, but it was not supported. Corroborating with the results of PGFM concentrations, the presence of conceptus did not attenuate the up-regulation of endometrial receptors and enzymes related to $PGF_{2\alpha}$ synthesis in EB-treated heifers. Expression of *OXTR* and *PGR* was upregulated in heifers treated with 1mg EB, regardless of the pregnancy status. For ERs, although expression of *ESR1* did not differ between pregnancy status and between treatment groups, the *ESR2* was reduced in pregnant heifers, regardless of EB treatment. This could indicate that expression of ERβ is down-regulated by bovine conceptus as early as day 13 post-ovulation. Previous reports, using ewes (MANSOURI-ATTIA et al., 2009; SPENCER AND BAZER, 1995) or beef cows (OLIVEIRA et al., 2022), reported that *ESR1* and *ESR2* expression are reduced in cycling animals treated with E2. Our thought about those differences could be explained by the dose and moment of the treatments. In the study by Oliveira et al. (2022), 3mg E2 in 50% ethanol infusion was given intravenously in beef cows, whereas our group used an intramuscular injection of 1mg EB in beef heifers. The higher dose of E2 may have caused down-regulation in *ESR1* and *ESR2* in the previous report. In addition, the time of evaluation and the type of sample were different between the studies. In the previous study (Oliveira et al., 2022), the samples were collected 15 days after estrus, 4 hours after treatment, by biopsy while in our study the samples were collected 13 days after ovulation, 3 hours after treatment by endometrial cytology. On day 15, receptor expression is expected to be lower than on day 13 (ROBINSON et al., 2001). Our results about the abundance of *OXTR*, agree with those observed by Oliveira et al., (2022), which was greater in animals treated with E2. The upregulation of *OXTR*, suggests, that E2 turns on *OXTR* to synthesize $PGF_{2\alpha}$ and lead to a luteolytic cascade (MCCRACKEN et al., 1999).

The synthesis of $PGF_{2\alpha}$ is induced by a series of coordinated events, which involve some specific enzymes. The arachidonic acid, derived from cleaved diacylglycerol or phospholipid, by phospholipase A2 (PLA2) through the

cyclooxygenase 2 (PTGS2) activity, is converted to prostaglandin H2 (PGH2). Finally, aldo-keto reductase 1 family member B1 (AKR1B1) and AKR1C4, act by converting PGH2 in $PGF_{2\alpha}$ (ASSELIN et al., 1997; FORTIER et al., 2008). In the present study, the abundance of the key enzymes (*AKR1C4*, *PTGS2*, *PLA2G4A)* for $PGF_{2\alpha}$ synthesis was not altered by E2 treatments. These results are according to previous studies in non-inseminated cattle (MALDONADO et al., 2021; OLIVEIRA et al., 2022). Nonetheless, more studies are necessary to evaluate the gene expression of the main enzymes involved in $PGF_{2\alpha}$ release, using *in vivo* experimental model, from animals treated with E2, on day 13 of pregnancy.

Besides the modulation of uterine transcriptome related to $PGF_{2\alpha}$ synthesis, in pregnant ruminants, IFN-t stimulates the transcription of several genes (ISGs) associated with immune modulation in the uterus, peripheral immune cells, and ovary (ROCHA et al., 2020). Expression of ISGs in the immune blood cells and uterus has been studied as an innovative and alternative method for early diagnosis of pregnancy, as soon as 20 days of pregnancy in beef (PUGLIESI et al., 2014a; DALMASO DE MELO et al., 2020) and dairy cattle (FERRAZ et al., 2021). In our experiment, expression of *ISG15* and *RSDA2* in the uterine cytological samples were not modulated by embryo presence on day 13 post-ovulation. Gene expression of *ISG15* and *RSDA2* in the endometrium was not affected by pregnancy status or treatment. In contrast, Spencer et al. (2013) showed a greater gene expression of *ISG15* and *RSAD2*, in the endometrium, in pregnant heifers compared to cyclic control heifers on day 13 after embryo transfer. The difference may be due to the type of sample. In this study, the samples were collected after slaughter from uterine washings mixed with endometrium whereas our study was the first to access the uterus (*in vivo*) of pregnant females during this period. Therefore, the determination of ISG expression by cytobrush procedure in the uterine body does not seem to be an effective method for detecting conceptus signaling at such an early stage.

Successful pregnancy has long been reported to be an anti-inflammatory phenomenon (Th_2), while pregnancy loss is associated with pro-inflammatory predominance (WEGMANN et al., 1993; RAGHUPATHY et al., 1997). Previous reports have shown a downregulation of a pro-inflammatory cytokine, *IL1-β,* in bovine uterine epithelial cells cultured with uterine flush collected on day 7 of pregnancy (RASHID et al., 2018) or treated with IFN-t (TALUKDER et al., 2018). Thus, it was expected in our study a downregulation of the *IL1-β* in the uterus of pregnant heifers. Although the IL1-

β expression was not modulated by the pregnancy status and was downregulated by E2 treatment. The mechanism by which E2 inhibits IL-1β-mediated responses by uterine epithelial cells appears to be the down-modulation of the IL-1R type I, thereby reducing the uterine epithelial cell's ability to respond to IL-1β (SCHAEFER et al., 2005).

An important factor that needs to be considered in the interpretation of the herein results indicating the EB stimulus on $PGF_{2\alpha}$ on day 13 of pregnancy is that the balance (ratio) between circulating E2 and P4 may have influenced this effect. In pregnant heifers, the application of 1mg of EB 14 days after TAI along with the insertion of an intravaginal P4 device did not induce $PGF_{2\alpha}$ release, and only the number of prominent PGFM pulses and maximum concentration were increased (MOTTA et al., 2021). Also, the treatment with 1 mg EB associated with a P4 device on days 13 or 14 after TAI for resynchronization did not affect the pregnancy rate of the first insemination (MOTTA et al., 2020; PALHAO et al., 2020). Thus, the insertion of a P4 device increases circulating P4 concentrations and may have influenced the modulation of endometrial receptors and stimulus to $PGF_{2\alpha}$ synthesis or resulted in better support for the maintenance of pregnancy, but these hypotheses need further investigation.

In conclusion, treatment with 1 or 2 mg EB, 13 days after ovulation in beef heifers results in increased plasma E2 concentrations and induces $PGF_{2\alpha}$ synthesis, regardless of the pregnancy status or the EB dose. The EB treatment anticipates luteolysis in about one day in non-pregnant animals as well as reduces the proportion of pregnant heifers. In Exp.2, heifers treated with 1 mg EB had an earlier structural luteolysis but did not reduce the proportion of pregnant heifers. Expression of *OXTR,* and *PGR* was upregulated in heifers treated with 1mg EB, regardless of the pregnancy status. The *ESR2* tended to be reduced in pregnant heifers, regardless of EB treatment. The abundance of the key enzymes (*AKR1C4*, *PTGS2*, *PLA2G4A)* for $PGF_{2\alpha}$ synthesis was not altered by E2 treatments, but IL1-β was downregulated in E2-treated heifers.

8 FINAL CONSIDERATIONS

In the first study, the pregnancy establishment was not affected, but the $PGF_{2\alpha}$ release was more stimulated and occurred earlier in bred-non-pregnant and pregnant heifers submitted to resynchronization of ovulation with an intravaginal P4 device combined with 1 mg E2 + 9 mg P4 than with 1 mg EB. Also, in pregnant heifers, 1 mg EB 14 days after TAI did not induce $PGF_{2\alpha}$ release, and only the number of prominent PGFM pulses and maximum concentration were greater than controls. However, in the Study 2, when 1 mg EB or 2 mg EB were given 13 days after ovulation without the P4 device, the proportion of pregnant heifers were reduced. This indicates that a dose dependent effect of EB could be also associated to the circulating P4 concentrations or E2/P4 ratio. The PGFM was greater in the EB-treated groups than in the control groups, regardless of EB doses. Therefore, our hypothesis is that when was necessary to treat females with E2 in the period before maternal recognition of pregnancy, it is preferable to use EB than E2 and always associate it with a source of P4.

In experiment 2, the administration of 1 or 2 mg EB anticipates the moment of lutetolysis in non-inseminated heifers, and reduced a pregnancy rate when compared to heifers that did not receive the treatment (0 mg of EB). We observed, the estrogen effects on uterine modulation, since OXTR and *PGR*, were affected by EB injection, being greater in animals treated with EB. Unexpectedly, the abundance of *ESR2* was lower in pregnant animals, regardless of E2 treatment. However, the abundance of the main enzymes involved in $PGF_{2\alpha}$ release was not affected by EB treatment. Thus, further studies are required to achieve a better understanding of the estrogen effects on uterine modulation, and the stimulation of the $PGF_{2\alpha}$ synthesis.

Finally, pregnancy status had no influence on the gene expression of *IL1-β,* but, surprisingly, animals treated with 1 mg of EB, had a downregulation of this target, evidencing, the importance of further studies to understand, the effects of an exogenous administration of estrogen, on the gene expression of proinflammatory cytokines in the endometrium on day 13 of the estrous cycle.

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