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Mechanisms involved in maintaining the corpus luteum during the first two months of pregnancy

Jéssica Nora Drum

Thesis presented to obtain the degree of Doctor in Science. Area: Animal Science and Pastures

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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DEDICATION

To my dear parents, Osmar and Tânia, my role models, for all the support. To my sister Anye, I always try to be better for you too.

You guys are the reason for all of it. I love you so much that hurts!

"Nothing in this world can take the place of persistence. Talent will not: nothing is more common than unsuccessful men with talent. Genius will not; unrewarded genius is almost a proverb. Education will not: the world is full of educated derelicts. Persistence and determination alone are omnipotent."

(Calvin Coolidge)

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SUMÁRIO

RESUMO

Mecanismos envolvidos na manutenção do corpo lúteo durante os dois primeiros meses de gestação

A progesterona (P4) produzida pelo corpo lúteo (CL), é essencial para a manutenção da gestação. Por sua vez, o interferon tau (IFNT) produzido pelo embrião durante o processo de alongamento, além de ser o sinal primário para reconhecimento e manutenção da gestação também é responsavel pela manutenção do CL durante a gestação inicial. A presença de receptores de ocitocina (OXTR) no endométrio no momento esperado da luteólise é determinante para liberação uterina de prostaglandina F2α (PGF), a qual é responsável pela regressão do CL. O IFNT evita a ocorrência da luteólise por meio da supressão da expressão de OXTR no endométrio. Entretanto, durante o segundo mês de gestação, CLs acessórios, principalmente contralaterais, são capazes de regredir, indicando que ocorre liberação de PGF pelo útero conforme a gestação avança, e os mecanismos que iniciam a luteólise são restabelecidos. Portanto, falhas na manutenção do CL podem causar luteólise e perdas gestacionais de 30 para 60 dias, um dos importantes problemas de eficiência reprodutiva em bovinos, principalmente quando embriões produzidos *in vitro* (PIV) são transferidos. Dois estudos foram delineados para estudar estes fatores, com foco em determinar o momento em que o útero gravídico retoma a liberação de PGF, e identificar prováveis diferenças entre estes mecanismos em gestações de embriões PIV ou de inseminação artificial (IA). O primeiro estudo avaliou a concentração circulante do metabólito de PGF (PGFM) após desafio com ocitocina durante os primeiros dois meses de gestação em vacas Holandesas lactantes. O tratamento com ocitocina não afetou a concentração de PGFM em vacas de d11 prenhes (P) e não-prenhes (NP), no d18 apresentou um ligeiro aumento em vacas P, enquanto aumentou cerca de duas vezes em relação ao nível basal em vacas NP. O aumento de PGFM induzido por ocitocina em vacas P no dia 25, foi maior que P em d18, entretanto foi menor que vacas P nos dias 53 e 60. Os dias 32, 39 e 46 da gestação tiveram resposta intermediária. O segundo estudo avaliou a PGFM circulante em resposta a ocitocina em vacas Nelore prenhes de embriões PIV ou IA, nos dias 17 e 31 de gestação, e sua associação com fatores que podem impactar no successo da prenhez, como P4 circulante, tamanho de concepto no d18, e o tamanho de embrião no d32. Além disso, foi avaliada e localizada a expressão de OXTR e do gene estimulado por interferon 15 (ISG15) no endométrio uterino. O tamanho de embrião no dia 32 e a P4 circulante no dia 31, foram maiores no grupo IA. Vacas do grupo PIV d17 apresentaram menor resposta a ocitocina na concentração de PGFM do que as de IA no mesmo dia, contudo no dia 31 ambos os grupos tiveram maior resposta do que PIV d17. As vacas do d31 dos dois grupos tiveram aumento na PGFM similar às vacas não-inseminadas (NI). Os OXTR foram altamente suprimidos nas vacas prenhes do d18, especialmente no grupo PIV, mas com alta expressão em vacas NI e no dia 32 para os dois grupos, sendo a IA com maior expressão que a PIV neste dia. O gene ISG15 apresentou expressão irrelevante em NI e d32 para IA e PIV, mas apresentou expressão extremamente alta no d18 nos dois grupos prenhes. Conclui-se que o CL na gestação inicial é mantido pela supressão da liberação de PGF,

enquanto que no segundo mês, ocitocina induz liberação de PGF, sugerindo que outros mecanismos regem a manutenção do CL a partir do dia 25. Além disso, nossos resultados demonstram que há diferenças entre a sinalização de gestações provenientes de embriões PIV e IA, que impactam no ambiente molecular e endócrino, influenciando a liberação de PGF nestes momentos.

Palavras-chave: Bovino; Concepto; Gestação; Ocitocina; Prostaglandinas

ABSTRACT

Mechanisms involved in maintaining the corpus luteum during the first two months of pregnancy

The progesterone (P4) produced by the corpus luteum (CL) is essential for maintenance of pregnancy. On the other hand, the interferon tau (IFNT) produced by the embryo during elongation process, besides being the primary signal for recognition, also is responsible for maintenance of the CL during early pregnancy. The presence of oxytocin receptors (OXTR) in endometrium during expected time of luteolysis is determinant for trigger the uterine release of prostaglandin F2α (PGF), which is in charge of CL regression. The IFNT avoid the luteolysis by suppressing the OXTR appearance. However, during second month, accessory CLs are able to regress, indicating that the PGF release occurs with the advancing of the pregnancy and the mechanisms that initiated luteolysis are recovered. Therefore, failures in maintenance of the CL can cause luteolysis and pregnancy loss during this period of 30 to 60 days, which is one of the most important problems in reproductive efficiency in cattle, specially when *in vitro* produced (IVP) embryos are transferred. Two experiments were designed to study this factors, focused on point when uterus recover its PGF release during pregnancy and to identify possible differences between those mechanisms on pregnancies from IVP or artificial insemination (AI) embryos. The first study evaluated circulating PGF metabolite (PGFM) after an oxytocin challenge throughout first two months of pregnancy in lactating Holstein cows. Treatment with oxytocin did not affected PGFM concentration in d11 pregnant (P) and non-pregnant (NP), on d18 had a little increase in P cows, while increased 2-fold in NP cows. Oxytocin-induced PGFM in P cows on day 25 was greater than d18 P, however was lower than P cows on d53 and d60. Days 32, 39 and 46 of pregnancy had intermediate response. The second study evaluated the oxytocin-induced PGFM in Nelore cows pregnant from AI or IVP embryos on days 17 and 31, and its association with factors that can impact in success of the pregnancy, such as P4 levels, conceptus length on d18 and size of the embryo on d32. Also, OXTR and interferon-stimulated gene 15 (ISG15) gene expression were evaluated and located in uterine endometrium. Embryo size on d32 and P4 on d31, were higher in AI than IVP. Cows from IVP on d17 presented lower oxytocin-induced PGFM than AI in the same day, however, d31 for both groups had higher PGF release after oxytocin. On d31 there was similar PGFM increase in synchronized non-inseminated group (NI). The OXTR are highly suppressed on pregnant cows on d18, especially in IVP group, but were high expressed in NI cows and on d32 for both groups, AI being higher than IVP at this day. The ISG15 had irrelevant expression on NI and d32 groups, while had extremely high expression in d18 pregnant cows for both groups. Concluding, the CL in early pregnancy is maintained by PGF release suppression, while during second month there is oxytocin-induced PGF release, suggesting that other mechanisms are responsible for maintaining CL after d25. In addition, these results demonstrate there are signaling differences between IVP and AI pregnancies, impacting the molecular and endocrine environment that influences PGF release during these time points.

Keywords: Bovine; Conceptus; Pregnancy; Oxytocin: Prostaglandins

1. INTRODUCTION

The use of assisted reproductive technologies in cattle is been increasing in the past few years. For example, fixed-timed artificial insemination (FTAI) procedures are responsible for 85% of all artificial insemination (AI) done in Brazil. Despite the numbers of *in vivo* derived (IVD) embryos tended to decrease in the last 10 years, *in vitro* production (IVP) of embryos has increased significantly, with more than 57% of all IVP embryos in the world produced in Brazil [1, 2].

Recent data from approximately 25,000 pregnancies in cattle, have shown there is around 12% pregnancy loss between 32 and 60 days of pregnancy [3]. Although the IVP has been improving significantly in technology and efficiency along the years, we still face big differences between conception rates (P/AI or P/ET) compared to FTAI (34.0% - 195/573 vs 50.3% – 174/346). In addition, by evaluating pregnancy loss from 30 to 60 days, the difference becomes even bigger (15.9% vs 5.2%) getting worst when healthy births per protocol of synchronization are evaluated (17,3% vs 39,6%, [2]). This suggests that those biotechnologies have greater failure in maintaining pregnancy during the period from 30 to 60 days of pregnancy and because of that, it is important to understand which factors determine pregnancy success or loss.

The primary hormone of gestation, progesterone (P4) which is responsible for the establishment and maintenance of pregnancy is produced by the temporary gland called corpus luteum (CL) [4, 5]. In cows, maintenance of pregnancy requires the presence of a functional CL through at least day 180-200 of gestation, yet little is known about CL maintenance after 25 days [6, 7]. Luteolysis during a normal estrous cycle, involves dynamic functional and structural changes in the CL that result in depletion of luteal P4 production and a complete collapse of CL integrity, blood flow, and tissue volume [8-10].

In ruminants, the secretion of prostaglandin F2α (PGF) from the uterus underlies the luteolytic process, with multiple distinct pulses of uterine PGF observed during luteolysis [8, 10-14]. These PGF pulses are initiated by oxytocin pulses secreted by the posterior pituitary gland [10, 15-17]. Acquisition of uterine oxytocin responsiveness is key to the timing of luteolysis in ruminants. The expression of oxytocin receptors in the endometrium occurs just before the time of normal luteolysis, initiated by follicular estradiol-17β binding to ESR1 (estrogen receptor alpha) in the endometrial cells [18-21]. Expression of endometrial oxytocin receptors allows oxytocin to activate a cascade of enzymes that release arachidonic acid from membrane phospholipids and convert it into PGF that is secreted in pulses [10, 22, 23]. Some

of the PGF from the oxytocin-induced pulses is transported locally from the utero-ovarian vein to the ipsilateral ovarian artery and these PGF pulses induce the luteolytic process [10, 14, 24, 25]. Thus, oxytocin pulses, endometrial oxytocin responsiveness, and oxytocin-induced pulses of PGF are central to the process that eliminates the CL in a non-pregnant ruminant and hence, initiation of a new estrous cycle.

During gestation though, the interferon-tau (IFNT) is responsible by maternal recognition of pregnancy in cattle [5] and by blockade of the luteolytic process [26, 27]. The IFNT is an antiviral, antiproliferative, and immunomodulatory molecule that is secreted by the trophectoderm of the ruminant conceptus, but only during a limited stage of pregnancy, with maximal secretion between d17 and 20 after breeding [28-32]. Early pregnancy or intra-uterine infusion of IFNT is able to inhibit the normal induction of endometrial oxytocin receptors, probably by inhibiting expression of endometrial estrogen receptors [21, 33, 34]. Other action of the IFNT is stimulating the expression of specific genes, including a group of characteristic genes, termed interferon-stimulated genes (ISGs). The ISGs have been detected during early ruminant pregnancy in many tissues including endometrium, CL, and peripheral blood leukocytes (PBL) and this ISGs expression can be used as a marker for diagnosing early pregnancy [35, 36].

Nonetheless, there is a dramatic decrease in ISGs detected in PBL by d25 of pregnancy [36, 37], reflecting the loss of IFNT expression in the embryo by d25 [28, 38]. Consistent with this idea, intrauterine infusion of ovine embryonic homogenates from d14-15 extended CL lifespan, whereas, homogenates of d21-25 embryos did not alter CL lifespan [39, 40]. Thus, IFNT is critical for blocking luteolysis and maintaining the CL of early pregnancy but is not present and is likely not responsible for maintaining the CL in the second month of pregnancy.

Considering everything previously mentioned, there are two reasons that can lead to pregnancy loss in this period: embryo death leading to early luteolysis, hence pregnancy loss, or the luteolysis can be initiated in an erroneous time leading to defect in maintenance of pregnancy [41].

Some studies sustain that the most common cause for late pregnancy loss in IVP embryos is placentary anomalies [42, 43], and it can be related to defective development of the placenta, specially due to abnormal epigenetic reprograming caused by IVP systems [44-46] leading to embryo death. Also, it has been reported that IVP systems are able to modify embryo gene expression profile, hence affecting development and signaling [47, 48]. Corroborating evidences that IVP embryos fail in maintaining pregnancy, there were more

degenerate or broken d16 embryos from IVP systems (7/28, 25%) than from AI (2/34, 5%). Moreover, embryonic discs on d16 embryos were smaller for the IVP group [44].

Regarding luteolysis as primary cause of pregnancy loss, it is known that an accessory CL when ipsilateral to the pregnancy rarely regresses, while almost all contralateral accessory CLs undergo luteolysis during the second month of pregnancy [49]. These findings reiterate the theory that pregnancy and CL are maintained by local mechanisms at the ipsilateral horn, and luteolysis mechanisms can be triggered contralaterally despite the pregnancy signals. One of the possibilities to explain the absence of luteolysis in the ovary ipsilateral to the pregnancy at the second month, is the luteoprotective action of some molecules produced by the embryo. Prostaglandin E (PGE) is a strong candidate for the job. Pregnant cows have been described as having higher concentration of PGE inside the uterine lumen than non-pregnant cows and PGE intrauterine infusion is able to avoid luteolysis even in the presence of PGF [50, 51]. However, PGF and its metabolite (PGFM) are also higher in pregnant cows, both in uterine lumen and circulation [50, 52], however, little is known about the pulsatility of PGF during this period.

Recently, pulsatile PGF patterns have been studied during the second month of pregnancy in dairy cattle suggesting that inhibition of uterine PGF release may not be the mechanism responsible for maintaining the CL after the first month of pregnancy. Evaluation of PGF release or PGFM concentrationafter oxytocin challenge has been an important method for assessing uterine oxytocin responsiveness in pregnant (P) and non-pregnant (NP) ruminants [18, 34, 53-55]. Treatment with oxytocin on day 18-20 of the cycle or pregnancy increased circulating PGFM in NP heifers, however, in P heifers this response was suppressed [53, 56, 57]. A similar suppression of oxytocin-induced PGFM has been observed when IFNT was infused into the uterus [58, 59]. Few studies have evaluated oxytocin action or oxytocin receptor expression after the first month of bovine pregnancy [60, 61].

Identification of uterine elements that lead to endocrine responses during gestation are important to understand the physiology of pregnancy. One important factor to be studied is the PGF release under oxytocin influence during pregnancy. Manipulative and descriptive studies and comparisons of IVP and AI embryos, can help to elucidate how these factors are acting, and how they affect the signaling for maternal recognition and maintenance of pregnancy during the second month and contribute to improve reproductive efficiency in cattle by reducing pregnancy loss.

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2. OXYTOCIN-INDUCED PROSTAGLANDIN F2-ALPHA RELEASE IS LOW IN EARLY BOVINE PREGNANCY BUT INCREASES DURING SECOND MONTH OF PREGNANCY

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Abstract

Circulating prostaglandin F2α metabolite (PGFM) after an oxytocin challenge was evaluated throughout the first 2 months of pregnancy in lactating Holstein cows. On d11, 18, and 25 after AI, and on d32, 39, 46, 53, and 60 of pregnancy, cows were challenged with 50 IU oxytocin, i.m. Blood was collected before (0 min), 30, 60, 90, and 120 min after oxytocin for plasma PGFM concentrations. Ultrasound evaluations were performed for pregnancy diagnosis on d32 to 60 post-AI. Nonpregnant (NP) cows on d18 were designated by lack of interferon-stimulated genes in peripheral blood leukocytes and Pregnant (P) based on d32 ultrasound. On d11, P and NP were similar with low PGFM and no effect of oxytocin on PGFM. On d18, oxytocin increased PGFM (3-fold) in NP with little change in P cows. Comparing only P cows from d11 to 60, basal circulating PGFM increased as pregnancy progressed, with d11 and d18, lower than all days from d25 to d60 of pregnancy. Oxytocin-induced PGFM in P cows on d25 was greater than P cows on d18 (2.9-fold). However, oxytocin-induced PGFM was lower on d25 compared to d53 and 60, with intermediate values on d32, 39, and 46 of pregnancy. Thus, the CL of early pregnancy (d11, d18) is maintained by suppression of PGF, as reflected by suppressed PGFM in this study. However, during second month of pregnancy, uterine PGF secretion was not suppressed since basal PGFM and oxytocin-induced PGFM secretion were elevated. Apparently, mechanisms other than suppression of oxytocin receptors maintain CL after d25 of pregnancy.

Keywords: Bovine; Conceptus; Pregnancy; Oxytocin; Prostaglandins

2.1. Introduction

Luteolysis involves dynamic functional and structural changes in the corpus luteum (CL) that result in elimination of luteal progesterone (P4) production in about a 24 h period

and a complete breakdown of CL integrity, blood flow, and tissue volume [1-3]. In ruminants, the secretion of Prostaglandin F2α (PGF) from the uterus clearly underlies the luteolytic process, with multiple distinct pulses of uterine PGF observed during luteolysis [1, 3-7]. These PGF pulses are initiated by oxytocin pulses secreted from the posterior pituitary gland [3, 8-10]. Acquisition of uterine oxytocin responsiveness is key to the timing of luteolysis in ruminants. The expression of oxytocin receptors in the endometrium occurs just before the time of normal luteolysis, initiated by follicular estradiol-17β binding to ESR1 (estrogen receptor alpha) in the endometrial cells [11-14]. Expression of endometrial oxytocin receptors allows oxytocin to activate a cascade of enzymes that release arachidonic acid from membrane phospholipids and convert it into PGF that is secreted in pulses [3, 15, 16]. Some of the PGF from the oxytocin-induced pulses is transported locally from the utero-ovarian vein to the ipsilateral ovarian artery and these PGF pulses induce the luteolytic process [3, 7, 17, 18]. Thus, oxytocin pulses, endometrial oxytocin responsiveness, and oxytocin-induced pulses of PGF are central to the process that eliminates the CL in a NP ruminant and leads to initiation of a new estrous cycle.

During early pregnancy, there is blockade of the luteolytic process due to secretion of interferon-tau (IFNT) by the elongating embryo. The IFNT is an antiviral, antiproliferative, and immunomodulatory molecule that is secreted by the trophectoderm of the ruminant conceptus, but only during a limited stage of pregnancy, with maximal secretion between d17 and 20 after breeding [19-23]. Early reports showed that early pregnancy or intra-uterine infusion of IFNT inhibited the normal induction of endometrial oxytocin receptors, probably by inhibiting expression of endometrial estrogen receptors [14, 24, 25]. In addition, IFNT stimulates the expression of specific genes, including a group of characteristic genes, termed the interferon-stimulated genes (ISGs). The ISGs have been detected during early ruminant pregnancy in many tissues including endometrium, CL, and peripheral blood leukocytes (PBL) [26]. Detection of ISGs in PBL, has been used as a marker of early pregnancy [27-32]. Nevertheless, there is a dramatic decrease in ISGs that are detected in PBL by d25 of pregnancy [27, 32], reflecting the loss of IFNT expression in the embryo by d25 [19, 33]. Consistent with this idea, intrauterine infusion of ovine embryonic homogenates from d14-15 extended CL lifespan, while, homogenates of d21-25 embryos did not alter CL lifespan [34, 35]. Thus, IFNT is critical for blocking luteolysis and maintaining the CL of early pregnancy but is not present and is likely not responsible for maintaining the CL in the second month of pregnancy.

The mechanisms that maintain the ruminant CL during the second month of pregnancy have not been adequately investigated. Recently, pulsatile PGF patterns have been found during the second month of pregnancy in dairy cattle suggesting that inhibition of uterine PGF secretion may not be the mechanism responsible for maintaining the CL after the first month of pregnancy [36]. The measurement of secreted PGF or PGFM after an oxytocin challenge has been an important method for evaluating uterine oxytocin responsiveness in P and NP ruminants [11, 25, 37-39]. Treatment with oxytocin on d18-20 of the cycle or pregnancy increased circulating PGFM in NP heifers, however, in P heifers this response was suppressed [37, 40, 41]. A similar suppression of oxytocin-induced PGFM has been observed when IFNT is infused into the uterus [42, 43]. Few studies have evaluated oxytocin action or oxytocin receptor expression after the first month of bovine pregnancy [44, 45]. One study reported that 100 IU of oxytocin, i.v. increased circulating PGFM on d50 of pregnancy but the response was much greater in cows treated with oxytocin on d150, 250, and 280 of pregnancy [44]. Similarly, oxytocin receptor was detectable but low on d50 of pregnancy and increased 6-fold by d280 of pregnancy [44]. In addition, endometrial oxytocin receptors were reported to be low on d20 (165 fmol/mg protein) and d50 (344 fmol/mg) of pregnancy but increased during later pregnancy [45]. No previous studies have systematically evaluated the changes in oxytocin responsiveness of the uterus during the first and second month of pregnancy.

Therefore, we hypothesized that oxytocin responsiveness, in terms of circulating PGFM profile after an oxytocin challenge, changes during the first 2 months of pregnancy in cattle. Our first specific hypothesis was that an oxytocin challenge would induce a much smaller increase in circulating PGFM on d18 of pregnancy compared to d18 NP cows. Our second hypothesis was that the PGFM response to an oxytocin challenge would increase as pregnancy progressed with d18 being lower than d25 and subsequent increases in PGFM response to oxytocin on d32 until d60 of pregnancy. Thus, the main objective of this study was to evaluate and characterize the profile of PGFM before and during an oxytocin challenge, using a week-by-week systematic approach, throughout the first 60 d of pregnancy in lactating dairy cows.

2.2. Material and methods

2.2.1. Experimental procedures

The experiment was conducted at Arlington Agricultural Research Station - University of Wisconsin - Madison, Arlington, WI. Animals were handled in accordance with the United States Department of Agriculture Guide for Care and Use of Agricultural Animals in Research, under an animal protocol that was approved by the Animal Care and Use Committee of the University of Wisconsin - Madison (RARC# A05712-A01). A total of 171 lactating Holstein cows (n = 14 primiparous and n = 157 multiparous), at 170.9 ± 4.1 DIM (ranging from 85 to 289), were enrolled in the experiment. Cows received the same TMR to meet or exceed the nutrient requirements for a lactating Holstein cow producing 50 kg of milk/d with 3.5% fat and 3.1% true protein when DM intake was 24 kg/d [46]. Cows were submitted to an Ovsynch protocol (GnRH – 7 d – PGF – 1 d – PGF – 32 h – GnRH – 16 h – AI [47]) and all cows received AI at a fixed time. On specific days after AI, d11 (n = 23), d18 (n = 23), d25 (n = 30), d32 (n = 13), d39 (n = 13), d46 (n = 12), d53 (n = 13), and d60 (n = 12), cows were enrolled in the experiment.

Independent of the day after AI, all cows were submitted to an oxytocin challenge as described in Figure 1. A blood sample was collected by puncture of the coccygeal vein or artery, at time 0, before challenge, in order to obtain basal circulating concentration of PGFM, P4, and pregnancy-specific protein B (PSPB). After collection of the first blood sample, cows received 50 IU i.m. of oxytocin (Agrilab, St. Joseph, MO) as described by Macuhová [48] and had serial blood samples collected at times 30, 60, 90, and 120 min after challenge. This treatment was chosen in order to provide an oxytocin challenge greater than what normally occurs during milking, however not as acute and high as if oxytocin were given i.v. and in higher doses.

Timeline Days after AI (N)	11 (n = 23)	18 (n = 23)	25 (n = 30)	32 (n = 13)	39 (n = 13)	46 (n = 12)	53 (n = 13)	60 (n = 12)
Non-Pregnant	Yes	Yes	No	No	No	No	No	No
Pregnant	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Oxytocin Challenge	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes
ISGs blood sampling	Yes	Yes	No	No	No	No	No	No
Pregnancy Diagnosis	No	No	No	Yes	Yes	Yes	Yes	Yes
PSPB blood sampling	No	No	Yes	Yes	Yes	Yes	Yes	Yes

Blood sampling for oxytocin challenge

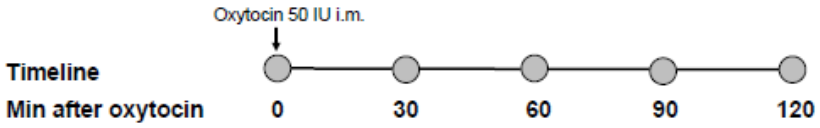

Figure 1. Schematic experimental design showing the measurements that were performed on different days and the timing of oxytocin treatment and blood sample collection.

Blood samples were collected into heparinized tubes (Vacutainer; Becton Dickinson, Franklin Lakes, NJ) that were placed on ice and kept refrigerated until transported to the laboratory within 4 to 5 h for processing. Blood tubes were centrifuged at 1,700 x g for 15 min at 4ºC for plasma separation. Aliquots of plasma were frozen at -20ºC until assayed.

On d18 for cows from groups d11 and d18, blood samples were collected into a TempusTM blood RNA tube (Applied Biosystems™, Foster City, CA) following manufacturer's instructions for ISGs analysis. On d32, 39, 46, 53 and 60 after AI, a transrectal ultrasound exam using a real-time B-mode ultrasound scanner (Ibex Pro+Lite, E. I. Medical Imaging, Loveland, CO) equipped with a 7.5 MHz linear-array transducer was performed in all cows by the same technician, in order to identify the amniotic vesicle and heartbeat of the embryo/fetus as pregnancy diagnosis. For d25, analyses were only done with cows that were diagnosed pregnant on d32 and d60. Information for cows that were collected on d25 but were NP were not used.

2.2.2. Hormone assays

Plasma samples were assayed for PGFM by an ELISA assay that was previously validated for use in bovine plasma [49] with some modifications as described by Ochoa et al.

[50]. The intra- and inter-assay CVs were 4.7% and 15.3%, respectively, and the sensitivity of the assay was 5.5 pg/mL.

Concentrations of P4 were determined using a solid-phase RIA kit containing antibody-coated tubes and 125I-labeled P4 (ImmuChem Coated Tube P4 125 RIA Kit, MP Biomedicals, Costa Mesa, CA) as described previously for mares [51] and validated for bovine plasma in our laboratory as reported [52]. The intra- and inter-assay CVs and the sensitivity were 4.4%, 8.1%, and 0.08 ng/mL, respectively.

The PSPB concentrations were analyzed on d25 to d60 of pregnancy at time 0 (before challenge) and on d53 for all samples during the oxytocin challenge. A commercially available quantitative ELISA assay was used (Biopryn, BioTracking LLC, Moscow, ID) as previously described [53]. The inter- and intra-assay CVs were 2.3% and 2.2%, respectively for the 2 plates.

All cows from d11 and 18 had whole blood collected into evacuated tubes for evaluation of ISGs in PBL (TempusTM Blood RNA tubes, Applied Biosystems, Foster City, CA). After collection, samples were stored at -20ºC until RNA extraction and DNAse treatment were performed using a commercial kit (TempusTM, Spin RNA isolation kit, Cat. No. 4380204, Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Isolated RNA was evaluated for concentration and purity using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Rockford, IL). A total of 250 ng of RNA were reverse transcribed to complementary DNA using a commercial kit (iScriptTM reverse transcription supermix for RT-qPCR, Cat. No. 1708841, BioRad, Hercules, CA) following manufacturer's instructions. After an initial activation at 60oC for 2 min followed by denaturation at 95oC for 10 min, the amplification protocol followed 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was evaluated in triplicate, and the specificity for amplification was verified by melting curve analysis. Four genes were investigated (Table 1), including the 2 reference genes, beta-actin (ACTB) and ribosomal protein L19 (RPL19), and 2 target genes, ISG15 and MX2.

Table 1. Gene, primer orientation, primer sequence (5' to 3′), and National Center for Biotechnology Information (NCBI) access number and sequence for primers used in RT-qPCR assay

Gene	Primer	Sequence (5' to 3')	NCBI sequence
ACTB	Forward	CTGGACTTCGAGCAGGAGAT	AY141970
	Reverse	GATGTCGACGTCACACTTC	
ISG15	Forward	GGTATCCGAGCTGAAGCAGTT	NM_174366
	Reverse	ACCTCCCTGCTGTCAAGGT	
RPL19	Forward	ATTGACCGCCACATGTATCA	NM_001040516
	Reverse	GCGTGCTTCCTTGGTCTTAG	
MX2	Forward	CTTCAGAGACGCCTCAGTCG	NM_173941
	Reverse	TGAAGCAGCCAGGAATAGTG	

2.2.3. Data handling and statistical analysis

The ISGs data were analyzed using the ΔΔCt method and blood samples from cows on d11 and 18 were used to confirm NP cows. Data from d11 were used as a negative control for ISGs. On d18, expression of ISGs from cows confirmed pregnant by ultrasound on d32 was used as a positive control. The highest value of ISG15 and MX2 expression in fold change on d11 was determined as a cut-off value (2.5 and 2.3, respectively). Cows on d11 with confirmed absence of any sign of an embryo on d32 were considered NP, although because all cows had been bred, a conceptus could have been present on d11 but died prior to pregnancy diagnosis. Cows on d18 that were diagnosed NP on d32 and that had higher expression of ISG15 and MX2 mRNA than the cut-off value were excluded from subsequent analyses. Cows were only considered NP if they had low expression of ISGs on d18 with confirmed absence of any sign of an embryo on d32. For groups d25, d32, d39, d46, d53, and d60, cows were diagnosed for pregnancy on d32 and had pregnancy confirmed weekly.

Continuous data were tested for normality of residuals using the UNIVARIATE procedure according to Shapiro-Wilk test. Data with residuals not normally distributed were transformed to logarithm, square root, or inverse scale before analysis if residual distribution was improved. In addition, outliers were removed when necessary, and then data were analyzed using the MIXED procedure of Statistical Analysis System (SAS, Version 9.4 for Windows, SAS Institute Inc., Cary, NC). Tukey honest significant difference test was performed to determine differences.

For P4, data were analyzed before oxytocin challenge (time 0) to obtain the basal concentrations. The P4 data on d11 and d18 at time 0 were analyzed separately to compare

potential differences between P and NP cows. The model included effect of pregnancy and day after AI and their interaction. Another comparison was made for P cows from d25 to 60 and included the effect of day of pregnancy.

In order to detect the effect of oxytocin on circulating P4, data were analyzed as repeated measures over time. The same comparisons and models used for basal P4 were performed, but included time (before and after oxytocin) as the repeated statement in which before was the circulating P4 at time 0 and after was the average of P4 concentration on 30, 60, 90, and 120 minutes after challenge.

In order to evaluate week-to-week differences in circulating PGFM in response to oxytocin challenge, comparisons were performed between specific days of pregnancy in which challenge time (0, 30, 60, 90, and 120 min) was considered the repeated statement, and the effects of day, pregnancy and their interaction were evaluated. Comparisons that were made included: d18 and d11 for P vs NP cows, d18-P vs d25, d25 vs d32, d32 vs d39, d39 vs d46 vs d53 vs d60.

To characterize the variation in circulating PGFM for d18 NP cows, the distribution of PGFM before (time 0) and after challenge (average of 30, 60, 90, and 120 min) was presented with data organized for each cow numbered from 1 to 8.

The basal PGFM, considering the circulating concentration at challenge time 0, was compared among groups. The model was composed of fixed effects of pregnancy, day and their interaction.

Another analysis evaluated circulating PGFM concentration in response to oxytocin, comparing the groups (d11 [P or NP], d18 [P or NP], d25, d32, d39, d46, d53 and d60 [P]). In order to obtain the isolated effect of oxytocin challenge, the baseline concentration of PGFM at time 0 (before challenge) was subtracted from averaged results of 60 to 120 min after oxytocin. Mean responses were compared among groups. In this analysis, the effects of day and pregnancy status, as well their interaction, were included in the model.

The basal PSPB before challenge (time 0) was compared among days of pregnancy, from d25 to 60. For this analysis, the effect of day was included.

To demonstrate the potential effect of the oxytocin challenge on circulating PGFM, P4 and PSPB, the circulating concentrations of all three variables from cows on d53 of pregnancy were analyzed during challenge times (0, 30, 60, 90, and 120 min). In this case, data were analyzed as repeated measures, using time as the repeated statement. However, since the analysis was performed using a single day, only the effect of challenge time was considered in the model.

Differences were considered significant for $P \leq 0.05$, whereas a tendency was designated when $P < 0.10$ and $P > 0.05$. Data are presented as least squares means ± SEM.Tukey honest significant difference post hoc test was performed to determine differences.

2.3. Results

Three cows from d18 that were diagnosed as NP on d32, had elevated ISGs on d18 and therefore were excluded from the analyses. One cow from the NP group, had low P4 on d18 (≤ 0.15 ng/mL), indicating that this cow had already undergone luteolysis and this cow was not used in the analyses of PGFM.

Basal circulating P4 (Time 0) tended to be greater for d18 compared to d11 (Figure 2A; P = 0.06) but there was no effect of pregnancy (P = 0.53). After d25 there was no effect of day (P = 0.76) on basal circulating P4 (Figure 2B). Challenge time 30 to 120 were averaged and compared to 0 min (basal concentration) to evaluate the effect of oxytocin challenge on circulating P4. As shown in Figure 3A, on d18 (before and after challenge), the cows had greater P4 than on d11 (P = 0.01), however there was no effect of oxytocin challenge (Challenge Time; P = 0.13) or pregnancy (P = 0.92) on circulating P4. Interactions among factors were not significant (see legend; P > 0.10). A similar comparison of circulating P4 after d25 of pregnancy (Figure 3B) detected no effect of day of pregnancy (P = 0.72) and no interaction between challenge time and day of pregnancy (P = 0.97). Curiously circulating P4 tended to be slightly greater after oxytocin challenge (P = 0.09), although there were no detectable differences before and after challenge for any day of pregnancy (analyzed within a day).

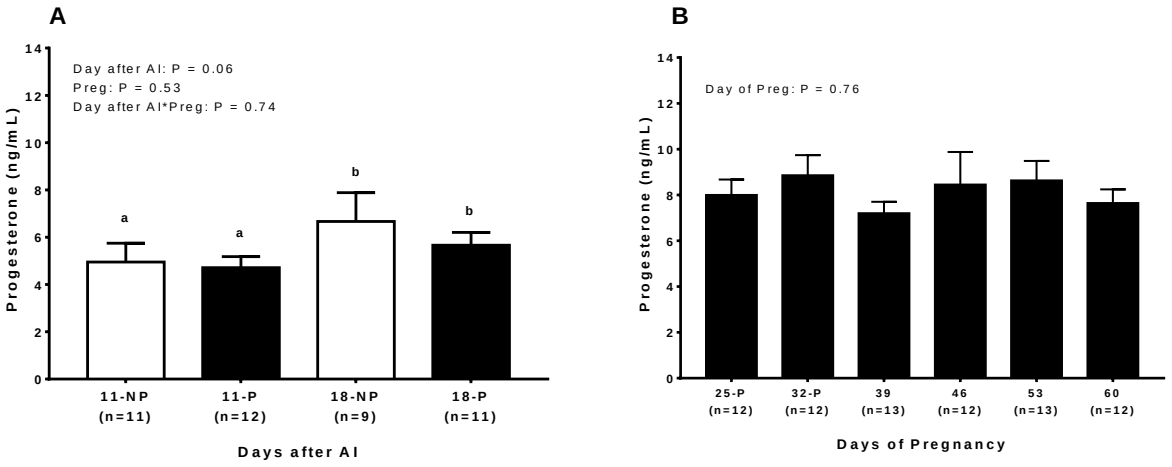

Figure 2. Basal concentrations of P4 for the groups. (A) Comparison between day 18 and 11, pregnant (P) and nonpregnant (NP). (B) Comparisons among groups on days of pregnancy from d25 to d60. Data are shown as least squares means ± SEM. a, b $P \leq 0.05$.

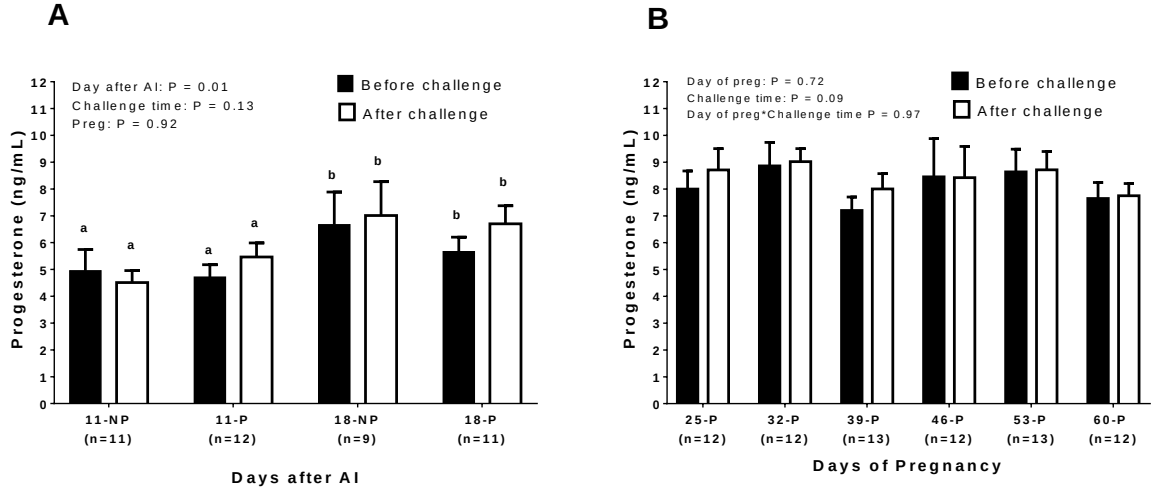

Figure 3. Circulating P4 concentrations before (time 0) and after (average of 30, 60, 90 and 120 min) oxytocin challenge. Interactions not shown in the figure are not significant: Day after AI* Challenge time*: P = 0.56; Day after AI*Preg: P = 0.44; Day after AI*Challenge time*Preg: P = 0.37. Data are shown as least squares means ± SEM. a, b $P \leq 0.05$.

In figure 4A, on d11 (P or NP), pregnancy (P = 0.12) and time had no effect (P = 0.93) on circulating PGFM, and there was no interaction of challenge time and pregnancy (P = 0.84), indicating that PGFM concentrations were constant before and after the oxytocin challenge. In contrast, on d18, there was an effect of challenge time* (P < 0.0001), pregnancy (P < 0.001), and an interaction of pregnancy and challenge time (Figure 4B, P = 0.05) on circulating PGFM. For example, at 30 min after oxytocin treatment on d18, NP cows had a

3.6-fold increase in circulating PGFM concentration compared to basal concentrations (50.2 ± 13.5 vs. 13.8 ± 2.3; P = 0.0001), and continued to be greater than basal concentrations at all other times (P ≤ 0.001): 3.2-fold greater at 60 min (44.7 ± 12.2), 5.7-fold at 90 min (79.4 ± 34.4), and 7.6-fold greater at 120 min after oxytocin (105.7± 35.3). On the other hand, P cows on d18 had a minimal increase in PGFM concentrations after oxytocin challenge with only the 30 min challenge time being different from basal concentrations (25.4 ± 4.1 vs 9.6 ± 1.2, P < 0.01). Concentrations of PGFM at all times after oxytocin challenge were greater in d18-NP than d18-P from 30 min until 120 min after oxytocin.

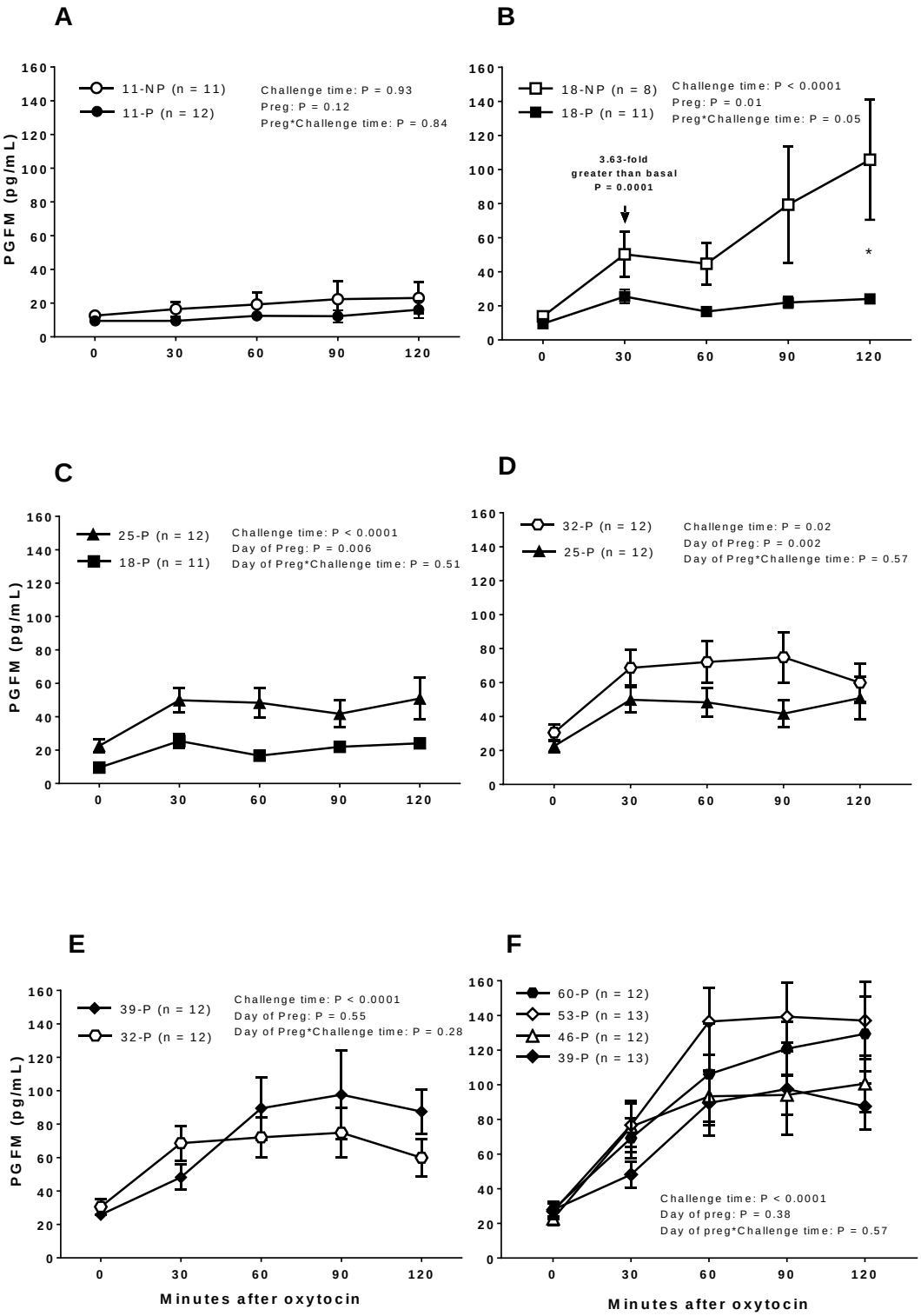

Figure 4. Response to the oxytocin challenge on circulating concentration of PGFM for cows on d11 to d60 after AI. (A) Comparison of d11 P vs NP cows; (B) Comparison of d18 P vs NP cows; (C) Comparison of d18-P vs d25-P; (D) Comparison of d25-P vs d32-P; (E) Comparison of d32-P vs d39-P, and (F) Comparison of d39-P, d46-P, d53-P, and d60-P. Data are shown as least squares means ± SEM. *P ≤ 0.05.

Comparison of P cows on d18 vs d25 (Figure 4C) showed that there was an effect of challenge time (P < 0.0001) and day of pregnancy (P = 0.006) but no interaction (P = 0.51). The oxytocin-induced PGFM in P cows on d25 (Figure 4C; 48.3 ± 8.6) was greater than in P cows on d18 (16.7 ± 2.9; P = 0.006), especially at 60 min after challenge (P = 0.01) when values were 2.9-fold greater on d25 than d18. Similarly, d32 P cows had greater circulating PGFM than d25 P cows (Figure 4D), as indicated by an effect of day of pregnancy (P = 0.002), with both groups having an increase in PGFM after oxytocin (P = 0.002). For example, there was more than a 2-fold increase in PGFM by 30 min after oxytocin compared to basal (d25 = 49.9 ± 7.3 vs 22.6 ± 3.7; d32 = 68.7 ± 10.5 vs 30.6 ± 4.8; P = 0.02). In contrast, there was no effect of day of pregnancy when comparing d32 vs. d39 P cows (P = 0.55; Figure 4E) or d39, d46, d53, and d60 of pregnancy (P = 0.38; Figure 4F) although there was an effect of time after oxytocin challenge in all groups.

Individual profiles for NP cows on d18 before and after challenge (average of 30, 60, 90, and 120 min combined) are shown in Figure 5. The variation in oxytocin-induced PGFM in individual cows is evident as shown by 3 cows having more than a 7-fold increase in PGFM after oxytocin compared to basal PGFM (#3 = 10.6X, #4 = 8.1X, and #5 = 7.1X), 3 cows having intermediate values (#2 = 2.4X, #6 = 3.9X, and #7 = 3.5X), and 2 cows having a low response with less than a 2-fold increase after oxytocin challenge (#1 = 1.4X and #8 = 1.6X). Moreover, even before the challenge, substantial variation in basal PGFM is apparent, ranging from 5.5 to 46.4 pg/mL.

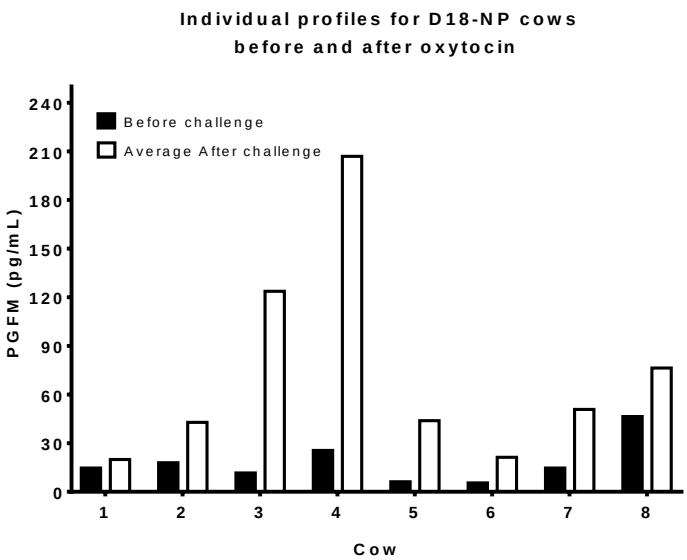

Figure 5. Individual profiles for NP cows from d18. Data demonstrate the variation in circulating PGFM in response to oxytocin for individual cows. Cows that were pregnant on d32 or that had high Interferon-stimulated gene expression (ISGs) on d18 are not included.

An analysis of circulating PGFM prior to the oxytocin challenge (0 min) indicated that baseline PGFM (Figure 6A) was affected by pregnancy (P = 0.05) and day of pregnancy (P < 0.0001), but there was no interaction (P = 0.94). Pregnant cows from d11 and d18, had lower basal PGFM (d11-P = 9.5 ± 2.3 and d18-P = 9.6 ± 1.2) than all groups of P cows after day 25. In addition, NP cows on d11 and d18 were lower (d11-NP = 13.7 ± 2.8 and d18-NP = 13.8 ± 2.3) than P cows on all days of pregnancy after d25, except d46 which had similar basal PGFM concentrations (22.7 ± 3.9) as found in NP cows on d11 and d18. From d25 to d60 of pregnancy (d25 = 22.6 ± 3.7; d32 = 30.6 ± 4.9; d39 = 27.9 ± 2.7; d46 = 22.7 ± 3.9; d53 = 29.0 ± 4.5; d60 = 28.0 ± 4.2) there was no difference among days of pregnancy (P > 0.05) for basal PGFM concentrations (Figure 6A).

Overall PGFM response to oxytocin increased throughout gestation and there was an interaction between day of pregnancy and challenge time (P < 0.001). To determine the increase in PGFM after the oxytocin challenge, the PGFM concentrations at 60, 90, and 120 min after oxytocin challenge were averaged for a given d after AI and the baseline PGFM was subtracted to provide an average oxytocin-induced response (Figure 6B). Minimal responses to oxytocin were observed for P and NP cows on d11 and for P cows on d18. In addition, the PGFM response in P cows on d25 P was intermediate and not different from d11-P, d11-NP, and d18-P, but it was also not different from d18-NP and D32-P. The greatest PGFM response to oxytocin was observed on d53 and d60 of pregnancy. The PGFM response to oxytocin was similar for d18-NP and P cows on d25, d32, d39, and d46 (Figure 6B).

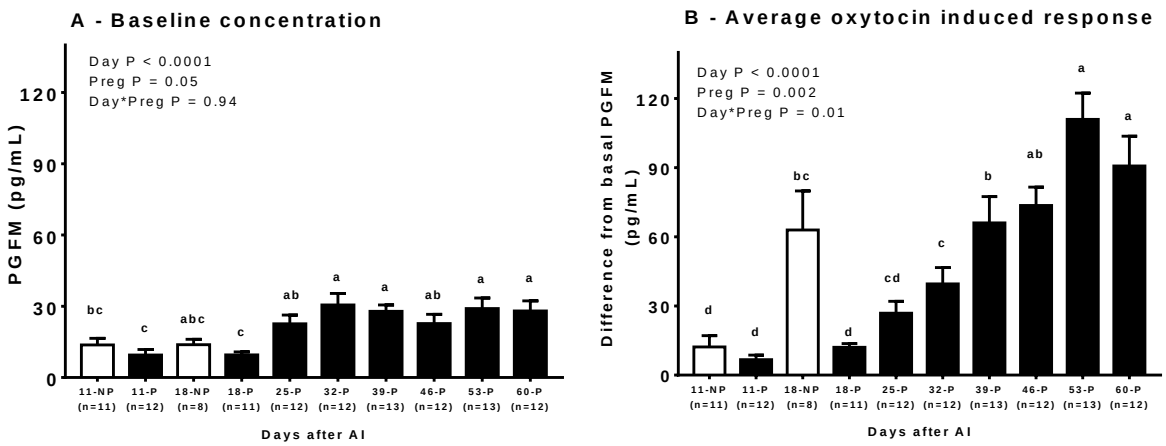

Figure 6. Basal circulating concentration of PGFM, before oxytocin challenge (0 min) during first 60 d of pregnancy (A) and the oxytocin-induced response in PGFM concentrations 60 to 120 min after oxytocin challenge (B). The PGFM concentrations at 60, 90, and 120 min after oxytocin were average and the basal concentration was subtracted to obtain the induced PGFM response as the pregnancy progresses. Data are shown as least squares means ± SEM. a, b, c, d P ≤ 0.05.

The PSPB concentrations were evaluated throughout pregnancy (Figure 7). On d25, PSPB was at the lowest concentrations, although d46 and d60 were not different from d25. The PSPB on d32 and d39 were greater than d25, d46, and d60 and tended to be greater than d53 ($P \leq 0.08$).

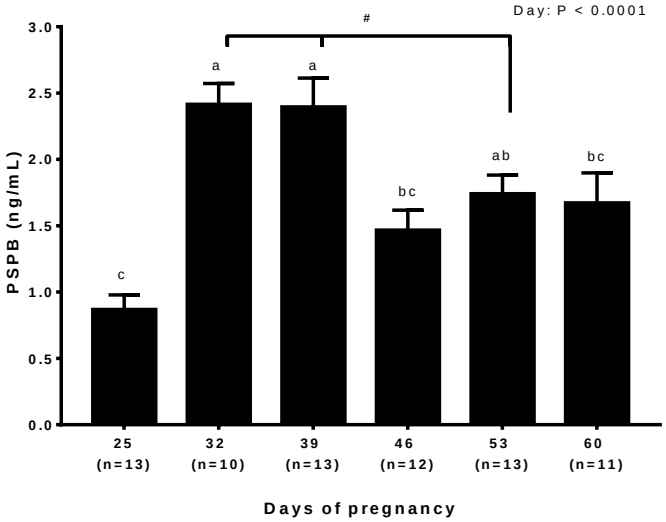

Figure 7. Circulating PSPB concentrations (ng/mL) at time 0 (before challenge) for pregnant cows from d25 until d60. Data are shown as least squares means ± SEM. a, b, c $P \leq 0.05$. # $P < 0.10$.

Since d53 had the most impressive response in PGFM after the oxytocin challenge (Figure 6B), the profiles for PGFM, P4, and PSPB were evaluated before and after the oxytocin challenge (Figure 8). Circulating PGFM was greater than 0 min (26.9 ± 4.6) at 30 min (76.7 ± 12.6; $P < 0.0001$), 60 min (136.5 ± 19.3), and 120 min (137.1 ± 22.2) after oxytocin (Figure 8A). In contrast, there was no effect of the oxytocin challenge on P4 ($P = 0.55$; (Figure 8B) or PSPB ($P = 0.40$; (Figure 8C) concentrations.

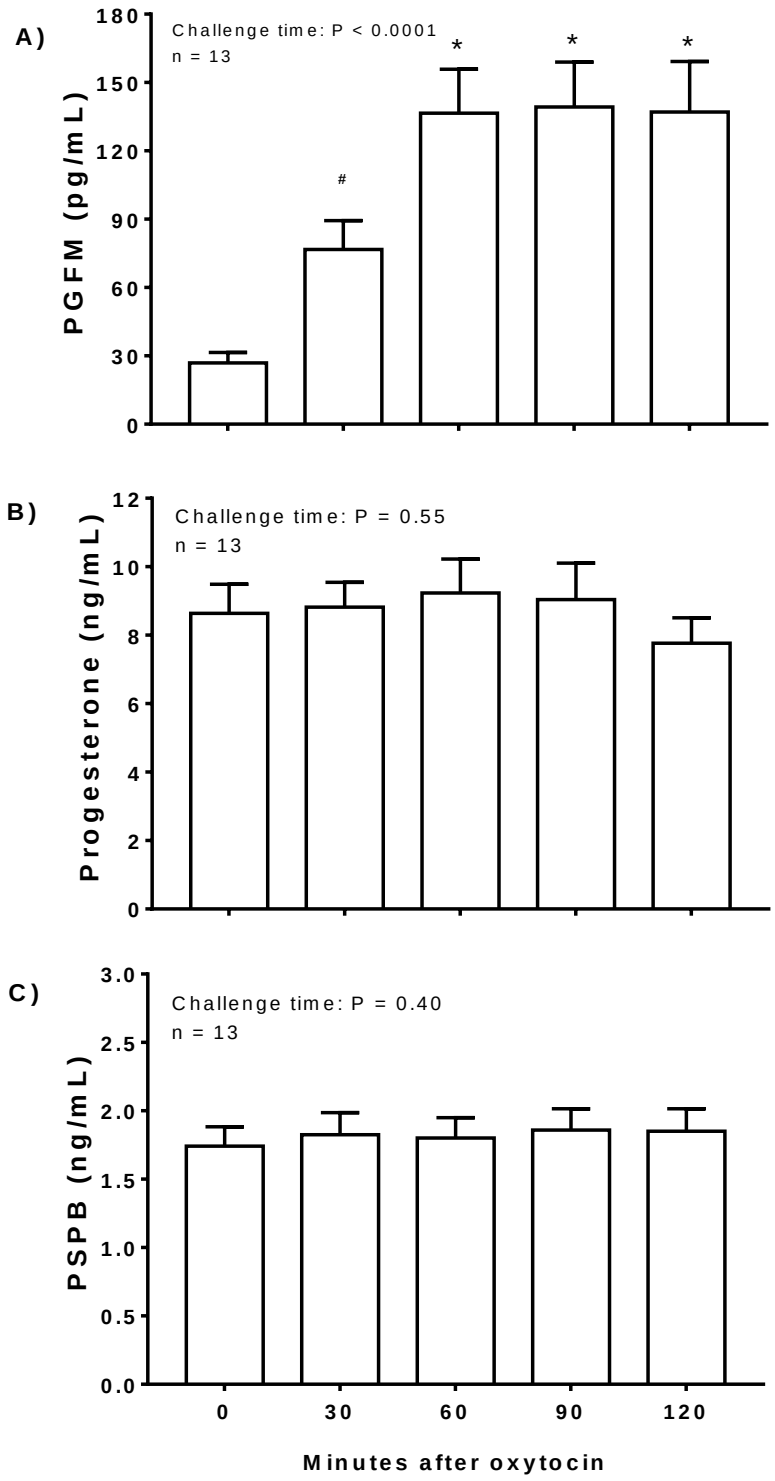

Figure 8. Comparison of changes in PGFM, P4, and PSPB in pregnant cows on d53 before and after the oxytocin challenge. Data are shown as least squares means ± SEM. * $P \leq 0.05$. # $P < 0.10$.

2.4. Discussion

The uniqueness of this study was that we could determine the precise timing during pregnancy when the uterus ends refractoriness and initiates responsiveness to oxytocin, as measured by increases in circulating PGFM in response to an exogenous oxytocin challenge. As expected, the uterus was unresponsive to oxytocin during the time period of early pregnancy when normal luteolysis occurs in NP cows, consistent with previous studies [37, 39-41, 54]. These data are also somewhat consistent with results in heifers without oxytocin stimulation in which pregnant heifers had lower average PGFM concentration and less prominent peaks of PGFM compared to nonpregnant heifers at the expected time of luteolysis (d16 to 18) [55]. However, as production of embryonic IFNT wanes by d25 of pregnancy [19], there was an increase in basal circulating PGFM and responsiveness to oxytocin began to increase. Indeed, responsiveness to oxytocin was similar in P cows after d25 as observed in NP cows during the normal luteolytic period (d18). After d50 of pregnancy, cows had even greater oxytocin responsiveness, as measured by circulating PGFM, compared to NP cows on d18. These results provide critical insights that will help in designing future experiments on the physiological mechanisms regulating CL function during ruminant pregnancy and are likely to be of considerable practical value in providing physiological targets for overcoming the substantial pregnancy losses that occur during this period in dairy cattle [56], beef cattle [57], and recipients of embryos [58].

The first hypothesis, that oxytocin responsiveness would be suppressed during early pregnancy (d18), was clearly supported by our results and by previous studies [37, 38, 40, 41]. The underlying physiological basis for these results depend on embryonic IFNT [34, 59, 60], suppressing PGFM response to oxytocin [40-43] due to suppression of endometrial expression of oxytocin receptor [61-63]. In contrast, NP cows have detectable endometrial oxytocin receptor [41] and a 3-fold greater response to oxytocin compared to pregnant cows on d16 after AI [37]. Nevertheless, there was substantial variation between animals in the magnitude of the oxytocin-induced PGFM increase perhaps indicating differences in endometrial oxytocin receptor between individuals. Previous studies have shown substantial variation in timing of luteolysis in individual cows, particularly in cows with two vs. three follicular waves [64, 65]. Induction of endometrial oxytocin receptors likely occurs in response to activation of ESR1 by estradiol secreted by the dominant follicle of either the second or third follicular wave [13, 65, 66]. Thus, in our study NP cows that were likely to have earlier luteolysis, associated with 2 follicular waves, probably had much greater uterine

oxytocin responsiveness than cows that had not yet obtained sufficient oxytocin responsiveness, i.e. cows that were likely to have 3 follicular waves.

Our second hypothesis, that oxytocin responsiveness would increase as pregnancy progressed, was clearly supported by our results. There was minimal increase in circulating PGFM after oxytocin treatment on d18 but increased basal and oxytocin-induced PGFM secretion by d25 with further increases by d53 of pregnancy. A previous study [44] reported increased PGFM response to oxytocin challenge as pregnancy progressed (50, 150, 250, or 280 d) with a 7-fold greater increase in circulating PGFM on d280 than on d50. In addition to the oxytocin challenge, presence of intercaruncular endometrial oxytocin receptors were found at day 50 of pregnancy [44]. Our results extend these previous findings by demonstrating that there is an increase in basal PGFM and oxytocin-induced PGFM within the first few days after loss of IFNT secretion by the embryo, d25 of pregnancy in our study. The previous study focused on the elevation in PGFM and oxytocin responsiveness as parturition approached, whereas our study focused on the timing of oxytocin-induced PGFM during the first 2 months of pregnancy in order to understand how the CL is maintained after IFNT secretion declines.

A previous study also detected PGFM in the posterior vena cava in pregnancies maintained by exogenous progestins or by an accessory CL that was induced during the second month of pregnancy [67]. The animals in our study all had pregnancy maintenance after the oxytocin challenge and did not have an accessory CL or treatment with exogenous progestin. Hence, our results, as well as some previous results are consistent with an increase in basal circulating PGFM during the second month of pregnancy, although they do not provide information on the source of PGF that leads to this increased basal PGFM during pregnancy.

The effects of exogenous oxytocin on circulating PGFM were dramatic during the second month of pregnancy with oxytocin-induced PGFM reaching concentrations that were as high or higher than those observed near the time of normal luteolysis in NP cows. Remarkably, in spite of the striking effect of oxytocin on circulating PGFM there was no detectable effect on circulating P4 and none of the pregnancies were lost after the oxytocin challenge. Previous studies have shown clear decreases in circulating P4 during a similar timeframe after administration of exogenous PGF [1, 4]. Thus, the pregnant uterus acquires clear oxytocin responsiveness during the late stages of the first month of bovine pregnancy with clear increases in this oxytocin responsiveness during the second month of pregnancy. The lack of CL regression in the face of PGFM pulses that are of a magnitude that would be

expected to be luteolytic in NP cows indicate that the CL during the second month of pregnancy is maintained by mechanisms other than suppression of uterine oxytocin receptors and corresponding PGF secretion, as occurs during early bovine pregnancy. Other data have previously suggested that a parsimonious explanation for the lack of CL regression in spite of uterine PGF secretion is that PGF does not reach the CL through local mechanisms due to the elevated blood flow in the uterine horn ipsilateral to the pregnancy during the second month of pregnancy [58]. This physiologic model may also explain regression of the contralateral accessory CL during 33-60 d of pregnancy, but continued maintenance of the pregnancy and ipsilateral CL [68] because blood flow in the uterine horn contralateral to the pregnancy increases at a slower rate than in the ipsilateral horn [69]. Thus, a local mechanism exists during the second month of pregnancy that protects the CL that is ipsilateral but not contralateral to the pregnancy.

Potential practical benefits could result from the future application of this fundamental research. Pregnancy loss prior to d35 appears to be initiated by death of the embryo [70]; however, during the second month of pregnancy, little is known how much is primarily related to embryonic death and what percentage is caused by inappropriate regression of the CL. It seems possible that the increase in uterine oxytocin responsiveness and PGFM secretion during the second month of pregnancy may be excessive in certain circumstances and that animals in these conditions may benefit from treatments directed at reducing PGF secretion. For example, it is well-established that recipients of cloned embryos have extremely high pregnancy loss during the second month of pregnancy with placental abnormalities and vascular problems being implicated as causative factors [71, 72]. In addition, pregnancy loss is substantial during the second month of pregnancy in lactating cattle and in recipients of *in vitro*-produced embryos [56, 73]. It seems likely that some of the pregnancy loss is due to inappropriate CL regression due to inadequacies in mechanisms maintaining the CL during this second month of pregnancy [74]. A delay in the mechanisms protecting the CL, such as a delayed increase in uterine blood flow, may result in inadequate inhibition of the increasing PGFM secretion that occurs at d25 of pregnancy and beyond and untimely CL regression. Development of physiologically rational methods to overcome this pregnancy loss could lead to substantial increases in reproductive efficiency [75]. Unfortunately, no studies have clearly differentiated if a defective embryo or inappropriate CL regression underlies pregnancy loss in the second month of pregnancy which could more effectively focus future research on the root cause of pregnancy losses in cattle.

In conclusion, consistent with the hypothesis in this study and previous reports, the CL of early pregnancy is maintained due to suppression of uterine oxytocin receptors and PGF secretion likely resulting from actions of embryonic IFNT. However, during the second month of pregnancy, uterine PGF secretion was not suppressed since basal PGFM and oxytocin-induced PGF secretion were greatly elevated (equal or greater than in d18 NP cows). These results indicate that there are alternative mechanisms for maintenance of the CL during the second month of pregnancy that do not involve suppression of uterine PGF secretion.

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3. ENDOMETRIAL GENE EXPRESSION, EMBRYO MORPHOLOGY, AND CIRCULATING PROSTAGLANDIN F2-ALPHA METABOLITE DIFFER BETWEEN ARTIFICIAL INSEMINATION AND *IN VITRO* PRODUCED EMBRYO PREGNANCIES ON DAYS 18 AND 32 OF *BOS INDICUS CATTLE*

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Abstract

Mechanisms that maintain the corpus luteum involve suppression of prostaglandin F2alpha (PGF) by embryonic interferon-tau near d18, however, uterine PGF secretion increases near the end of the first month of pregnancy. The aim of this study was to evaluate differences in oxytocin-induced prostaglandin F metabolite (PGFM), uterine gene expression for oxytocin receptors (OXTR) and interferon-stimulated gene 15 (ISG15), and circulating P4 in cows that were non-pregnant (d18) or pregnant from artificial insemination (AI) or *in vitro* produced (IVP) embryos on d18 and d32 of pregnancy. Conceptus length on d18 and embryo size on d32 were also evaluated. Non-lactating Bos indicus (Nelore) cows (n=142) were submitted to a synchronization protocol, and randomly assigned to one of the following groups: non-inseminated (NI), AI on d0 (48 h after implant removal), or received an IVP embryo on d6.5. Thereafter, NI, AI and IVP cows were slaughtered on d18 or d32, according to AI or IVP groups. One day before slaughter (d17 and d31) cows were challenged with 50 IU oxytocin, i.m., and blood samples were collected before (0 min), 60 and 120 min after oxytocin for circulating PGFM analysis. Samples for P4 were collected on d6.5 and on the day of oxytocin for all groups. After slaughter, the uterus was collected and dissected for conceptus, embryo and gene expression analysis. There was no difference (P>0.05) between AI vs IVP for conceptus length on d18 (44.6±4.3 vs 53.3±5.9 cm), or P4 on d6.5 and d17. However, crown-rump on d32 (1.8±0.2 vs 1.3±0.1 cm) was bigger, and P4 on d31 (8.5±0.9 vs 6.6±0.5 ng/mL; P=0.07) tended to be

higher in AI than IVP. For basal circulating PGFM on d31, AI and IVP were similar (47.3±6.4 vs 39.8±4.8 pg/mL), and greater than NI (15.7±3.6 pg/mL) and IVP on d17 (17.9±3.9 pg/mL) with intermediate basal PGFM for AI on d17 (33.3±6.6 pg/mL). The greatest increase in PGFM after challenge was detected at 60 min for all groups. The NI cows had greater PGFM increase after oxytocin compared to IVP (7.6- vs 0.2-fold increase) with intermediate response for AI group. Cows from IVP on d17 or d31 had greater oxytocin-induced PGFM than other groups. In addition, OXTR mRNA was elevated in NI cows and AI or IVP cows on d32 but was, suppressed in AI or IVP on d18. In contrast, ISG15 mRNA was elevated in d18 AI or IVP cows but suppressed in NI or d32 pregnant cows. In conclusion, pathways for PGF production are induced near luteolysis in non-pregnant cows but suppressed by interferon-tau near d18; however, near the end of the first month of pregnancy PGF production pathways are induced in the uterus, indicating alternative mechanisms for CL maintenance besides PGF suppression. Moreover, there were subtle differences between IVP and AI pregnancies that could underlie fertility differences.

Keywords: Bovine: Prostaglandin; Conceptus; Embryo; *in vitro* fertilization; Oxytocin

3.1. Introduction

Pregnancy loss is a substantial problem for reproductive efficiency in dairy cattle [1], beef cattle [2] and embryo recipients [3]. Data from approximately 25,000 pregnancies in dairy cattle indicated about 12% pregnancy loss between 32 and 60 d of pregnancy [4]. Most of these losses appeared to occur after expression of interferon-stimulated gene 15 (ISG15) on d19 and d32 pregnancy diagnosis by ultrasound (31%, 157/499; [5]). There continue to be improvements in *in vitro* embryo production (IVP) techniques has being adjusted, although variation in pregnancy per ET (P/ET) is still substantial. A fair comparison between embryo transfer (ET) and fixed-time AI (FTAI) has reported P/ET of 34% [195/573] for ET vs. P/AI of 50.3% [174/346] for FTAI [3]. In addition, pregnancy loss from 30 to 60 d was much greater for ET than AI (15.9 vs 5.2%; [3]). Pregnancy loss is particularly costly in dairy farms because of the increase in feeding costs and days open [6].

Pregnancy loss following transfer of *in vivo* derived (IVD) or IVP embryos frequently occurs prior to 21 d of gestation or within 2 weeks of transfer [7]. The losses in this period can be caused by two reasons: embryo death leading to luteolysis or premature luteolysis leading to pregnancy loss [8].

During early gestation, from about d30 through d90, the loss of IVP embryos/fetuses may be attributed to failures in development of placental membranes and reduced placental blood vessel development [9, 10], especially due to abnormal epigenetic reprograming caused

by the IVP systems [11-13]. In addition, it has been reported that IVP systems are able to modify embryo gene expression profiles, hence affecting development and signaling [14]. The IVP embryos have been reported to differ at the transcriptomic level from their *in vivo* counterparts [15]. Other evidence that IVP embryos are deteriorating during pregnancy, were provided by flushing of embryos on d16 of pregnancy from recipients of IVP or IVD embryos with more degenerate embryos (25 [7/28] vs 5% [2/34]) and smaller embryonic discs for IVP than IVD embryos [12]. Death of the embryo or inadequate communication among the conceptus, uterus, and ovary can lead to inadequate signals for maintenance of the CL [16] and eventual luteolysis. Loss of pregnancy prior to d25 can induce no change in time of luteolysis or a delay in luteolysis [1].

It is well known that interferon-tau (IFNT) secretion is responsible for pregnancy maintenance during the first month of gestation. At the expected time of expected luteolysis (d16 to d20 after estrus), IFNT acts by suppression of estradiol-induced expression of oxytocin receptor (OXTR) in the endometrium [17, 18] probably due to inhibition of transcription of the estrogen receptor, ESR1, by IFNT resulting in a lack of induction OXTR during the late luteal phase [19]. The oxytocin-stimulated secretion of luteolytic pulses of prostaglandin F2α (PGF) is thereby inhibited [20].

Administration of IFNT into the sheep uterine lumen or into the systemic circulation during the maternal recognition period causes extended CL lifespan [21-23]. In addition, the presence of higher concentrations of interferon stimulated genes (ISGs) in pregnant CL suggests extra-uterine, endocrine effects of IFNT [21, 23-25]. Nonetheless, there is a significant decrease in ISGs detected in peripheral blood cells (PBL) by d25 of pregnancy [26, 27], reflecting the loss of IFNT expression in the embryo by d25 [28, 29]. Consistent with this idea, intrauterine infusion of homogenates from d14-15 ovine embryos extended CL lifespan, whereas, homogenates from d21-25 embryos did not alter CL lifespan [30, 31]. Thus, the mechanisms that maintain the CL after termination of embryonic IFNT are not yet defined.

Recently, a study in dairy cattle from our group described, in a week-by-week approach, a progressive increase in oxytocin-induced PGFM throughout the first 60 d of pregnancy. Of particular interest, cows by d32 of pregnancy had a similar response to oxytocin as non-pregnant cows near the time of luteolysis (d18), but without any detectable effect on circulating P4 (Drum et al., in press). Indeed, another recent study has shown a dramatic increase in PGF pulsatility during the second month of pregnancy. More PGFM pulses were observed in cows during the second month of pregnancy than during the first month, and cows in the second month were similar in pulse frequency to cows undergoing CL

regression [32]. These observations help explain the high percentage of cows that undergo regression of the contralateral accessory CL during d33-60 of pregnancy, even in the presence of maintenance of the ipsilateral CL and the pregnancy [33].

Therefore, this study was designed to quantify OXTR expression in the uterus during pregnancies produced by AI or by transfer of an IVP embryo. Furthermore, we evaluated non-bred cows and pregnant cows on d18 and d32 for basal and oxytocin-induced PGFM release, size of the embryos, and expression of ISGs in the ipsilateral and contralateral horns.

Our primary biological hypothesis was that the CL was maintained during early pregnancy (~d18) by suppression of the uterine PGF production pathways including OXTR but that these inhibitory mechanisms are lost by the end of the first month of pregnancy (d32) after cessation of embryonic IFNT production. To test this biological hypothesis, we formulated four specific experimental hypotheses. First, we hypothesized that circulating PGFM would increase after oxytocin challenge in non-pregnant cattle on d18 as well as in pregnant cows on d32, but not in pregnant cows on d18, indicating that PGF production pathways are suppressed in pregnant cows during the period of expected luteolysis but not during later pregnancy. The second hypothesis was that OXTR mRNA would be increased in endometrium of d18 non-pregnant cows and on d32 of pregnancy; but would be suppressed in the pregnant uterus on d18, providing a potential mechanism for suppression of PGF in early but not later pregnancy. The third hypothesis was that ISG15 mRNA would be very low in non-pregnant cows and in cows on d32 of pregnancy but would be elevated in pregnant cows on d18, consistent with the expected IFNT secretion during early pregnancy. The fourth hypothesis was that IVP embryos would be smaller and more variable on d18 and d32 compared to AI-produced embryos and that there would be corresponding differences in endocrine patterns and uterine gene expression in AI vs. IVP pregnancies.

3.2. Material and methods

The experiment was conducted at the Experimental Station Hildegard Georgina Von Pritzelwiltz, located in Londrina, PR, Brazil. The Animal Research Ethics Committee of “Luiz de Queiroz” College of Agriculture (ESALQ)/University of São Paulo approved all procedures involving cows in this study (Protocol #2018.5.1252.11.5, nº CEUA – 2018-21).

A total of 182 non-lactating, multiparous, Nelore cows with BCS 3.0 ± 0.04 were enrolled in the experiment, 142 synchronized and 40 for ovum pick-up (OPU) procedure.

Cows were kept in a feedlot receiving the same TMR to meet or exceed the nutrient requirements [34], and had ad libitum access to water.

3.2.1. Experimental design

The experiment was conducted in four replicates in a completely randomized design. All experimental procedures are described in Figure 1. On d-9, 40 cows were randomly selected for OPU, whereas remaining cows (n = 142) were submitted to a synchronization protocol for FTAI or ET. Cows received a disinfected intravaginal implant containing 1.0 g P4 previously used for 7 d and 2.0 mg estradiol benzoate (EB) i.m. Seven d later (d-2) 0.526 mg cloprostenol sodium (PGF), 300 IU equine chorionic gonadotropin (eCG) and 0.5 mg estradiol cypionate (EC) were administered i.m., concomitant with implant withdrawal. On d0, 8.4 µg buserelin acetate (GnRH) was administrated i.m. All cows were identified using an ear tag with a number and were assigned to the following treatments: Synchronized non-inseminated group (NI; n = 10); Artificial insemination group (AI; n = 50); and IVP embryo transfer group (IVP, n = 82).

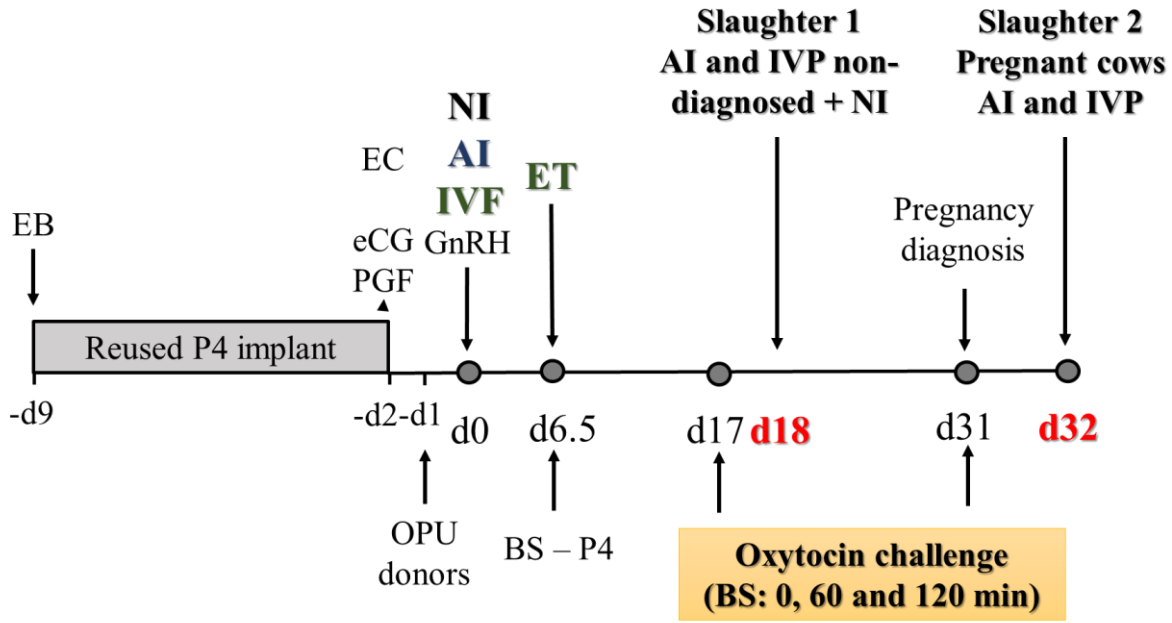

Figure 1. Experimental procedures described in a timeline**.** Schematic experimental design representing the synchronization protocol used, blood sample collections (BS), timing of oxytocin challenges and ultrasound evaluations performed. Reused P4 device = progesterone implant (1.0 g) previously used for 7 d; EB = estradiol benzoate (2.0 mg); EC = estradiol cypionate (0.5 mg); eCG = equine chorionic gonadotropin (300 IU); PGF = cloprostenol sodium (0.526 mg); GnRH = buserelin acetate (8.4 µg); OPU = ovum pick-up; FTAI = fixed-time artificial insemination; IVF = *in vitro* fertilization; ET = embryo transfer; IVP = *in vitro* produced embryo group; AI = artificially inseminated group; NI = non-inseminated group.

On d0, cows from the AI group were inseminated using frozen/thawed semen from two high fertility Aberdeen Angus bulls (Alta Genetics, Uberaba, Brazil) by the same technician. On d6.5, CL presence and side were evaluated in all cows by transrectal ultrasonography with a 7.5MHz linear transducer (Mindray DP 2200, Mindray Bio-Medical Electronics Co. Ltd., Shenzen, China). Then cows from the IVP group received a viable embryo transferred by the same trained technician, into the tip of the uterine horn ipsilateral to the CL.

3.2.2. Oxytocin challenge and blood sampling

Blood samples for P4 analysis were collected 6.5 d after AI (d6.5) and 1 d before slaughter of the cows (d17 and d31). On the same d before slaughter, d17 and d31, cows were submitted to an oxytocin challenge and had serial blood collections (Figure 1).

Oxytocin challenge with 50 IU i.m. was performed as previously described (Drum et al. in press) with some modifications. Blood samples were collected at 0 (before challenge), 60 and 120 min after challenge by puncture of the coccygeal vein or artery into evacuated 10 mL tubes containing sodium heparin (Vacutainer, Dickinson, Franklin Lakes, NJ). Immediately after collection, the tubes were placed on ice and kept refrigerated until processing. Blood samples were centrifuged at 1,700 x g for 15 min and aliquots of plasma were frozen and stored in duplicates at -20ºC until assayed for P4 and PGFM.

3.2.3. Ovum pick-up and *in vitro* embryo production

Eight d after the beginning of the synchronization protocols (d-1), 40 cows previously selected based on antral follicle count were submitted to OPU by a trained technician following the procedure previously described [35]. Briefly, visible follicles > 2 mm in diameter were aspirated using a real-time B-mode ultrasound scanner, equipped with a 7.5-MHz convex array transducer (Mindray DP 2200, Mindray Bio-Medical Electronics Co. Ltd., Shenzen, China) fitted into an intravaginal guide and a stainless steel guide. Follicular puncture was performed using a disposable 19-gauge hypodermic needle connected to a 50-mL conical tube via silicon tubing (0.8 m; 2.0 mm id). Aspiration was performed using a vacuum pump (WTA, Watanabe, Cravinhos, SP, Brazil) with a negative pressure of 75 mm

Hg. The collection medium used was phosphate buffer solution (PBS, Nutricell, Campinas, SP, Brazil) with 10,000 IU/L sodium heparin (Sigma H-3149).

The procedure IVP after OPU was also the same as previously described [35]. The content recovered was immediately washed with PBS and filtered through an embryo filter. The washes were placed into a petri dish and cumulus oocyte complexes (COCs) were recovered. The COCs were classified according to the presence of cumulus cells layers and oocyte quality using the criteria: good, more than three layers of cumulus cells; regular, at least one layer of cumulus cells; denuded; and atretic with dark cumulus oophorus and signs of cytoplasmic degeneration. Good and regular oocytes were considered viable and used, whereas denuded and atretic oocytes were discarded. Procedures for *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* culture (IVC) were performed as previous described by Seneda et al. and Silva-Santos et al. [35, 36].

Frozen/thawed sperm (2×106dose) were used from the same two Aberdeen Angus sires used for AI. By the end of IVC, embryos were evaluated on d6 (d0 = d of IVF and day of AI for the other group) according to IETS criteria [37].

3.2.4. Pregnancy diagnosis and tissue processing

As described on Figure 1, cows were slaughtered on d18 and d32 after AI (d0). On the given days, whole female reproductive tract (uterus, cervix and both ovaries) were collected immediately after slaughter. The tracts were placed in plastic bags, identified using a tag by slaughter order, sealed and kept on ice until processing. All uteri were taken from ice immediately before processing. For d18, on AI and IVP groups, each horn was flushed using 10 mL of sterile saline solution. Pregnancy was confirmed by the presence of an elongated embryo in uterine flushes and the presence of a functional CL (later confirmed by P4 measurement). For cows to be slaughtered on d32, 1 d before slaughter (d31), pregnancy diagnosis was performed by a trained technician using ultrasonography to detect embryo heart beating. Only pregnant cows had their uterus collected.

Elongated embryos from d18 after recovery, were disposed linearly in a petri dish and measured using a ruler. To obtain embryos from d32, the amniotic vesicle was carefully extracted and the cranio-caudal measurement (crown-rump) was performed using a ruler.

All cows assigned in the NI group had their uterus collected and processed. Each uterus (from pregnant and from NI group), was divided as ipsilateral (ipsi) or contralateral (contra) to the CL, and then separated as proximal or distal to the ovary. Endometrial samples

from the proximal region of each side (ipsi and contra) were collected using curved scissors and tweezers, placed in identified cryotubes, then frozen in liquid nitrogen right after collection and stored at -80°C for subsequent RNA extraction.

3.2.5. Hormonal assays

Circulating concentrations of P4 were determined using a solid-phase RIA kit containing antibody-coated tubes and 125I-labeled P4 (ImmuChem Coated Tube P4 125 RIA Kit, MP Biomedicals, Costa Mesa, CA) as previously described for mares [38] and validated for bovine plasma [39]. The intra- and inter assay CVs were 2.1% and 2.2% respectively, and the sensitivity was 0.05 ng/mL.

Plasma samples were assayed for PGFM by an ELISA assay that was previously validated for use in bovine plasma [40] with some modifications as described by Ochoa et al. [41]. The intra- and inter-assay CVs were 5.9% and 16.6% respectively, and the sensitivity of the assay was 6.5 pg/mL.

3.2.6. RT-PCR

Total RNA was isolated from tissue using Tri Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Isolated RNA was evaluated for concentration and purity using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Rockford, IL), following manufacturer's instructions. A maximum of 1.0 µg RNA was used in reverse transcription to cDNA by means of a commercially available kit (High-Capacity cDNA Reverse Transcription Kit, Thermo Fisher Scientific, Rockford, IL) with RNase inhibitor (New England Biolabs Inc. Ipswich, MA) following manufacturer's instructions. Using CFX Connect Real-Time System (Biorad, Hercules, CA, USA), steady-state concentrations of mRNA for OXTR and ISG15 were measured using SsoFast EvaGreen Supermix (BioRad, Hercules, CA, USA). The thermal cycling conditions were: 95ºC for 3 min, then 40 cycles of 95ºC for 10 sec and 60ºC for 30 sec. All primers had its amplification efficiencies evaluated and ranged from 95% to 105% efficiency, and primer specificity was assessed by the presence of a single temperature dissociation peak. Each sample was evaluated in duplicate. The ribosomal protein S18 (RPS18) was used as housekeeping gene and the 2-ΔΔCt method was used to calculate relative gene expression [42]. The sequences of

primers used for quantitative qPCR were chosen based on previous reports [43, 44] and are listed in Table 1

Table 1. Gene, primer orientation, primer sequence (5′ to 3′), and National Center for Biotechnology Information (NCBI) access number and sequence for primers used in RT-qPCR assay

Gene symbol	Gene name	Forward primer	GenBank ID
OXTR	Oxytocin receptor	F: AAGATGACCTTCATCGTCGTG R: CGTGAAGAGCATGTAGATCCAG	NM_174134.2
RPS18	Ribosomal Protein S 18	F: GTGGTGTTGAGGAAAGCAGACA R: TGATCACACGTTCCACCTCATC	NM_001033614.2
ISG15	Interferon-stimulated gene 15	F: GGTATCCGAGCTGAAGCAGTT R: ACCTCCCTGCTGTCAAGGT	NM_174366

3.2.7. Data handling and statistical analysis

Three cows from AI group on d18, despite detection of an elongated embryo, presented small conceptus length (<17 cm), which was not compatible with the developmental stage expected for d18. Two of them, also had anomalous response to oxytocin challenge, approximately 10-fold greater circulating PGFM 60 min after oxytocin injection. All three cows were excluded from analyzes.

Two cows from d18 (one each from AI and IVP groups), by the time of uterine flushing, had an elongated embryo, however it was broken into small parts and could not be accurately measured. Furthermore, one cow from d32, in spite of the positive pregnancy diagnosis on d31, the embryo appeared to be dead on d32 and had a broken embryonic vesicle. These animals were excluded from all analyses.

Two cows from NI group had low circulating P4 on d17 and high basal circulating PGFM and a greater response to oxytocin treatment than all other cows in this group. These observations indicated that these cows were already undergoing luteolysis and therefore these cows were excluded from gene expression analyses.

All data were tested for normality of residuals using the UNIVARIATE procedure of Statistical Analysis System (SAS, Version 9.4 for Windows, SAS Institute Inc., Cary, NC) according to Shapiro-Wilk or Koromorov-Smirnov tests. Data with residuals not normally

distributed were transformed to logarithm, square root, or inverse scale before analysis if this transformation improved residual distribution. In addition, outliers were removed, when necessary.

The initial model for d17 conceptus or d31 embryo crown-rump length included: Group, P4 on d7 and P4 on d17 or d31. No P4 results had an interaction with group and, therefore, P4 data were eliminated from the model. Final models were determined using backward elimination, and variables with $P > 0.15$ were gradually excluded in order to choose the best final model based on the lowest value for Akaike information criterion (AIC).

Continuous data, such as circulating P4 on d6.5, P4 on d17, P4 on d31, conceptus length, crown-rump length, PGFM before oxytocin challenge, PGFM after challenge and fold-changes in gene expression, were analyzed for Group effects using the MIXED procedure with models fitting a Gaussian distribution. For gene expression results, effect of Group and uterine horn side were kept in the model.

Circulating PGFM after oxytocin challenge, as continuous data with repeated measures over time, was also analyzed using the MIXED procedure with models fitting a Gaussian distribution. Effects of Group, Time and interaction Group*Time were used in the model.

The relationships among circulating P4 on d6.5, P4 on d17, P4 on d31, conceptus length, crown-rump length, circulating PGFM before challenge, PGFM induced by oxytocin, OXTR and ISG15 fold-change were calculated by the CORR method. For OXTR and ISG15 an average of both sides were used. Two analyses were performed separately, one for cows slaughtered on d18 using P4 on 6.5, P4 on d17, basal PGFM, PGFM induced, OXTR and ISG15; and another for cows slaughtered on d32 using P4 on 6.5, P4 on d31, basal PGFM, PGFM induced, OXTR and ISG15. Pearson's correlation coefficients were considered for data with normal distribution. For not normalized data, we considered the correlations of Spearman, according to the statistical methodology described by Sampaio [45]. Values of r with $P \leq 0.05$ were considered significant.

Tukey honest significant difference post hoc test was performed to determine differences. Differences were considered significant for $P \leq 0.05$, whereas a tendency was designated when $P < 0.10$ and $P > 0.05$. Data are presented as least squares means ± SEM.

3.3. Results

By evaluating the IVP efficiency, from all replicates, we obtained an average of 27.0% blastocyst rate per total number of cumulus-oocyte-complexes (COCs) collected and 34.2% blastocyst rate per viable COCs. A total of 73 high quality embryos (only grade 1) were transferred, of which, 38 resulted in pregnancy (52% P/ET). The AI group had 30 pregnancies from 50 inseminations (60% P/AI).

3.3.1. Circulating P4

On d6.5 after expected time of AI (Figure 2), circulating P4 did not differ among groups (NI d17: 3.39 ± 0.42; AI d17: 3.19 ± 0.59; IVP d17: 2.61 ± 0.18; AI d31: 3.76 ± 0.62; and IVP d31: 2.14 ± 0.15; P > 0.05).

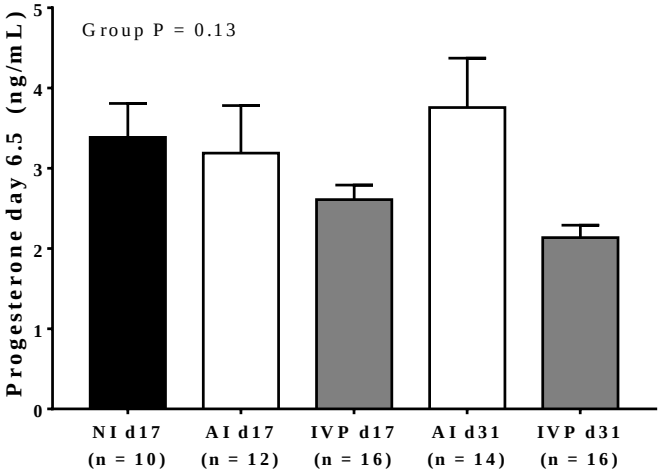

Figure 2. Circulating P4 concentration 6.5 d after AI (d6.5), or at the time of ET, compared among five experimental groups: Non-inseminated on d17 of the estrous cycle (NI d17); Pregnant from AI on d17 (AI d17); pregnant from an IVP embryo on d17 (IVP d17); Pregnant from AI on d31 (AI d31) and pregnant from an IVP embryo on d31 (IVP d31). Data are shown as means ± SEM.

Circulating P4 measured from blood samples collected on d17 (Figure 3A) was not different among groups slaughtered on d18 (NI: 5.7 ± 1.1; AI: 5.7 ± 0.6; IVP: 6.0 ± 0.4; P > 0.05). However, on d31, pregnant cows from AI tended to have greater circulating P4 than pregnant cows from IVP embryos (Figure 3B; AI: 8.5 ± 0.9 vs IVP: 6.6 ± 0.5; P = 0.07).

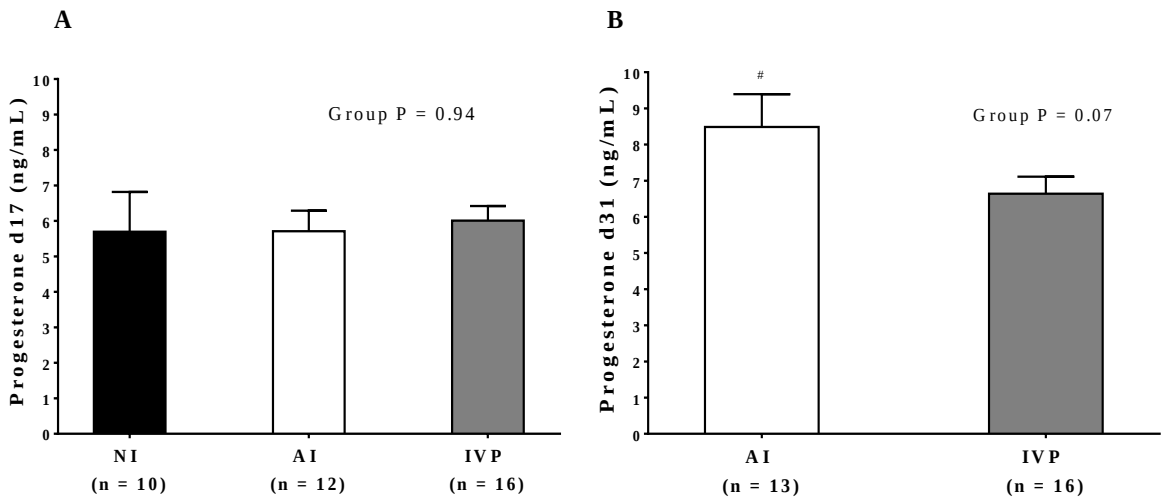

Figure 3. Circulating P4 concentration (ng/mL) on d17 (A) and d31 (B). (A) Comparison among: Non-inseminated on d17 of the estrous cycle (NI d17); Pregnant from AI on d17 (AI d17) and pregnant from an IVP embryo on d17 (IVP d17). (B) Comparison between Pregnant from AI on d31 (AI d31) and pregnant from an IVP embryo on d31 (IVP d31). Data are shown as means ± SEM.

3.3.1. Circulating PGFM before and after oxytocin challenge

The first comparison that was performed was to compare the PGFM profiles for all groups (NI, AI or IVP) that were on d17 (Figure 4A). There was an effect of Group ($P = 0.0003$), Time ($P < 0.0001$) and interaction between Group*Time ($P < 0.0001$) on circulating PGFM in response to oxytocin challenge. The greatest increase in PGFM was detected 60 min after challenge for all groups ($P < 0.04$). The NI cows had a greater PGFM response to oxytocin compared to IVP cows, with 7.6-fold increase vs 0.2-fold increase. The AI group on d17 had an intermediate response, which did not differ from the other groups ($P > 0.05$). None of the groups changed PGFM concentration between 60 and 120 min after challenge ($P > 0.05$). However, only the NI group maintained PGFM concentration higher than basal at 120 min after challenge.

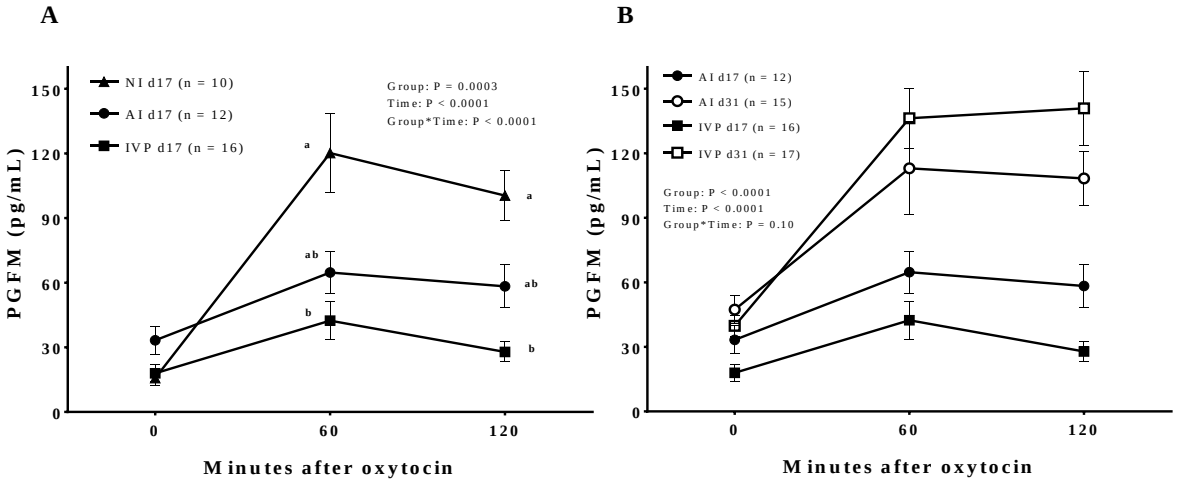

Figure 4. Response to the oxytocin challenge on circulating concentration of PGFM (pg/mL) during the first 2 h (0 before challenge, 60 and 120 min after challenge). (A) Comparison among: Non-inseminated on d17 of the estrous cycle (NI d17); Pregnant from AI on d17 (AI d17) and pregnant from an IVP embryo on d17 (IVP d17); (B) Comparison between: Pregnant from AI on d31 (AI d31) and pregnant from an IVP embryo on d31 (IVP d31). Data are shown as means ± SEM. a, b, c $P \leq 0.05$ comparison at the same time point (60 or 120 min after challenge).

Figure 4B shows the PGFM profiles during oxytocin challenge for pregnant cows on d17 and d31 for both groups. There was effect of Group ($P < 0.0001$) and Time ($P < 0.0001$), however there was no interaction between Group*Time ($P = 0.10$). On average, AI and IVP on d31 had similar elevated responses ($P > 0.05$) and the response was greater in both groups that in AI or IVP on d17 ($P < 0.05$). Furthermore, AI on d17 had greater response than IVP on d17. The time effect was present in all treatments with most important increase at 60 min after challenge. Again, none of the groups presented significant difference between 60 and 120 min after challenge.

The group effect was also present when only basal circulating PGFM prior to oxytocin challenge was directly compared for all groups (Figure 5A; Group: $P < 0.0001$). On d31, AI and IVP were similar (AI d31: 47.3 ± 6.4 vs IVP d31: 39.8 ± 4.8; $P < 0.05$), and both were respectively 3 and 2.5-fold greater than NI, and 2.6 and 2.2-fold greater than IVP on d17 (NI d17: 15.7 ± 3.6; IVP d17: 17.9 ± 3.9; $P < 0.05$). The AI group on d17 had intermediate basal concentration (AI d17: 33.3 ± 6.6) which was greater than IVP on d17 ($P = 0.03$), but similar to NI and both groups on d32 ($P > 0.05$). Figure 5C demonstrates the distribution of circulating basal PGFM concentration for individual cows in each group.

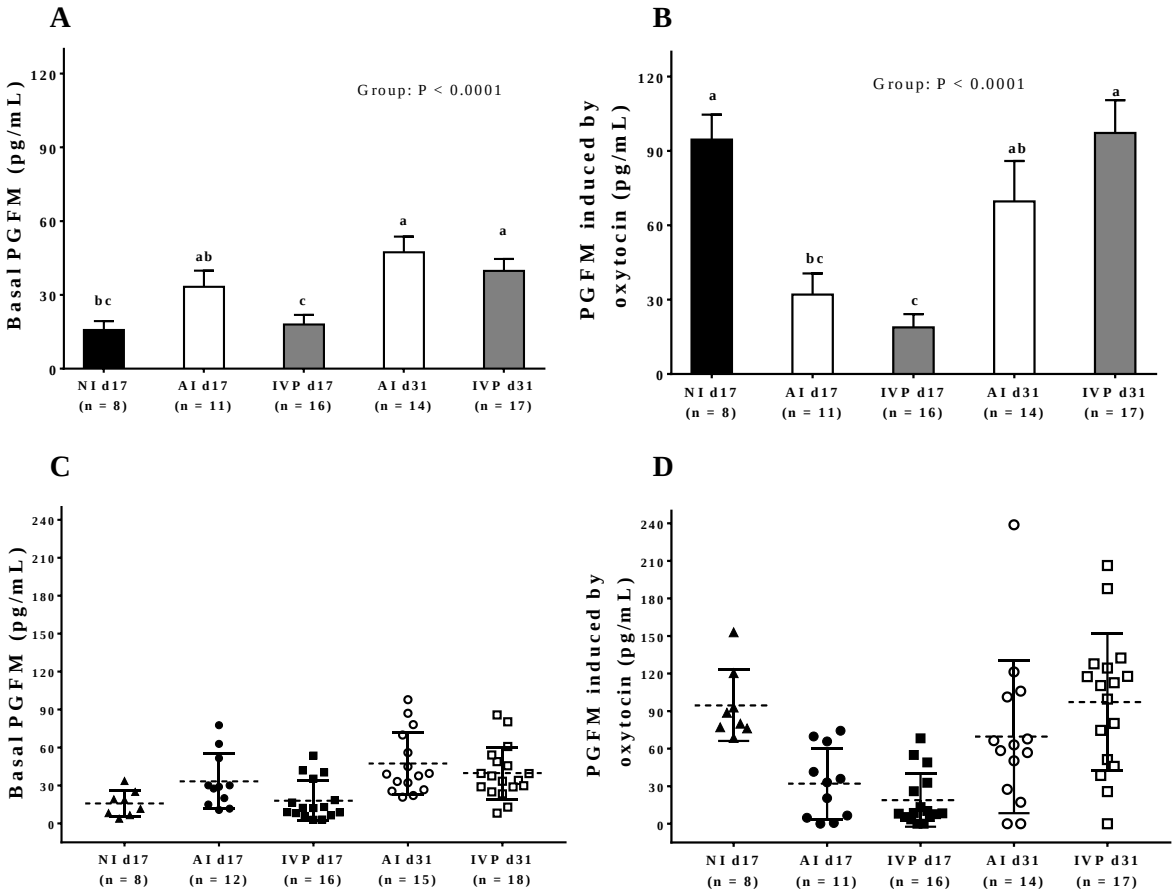

Figure 5. Basal circulating concentration of PGFM, before oxytocin challenge (0 min) for all experimental groups (A) and the oxytocin-induced response in PGFM concentrations 60 and 120 min after oxytocin challenge (B). The PGFM concentrations at 60 and 120 min after oxytocin were averaged and the basal concentration was subtracted to obtain the induced PGFM response. (C) Distribution of basal concentrations of PGFM and for induced PGFM increase after oxytocin (D) in all experimental groups. Dashed lines represent the average for each group. Data are shown as means ± SEM. a, b, c, d $P \leq 0.05$.

In order to determine the increase in PGFM after the oxytocin challenge, the PGFM concentrations at 60 and 120 min after oxytocin were averaged for each animal and the baseline oxytocin was subtracted to provide an average oxytocin-induced response (Figure 5B) and the distribution of oxytocin responses for each individual cow (Figure 5D). The NI d17 cows had a greater response to the oxytocin challenge than either of the pregnant groups on d17. The groups on d31 of pregnancy were similar to each other and to the NI group. The AI and IVP groups on d17 were similar to each other and were different from the groups on d31 of pregnancy. The variability in response to oxytocin in individual cows within each group was substantial with some cows in all of the pregnant groups having no respone or an elevated response to oxytocin (Figure 5D).

3.3.2. Conceptus/embryo development

The length on d18 of pregnancy of each conceptus recovered is represented by a scatter plot (Figure 6A). There was no difference between the mean of AI and IVP (AI d18d18: 44.6 ± 4.4 vs IVP d18: 53.3 ± 5.9 cm; P = 0.28).

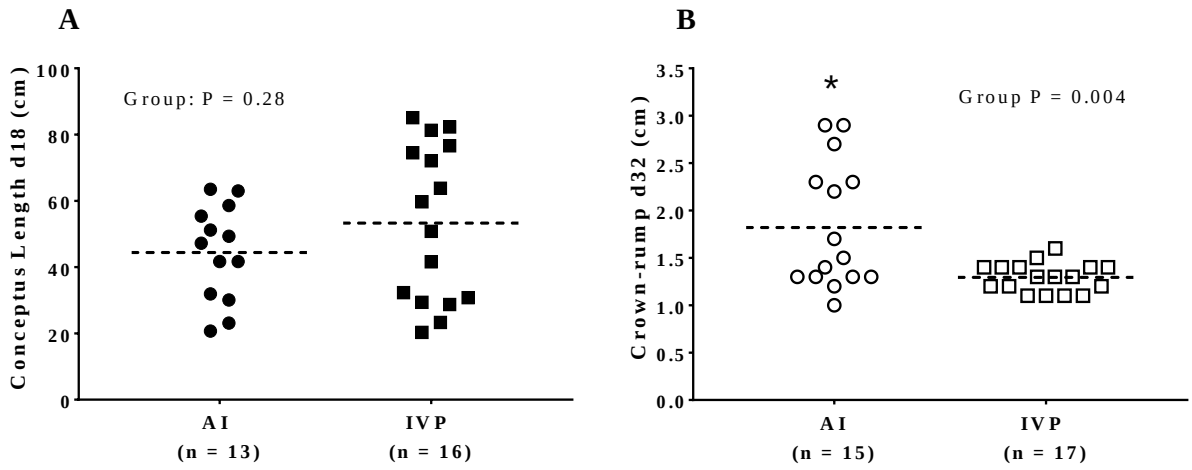

Figure 6. Distribution of the length of conceptuses recovered from uterine lumen on d18 (A). Distribution of crown-rump measurements of embryos recovered on d32 (B) Mean conceptus length and mean crown-rump measurement is represented by the dashed line. * P ≤ 0.05 for the mean (dashed line).

On d32 of pregnancy, AI embryos were significantly larger, on average, when crown-rump was measured (Figure 6B; AI d32: 1.8 ± 0.2 vs IVP d32: 1.3 ± 0.1 cm; P = 0.0004). In addition, it was possible to see the different distribution of embryo sizes between the two groups.

3.3.3. Gene expression

The mRNA relative expression of OXTR in endometrial tissue, demonstrated by fold change, was similar between ipsilateral and contralateral uterine horns in relation to CL side (Figure 7B; Side, P = 0.18) with no interaction between Side*Group (P > 0.05). However, a significant difference among groups was detected (Figure 7A; P < 0.0001). The OXTR expression was lower in IVP d18 (0.07 ± 0.01). Pregnant cows from AI on d18 had higher OXTR expression (0.41 ± 0.08) than IVP on the same day but lower than NI on d18 (1.42 ± 0.30) and pregnant cows from AI on d32 (1.43 ± 0.21). Pregnant cows from IVP embryo on

d32 (0.82 ± 0.17) had lower expression than AI on d32, and tended to be lower than NI on d18 (P = 0.06), and were similar to AI on d18.

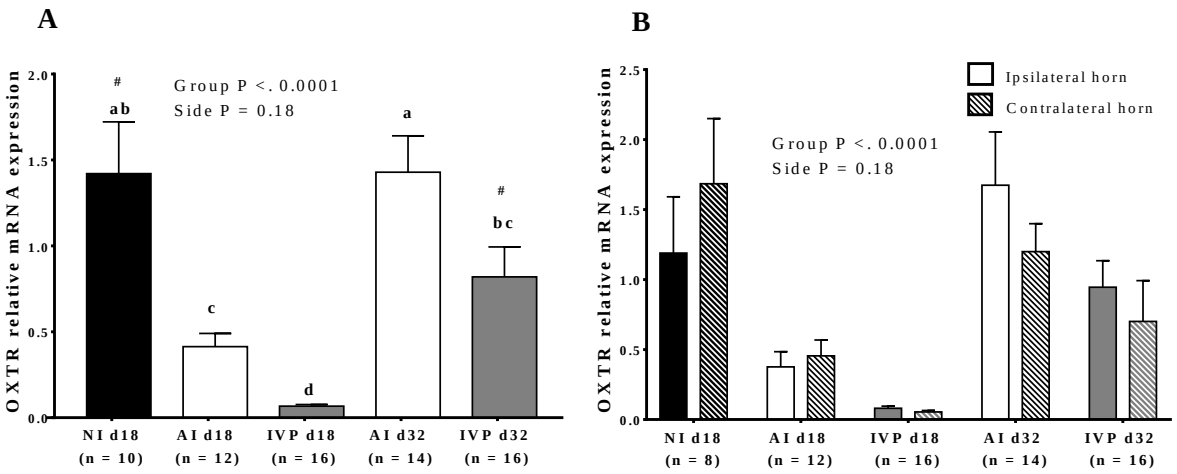

Figure 7. Relative abundance of mRNA for oxytocin receptors (OXTR) presented as fold change. Data from endometrial tissue collected from cows in their respective groups: Non-inseminated on d18 of the estrous cycle (NI d18); Pregnant from AI on d18 (AI d18); pregnant from an IVP embryo on d18 (IVP d18); Pregnant from AI on d32 (AI d32) and pregnant from an IVP embryo on d32 (IVP d32). (A) Representative figure of main effect of group (P < 0.0001). (B) Representative figure of effect of uterine horn side in relation to CL: ipsilateral horn vs. contralateral horn (P = 0.18). Group*Side: P = 0.53. Data are shown as means ± SEM. a, b, c, d P ≤ 0.05. # P < 0.10.

The ISG15 mRNA relative expression in endometrial tissue is illustrated in Figure 8A and 8B. There was a Group effect (P < 0.0001) but no Side effect (Figure 8B, P = 0.54) and no interaction of Group*Side (P > 0.05). Pregnant cows on d18 in both groups had greater (P < 0.001) ISG15 mRNA (AI d18: 109.65 ±.20.85 and IVP d18: 89.08 ± 23.92) than the other groups. All other groups (NI, AI d32, IVP d32) did not differ (P > 0.05) between each other and had very low ISG15 mRNA concentrations (NI d18: 2.48 ± 0.92; AI d32: 13.11 ± 3.70 and IVP d32: 8.55 ± 3.40).

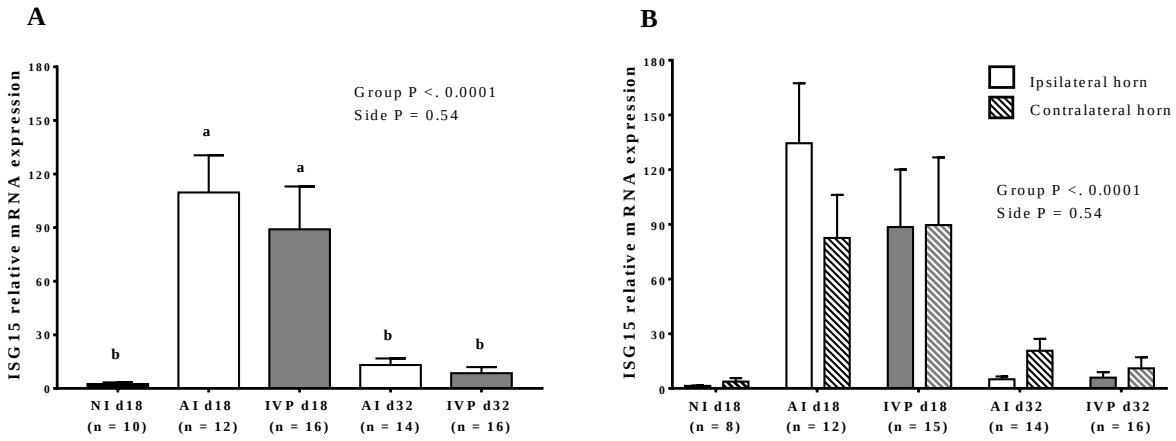

Figure 8. Relative abundance of mRNA for interferon-stimulated gene 15 (ISG15) presented as fold change. Data from endometrial tissue collected from cows in their respective groups: Non-inseminated on d18 of the estrous cycle (NI d18); Pregnant from AI on d17 (AI d18); pregnant from an IVP embryo on d18 (IVP d18); Pregnant from AI on d32 (AI d32) and pregnant from an IVP embryo on d32 (IVP d32). (A) Representative figure of main effect of group ($P < 0.0001$). (B) Representative figure of effect of uterine horn side in relation to CL: ipsilateral horn vs contralateral horn ($P = 0.54$). Group*Side $P = 0.26$. Data are shown as means ± SEM. a, b, c, d $P \leq 0.05$.

3.3.4. Correlation between circulating P4, circulating PGFM and embryo development

The relationship between variables such as circulating P4 on D6.5, P4 on d17, basal PGFM concentration on d17, conceptus length on d18, induced PGFM, OXTR and ISG15 was evaluated and is shown in Table 2 and 3. It was detected a significant ($P < 0.01$) and positive correlation ($R = 0.45$-0.48) between conceptus length on d18 and circulating P4 on d17 or circulating basal PGFM, independent if embryos were from AI or IVP. There was no significant relationship between circulating P4 on d6.5 and other variables ($P > 0.05$).

Table 2. Pearson / Spearman correlation coefficients representing the relationship among variables for data from cows on group d18.

	Conceptus length d18	Circulating P4 d6.5	Circulating P4 d17	Basal PGFM	Induced PGFM	OXTR	ISG15
Conceptus length d18	1.00	-0.21	0.29	0.41*	-0.34*	-0.33#	-0.02
Circulating P4 d6.5	-	1.00-	0.44*	-0.36*	0.23	0.38*	-0.09
Circulating P4 d17	-	-	1.00-	0.01	0.17	0.17	0.02
Basal PGFM	-	-	-	1.00	-0.23	-0.22	0.35*
Induced PGFM	-	-	-	-	1.00	0.44*	-0.31*
OXTR	-	-	-	-	-	1.00	-0.20
ISG15	-	-	-	-	-	-	1.00

* $P < 0.05$. # $P < 0.10$.

Table 3. Pearson / Spearman correlation coefficients representing the relationship among variables for data from cows on group d32.

	Crown-rump	Circulating P4 d6.5	Circulating P4 d31	Basal PGFM	Induced PGFM	OXTR	ISG15
Crown-rump	1.00	0.07	0.15	0.03	-0.09	0.08	0.06
Circulating P4 d6.5	-	1.00-	0.66*	0.07	-0.0005	0.07	0.009
Circulating P4 d31	-	-	1.00-	0.15	0.05	-0.0006	0.13
Basal PGFM	-	-	-	1.00	-0.26	0.03	0.28
Induced PGFM	-	-	-	-	1.00	-0.23	-0.09
OXTR	-	-	-	-	-	1.00	0.35*
ISG15	-	-	-	-	-	-	1.00

* P < 0.05. # P < 0.10.

3.4. Discussion

This study provided new information regarding the endocrine and molecular environment through early and post-IFNT releasing period during pregnancy. Several studies have described the differences between IVP vs IVD embryos, especially comparing first days of development [15, 46-49]. However, only few studies compared these embryos during the elongation period, maternal recognition time, and after the first month of pregnancy, during the peri-implantation period. Our findings may contribute to elucidate the possible mechanisms that drive a successful or the early interruption of a pregnancy.

Lack of synchrony between embryonic gastrulation and trophoblastic elongation has been suggested as a potential developmental defect that may produce pregnancy loss during early pregnancy [50]. Some of the most dramatic and well-described disabilities in development during this period occur in IVP embryos [10, 51] or clones using somatic cell

nuclear transfer (SCNT) [10, 52-54]. These embryos have increased pregnancy loss between 30-60 days. For example, 25% of the pregnancies from IVP embryos are lost during this period, whereas, this number can be as high as 80% in clones [55].

The effects of circulating P4 on embryo development have been strongly discussed in the past years. Supplementation of P4 prior to d7 after AI is associated with increase in d14 embryo size for AI and IVP embryos [56, 57]. Circulating P4 on d6.5 after AI or by the day of ET, in our data, was not different between IVP and AI groups. Since all cows were submitted to the same protocol and were randomly distributed between treatments, this result was expected. In addition, circulating P4 on d6.5 did not have any correlation with other variables. In fact, P4 seems to be important for conceptus elongation in some specific time-points. Suboptimal patterns of circulating P4 during early luteal phase can lead to gene expression alterations on uterine endometrial cells, suboptimal growth of the embryo, and reduced pregnancy success [58, 59]. One study that increased circulating P4 concentration from 3-7 d after AI showed increased conceptus length on d16 [56]. However, all of these studies used Bos taurus as experimental models. In this specific case, they achieved maximum circulating P4 close to 3-4 ng/mL on d7 after supplementation [56], which is similar to the range concentration of P4 related in Bos indicus cattle on d6.5 in our study. For this reason, it is uncertain the effect of P4 on conceptus for this genetic group, and it may justify the lack of association between P4 on d6.5 and conceptus length in our results. Furthermore, other authors reported no consistent relationship between P4 concentration on d6.5 and conceptus length at recovery on d17 and suggested that single assessment of P4 environment is not necessarily predictive of conceptus length on d17 [60]. On the other hand, some authors discarded the importance of P4 concentration for ET pregnancies. Whereas size of ovulatory follicle or P4 concentration on d7 had a significant effect on probability of pregnancy on d28 for AI procedures, it did not affect the probability of pregnancy for ET in Holstein cows [61, 62].

In our results, IVP and AI embryos were similar in length on d18. During a study from Bertolini et al. [12], IVP embryos were smaller than IVD on d16 (37.3 vs. 75.1 cm, on average), and had lower embryonic discs (ED; 0.10 vs 0.56 mm). However, in other data set, no differences between mean or median conceptus lengths on d17, comparing IVP vs IVD, were found. These authors emphasized that 4 of 23 (17.4%) *in vitro* produced conceptuses were longer than the longest IVD conceptus, and the IVP group contained numerically the longest conceptuses [60]. It is also comparable to our data, in which 6 of 16 (37%) IVP embryos were longer than the longest AI embryo, which give the idea that the IVP embryos

were elongating as much as or more than their IVD equivalent. Other reports demonstrated that conceptuses derived from IVP embryos were longer on d12 [63] and shorter on d13 [64] compared with IVD controls. Farin et al [65], with another contrasting result, reported that IVP conceptuses were longer (25.5 vs 16.6 cm) than conceptuses from IVD, and in IVP embryos, the class that had larger conceptuses were expanded blastocysts (41.3 cm). In our data, pregnancies obtained from the IVP group were mostly from expanded blastocysts (22/36) but unintentionally, all pregnancies obtained on d18 were from expanded blastocysts. The conflicting results among studies could be attributed to differences between *in vitro* conditions used to produce embryos or the timing of recovery relative to the onset of maternal recognition of pregnancy.

On d17 in our findings, circulating P4 was similar between groups, and had significant correlation with conceptus length on d18. This finding corroborated other information from Angus heifers as recipients. The study examined the relationship between recipient serum concentrations of P4, at the time of ET (d7) and at conceptus recovery (d17), on conceptus development from IVD or IVP embryos. Based on regression analyses performed, no significant relationships were identified between P4 concentrations at the time of embryo transfer and conceptus length at recovery on d17 (IVD: 6.1 vs IVP: 7.6 ng/mL). The relationship between P4 on d17 and conceptus length was not evaluated on that study, but there was also no difference in circulating P4 on d17 between IVD and IVP groups [60].

On d31, AI pregnancies presented more circulating P4 and on d32 had greater embryo size than IVP. Bertolini et al. [12] recovered and measured the ED using an ocular micrometer eyepiece and showed that ED were detected in 37% (7/19) of IVD control group and 35% (6/17) of IVP d16 intact conceptus. Nevertheless, the IVP group presented smaller ED diameters, supporting our data on later pregnancies. *In vitro*-cultured embryos seem to be less developmentally competent than IVD-cultured. This is confirmed by the deficiency in genes controlling transcription of IVP-cultured embryos and by the overall level of transcription in IVP-cultured embryos, which was reduced compared to IVD-cultured embryos [46].

Some authors that described the circulating P4 behavior during pregnancy, have shown approximately 8-11 ng/mL of circulating P4 on d 30-35 of pregnancies [66-68], similarly to our results for the AI group. None of previous studies compared IVD vs IVP pregnancies regarding circulating P4 increase rate. However, the similarity between P4 concentration described in previous studies and in ours, indicates that besides to be

developmentally incompetent, IVP embryos may also be inefficient also in keeping circulating P4 concentrations high later in pregnancy.

There was also higher basal circulating PGFM for pregnant cows, and high correlation between conceptus length and basal PGFM. These results may be explained by the embryo and endometrium synergism increasing prostaglandin release. In fact, some authors previously described that the embryo produces PGF during early pregnancy, demonstrated by uterine fluid concentrations [69, 70] or even by evaluating production by embryos cultivated *in vitro* [69, 71]. A study in ewes, collected embryos from d13 and d15, and endometrial tissues and cultured *in vitro* for analysis of prostaglandin released in culture media. In fact, embryos produced high concentrations of PGF in the media, releasing 1833 ng during 8 h incubation, and it did not depend upon the presence of endometrial tissues [69]. In addition, a study evaluating bovine conceptus and endometrium cultivated for 24 h, described higher concentration of PGF, PGFM and PGE2 in uterine flushings of pregnancies from d19 than from d16, and for all these measurements, pregnant cows had almost 3-fold higher concentration than non-pregnant cows [71].

Our data showed that circulating PGFM increased after oxytocin challenge, partially confirming our hypothesis. Similar to the results in this study, a previous experiment from our group (Drum et al., in press), described the increase in oxytocin-induced PGFM on d32 similar to non-pregnant cows on d18 (near the time of luteolysis). Our results from induced PGFM in AI on d17, although lower than NI cows and IVP on d31, were not different than AI d31. An important difference between the current study and our previous report for AI, was the basal level of PGFM, which presented similar concentration to d31, contradicting our previous findings. We attribute this difference mainly to the genetic group, which has been reported to have relevant differences especially regarding hormone levels and reproductive efficiency [36, 72-75]. A recent study with PGFM-induced in Bos indicus on d30, using a similar ELISA assay, has reported higher response than ours, even in this study, achieving 739 pg/mL 2 h after challenge [76]. It confirms that Bos indicus cattle have substantial differences regarding hormone concentrations even for prostaglandins, although in this present study no comparison between Bos taurus and Bos indicus was performed.

A study performed in Holstein heifers had similar experimental design as ours [77]. Two groups of cattle were selected using the amplitude of their prostaglandin response to an oxytocin challenge (100 IU, i.v.) as a potential indicator of the ability to achieve a successful pregnancy. Heifers were submitted to ET on d7 after estrus and had their conceptuses recovered on d17. Circulating PGFM concentration in the previous cycle, in fact did not

represent a good indicator for successful pregnancy. However, when separated from small and large embryos they found interesting results. Corroborating previous mentioned data, the circulating P4 on d3 and d5 after estrus were lower on cows that presented smaller conceptuses, however on d7 this difference disappeared [77], which confirms the absence of correlation between conceptus length and circulating P4 on d6.5 in our data.

Similar to our results, the same authors described no difference in OXTR expression between uterine horns, indicating that the elongated embryo signaled in whole uterus. Supporting our data on d18, non-pregnant cows also had higher OXTR, approximately 2.5-fold greater expression than pregnant cows on d17 [77]. Interestingly, in a universe of 30 pregnancies there were 10 conceptuses smaller than 12 cm, different from our results that only had three conceptuses with small size.

Suppression of OXTR near d18 has being extensively described by collecting tissue directly from the uterus [78-80] or by *in vitro* culture of uterine tissues [20]. However, our results described for the first time the abundance of OXTR expression in pregnant uterus from d32. Similarly, in a previous report, OXTR was detected in low amounts on d50 of pregnancy and increased 6-fold by d280 of pregnancy [81], indicating the impact of the days of pregnancy on the sensitivity to oxytocin. In addition, another study reported that endometrial OXTR are low on d20 (165 fmol/mg protein) and on d50 (344 fmol/mg) of pregnancy but increase during later pregnancy close to the time of parturition [82].

A speculative possible reason for IVP gestation having smaller responses to oxytocin, as well as lower oxytocin receptor expression in the endometrium on d18 and d32, is IFNT secretion. Some authors have described higher IFNT expression in IVP embryos compared to IVD [14] which can be causing higher OXTR suppression and for longer periods. Besides higher secretion of IFNT seems to be important for a successful pregnancy, a delay in OXTR recovery during the second month, may jeopardize PGF release by the uterus during pregnancy, which also seems to be important to maintaining pregnancy, especially because it increases during pregnancy [32, 70, 83].

A possibility suggested from some groups, is that prostaglandin E (PGE) acts as a luteoprotective factor during pregnancy, and some evidences confirmed changes in PGE behavior during maternal recognition. It is known that IFNT stimulates PGE sintase (PGES) in the uterus and especially in CL of ewes [84]. Also, throughout the estrous cycle in cows, PGES protein was highly expressed in CL before the expected time of luteolysis [85]. Another strong evidence that corroborate the prostaglandins importance for pregnancy is the temporal and tissue-specific expression of PGE and PGF receptors and COX-1 and -2 at the

maternal-fetal interface. It suggests a possible selective and distinctive role for PGE2 and PGF2 in uterine activities during gestation in bovine [86]. Besides these data, nothing else was reported in this regard, comparing AI, IVP or IVD embryos.

An interesting study was designed to test the capacity of the CL from pregnancy to be maintained only using exogenous P4 or by a new induced CL before or after d36 of pregnancy. Corroborating data previously mentioned in this paper, ipsilateral induced CL were more likely to maintain the pregnancy (13/13 vs 2/6). In addition, maintenance of pregnancy tended to be greater in cows with high concentrations of PGF and low concentrations of estradiol-17β during d31 through 35. Also, it was reported that concentrations of PGF2α were relatively constant, between 200 and 400 pg/mL, rather than secretory episodes of > 1.5 ng/mL that induced luteal regression. The stage of pregnancy in which CL were induced affected the development and function of the induced CL, and the proportion of pregnancies maintained, with greater success after d36 of pregnancy [87]. These results also suggested that a second signal from the embryo during the second month of pregnancy may be required to complete maternal recognition of pregnancy and maintenance of the CL.

Another conceivable signal from the embryo that does not discard a luteoprotective factor and may be the second month factor previously suggested, is that in spite of uterine PGF secretion, PGF does not reach the CL through local mechanisms due to the elevated blood flow in the uterine horn ipsilateral to the pregnancy during the second month of pregnancy [3]. Thereby, a local mechanism exists during the second month of pregnancy that protects the CL that is ipsilateral but not contralateral to the pregnancy, explaining regression of the contralateral accessory CL during d33-60 of pregnancy, but continued maintenance of the pregnancy and ipsilateral CL [33]. Blood flow in the uterine horn contralateral to the pregnancy increases at a slower rate than in the ipsilateral horn [88], and maybe a deficient capacity of increasing blood flow, could be a factor that impacts the efficiency of IVP embryos to establish pregnancies.

In conclusion, our study supported previous data that described the early pregnancy suppression of OXTR and its restablishment in uterus during later pregnancy. Moreover, our data confirmed our hypothesis that this phenomenon happened after the expected time of IFNT release, by the end of the first month as confirmed by lack of IFNT uterine signaling represented by ISG15. In addition, IVP and AI embryos were associated to different paracrine effects, such as for OXTR on d18 and 32 and basal PGFM on d17. In spite of that and contrary to our hypothesis, the variation in conceptus length on d18 was similar such as the

oxytocin-induced PGF release. Although, on d32 the variation in crown-rump seems to be higher in AI embryos.

Future studies may be designed aiming to identify if in fact there is a luteoprotective factor, besides IFNT, acting in CL maintenance or if local and biomechanical factors such as blood flow changes throughout gestation are responsible for keeping pregnancies and CL viable after the first month of gestation. Further studies should be performed to compare IVP vs AI in this regard, and can be an important tool for understanding pregnancy loss.

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4. FINAL CONSIDERATIONS

The studies presented in this thesis, contributed to the understanding of the mechanisms of pregnancy maintenance and loss in dairy and beef cattle. Nowadays, pregnancy losses between d30 and 60 are one of the most challenging problems faced by dairy and beef herds, but little is known about the mechanisms that lead to that. Our experiments were designed to understand one of the suggested mechanisms that maintain the CL after maternal recognition of pregnancy, when IFNT is no longer secreted, the PGF release pattern. It has been concluded that PGF is released by d32 of pregnancy and exhibiting the luteolytic machinery able to regress contralateral CL, represented by the increase in OXTR at this point. However, the role of PGF during pregnancy is still unknown. In spite of that, from the data obtained in this thesis, we could not detect any local uterine effects (contralateral vs ipsilateral). In addition, *in vitro* embryos seem to cause different behavior in PGF suppression and release during pregnancy.

The PGF role on pregnancy is the primary next step for undersanding these results. Cause-effect experiments, for example, blocking the PGF release by the uterus or even OXTR could be a good way to detect specific effects of PGF release during the second month of pregnancy. A possible effect of PGF is acting as a stimulus for development of the attachment between the embryo and uterus, maybe related to PAGs release. Another possibility but less exciting, is that the uterus is starting the preparation for calving, which demands a massive contraction of the uterine smooth muscle stimulated by oxytocin.

Another topic related to our results that should be studied, is the difference between breeds, since the physiology for *Bos indicus* (Nelore) is quite different from *Bos taurus* (Holstein) cattle, as represented by much lower pregnancy loss in AI. The next step in this topic should be another experiment with challenge of oxytocin in other days of pregnancy for Nelore. Furthermore, to test multiple challenges in the same animal, which can confirm that the oxytocin is not able to cause pregnancy losses despite the increase in PGFM after oxytocin even on d17 in Nelore. In addition, how much crossbred embryos like the ones used in our second experiment, are signaling in an endocrine and paracrine manner during pregnancy, and how it can affect the maintenance of pregnancy.

It is clear that *in vitro* manipulation causes remarkable effects on embryos, and our second study provided evidences of these effects even at endocrine levels. The *in vitro* embryos, though, did not present different variability on d18 as previously suggested by other studies. For this reason, it seems that the embryo morphology is not that much representative

of embryo communication to the surrounding environment. Even though, IVP embryos also seem to communicate differently than AI-derived embryos, as demonstrated by differences in OXTR and PGFM concentrations reported in our study. More studies should be performed to characterize the effect of the embryo in uterine environment. First of all, describing the profile of molecular secretion by the embryo during this time period comparing AI and IVP to determine what else is differencially expressed and produced by them. It could be interesting to detect even new molecules that can be responsible for communication during this period. Later, to test cause-effect of this molecules in maintenance of pregnancy and CL. Experiments comparing IVP vs AI, like ours, contribute to the improvement of *in vitro* systems by detecting and correct possible drawbacks associated to this technique, contributing to their ablicability in the future.

Our data also discarded continuous secretion of ISGs on d32, agreeing with previous suggestions of another local luteoprotective mechanism in favor of pregnancy maintenance after the first month. Considering that most known studies were performed in ewes, other studies using cows could be designed to find candidate molecules for this role. However, the evidences of local hemodynamich effect can not be discarded. Thereafter, next experiments in this field should be designed to obtain conceivable data related to local effects of pregnancy. First of all, it is important to develop a precise method to quantify the blood flow in the uterus and verify if it is changing during pregnancy.