THIAGO MARTINS

Luteolytic and embryotrophic effects of progesterone supplementation in beef cattle

Pirassununga
2018

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Luteolytic and embryotrophic effects of progesterone supplementation in beef cattle

Thesis presented to the Graduates School in Animal Reproduction of the School of Veterinary Medicine and Animal Science of the University of São Paulo as requirement for title of Doctor in Science.

Department
Animal Reproduction

Concentration Area:
Animal Reproduction

Advisor:
Prof. Dr. Mario Binelli

In accordance:_____
Advisor

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CERTIFICADO

Certificamos que o Projeto intitulado "Efeito da suplementação com Progesterona injetável 4 dias pós IATF sobre a taxa de prenhez de vacas lactantes; ", protocolado sob o CEUA nº 6236220316, sob a responsabilidade de **Mario Binelli** *e equipe; Thiago Martins* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovado** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 18/05/2016.

We certify that the proposal "Effect of progesterone supplementation 4 days after TAI on the pregnancy rate of lactating cows", utilizing 1172 Bovines (1172 females), protocol number CEUA 6236220316, under the responsibility of **Mario Binelli** *and team; Thiago Martins* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the University of São Paulo (CEUA/FMVZ) in the meeting of 05/18/2016.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 12/2014 a 04/2015 Área: Reprodução Animal

Procedência:	Animais de proprietários						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	2 a 12 anos	N:	1172
Linhagem:	Bos indicus (Nelore)			Peso:	400 a 600 kg		

Resumo: Incrementos na taxa de prenhez após o uso de IATF serão sempre necessários visando contemplar a crescente necessidade de aumento da eficiência reprodutiva e rentabilidade do sistema. O principal entrave para esse aumento pode estar associado aos elevados índices de mortalidade embrionária (? 30%) entre os Dias 8 e 16 da gestação. A administração de cipionato de estradiol no período pré-ovulatório e/ou progesterona (P4) injetável de longa ação pode favorecer o desenvolvimento e a manutenção da gestação. Estudo prévios demonstraram que a administração de cipionato de estradiol no final do protocolo de sincronização ovulação resultou em aumento das taxas de prenhez de fêmeas de corte (Theriogenology, 2011, 76:455-63; J. Anim. Sci. 2013, 93:1176-85). Recentemente observamos que a administração de P4 de longa ação no diestro inicial aumentou as taxas de prenhez de fêmeas de corte em anestro (Theriogenology, 2016, in press). Assim presente estudo tem como objetivos (1) determinar se a resposta à suplementação com P4 injetável é influenciada positivamente pela administração de cipionato de estradiol e (2) estabelecer se a condição ovariana do animal no início do protocolo de sincronização (i.e. anestro ou ciclando) altera a resposta ao tratamento com P4 injetável. Busca-se com este estudo, dar maior suporte para o uso eficiente e seguro da suplementação com P4 injetável de longa ação.

Local do experimento: Munícipio de Rochedo / MS

São Paulo, 22 de julho de 2016

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

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

CERTIFICADO

Certificamos que a proposta intitulada "Efeito do embrião sobre o processo luteolítico antecipado em vacas de corte suplementadas com P4", protocolada sob o CEUA nº 4664220316, sob a responsabilidade de **Mario Binelli** *e equipe; Thiago Martins* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 27/10/2016.

We certify that the proposal "Embryo effect on the early luteolitic process of beef cows supplemented with P4", utilizing 53 Bovines (53 females), protocol number CEUA 4664220316, under the responsibility of **Mario Binelli** *and team; Thiago Martins* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 10/27/2016.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 06/2016 a 12/2016 Área: Reprodução Animal

Origem:	Prefeitura do Campus da USP de Pirassununga						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	2 a 15 anos	N:	53
Linhagem:	Nelore			Peso:	400 a 650 kg		

Resumo: A suplementação com progesterona (P4) no início do diestro aumenta as taxas de prenhez, porém, também antecipa a regressão funcional e estrutural do corpo lúteo (CL) que pode prejudicar o sucesso da estratégia. Especulamos que essa antecipação (i.e. luteólise precoce) pode ser evitada pela presença do concepto (embrião + membranas anexas), que é o principal agente responsável pela manutenção do CL. Porém, esse efeito ainda não é conhecido e o presente estudo tem como objetivo investiga-lo. Para isso, foram propostos experimentos para testarem as seguintes hipóteses: (experimento 1) vacas inseminadas e suplementadas com P4 apresentam menor frequência de luteólise precoce que vacas não inseminadas e suplementadas com P4; (experimento 2) a presença do embrião em vacas suplementadas com P4 aumenta a probabilidade de concepção, por conseguinte, o processo luteolítico antecipado ocorrerá em menor proporção ou será anulado comparado com a ausência de embrião; (experimento 3) em vacas suplementadas com P4 a presença do embrião diminui a expressão dos receptores que controlam a liberação de PGF2? e desloca a síntese de PGF2? para PGE2 no endométrio. No experimento 1, vacas Nelore multíparas não lactantes detectadas em cio serão selecionadas para receberem 3 tratamentos: IA e 150 mg de P4 de longa ação (P4-LA) no Dia 3 (Dia 0: ovulação); diluente no momento da IA e 150 mg de P4-LA no Dia 3 (Controle positivo) ou diluente no momento da IA e placebo no Dia 3 (Controle negativo). No experimento 2, os animais serão selecionados no Dia 3 para 2 receberem P4-LA ou placebo e transferência de 0, 1 ou 5 embriões (TE) no Dia 7 (fatorial 2x3). A função luteal nos experimentos 1 e 2 será monitorada diariamente por exame ultrassonográfico e Doppler e por dosagem de concentrações plasmáticas de P4 nos Dias 3, 9, 11, 13, 15, 17, 19 e 21. No experimento 3, as vacas serão tratadas no Dia 3 com P4-LA ou placebo e transferência de 0 ou 5 embriões no Dia 7 (fatorial 2x2). Fragmentos de tecido endometrial post-mortem serão coletados no dia que precede o momento de maior ocorrência de luteólise precoce, previamente estabelecido no experimento 2. Será analisada a abundância dos transcritos ligados ao controle do momento da luteólise [i.e. progesterone receptor (PGR), estrogen receptor 1 (ESR1), estrogen receptor 2 (ESR2), oxytocin receptor (OXTR)], à via de síntese de prostaglandinas (PGs) [i.e, phospholipase A2, grupo X (PLA2G10), prostaglandin-endoperoxidase synthase 2 (PTGS2)], à síntese de PGF2? [aldo-keto reductase, family 1, member B1 (AKR1B1), aldo-keto reductase, family 1, member C3 (AKR1C3), aldo-keto reductase, family 1, member C4 (AKR1C4), carbonyl reductase 1 (CBR1))] e à síntese de PGE2 [i.e. prostaglandin E synthase (PGES), prostaglandin E synthase 2 (PTGES2) e prostaglandin E synthase 3 (PTGES3)]. Nas vacas estimuladas com P4 espera-se que a incidência de luteólise precoce seja atenuada pela IA e TE ou até mesmo anulada pela transferência de 5 embriões. No endométrio das vacas estimuladas pela P4 a presença do concepto pode diminuir a expressão dos genes ESR1, ESR2 e OXTR, não alterar a dos PLA2G10 e PTGS2 (i.e, permanecem aumentados) e aumentar a expressão das PGE sintases (i.e. PGES, PGES2 e PGES3) em detrimento aos genes envolvidos com a síntese de PGF2? (i.e. AKR1B1, AKR1C3, AKR1C4 e CBR1). Busca-se com este estudo, dar maior suporte para o uso eficiente e seguro da suplementação com P4 injetável de longa ação.

Local do experimento: Departamento de Reprodução Animal (VRA-USP) - Pirassununga

São Paulo, 30 de outubro de 2016

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

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

CERTIFICADO

Certificamos que a proposta intitulada "Histotrofo e receptividade uterina", protocolada sob o CEUA nº 9585220316, sob a responsabilidade de **Mario Binelli** *e equipe; Thiago Martins* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 18/05/2016.

We certify that the proposal "Histotroph and uterine receptivity", utilizing 32 Bovines (32 females), protocol number CEUA 9585220316, under the responsibility of **Mario Binelli** *and team; Thiago Martins* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of July 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 05/18/2016.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 05/2015 a 09/2015 Área: Reprodução Animal

Origem:	Prefeitura do Campus da USP de Pirassununga						
Espécie:	Bovinos	sexo:	Fêmeas	idade:	2 a 12 anos	N:	32
Linhagem:	Nelore			Peso:	400 a 650 kg		

Resumo: Antes da placentação o sucesso gestacional está vinculado a um ambiente uterino capaz de prover um fluído com metabólitos em concentrações adequadas para a manutenção do embrião. A composição desse fluído conhecido como histotrofo, está diretamente relacionado ao desenvolvimento embrionário e estabelecimento da gestação. Apesar da importância do histotrofo para o embrião pouco se sabe sobre sua composição e dinâmica ao longo do ciclo estral. Portanto, o presente estudo tem como objetivos (1) determinar as alterações provocada nas concentrações de proteínas secretadas pelo endométrio após lavagens uterinas realizadas em dias específicos do ciclo estral (2) determinar a taxa de concepção em receptoras de embrião submetidas a lavagens uterinas prévias.

Local do experimento: Departamento de Reprodução Animal (VRA-USP) - Campus Fernando Costa

São Paulo, 18 de dezembro de 2017

Profa. Dra. Anneliese de Souza Traldi
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

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EVALUATION FORM

Author: MARTINS, Thiago

Title: **Luteolytic and embryotrophic effects of progesterone supplementation in beef cattle**

Thesis presented to the Graduates School in Animal Reproduction of the School of Veterinary Medicine and Animal Sciences of the University of São Paulo as a requirement for the title of Doctor in Science.

Date: _____/_____/_____

Examination Board

Prof. Dr._____

Institution:_____ Verdict:_____

Prof. Dr._____

Institution:_____ Verdict:_____

Prof. Dr._____

Institution:_____ Verdict:_____

Prof. Dr._____

Institution:_____ Verdict:_____

Prof. Dr._____

Institution:_____ Verdict:_____

I dedicate this doctoral Thesis to my mother, Norma H. A. Lacerda. The one who taught me to be persistent and the value of hard working.

Thank you for everything. I love you.

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First of all, I would like to thank God for always being with me, giving me strength and blessing my life.

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Finally, I express my deepest gratitude for all those mentioned here and those that I failed to mention, but that by one way or another contributed to my education and to my professional career.

"Remember to look upwards, to the stars, and not down, to your feet"

Stephen Hawking

RESUMO

MARTINS, T. **Efeitos luteolíticos e embriotróficos da suplementação de progesterona em bovinos de corte.** [Luteolytic and embryotrophic effects of progesterone supplementation in beef cattle]. 2018. 168 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, 2018.

Um ambiente uterino deficiente é uma das principais causas de falha gestacional em bovinos. A suplementação com progesterona (P4) durante o diestro inicial estimula a receptividade uterina, mas também pode encurtar a fase luteal, prejudicando a fertilidade. Os fatores que levam a um dos dois resultados não são claros. Nesta tese, quatro estudos foram conduzidos para testar a hipótese principal de que o concepto pré-implantacional bloqueia o efeito luteolítico antecipado causado pela P4 suplementar. Em um quinto estudo, avaliou-se a importância do ambiente uterino no estabelecimento da gestação. A suplementação com P4 foi realizada pela administração de 150 mg de P4 injetável (iP4) de longa ação 3 dias após a ovulação. No último estudo, lavados uterinos foram coletados nos dias 1, 4 e/ou 7 após o estro, com o objetivo de depletar o ambiente uterino. Nestes estudos, o útero desempenhou um papel crucial na determinação da resposta luteolítica da iP4. O primeiro experimento demonstrou que a exposição uterina a maiores concentrações de estradiol (E2) durante o período pré-ovulatório bloqueou efeito luteolítico da iP4, mas não resultou em incremento na taxa de prenhez. Análises complementares revelaram que a suplementação de iP4 teve efeito positivo na fertilidade quando houve uma ótima exposição do útero ao E2 pré-ovulatório. Vacas com maiores folículos pré-ovulatórios ou com pequenos folículos, mas suplementadas com estradiol exógeno, apresentaram maiores taxas de prenhez. A exposição sub-ótima ou exagerada ao E2 foi deletéria ao efeito embriotrófico da P4. Em seguida, demonstramos que a iP4 prejudicou o desenvolvimento luteal, porém, esse não foi o principal fator relacionado com a antecipação da luteólise. Aproximadamente metade das vacas suplementadas com iP4 apresentaram luteólise precoce, que ocorreu no dia 15 após a ovulação. O efeito do embrião sobre o processo luteolítico antecipado foi dependente da sua capacidade de estabelecer a gestação. No terceiro estudo, verificamos que a redução do desenvolvimento do folículo dominante da primeira onda esteve associado à ocorrência de luteólise precoce, e isso foi independente do

número de ondas no ciclo (dois vs. três). No entanto, ciclos de três ondas favoreceram a capacidade embrionária de inibir a luteólise precoce. No quarto estudo, nós não evidenciamos um efeito embriotófico da iP4 reduzindo a mortalidade embrionária, mesmo quando 5 embriões foram transferidos para cada receptora, com objetivo de maximizar a sinalização embrionária. No último estudo, a composição do ambiente uterino foi alterada por lavagens uterinas e isso afetou negativamente a prenhez, porém não aniquilou as chances de sobrevivência embrionária. A partir dos resultados obtidos nessa tese, concluímos que a variabilidade nas taxas de fertilidade após a suplementação com P4 é, em grande parte, determinada pela complexidade na programação da função uterina pelos hormônios ovarianos, em vez de ser causada pela incidência de luteólise precoce. Além disso, concluímos que uma composição sub-ótima do ambiente uterino é um dos principais contribuintes para as perdas embrionárias em bovinos de corte.

Palavras-chave: Útero. Estradiol. Folículo. Corpus Luteum. Embrião.

ABSTRACT

MARTINS, T. **Luteolytic and embryotrophic effects of progesterone supplementation in beef cattle.** [Efeitos luteolíticos e embriotróficos da suplementação de progesterona em bovinos de corte]. 2018. 168 p. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, Pirassununga, 2018.

Inadequate uterine environment is one of the main causes of pregnancy failure in cattle. Progesterone (P4) supplementation during early diestrus may either induce a receptive uterine status or shorten luteal lifespan and reduce fertility. Regulation that leads to either outcome is currently unclear. In this thesis, four studies were conducted to test the main hypothesis that the pre-implantational conceptus plays a major role on preventing P4-luteolytic effects in *Bos indicus* beef cows, due the supplementary P4. In a fifth study the importance of the uterine luminal milieu on the establishment of pregnancy was evaluated. P4 was supplemented by injecting 150 mg of long acting injectable P4 (iP4) on 3 days post-ovulation. In the last study, uterine luminal flushings were performed on days 1, 4 and/or 7 post-estrus, aiming to deplete the uterine milieu. In this thesis, we evidenced that uterus played a key role on determining the iP4-luteolytic response. In this sense, first study revealed that higher uterine exposure to estradiol (E2) during pre-ovulatory period prevented P4-luteolytic effect, but did not increase overall pregnancy outcome. Further analysis, revealed that optimal uterine estradiol exposure is required for beneficial effects of iP4. Cows with large preovulatory follicle or with small follicle, but exposed to the exogenous estradiol presented higher pregnancy rates. Next, we demonstrated that iP4 hindered CL formation, but this had a minor impact on iP4-induced luteolysis. About half of iP4 supplemented cows presented early luteolysis, which occurred by day 15 post-ovulation. Role of the embryo to inhibit iP4-induced early luteolysis relied on its capability to establish pregnancy. In the third study, we demonstrated that iP4-inhibition of growth of the first-wave dominant follicle was related to early luteolytic onset, and this was independent of number of waves in the cycle (two vs. three). Three-wave cycles favored embryonic capacity to inhibit early luteolysis. In the fourth study, we failed to demonstrate that P4 supplementation supported embryonic survival. This was despite of the transfer of 5 embryos to each recipient cow to

maximize embryonic signaling. In the last study we disturbed the composition of the uterine environment and negatively affected, but did not abolish, embryonic survival. Overall, from the results obtained in the course of this thesis, we conclude that variability in fertility rates after P4 supplementation, are in part attributable to the complexity of uterine function programming by sex steroids, rather than caused by the incidence of early luteolysis. Furthermore, we highlighted that a sub-optimal composition of the uterine environment is a major contributor to embryonic losses in beef cattle.

Keywords: Uterus. Estradiol. Follicle. Corpus Luteum. Embryo.

SUMMARY

GENERAL INTRODUCTION

Improvements in reproductive efficiency during the breeding season can increase the profitability of beef farms [1]. The reproductive performance of a breeding season is generally measured as the number of females that conceive during the first 30 days of that season. This is an important benchmark that governs calf-crop system and requires the use of timed AI and breeding management programs. The importance of adhering to a tight breeding window is highlighted by the fact that increasing the proportion of animals that conceive earlier in the season lead to a reduction in post-calving mortality, an increase in calf weaning weight, and a reduction in the age at which heifers reach puberty [2]. In addition, AI gives farmers the opportunity to infuse superior genetics into their operations. Thus it is desirable that more animals became pregnant from AI. The TAI programs are all well-established and generate pregnancy rates of around 50% after the first AI, when applied to beef herds with acceptable body condition scores [3–5]. Currently, several strategies have been proposed to improve pregnancy outcomes to basic TAI protocols [4,6]. The majority of those strategies are based on hormonal manipulations during the estrus synchronization protocol that aim to achieve pregnancy rates above the 50% mark.

One important factor that limits fertility gain in cattle is the incidence of embryonic mortality, that reaches 40 to 50% during the first three weeks of pregnancy [7,8]. During this period, the developing embryo depends exclusively on secretions from the oviduct and uterus (i.e., histotroph) for development. Failure of the uterine environment to support embryonic development is deemed to be one of major cause of pregnancy loss during these early stages [7,8]. The uterine secretion process, as well as the quality of such secretions, is programmed by the sequential exposure of the reproductive tract to estradiol (E2), during proestrus and estrus, and to progesterone (P4), during early diestrus. This justifies the use of hormonal strategies to create optimum oviductal and uterine environments for embryo development.

From the perspective of the maternal system, a key regulator of uterine function and histotroph composition is the circulating P4 concentrations during diestrus after AI. Indeed, a vast number of studies in the literature show that suboptimal patterns of circulating P4 compromise uterine functions resulting in poor fertility in cattle. It is also well-known that a more receptive uterine status can be generated through a pharmacological increase in circulating P4 concentrations at early diestrus. Accordingly, elevation of P4 at this time, stimulates endometrial secretions [10], accelerates conceptus elongation and subsequent release of interferon-tau into the uterine lumen [11–13], which favors the estabilishment of pregnancy. However, despite a clear positive role of P4 in the establishment of pregnancy in cattle, the fertility outcome after P4 supplementation is remarkably variable [14,15]. Such inconsistency in fertility results is a critical limitation of this strategy.

The reason for the negative or null effects of P4 supplementation in fertility has been classically associated to a greater incidence of shortened luteal lifespan, characterized by the onset of luteolysis before day 15 post-estrus. Early luteolysis results from P4–driven luteal formation impairment [16,17] and/or advancement of endometrial prostaglandin F2α (PGF2α) release [18]. It is deemed that advancing the time at which luteolysis takes place impairs the conceptus-maternal cross-talk during the critical time of maternal recognition of pregnancy. This is because the developing conceptus would not have achieved the capacity to secrete enough interferon-tau (IFNt) to block the PGF2α luteolytic pulses from the surrounding endometrium [19,20]. In contrast, P4 supplementation also accelerates conceptus elongation [11–13], which is expected to result in earlier signaling from the conceptus to the maternal unit, thereby preventing the early onset of luteolysis. Indeed, the incidence of early luteolysis seems to be more frequent in studies that use non-inseminated cows in their design [17,21,22], suggesting that the presence of an embryo decreases the chances of this process taking place. However, there is currently little information on the potential role of the elongating conceptus in the reproductive outcome of P4-supplemented animals.

Taking all this information into account, this thesis focused on the study of the factors related to the P4-luteolytic and P4-embryotropic effects, and the role of embryo on changing the P4-luteolytic effect. By determining the underlying mechanism that drives this complex interaction and elucidating the role of the embryo

we aimed to improve the way in which the P4-supplementation strategy is implemented and, consequently, decrease the proportion of early embryo losses. Furthermore, a secondary objective of this thesis was to evaluate the requirement of a proper uterine environment during the early diestrus for the establishment of pregnancy. **The present thesis tested the following three Central Hypotheses: (i) pre-ovulatory estradiol prevent early onset of luteolysis, improving fertility response to the P4 supplementation at early diestrus; (ii) preimplantational conceptus abrogates the P4-luteolytic effect; and finally (iii) an undisturbed uterine environment is required for pregnancy establishment.** To test the first hypothesis, an experimental model was developed in which pre-ovulatory estradiol concentration was manipulated through administration of estradiol cypionate at the end of a TAI protocol. During early diestrus, cows received or not a single injection of long-acting P4 (iP4). Because of effects of pre-ovulatory E2 on uterine programming, we rationalized that greater E2 concentrations could prevent the P4-luteolytic effect, improving the fertility response obtained after diestrus iP4 supplementation. To test the second hypothesis, two experiments were carried out. In the first experiment, cows were submitted or not to conventional AI follow by iP4 supplementation; cows not AI and non-supplemented with iP4 served as Control. Ovarian dynamics were followed for 21 days and P4 concentration was measured at the end of diestrus. In the second experiment, cows were assigned to receive or not 5 *in vitro* produced embryos and receive or not iP4. Again, ovarian dynamics were followed for 21 days, P4 concentration was measured at the end of diestrus, and abundance of a gene stimulated by IFNt was determined in peripheral blood mononuclear cells. To test the third hypothesis, uterine luminal flushing (ULF) was collected on days 1, 4 and/or 7, aiming to deplete the uterine milieu. On day 7.5, protein content of the recovered ULF was measured and in a second trial three embryos were transferred and pregnancy was evaluated.

The results obtained from these experiments are presented in five chapters. Each section is organized in a journal manuscript format. Chapters 1 to 5 are stand-alone manuscripts that contain the following subsections: introduction, materials and methods, results, discussion, and references. The manuscript that addresses the first hypothesis of this thesis (Chapter 1) was published in Theriogenology 2017 (Martins et al., 2017; Appendices A and B). The results obtained from the second experiment performed to answer the second hypothesis are divided into two manuscripts

(Chapters 2 and 3) and have been submitted to Domestic Animal Endocrinology (Appendices C and D; Proof of submission). Results from the second experiment carried out to address the second hypothesis are presented in Chapter 4, the manuscript is in the final process of corrections before submission. Chapter 5 manuscript addresses the third hypothesis and has been submitted to Journal of Animal Science and Biotechnology (Appendix E; Proof of submission). The final pages of this PhD Thesis have been reserved for a general discussion, which summarizes the studies carried out and the results obtained in the previous chapters, and provides directions for future research.

REFERENCES

1. Vasconcelos JLM, de Sá Filho OG, Cooke RF. Impacts of Reproductive Technologies on Beef Production in South America BT - Current and Future Reproductive Technologies and World Food Production. In: Lamb GC, Di Lorenzo N, editors. New York, NY: Springer New York; 2014. p. 161–80. Available from: https://doi.org/10.1007/978-1-4614-8887-3_8

2. Lamb GC. The 30-day game changer - reproductive management strategies to increase the percentage of calves born earlier in the calving season. In: XXII Novos Enfoques na Produção e Reprodução de Bovinos. Uberlândia 2017.

3. Meneghetti M, Filho OGS, Peres RFG, Lamb GC, Vasconcelos JLM. Fixed-time artificial insemination with estradiol and progesterone for Bos indicus cows I: Basis for development of protocols. Theriogenology. 2009;72:179–89.

4. Stevenson JS, Hill SL, Bridges GA, Larson JE, Lamb GC. Progesterone status, parity, body condition, and days postpartum before estrus or ovulation synchronization in suckled beef cattle influence artificial insemination pregnancy outcomes. J Anim Sci [Internet]. 2015;93:2111–23. Available from: https://www.animalsciencepublications.org/publications/jas/articles/93/5/2111

5. Vasconcelos JLM, Carvalho R, Peres RFG, Rodrigues ADP, Cooke RF, Pohler KG. Reproductive programs for beef cattle : incorporating management and reproductive techniques for better fertility. 2017;

6. Lamb GC, Mercadante VRG, Henry DD, Fontes PLP, Dahlen CR, Larson JE, DiLorenzo N. Advantages of current and future reprodutive technologies for world-wide beef cattle production. In: XXII Novos Enfoques na Produção e Reprodução de Bovinos. Uberlândia 2017.

7. Diskin MG, Sreenan JM. Fertilization and embryonic mortality rates in beef heifers after artificial insemination. J Reprod Fertil. 1980;59:463–8.

8. Wiltbank MC, Baez GM, Garcia-Guerra A, Toledo MZ, Monteiro PLJ, Melo LF, et al. Pivotal periods for pregnancy loss during the first trimester of gestation in lactating dairy cows. Theriogenology [Internet]. Elsevier Inc; 2016;86:239–53. Available from: http://dx.doi.org/10.1016/j.theriogenology.2016.04.037

9. Diskin MG, Parr MH, Morris DG. Embryo death in cattle: An update. Reprod Fertil Dev. 2012;24:244–51.

10. Garrett JE, Geisert RD, Zavy MT, Morgan GL. Evidence for maternal regulation of early conceptus growth and development in beef cattle. J Reprod Fertil. 1988;84:437–46.

11. Carter F, Forde N, Duffy P, Wade M, Fair T, Crowe M a., et al. Effect of increasing progesterone concentration from Day 3 of pregnancy on subsequent embryo survival and development in beef heifers. Reprod Fertil Dev. 2008;20:368–75.

12. O'Hara L, Forde N, Carter F, Rizos D, Maillo V, Ealy AD, et al. Paradoxical effect of supplementary progesterone between day 3 and day 7 on corpus luteum function and conceptus development in cattle. Reprod Fertil Dev. 2014;26:328–36.

13. Clemente M, De La Fuente J, Fair T, Al Naib A, Gutierrez-Adan A, Roche JF, et al. Progesterone and conceptus elongation in cattle: A direct effect on the embryo or an indirect effect via the endometrium? Reproduction. 2009;138:507–17.

14. Mann GE, Lamming GE. The Influence of Progesterone During Early Pregnancy in Cattle. Reprod Domest Anim. 1999;34:269–74.

15. Yan L, Robinson R, Shi Z, Mann G. Efficacy of progesterone supplementation during early pregnancy in cows: A meta-analysis. Theriogenology [Internet]. Elsevier Inc; 2015;1–9. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0093691X15007025

16. O'Hara L, Forde N, Duffy P, Randi F, Kelly AK, Valenza A, et al. Effect of combined exogenous progesterone with luteotrophic support via equine chorionic gonadotrophin (eCG) on corpus luteum development, circulating progesterone

concentrations and embryo development in cattle. Reprod Fertil Dev [Internet]. 2014;269–77. Available from: http://www.publish.csiro.au/?paper=RD14019

17. Ginther OJ. Effect of progesterone on length of estrous cycle in cattle. Am J Vet Res. 1970;31:493–6.

18. Garrett JE, Geisert RD, Zavy MT, Gries LK, Wettemann RP, Buchanan DS. Effect of exogenous progesterone on prostaglandin F2α release and the interestrous interval in the bovine. Prostaglandins. Elsevier; 1988;36:85–96.

19. Spencer TE, Johnson GA., Bazer FW, Burghardt RC, Palmarini M. Pregnancy recognition and conceptus implantation in domestic ruminants: Roles of progesterone, interferons and endogenous retroviruses. Reprod Fertil Dev. 2007;19:65–78.

20. Binelli M, Thatcher WW, Mattos R, Baruselli PS. Antiluteolytic strategies to improve fertility in cattle. Theriogenology. 2001;56:1451–63.

21. Burke CR, Mihm M, Macmillan KL, Roche JF. Some effects of prematurely elevated concentrations of progesterone on luteal and follicular characteristics during the oestrous cycle in heifers. Anim Reprod Sci. 1994;35:27–39.

22. Pugliesi G, Oliveria ML, Scolari SC, Lopes E, Pinaffi FV, Miagawa BT, et al. Corpus luteum development and function after supplementation of long-acting progesterone during the early luteal phase in beef cattle. Reprod Domest Anim. 2014;49:85–91.

CHAPTER 1

IMPACT OF ESTRADIOL CYPIONATE PRIOR TO TAI AND PROGESTERONE SUPPLEMENTATION AT INITIAL DIESTRUS

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Appendices A and B.

1 IMPACT OF ESTRADIOL CYPIONATE PRIOR TO TAI AND PROGESTERONE SUPPLEMENTATION AT INITIAL DIESTRUS

1.1 ABSTRACT

In cattle, early diestrus progesterone (P4) supplementation modulates endometrial function to exert pro- and anti-pregnancy establishment effects; specifically, P4 stimulates conceptus growth, but also induces early onset of luteolysis. This paradoxical effect is frequently related to the inconsistent fertility outcomes that result from P4 supplementation experiments. Aim was to investigate the impact of exogenous estradiol (E2) treatment at the end of timed fixed AI (TAI) on frequency of early luteolysis and pregnancy of beef cows supplemented with P4. Ovulations (D0 of study) of suckled multiparous (n=643) and primiparous (n=193) Nelore cows (*Bos taurus indicus*) were synchronized with an E2/P4-based protocol for TAI and assigned to receive 1.0 mg of estradiol cypionate (CP) or nothing (NoCP) on D–2 and 150 mg of injectable long-acting P4 (iP4) or Placebo (NoiP4) on D4 on a 2 x 2 factorial arrangement. On D15, the iP4 supplementation increased (P<0.05) the frequency of early luteolysis (NoCP+iP4: 26.0%; [13/50] vs. NoCP: 8.0% [4/50]), but CP prevented this effect (CP+iP4: 8.3% [4/48] and CP: 6.4% [3/47]). The CP improved pregnancy/AI (P/AI) of multiparous (CP: 51.6% [165/320] and NoCP: 35.0% [113/323]; P<0.001) and primiparous cows (CP: 40.4% [40/99] and NoCP: 24.5% [23/94], P<0.05), regardless of iP4 treatment. The iP4 supplementation affected P/AI of CP and NoCP treated cows according to follicle size at TAI. For the CP treated cows, the iP4 supplementation improved P/AI of sub-populations of cows with follicles <12.35 mm (42.0% [34/81] vs. 53.1% [34/64]), while for NoCP treated cows, the improvements occurred in subpopulations of cows with follicles ≥12.35 mm (46.1% [35/76] vs. 58.7% [37/63]). In conclusion, strategies associating E2 and P4 supplementation decrease the incidence of early onset of luteolysis and improve P/AI of suckled beef cows with smaller follicles.

1.2 INTRODUCTION

In addition to genetic gains, timed artificial insemination (TAI) programs in beef cattle improves reproductive efficiency because it overcomes challenges associated to long anestrous periods and estrus detection, that delay the time to first service post-partum. Despite of such benefits, cows induced to ovulate dominant follicles smaller than 11.0 mm at TAI present low pregnancy per AI (P/AI) [1–3]. Such poor results are mainly attributable to the insufficient uterine exposure to estradiol (E2) and progesterone (P4) at the pre-ovulatory (proestrus/estrus) and post-ovulatory (diestrus) periods [4,5], respectively. Indeed, aiming to achieve an adequate sequential uterine exposure to E2 and P4, different strategies to stimulate follicle growth in beef cattle submitted to TAI were proposed [6,7]. For example, consistent fertility gains in TAI protocol have been achieved by extending the proestrus period [8,9] or adding exogenous E2 [5,10,11]. An adequate uterine exposure to E2 affects positively the fertilization process [12], reduces the incidence of early luteolysis [13,14], and provides an uterine environment favorable to the establishment of pregnancy [15,16]. Regarding P4 concentrations during diestrus, the relationship between P4 concentrations and fertility are generally positive [3,6,17]. Progesterone is critical for successful maternal recognition and maintenance of pregnancy [18,19]. This steroid stimulates endometrial secretions [20] associated with conceptus growth [21–23] and interferon-t production [24]. Therefore, manipulation of the endocrine environment by the addition of exogenous E2 and/or P4 can potentially improve fertility outcomes in TAI programs.

Forde *et al*. [23] demonstrated that insertion of an intravaginal P4-releasing device between days 3 and 7 after estrus advanced expression of endometrial genes related to energy provision and histotroph constituents. Such alterations seems to be associated with greater conceptuses growth during late diestrus, that was also observed by others using a similar approaches [22,23]. Commonly, the benefits of P4 supplementation on fertility are observed when administration occur at early diestrus (i.e., days 3 to 7 after ovulation) [26,27]. However, in many studies, P4 supplementation at this period causes a greater incidence (30 to 35%) of early luteolysis (i.e., by day 14), that may impair maternal recognition and maintenance of pregnancy [24,28]. This paradoxical effect is one of the main explanations for the

inconsistent fertility results obtained in response to different strategies to supplement P4 at early diestrus. Indeed, studies reported no effect [29–31], a negative effect [29,32,33], or a positive effect [34–36] of P4 supplementation on P/AI of beef and dairy cattle.

One plausible explanation for the incidence of early luteolysis in P4-supplemented cows is that the early increase in circulating P4 is related to advances in the timing of nuclear P4 receptor (PGR) downregulation in the endometrial epithelia [37]. The downregulation of the PGR is closely followed by an increase in epithelial estrogen receptor alpha (ERα) and oxytocin receptors (OXTR), resulting in the pulsatile release of PGF2α, which induces luteolysis [38,39]. Furthermore, inconsistent fertility results can be a consequence of differences in the timing, duration, source and dose of P4 treatments [26,27].

Recently, a long-acting injectable P4 formulation (iP4) was evaluated by Pugliesi *et al*. [40] to supplement P4 at early diestrus in a manner that would be more practical than the use of the P4 intravaginal device or multiple P4 injections. By a single administration of 150 or 300 mg iP4 on days 2 or 3 post-ovulation, the authors observed that supplementation efficiently increased the circulating P4 concentrations for ≥3 days during early luteal phase in non-suckled Nelore cows, but also increased the frequency of early luteolysis (0% vs. 40.7%). Despite of this apparent negative aspect, in a subsequent fertility trial using an E2/P4-based TAI protocol, Pugliesi *et al*. [35] verified that 150 mg of iP4 administered 4 days post TAI (~3 days post-ovulation) increased the P/AI of anestrous suckled beef cows by 20%. Thus, our recent findings highlight the somewhat paradoxical effects of P4 supplementation when given in the early diestrus, as shown previously by others [24]; there are both beneficial effects regarding uterine receptivity and conceptus elongation and potentially negative effects associated with reduced CL lifespan. Thus, it is critical to find strategies to minimize the negative, while emphasizing the beneficial effects of P4 supplementation to maximize efficiency of this technology. Here, we propose to test the effects of supplementing E2 concurrent with the withdrawal of the P4-releasing device (i.e., at the beginning of proestrus) as a strategy to support the beneficial effects of exogenous P4 administration to improve fertility in beef cattle.

The exposure of the endometrium to the rising proestrus concentrations of E2 stimulates ERα and PGR [41,42], and this is indirectly responsible for the decrease of OXTR during metaestrus and early diestrus [13]. Thus, it is possible that E2

supplementation could cause a greater increase of ERα and PGR than the endogenous E2 pre-ovulatory surge. A greater initial rise in PGR could compensate the advanced disappearance of PGR caused by P4 supplementation. This represents a possible alternative to bypass the detrimental aspects of P4 supplementation. In fact, there is evidence for a positive role of proestrus E2 alone on uterine [15] and luteal [8,13,15] functions during diestrus, establishment of pregnancy [5,43,44] and fertility [5,10,11]. However, the associated response to supplemental E2 and P4 is unknown. Therefore, in this study, we aimed to evaluate the role of E2 supplementation at the P4 device withdrawal on the incidence of advanced luteolysis and fertility outcome after iP4 supplementation at early diestrus. Specifically, we tested the hypothesis that the E2 supplementation (1) decreased the incidence of short luteal lifespan and (2) improved the fertility response obtained by diestrus iP4 supplementation.

1.3 MATERIALS AND METHODS

1.3.1 Animals

This experiment was carried out during the summer and early fall on a commercial beef operation located in Mato Grosso do Sul, Brazil. Suckled multiparous (n= 643) and primiparous (n= 193) Nellore cows used in this study exhibited an average of days of postpartum of 52.1 and 50.8 (range, 31 to 85) and body condition scores of 3.48 and 3.00 (BCS, range, 2.25 to 5.00; 1 = emaciated to 5 = obese [45], using 0.25 increments), respectively. The cows were kept in grazing conditions (*Brachiaria brizantha*) with *ad libitum* access to water and minerals. The multiparous cows were split into 5 and 2 allotments from farms 1 (n= 524) and 2 (n= 119), respectively, while the primiparous cows were split into 4 allotments on farm 1.

All animal procedures were approved by the Ethics and Animal Handling Committee of the School of Veterinary Medicine and Animal Science of the University of Sao Paulo under the protocol number CEUA-6236220316.

1.3.2 Experimental design

Within each allotment, cows were subjected to an estrus synchronization protocol based on a single administration of 2 mg of estradiol benzoate (2.0 mL, i.m., Sincrodiol® Ourofino Saúde Animal) and insertion of an intravaginal P4-releasing device (1.0 g, Sincrogest® Ourofino Saúde Animal) followed by visual evaluation of BCS, on day –10. On this day, a transrectal ultrasonography exam was performed to exclude any cows with abnormalities of the reproductive tract and to establish the ovarian status. The ovarian status was determined based in three predefined categories, presence of CL, absence of CL and presence of follicles < 8.0 mm and absence of CL and presence of follicles ≥ 8.0 mm.

On day –2, P4 devices were removed, and cows received 0.53 mg of sodium cloprostenol (2.0 mL, i.m., Sincrocio® Ourofino Saúde Animal) followed by administration of 300 IU of equine chorionic gonadotropin (1.5 mL, i.m., SincroeCG® Ourofino Saúde Animal). At the time of device removal, Estrotect™ patches (Western Point Inc., Apple Valley, MN) were applied halfway between the hip and tail head to determine the occurrence of mounting behavior associated with estrus. Concurrent with TAI (day 0), all cows received 10 μg of gonadotropin releasing hormone analog (buserelin acetate, 2.5 mL, i.m., Sincroforte® Ourofino Saúde Animal).

Cows were blocked based on the BCS (low: L-BCS, 2.00 to 2.50, moderate: M-BCS, 2.75 to 3.50 or high: H-BCS, 3.75 to 5.00) to receive one of four treatments: injection of 1.0 mg of estradiol cypionate (CP, 1.0 mL, i.m, SincroCP® Ourofino Saúde Animal) or none (NoCP) on day –2 and supplementation with 150 mg of long-acting P4 (iP4, 1.0 mL, i.m., Sincrogest® injectable Ourofino Saúde Animal) or placebo (NoiP4, Sincrogest® injectable vehicle) on day 4, on a 2 by 2 factorial arrangement of treatments (CP, CP+iP4, NoCP and NoCP+iP4). Administration of CP at the time of P4 device withdrawal was based on previous publications [46,47]. Compared to estradiol benzoate, CP releases estradiol more slowly. Thus, it may be injected earlier in the protocol, at the same moment as the P4-releasing device withdrawal. This is advantageous for management because it reduces one handling of the animals and allows easy supplementation of estradiol.

On the day of TAI (day 0), cows were assigned randomly within treatments to receive a single insemination by one of four experienced operators using frozen-

thawed commercial semen from 12 and three sires on farm 1 and farm 2, respectively. At the time of TAI, the cows with approximately 50% or more of the silver rub-off coating removed from the Estrotect device were considered to have been in estrus between days -2 and 0.

1.3.3 Ultrasound examinations

Ultrasonography exams were performed in B-mode with a 7.5 MHz linear-array transrectal transducer by the same operator on days –10, 0 and 4.

On day 0, the diameter of the largest ovarian follicle observed in the B-mode still image was determined by taking the average between measurements of its two perpendicular axes. These measurements were taken only in cows with follicles ≥ 6.5 mm (n= 561 and n= 154 for multiparous and primiparous cows, respectively). On day 4, the ovaries were evaluated for detection of a newly formed CL, and its maximum area was determined by B-mode still image and the tracing function. For CL with an anechoic fluid-filled cavity, the area of the cavity was subtracted from the total area [48]. For primiparous cows, the evaluations on day 4 were performed in only 94 out of 193 animals due to operational constraints.

For determining early onset of luteolysis after P4 supplementation, a subgroup of ovulated multiparous cows (n= 195) were evaluated by Color Doppler ultrasonography on day 15. The CL scanning was performed using an ultrasound equipped with pulse-wave color Doppler function and a multi-frequency linear transducer. The evaluation and proportion of color signals of luteal blood flow were performed and determined as described previously in cattle [49]. A scale from 0 to 100% with 5% interval points was used for visually determining the proportion of the luteal area with blood flow signals. All scans were performed at a constant color-gain setting and a velocity setting of 5.4 cm/s by an operator unaware of the treatment allocation of animals. The criteria for describing a cow that underwent early structural luteolysis were: (1) the presence of a CL area < 2.0 cm^2 and (2) CL blood flow signals that covered ≤ 25% of the total luteal area on day 15, as reported previously [34,40,49].

Pregnancy diagnosis was conducted by transrectal ultrasonography 30 to 35 days after TAI. Conception rate was calculated as the proportion of ovulated cows (i.e. with CL on day 4) that became pregnant due to TAI, and P/AI was calculated as the proportion of total cows inseminated that were pregnant.

1.3.4 Blood sampling for analysis of serum P4 concentrations

Blood sampling for determination of circulating concentrations of P4 was taken from the subgroup of cows that were submitted to ovarian ultrasonography on day 15 (n=195). The serum P4 concentrations were used for identification of functional luteolysis, defined as P4 concentrations < 1.0 ng/mL [40]. Blood samples were collected from coccygeal vessels, centrifuged at 1500 g for 10 min at room temperature; serum was stored at –20°C until hormonal assays were performed.

Serum P4 was assayed by solid-phase radioimmunoassay using an Immuchem^{TM} Double Antibody Progesterone Kit (Cat. 07-170105, MP Biomedicals, NY, USA) according to manufacturer's instructions. The detection limit (sensibility) of the assay was 0.1 ng/mL. The intra-assay coefficients of variation (CV), were 0.17% (low) and 7.39% (high), respectively. The inter-assay CVs were 14.48% (low) and 9.95% (high), respectively.

1.3.5 Statistical analyses

All statistical analyses were carried out using SAS (version 9.3, SAS Institute Inc., Cary, NC, USA). Cows were the experimental units in all models. The analyses of datasets containing primiparous and multiparous cows were ran separately.

For continuous dependent variables (follicle diameter, CL area, and P4 concentrations), the assumptions of normality of residues and homogeneity of variance were checked by histograms, q-q plots, and formal statistical tests as part of GLM and the UNIVARIATE procedure of SAS. The natural logarithmic transformation and square root were respectively used to normalize the data distribution of the CL

area and P4 concentrations. Non-transformed data were shown for clarity. These variables were analyzed by ANOVA using the MIXED procedure of SAS. Follicle diameter on day 0 and CL area on day 4 were measured before P4 supplementation. For these variables, only the effect of CP was included into the model as a fixed effect. Area of the CL on day 15 was analyzed according to the fixed effects of CP, iP4 supplementation and their interaction. On additional analyses, the variable estrous behavior and the appropriate interactions were included as fixed effects into the model, but only for multiparous cows, due the limited number of primiparous cows detected in estrus.

Binomial, dependent variables were analyzed by the GLIMMIX procedure of SAS, using binomial distribution. For rates of ovulation and estrus, only the fixed effect of CP it was included in the model. For rates of conception and pregnancy, the fixed effect of CP, iP4 supplementation and their interaction were included in the model. For primiparous cows, the conception rate analysis was not performed because the ovulation was checked only in a limited number of animals on day 4 (n=94). For further analyses of pregnancy, the variables estrous behavior (only for multiparous) or ovarian status at day –10 (i.e., presence or absence of CL), and the appropriate interactions were included as fixed effects in the model.

For continuous and binomial dependent variables, the effects of BCS block, farm, and allotment nested within farm were included as random effects in the model. For primiparous cows, the farm effect was not included because they belonged to a single farm. To determine the denominator degrees of freedom for tests of fixed effect, the options Kenward-Roger and Between-Within degrees was used into the models of continuous and binomial dependent variables, respectively.

To evaluate the effect of follicle diameter (day 0) and CL area (day 4) on pregnancy rate in the model described previously, each variable was manually included as a covariate, and the appropriate interactions were considered. According to the covariate effects, the GLM procedure of SAS was used to establish whether the effect was linear, quadratic, or cubic. The significant, more complex model was selected. Then, the logistical regression models were designed using the intercept and slope value generated by the LOGISTIC DESC procedure of SAS for the following equation: Probability = $(e^{\text{logisticequation}})/(1+^{\text{elogistic equation}})$. Subsequently, the same analysis was conducted, but each continuous variable was divided in two classes according to the median (< m or ≥ m) to be included as fixed effects in the

models. None of these latter analyses were performed for primiparous cows due the limited number of animals.

The effects of treatments were determined by F-tests using Type III sums of squares. When necessary, means across treatments were compared using Fisher's protected least significant difference (LSD, i.e., the DIFF option of the LSMEANS statement). Results of continuous variables were reported as LSMEANS ± S.E.M and from binomial variables as means. A probability of $P \leq 0.05$ indicated that a difference was significant, and a probability of $0.05 > P \leq 0.10$ indicated that significance was approached.

The proportion of the luteal area containing blood flow signals was analyzed using the non-parametrical Kruskal-Wallis test of the NPAR1WAY procedure of SAS. The comparisons of frequency of luteolysis between treatments were performed with the FREQ procedure using the Chi-squared distribution of SAS.

1.4 RESULTS

1.4.1 Effects of supplementation of CP prior to TAI and iP4 at initial diestrus on fertility of suckled beef cows

An effect of CP treatment on the conception rate was detected for multiparous cows (CP: 59.1% [165/279] vs. NoCP: 41.2% [113/274]; $P < 0.001$, Table 1). The P/AI was also influenced positively by CP treatment both in multiparous (CP: 51.6% [165/320] vs. NoCP: 35.0% [113/323]; $P < 0.001$) and primiparous (CP: 40.4% [40/99] vs. NoCP: 24.5% [23/94]; $P= 0.03$) cows. There was no main effect of iP4 nor a CP by iP4 interaction ($P > 0.10$; Table 1).

The CP increased the proportion of cows showing estrus at TAI (Table 2), and this was associated positively with P/AI. In fact, multiparous cows detected in estrus presented greater ($P < 0.001$) conception (67.4% [145/215] vs. 39.3% [133/338]) and P/AI (65.0% [145/223] vs. 31.2% [133/420]), regardless of treatment. Multiparous cows in estrus presented larger follicle diameters at TAI (Estrus: 13.55 ± 0.29 vs. Noestrus: 11.62 ± 0.27 mm; $P < 0.001$), regardless of treatment.

Table 1. Effect of CP supplementation 2 days prior to TAI and/or iP4 supplementation 4 days post-TAI on fertility of suckled beef cows.

Variables/Category	CP		NoCP		P value		
	NoiP4	iP4	NoiP4	iP4	CP	iP4	CP*iP4
Multiparous							
Conception/AI,% (n/n)[1]	60.1 (83/138)	58.2 (82/141)	38.3 (54/141)	44.4 (59/133)	<0.001	0.62	0.36
P/AI, % (n/n)[2]	51.2 (83/162)	51.9 (82/158)	32.9 (54/164)	37.1 (59/159)	<0.001	0.51	0.65
Primiparous[3]							
P/AI, % (n/n)[2]	48.0 (24/50)	32.7 (16/49)	25.5 (12/47)	23.4 (11/47)	0.03	0.23	0.41

Suckled primiparous (n = 193) and multiparous (n = 643) beef cows were assigned to receive an intravaginal P4 implant device plus 2 mg of EB at initiation of synchronization protocol for TAI (D–10). Devices were removed 8 days later (D–2) and animals received 300 IU of eCG and 0.53 mg of PGF and were selected to receive nothing or 1 mg of CP. On D0, all cows were TAI and received 100 µg of a GnRH analogue. On D4, animals were further sub-divided to receive placebo or 150 mg of iP4, on a 2 by 2 factorial arrangement of treatments. Pregnancy diagnosis was performed by transrectal ultrasonography between days 30 and 35.

[1]Conception/AI= number of pregnant cows divided by the number of cows detected with a CL 4 days after TAI;

[2]P/AI= number of pregnant cows divided by the total number of cows that received TAI;

[3]Conception analysis was not performed for primiparous because ovulation was checked only in a sub-sample of animals on D4 (n = 94).

Table 2. Effect of CP supplementation prior TAI on ovarian characteristics and estrus behavior

Variables	Multiparous		P value	Primiparous		P value
	CP	NoCP		CP	NoCP	
FD on D0 (mm)	12.32±0.33	12.37±0.33	0.83	11.06±0.29	11.16±0.30	0.75
Estrus on D0, % (n/n)	53.4 (171/320)	16.1 (52/323)	< 0.001	31.3 (31/99)	3.2 (3/94)	< 0.001
CL area D4 (cm^2)	1.31±0.13	1.22±0.13	0.006	1.07±0.06	0.95±0.07	0.13
Ovulation, % (n/n)[1]	87.2 (279/320)	84.3 (274/323)	0.38	85.7 (42/49)	82.2 (37/45)	0.59
Double ovulation, % (n/n)	2.7 (8/279)	1.4 (4/274)	0.27	2.2 (1/46)	5.3 (2/38)	0.46

Abbreviations: FD on D0= diameter of the largest ovarian follicle at TAI.

Suckled primiparous (n= 193) and multiparous (n= 643) beef cows underwent to TAI (D0) were treated 2 days prior TAI with 1 mg of CP or nothing (NoCP) and received an estrotect device. On D0, all cows received 100 □g of a GnRH analogue. On D0, the diameter of the largest ovarian follicle at TAI was determined with ultrasound and cows with estrotect device activated were considered to have been in estrus. On D4, the ovaries were evaluated with ultrasound for detection of a newly formed CL, and determination of its maximum area. Dataset of primiparous and multiparous cows were analyzed separately. [1]On D4, the ultrasound exam was performed only in 94 out of 193 primiparous cows due to operational constraints.

There was an interaction (P=0.04) between the covariate follicle diameter at TAI and the variables CP and iP4 on pregnancy rate of multiparous cows. The relationship that better explained the effect of this covariate on pregnancy rate according to treatment was linear for NoCP and NoCP+iP4 treatments and quadratic

for CP and CP+iP4 treatments (Figure 1). The iP4 supplementation appears to improve probability of pregnancy when follicle diameters were greater in cows not treated with CP (NoCP+iP4 group), while for cows treated with CP (CP+iP4 group) the apparent positive effect occurred when cows presented smaller follicle diameter.

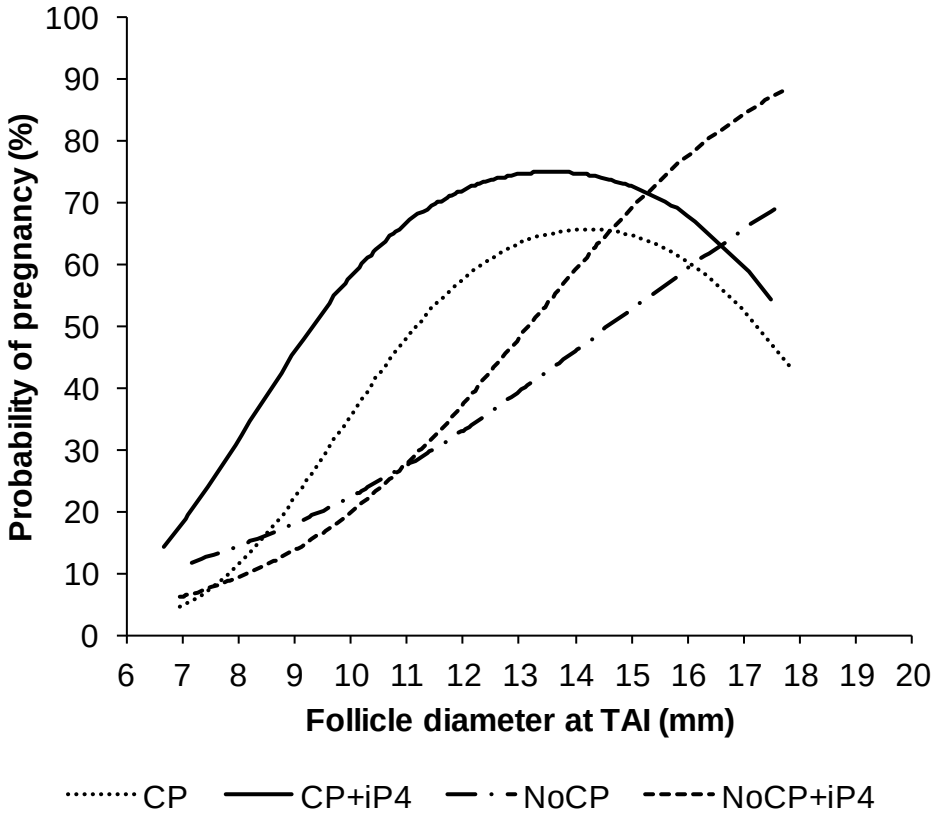

Figure 1. Relationships between follicle diameter at TAI (D0) and probability of pregnancy of suckled multiparous beef cows (n= 561) synchronized with E2/P4 based protocol and assigned to receive nothing or 1 mg of CP on D–2 and placebo or 150 mg of iP4 on D4 on a 2 by 2 factorial arrangement of treatments. The effect of treatments on probability of pregnancy varied according to follicle diameter (P= 0.04). The regression equations were as follows: **CP**= exp (-0.07x^2 +1.99x -13.49)/1+exp (-0.07x^2 +1.99x -13.49); **CP+iP4**= exp (-0.06x^2 +1.63x -9.97)/1+exp (-0.06x^2 +1.63x -9.97); **NoCP**= exp (0.27x –3.94)/1+exp(0.27x –3.94); **NoCP+iP4**= exp (0.44x –5.79)/ 1+exp (0.44x –5.79).

For clarity, when follicles were categorized according to the median (12.35 mm) in the classes < 12.35 and ≥ 12.35 mm, the treatment by follicle size interaction approached significance (P= 0.06; Figure 2). For follicles that were ≥ 12.35 mm, the mean group comparisons demonstrated that pregnancy rate of NoCP+iP4 group was intermediate between the NoCP and CP groups. This indicated an increase in fertility promoted by iP4 supplementation, compatible with the observations of results from Figure 1 for this group. For each group, the comparisons between classes of follicles (i.e. <12.35 and ≥12.35 mm), demonstrated that except for the CP+iP4 group, every

other group presented a smaller pregnancy rate in the class of follicles < 12.35 mm. This indicated that the positive effect of iP4 supplementation for CP treated cows occurred for cows with smaller follicles (<12.35 mm).

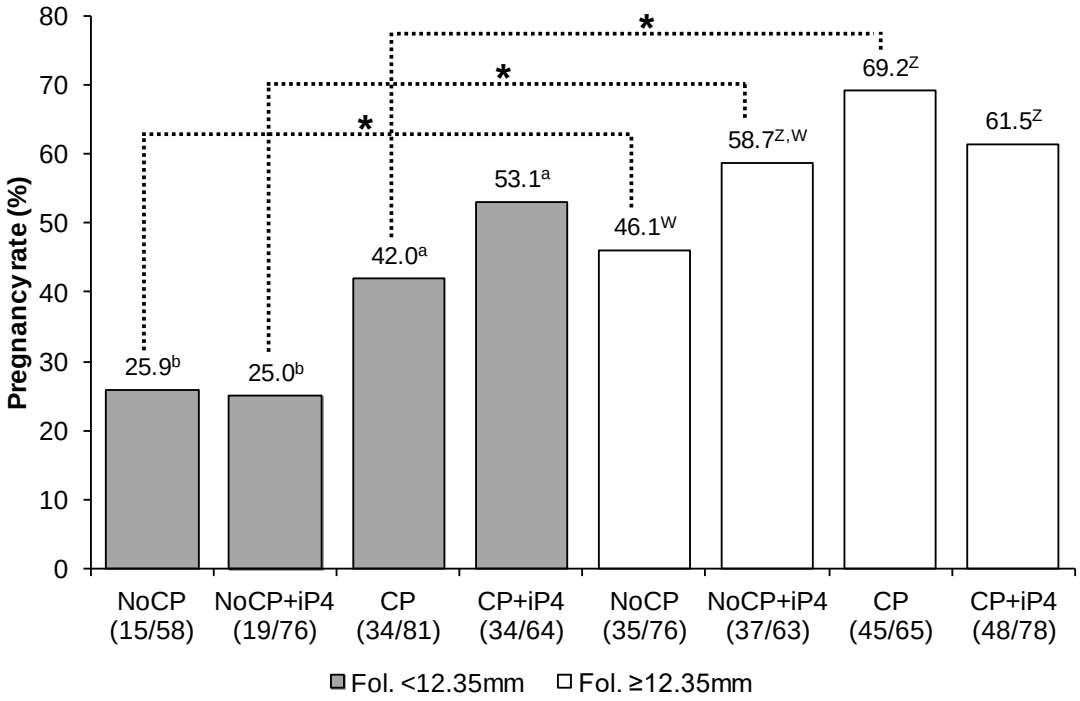

Figure 2. Effect of classes of follicle diameter at TAI (D0) on pregnancy rates of suckled multiparous beef cows (n= 561) synchronized with E2/P4 based protocol and assigned to receive nothing or 1 mg of CP on D–2 and placebo or 150 mg of iP4 on D4 on a 2 by 2 factorial arrangement of treatments. The effect of treatments on pregnancy rates varied according to follicle diameter (P= 0.06).*Significant difference (P < 0.01) for comparisons between class of follicle within each group. ^{a,b}Values without a common superscript differed (P ≤ 0.05) for comparisons performed within class of follicles <12.35 mm. ^{Z,W}Values without a common superscript differed (P ≤ 0.05) for comparisons performed within class of follicles ≥12.35 mm.

Contrary to the follicle analysis, the interaction between the covariate CL size and the variables CP and iP4 on conception rate was not significant (P = 0.79). Regardless of treatment, the conception rate was affected by CL size (P = 0.007; Figure 3). Curiously, the equation that better explained the relationship between the covariate CL size and pregnancy rate was of a cubic order. When CLs were categorized according to the median (1.17 cm^2) in the classes <1.17 and ≥ 1.17 cm^2, again, independent of treatment an effect of CL size was found (P = 0.002). The pregnancy rate of cows with CL ≥ 1.17 cm^2 was greater than those with CL < 1.17 cm (57.3% [160/279] vs. 41.7% [115/276]), which reflects the effects observed in Figure 3 more clearly.

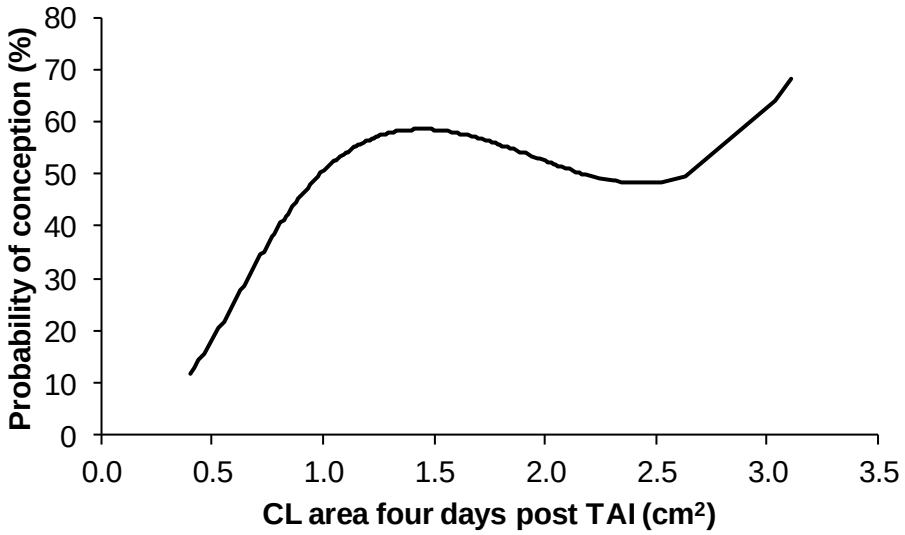

Figure 3. Relationship between CL area four days post TAI (D0) and the probability of pregnancy of suckled multiparous beef cows (n=553) synchronized with E2/P4 based protocol and assigned to receive nothing or 1 mg of CP on D–2 and placebo or 150 mg of iP4 on D4 on a 2 by 2 factorial arrangement of treatments. Independent of treatments, the CL area affected (P= 0.007) the probability of pregnancy in a cubic order [exp ($0.85x^3$ $-4.95x^2$ +8.98x -4.86)/ 1+exp($0.85x^3$ $–4.95x^2$ + 8.98x –4.86)].

In addition, at the start of the estrus synchronization protocol 45.4% [292/643] of multiparous and 8.8% [17/193] of primiparous cows presented CL, however, the presence or absence of a CL did not affect (P > 0.10) P/AI.

1.4.2 Interactions between CP addition prior to TAI and iP4 supplementation at initial diestrus on luteal function and lifespan of suckled beef cows

In the absence of CP supplementation, iP4 injection on day 4 caused the greatest proportion of early luteolysis (26 and 20% for structural and functional luteolysis, respectively, for the NoCP+iP4 group; Table 3). The addition of CP prior to TAI prevented the incidence of early luteolysis triggered by iP4 supplementation on day 4 (P < 0.05). Indeed, CP+iP4 treatment resulted in a proportion of cows showing early luteolysis similar to that of groups that did not receive iP4 supplementation.

For cows with a functional CL on day 15, i.e. no structural or functional luteolysis detected, no effect of iP4 supplementation was observed on CL area or blood flow (Table 3). However, a CP by iP4 interaction approaching significance (P= 0.08) indicated that the NoCP group presented a lower (P < 0.05) serum P4 concentration than the CP group (Table 3). In addition, cows showing estrus had

greater serum P4 concentrations (6.77 ± 0.43 vs. NoEstrus: 5.38 ± 0.24 ng/mL; P = 0.006).

Table 3. At day 15, effects of CP addition 2 days prior TAI and/or iP4 supplementation 4 days post-TAI on incidence of luteolysis, CL area, CL blood flow and serum P4 concentrations of suckled beef cows.

Variables on D15	CP		NoCP		P value		
	NoiP4	iP4	NoiP4	iP4	CP	iP4	CP*iP4
Structural lut., % (n/n)[1]	6.4[b] (3/47)	8.3[b] (4/48)	8.0[b] (4/50)	26.0[a] (13/50)	.	.	.
Functional lut., % (n/n)[2]	2.1[b] (1/47)	4.2[b] (2/48)	8.0[a,b,Y] (4/50)	20.0[a,X] (10/50)	.	.	.
CL area (cm^2)	2.80±0.08	2.67±0.08	2.65±0.08	2.67±0.09	0.34	0.59	0.29
CL blood flow, %[3]	44.8±0.02	43.8±0.02	44.1±0.02	44.1±0.02	.	.	.
P4 conc. (ng/mL)[4]	6.14±0.35[a]	5.84±0.35[a,b,X]	4.95±0.35[b,Y]	5.84±0.39[a,b,X]	0.11	0.37	0.08

Abbreviations: Structural lut.= structural luteolysis; Functional luteolysis= functional luteolysis; P4 conc.= concentrations of P4;

Suckled beef cows were assigned to receive an intravaginal P4 implant device plus 2 mg of EB at initiation of synchronization protocol for TAI (D–10). Devices were removed 8 days later (D–2) and animals received 300 IU of eCG and 0.53 mg of PGF and were selected to receive nothing or 1 mg of CP. On D4, animals were further sub-divided to receive placebo or 150 mg of iP4 , on a 2 by 2 factorial arrangement of treatments. On D0, all cows were TAI and received 100 µg of a GnRH analogue. On D15, CLs were examined by ultrasonography to determine CL area, proportion of the CL area that contained color-doppler signals of blood flow and a blood sample was collected for P4 assay. Cows that had undergone structural or functional luteolysis were removed from the analysis of CL area, CL blood flow and P4 concentrations.

[1]Structural luteolysis: CL area < 2.0 cm^2 and blood flow rate ≤ 25%. [2]Functional luteolysis: concentration of P4 < 1.0 ng/mL. The analyses were performed using Chi-Square test;

[3]Analyzed by Kruskal Wallis test of SAS. The sum of scores were 3928.50, 3744.0, 3800.50 and 3233.0 for the CP, CP+iP4, NoCP and NoCP+iP4 respectively (P = 0.93);

[a,b] Values without a common superscript differed between treatments (P ≤ 0.05).

[X,Y] Values without a common superscript tended to differ (0.05 > P ≤ 0.10).

1.5 DISCUSSION

The main motivation of the present study was to verify whether the combined supplementation of CP and iP4 would increase P/AI in suckled beef cows. Expectation was that supplementation of CP at the beginning of proestrus (day of P4-releasing device removal) in cows supplemented with long-acting P4 at early diestrus would prevent the incidence of iP4-induced early luteolysis. In general, we found that CP treatment: 1) improved the incidence of estrus; 2) increased size of CL at early diestrus; 3) increased P4 concentrations at late diestrus; 4) prevented iP4-induced

early onset of luteolysis and 5) improved fertility of both multiparous and primiparous beef cattle submitted to TAI, independent of iP4. The fact that early luteolysis was suppressed but P/AI was not further increased after combined CP and iP4 supplementation was intriguing. Further examination of the data after partitioning cows according to size of the preovulatory follicle revealed that the fertility response to CP and iP4 is complex. Complexity is probably due to the isolated and combined effects of CP and iP4 on the preovulatory follicle, the subsequent CL, the reproductive tract and the embryo/conceptus functions.

The CP supplementation prevented the incidence of iP4-stimulated early luteolysis. Shortened luteal lifespan in P4-supplemented cows [24,28,40] is one explanation for the historically inconsistent fertility results obtained in response to supplementation of P4 at early diestrus. Here, we successfully demonstrated that an injection of CP 48 h before TAI suppressed the increased incidence of early luteolysis associated with P4 supplementation. Early luteal regression has two main explanations: advanced release of PGF2α pulses from the endometrium, leading to regression, and luteal insufficiency or sub-functionality. Regarding the endometrium, although we did not evaluate the molecular mechanisms underlying this response, we propose that the CP effect might be a consequence of the supplemental pre-ovulatory E2 stimulating an increase in the expression of endometrial ESRα and PGR [41,42] at estrus. We rationalized that perhaps a greater starting abundance of PGR would take longer to become down-regulated during early diestrus, and this might counteract the acceleration of PGR loss from the uterine epithelia caused by supplemental P4 [37]. In such scenario, timing of onset of the luteolytic cascade could be reset to that expected on a normal estrous cycle, which would provide enough time for anti-luteolytic signaling from the conceptus [38,39]. This preposition needs specific experimental testing.

Regarding a CP effect on luteal function, we observed that P4 concentration on day 15 of the CP group was 19% greater than NoCP group, and this was consistent with a 13% greater CL volume on day 4. Interestingly, increases in CL growth and function occurred despite the fact that pre-ovulatory follicle sizes were similar among groups. These responses could be related to the longer duration of the LH surge stimulated by CP in comparison to the surge stimulated by the GnRH injection alone. The LH remains elevated up to 16.5 h after CP-stimulation [46] instead of 6h when a GnRH analogue is injected [50]. Indeed, variations in the

magnitude of the LH surge around the time of ovulation were reported to be important for early luteal function [51,52]. In this regard, a possible cause of early luteolysis onset caused by P4 supplementation is because of failures on CL development due to inadequate luteotrophic support [28,53]. Administration of human [53] or equine [28] chorionic gonadotrophin concurrent with P4 supplementation prevented early onset of luteolysis in cows. Thus, we infer that CLs from CP-group may be more resilient to the LH pulse frequency inhibition caused by supplemental P4. The possible association between the LH-release dynamics during proestrus and the incidence of short luteal lifespan warrants further investigation.

In the present paper, lack of a main effect of iP4 supplementation and lack of an effect of the interaction CP by iP4 on P/AI and conception rate led to the conclusion that iP4 did not affect fertility. However, a closer look at the data, after stratification by size of the pre-ovulatory follicle, allowed the identification of sub-populations of cows responsive to supplemental P4. Specifically, when follicles were ≥ 12.35 mm, pregnancy rates increased from 46.1 to 58.7% after administration of iP4 to cows not treated with CP. Furthermore, when follicles were < 12.35 mm, pregnancy rates increased from 42.0 to 53.1% after administration of iP4 to cows treated with CP (Figure 2). Thus, in a scenario of presumed low E2 exposure during proestrus, for example in the presence of smaller pre-ovulatory follicles, (Figure 1), the CP treatment favored the iP4 supplementation fertility response (i.e. CP+iP4 group); in contrast, CP reduced iP4 benefits when concentrations of E2 were already elevated during proestrus, such as in the presence of a larger follicle. Furthermore, when the pre-ovulatory E2 milieu was generated only by size of follicle (i.e., NoCP groups), the response to iP4 stimulus was verified only for cows with larger follicles. In support of this observation, Madsen *et al* [43] demonstrated that lack of E2 exposure during proestrus period impaired the capability of ovariectomized beef cows treated with P4 to sustain a pregnancy. The authors verified that previous E2 exposure during the proestrus period was required to sustain embryonic growth and or/placental attachment. Moreover, Davoodi *et al* [15] observed that at similar P4 concentrations during diestrus, beef cows that showed estrus at TAI presented longer conceptuses compared to those that did not come in estrus. Collectively, consistent with previous reports, we show here that P4 supplementation may exert embryotrophic [22], luteolytic [24,40] or no effect on the probability of conception of an individual cow. Furthermore, such effects are modulated by exposure to follicular,

endogenous and exogenous estradiol. The interacting effects of E2 and P4 coordinate shifts in sub-populations of cows between pregnant and non-pregnant, to yield the overall pregnancy rate. This phenomenon is well illustrated on Figure 1.

Interpretation of this complex scenario may help us to understand why, contrary to our initial hypothesis, the suppression of short luteal lifespan by CP (Table 3) did not improve the proportion of pregnant cows after iP4 supplementation (Table 1 and Figure 2). For example, the 18% increase in the incidence of early luteolysis in the NoCP+iP4 vs. the NoCP group (Table 3) did not impair P/AI (Table 1). Indeed, numerically, the P/AI of NoCP+iP4 group (37.1%) was even greater than NoCP (32.9%). However, magnitude of iP4 effects were clearly affected by size of the pre-ovulatory follicle. For example, the iP4 benefits for NoCP treated cows with follicles ≥ 12.35 mm (12.6% increase in pregnancy rate; Figure 2) probably counterbalanced the overall iP4-luteolytic effect. The numerical improvement on P/AI indicates that iP4 benefits in cows with follicles ≥ 12.35 mm compensated partially the fertility limitations, associated with sub-maximal exposure to pre-ovulatory E2 and incidence of early luteolysis. In fact, the pregnancy rates of NoCP+iP4 group (58.7%) was intermediary between groups NoCP (46.1%) and CP (69.2%), Figure 2. For the CP treated cows, the absence of iP4-induced early luteolysis (Table 3) associated to iP4 fertility benefits (11.1% increase in pregnancy rate; Figure 2) for cows with follicles < 12.35 mm did not result in gains in the P/AI (Table 1). This was probably due to the reduced benefits of iP4 when follicles were larger (Figure 1). In this regard, for animals treated with CP, the numerical decrease (7.7%) in the pregnancy rate of cows with follicles ≥ 12.35 mm treated with iP4 (Figure 2) suggest that the positive effect of iP4 was abrogated in these sub-populations of cows, but this was not because of iP4-induced early luteolysis. Thus, our findings indicated that success of iP4 supplementation in TAI programs depends on the endocrine profiles resulting from the TAI programs. This implies that an iP4 treatment designed specifically to certain populations of cows within TAI programs should be considered in future investigations.

The present results involving CP supplementation and size of follicles at TAI confirm, respectively, the strong positive association between (i) the circulating estradiol concentrations during proestrus and fertility [5,8,10,44] and (ii) the follicle diameter at TAI and fertility [1–3,6].

In conclusion, the present data highlighted the complex interplay among endogenous and exogenous estradiol and supplementary P4 on beef cow fertility. The association of supplemental E2 and P4 can increase pregnancy rates in sub-populations of cows with smaller follicles at TAI and this may be partially due to a decrease in the incidence of early onset of luteolysis. The precise mechanisms by which CP induces these effects need to be determined. From a practical standpoint, exogenous E2 addition at the beginning of proestrus reduces the occurrence of early luteolysis in P4-supplemented cows at early diestrus and, strategies associating E2 and P4 supplementation have a potential positive impact on the reproductive performance of suckled beef cows.

1.6 REFERENCES

[1] Sá Filho MF, Crespilho AM, Santos JEP, Perry GA, Baruselli PS. Ovarian follicle diameter at timed insemination and estrous response influence likelihood of ovulation and pregnancy after estrous synchronization with progesterone or progestin-based protocols in suckled *Bos indicus* cows. Anim Reprod Sci 2010;120:23–30. doi:10.1016/j.anireprosci.2010.03.007.

[2] Perry GA, Smith MF, Roberts AJ, MacNeil MD, Geary TW. Relationship between size of the ovulatory follicle and pregnancy success in beef heifers. J Anim Sci 2007;85:684–9. doi:10.2527/jas.2006-519.

[3] Martins T, Peres RFG, Rodrigues ADP, Pohler KG, Pereira MHC, Day ML, *et al*. Effect of progesterone concentrations, follicle diameter, timing of artificial insemination, and ovulatory stimulus on pregnancy rate to synchronized artificial insemination in postpubertal Nellore heifers. Theriogenology 2014;81:446–53. doi:10.1016/j.theriogenology.2013.10.020.

[4] Perry GA, Smith MF, Lucy MC, Green JA, Parks TE, MacNeil MD, *et al*. Relationship between follicle size at insemination and pregnancy success. Proc Natl Acad Sci U S A 2005;102:5268–73. doi:10.1073/pnas.0501700102.

[5] Jinks EM, Smith MF, Atkins JA, Pohler KG, Perry GA, MacNeil MD, *et al*. Preovulatory estradiol and the establishment and maintenance of pregnancy in suckled beef cows. J Anim Sci 2013;91:1176–85. doi:10.2527/jas.2012-5611.

[6] Peres RFG, Júnior IC, Filho OGS, Nogueira GDP, Vasconcelos JLM. Strategies to improve fertility in *Bos indicus* postpubertal heifers and nonlactating cows submitted to fixed-time artificial insemination. Theriogenology 2009;72:681–9. doi:10.1016/j.theriogenology.2009.04.026.

[7] Dias CC, Wechsler FS, Day ML, Vasconcelos JLM. Progesterone concentrations, exogenous equine chorionic gonadotropin, and timing of prostaglandin F2α treatment affect fertility in postpuberal Nelore heifers. Theriogenology 2009;72:378–85. doi:10.1016/j.theriogenology.2009.03.006.

[8] Bridges GA, Mussard ML, Burke CR, Day ML. Influence of the length of proestrus on fertility and endocrine function in female cattle. Anim Reprod Sci 2010;117:208–15. doi:10.1016/j.anireprosci.2009.05.002.

[9] Bridges GA, Helser LA, Grum DE, Mussard ML, Gasser CL, Day ML.

Decreasing the interval between GnRH and PGF2α from 7 to 5 days and lengthening proestrus increases timed-AI pregnancy rates in beef cows. Theriogenology 2008;69:843–51. doi:10.1016/j.theriogenology.2007.12.011.

[10] Sá Filho MF, Santos JEP, Ferreira RM, Sales JNS, Baruselli PS. Importance of estrus on pregnancy per insemination in suckled *Bos indicus* cows submitted to estradiol/progesterone-based timed insemination protocols. Theriogenology 2011;76:455–63. doi:10.1016/j.theriogenology.2011.02.022.

[11] Pereira MHC, Rodrigues ADP, Martins T, Oliveira WVC, Silveira PSA, Wiltbank MC, *et al*. Timed artificial insemination programs during the summer in lactating dairy cows: Comparison of the 5-d Cosynch protocol with an estrogen/progesterone-based protocol. J Dairy Sci 2013;96:6904–14. doi:http://dx.doi.org/10.3168/jds.2012-6260.

[12] Perry GA, Perry BL. Effects of standing estrus and supplemental estradiol on changes in uterine pH during a fixed-time artificial insemination protocol. J Anim Sci 2008;86:2928–35. doi:10.2527/jas.2008-1181.

[13] Mann GE, Lamming GE. The role of sub-optimal preovulatory oestradiol secretion in the aetiology of premature luteolysis during the short oestrous cycle in the cow. Anim Reprod Sci 2000;64:171–80. doi:10.1016/S0378-4320(00)00205-0.

[14] Kieborz-Loos KR, Garverick HA, Keisler DH, Hamilton SA, Salfen BE, Youngquist RS, *et al*. Oxytocin-induced secretion of prostaglandin F2alpha in postpartum beef cows: effects of progesterone and estradiol-17beta treatment. J Anim Sci 2003;81:1830–6.

[15] Davoodi S, Cooke RF, Fernandes ACC, Cappellozza BI, Vasconcelos JLM, Cerri RLA. Expression of estrus modifies the gene expression profile in reproductive tissues on day 19 of gestation in beef cows. Theriogenology 2015;85:645–55. doi:10.1016/j.theriogenology.2015.10.002.

[16] Mesquita FS, Ramos RS, Pugliesi G, Andrade SCS, Van Hoeck V, Langbeen A, *et al*. The Receptive Endometrial Transcriptomic Signature Indicates an Earlier Shift from Proliferation to Metabolism at Early Diestrus in the Cow. Biol Reprod 2015;93:1–12. doi:10.1095/biolreprod.115.129031.

[17] Frade MC, Frade C, Cordeiro MB, Sá Filho MF, Mesquita FS, Nogueira GDP, *et al*. Manifestation of estrous behavior and subsequent progesterone concentration at timed-embryo transfer in cattle are positively associated with

pregnancy success of recipients. Anim Reprod Sci 2014;151:85–90. doi:10.1016/j.anireprosci.2014.09.005.

[18] Mann GE, Lamming GE. Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. Reproduction 2001;121:175–80. doi:10.1530/reprod/121.1.175.

[19] Spencer TE, Forde N, Lonergan P. The role of progesterone and conceptus-derived factors in uterine biology during early pregnancy in ruminants. J Dairy Sci 2015;99:1–10. doi:10.3168/jds.2015-10070.

[20] Spencer TE, Johnson GA, Bazer FW, Burghardt RC. Implantation mechanisms: Insights from the sheep. Reproduction 2004;128:657–68. doi:10.1530/rep.1.00398.

[21] Garrett JE, Geisert RD, Zavy MT, Gries LK, Wettemann RP, Buchanan DS. Effect of exogenous progesterone on prostaglandin F2α release and the interestrous interval in the bovine. Prostaglandins 1988;36:85–96.

[22] Carter F, Forde N, Duffy P, Wade M, Fair T, Crowe MA, *et al*. Effect of increasing progesterone concentration from Day 3 of pregnancy on subsequent embryo survival and development in beef heifers. Reprod Fertil Dev 2008;20:368–75. doi:10.1071/RD07204.

[23] Clemente M, De La Fuente J, Fair T, Al Naib A, Gutierrez-Adan A, Roche JF, *et al*. Progesterone and conceptus elongation in cattle: A direct effect on the embryo or an indirect effect via the endometrium? Reproduction 2009;138:507–17. doi:10.1530/REP-09-0152.

[24] O'Hara L, Forde N, Carter F, Rizos D, Maillo V, Ealy AD, *et al*. Paradoxical effect of supplementary progesterone between day 3 and day 7 on corpus luteum function and conceptus development in cattle. Reprod Fertil Dev 2014;26:328–36. doi:10.1071/RD12370.

[25] Forde N, Carter F, Fair T, Crowe MA, Evans ACO, Spencer TE, *et al*. Progesterone-Regulated Changes in Endometrial Gene Expression Contribute to Advanced Conceptus Development in Cattle. Biol Reprod 2009;81:784–94. doi:10.1095/biolreprod.108.074336.

[26] Mann GE, Lamming GE. The Influence of Progesterone During Early Pregnancy in Cattle. Reprod Domest Anim 1999;34:269–74. doi:10.1111/j.1439-0531.1999.tb01250.x.

[27] Yan L, Robinson R, Shi Z, Mann G. Efficacy of progesterone supplementation during early pregnancy in cows: A meta-analysis. Theriogenology 2016;85:1390–8. doi:10.1016/j.theriogenology.2015.12.027.

[28] O'Hara L, Forde N, Duffy P, Randi F, Kelly AK, Valenza A, *et al*. Effect of combined exogenous progesterone with luteotrophic support via equine chorionic gonadotrophin (eCG) on corpus luteum development, circulating progesterone concentrations and embryo development in cattle. Reprod Fertil Dev 2014:269–77. doi:10.1071/RD14019.

[29] Monteiro PLJ, Nascimento AB, Pontes GCS, Fernandes GO, Melo LF, Wiltbank MC, *et al*. Progesterone supplementation after ovulation: Effects on corpus luteum function and on fertility of dairy cows subjected to AI or ET. Theriogenology 2015;84:1215–24. doi:10.1016/j.theriogenology.2015.06.023.

[30] Stevenson JS, Mee MO. Pregnancy Rates of Holstein Cows After Postinsemination Treatment with a Progesterone-Releasing Intravaginal Device. J Dairy Sci 1991;74:3849–56. doi:10.3168/jds.S0022-0302(91)78577-9.

[31] Monteiro PLJ, Ribeiro ES, Maciel RP, Dias ALG, Solé E, Lima FS, *et al*. Effects of supplemental progesterone after artificial insemination on expression of interferon-stimulated genes and fertility in dairy cows. J Dairy Sci 2014;97:4907–21. doi:10.3168/jds.2013-7802.

[32] Parr MH, Crowe MA, Lonergan P, Evans ACO, Rizos D, Diskin MG. Effect of exogenous progesterone supplementation in the early luteal phase post-insemination on pregnancy per artificial insemination in Holstein–Friesian cows. Anim Reprod Sci 2014;150:7–14. doi:10.1016/j.anireprosci.2014.08.008.

[33] Van Cleeff J, Macmillan KL, Drost M, Lucy MC, Thatcher WW. Effects of administering progesterone at selected intervals after insemination of synchronized on pregnancy rates and resynchronization of returns to service. Theriogenology 1996;46:1117–30. doi:10.1016/S0093-691X(96)00284-1.

[34] Pugliesi G, Santos FB, Lopes E, Nogueira É, Maio JRG, Binelli M. Improved fertility in suckled beef cows ovulating large follicles or supplemented with long-acting progesterone after timed-AI. Theriogenology 2016;85:1239–48. doi:10.1016/j.theriogenology.2015.12.006.

[35] Robinson NA, Leslie KE, Walton JS. Effect of Treatment with Progesterone on Pregnancy Rate and Plasma Concentrations of Progesterone in Holstein

Cows. J Dairy Sci 1989;72:202–7. doi:http://dx.doi.org/10.3168/jds.S0022-0302(89)79098-6.

[36] Johnson KR, Barth EJ, Science A, Soc SREA. Effect of 17α- hydroxyprogesterone 17- n- caproate on the reproductive performance of cattle. Ann N Y Acad Sci 1958;71:577–9.

[37] Okumu LA, Forde N, Fahey AG, Fitzpatrick E, Roche JF, Crowe MA, *et al*. The effect of elevated progesterone and pregnancy status on mRNA expression and localisation of progesterone and oestrogen receptors in the bovine uterus. Reproduction 2010;140:143–53. doi:10.1530/REP-10-0113.

[38] Spencer T. Conceptus signals for establishment and maintenance of pregnancy. Anim Reprod Sci 2004;15:1–15. doi:10.1016/S0378-4320(04)00070-3.

[39] Spencer TE, Johnson GA, Bazer FW, Burghardt RC, Palmarini M. Pregnancy recognition and conceptus implantation in domestic ruminants: Roles of progesterone, interferons and endogenous retroviruses. Reprod Fertil Dev 2007;19:65–78. doi:10.1071/RD06102.

[40] Pugliesi G, Oliveria ML, Scolari SC, Lopes E, Pinaffi FV., Miagawa BT, *et al*. Corpus luteum development and function after supplementation of long-acting progesterone during the early luteal phase in beef cattle. Reprod Domest Anim 2014;49:85–91. doi:10.1111/rda.12231.

[41] Ing NH, Tornesi MB. Estradiol up-regulates estrogen receptor and progesterone receptor gene expression in specific ovine uterine cells. Biol Reprod 1997;56:1205–15.

[42] Robinson RS, Mann GE, Lamming GE, Wathes DC. Expression of oxytocin , oestrogen and progesterone receptors in uterine biopsy samples throughout the oestrous cycle and early pregnancy in cows. Reproduction 2001;122:965–79.

[43] Madsen CA, Perry GA, Mogck CL, Daly RF, MacNeil MD, Geary TW. Effects of preovulatory estradiol on embryo survival and pregnancy establishment in beef cows. Anim Reprod Sci 2015;158:96–103. doi:10.1016/j.anireprosci.2015.05.006.

[44] Pereira MHC, Wiltbank MC, Vasconcelos JLM. Expression of estrus improves fertility and decreases pregnancy losses in lactating dairy cows that receive artificial insemination or embryo transfer. J Dairy Sci 2016;99:2237–47.

doi:http://dx.doi.org/10.3168/jds.2015-9903.

[45] Houghton PL, Lemenager RP, Moss GE, Hendrix KS. Prediction of postpartum beef cow body composition using weight to height ratio and visual body condition score. J Anim Sci 1990;68:1428–37.

[46] Sales JNS, Carvalho JBP, Crepaldi GA, Cipriano RS, Jacomini JO, Maio JRG, *et al*. Effects of two estradiol esters (benzoate and cypionate) on the induction of synchronized ovulations in *Bos indicus* cows submitted to a timed artificial insemination protocol. Theriogenology 2012;78:510–6. doi:10.1016/j.theriogenology.2012.02.031.

[47] Colazo MG, Kastelic JP, Mapletoft RJ. Effects of estradiol cypionate (ECP) on ovarian follicular dynamics, synchrony of ovulation, and fertility in CIDR-based, fixed-time AI programs in beef heifers. Theriogenology 2003;60:855–65. doi:10.1016/S0093-691X(03)00091-8.

[48] Pugliesi G, Beg MA, Carvalho GR, Ginther OJ. Induction of PGFM pulses and luteolysis by sequential estradiol-17β treatments in heifers. Theriogenology 2012;77:492–506. doi:10.1016/j.theriogenology.2011.08.020.

[49] Pugliesi G, Miagawa BT, Paiva YN, Franca MR, Silva LA, Binelli M. Conceptus-Induced Changes in the Gene Expression of Blood Immune Cells and the Ultrasound-Accessed Luteal Function in Beef Cattle: How Early Can We Detect Pregnancy? Biol Reprod 2014;91:1–12. doi:10.1095/biolreprod.114.121525.

[50] Picard-Hagen N, Lhermie G, Florentin S, Merle D, Frein P, Gayrard V. Effect of gonadorelin, lecirelin, and buserelin on LH surge, ovulation, and progesterone in cattle. Theriogenology 2014;84:177–83. doi:10.1016/j.theriogenology.2015.03.004.

[51] Fields SD, Perry BL, Perry GA. Effects of GnRH treatment on initiation of pulses of LH, LH release, and subsequent concentrations of progesterone. Domest Anim Endocrinol 2009;37:189–95. doi:10.1016/j.domaniend.2009.04.006.

[52] Quintal-Franco JA, Kojima FN, Melvin EJ, Lindsey BR, Zanella E, Fike KE, *et al*. Corpus luteum development and function in cattle with episodic release of luteinizing hormone pulses inhibited in the follicular and early luteal phases of the estrous cycle. Biol Reprod 1999;61:921–6. doi:10.1095/biolreprod61.4.921.

[53] Ginther OJ. Effect of progesterone on length of estrous cycle in cattle. Am J Vet Res 1970;31:493–6.

CHAPTER 2

SUPPLEMENTATION WITH LONG-ACTING PROGESTERONE IN EARLY DIESTRUS IN BEEF CATTLE: I. EFFECT OF ARTIFICIAL INSEMINATION ON ONSET OF LUTEOLYSIS

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2 SUPPLEMENTATION WITH LONG-ACTING PROGESTERONE IN EARLY DIESTRUS IN BEEF CATTLE: I. EFFECT OF ARTIFICIAL INSEMINATION ON ONSET OF LUTEOLYSIS

2.1 ABSTRACT

In beef cattle, progesterone (P4) supplementation in early diestrus advances changes in the endometrial transcriptome causing detrimental (early onset of luteolysis) and stimulatory (accentuated conceptus growth) effects on fertility. An overall positive pregnancy outcome will occur when maternal-embryonic interactions overcome possible failures resulting from P4-stimulated luteolytic effects. Hypothesis was that AI decreases the incidence of early luteolysis in cows supplemented with P4 at early diestrus. Non-suckled beef cows received AI 12h after estrus (D0: ovulation) or were not inseminated (no-AI). On D3, the AI cows were assigned to receive a single dose of 150 mg of injectable long-acting P4 (AI+iP4; n=23) and the no-AI cows were assigned to receive long-acting P4 (iP4; n=21) or saline (Control, n=22). The CL development and regression were determined by ultrasonography (US) on B and color Doppler modes, between D3 and D21. Blood P4 concentrations were measured on D3 and every other day from D9 to D21. Pregnancy status was determined by US between D28 and D32. The iP4 treatment reduced CL development between D5 and D10 compared to the control group. Proportion of cows undergoing luteolysis by D15 in the iP4 group (47.6% [10/21]) was greater than in the Control group (13.6% [3/22]) and proportion on the AI+iP4 group was intermediate (26.1% [6/23]). Interval to luteolysis was similar between groups iP4 (16.38±0.46 days) and AI+iP4 (15.50±0.66 days) but smaller than the Control group (17.38±0.40 days; effect of treatment P=0.05). Consistently, plasma P4 concentrations on D15 of the iP4 group (2.10±0.47 ng/mL) were lower (P<0.05) than those of control (4.40±0.46 ng/mL) and AI+iP4 (3.70±0.45 ng/mL) groups. Except for one cow, luteolysis occurred between D14 and D21. The effects of AI reducing frequency of early luteolysis was explained by the cows that became pregnant (n=7) in the AI+iP4 group. Remaining animals in this

group showed frequency of luteolysis by D15 (37.5% [6/16]) and interval to luteolysis similar to the iP4 group. When cows were partitioned according to the day of luteolysis (≤ 15d or > 15d), the CL development from D3 to D10 was similar between these sub-groups. In conclusion, AI was only able to overcome iP4-induced early luteolysis if pregnancy was established and maintained. Also, iP4 deleterious effects on CL development were not associated with early luteolysis.

2.2 INTRODUCTION

Progesterone (P4) is a central hormone to the success of pregnancy, and its reduced circulating concentrations at early diestrus are associated with low conception rates in cattle [1–3]. In fact, P4 stimulates endometrial secretions, favoring embryo development [4] and pregnancy establishment [3]. In cattle, a major contributor of pregnancy failure is embryonic mortality, that occurs in the first 3 weeks after estrus and reaches 70–80% of inseminated animals [2,5,6]. Due to the importance of P4 at this initial phase [7], a number of studies have focused on the supplementation of P4 as a means to overcome the challenge of embryonic loss and to gain further understand on the role of P4 on embryo development.

The embryotrophic effect of P4 was reported previously [8–10]. Specifically, insertion of a P4-releasing intravaginal device on day 3 post-estrus stimulated conceptus elongation and increased interferon-tau (IFNt). These effects were mediated through the action of P4 on the uterine environment [10] by advancing transcription of genes related to energy sources and histotroph components in the endometrium [11]. In contrast, elevation of circulating P4 concentrations by daily injection of P4 or by the use of P4-releasing devices during early diestrus (ranging from 0 to 7 days post-estrus) shortened the estrous cycle of cows [9,12,13] due to a greater proportion of cows showing premature luteolysis (30–35% vs. less than 10% in controls [9,14]). This undesirable effect may be related to inadequate luteotrophic support to the CL, due to a P4-mediated acute reduction in LH pulse frequency [12,14]. Alternatively, supplemental P4 could re-program the endometrial PGF2α-synthesizing machinery to advance release of PGF2α in non-pregnant cows [15]. Thus, P4-supplementation at early diestrus causes paradoxical effects; there are

both beneficial downstream effects on uterine receptivity and conceptus elongation, and potentially negative effects on CL lifespan. As expected, compilation studies [3,16] show that the effects of P4 supplementation during diestrus on fertility are inconsistent.

In this paradoxical scenario, the role of the elongating conceptus in the pregnancy outcome in response to P4 is poorly understood. In ruminants, recognition of the embryo by the maternal tissues is reported to be a response to IFNt secreted by the elongating conceptus [17]. In fact, modifications in the endometrial transcriptome of cattle, caused by the presence of an embryo, were initially detectable on day 7 [18], but became evident on day 16 post-estrus [19]. This was largely due to the increasing effects of IFNt [19,20]. Mechanism includes IFNt-mediated inhibition of luteolytic pulses of PGF2α by the surrounding endometrium [21]. However, P4-induced shortening of the CL lifespan is frequently reported to occur before maternal recognition of pregnancy (i.e., day 16 after estrus). This indicates that lack of appropriate signaling by the conceptus may result in negative effects on pregnancy rates of P4-supplemented cows [22,23]. Furthermore, the occurrence of early luteolysis seems to be more prominent in studies that used non-inseminated cows in the design [12,13, 24], meaning that the effect may be less when an embryo is present.

Recently, a novel, practical method to supplement P4 to cattle was reported [24,25]. Supplementation was through a single dose of injectable long-acting P4 (iP4). Current results [24] indicated that, compared to controls, the administration of 150 or 300 mg of iP4 to non-inseminated cows on days 2 or 3 post-ovulation increased the proportion of cows that showed luteolysis by day 15 post-ovulation (0.0% vs. 40.7%). The effect was more intense when the iP4 dose was greater and injection was given earlier; thus, the dose of 150 mg on day 3 was chosen as the strategy to supplement cows in subsequent studies. While the luteolytic effect was evident in non-inseminated cows, the embryotrophic aspect was highlighted in a subsequent study [25]. Anestrous suckled beef cows that received timed artificial insemination (TAI) and were treated with iP4 presented a 20% increase in conception in comparison to non-treated controls. Collectively, P4 supplementation effects on fertility are complex because of the multiple potential P4 targets; specifically, the CL (via reduced LH pulse frequency), the endometrium and the conceptus. To the best

of our knowledge, this is the first study designed to sort out the conceptus effects on P4-induced early luteolysis in cattle. We aimed to evaluate the role of AI on the incidence of premature luteolysis induced by iP4 supplemented at early diestrus. Specifically, we tested the hypothesis that supplementation with iP4 at early diestrus shortens the CL lifespan of non-inseminated cows, while AI attenuates this process.

2.3 MATERIALS AND METHODS

2.3.1 Animals

Cycling, multiparous, non-lactating Nelore cows (n=90) with an average age of 6.5 years old (range: 3 to 12 years); and weight of 591 kg; SEM: 7.21 (range: 430-725 kg) were used between November and February in the Southern hemisphere tropics at the Pirassununga Campus of the University of Sao Paulo (USP) in Brazil. Experimental animals had no apparent abnormalities in the reproductive tract and had a CL in at least one of the weekly ultrasound scans. Animals were kept under grazing conditions supplemented with sugarcane and concentrate; they had free access to water and mineralized salt. Handlings were done in accordance with the Institutional Animal Care and Use Committee (CEUA-FMVZ/USP, n° 4664220316).

2.3.2 Experimental design and treatments

Follicular growth was synchronized with the insertion of an intravaginal P4 device (1.0 g P4; Sincrogest®, Ourofino Saude Animal, Cravinhos, SP, Brazil), along with intramuscular (i.m.) injections of estradiol benzoate (2.0 mg; i.m.; Sincrodiol®, Ourofino Saude Animal) and PGF2α analogue (500 μg of sodium cloprostenol; i.m.; Sincrocio®, Ourofino Saude Animal). Eight days later, the P4 devices were removed, and cows were given another dose of PGF2α analogue. At the time of P4 device removal, Estrotect™ patches (Western Point Inc., Apple Valley, MN) were applied halfway between the hip and tail head to aid in estrus detection. Cows were checked

for signs of estrus twice daily for 40 min at 07:30 and 16:30 h over a period of 4 days, commencing 36 h after P4 device removal. Cows seen in standing estrus and/or with approximately 50% or more of the silver rub-off coating removed from the Estrotect patch were considered to be in estrus. One-third of cows observed in estrus were inseminated by a single AI technician 12 h later, using the same batch of frozen-thawed commercial semen of a Nelore bull with proven fertility. In the remaining two-thirds of cows, the AI procedure was performed with straws that contained only the semen extender.

At the time of AI, the diameter of the dominant follicle was recorded by transrectal ultrasound scan and tracked every 12 h to check ovulation. The moment of ovulation was defined as day 0 of the study (D0). Cows that ovulated within 36 h after estrus detection were assigned to one of three treatment groups on D3 post-ovulation: (i) those that underwent AI with semen were supplemented with 150 mg of a single long-acting P4 (iP4; i.m.; Sincrogest® injectable, Ourofino Saude Animal; AI+iP4 group); (ii) those that underwent AI only with semen extender were randomly split to be injected with the same volume of the saline solution at 0.9% (1 mL; Control group) or (iii) with iP4 (iP4 group). A total of 66 cows detected in estrus were allocated in the treatment groups (Control, n=22; AI+iP4, n=23 and iP4, n=21).

2.3.3 Ultrasound examinations

The diameter of the dominant follicle observed in the B-mode still image with a linear transrectal transducer set to 7.5 MHz (Mindray M5 equipped with multifrequency linear transducer) was assessed by taking the average between measurements of its two perpendicular axes. The moment of ovulation was defined by the disappearance of the dominant follicle seen in the previous scan, and confirmed on D3 by the presence of a hemorrhagic corpus luteum (CL) at the same approximate topographical location on the ovary. Between D3 and D21, the ovaries were scanned by transrectal ultrasonography to evaluate the development and regression of the CL. The CL scanning was performed using the B-mode and pulse-wave color Doppler functions for evaluations of total CL area and CL area containing color signals of luteal blood flow, respectively.

For the CL area, the linear transducer set to 7.5 MHz was passed over the entire CL, and the images were stored on film by a single operator. After recording images from all cows and days, the film clips were evaluated to assess the CL area. The maximum CL area was determined using a B-mode still image and the tracing function. For CL with an anechoic fluid-filled cavity, the area of the cavity was subtracted from the total area [26].

The percentage of CL area with color signals of luteal blood flow (CLBF) were visually estimated during CL scanning using the same criteria described in cattle by Pugliesi *et al*. [24,27]. Accordingly, a scale from 0 to 100%, with 5 interval points was used for determination of the percentage of the luteal area with CLBF. All scans were performed at a constant color-gain setting (6.5 MHz, Gain: 62 and Pulse Repeated Frequency: 5.3 kHz) and a velocity setting of 5.4 cm/s by the same operator.

A continuous decrease of the CL area (cm^2) and CLBF was defined as structural luteolysis. Based on a previous report [24,27] in *Bos taurus indicus* beef cattle, the day of structural luteolysis was defined as the day between D8 and D21 when the maximum CL area (cm^2) and the luteal blood flow decreased by 25% and 50%, respectively, from the respective mean values recorded on D8 and D9.

The pregnancy status of cows from the AI+iP4 group was determined by transrectal ultrasonography, detecting an embryo with heartbeat between 28 and 32 days after AI.

2.3.4 Blood sampling for analysis of plasma P4 concentrations

Blood samples were taken from the jugular vein into heparinized Vacutainer tubes (Vacuette, Greiner bio-one, Sao Paulo, SP, Brazil) at D3 and every 2 days between D9 and D21 to measure changes in circulating P4 concentration associated with functional luteolysis, i.e., a progressive decrease in P4 concentrations to <1.0 ng/mL.

The blood samples were immediately placed in a box with chopped ice before centrifuging (2700 x g for 15 min at 4°C). Plasma was separated and stored at –20°C until assayed to determine P4 concentrations by solid phase RIA using a RIA kit (Immuchem™ Double Antibody Progesterone Kit; Cat. 07-170105, MP Biomedicals,

NY, USA). The sensitivity of the assay was 0.1 ng/mL. The intra-assay coefficient of variation (CV) for quality control samples was 0.76% (low) and 2.42% (high). The inter-assay CV was 19.0% (low) and 8.38% (high).

2.3.5 Statistical analyses

Data from the CL area, CL blood flow, and P4 concentrations were analyzed as a repeated measures design using a mixed model. Fixed effect included experimental groups, day, and their interaction. The random effect of cows nested within group was used as an error term for the effect of group using the MIXED procedure of SAS (version 9.3). The type of variance–covariance structure used was chosen based on smaller magnitude of the corrected Akaike's information criterion (AICC). The residual and influencing diagnostics outputs from the MIXED procedure were checked for the assumption of normality of the data. Further, studentized residual error outputs were also checked for normality by the Shapiro–Wilk test using the UNIVARIATE procedure of SAS. One cow from AI+iP4 group presented very early luteolysis (i.e., D8) and consequently skewed the residual error variances of the CL blood flow and P4 concentrations. She was excluded from both analyses. The assumptions underlying all models and tests were met. The Kenward-Rogers degrees of freedom approximation option of SAS was used to determine the denominator degrees of freedom for tests of fixed effects. Significance of effects was determined by F-tests using Type III sums of squares. When fixed effects were significant, means across a given treatment were compared using Fisher's protected least significant difference (LSD, i.e., the DIFF option of the LSMEANS statement). In case of interaction, the slice command was incorporated into the procedure to determine which days the treatment effect occurred.

Secondary analyses were run separately for each group to assess the differences in the CL area from D3 to D10 between cows presenting luteolysis by D15 or afterwards. A similar analysis was run for AI+iP4 group to measure the difference in CL area between pregnant and non-pregnant cows. The fixed effects were the effect of class, day, and their interaction. The random effect of cows nested within class was used as an error term to test the effect of class.

Survival analyses were performed, using the LIFETEST procedure of SAS to establish the effects of group on luteolysis occurrence between D6 and D21. Survival analysis regresses the proportion of luteolysis on the day of its occurrence, and curves were compared using the Wilcoxon test. The variables days to structural/functional luteolysis and days to estrus occurrence were analyzed with NPAR1WAY procedure using the non-parametrical Kruskal-Wallis test of SAS. On D15, the effect of group on the frequency of luteolysis was assessed by the chi-squared test, using the FREQ procedure of SAS.

Effects and differences were declared significant at $P \leq 0.05$ and approaching significance at $0.05 > P \leq 0.10$.

2.4 RESULTS

2.4.1 Animals and treatments

The age (Control: 6.6 ± 0.5; AI+iP4: 6.3 ± 0.4 and iP4: 6.7 ± 0.5 years old; P = 0.82), weight (Control: 576.8 ± 12.4; AI+iP4: 594.7 ± 12.4 and iP4: 602.1 ± 12.7 kg; P = 0.35) and size of pre-ovulatory follicle (Control: 14.0 ± 0.4; AI+iP4: 13.5 ± 0.4 and iP4: 13.6 ± 0.4 mm; P = 0.62) were similar among groups.

2.4.2 Effect of P4 supplementation and AI on CL area and CLBF during diestrus

There was a group by day interaction in luteal tissue area ($P < 0.01$). For cows receiving iP4 (i.e. iP4 and AI+iP4 groups), the CL area was less between D5 and D9, compared to that of the Control group (Figure 1). From D15 to D18, CL area of the iP4 group was smaller than that of the Control group. CL area of the AI+iP4 group was intermediate. From D19 on, the CL area of the AI+iP4 group stopped decreasing and remained constant, while CL area from other groups decreased progressively up to D21.

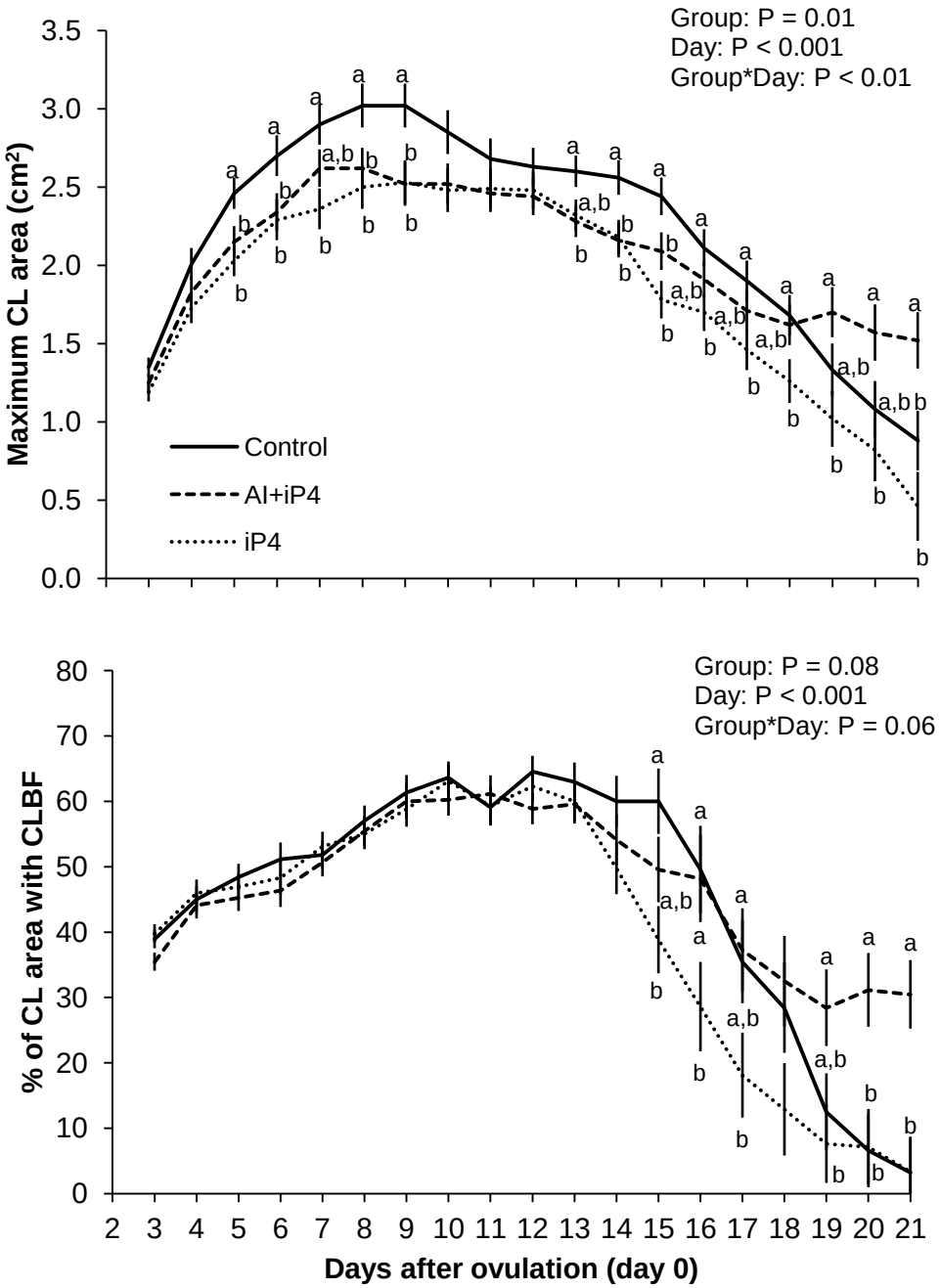

Figure 1. Least squares means ± SEM of CL area (cm^2) and percentage of CL with colour signals of luteal blood flow (CLBF) of beef cows submitted to AI 12 h post-estrus and supplemented with 150 mg of long acting P4 on D3 (AI+iP4; n=23), or non-AI and supplemented (iP4; n=21) or not with iP4 (Control; n= 22). Main effect of group, day and their interaction (group*day) are indicated. [a,b]Values within a day without a common superscript are different between groups (P ≤ 0.05).

For the proportion of luteal area with CLBF, there was a group by day interaction that approached significance (P = 0.06). In contrast to the CL area, there was no effect of long-acting P4 treatment on percentage of CLBF during CL development phase. However, during the CL regression phase, specifically from D15

on, percentage of CLBF followed a similar pattern to that described for CL area. CLBF started to decrease earlier for the iP4 treated groups, but was less prominent for the AI+iP4 group. After D16, rate of CLBF decrease switched between the AI+iP4 and the control group. CLBF continued to decrease until it reached basal levels for the iP4 and control groups on D21. In contrast, CLBF for the AI+iP4 group stop decreasing by D19 and remained constant thereafter.

The CL development between D3 to D10 for cows in which luteolysis occurred by D15 or after D15 was analyzed within each group (Figure 2). For the Control group, the CL development of cows presenting luteolysis by D15 was numerically lower than those presenting luteolysis after D15 (Figure 2). In contrast, CL development was strikingly similar for iP4 group regardless of timing of luteolysis. It was noteworthy that a single iP4-treated cow, from the AI+iP4 group, showed a severe impairment of luteal development that resulted in luteolysis on D8 (Figure 3). The CL area and CLBF during CL development of the AI+iP4 group were analyzed according to pregnancy status (Figure 4). CL area and CLBF were similar between pregnant and non-pregnant cows.

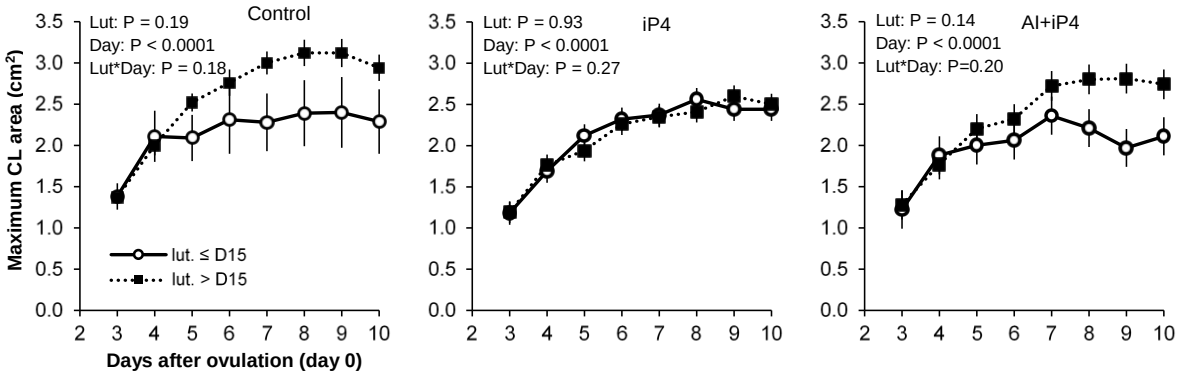

Figure 2. Least squares means ± SEM of CL area (cm2) of beef cows detected in luteolysis by D15 (lut. ≤ D15) or after (lut. > D15). Cows were submitted to AI 12 h post-estrus and supplemented with 150 mg of long acting P4 on D3 (AI+iP4 group; non-pregnant cows: lut. ≤ D15, n=6 and lut. > D15, n=10), or they were non-AI and supplemented (iP4 group; lut. ≤ D15, n=10 and lut. > D15, n=11) or not with iP4 (Control; lut. ≤ D15, n=3 and lut. > D15, n=19). Main effect of day of luteolysis (lut.), day (D) and their interaction is indicated.

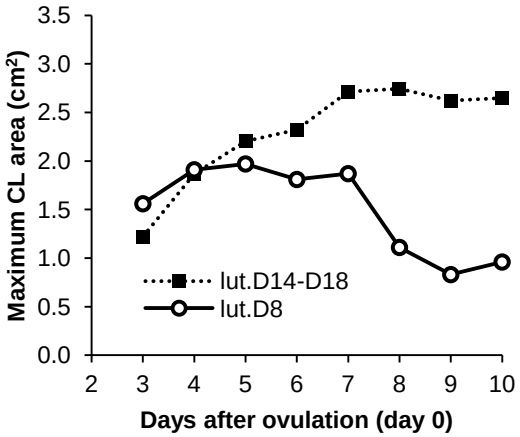

Figure 3. CL area of a cow that showed luteolysis on D8 (n=1), and the mean from those that showed luteolysis later (n=13). Beef cows were AI 12h after estrus detection and were treated with 150 mg of long acting P4 on D3 (AI+iP4 group).

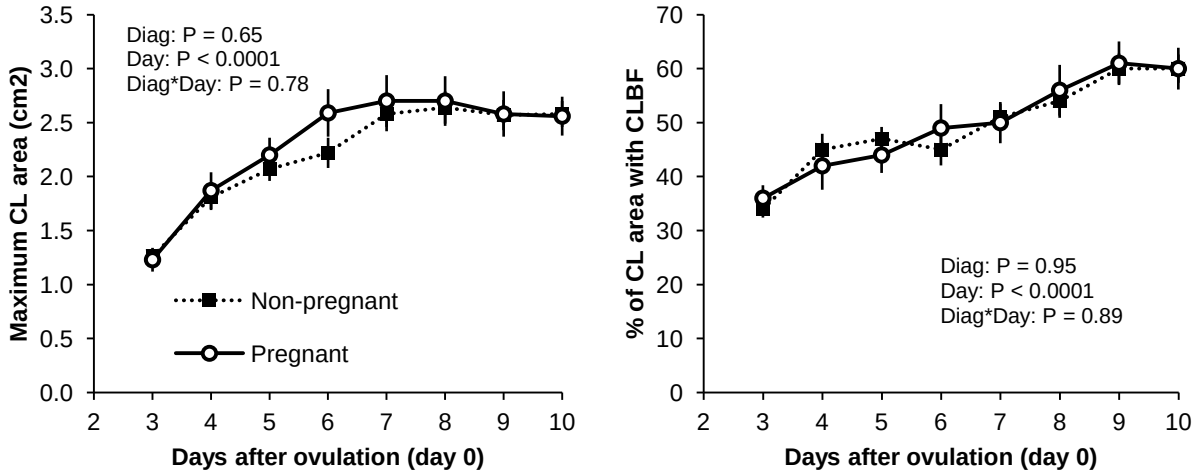

Figure 4. Least squares means ± SEM of CL area (cm2) and percentage of CL with colour signals of luteal blood flow (CLBF) of AI beef cows treated with 150 mg of long acting P4 on D3 (AI+iP4), diagnosed as non-pregnant (n=16) or pregnant (n=7) 28 to 32 days after AI (Diag.). Main effect of Diag., day and their interaction (Diag*day) are indicated.

2.4.3 Effect of P4 supplementation and AI on the onset of luteolysis and on plasma P4 concentrations at late diestrus

Proportion of functional CLs between D6 and D21 was affected (P = 0.05) by treatment groups (Figure 5). The survival curve of AI+iP4 group was different (P = 0.03) from iP4 group but did not differ (P = 0.74) from that of the Control group. The iP4 treatment hastened (P = 0.06) the occurrence of structural luteolysis compared to the Control group (Figure 5, Panel A). The AI retarded iP4-stimulated structural

luteolysis. In a subsequent retrospective analysis, removing the pregnant cows from the AI+iP4 group (Figure 5, Panel B), the effect of group only approached significance (P = 0.10), and the difference between AI+iP4 and iP4 groups was not significant. Therefore, the effect of AI (i.e., a potential embryo) on the dynamics of luteolysis in non-pregnant cows was undetectable.

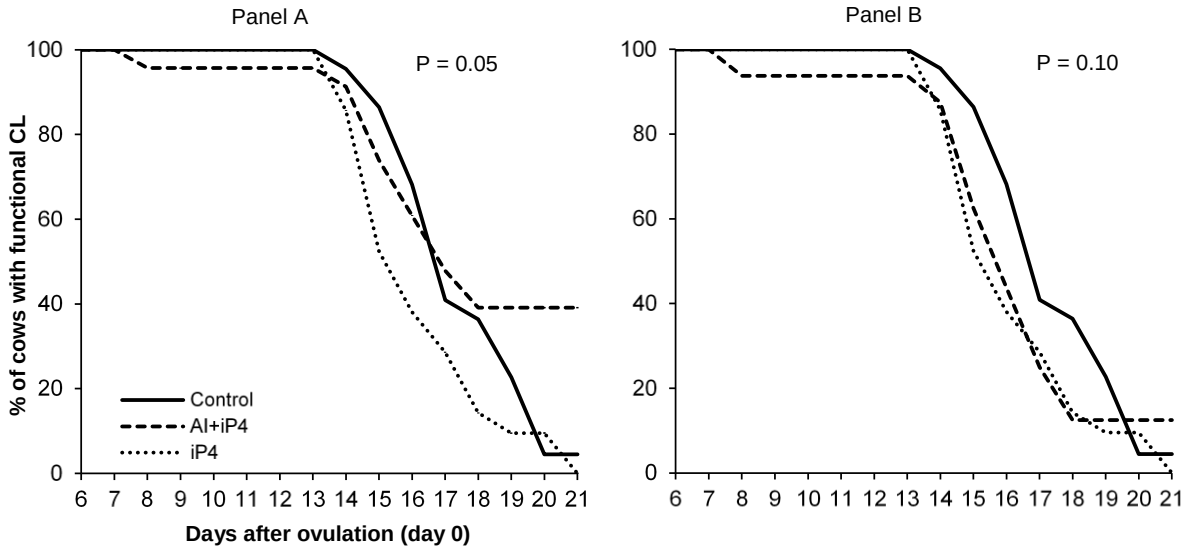

Figure 5. Survival curves of the proportion of cows with a functional CL across the experiment, considering all cows from AI+iP4 group (Panel A) or only those non-pregnant (Panel B). Beef cows were submitted to AI 12 h post-estrus and supplemented with 150 mg of long acting P4 on D3 (AI+iP4; n=23), or they were non-AI and supplemented or not with iP4 (iP4; n=21 and Control; n= 22, respectively). In the panel A, the iP4 group showed hastened (P = 0.06) luteolysis occurrence compared to the control group. The curve of AI+iP4 group was different (P = 0.03) to the iP4 group and similar to the control group (P = 0.74). In the panel B (only with non-pregnant cows), the effect of AI+iP4 group compared to the iP4 group was lost (P = 0.60).

Because of alterations in the time dynamics of luteolysis, supplementation of long-acting P4 (i.e. iP4 and AI+iP4 groups) shortened the interval from ovulation to structural/functional luteolysis (P = 0.05; Table 1) compared to the Control group.

Day 15 was chosen as the end-point for analysis of the frequency of premature luteolysis (Table 1). On D15, the frequency of animals that underwent structural luteolysis was affected by group (P = 0.05). The iP4 group presented a greater frequency of premature luteolysis than the Control group. For the AI+iP4 group, the frequency of luteolysis was intermediate between iP4 and Control groups, but numerically and statistically was closer to the Control group (P = 0.30) than the iP4 group (P = 0.14). If pregnant cows are excluded from the AI+iP4 group, a group effect is detected (P = 0.05). In this scenario, frequency of luteolysis of AI+iP4 group

(37.5%; [6/16]) became numerically and statically closer to frequencies in the iP4 group (P = 0.54) than the Control group (P = 0.13). This further indicates the lack of effect of AI (i.e. embryo) in the incidence of luteolysis in cows that failed to become pregnant.

Table 1. Variables associated with timing of the onset of luteolysis in cows treated with 0 (Control) or 150 mg of long-acting P4 on day 3 post-ovulation.

Variable	Groups[1]			P value
	Cont. (n = 22)	AI+iP4 (n = 23)	iP4 (n = 21)	
Ovulation to structural luteolysis (days)[2]	$17.38^{a,X} \pm 0.40$	$15.50^{b} \pm 0.66$	$16.38^{a,b,Y} \pm 0.46$	0.05
Day of P4 < 1.0 ng/mL (days)[3]	$17.76^{a} \pm 0.35$	$16.14^{b} \pm 0.69$	$16.60^{b} \pm 0.37$	0.04
Number of cows having structural luteolysis by day 15	3^{b} (13.6%)	$6^{a,b}$ (26.1%)	10^{a} (47.6%)	0.05
Ovulation to subsequent estrus (days)[4]	$20.59^{a,X} \pm 0.29$	$19.44^{a,b,Y} \pm 0.53$	$19.13^{b} \pm 0.31$	<0.01

[1] Beef cows were submitted to AI 12 h post-estrus and supplemented with 150 mg of long acting P4 on D3 (AI+iP4; n=23), or they were non-AI and supplemented or not with iP4 (iP4; n=21 and Control; n= 22, respectively);
[2] Luteolysis was defined as the day between D8 and D21 when the maximum CL area (cm^2) and the luteal blood flow decreased by 25% and 50%, respectively, from mean of D8 and D9;
[3] Blood sampling was taken from the jugular vein every 2 days between D9 and D21, aiming to identify plasma P4 concentrations < 1.0 ng/mL, as an indicative of functional luteolysis;
[4] 42 cows were detected in estrus along the D17 to D23 (control; n=18, AI+iP4; n=9 and iP4; n=15);
[a,b] Means within a row without a common superscript are different between groups (P ≤ 0.05);
[X,Y] Means within a row without a common superscript tended to differ (0.05 < P < 0.10).

The interval between pre-treatment ovulation and post-treatment estrus (Table 1) was less in iP4-treated cows compared to the control group (group effect; P < 0.01). There was no difference between the iP4 and AI+iP4 group.

Before iP4 treatment, plasma P4 concentrations were similar between groups (Control: 0.95 ± 0.08 ng/mL; AI+iP4: 0.83 ± 0.08 ng/mL; and iP4: 0.81 ± 0.08 ng/mL; P = 0.42). There was a treatment by day interaction (P < 0.001) on plasma P4 concentrations between D9 and D21 (Figure 6). Decrease of P4 concentrations associated with luteolysis was earlier in cows from the iP4 group, associated with the overall earlier onset of luteolysis in that group. Maintenance of pregnancy in the AI+iP4 group reversed this iP4 effect. Indeed, by D19, P4 concentrations were greatest in the AI+iP4 group.

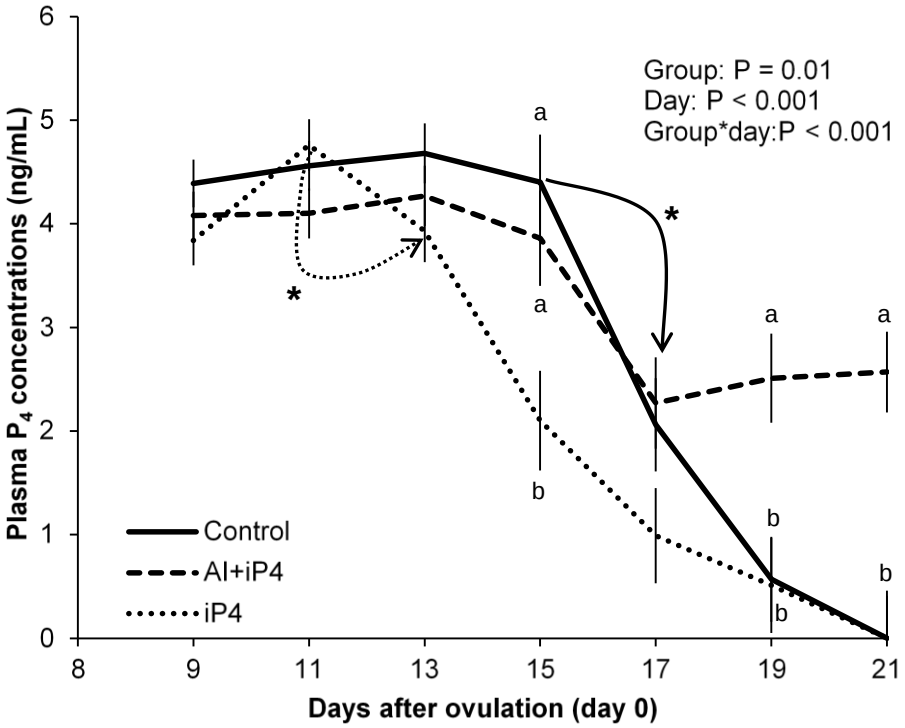

Figure 6. Least squares means ± SEM of plasma P4 concentrations (ng/mL) of beef cows submitted to AI 12 h post-estrus and supplemented with 150 mg of long acting P4 on D3 (AI+iP4; n=23), or non-AI and supplemented (iP4; n=21) or not with iP4 (Control; n= 22). Main effect of treatment group (group), day and their interaction (group*day) are indicated. [a,b]Values within a day without a common superscript are different between groups (P ≤ 0.05). For the Control and AI+iP4 groups, the first significant decrease of plasma P4 concentrations, represented by a solid-line arrow, occurred between D15 and D17 (P < 0.01). Whereas for iP4 group, the first significant decrease, represented by a dotted line arrow, occurred between D11 and D13 (P < 0.01).

2.5 DISCUSSION

The main findings of the present study are that (1) a single i.m. injection of 150 mg long-acting P4 on day 3 after ovulation reduced CL growth at the development phase and hastened the onset of luteolysis; (2) the reduced CL development had no relationship with the interval to luteolysis, indicating that the impairment on luteal development was not the major cause of the reduced CL lifespan; and (3) the effect of AI on reducing the earlier onset of luteolysis was associated with the cows that remained pregnant. Therefore, the variability of response to iP4 supplementation was contingent on the embryo's capability to properly interact with the maternal environment and establish pregnancy.

Regarding the induction of earlier CL regression by P4 treatment, our results are consistent with previous studies in cattle [9,12,13] and sheep [28] that reported an earlier onset of luteolysis after supplementation with P4 at early diestrus. In such studies, the insertion of a P4 device [9,13] or daily intramuscular P4 injection [12,28] were used to increase circulating P4 concentration during a period that varied from days 0 to 7 post-estrus. Collectively, those authors showed reduced CL lifespans or short cycles (by days 5 to 14 post-estrus) and short interestrous interval (15 to 18 days length) in response to such treatments. In the present study, the CL lifespan was as short as observed by others authors in response to injectable long-acting P4. Accordingly, the interval between ovulation to structural/functional luteolysis (Table 1) was approximately 1 day earlier in iP4-treated cows (~day 16) than Control (~day 17) and this trend was also similar for the interval between ovulation to estrus (iP4: 19.1, AI+iP4: 19.4 and Control: 20.6). In support to our current findings, when 150 mg of long-acting P4 was administered on day 3 post-ovulation, Pugliesi *et al*. [24] also verified similar interval to structural luteolysis (day 16), that was shorter than controls (day 18.6). In the current report, except for one cow that exhibited abnormally early luteolysis (day 8), the iP4 treatment mainly produced a shortened cycle rather than a short cycle. This indicated that the detrimental aspect of the iP4 supplementation seemed to be less intense than those reported elsewhere.

Causes for short cycles induced by P4 supplementation have most commonly been attributed to luteal and/or uterine components. Regarding the luteal component, luteinizing hormone (LH) stimulates function of the CL at most stages of diestrus [29,30]. Accordingly, treatment with LH antagonist for 7 days before, during, and after the preovulatory LH surge impaired CL development and function [31]. Furthermore, it is well known that elevated P4 concentrations play a negative feedback role in LH secretion [32]. Thus, inadequate luteotrophic support caused by the P4 treatment is deemed to be the cause of premature CL demise [12,14]. Conversely, simultaneous injections of human [12] or equine [14] chorionic gonadotrophin during metaestrus to P4-treated cows prevented the cycle-shortening effect. Here, we verified that long-acting P4 administration reduced the CL area at the development phase (days 5 to 10 post-ovulation) possibly because the luteal cells were deprived of LH support. On the other hand, at this initial phase, no alterations in CL blood flow perfusion were observed, which corroborate with previous findings. Despite the iP4 effects to decrease CL growth, we did not detect a significant association between CL size and

occurrence of earlier luteolysis in the iP4 group (Figure 2). Only one out of 44 iP4-treated cows (2.3%) presented luteolysis on day 8 post-ovulation (Figure 3). In that particular case, we presume that CL support failed and that caused early demise. Thus, the present results indicate that, in the majority of iP4 treated cows, failure on CL formation were not sufficient to cause regression, nor the consequent short cycle. Rather, an uterine component could be involved as the major cause of iP4-induced early luteolysis.

Regarding the uterine component, Woody and Ginther [33] nicely demonstrated that the absence of the uterine horn on the side of the CL-bearing ovary prevented the ability of exogenous P4 (given between days 1 and 10 post-estrus) to reduce the CL lifespan. Moreover, exogenous P4 treatment at early diestrus advances the release of PGF2α in non-pregnant cows [15]; while at the same time advances the secretory capacity of the pregnant uterus [4,34], favoring conceptus elongation [4,8] and IFNt secretion [9]. This paradoxical effect was confirmed in the present study. The CL survival curve of the AI treatment was intermediate in relation to the curves generated from cows supplemented with P4 or those that were not supplemented. Interpretation is that in a proportion of cows, the P4-embryotrophic effects downregulated the mechanism involved in the earlier onset of the luteolytic process triggered by the exposure of the uterus to iP4. Such positive effect of iP4 to increase CL lifespan was limited to cows that maintained pregnancy, indicating that conceptus signaling must occur properly to prevent early luteolysis. This became clear when CL survival curves were generated for the AI+iP4 group after pregnant cows were removed. This new curve was parallel to that of the iP4 group. Thus, in our study, the negative effect of exogenous P4 to hasten luteolysis was present in cows that did not maintain pregnancy. This suggests that if an embryo was present, its effect was insufficient to block early luteolysis in these cows. Similarly, Wijma *et al*. [35] very recently demonstrated in dairy cows that embryo development needs to continue beyond 22 days after AI to effectively prevent luteolysis and substantially extend the luteal phase.

In conclusion, the results of this study have demonstrated that administration of iP4 on day 3 post-ovulation reduced luteal growth and hastened the interval to structural and functional luteolysis. The shortened luteal lifespan was most likely a result of the pharmacologically earlier exposure of the uterus to P4 that advanced the luteolytic cascade, rather than caused by failures in luteal development. This

observation was further confirmed when cows received AI, since the P4-embryotrophic effects were capable of downregulating the mechanism involved in the earlier luteolytic process. However, this effect was determined by the embryo's capability to properly interact with the maternal environment and to establish pregnancy.

2.6 REFERENCES

[1] Frade MC, Frade C, Cordeiro MB, Sá Filho MF de, Mesquita FS, Nogueira G de P, *et al*. Manifestation of estrous behavior and subsequent progesterone concentration at timed-embryo transfer in cattle are positively associated with pregnancy success of recipients. Anim Reprod Sci 2014;151:85–90. doi:10.1016/j.anireprosci.2014.09.005.

[2] Diskin MG, Morris DG. Embryonic and Early Foetal Losses in Cattle and Other Ruminants. Reprod Domest Anim 2008;43:260–7. doi:10.1111/j.1439-0531.2008.01171.x.

[3] Mann GE, Lamming GE. The Influence of Progesterone During Early Pregnancy in Cattle. Reprod Domest Anim 1999;34:269–74. doi:10.1111/j.1439-0531.1999.tb01250.x.

[4] Garrett JE, Geisert RD, Zavy MT, Morgan GL. Evidence for maternal regulation of early conceptus growth and development in beef cattle. J Reprod Fertil 1988;84:437–46. doi:10.1530/jrf.0.0840437.

[5] Diskin MG, Sreenan JM. Fertilization and embryonic mortality rates in beef heifers after artificial insemination. J Reprod Fertil 1980;59:463–8. doi:10.1530/jrf.0.0590463.

[6] Diskin MG, Parr MH, Morris DG. Embryo death in cattle: An update. Reprod Fertil Dev 2012;24:244–51. doi:10.1071/RD11914.

[7] Spencer TE, Forde N, Lonergan P. The role of progesterone and conceptus-derived factors in uterine biology during early pregnancy in ruminants. J Dairy Sci 2015;99:1–10. doi:10.3168/jds.2015-10070.

[8] Carter F, Forde N, Duffy P, Wade M, Fair T, Crowe MA, *et al*. Effect of increasing progesterone concentration from Day 3 of pregnancy on subsequent embryo survival and development in beef heifers. Reprod Fertil Dev 2008;20:368–75. doi:10.1071/RD07204.

[9] O'Hara L, Forde N, Carter F, Rizos D, Maillo V, Ealy AD, *et al*. Paradoxical effect of supplementary progesterone between day 3 and day 7 on corpus luteum function and conceptus development in cattle. Reprod Fertil Dev 2014;26:328–36. doi:10.1071/RD12370.

[10] Clemente M, De La Fuente J, Fair T, Naib AA, Gutierrez-Adan A, Roche JF, *et*

al. Progesterone and conceptus elongation in cattle: A direct effect on the embryo or an indirect effect via the endometrium? Reproduction 2009;138:507–17. doi:10.1530/REP-09-0152.

[11] Forde N, Carter F, Fair T, Crowe MA, Evans ACO, Spencer TE, *et al*. Progesterone-Regulated Changes in Endometrial Gene Expression Contribute to Advanced Conceptus Development in Cattle. Biol Reprod 2009;81:784–94. doi:10.1095/biolreprod.108.074336.

[12] Ginther OJ. Effect of progesterone on length of estrous cycle in cattle. Am J Vet Res 1970;31:493–6.

[13] Burke CR, Mihm M, Macmillan KL, Roche JF. Some effects of prematurely elevated concentrations of progesterone on luteal and follicular characteristics during the oestrous cycle in heifers. Anim Reprod Sci 1994;35:27–39. doi:10.1016/0378-4320(94)90004-3.

[14] O'Hara L, Forde N, Duffy P, Randi F, Kelly AK, Valenza A, *et al*. Effect of combined exogenous progesterone with luteotrophic support via equine chorionic gonadotrophin (eCG) on corpus luteum development, circulating progesterone concentrations and embryo development in cattle. Reprod Fertil Dev 2014:269–77. doi:10.1071/RD14019.

[15] Garrett JE, Geisert RD, Zavy MT, Gries LK, Wettemann RP, Buchanan DS. Effect of exogenous progesterone on prostaglandin F2α release and the interestrous interval in the bovine. Prostaglandins 1988;36:85–96.

[16] Yan L, Robinson R, Shi Z, Mann G. Efficacy of progesterone supplementation during early pregnancy in cows: A meta-analysis. Theriogenology 2015:1–9. doi:10.1016/j.theriogenology.2015.12.027.

[17] Spencer TE, Johnson GA, Bazer FW, Burghardt RC, Palmarini M. Pregnancy recognition and conceptus implantation in domestic ruminants: Roles of progesterone, interferons and endogenous retroviruses. Reprod Fertil Dev 2007;19:65–78. doi:10.1071/RD06102.

[18] Sponchiado M, Gomes NS, Fontes PK, Martins T, del Collado M, Pastore A de A, *et al*. Pre-hatching embryo-dependent and -independent programming of endometrial function in cattle. PLoS One 2017;12:e0175954. doi:10.1371/journal.pone.0175954.

[19] Forde N, Duffy GB, McGettigan PA, Browne JA, Mehta JP, Kelly AK, *et al*. Evidence for an early endometrial response to pregnancy in cattle: both

dependent upon and independent of interferon tau. Physiol Genomics 2012;44:799–810. doi:10.1152/physiolgenomics.00067.2012.

[20] Forde N, Carter F, Spencer TE, Bazer FW, Sandra O, Mansouri-Attia N, *et al*. Conceptus-induced changes in the endometrial transcriptome: how soon does the cow know she is pregnant? Biol Reprod 2011;85:144–56. doi:10.1095/biolreprod.110.090019.

[21] Binelli M, Thatcher WW, Mattos R, Baruselli PS. Antiluteolytic strategies to improve fertility in cattle. Theriogenology 2001;56:1451–63. doi:10.1016/S0093-691X(01)00646-X.

[22] Parr MH, Crowe MA, Lonergan P, Evans ACO, Rizos D, Diskin MG. Effect of exogenous progesterone supplementation in the early luteal phase post-insemination on pregnancy per artificial insemination in Holstein–Friesian cows. Anim Reprod Sci 2014;150:7–14. doi:10.1016/j.anireprosci.2014.08.008.

[23] Monteiro PLJ, Nascimento AB, Pontes GCS, Fernandes GO, Melo LF, Wiltbank MC, *et al*. Progesterone supplementation after ovulation: Effects on corpus luteum function and on fertility of dairy cows subjected to AI or ET. Theriogenology 2015;84:1215–24. doi:10.1016/j.theriogenology.2015.06.023.

[24] Pugliesi G, Oliveria ML, Scolari SC, Lopes E, Pinaffi FV, Miagawa BT, *et al*. Corpus luteum development and function after supplementation of long-acting progesterone during the early luteal phase in beef cattle. Reprod Domest Anim 2014;49:85–91. doi:10.1111/rda.12231.

[25] Pugliesi G, Santos FB, Lopes E, Nogueira É, Maio JRG, Binelli M. Improved fertility in suckled beef cows ovulating large follicles or supplemented with long-acting progesterone after timed-AI. Theriogenology 2016;85:1239–48. doi:10.1016/j.theriogenology.2015.12.006.

[26] Pugliesi G, Beg MA, Carvalho GR, Ginther OJ. Induction of PGFM pulses and luteolysis by sequential estradiol-17β treatments in heifers. Theriogenology 2012;77:492–506. doi:10.1016/j.theriogenology.2011.08.020.

[27] Pugliesi G, Miagawa BT, Paiva YN, Franca MR, Silva LA, Binelli M. Conceptus-Induced Changes in the Gene Expression of Blood Immune Cells and the Ultrasound-Accessed Luteal Function in Beef Cattle: How Early Can We Detect Pregnancy? Biol Reprod 2014;91:95–95. doi:10.1095/biolreprod.114.121525.

[28] Pope WF, Cárdenas H, Wiley TM, McClure KE. Dose-response relationships of exogenous progesterone shortly after ovulation on estrous cycle length,

blastocyst development and fertility in sheep. Anim Reprod Sci 1995;38:109–17. doi:10.1016/0378-4320(94)01348-P.

[29] Donaldson LE, Hansel W, Vanvleck LD. Luteotropic Properties of Luteinizing Hormone and Nature of Oxytocin Induced Luteal Inhibition in Cattle. J Dairy Sci 1965;48:331–7. doi:10.3168/jds.S0022-0302(65)88223-6.

[30] Armstrong DT, Black DL. Influence of Luteinizing Hormone on Corpus Luteum Metabolism and Progesterone Biosynthesis Throughout the Bovine Estrous Cycle1. Endocrinology 1966;78:937–44.

[31] Quintal-Franco JA, Kojima FN, Melvin EJ, Lindsey BR, Zanella E, Fike KE, *et al*. Corpus luteum development and function in cattle with episodic release of luteinizing hormone pulses inhibited in the follicular and early luteal phases of the estrous cycle. Biol Reprod 1999;61:921–6. doi:10.1095/biolreprod61.4.921.

[32] Kojima FN, Bergfeld EGM, Wehrman ME, Cupp AS, Fike KE, Mariscal-Aguayo DV, *et al*. Frequency of luteinizing hormone pulses in cattle influences duration of persistence of dominant ovarian follicles, follicular fluid concentrations of steroids, and activity of insulin-like growth factor binding proteins. Anim Reprod Sci 2003;77:187–211. doi:10.1016/S0378-4320(03)00038-1.

[33] Woody CO, Ginther OJ. Effect of exogenous progesterone on corpora lutea in unilaterally hysterectomized heifers. J Anim Sci 1968;27:1387–90.

[34] Forde N, Carter F, Fair T, Crowe M a, Evans ACO, Spencer TE, *et al*. Progesterone-regulated changes in endometrial gene expression contribute to advanced conceptus development in cattle. Biol Reprod 2009;81:784–94. doi:10.1095/biolreprod.108.074336.

[35] Wijma R, Stangaferro ML, Kamat MM, Vasudevan S, Ott TL, Giordano JO. Embryo Mortality Around the Period of Maintenance of the Corpus Luteum Causes Alterations to the Ovarian Function of Lactating Dairy Cows. Biol Reprod 2016. doi:10.1095/biolreprod.116.142075.

CHAPTER 3

SUPPLEMENTATION WITH LONG-ACTING PROGESTERONE IN EARLY DIESTRUS IN BEEF CATTLE: II. RELATIONSHIPS BETWEEN FOLLICLE GROWTH DYNAMICS AND LUTEOLYSIS

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3 SUPPLEMENTATION WITH LONG-ACTING PROGESTERONE IN EARLY DIESTRUS IN BEEF CATTLE: II. RELATIONSHIPS BETWEEN FOLLICLE GROWTH DYNAMICS AND LUTEOLYSIS

3.1 ABSTRACT

The aims were to characterize follicular growth and timing of luteolysis in cows supplemented with injectable progesterone (iP4) at early diestrus and submitted or not to AI. Non-suckled beef cows detected in estrus were assigned to receive AI or to remain no-AI. Three days after ovulation (i.e., D3), AI cows received 150 mg of iP4 (AI+iP4; n=22) and the no-AI cows were assigned to receive iP4 (n=19) or saline (Control, n=19). Between D3 and D21, growth dynamics of the dominant follicles (DF) was monitored by ultrasonography. Plasma P4 concentrations measured every other day from D9 to D19. Pregnancy was diagnosed on D30 by ultrasound (P: pregnant and NP: non-pregnant). iP4 decreased average maximum diameter of first-wave DF (DF1). Daily mean diameter of DF1 in iP4-treated cows was smaller when luteolysis occurred by day 15 compared to a later onset. iP4 supplementation did not affect the day of emergence of subsequent follicular waves, nor the proportion of two- or three-wave cycles. Daily mean diameter of DF2 and DF3 was similar between Control and iP4 groups. P4 concentrations between D9 to D19 decrease earliest in the iP4 group, latest in the Control group and intermediate for the NP-AI+iP4 group. Also, three-wave cycles presented a delayed decrease on plasma P4 concentrations than two-wave cycles. On two-wave cycles, P4 concentrations on D15 were lowest in the iP4 and NP-AI+iP4 animals compared to the Control and P-AI+iP4 groups. Conversely, for three-wave cycles, on D15, P-AI+iP4, NP-AI+iP4 and Controls had greater P4 concentrations than the iP4 group. In summary, our data indicate that in iP4-supplemented cows, DF1-estradiol inhibits iP4-induced early luteolysis and that three-wave cycles post-AI are more supportive of pregnancy maintenance after iP4. We speculate that such characteristics are critical to define the embryonic ability to inhibit iP4-induced early luteolysis, as reported in part I of this series.

3.2 INTRODUCTION

Embryonic losses during the first 3 weeks of pregnancy accounts for up to 80% of all losses in beef cattle [1]. The circulating progesterone (P4) concentrations during the first week after conception are positively correlated with conception rates in cattle [2,3]. Because of the importance of P4 for embryonic development at this initial phase [4], a number of studies have focused on the supplementation of P4 as a mean to reduce the elevated incidence of early embryonic mortality and improve pregnancy rates. P4 supplementation in the first week after ovulation advances temporal changes that occur in endometrial gene expression [5]. Changes have been associated to increased conceptus elongation [6,7] and to greater production of interferon-tau (IFNt) [7], that are critical for the establishment and maintenance of pregnancy in ruminants [8]. Despite of such beneficial effects for conceptus elongation, increased P4 during early luteal phase can also shorten the luteal lifespan [7,9,10]. Such adverse effect may compromise the establishment of pregnancy, ultimately leading to increased embryonic loss. As a consequence, compilation studies have reported inconsistent fertility results in response to supplemental P4 [3,11].

The paradoxical aspects of supplemental P4 [7] were recently confirmed by us in series of studies using *Bos taurus indicus* cattle treated with a long-acting P4 formulation (iP4). Pugliesi *et al*. [10] observed that a single administration of 150 or 300 mg iP4 on days 2 or 3 post-ovulation increased the circulating P4 concentrations for ≥3 days during the early luteal phase in non-suckled Nelore cows. Additionally, iP4 increased the frequency of early luteolysis by day 15 (0% vs. 40.7%). In a subsequent fertility study, Pugliesi *et al*. [12] used an estradiol (E2)/P4-based timed AI (TAI) protocol to verify that 150 mg of iP4 administered 4 days post TAI (~3 days post-ovulation) increased the P/AI of anestrous suckled beef cows by 20%. In contrast, Martins *et al*. [13] failed to find a beneficial effect of iP4 on the overall pregnancy rate of suckled beef cows submitted to TAI, and this was in part explained by the incidence of shortened luteal lifespan in a proportion of cows. Collectively, the net fertility outcome of iP4 supplementation results from the beneficial effects on conceptus development and the incidence of early luteolysis.

Shortened corpus luteum (CL) lifespan in response to P4 supplementation can be associated to an inadequate luteotrophic support to the CL, caused by P4-driven inhibition of LH pulse frequency [14,15]. Alternatively, early diestrus P4 supplementation may advance the release of pulsatile endometrial PGF2□ in non-pregnant cows, causing early CL demise [16]. In this last scenario, the conceptus plays a critical role, because secretion of IFNt by day 16 post-breeding blocks the luteolytic PGF2α pulses, thereby maintaining the CL and the pregnancy [17]. Modifications in the endometrial transcriptome of cattle in response to the presence of an embryo were detectable as early as day 7 [18], but they were only evident on day 16 post-estrus, largely due to the effect of increasing production of IFNt [19,20]. Thus, ability of the conceptus to respond to the supplemental P4-primed endometrium will determine whether pregnancy will be maintained or not.

In the first paper of this series, we aimed to show that AI attenuates the incidence of premature luteolysis induced by iP4 supplementation, due to embryo-driven mechanisms. Our investigation demonstrated that this effect was limited by the embryo's capability to properly interact with the maternal environment and to establish pregnancy. Specifically, all cows that received AI but were detected not-pregnant at a D32 pregnancy check failed to block early luteolysis. However, another important component involved in the control of the onset of luteolysis in ruminants is timing and magnitude of follicular E2 produced during the luteal phase [21,22]. Follicular E2 may influence the embryo's capability to establish pregnancy. Specifically, E2 produced by dominant follicles (DF) at the mid- to late- luteal phase, stimulates oxytocin-induced release of uterine PGF2□, thereby regulating timing of luteolysis in ruminants [21,22]. Indeed, the inhibition of follicular growth by x-ray irradiation [23,24], suppression of FSH secretion [25] or follicle ablation [26], extended the CL lifespan in heifers. Collectively, it is expected that an early-elongating conceptus, with limited IFNt-secretory capacity, will be less able to block the E2/oxytocin-induced PGF2α pulses and luteolysis.

Timing of luteolysis is influenced by the number of follicular waves in the estrous cycle. In two-wave cycles, luteolysis occurred 2 or 3 days earlier than in three-wave cycles [27–29]. The influence of the pattern of follicular growth on timing of luteolysis in response to supplementary P4 remains poorly investigated. To the best of our knowledge, only Burke *et al*. [9] addressed this issue in non-AI beef heifers. Accordingly, insertion of an intravaginal P4 device between days 1 and 5

post-estrus reduced the growth of first follicular wave and increased the frequency of two- rather than three- follicular waves, compared to the control. Furthermore, P4 treatment stimulated premature luteolysis. Potential influences of the early embryo have not been addressed to date.

Improving the current knowledge about the effects of supplemental P4 on follicular growth dynamics and the potential implications for regulation of the onset of luteolysis and establishment of pregnancy can provide clues to help reducing the variable fertility outcomes associated to P4 supplementation. Thus, aims of the present study were to characterize follicular growth alterations associated to early diestrus-P4 supplementation and the potential consequences for the timing of luteolysis in P4-treated cows submitted or not to AI.

3.3 MATERIALS AND METHODS

3.3.1 Source of data

The present report is part of an experiment designed to evaluate the effect of AI on occurrence of early luteolysis following iP4 supplementation at early diestrus. Animals, treatments and design were described in the accompanying paper (Part I). Animal handling was conducted according to the institutional Animal Care and Use Committee (CEUA-FMVZ/USP, n° 4664220316). Briefly, the ovulation of non-suckled, cycling, multiparous Nelore cows (n=90), averaging 6.5 years old and 591 kg, were synchronized by inserting an intravaginal P4 device (1.0 g P4; Sincrogest®, Ouro Fino Saude Animal, Cravinhos, SP, Brazil) and injecting estradiol benzoate (2.0 mg; i.m.; Sincrodiol®, Ouro Fino Saude Animal) and a PGF2α analogue (500 µg of sodium cloprostenol; i.m.; Sincrocio®, Ouro Fino Saude Animal). Eight days later, the P4-releasing device was withdrawn, cows received another dose of PGF2α analogue and were fitted with Estrotect® patches as a heat detection aid. After 36 h, estruses were detected twice daily, over a period of 4 days. Cows observed in standing estrus and/or presenting an activated Estrotect (n=66) received AI 12 h later using frozen-thawed commercial semen of the same batch from a single Nelore bull (n=23) or only semen extender (n=43). Ovulations (day 0 of study; D0) were monitored each 12h by transrectal ultrasonography. On D3, the AI cows were supplemented with 150 mg of

long acting P4 (iP4; 1 mL; i.m.; Sincrogest® injectable, Ouro Fino Saude Animal, Cravinhos, Brazil; AI+iP4 group; n=23) and those that received semen extender were assigned randomly to receive saline solution (1 mL; control group; n=22) or iP4 (iP4 group; n=21). Animals were kept under grazing conditions supplemented with sugarcane and concentrate; they had free access to water and mineralized salt.

Ovaries were scanned by transrectal ultrasonography in B mode using a linear transducer set to 7.5 MHz. Images were stored on film clips from D3 to D21, by a single operator. Also, CLs were scanned using Doppler mode to evaluate the percentage of CL area with color signals of luteal blood flow, as described by Pugliesi *et al*. [10,30]. As described in the Part I paper of this series, film clips were used to assess the CL area aiming to determine the effects of P4 supplementation on CL growth and regression. The day of luteolysis was defined as the day when the maximum CL area (cm2) and the luteal blood flow decreased by 25% and 50%, respectively, from the mean area and blood flow measured on D8 and D9 [10,30]. Blood P4 concentrations were measured on D3 and every other day from D9 to D21. The pregnancy status was determined by transrectal ultrasonography, detecting an embryo with heartbeat between 28 and 32 days after AI.

In the present report, we used follicular growth data taken from the film clips and plasma P4 concentration between D9 and D19 and of time of luteolysis that were reported in Part I.

3.3.2 Follicle data set

Individual follicle diameters were calculated as the average between measurements of two perpendicular axes using a B-mode still image and caliper function of the ultrasound equipment. Patterns of growth and regression of DF from each follicular wave were determined, as reported by Savio *et al*. [31] and Sirois and Fortune [32]. Only cows showing two or three waves of follicle growth were analyzed in the present study. Day of emergence was identified retrospectively to the day when the DF was first detected with a diameter of 4 to 5 mm. All ultrasonographic examinations and review of film clips were performed, blindly of treatments, by the same experienced operator.

3.3.3 Statistical analyses

Data of DF diameter and plasma P4 concentrations were analyzed as a repeated measures design using the MIXED procedure of SAS (version 9.3). For the analysis of DF diameter from the first follicular wave (DF1) and plasma P4 concentrations, the model included the fixed effect of experimental groups, number of follicular waves (two- or three-wave cycles), day and their interactions. The random effect of cows nested within the combination of groups and number of follicular waves was used as an error term for the effect of group and number of follicular waves. To analyze growth of the DF from second (DF2) and third follicular (DF3) waves, models included the fixed effect of experimental groups, day and their interaction. Error term was the effect of cow nested within group. The type of variance–covariance structure used for determination of residual error was the one presenting the smaller magnitude of the corrected Akaike's information criterion (AICC). The residual and the influencing diagnostics outputs from the MIXED procedure and the Shapiro-Wilk test from PROC UNIVARIATE were checked for the assumption of normality of the data. When necessary, the natural logarithmic or the square root transformation was used to normalize the data distribution. Non-transformed data were shown for clarity. The Kenward-Rogers degrees of freedom approximation option of SAS was used to determine the denominator degrees of freedom for tests of fixed effects.

Subsequent analyses were run separately for each experimental group to assess the differences in the growth pattern of DF1 for cows having luteolysis by D15 or afterwards (two classes). For AI+iP4 group, additional analyses were run to evaluate the differences in the pattern of growth of DF1, DF2 and DF3 between pregnant and open cows (two classes). In these analyses, the model included the fixed effect of class, day and their interaction. The effect of cow nested within class was used as the error term.

The dependent variables: day of DF emergence, DF diameter at day of luteolysis, day of luteolysis and plasma P4 concentrations on D15, were analyzed using PROC MIXED of SAS. The model included the fixed effect of experimental group, number of follicular waves and their interaction. For the variable plasma P4

concentrations on D15, a subsequent analysis was run using the same model, but dividing the AI+iP4 group in two subgroups according to the pregnancy status, i.e. pregnant and non-pregnant cows. The assumptions of normality of residues and homogeneity of variances (Welch's test) were checked prior to the ANOVA.

Statistical significance of effects was determined by the F-test, using Type III sums of squares. Means across a given treatment were compared using Fisher's protected least significant difference (LSD, i.e., the DIFF option of the LSMEANS statement). The slice command was incorporated into the procedure to determine the effect of treatment by day interaction, when significant. In all instances, significant differences were declared at $P \leq 0.05$ and approaching significance at $0.05 > P \leq 0.10$.

3.4 RESULTS

Two and three cows from the iP4 and Control groups, respectively, showed four follicular waves and presented luteolysis between D19 and D21. One cow from AI+iP4 group presented luteolysis on D8 and developed a persistent DF1 followed by the emergence of an ovulatory DF. Per design, these cows were excluded from subsequent analyzes.

Luteolysis was not detected between D8 and D21 in one cow from the Control group and in two non-pregnant cows from AI+iP4 group. All of these 3 cows had three-follicular waves. The proportion of pregnant cows from the AI group was 30.4% (7/23); three and four pregnant cows, presented, respectively, two and three follicular waves between D3 and D21.

3.4.1 First dominant follicle (DF1)

DF1 diameter changed over time (effect of day; $P < 0.0001$; Figure 1). There was an expected increase in diameter between D3 to D7 post ovulation, followed by a progressive reduction in diameter associated with loss of dominance. Daily mean

diameters of DF1 were significantly reduced in iP4-supplemented cows (i.e. iP4 and AI+iP4 groups) compared to the Control group (effect of treatment; P < 0.001). The average maximum diameter of the DF1 (between D3 and D12 post ovulation) was smaller (P < 0.001) in the iP4 (9.50 ± 0.29 mm) and AI+iP4 (9.48 ± 0.27 mm) groups compared with the Control group (11.00 ± 0.30 mm).

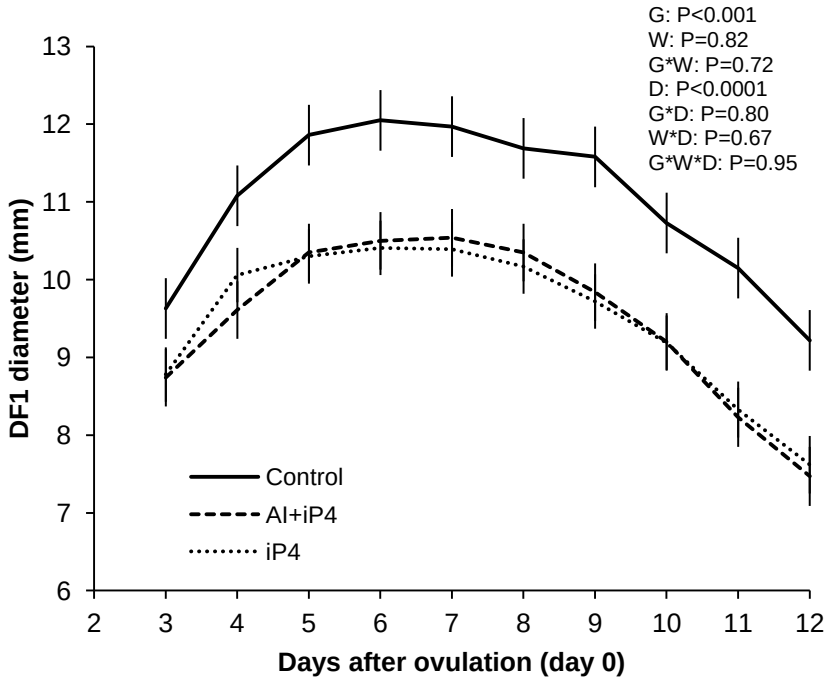

Figure 1. Least squares means ± SEM of CL area (cm2) and percentage of CL with colour signals of luteal blood flow (CLBF) of beef cows submitted to AI 12 h post-estrus and supplemented with 150 mg of long acting P4 on D3 (AI+iP4; n=23), or non-AI and supplemented (iP4; n=21) or not with iP4 (Control; n= 22). Main effect of group, day and their interaction (group*day) are indicated. [a,b]Values within a day without a common superscript are different between groups (P ≤ 0.05).

There was a time of onset of luteolysis (early: ≤ D15; late: > D15) by day interaction for diameter of DF1 for the iP4 (P = 0.05) and AI+iP4 (P < 0.001) groups (Figure 2). Specifically, reduced mean diameters of DF1 were associated with earlier onset of luteolysis in iP4-supplemented cows. There was no difference on DF1 development between pregnant and non-pregnant cows from the AI+iP4 group (Figure 5, Panel A). This indicates that the iP4-induced reduction on DF1 growth was not associated to the establishment of pregnancy.

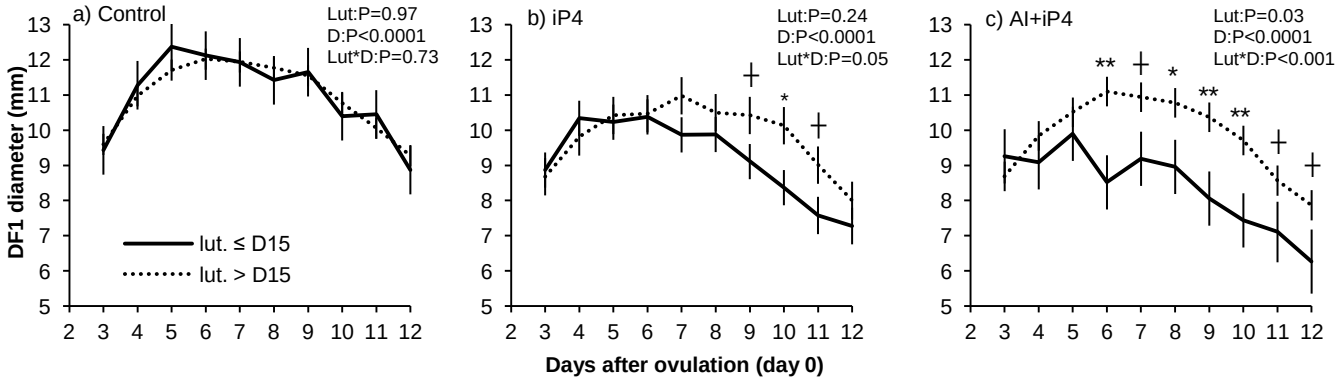

Figure 2. Least squares means ± SEM daily diameter of the first dominant follicle (DF1) for beef cows detected in luteolysis by D15 (lut. ≤ D15) or after (lut. > D15). Cows non-supplemented with P4 (a: Control; lut. ≤ D15, n=3 and lut. > D15, n=16) or supplemented with 150 mg of long acting P4 and non-AI ((b: iP4; lut. ≤ D15, n=10 and lut. > D15, n=9) or previously submitted to AI 12h post-estrus (c: AI+iP4; lut. ≤ D15, n=5, lut. > D15, n=10, and pregnant, n=7). Main effect of day of luteolysis (Lut.), day (D) and their interaction is indicated. Values within a day was different at *P<0.05 or **P<0.01. ┼Values within a day tended to differ (0.05<P<0.10).

3.4.2 Second and third dominant follicles (DF2 and DF3)

For two-wave cycles, the average maximum diameter of the DF2 (between D0 and D10 after emergence) was smaller in the AI+iP4 group (8.42 ± 0.42 mm) compared with iP4 (9.62 ± 0.40 mm) and Control (9.57 ± 0.50 mm) groups (treatment effect; P = 0.07; Figure 3, panel A). For three-wave cycles, DF2 from AI+iP4 group was smaller than Control between D2 and D5 after emergence and smaller than iP4 group on D2 and D3 after emergence (treatment by day interaction; P = 0.03; Figure 3, Panel B). Whereas for iP4 group, DF2 was intermediate on D4 after emergence and on D5, it was smaller than Control and similar to the AI+iP4 group (Figure 3, Panel B). Specifically within the AI+iP4 group, DF2 daily mean diameter was similar between pregnant and non-pregnant cows (Figure 5, Panels B and C).

Growth of the DF3 was not affected by treatment (Figure 4). Analysis of the AI+iP4 group alone showed that on D4 and D5 after emergence, the DF3 diameter of pregnant cows was smaller than those of non-pregnant cows (pregnancy by day interaction; P = 0.09; Figure 5, Panel D).

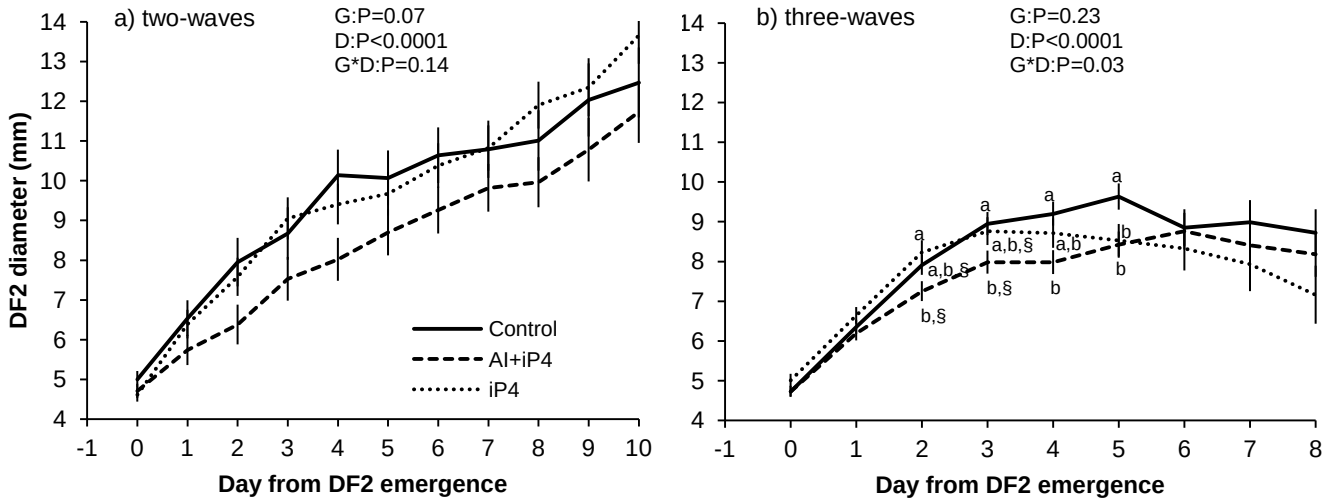

Figure 3. Least squares means ± SEM daily diameter of the second dominant follicle (DF2) in two- (a) or three- (b) follicular wave cycles. Beef cows were submitted to AI 12 h post-estrus and supplemented with 150 mg of long acting P4 on D3 post-ovulation (AI+iP4; 2 waves, n=10 and 3 waves, n=12), or non-AI and supplemented (iP4; 2 waves, n=11 and 3 waves, n=8) or not with iP4 (Control; 2 waves, n=7 and 3 waves, n=12). Mean day of emergence of DF2 in two- and three- wave cycles were, respectively, 8.52 and 7.56 days post-ovulation. Main ffect of group (G), day (D) and their interaction is indicated. Different letters within a day was different at ^{a,b}P ≤0.05. §Values within a day tended to differ 0.05<P<0.10.

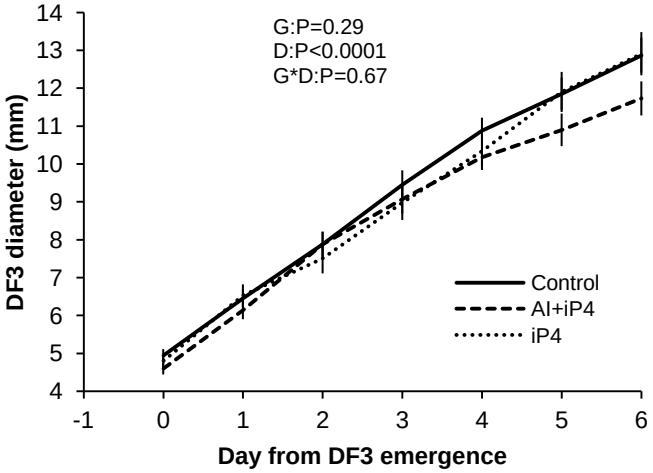

Figure 4. Least squares means ± SEM daily diameter of the third dominant follicle (DF3) for beef cows submitted to AI 12 h post-estrus and supplemented with 150 mg of long acting P4 on D3 post-ovulation (AI+iP4; n=12), or non-AI and supplemented (iP4; n=8) or not with iP4 (Control; n=12). Mean day of emergence of DF3 was 13.5 days post-ovulation. Main effect of group (G), day (D) and their interaction is indicated.

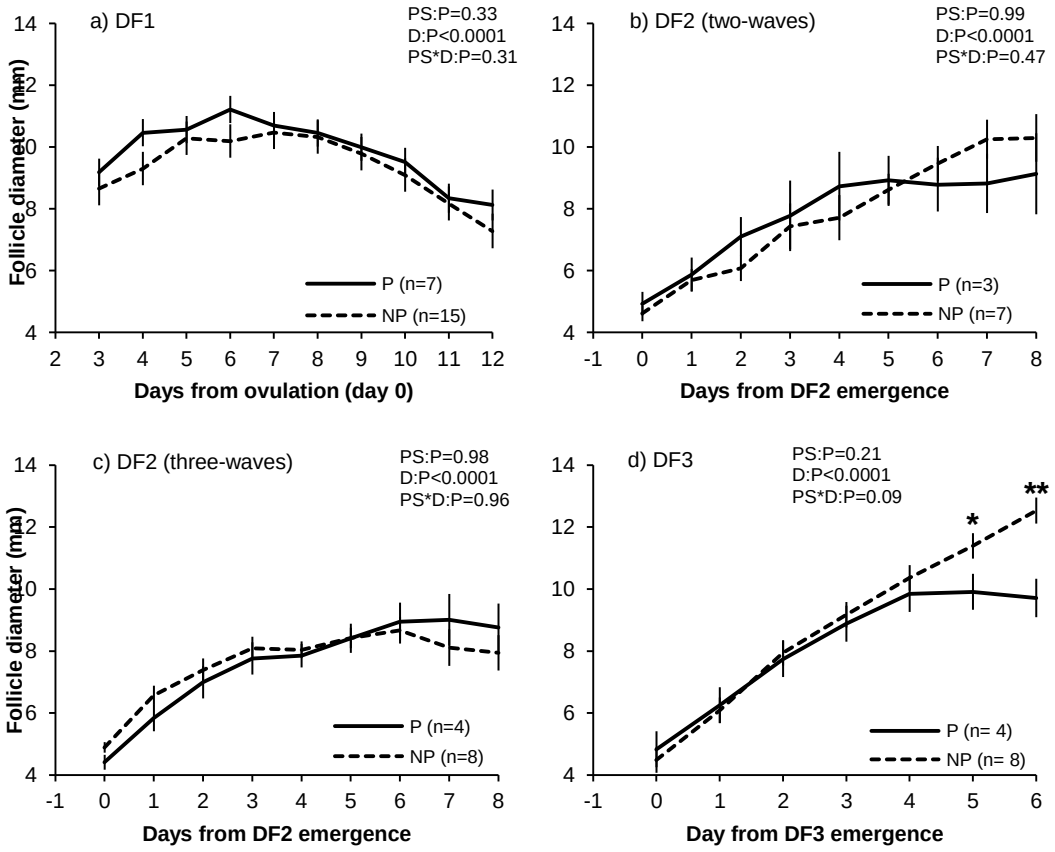

Figure 5. Least squares means ± SEM daily diameter of the dominant follicle (DF) according to pregnancy status on 30 d post-ovulation (pregnant [P] and non-pregnant [NP]) and development of the DF. Mean day of emergence of DF2 in two- and three- wave cycles were, respectively, 8.52 and 7.56 days post-ovulation and of DF3 was 13.5 days post-ovulation. Beef cows were submitted to AI 12h post-estrus and supplemented with 150 mg of long acting P4 on D3 post-ovulation. Main effect of pregnancy status (PS), day (D) and their interaction is indicated. Values within a day was different at *P=0.05 and **P=0.001.

3.4.3 Characteristics of follicular waves

Day of emergence of DF2 was earlier in three-wave cycles (Table 1; P = 0.04). There was no effect of treatment on days of emergence of the second or third waves.

The DF diameter at time of luteolysis in two-wave cycles (10.44 ± 0.43 mm) were larger than in three-wave cycles (9.27 ± 0.43 mm), regardless of treatment (effect of number of waves; P= 0.06; Table 2). Time of onset of luteolysis was similar between two- and three-wave cows, regardless of group. Plasma P4 concentrations on D15 were similar in Control (4.14 ± 0.52 ng/mL) and AI+iP4 (3.83 ± 0.46 ng/mL) groups, but were both greater than the iP4 group (1.75 ± 0.50 ng/mL; effect of

treatment; P = 0.004). Consistently, numbers of cows that underwent luteolysis by D15 were greater in the iP4 group that in the other groups.

Table 1. Day of emergence of the second dominant follicle (DF2), in two- and three-waves cycles, and of the third dominant follicle (DF3) following supplementation with long acting P4 at 3 days post-ovulation

Variable	2 waves			3 waves			P value[ii]		
	Control	AI+iP4	iP4	Control	AI+iP4	iP4	G	W	G*W
Distribution of cows[i]	7	10	11	12	12	8	.	.	.
Emergence of DF2 (days)	8.57±0.63	8.80±0.53	8.18±0.50	7.92±0.48	7.18±0.50	7.57±0.63	0.36	0.04	0.33
Emergence of DF3 (days)	.	.	.	13.75±0.44	13.50±0.44	13.13±0.54	0.67	.	.

Abbreviations: DF2 and DF3= dominant follicle from second and third follicular wave, respectively
[i]Beef cows were submitted to AI 12 h post-estrus and supplemented with 150 mg of long acting P4 on D3 post-ovulation (AI+iP4), or non-AI and supplemented (iP4) or not with iP4 (Control). Two- or three-waves cycles were determined by ultrasound scans between D3 and D21 post-ovulation.
[ii]Main effect of group (G), two- or three- waves cycles (W) and their interactions are indicated.

Table 2. Diameter of dominant follicle (DF) at luteolysis, day of luteolysis and P4 concentrations on day 15 post-ovulation in beef cows supplemented with P4 at early diestrus

Variable	2 waves[i]			3 waves[i]			P value[ii]		
	Control (n=7)	AI+iP4 (n=10)	iP4 (n=11)	Control (n=12)	AI+iP4 (n=12)	iP4 (n=8)	G	W	G*W
DF at lut. (mm)	10.91±0.79	8.99±0.79	11.41±0.63	9.19±0.63	9.61±0.86	9.01±0.74	0.46	0.06	0.14
Luteolysis (days)	16.57±0.58	15.71±0.58	15.73±0.46	17.27±0.46	16.50±0.63	16.13±0.54	0.14	0.17	0.93
P4 conc. D15 (ng/mL)	3.98±0.82	3.43±0.68	1.71±0.65	4.30±0.62	4.22±0.63	1.79±0.77	0.004	0.31	0.83
Number of lut. by D15	2 (28.6%)	3 (30.0%)	6 (54.5%)	1 (8.3%)	2 (16.7%)	4 (50.0%)	.	.	.

Abbreviations: DF at lut.= diameter of dominant follicle (DF) at day of structural luteolysis; P4 conc. D15= plasma progesterone concentrations on D15; Number of lut.= number of cows having structural luteolysis by D15;
[i]Beef cows were submitted to AI 12 h post-estrus and supplemented with 150 mg of long acting P4 on D3 post-ovulation (AI+iP4), or non-AI and supplemented (iP4) or not with iP4 (Control). Two- or three-waves cycles were determined by ultrasound scans between D3 and D21 post-ovulation. Structural luteolysis was detected according to criteria defined in the manuscript part I;
[ii]Main effect of group (G), two- or three- waves cycles (W) and their interactions are indicated.

3.4.4 Plasma P4 concentrations at late diestrus

For two-wave cycles, plasma P4 concentrations on D15 were similar between iP4 group and the non-pregnant cows of the AI+iP4 group, and they were lower than the Control and AI+iP4 pregnant groups. In contrast, for three-wave cycles, the P4 concentrations of AI+iP4 non-pregnant group was sustained at similar concentrations to those of the Control and AI+iP4 pregnant group, and they were greater than the iP4 group (Figure 6; wave by group interaction; P = 0.03). Temporal changes on plasma concentrations of P4 showed that the decrease associated with luteolysis, between D13 and D19 of the experiment, occurred earliest in the iP4 group, latest in the Control group and intermediate for the non-pregnant cows of the AI+iP4 group (Figure 7; day by treatment interaction; P = 0.02). Specifically, on D15, P4 concentrations of iP4 group were lower than Control, whereas concentrations of the AI+iP4 group were intermediate. Regardless of group, on D17 and D19, 71.7% and 90.6% of cows had undergone functional luteolysis. Interestingly, three-wave cycles presented a delayed decrease on plasma P4 concentrations than two-wave cycles (Figure 7; day by wave interaction; P = 0.07). Specifically, on D15 and D17, the two-wave cycles had lower plasma P4 concentrations than three-wave cycles.

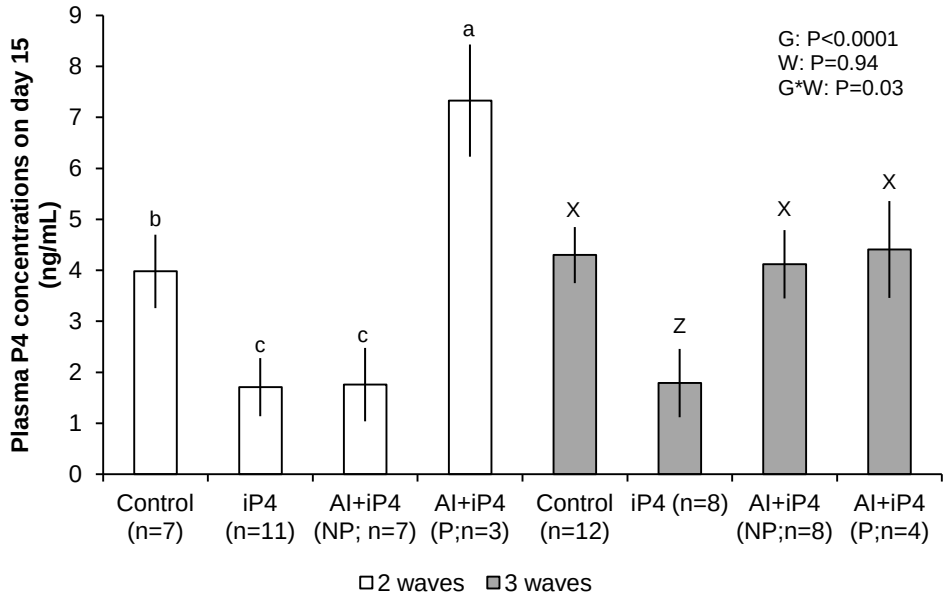

Figure 6. Least squares means ± SEM of plasma progesterone (P4) concentrations (ng/mL) on D15 post-ovulation for beef cows submitted to AI 12 h post-estrus and supplemented with 150 mg of long acting P4 on D3 post-ovulation (AI+iP4) or non-AI and supplemented (iP4) or not with iP4 (Control). The AI+iP4 group was divided in pregnant (P) and non-pregnant (NP) cows according to the pregnancy status on D30 post-ovulation. Two- or three-waves cycles were determined by ultrasound scans between D3 and D21 post-ovulation. Main effect of group (G), two- or three-waves cycles (W) and their interactions are indicated. ^{a,b}Values within a 2 waves according to the treatment was different at 0.01 < P < 0.05. ^{X,Z}Values within a 3 waves according to the treatment was different at P < 0.05.

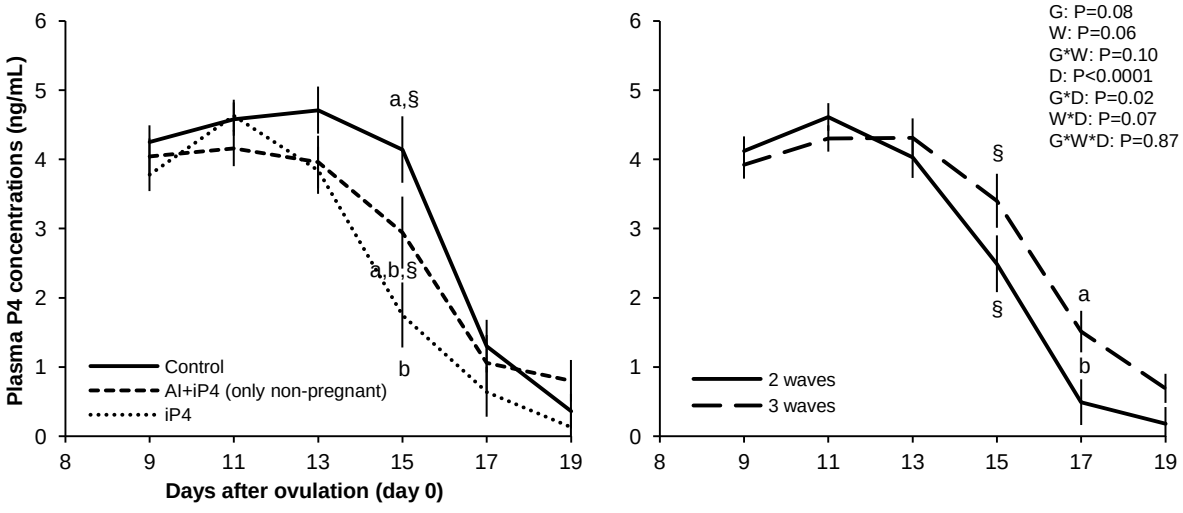

Figure 7. Least squares means ± SEM plasma P4 concentrations (ng/mL) according to the effects of group and number of follicular waves. Beef cows were submitted to AI 12 h post-estrus and supplemented with 150 mg of long acting P4 on D3 (AI+iP4; n= 15), or non-AI and supplemented (iP4; n= 19) or not with iP4 (Control; n=19). Only non-pregnant cows from AI+iP4 group were used in this analysis. Two- or three-waves cycles were determined by ultrasound scans between D3 and D21 post-ovulation. Main effect of group (G), day (D), two- or three- waves cycles (W) and their interactions are indicated. Different letters within a day was different at ^{a,b}P ≤0.05. §Values within a day tended to differ 0.05 < P < 0.10.

3.5 DISCUSSION

Here, we report that early diestrus P4 supplementation alters follicular growth dynamics in association with the incidence of early luteolysis. We showed for the first time that iP4-induced suppression of DF1 growth was associated with early onset of luteolysis. It is possible that concentrations of estradiol during the first follicle wave play a role on programming timing of luteolysis. Unexpectedly, there was no subsequent effect of P4-supplementation on the growth of second or third-waves DF, except for AI cows supplemented with P4, in which growth of DF2 and DF3 was reduced. This supports the notion that there are yet unidentified pregnancy-induced mechanisms that modulate DF growth. Finally, incidence of early luteolysis was reduced in three-wave cows that received AI, suggesting an effect of the developing conceptus to counteract iP4 luteolytic effects.

The reduced DF growth in the first follicular wave in response to iP4 was associated to an earlier onset of luteolysis. Similarly, Burke *et al*. [9] reported a reduction in growth of the DF from the first-wave when an intravaginal P4-releasing device was inserted between days 1 and 5 post-estrus and associated their findings with the occurrence of shortened estrous cycles. The negative feedback effects of elevated circulating P4 on LH secretion from the anterior pituitary [33] was probably the cause of reduced DF growth, since final follicular growth is LH-dependent [34]. The same reduction in LH pulse frequency can also potentially affect early CL development. Indeed, in the Part I manuscript of this series, we showed that CL development was affected negatively by iP4. However, we could not detect an association between CL growth and CL lifespan. Collectively, we propose that there is a major role of DF1 growth to regulate timing of the onset of luteolysis. Possible mechanisms include effects of follicular E2 on programming uterine function. The first-wave DF produces E2 [35], and plasma E2 concentrations increase during the early stages of the estrous cycle [36,37]; iP4-induced impairment of DF growth may have lowered the uterine exposure to E2 at this initial phase. To the best of our knowledge, the role of early estrous cycle exposure to E2 on the duration of estrous cycle is currently unknown and deserves investigation.

The growth pattern and characteristics of the second and third follicular waves were not significantly altered by iP4 and, consequently, they were not associated with

iP4-induced early onset of luteolysis. This observation is important because ovarian follicle growth at mid to late diestrus has been associated with the timing of luteolysis. Indeed, inhibition of follicular growth at mid-cycle by x-ray irradiation [23,24], suppression of FSH secretion [25] or follicle ablation [26], extended the CL lifespan in heifers. These findings support that mid-cycle follicular E2 regulates timing of uterine PGF2□ secretion [23,26] and, thereby, luteolysis. Thus, we anticipated that perhaps iP4-induced early luteolysis could be associated with an increased E2 tone originating from larger or longer-lasting dominant follicles from the second wave. Contrary to our expectations, moment of second wave emergence and duration was not affected by treatments. This suggests that mid-cycle E2 regulation of PGF2□ secretion was not a critical factor for explaining iP4-induced early luteolysis.

In the Part I of this series, the effect of AI+iP4 group on reducing early luteolysis incidence was limited by the embryo's capability to establish pregnancy. Accordingly, temporal changes of P4 concentrations in the non-pregnant subgroup were intermediate between Control and iP4 groups (Figure 7, Panel A). This suggested that the effect of AI+iP4 group was not only related to the pregnant subgroup. Interestingly, this effect was restricted to the three-follicular waves cycles (Figure 6). The AI minimized the iP4-induced early luteolysis in cows having three- vs. two-waves, evidenced by analysis of plasma P4 concentrations on day 15. Perhaps this effect was associated with the delayed decrease in P4 concentrations for three- vs. two-wave cycles (Figure 7, Panel B). It is possible that conceptus exposed to elevated P4 for a longer period of time, such as in three-wave cycles, are better able to initiate maternal recognition responses in the endometrium. In this regard, Ahmad *et al*. [29] verified a greater conception rate in beef cattle with three-wave (96%) versus two-wave (70%). The authors attributed this effect to the longer duration of CL in three-wave cows, which may favor the maternal recognition of pregnancy by the embryo. Collectively, early onset of luteolysis in P4-supplemented cows was similar between cows with two or three-wave cycles, but AI minimized the P4-luteolytic effect in three-wave cows. This implies that fertility response to supplemental P4 may be influenced by the number of follicular waves post-AI.

DF2 growth was reduced in the AI+iP4 group, suggesting an effect of the embryo on follicular growth, possibly to maintain the plasma P4 elevated (Figure 3). In fact, analysis of plasma P4 concentrations between days 9 and 21 and CL development between days 3 and 21 evidenced an effect of AI+iP4 group to maintain

CL function and P4 concentrations from day 17 (Part I of this series). Intriguingly, these data revealed that most of DF2 growth from AI+iP4 group occurred at levels of P4 concentrations comparable to the remaining groups, considering the mean day of emergence in two- (8.52d) and three- (7.56d) wave cycles (Table 1). This excludes a P4-mediated suppression in follicle growth in the AI+iP4 group. Furthermore, this follicle growth suppressive-effect of the early embryo occurred despite of final pregnancy outcome, since DF2 growth was similar between pregnant and non-pregnant cows (Figure 5, Panels B and C). Collectively, these data suggest that not previously-described mechanisms activated by the embryo are probably in place to result in reduced DF2 growth. Our findings are consistent with early studies [38–40] in cattle that described an effect of the gravid uterus to prevent the development of large follicles by inducing follicular atresia. Those authors concluded that inhibition of follicular growth would contribute to the maintenance of luteal function during early pregnancy. In addition, the reduction on DF2 growth may contribute to the observed effect of AI on inhibiting the iP4-induced luteolytic effect in three-wave cycles, as discussed above (Figure 6). In contrast, for two-wave cycles, decreased DF2 growth seemed to be irrelevant or insufficient to affect the iP4-induced luteolytic process. Future studies need to specifically test the embryo effect on growth of second follicular wave and the subsequent impact on timing of luteolysis.

In summary, the iP4 at day 3 post-ovulation reduced DF size from first-wave, which was related to shortened CL lifespan in cows submitted or not to AI. Two- or three-wave pattern of follicular growth were not involved in the incidence of early luteolysis in non-AI cows supplemented with P4. However, the AI decreased the P4-luteolytic effect in three-wave non-pregnant cows, suggesting that number of follicular waves post-AI play a role on P4-suplementation fertility response. Overall, findings of part I demonstrated the iP4 supplementation did not produce a classical short cycle (i.e., CL regression within 2 weeks post-estrus), although it accelerated luteolysis onset by one day. This mild iP4-luteolytic effect was probably a result of advances on uterine luteolytic cascade, rather than caused by failures on luteal development. Apparently, impairment of CL development becomes determining for the iP4-luteolytic effect when early luteal demise occurs at the very early stages of diestrus. Findings of Part II support the concept that follicular growth dynamics and function are involved in iP4-induced early luteolysis, perhaps through regulation of uterine function. Indeed, DF1 growth, and potentially E2 production, was less in cows

showing early luteolysis. A resulting dysregulated sex-steroid pattern (i.e., greater exposure to P4 and decreased exposure to E2) may have altered endometrial programming that regulates onset of luteolysis. Interesting, number of follicular waves during mid- to late diestrus did not affect iP4-luteolytic outcome. However, three-wave cycles were associated positively with the antiluteolytic capacity of the embryo, suggesting that ovarian features during mid- to late diestrus play role changing iP4-induced embryotrophic responses. A collective conclusion from Parts I and II is that ovarian features at early diestrus drive iP4-induced luteolytic response and those at mid- to late diestrus drive the P4-embryotropic responses. The effectiveness of embryo to block iP4-induced early luteolysis is limited by its competence to properly interact with the iP4-modulated uterine environment, elongate, send anti-luteolytic signals and establish pregnancy. Collectively, these results are summarized on Figure 8.

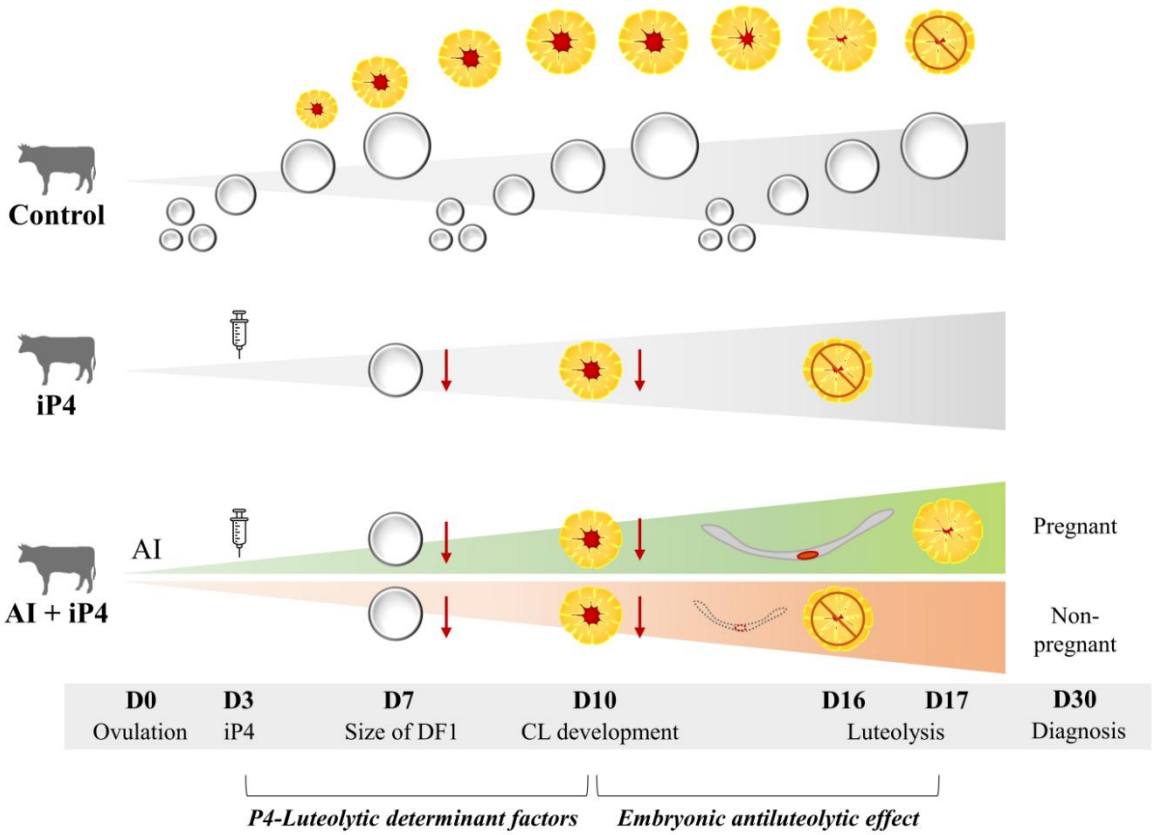

Figure 8. This schematic summarizes the effect of long acting P4 injection (iP4) 3 days after ovulation over the estrous cycle and consequences for early luteal demise in cows submitted or not to AI. iP4 supplementation accelerated luteolysis timing, but it did not produce very short cycle. Average time of luteolysis was shortened by only one day. This mild iP4-luteolytic effect was probably a result of advances on uterine luteolytic cascade, rather than caused by failures on luteal development. One cow of 44 presented very early CL demise (D8), in this single case, probably the P4-luteolytic effect was toward reducing the luteotrophic support to luteal function. A screening of ovarian structures demonstrated that supplementary P4 reduced diameter of first dominant follicular (DF1) and area of CL during their development. No other alterations on pattern of follicular growth were associated to supplementary P4. Further analysis revealed that occurrence of luteolysis before D15 was related to a reduced DF1 growth, but it was not related to reduced CL growth. Two- or three-waves cycles were irrelevant to the P4-luteolytic effect. The embryo role was determined by its inherent ability to properly interact with the iP4-stimulated uterine environment to establish pregnancy. In this interplay, our analysis revealed that three-wave cycles favored the preparation of antiluteolytic response of the embryo, indicating that ovarian features during mid- to late diestrus modulate iP4-induced embryotrophic responses. Overall, ovarian features at early diestrus seem to be critical for iP4-induced luteolytic effects, whereas those at late diestrus influenced the iP4-induced embryotrophic effects.

3.6 REFERENCES

[1] Diskin MG, Sreenan JM. Fertilization and embryonic mortality rates in beef heifers after artificial insemination. J Reprod Fertil 1980;59:463–8. doi:10.1530/jrf.0.0590463.

[2] Diskin MG, Morris DG. Embryonic and Early Foetal Losses in Cattle and Other Ruminants. Reprod Domest Anim 2008;43:260–7. doi:10.1111/j.1439-0531.2008.01171.x.

[3] Mann GE, Lamming GE. The Influence of Progesterone During Early Pregnancy in Cattle. Reprod Domest Anim 1999;34:269–74. doi:10.1111/j.1439-0531.1999.tb01250.x.

[4] Spencer TE, Forde N, Lonergan P. The role of progesterone and conceptus-derived factors in uterine biology during early pregnancy in ruminants. J Dairy Sci 2015;99:1–10. doi:10.3168/jds.2015-10070.

[5] Forde N, Carter F, Fair T, Crowe MA, Evans ACO, Spencer TE, *et al*. Progesterone-Regulated Changes in Endometrial Gene Expression Contribute to Advanced Conceptus Development in Cattle. Biol Reprod 2009;81:784–94. doi:10.1095/biolreprod.108.074336.

[6] Clemente M, De La Fuente J, Fair T, Naib AA, Gutierrez-Adan A, Roche JF, *et al*. Progesterone and conceptus elongation in cattle: A direct effect on the embryo or an indirect effect via the endometrium? Reproduction 2009;138:507–17. doi:10.1530/REP-09-0152.

[7] O'Hara L, Forde N, Carter F, Rizos D, Maillo V, Ealy AD, *et al*. Paradoxical effect of supplementary progesterone between day 3 and day 7 on corpus luteum function and conceptus development in cattle. Reprod Fertil Dev 2014;26:328–36. doi:10.1071/RD12370.

[8] Spencer TE, Johnson GA, Bazer FW, Burghardt RC, Palmarini M. Pregnancy recognition and conceptus implantation in domestic ruminants: Roles of progesterone, interferons and endogenous retroviruses. Reprod Fertil Dev 2007;19:65–78. doi:10.1071/RD06102.

[9] Burke CR, Mihm M, Macmillan KL, Roche JF. Some effects of prematurely elevated concentrations of progesterone on luteal and follicular characteristics during the oestrous cycle in heifers. Anim Reprod Sci 1994;35:27–39. doi:10.1016/0378-4320(94)90004-3.

[10] Pugliesi G, Oliveria ML, Scolari SC, Lopes E, Pinaffi FV, Miagawa BT, *et al*. Corpus luteum development and function after supplementation of long-acting progesterone during the early luteal phase in beef cattle. Reprod Domest Anim 2014;49:85–91. doi:10.1111/rda.12231.

[11] Yan L, Robinson R, Shi Z, Mann G. Efficacy of progesterone supplementation during early pregnancy in cows: A meta-analysis. Theriogenology 2015:1–9. doi:10.1016/j.theriogenology.2015.12.027.

[12] Pugliesi G, Santos FB, Lopes E, Nogueira É, Maio JRG, Binelli M. Improved fertility in suckled beef cows ovulating large follicles or supplemented with long-acting progesterone after timed-AI. Theriogenology 2016;85:1239–48. doi:10.1016/j.theriogenology.2015.12.006.

[13] Martins T, Talamoni JP, Sponchiado M, Maio JRG, Nogueira GP, Pugliesi G, *et al*. Impact of estradiol cypionate prior to TAI and progesterone supplementation at initial diestrus on ovarian and fertility responses in beef cows. Theriogenology 2017;104:156–63. doi:10.1016/j.theriogenology.2017.08.017.

[14] Ginther OJ. Effect of progesterone on length of estrous cycle in cattle. Am J Vet Res 1970;31:493–6.

[15] O'Hara L, Forde N, Duffy P, Randi F, Kelly AK, Valenza A, *et al*. Effect of combined exogenous progesterone with luteotrophic support via equine chorionic gonadotrophin (eCG) on corpus luteum development, circulating progesterone concentrations and embryo development in cattle. Reprod Fertil Dev 2014:269–77. doi:10.1071/RD14019.

[16] Garrett JE, Geisert RD, Zavy MT, Gries LK, Wettemann RP, Buchanan DS. Effect of exogenous progesterone on prostaglandin F2α release and the interestrous interval in the bovine. Prostaglandins 1988;36:85–96.

[17] Binelli M, Thatcher WW, Mattos R, Baruselli PS. Antiluteolytic strategies to improve fertility in cattle. Theriogenology 2001;56:1451–63. doi:10.1016/S0093-691X(01)00646-X.

[18] Sponchiado M, Gomes NS, Fontes PK, Martins T, del Collado M, Pastore A de A, *et al*. Pre-hatching embryo-dependent and -independent programming of endometrial function in cattle. PLoS One 2017;12:e0175954. doi:10.1371/journal.pone.0175954.

[19] Forde N, Carter F, Spencer TE, Bazer FW, Sandra O, Mansouri-Attia N, *et al*. Conceptus-induced changes in the endometrial transcriptome: how soon does

the cow know she is pregnant? Biol Reprod 2011;85:144–56. doi:10.1095/biolreprod.110.090019.

[20] Forde N, Duffy GB, McGettigan PA, Browne JA, Mehta JP, Kelly AK, *et al*. Evidence for an early endometrial response to pregnancy in cattle: both dependent upon and independent of interferon tau. Physiol Genomics 2012;44:799–810. doi:10.1152/physiolgenomics.00067.2012.

[21] Silvia WJ, Lewis GS, Mccracken JA, Thatcher WW, Wilson L. Hormonal Regulation of Uterine Secretion of Prostaglandin F2α during Luteolysis in Ruminants1. Biol Reprod 1991;45:655–63. doi:10.1095/biolreprod45.5.655.

[22] Weems CW, Weems YS, Randel RD. Prostaglandins and reproduction in female farm animals. Vet J 2006;171:206–28. doi:10.1016/j.tvjl.2004.11.014.

[23] Fogwell RL, Cowley JL, Wortman JA, Ames NK, Ireland JJ. Luteal function in cows following destruction of ovarian follicles at midcycle. Theriogenology 1985;23:389–98. doi:10.1016/0093-691X(85)90041-X.

[24] Villa-Godoy A, Ireland JJ, Wortman JA, Ames NK, Hughes TL, Fogwell RL. Effect of ovarian follicles on luteal regression in heifers. J Anim Sci 1985;60:519–27.

[25] Garverick A, Salfen BE, Cresswell JR, Xu ZZ, Bao B, Garverick HA. Effects of the presence of a dominant follicle and exogenous oestradiol on the duration of the luteal phase of the bovine oestrous cycle. J Reprod Fertil 1999;115:15–21.

[26] Araujo RR, Ginther OJ, Ferreira JC, Palhão MM, Beg MA, Wiltbank MC. Role of follicular estradiol-17beta in timing of luteolysis in heifers. Biol Reprod 2009;81:426–37. doi:10.1095/biolreprod.108.073825.

[27] Ginther OJ, Knopf L, Kastelic JP. Temporal associations among ovarian events in cattle during oestrous cycles with two and three follicular waves. J Reprod Fertil 1989;87:223–30. doi:10.1530/jrf.0.0870223.

[28] Figueiredo RA, Barros CM, Pinheiro OL, Soler JMP. Ovarian follicular dynamics in nelore breed (*Bos indicus*) cattle. Theriogenology 1997;47:1489–505. doi:https://doi.org/10.1016/S0093-691X(97)00156-8.

[29] Ahmad N, Townsend EC, Dailey RA, Inskeep EK. Relationships of hormonal patterns and fertility to occurrence of two or three waves of ovarian follicles, before and after breeding, in beef cows and heifers. Anim Reprod Sci 1997;49:13–28. doi:https://doi.org/10.1016/S0378-4320(97)00057-2.

[30] Pugliesi G, Miagawa BT, Paiva YN, Franca MR, Silva LA, Binelli M. Conceptus-

Induced Changes in the Gene Expression of Blood Immune Cells and the Ultrasound-Accessed Luteal Function in Beef Cattle: How Early Can We Detect Pregnancy? Biol Reprod 2014;91:95–95. doi:10.1095/biolreprod.114.121525.

[31] Savio JD, Keenan L, Boland MP, Roche JF. Pattern of growth of dominant follicles during the oestrous cycle of heifers. J Reprod Fertil 1988;83:663–71. doi:10.1530/jrf.0.0830663.

[32] Sirois J, Fortune JE. Ovarian Follicular Dynamics during the Estrous Cycle in Heifers Monitored by Real-Time U Itrasonography. Biol Reprod 1988;39:308–3. doi:10.1095/biolreprod39.2.308.

[33] Kojima FN, Bergfeld EGM, Wehrman ME, Cupp AS, Fike KE, Mariscal-Aguayo DV, *et al*. Frequency of luteinizing hormone pulses in cattle influences duration of persistence of dominant ovarian follicles, follicular fluid concentrations of steroids, and activity of insulin-like growth factor binding proteins. Anim Reprod Sci 2003;77:187–211. doi:10.1016/S0378-4320(03)00038-1.

[34] Ginther OJ, Wiltbank MC, Fricke PM, Gibbons JR, Kot K. Minireview Selection of the Dominant Follicle in Cattle. Biol Reprod 1996;1194:1187–94.

[35] Badinga L, Driancourt MA, Savio JD, Wolfenson D, Drost M, de la Sota RL, *et al*. Endocrine and ovarian responses associated with the first-wave dominant follicle in cattle. Biol Reprod 1992;47:871–83. doi:10.1095/biolreprod47.5.871.

[36] Savio JD, Thatcher WW, Badinga L, de la Sota RL, Wolfenson D. Regulation of dominant follicle turnover during the oestrous cycle in cows. J Reprod Fertil 1993;97:197–203.

[37] Kulick LJ, Kot K, Wiltbank MC, Ginther OJ. Follicular and hormonal dynamics during the first follicular wave in heifers. Theriogenology 1999;52:913–21. doi:https://doi.org/10.1016/S0093-691X(99)00182-X.

[38] Driancourt MA, Thatcher WW, Terqui M, Andrieu D. Dynamics of ovarian follicular development in cattle during the early estrous cycle, early pregnancy and in response to PMSG. Dom Anim Endocr 1991;8:209–21.

[39] Thatcher WW, Driancourt MA, Terqui M, Badinga L, Thatcher WW, Terqui M, *et al*. Dynamics of ovarian follicular development in cattle following hysterectomy and during early pregnancy. Domest Anim Endocrinol 1991;8:223–34. doi:https://doi.org/10.1016/0739-7240(91)90058-R.

[40] Guilbault LA, Dufour JJ, Thatcher WW, Drost M, Haibel GK. Ovarian follicular development during early pregnancy in cattle. J Reprod Fertil 1987;78:127–35.

CHAPTER 4

EXACERBATED CONCEPTUS SIGNALLING DO NOT FAVOUR ESTABLISHMENT OF PREGNANCY IN BEEF CATTLE

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4 EXACERBATED CONCEPTUS SIGNALLING DO NOT FAVOUR ESTABLISHMENT OF PREGNANCY IN BEEF CATTLE

4.1 ABSTRACT

Inadequate, insufficient production of anti-luteolytic signals by the pre-attachment embryo is considered a major cause of pregnancy failure in cattle. We tested the hypothesis that multiple-embryo transfers (n=5/recipient) and progesterone supplementation amplify anti-luteolytic signaling and reduce embryonic losses in beef cattle. Cows detected in estrus (D0; n=104) were assigned randomly to receive 150 mg of injectable long-acting P4 (iP4) or vehicle (non-iP4) on D4 and transcervical transfer of none or 5 embryos produced *in vitro*, on D7. Luteal development and time of structural luteolysis was monitored by ultrasound scans. Plasma P4 concentrations were determined on D4, D5 and D7, and daily between D14 and D20. Embryo signaling was monitored by abundance of interferon-stimulated gene 15 (*ISG15)* in peripheral blood mononuclear cells isolated on D14, D16, D18 and D20. Early embryonic mortality (EEM) was defined as the absence of *ISG15* mRNA upregulation over time and/or luteal regression up to D20. Late embryonic mortality (LEM) was defined as the absence of embryonic vesicles with a heartbeat on pregnancy diagnosis at D30 (PD) after observing upregulation of *ISG15* mRNA and extension of luteal lifespan. Cows with viable embryonic vesicle(s) were considered pregnant. On D5, iP4-treated cows had P4 concentrations 2.07-fold greater than non-iP4 treated (P<0.001). On D7, P4 concentrations were similar. Pregnant and LEM animals had a progressive increase on the abundance of *ISG15* from D14 to D20. iP4-treated cows detected pregnant at PD had 1.53-fold greater abundance of *ISG15* mRNA between D14 and D20 than non-iP4 treated cows (P= 0.05). iP4 treatment did not shorten CL lifespan, but hindered CL development. iP4 doubled the frequency of EEM while it did not affect LEM. At PD, embryonic survival was 37.0% vs. 55.6% for iP4-treated vs. control cows. A substantial proportion of cows presented EEM (31%) and LEM (20%) after transferring 5 embryos produced *in vitro*. This argues that mortality is due

to poor uterine receptivity, pre-established at the time of transfer and could not be reversed by supplementary P4. Further, potentiating of embryonic signaling through supplementing P4 and transfer multiple embryos cannot overcome such deficits.

4.2 INTRODUCTION

Embryonic mortality during the first three weeks post-insemination accounts for up to two-thirds of overall pregnancy losses in cattle [1–3]. Explanation for this massive loss is related to a functional incompetence of the uterus to support embryonic development, embryonic incompetence or both [4]. A clear distinction among these possibilities has proven to be both a scientific and practical challenge.

In cattle, the uterine unit plays a central role on driving embryonic growth and elongation at early stages of pregnancy [5–7]. Accordingly, between days 4 and 16 after estrus, uterine luminal fluid (histotroph) secreted by the endometrial epithelia drives growth and elongation of pre-attachment embryos [5,6]. Subsequently, the uterus must be able to respond to the elongated conceptus-initiated interferon-tau (IFNT) signaling [8] by not activating the PGF2α synthesis cascade which will prevent luteal regression. Thus, manipulations that aim to stimulate uterine functions to support embryo elongation are expected to increase gestational success. For example, a more receptive-uterine status can be generated through increasing circulating progesterone (P4) concentrations during early diestrus. Indeed, increased concentrations of P4 stimulate endometrial secretions [9], accelerate conceptus elongation and subsequent release of IFNT into the uterine lumen [10–12] in cattle. Despite such clear positive effects of P4 on establishment of pregnancy, the reported fertility outcome to early diestrus P4 supplementation is variable [13,14]. Inconsistent fertility results are associated with the incidence of early luteolysis (i.e., before D16), which impairs the proper cross-talk between the maternal unit and the conceptus during the establishment of pregnancy. Early luteolysis is related to the P4–driven impairment of luteal formation [15,16] and/or the advancement of endometrial PGF2α release [17]. Alternatively, variable fertility results could be consequence of asynchrony between the stimulus required by the developing embryo and provided by the uterine environment [18]. Lack of competence of some embryos to timely

respond to the P4-induced functional changes that occur in the uterus during diestrus may account for a proportion of pregnancy failures due to supplementary P4.

The competence of an individual embryo to develop, elongate and signal is intrinsically confounded with the ability of the uterus to support embryonic development. Thus, multiple embryo transfer (MET) has been used as an experimental model to sort embryo vs. uterine effects [19,20]. For example, by transferring 3 to 8 high grade bovine embryos produced *in vitro* to recipients, Berg et al. [20] estimated that the fraction of pregnancy failures attributed to embryonic incompetence is expected to be minimum, and this would allow an estimation of the fraction of losses associated with the uterine component. However, embryonic responses to a given uterine environment are variable. For example, Betteridge et al. [21] observed striking differences in the size of embryos recovered from superovulated donors on D14 (0.226–57 mm) and on D16 (0.232–150 mm) and this was also true for an individual donor (e.g., there was a range of 4–40 mm from one Day 14 donor). Similarly, Garret et al. [17] verified that conceptuses recovered after natural mating from Day 14 uteri of P4-treated cows (37.3 ± 14.9 mm) were longer than those recovered from controls (3.8 ± 1.9 mm); however, they noticed a large variability within treatments (control: 1–13 mm; P4 treated: 3–119 mm). Furthermore, Clemente et al. transferred 20 embryos produced *in vitro* to D7 post-estrus heifers and verified that supplementation with P4 increased both the mean length and the variability of length of conceptus recovered on D14, compared to non-supplemented recipients [12]. Similar findings were reported for non P4 supplemented cattle [20] and in sheep [22]. Collectively, these data suggest that embryonic competence to establish a gestation is variable.

Rationale for the present study was to (1) stimulate uterine functions to support embryo elongation and survival, by treating recipients with supplemental P4, (2) decrease the random chance of an incompetent conceptus to fail development by transferring multiple embryos and (3) increase potency of embryonic signaling by the additive secretory capacity of multiple embryos. Combination of these ingredients was expected to increase the intensity of conceptus signaling (i.e., production of IFNT) and pregnancy success. Hypothesis was that P4 supplementation at early diestrus and MET reduce early (i.e. between days 8 and 20) and late (i.e. after day 20) embryonic mortality. Embryonic survival and signaling potency around the maternal recognition period were monitored through transcript abundance of

interferon-stimulated gene 15-kDa protein (*ISG15*) in peripheral blood mononuclear cells (PBMCs). A side-effect of P4 supplementation at early diestrus is an increased proportion of cows that undergo early luteolysis. A second hypothesis tested in the present report was that stimulating embryo signaling (by P4 supplementation and MET) reduces the incidence of early luteolysis.

4.3 MATERIALS AND METHODS

4.3.1 Animals

Cycling, non-lactating Nelore cows (*Bos taurus indicus*; n = 50; 569.9 ± 10.1 kg and 6.1 ± 0.3 years old) were used in three replicates performed in the summer of 2016-2017 in the Southern hemisphere at Fernando Costa Campus of the University of São Paulo (Pirassununga, São Paulo, Brazil). Experimental animals had no apparent abnormalities in the reproductive tract and had a CL in at least one of the weekly ultrasound scans. Animals were kept under grazing conditions and supplemented with corn silage, concentrate and minerals to fulfill their maintenance requirements, and water *ad libitum*. Handlings were done in accordance with the Ethics and Animal Handling Committee of the School of Veterinary Medicine and Animal Science of the University of São Paulo (CEUA-FMVZ/USP, n° 4664220316).

4.3.2 Experimental designs

Estrous cycles were synchronized using an P4-releasing intravaginal device for 8 days (1.0 g; Sincrogest®, Ourofino Saúde Animal, Cravinhos, SP, Brazil), along with an intramuscular (i.m.) injection of estradiol benzoate (2.0 mg; Sincrodiol®, Ourofino Saúde Animal), followed by an i.m. administration of PGF2α analogue (500 μg of sodium cloprostenol; Sincrocio®, Ourofino Saúde Animal) given on the day before P4-device removal. Cows were checked for signs of estrus twice a day between 36 and 96h after P4-releasing device withdrawal with aid of heat detector

patches (Estrotect™; Western Point Inc., Apple Valley, MN). Cows observed in standing estrus and/or presenting an activated heat detector patch were considered in estrus (D0 of study; n = 104). Animals were assigned randomly to one of the four treatment combinations on a two by two factorial arrangement of the following treatments: vehicle or supplementation with 150 mg of long-acting P4 (iP4, 1.0 mL, i.m., Sincrogest®, Ourofino Saúde Animal) on D4 and transfer of none (0-ET) or 5 *in vitro*-derived embryos on D7 (ET). Thus, the experimental groups were: Non-iP4+0-ET (n= 24); iP4+0-ET (n= 26); Non-iP4+5-ET (n= 27) and iP4+5-ET (n= 27). Usage of this dosage of iP4 on this selected day have produced both embryotropic and luteolytic stimulus in previous studies [23–25]. We used five embryos in our MET model because based on a previous report, a greater number of embryos could potentially reduce the recovery rates and conceptus growth [20].

Blood samples were collected via jugular venipuncture into 9 mL heparinized evacuated tubes and placed on ice until centrifugation on D4, D5 and D7 post estrus and daily between D14 and D20 to determine plasma P4 concentrations. Additional blood samples (18 mL) were collected on D14, D16, D18 and D20 for determination of *ISG15* expression in PBMCs. Samples for PBMC isolation were kept at controlled 24 °C until processing on the same day.

4.3.3 Ultrasound scanning

Ultrasound scanning was performed by transrectal B-mode ultrasonography (Mindray M5 equipped with multifrequency linear transducer set to 7.5 MHz) starting when estrus was detected and every 12 h to record the diameter of the dominant follicle and to confirm ovulation. To evaluate the CL development and regression, the ovaries were scanned between D4 and D20 via transrectal B-mode and pulse-wave color Doppler ultrasonography. Total CL area and CL area containing color signals of luteal blood flow (CLBF) were also measured. For determination of maximum CL area, the images were recorded in B-mode as film clips and measured a posteriori using the tracing function. In CLs having an anechoic fluid-filled cavity, the area of the cavity was subtracted from the total area [26]. Percentage of CL area with CLBF were estimated visually during CL scanning using the same criteria as described

previously [25,27]. Accordingly, a scale from 0 to 100%, with 5 interval points was used for determination of the percentage of the luteal area with CLBF. All scans were performed at a constant color-gain setting (6.5 MHz, Gain: 62 and Pulse Repeated Frequency: 5.3 kHz) and a velocity setting of 5.4 cm/s. Structural luteolysis was defined as the day between D11 and D20 when the maximum CL area (cm^2) and the luteal blood flow decreased by 25% and 50%, respectively, from the respective mean values recorded on days 10 and 11, based on previous reports (Pugliesi et al., 2014a, 2014b).

On D30, pregnancy status was checked through the detection of viable conceptus (presence of heartbeat) by transrectal B-mode ultrasonography. Numbers of viable embryonic vesicles were counted during the pregnancy diagnosis.

4.3.4 Embryo transfer procedure

In vitro derived embryos were obtained from a commercial supplier. Cumulus oocyte complexes used to produce the embryos were aspirated from ovaries collected in a local slaughterhouse. Embryos were produced according to a standard protocol for *in vitro* embryo production [28]. Five D7 freshly produced embryos (i.e., not frozen) were placed in straws containing holding medium and transported to our laboratory at 37 °C.

On D7, recipient cows received a caudal epidural anesthesia (2% lidocaine solution; Lidovet®, Bravet, Engenho Novo, RJ, Brazil) immediately before the embryo transfer (ET) procedure. Five *in vitro* produced grade 1 blastocysts were placed transcervically in the middle to the cranial third of the uterine horn ipsilateral to the ovary containing the CL, using standard non-surgical technique. For the 0-ET groups, all the procedures were performed but only holding medium was deposited in the uterine lumen.

4.3.5 Progesterone concentration measurements

Plasma was collected from blood samples by centrifugation at 2700 g for 15 min at 4°C. Progesterone was assayed by solid-phase radioimmunoassay (Immuchem™ Double Antibody Progesterone Kit; Cat. 07-170105, MP Biomedicals, NY, USA). The sensitivity of the assay was 0.5 ng/mL. The intra-assay coefficient of variation (CV) for quality control samples was 0.3% (low) and 3.08% (high). The inter-assay CV were 2.48% (low) and 14.24% (high).

Plasma P4 concentration profiles between D4 and D7 were used to evaluate the effect of iP4 supplementation on circulating P4 levels, and between D14 and D20 to determine the day of functional luteolysis. Functional luteolysis was defined as the day when P4 concentrations decreased > 2 ng/ml between daily samples and that was followed by a progressive decrease in concentrations to < 1 ng/ml [25].

4.3.6 Isolation of PBMCs, total RNA extraction and cDNA synthesis

PBMCs were isolated by Ficoll gradient as described by Pugliesi et al. [27]. Briefly, whole blood was mixed with an equal volume of PBS, and the solution was layered onto 15 ml of Ficoll-Paque solution (GE Healthcare), placed in a 50-ml conical tube, and then centrifuged at 1100 X g for 30 min to obtain the PBMC layer. Mononuclear cells were collected and centrifuged with PBS at 900 X g for 15 min and the contaminating red blood cells were lysed in hypertonic solution for approximately 10 min. Isotonicity was restored by suspending the cells in PBS and centrifuging at 900 X g for 15 min. The resulting pellet was suspended in PBS and the final solution was transferred to a 1.5-ml conical tube. The isolated PBMCs were centrifuged at 3300 X g for 2 min, the supernatant was removed, and the pellet containing PBMCs was stored in a 1.5-ml conical tube at –80 °C until RNA extraction.

Total RNA extraction of isolated PBMCs was performed using 1 mL of Trizol™ reagent (Invitrogen, Carlsbad, CA, USA) in accordance to manufacturer's guidelines. Total RNA extracted was eluted with 40 µL of RNase free water. Concentration and purity of total RNA in extracts were evaluated using spectrophotometry (NanoVueTM

Plus Spectrophotometer, GE Healthcare, UK) by the absorbance at 260 nm and the 260/280 nm ratio, respectively. Absorbance ratios values ranged between 1.7 and 1.9.

Before the reverse transcription, the isolated RNA samples were treated with DNase I (deoxyribonuclease I, Amplification Grade; Invitrogen, Carlsbad, CA, USA) for genomic DNA contamination as per manufacturer's instructions. Briefly, the treatment with DNase was done at room temperature using 1 μg of total RNA in a 10-μL reaction volume. After 15 min of incubation at room temperature, 1 μL of EDTA (25mM) was added to stop the enzyme activity and samples were warmed to 65 °C for 10 min. Synthesis of cDNA was performed using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies Corporation, Frederick, MD, USA). A master mix (9 μL) containing random primers, reverse transcriptase enzymes and deoxynucleotides were added to 11 μL of the treated samples. Samples were incubated at 25 °C for 10 min and then at 37 °C for 2 h, subjected to reverse transcriptase inactivation at 85 °C for 5 min, and stored at –20 °C until PCR analysis.

4.3.7 Real Time PCR

Analyses of transcript relative abundance were performed using Power SYBR Green PCR Master Mix (Life Technologies) for the amplification reactions in a Step One Plus thermocycler (Applied Biosystems Real-Time PCR System; Life Technologies). The primers used for qPCR were obtained from our previous report [29] as follows: 5'-AGAGAGCCTGGCACCAGAAC-3', forward, and 3'-TTCTGGGCGATGAACTGCTT-5', reverse, for *ISG15* (NM_174366.1); and 5'-GCCATGGAGCGCTTTGG-3', forward, and 3'-CCACAGTCAGCAATGGTGATCT-5', reverse, for *PPIA* (Cyclophilin A; NM_178320.2). Reactions were run in triplicate on 96-well plates sealed with a MicroAmp optical adhesive cover (Life Technologies) using the same qPCR settings as described previously [29]. The raw fluorescence data was extracted from the Step One Plus software with no baseline correction and analyzed using the LinReg PCR software (www.hartfaalcentrum.nl/index.php?main=files&sub=LinRegPCR) for baseline correction and cycle thresholds (Cts) determination, as described by Ruijter et al.

[30]. For the latter, the log-linear portion of the amplification curve containing four to six points with the highest R^2 value was considered. Relative transcripts abundance was obtained after normalization of *ISG15* Cts by the endogenous control *PPIA* Ct values, using the equation described by Pfaffl et al. [31].

4.3.8 Criteria for determining embryo survival or mortality

Cows that received five embryos on D7 (5-ET groups) were classified retrospectively as experiencing early embryonic mortality (EEM), late embryonic mortality (LEM) or confirmed pregnant on the day of pregnancy diagnosis (i.e., D30). Cows were included in the EEM group when there was no evidence of embryo presence up to D16 according to absence of *ISG15* mRNA increase between D14 and D20 and/or incidence of luteolysis before D20. Conversely, cows classified as LEM presented a clear increase on *ISG15* abundance, functional CL on D30, but no evidence of viable embryo on D30. Confirmed pregnant cows and those that received no embryos (0-ET or cyclic group) were used as positive and negative controls, respectively, for comparisons of *ISG15* expression among EEM and LEM groups.

4.3.9 Statistical analyses

Data from CL area, CL blood flow, P4 concentrations and *ISG15* expression was analyzed by split-plot ANOVA using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC, USA) version 9.3. Model included fixed effects of iP4 supplementation, ET treatment, day and their interactions for analysis of CL characteristics and P4 concentrations. For analysis of *ISG15* mRNA relative abundance, the model included fixed effects of iP4 supplementation, group (EEM, LEM, pregnant and cyclic cows), day and their interactions. Random effect of cows nested within treatment combinations was used as error term. The type of variance–covariance structure used was chosen based on smaller magnitude of the corrected Akaike's information criterion (AICC). Kenward-Rogers degrees of freedom

approximation option of SAS was used to determine the denominator degrees of freedom for tests of fixed effects. The residual and influencing diagnostics outputs from the MIXED procedure were checked for the assumption of normality of the data. Data that was not normally distributed was transformed by the natural logarithmic before analyses. Significance of effects was determined by F-test using Type III sums of squares. When treatment by day interactions were significant, the slice command was incorporated into the procedure to determine in which days the treatment effect occurred. When necessary, the DIFF command incorporating the Tukey test correction was applied to evaluate pairwise comparisons between treatment means.

Secondary analyses were performed using only cows submitted to 5-ET aiming to assess the difference in CL features between D4 and D14 and P4 concentrations according to pregnancy status (group). Model included the fixed effects of iP4 supplementation, group, day, and their interactions. To assess the effect of iP4 supplementation on *ISG15* relative abundance, an additional analysis was run considering only cows diagnosed as pregnant. Model included the fixed effects of iP4 supplementation, day, and their interaction.

Variables days to structural/functional luteolysis and day from estrus to P4 < 1.0 ng/mL were analyzed with MIXED procedure considering fixed effects of iP4 supplementation, ET treatment and their interaction. A new variable was generated by considering the mean of P4 concentrations and *ISG15* expression over time to assess effects according to pregnancy status. Model included the fixed effect of group, iP4 supplementation and their interaction. Finally, the effect of iP4 supplementation on proportion of pregnant cows was assessed by the chi-squared test, using the FREQ procedure of SAS. In all instances, a probability of $P \leq 0.05$ indicates a significant difference and a probability of $0.05 > P \leq 0.10$ indicates an approached significance.

4.4 RESULTS

4.4.1 Animals and groups

The mean ± SEM of the variables size of pre-ovulatory follicle (13.8 ± 0.16 mm) and time from estrus detection to ovulation (25.06 ± 0.61 h) were similar among groups (P > 0.10), as expected.

4.4.2 Effect of P4 supplementation and embryo transfer on ovarian and endocrines variables

Circulating P4 concentrations between D4 and D7 showed significant day by iP4 treatment interaction (Figure 1). The interaction effect was a consequence of an expected, acute increase (P < 0.0001) on average plasma P4 concentration on D5 in iP4 (5.16 ± 0.35 ng/mL) versus non-iP4 (2.49 ± 0.35 ng/mL) supplemented cows. On D7, plasma P4 concentrations between these two groups were similar.

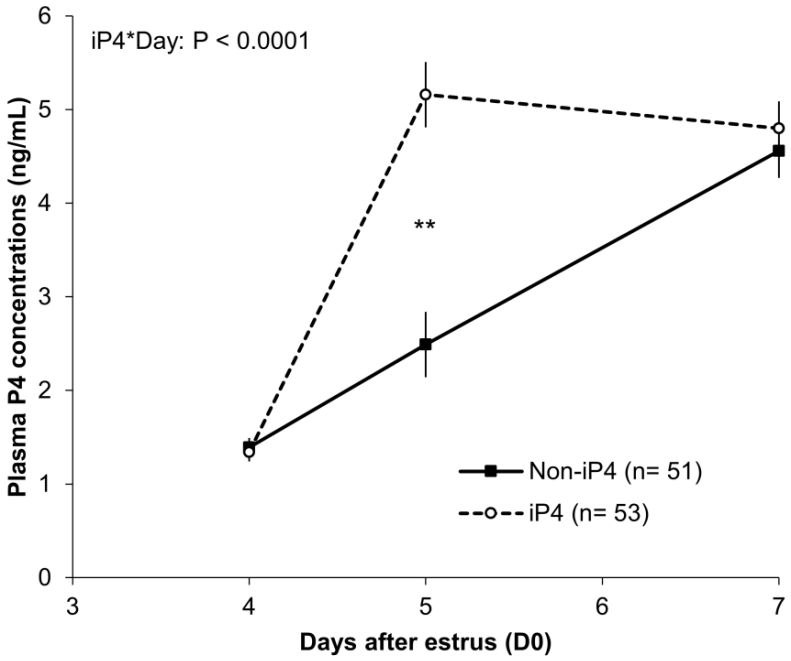

Figure 1. Effect iP4 supplementation on plasma progesterone concentrations. Beef cows detected in estrus (D0) were assigned randomly to receive a single injection of vehicle (Non-iP4) or 150 mg of long acting progesterone (iP4) on D4 and transfer of none or 5 *in vitro* derived embryos on D7. Data were analyzed by split-plot ANOVA and only the significant effect was reported. Data are represented as Least squares means ± SEM. Difference within a day is indicated by asterisks (**P < 0.0001).

The advanced increase on P4 concentrations experienced by the iP4 group affected CL development during diestrus (Figure 2). A day by iP4 treatment interaction indicated an effect of supplementary P4 on the expected time of CL development (D4 to D11) and regression (D14 to D20). The CL area of iP4 treated cows was smaller between D9 and D11 and between D19 and D20, compared to non-iP4 animals. There was no effect of iP4 on the proportion of luteal area with CLBF nor the concentrations of P4 between D14 and D20 ($P > 0.10$).

Regardless of iP4 supplementation, there was a day by ET treatment interaction effect on CL area (Figure 2), proportion of luteal area with CLBF and concentrations of P4 between D14 and D20 (Figure 3). Starting on day 18, the average of all these variables was greater in the 5-ET groups than 0-ET groups. The interaction reflected a positive effect of embryo on the maintenance of CL functionality in cows submitted to MET.

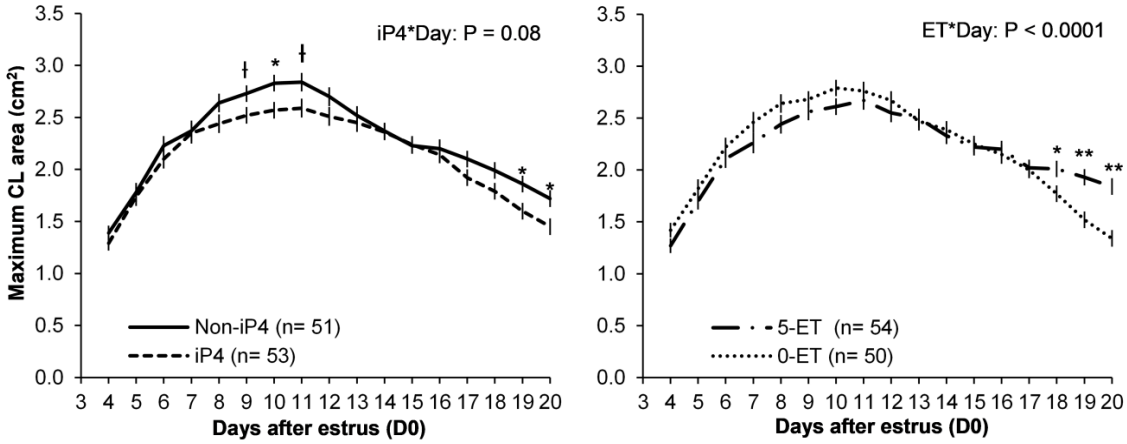

Figure 2. Effect of iP4 supplementation and embryo transfer on the corpus luteum (CL) along diestrus. Beef cows detected in estrus (D0) were assigned randomly to receive a single injection of vehicle (Non-iP4) or 150 mg of long acting progesterone (iP4) on D4 and transfer of none (0-ET) or 5 in vitro-derived-embryos (5-ET) on D7. Maximum area of CL was used for evaluation of CL development and regression. Data were analyzed by split-plot ANOVA and only the significant effects were reported. Data are represented as Least squares means ± SEM. Differences within a day are indicated by asterisks (* $P < 0.05$ and ** $P < 0.001$) or cross (ǂ $P < 0.10$).

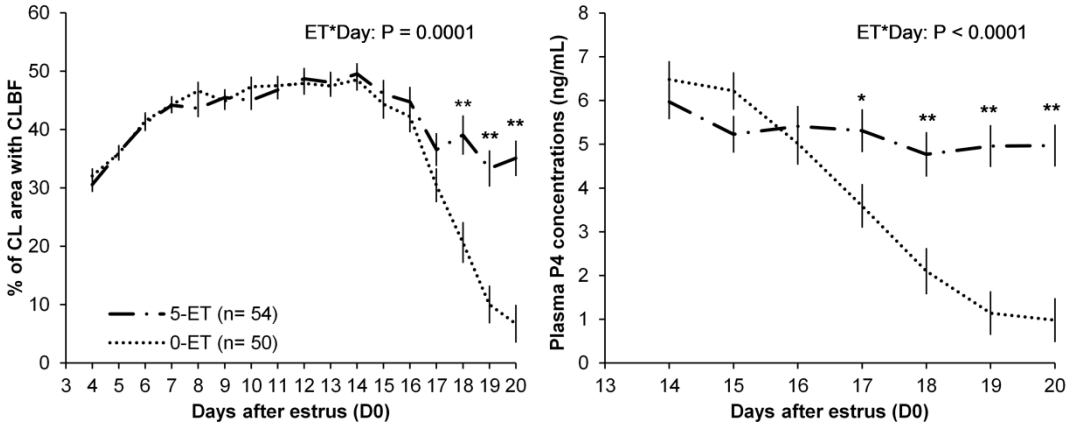

Figure 3. Effect of embryo transfer on maintenance of corpus luteum (CL) functionality. Beef cows detected in estrus (D0) were assigned randomly to receive a single injection of vehicle (Non-iP4) or 150 mg of long acting progesterone (iP4) on D4 and transfer of none (0-ET) or 5 in vitro-derived-embryos (5-ET) on D7. Assessment of proportion of CL area containing color signals of luteal blood flow (CLBF) and measurement of plasma P4 concentrations were used for determination of time of luteolysis. Data were analyzed by split-plot ANOVA and only the significant effects were reported. Both variables were affected by ET factor during CL regression. Data are represented as Least squares means ± SEM. Differences within a day are indicated by asterisks (* $P < 0.05$ and ** $P < 0.0001$).

The functional characteristics of CLs and P4 concentration profile between D14 to D20 were used to calculate the time of structural and functional luteolysis, respectively (Table 1). Intriguingly, both criteria indicated an approached or significant effect of ET treatment on time of luteolysis, regardless of iP4 supplementation. Time of structural luteolysis in 5-ET cows (16.95 ± 0.39 d) tended to be shorter than 0-ET cows (17.75 ± 0.24 d). Time of functional luteolysis (5-ET: 16.30 ± 0.35 vs. 0-ET: 17.03 ± 0.21 d) and between estrus to P4 < 1.0 ng/mL (5-ET: 16.87 ± 0.39 vs. 0-ET: 17.81 ± 0.23 d) indicated a similar effect. Controversially, iP4 supplementation did not shorten luteal lifespan, despite the impairment of the corpus luteum formation.

Table 1 Effect of iP4 supplementation and/or embryo transfers on the CL regression in beef cows.

Variable	Non-iP4		iP4		P value		
	None-ET (n = 24)	5-ET (n = 27)	None-ET (n = 26)	5-ET (n = 27)	iP4	ET	iP4*ET
Number of cows having functional CL on D20[1]	4 (16.7%)	21 (77.8%)	2 (7.7%)	16 (59.3%)	.	.	.
Estrus to structural lut. (days)[2]	17.58 ± 0.35	17.00 ± 0.63	17.92 ± 0.31	16.91 ± 0.46	0.79	0.09	0.64
Estrus to functional lut. (days)[3]	17.11 ± 0.31	16.50 ± 0.56	16.96 ± 0.28	16.09 ± 0.41	0.50	0.08	0.75
Estrus to P4 < 1.0 ng/mL (days)[4]	17.79 ± 0.35	16.83 ± 0.62	17.83 ± 0.31	16.91 ± 0.46	0.90	0.04	0.97
Number of pregnant cows on D30[5]	.	15 (55.6%)	.	10 (37.0%)	0.16	.	.
Number of non-pregnant cows having functional CL on D30[6]	.	6 (22.2%)	.	5 (18.5%)	0.74	.	.

Abbreviation: lut. = Luteolysis

Beef cows detected in estrus (D0) were assigned randomly to receive a single injection of vehicle (Non-iP4) or 150 mg of long acting progesterone (iP4) on D4 and transfer of none or 5 *in vitro* derived embryos on D7;

[1]Cows that did not present any of characteristics described in the items 2, 3 and 4;

[2]Structural luteolysis was defined as the day between D11 and D21 when the maximum CL area (cm^2) and the luteal blood flow decreased by 25% and 50%, respectively, from mean of D10 and D11;

[3]Functional luteolysis was defined as occurring at the sample when plasma P4 concentrations decreased > 2 ng/mL between samples collected from D14 to D20, and was followed by a progressive decrease in plasma P4 concentrations to < 1 ng/mL;

[4]Day when plasma P4 concentrations first reaches concentrations < 1.0 ng/mL;

[5,6]On D30, pregnancy status of those cows that had a functional CL on day 20 were checked through the detection of embryonic vesicle viable (presence of heartbeat) by transrectal B-mode ultrasonography. In non-pregnant cows, the functionality of the CL from day 20 was checked according to criteria established for detection of structural luteolysis.

4.4.3 Effect of P4 supplementation on variables related to pregnancy and on *ISG15* relative abundance

On D20, 68.5% of 5-ET cows (n = 37/54) had a functional CL (Table 1). On D30, the presence of at least one embryo with heartbeat was confirmed by ultrasound examination in 67.6% of cows with a functional CL (n = 25/37), whereas in those in which a viable embryo was not detected, the CL remained functional on D30, with the exception of one iP4 supplemented cow that did not have a functional CL. In 63.6% (n = 7/11) of cows experiencing LEM, a small amount of liquid in the uterine horn ipsilateral to the ovary containing the functional CL was evident. In pregnant cows, a single embryonic vesicle was verified in 73.3% (11/15) of non-iP4 supplemented cows and in 66.6% (6/9) of iP4 supplemented cows. Two non-iP4 supplemented cows had three embryonic vesicles, while the remaining (n = 5) had two embryonic vesicles. Overall, as represented in Figure 4, iP4 supplementation

doubled the frequency of EEM (iP4: 44.4% vs. Non-iP4: 22.2%) while it did not affect LEM (iP4: 18.6% vs. Non-iP4: 22.2%). Consequently, proportion of pregnancy in iP4 group was numerically lower than that of non-iP4 group (Table 1). Characteristics of CL area and CLBF between D4 and D14 were not related to the pregnancy status, nor to P4 concentrations. Analysis considering the mean P4 concentrations over the diestrus, revealed similar results (Table 2). Thus, there was not a specific profile of CL area, CLBF and P4 concentrations related to the cows experiencing EEM, LEM or that became pregnant.

Table 2 Mean plasma P4 concentrations and *ISG15* abundance according to reproductive status in beef cows

Variable	iP4[1]			Non-iP4			P value		
	Pregnant[2] (n = 10)	EEM (n = 12)	LEM (n = 5)	Pregnant (n = 15)	EEM (n = 6)	LEM (n = 6)	iP4	Group	iP4*Group
Mean plasma P4 concentrations (ng/mL)									
D4, D5 and D7	3.21 ± 0.44	4.01 ± 0.38	2.77 ± 0.59	3.17 ± 0.34	2.56 ± 0.54	2.01 ± 0.54	0.04	0.11	0.22
D14 to D20	7.19 ± 0.70	.	4.89 ± 0.94	6.79 ± 0.58	.	6.76 ± 0.86	0.35	0.15	0.16
Mean *ISG15* abundance in PBMCs									
D14 to D20	5.02 ± 0.58	0.76 ± 0.53	3.42 ± 0.82	3.49 ± 0.47	0.76 ± 0.74	3.78 ± 0.74	0.48	<0.001	0.37
D20	8.56 ± 1.24	1.27 ± 1.31	5.56 ± 1.76	5.79 ± 1.02	0.72 ± 1.97	6.39 ± 1.61	0.50	<0.001	0.52

Abbreviations: EEM = early embryonic mortality; LEM= late embryonic mortality

[1]Beef cows detected in estrus (D0) were assigned randomly to receive a single injection of vehicle (Non-iP4) or 150 mg of long acting progesterone (iP4) on D4 and transfer of 5 *in vitro* derived embryos on D7. Embryo signaling was monitored by abundance of ISG15 in peripheral blood mononuclear cells (PBMCs) isolated on D14, D16, D18 and D20. Blood samples were collected on D4, D5 and D7 and from D14 to D20, every day, for P4 assay.

[2]Cows were classified retrospectively as experiencing early embryonic mortality (EEM), late embryonic mortality (LEM) or confirmed pregnant on the day of pregnancy diagnosis (D30). Cows were included in the EEM group when there was no evidence of embryo presence up to D16 according to absence of *ISG15* mRNA increase between D14 and D20 and/or incidence of luteolysis before D20. Conversely, cows classified as LEM presented a clear increase on *ISG15* abundance, functional CL on D30, but no evidence of viable embryo on D30. Means of variables over time were considered for analysis including the effect of iP4 supplementation, group and interaction. Results are reported as Least squares means ± SEM.

The relative abundance of *ISG15* in PBMCs during early pregnancy showed a significant day by group interaction effect (Figure 5). The interaction reflected the expected effect of the embryos on the progressive increase of *ISG15* expression over time in the pregnant and LEM cows. The *ISG15* abundance over time in EEM group remained low and similar to the cyclic group. Embryo signalling was first detectable on D16, but only became evident on D18 and D20 (Figure 5). Consistently, mean *ISG15* abundance over time for pregnant and LEM group was

similar and they were 3.49- and 2.84- fold greater than EEM group, respectively. Similar results were found on D20 (table 2).

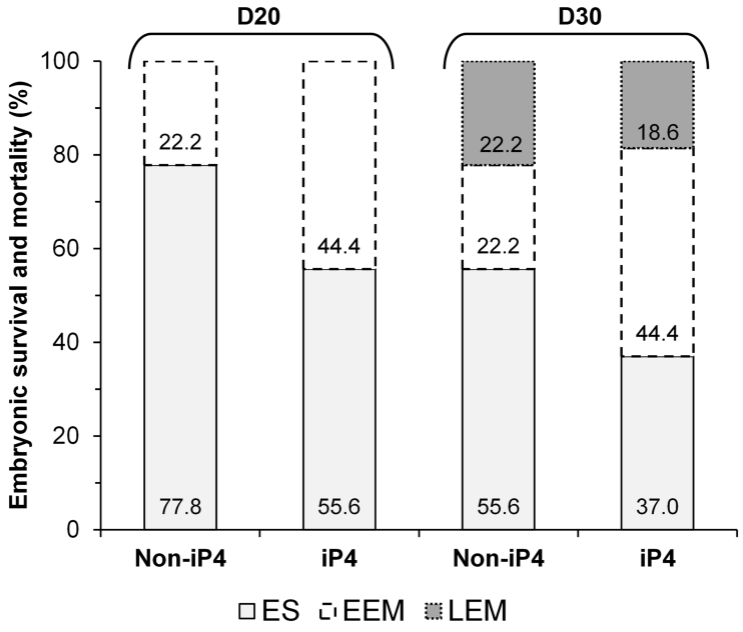

Figure 4 Frequency of pregnancy losses in beef cattle supplemented or not with iP4. Cows detected in estrus (D0) were assigned randomly to receive a single injection of vehicle (Non-iP4; n= 27) or 150 mg of long acting progesterone (iP4; n= 27) on D4 and transfer of 5 *in vitro* derived embryos on D7. These animals were classified retrospectively as experiencing early embryonic mortality (**EEM**), late embryonic mortality (**LEM**) or confirmed pregnant on the day of pregnancy diagnosis (D30).

Thirty six out of 37 5-ET cows having a functional CL on D20 also presented an upregulation on ISG15 abundance over time and a functional CL on D30. The exception was a cow from the iP4 group from which the relative abundance of *ISG15* on D16 (0.97-fold) and D20 (0.50-fold) was essentially unchanged. When only data from pregnant cows were analysed to evaluate the effect of iP4 supplementation on *ISG15* expression, there was an effect of iP4 supplementation, regardless of day (Figure 6). The average relative *ISG15* abundance (between D14 and D20 post-estrus) was 1.44-fold greater ($P = 0.05$) in group iP4 compared to the non-iP4.

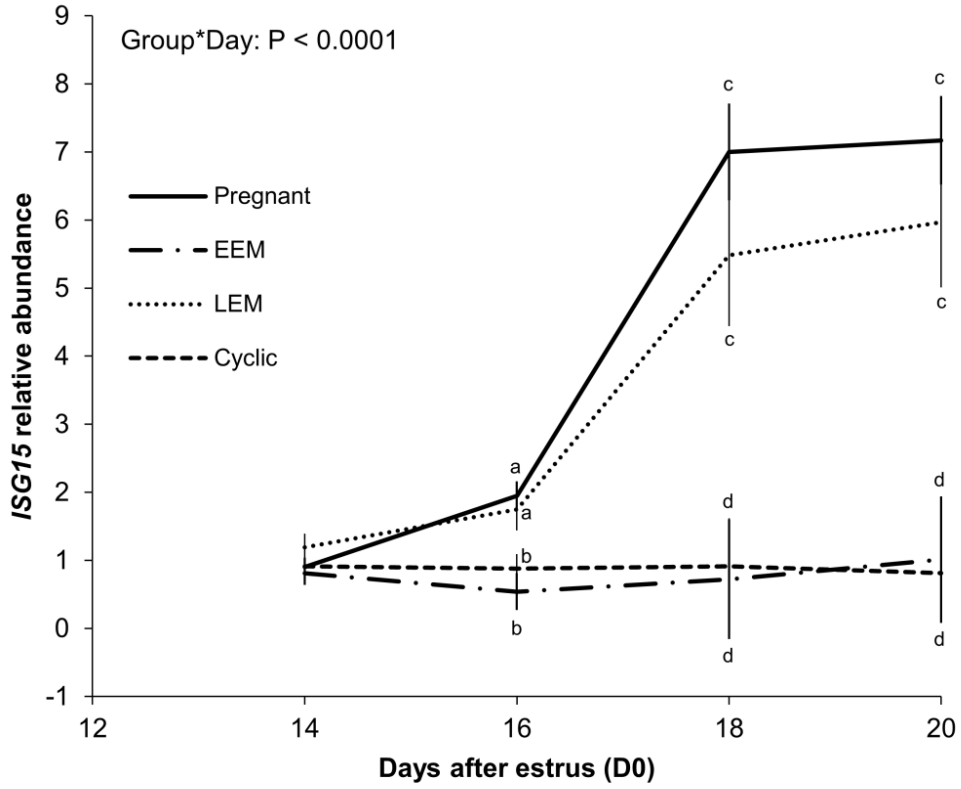

Figure 5. *ISG15* mRNA expression in peripheral blood mononuclear cells according to reproductive status of beef cows. Animals detected in estrus (D0) were assigned randomly to receive a single injection of vehicle (Non-iP4) or 150 mg of long acting progesterone (iP4) on D4 and transfer of none or 5 *in vitro* derived embryos on D7. Cows that received five embryos on D7 were classified retrospectively as experiencing early embryonic mortality (EEM; n= 18), late embryonic mortality (LEM; n= 11) or confirmed pregnant on the day of pregnancy diagnosis, D30 (n= 25). Cows receiving no embryos were classified as cyclic (n=25). Data were analyzed by split-plot ANOVA and only the significant effect was reported. Data are represented as Least squares means ± SEM. Values within a day without a common superscript are different between groups at $^{a,b}P < 0.05$ or $^{c,d}P < 0.0001$.

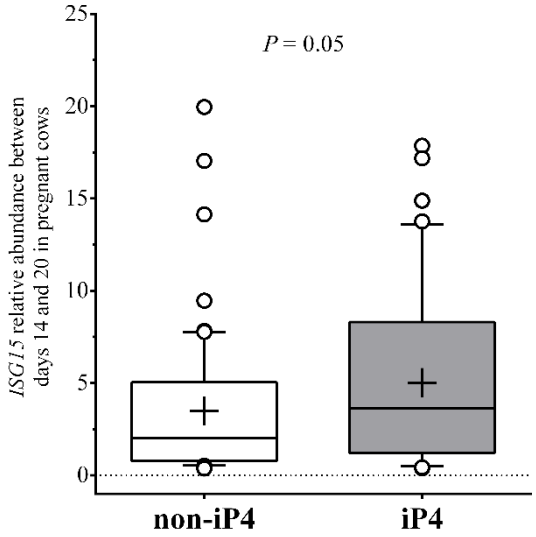

Figure 6. Effect of iP4 supplementation on *ISG15* mRNA expression in PBMCs of beef cows. Animals detected in estrus (D0) were assigned randomly to receive a single injection of vehicle (Non-iP4) or 150 mg of long acting progesterone (iP4) on D4 and transfer of 5 *in vitro* derived embryos on D7. Embryo signaling in cows confirmed pregnant at D30 through ultrasound (iP4; n= 10 and Non-iP4; n= 15) was monitored by abundance of ISG15 in peripheral blood mononuclear cells (PBMCs) isolated on D14, D16, D18 and D20. Data were analyzed by split-plot ANOVA and only the significant effect was reported. Data are represented as Box-plot using least squares means ± SEM over the time. The average abundance of *ISG15* (between days 14 and 20) was greater in iP4 supplemented cows than those Non-iP4 supplemented (mean is represented by the cross in the graphic).

4.5 DISCUSSION

In cattle, early embryonic mortality can be attributed to an incompetent embryo, a non-receptive reproductive tract (i.e., oviduct and/or uterus), a combination of both or a lack of compatibility between the embryonic and maternal units. Here, we used a MET approach, aiming to minimize pregnancy failures caused by the potential random developmental inability of a single embryo. Furthermore, embryonic competence was tested in the presence of supplemental P4 (iP4), that has both positive, embryo-elongating effects as well as negative, early-luteolytic effects. Our overall expectation was that using that model we would minimize the probability of an incompetent embryo to affect pregnancy. Furthermore, iP4 detrimental effects were expected to be suppressed by strong antiluteolytic signaling provided by iP4-stimulated embryos. Based on the greater ISG15 expression in response to iP4,

there was evidence of a greater antiluteolytic activity in these cows. However, the benefit of supplementary P4 on embryo signaling did not prevent early nor late embryonic losses; neither improved the embryo retention or P/ET. These effects were despite of absence iP4-induced short CL lifespan. Our data provided novel insights into early pregnancy biology: (1) EEM in non-iP4 cows indicated that a non-receptive uterus may prevent embryo development and signaling; (2) excessive EEM in iP4-treated cows indicated that antiluteolytic signaling was even further disrupted after iP4-priming, and this was not related to early onset of luteolysis; (3) LEM occurred despite successfully antiluteolytic signaling during maternal recognition of pregnancy, and was not influenced by the iP4 treatment.

Contrary to our expectation, frequency of EEM (Non-iP4+5-ET: 22.2%) was not substantially reduced by MET model and it approached the incidence of EEM (30%) currently described in the literature in cattle [1–3]. This supports the notion that embryo mortality was caused by a functionally incompetent uterus, that was unable to support embryo development at the time of embryo transfer. Results from iP4+5-ET group corroborated this concept, because supplementation with P4 doubled EEM frequency (44.4%), revealing to be detrimental rather than beneficial for embryonic survival. This adverse result occurred in spite of the P4-stimulated increase of relative *ISG15* abundance in PBMCs (Figure 6). Such P4 effects indicated that, at least in a proportion of animals, supplemental P4 stimulated conceptus elongation, amplifying intrauterine IFNT signalling. Accordingly, Matsuyama et al. [32] showed a positive relationship between increasing amounts of IFNT infused into the uterus of cows and abundance of *ISG15* mRNA in PBMCs. The fact that iP4 supplementation increased plasma P4 concentrations on day 5 (2.7 ng/mL increase) but not on day 7 (day of embryo transfer) further supports the notion that P4 actions changed the uterine milieu to affect embryonic development, as reported previously [11,12]. The effects of iP4 were probably through advancing the normal temporal changes that occur in the endometrial transcriptome along diestrus [33]. Such changes both benefitted (i.e., stimulated ISG abundance) and impaired (greater EEM) embryo survival, and this was through unknown uterine responses. We and others reported earlier that early elevation of P4 concentrations during diestrus have increased the incidence of early luteolysis [11,24,25,34]. However, in the present study, iP4 treatment did not reduce luteal lifespan of any animal. Furthermore, MET likely reduced chances of asynchrony generated between the P4-induced functional

changes on uterine environment and incapability of a single embryo to timely respond to such changes. Collectivelly, we propose that a functionally incompetent uterus hindered conceptus development.

Frequency of LEM was not impacted by the effect of greater P4 concentrations that potentiated embryonic signaling (iP4: 18.5% and Non-IP4: 22.2%). Our results were compatible to the reported incidence of LEM in dairy cows submitted to AI (~20%) [1,35] or to crossbred recipient heifers that received one or two *in vitro* produced embryos (14%) [36]. Incidence of late embryonic or fetal mortality (after day 28) varies between 8% and 11% for beef cows submitted to AI [2,37] or crossbred heifers transferred with one *in vitro* derived embryo [18,38]. Causes of LEM can be related to the pre-implantation development phase, such as insufficient growth. Indeed, IFNT acts on the endometrium regulating genes that may be important for implantation and placentation [5,39,40]. In a previous study, expression of *ISG15* in PBMCs of cows experiencing LEM after ET was less than that of pregnant cows [32], suggesting that reduced or delayed IFNT secretion is associated with embryonic loss after maternal recognition of pregnancy. In this sense, we had anticipated that LEM incidence would be attenuated in the iP4 group due amplification of IFNT signaling. However, we were unable to identify significant differences on *ISG15* abundance between cows experiencing LEM and those pregnant (Figure 4; Table 2), regardless of iP4 supplementation. Collectively, these results showed that even if the conceptus is able to release IFNT, pregnancy is not always maintained.

The timing of increase of *ISG15* mRNA abundance was compatible with a previous study using AI [27], indicating that neither MET model nor supplementary P4 disrupted the normal timing of IFNT production and sensing by PBMCs, that started around D16 [8]. However, MET induced unexpectedly earlier luteal demise. Apparently, MET generated signals to the endometrium stimulated, rather that inhibited, the luteolytic cascade, in cows experiencing EEM. In both sheep and cattle, the conceptuses and endometrium synthesize a variety of prostaglandins (PGs), as PGE2 and PGF2α, during early pregnancy [41,42]. Indeed, the endometrium produces substantially more PGs during early pregnancy than during the estrous cycle, although release is not pulsatile [43,44]. Accordingly, prostaglandin synthase two (PTGS2) a key enzyme in PG synthesis, was upregulated in the endometrium of sheep and cows collected at various days of early pregnancy (D10 to D24)

[42,45,46]. In addition, PTGS2 was induced in the endometrium by IFNT stimulus [46,47] and by P4 treatment in ovariectomized ewes [42]. Thus, uterine exposure to multiple-embryos may have enhanced PTGS2 expression, favoring the luteolytic process in cows experiencing EEM. Furthermore, intraluminal uterine concentration of PGs from embryonic and endometrium origin was probably enhanced in such cows. Despite the clear importance of PGs for embryonic development and maintenance of pregnancy in ruminants [43,44,47], PGF2α can also impair early embryonic development [48] and pregnancy rates [49] in cattle. In this regards, the excessive production of PGs and embryonic oversignaling may be deleterious to embryonic survival. Therefore, the iP4 supplementation may have potentiated these side-effects of MET model, hindering embryonic survival. This also suggests that exacerbated embryonic signaling is not capable to overcome inherent uterine deficiencies.

Finally, in this study the pregnancy per ET (iP4: 37.0% and Non-iP4: 55.6%) was compatible with previous reports (37.4% to 56%) for *in vitro* derived embryos [18,38,50], restating the absence of positive effect of MET model and P4 supplementation on reducing pregnancy losses.

In conclusion, a substantial number of cows had early (31%) and late (20%) embryonic mortality after transferring 5 embryos produced in-vitro. This argues that mortality is due to poor uterine receptivity, pre-established at the time of transfer and could not be reversed by a single injection of 150 mg of long acting P4 on day 4 post-estrus (~day 3). Receptive uteri that responded positively to iP4 resulted in improved embryo signaling during the maternal recognition of pregnancy window, evidenced by the elevated abundance of *ISG15* in PBMCs of pregnant cows. Further, luteal lifespan was not shortened by iP4 supplementation, demonstrating that iP4-induced earlier luteolysis was not the cause of embryo mortality. Collectively, our results indicated that deficiencies on uterine enviroment are the main cause of embryonic losses in cattle. Potentiating embryonic signaling through supplementing P4 and transfer of multiple embryos were not capable to overcome uterine dysfunction.

4.6 REFERENCES

1. Wiltbank MC, Baez GM, Garcia-Guerra A, Toledo MZ, Monteiro PLJ, Melo LF, et al. Pivotal periods for pregnancy loss during the first trimester of gestation in lactating dairy cows. Theriogenology 2016;86:239–53. Available from: http://dx.doi.org/10.1016/j.theriogenology.2016.04.037

2. Diskin MG, Sreenan JM. Fertilization and embryonic mortality rates in beef heifers after artificial insemination. J Reprod Fertil. 1980;59:463–8.

3. Dunne LD, Disken MG, Streenan JM. Embryo and fetal loss in beef heifers between 14 d of gestation and full term. Anim Reprod Sci. 2000;58:39-44.

4. McMillan WH. Statistical models predicting embryo survival to term in cattle after embryo transfer. Theriogenology. 1998;50:1053–70.

5. Spencer TE, Johnson GA., Bazer FW, Burghardt RC, Palmarini M. Pregnancy recognition and conceptus implantation in domestic ruminants: Roles of progesterone, interferons and endogenous retroviruses. Reprod Fertil Dev. 2007;19:65–78.

6. Bazer FW, Spencer TE, Johnson G a., Burghardt RC, Wu G. Comparative aspects of implantation. Reproduction. 2009;138:195–209.

7. Bridges GA, Day ML, Geary TW, Cruppe LH. Triennial Reproduction Symposium: Deficiencies in the uterine environment and failure to support embryonic development. J Anim Sci 2013;91:3002–13. Available from: http://dx.doi.org/10.2527/jas.2013-5882

8. Thatcher WW, Binelli M, Burke J, Staples CR, Ambrose JD, Coelho S. Antiluteolytic signals between the conceptus and endometrium. Theriogenology. 1997;47:131–40.

9. Garrett JE, Geisert RD, Zavy MT, Morgan GL. Evidence for maternal regulation of early conceptus growth and development in beef cattle. J Reprod Fertil. 1988;84:437–46.

10. Carter F, Forde N, Duffy P, Wade M, Fair T, Crowe MA, et al. Effect of increasing progesterone concentration from Day 3 of pregnancy on subsequent embryo survival and development in beef heifers. Reprod Fertil Dev. 2008;20:368–75.

11. O'Hara L, Forde N, Carter F, Rizos D, Maillo V, Ealy AD, et al. Paradoxical effect of supplementary progesterone between day 3 and day 7 on corpus luteum function and conceptus development in cattle. Reprod Fertil Dev. 2014;26:328–36.

12. Clemente M, De La Fuente J, Fair T, Al Naib A, Gutierrez-Adan A, Roche JF, et al. Progesterone and conceptus elongation in cattle: A direct effect on the embryo or an indirect effect via the endometrium? Reproduction. 2009;138:507–17.

13. Mann GE, Lamming GE. The influence of progesterone during early pregnancy in cattle. Reprod Domest Anim. 1999;34:269–74.

14. Yan L, Robinson R, Shi Z, Mann G. Efficacy of progesterone supplementation during early pregnancy in cows: A meta-analysis. Theriogenology 2015;85:1390–8. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0093691X15007025

15. O'Hara L, Forde N, Duffy P, Randi F, Kelly AK, Valenza A, et al. Effect of combined exogenous progesterone with luteotrophic support via equine chorionic gonadotrophin (eCG) on corpus luteum development, circulating progesterone concentrations and embryo development in cattle. Reprod Fertil Dev. 2014;28:269–77. Available from: http://www.publish.csiro.au/?paper=RD14019

16. Ginther OJ. Effect of progesterone on length of estrous cycle in cattle. Am J Vet Res. 1970;31:493–6.

17. Garrett JE, Geisert RD, Zavy MT, Gries LK, Wettemann RP, Buchanan DS. Effect of exogenous progesterone on prostaglandin F2α release and the interestrous

interval in the bovine. Prostaglandins. Elsevier; 1988;36:85–96.

18. Randi F, Fernandez-Fuertes B, McDonald M, Forde N, Kelly AK, Bastos Amorin H, et al. Asynchronous embryo transfer as a tool to understand embryo–uterine interaction in cattle: is a large conceptus a good thing? Reprod Fertil Dev. 2016;28:1999–2006. Available from: https://doi.org/10.1071/RD15195

19. Gomez, E; Munõz M. Multiple embryo transfer for studying very early maternal-embryo interactions in cattle. Reproduction. 2015;11:1–33.

20. Berg DK, van Leeuwen J, Beaumont S, Berg M, Pfeffer PL. Embryo loss in cattle between Days 7 and 16 of pregnancy. Theriogenology. 2010;73:250–60.

21. Betteridge KJ, Eaglesome MD, Randall GCB, Mitchell D. Collection, description and transfer of embryos from cattle 10-16 days after oestrus. J Reprod Fertil 1980;59:205–16.

22. Rowson LE, Moor RM. Development of the sheep conceptus during the first fourteen days. J Anat. 1966;100:777–85.

23. Pugliesi G, Santos FB, Lopes E, Nogueira É, Maio JRG, Binelli M. Improved fertility in suckled beef cows ovulating large follicles or supplemented with long-acting progesterone after timed-AI. Theriogenology 2016;85:1239–48. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0093691X15006792

24. Martins T, Talamoni JP, Sponchiado M, Maio JRG, Nogueira GP, Pugliesi G, et al. Impact of estradiol cypionate prior to TAI and progesterone supplementation at initial diestrus on ovarian and fertility responses in beef cows. Theriogenology. 2017;104:156-63.

25. Pugliesi G, Oliveria ML, Scolari SC, Lopes E, Pinaffi FV., Miagawa BT, et al. Corpus luteum development and function after supplementation of long-acting progesterone during the early luteal phase in beef cattle. Reprod Domest Anim. 2014;49:85–91.

26. Pugliesi G, Beg MA, Carvalho GR, Ginther OJ. Induction of PGFM pulses and luteolysis by sequential estradiol-17 treatments in heifers. Theriogenology [Internet]. Elsevier Inc.; 2012;77:492–506. Available from: http://dx.doi.org/10.1016/j.theriogenology.2011.08.020

27. Pugliesi G, Miagawa BT, Paiva YN, Franca MR, Silva LA, Binelli M. Conceptus-induced changes in the gene expression of blood immune cells and the ultrasound-accessed luteal function in beef cattle: How early can we detect pregnancy? Biol Reprod. 2014;91:95–95. Available from: http://www.biolreprod.org/cgi/doi/10.1095/biolreprod.114.121525

28. Pontes JHF, Melo Sterza FA, Basso AC, Ferreira CR, Sanches BV., Rubin KCP, et al. Ovum pick up, in vitro embryo production, and pregnancy rates from a large-scale commercial program using Nelore cattle (Bos indicus) donors. Theriogenology 2011;75:1640–6. Available from: http://dx.doi.org/10.1016/j.theriogenology.2010.12.026

29. Sponchiado M, Gomes NS, Fontes PK, Martins T, Del Collado M, Pastore A de A, et al. Pre-hatching embryo-dependent and -independent programming of endometrial function in cattle. PLoS One 2017;12:e0175954. Available from: http://dx.plos.org/10.1371/journal.pone.0175954

30. Ruijter JM, Ramakers C, Hoogaars WMH, Karlen Y, Bakker O, Van Den Hoff MJB, et al. Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res. 2009;37:e45.

31. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001;29:e45.

32. Matsuyama S, Kojima T, Kato S, Kimura K. Relationship between quantity of IFNT estimated by IFN-stimulated gene expression in peripheral blood mononuclear cells and bovine embryonic mortality after AI or ET. Reprod Biol Endocrinol 2012;10:2-10. Available from: http://www.rbej.com/content/10/1/21

33. Forde N, Carter F, Fair T, Crowe MA, Evans ACO, Spencer TE, et al. Progesterone-regulated changes in endometrial gene expression contribute to advanced conceptus development in cattle. Biol Reprod [Internet]. 2009;81:784–94. Available from: http://www.biolreprod.org/cgi/doi/10.1095/biolreprod.108.074336

34. Burke CR, Mihm M, Macmillan KL, Roche JF. Some effects of prematurely elevated concentrations of progesterone on luteal and follicular characteristics during the oestrous cycle in heifers. Anim Reprod Sci. 1994;35:27–39.

35. Wijma R, Stangaferro ML, Kamat MM, Vasudevan S, Ott TL, Giordano JO. Embryo mortality around the period of maintenance of the corpus luteum causes alterations to the ovarian function of lactating dairy cows. Biol Reprod. 2016;95:112–112. Available from: https://academic.oup.com/biolreprod/article-lookup/doi/10.1095/biolreprod.116.142075

36. Reichenbach HD, Liebrich J, Berg U, Brem G. Pregnancy rates and births after unilateral or bilateral transfer of bovine embryos produced in vitro. Reproduction 1992;95:363–70. Available from: http://www.reproduction-online.org/content/95/2/363.abstract

37. Pohler KG, Geary TW, Johnson CL, Atkins JA, Jinks EM, Busch DC, et al. Circulating bovine pregnancy associated glycoproteins are associated with late embryonic/fetal survival but not ovulatory follicle size in suckled beef cows. J Anim Sci. 2013;91:4158–67.

38. Pontes JHF, Nonato-Junior I, Sanches B V., Ereno-Junior JC, Uvo S, Barreiros TRR, et al. Comparison of embryo yield and pregnancy rate between in vivo and in vitro methods in the same Nelore (Bos indicus) donor cows. Theriogenology. 2009;71:690–7.

39. Bazer FW, Burghardt RC, Johnson GA, Spencer TE, Wu G. Interferons and progesterone for establishment and maintenance of pregnancy: Interactions among novel cell signaling pathways. Reprod Biol. 2008;8:179–211. Available from: http://dx.doi.org/10.1016/S1642-431X(12)60012-6

40. Spencer TE, Sandra O, Wolf E. Genes involved in conceptus-endometrial interactions in ruminants: Insights from reductionism and thoughts on holistic approaches. Reproduction. 2008;135:165–79.

41. Lewis GS, Thatcher WW, Bazer FW, Curl JS. Metabolism of Arachidonic Acid In Vitro by Bovine Blastocysts and Endometrium. Biol Reprod. 1982;27:431–9. Available from: https://academic.oup.com/biolreprod/article-lookup/doi/10.1095/biolreprod27.2.431

42. Charpigny G, Reinaud P, Tamby JP, Creminon C, Martal J, Maclouf J, et al. Expression of cyclooxygenase-1 and -2 in ovine endometrium during the estrous cycle and early pregnancy. Endocrinology. 1997;138:2163–71.

43. Ellinwood WE, Nett TM, Niswender GD. Maintenance of the corpus luteum of early pregnancy in the ewe. II. Prostaglandin secretion by the endometrium in vitro and in vivo. Biol Reprod 1979; 21:845–856.

44. Marcus GJ, Agriculture KIA, Canada C. Prostaglandin formation by the sheep embryo and endometrium as an indication of maternal recognition of pregnancy. Biol Reprod. 1981;25:56–64. Available from: http://dx.doi.org/10.1095/biolreprod25.1.56

45. Emond V, MacLaren LA, Kimmins S, Arosh JA, Fortier MA, Lambert RD. Expression of cyclooxygenase-2 and granulocyte-macrophage colony-stimulating factor in the endometrial epithelium of the cow is up-regulated during early pregnancy and in response to intrauterine infusions of interferon-tau. Biol Reprod. 2004;70:54–64. Available from: http://www.ncbi.nlm.nih.gov/pubmed/13679318

46. Kim S, Choi Y, Spencer TE, Bazer FW. Effects of the estrous cycle, pregnancy and interferon tau on expression of cyclooxygenase two (COX-2) in ovine endometrium. Reprod Biol Endocrinol. 2003;1:58. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=194659&tool=pmcentrez&rendertype=abstract

47. Dorniak P, Bazer FW, Spencer TE. Prostaglandins regulate conceptus elongation and mediate effects of interferon tau on the ovine uterine endometrium. Biol Reprod. 2011;84:1119–27. Available from: http://www.biolreprod.org/cgi/doi/10.1095/biolreprod.110.089979

48. Scenna FN, Edwards JL, Rohrbach NR, Hockett ME, Saxton AM, Schrick FN. Detrimental effects of prostaglandin F2α on preimplantation bovine embryos. Prostaglandins Other Lipid Mediat. 2004;73:215–26.

49. Scenna FN, Hockett ME, Towns TM, Saxton AM, Rohrbach NR, Wehrman ME, et al. Influence of a prostaglandin synthesis inhibitor administered at embryo transfer on pregnancy rates of recipient cows. Prostaglandins Other Lipid Mediat. 2005;78:38–45.

50. Hasler JF, Henderson WB, Hurtgen PJ, Jin ZQ, McCauley AD, Mower SA, et al. Production, freezing and transfer of bovine IVF embryos and subsequent calving results. Theriogenology. 1995;43:141–52.

CHAPTER 5

PERTURBATIONS IN THE UTERINE LUMINAL FLUID COMPOSITION ARE DETRIMENTAL TO PREGNANCY ESTABLISHMENT IN CATTLE

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5 PERTURBATIONS IN THE UTERINE LUMINAL FLUID COMPOSITION ARE DETRIMENTAL TO PREGNANCY ESTABLISHMENT IN CATTLE

5.1 ABSTRACT

A major, unresolved issue is how the uterine microenvironment determines pregnancy success in cattle. Before implantation, conceptus development depends on the uterine secretome (i.e., histotroph). Despite its pivotal role, little is known about the dynamics of histotroph synthesis and changes in composition throughout the early diestrus and the relevance to pregnancy establishment. We hypothesize that disturbances on histotroph composition affect the establishment of pregnancy. Aim was to disturb histotroph composition at early diestrus and verify the effects on: (Exp. 1) timing to restore its composition; and (Exp. 2) pregnancy rate after multiple-embryo transfer. Estrous cycle of multiparous Nelore cows were synchronized and estrus was considered d 0 (D0) of the experiments. Disturbance was through flushing each uterine horn with 30 mL of DMPBS and collecting the resulting uterine luminal flushing (ULF) on D1; D4; D7; D1+D4+D7. Control group remained not-collected. In Exp. 1, ULF was collected on D7.5 from all animals and used for quantification of total protein concentration and abundance of albumin. In Exp. 2, three *in vitro*-produced embryos were transferred to the uterine horn ipsilateral to the ovary containing the CL on D7.5 and pregnancy was checked on D25 by ultrasound. In Exp. 1, ULF collection on D4 or D7 increased (1.5 to 2.2-folds) the total protein concentration and albumin abundance. ULF collection on D1 did not alter ($P > 0.10$) these endpoints. In Exp. 2, ULF collected on D4 or D7 decreased pregnancy rates to approximately half of that measured in the remaining groups. Subtle perturbations imposed to the native intrauterine milieu, such as those caused by a single, low-volume collection of ULF, profoundly disturbs intrauterine composition and pregnancy success. At least 4 d were necessary for the uterus to recover its composition and the functional capacity to carry post-implantation gestation.

5.2 INTRODUCTION

In eutherian mammals, there are two manners for providing nutrients to the conceptus, histotrophic and hemotrophic nourishment. In ruminants, the histotrophic mode is of special importance, because preimplantation embryo development takes approximately 20 d [1,2]. During the preimplantation period, the bovine embryo development occurs independently of a direct connection to the blood supply. Development relies solely on a complex fluid secreted by the epithelium lining the reproductive tract (i.e., oviductal and uterine fluid) to supply basic energy needs [1,2]. Specifically, the fluid secreted into the uterine lumen, termed histotroph, is a complex mixture of growth factors, hormones, enzymes, transport proteins, ions, lipids, glucose, amino acids and other molecules, that are dynamically synthesized by the endometrial glandular and luminal epithelia as well as selectively transported from blood [2–4]. In cattle, the establishment of pregnancy is dependent on the presence of an elongated conceptus, capable of signaling to the endometrium, mainly through interferon-tau (INFT) that is released in increasing amounts from d 15-16 of gestation [2,5]. The IFNT acts on the endometrium and inhibits the biosynthetic cascade of prostaglandin F2α and thereby luteolysis. Conceptus signaling together with continuing P4 exposure further induces endometrial differentiation to support receptivity to the conceptus and implantation. Thus, the histotroph is pivotal for the maintenance and development of preimplantation conceptus in cattle, allowing the establishment of pregnancy.

There is convincing evidence that the phase of histotrophic nutrition is critical for the establishment of pregnancy in cattle. About 2/3 of the overall embryonic death loss (~30%) occurs between d 8 and 16 post-insemination [6–8]. Part of the elevated embryonic mortality that occurs during the preimplantation period is deemed to result from a functional incapacity of the uterine luminal milieu to properly support conceptus survival and elongation.

In fact, the importance of histotroph for the periimplantation conceptus development and survival was nicely demonstrated by Gray et al. [3–4] using an ovine uterine gland knockout model. The authors verified that the absence of

endometrial gland secretions in sheep uterus compromises conceptus survival and elongation, resulting in impaired conception.

The histotroph composition is temporally-regulated by estradiol (E2) and progesterone (P4), along the estrous cycle. In effect, the endometrium undergoes marked functional changes in response to the ovarian-endocrine stimulus and to pregnancy, as demonstrated in transcriptomic studies [9–11]. Furthermore, studies in cattle [12–15] indicated that the histotroph composition also undergoes dynamic changes along the estrous cycle and is altered by different P4 concentrations and pregnancy. Accordingly, proteomic [12,13], amino acids [14–16] and components of the redox system [17] were contrasting between uterine fluids recovered across the diestrus. The expression of a number of proteins during the cycle was related to P4 concentrations from d 3 to 7 post-estrus [12] or it was increased when P4 concentrations were exogenously elevated [14,15]. Altogether, these findings support the notion that histotroph composition results from dynamic changes in endometrial function, orchestrated by sex-steroids and the embryo/conceptus, to provide optimal uterine environments that support stage-specific requirement of embryo/conceptus development. Evidence for this well-regulated environment might be verified by a stringent requirement of synchrony between the developing embryo and the recipient (approximately 24 h) for optimal pregnancy rates [18,19] in cattle. Indeed, transfer of a single d 7 embryo towards a synchronous d 7 uterus resulted in greater pregnancy rate compared to transfer towards an asynchronous uterus [20]. Specifically, transfer of a single d 7 embryo to a d 5 or d 8 uterus moderately impacted pregnancy rate (~6% reduction), but transfer of a single d 7 embryo to a d 4 or d 9 uterus had negative impact on pregnancy rate (~22% reduction). In addition, indirect modifications introduced in the uterine environment, for example as caused by supplementation of P4 at early diestrus, advances conceptus elongation [21] and influences embryonic survival and the subsequent fertility [22]. However, the dynamics of histotroph changes along the estrous cycle are largely not known. Furthermore, understanding how and to which extent disturbances in this native environment influence pregnancy establishment in cattle is limited. Thus, in the present study we aimed to disturb the histotroph composition at early diestrus and verify the effect on (1) timing to recover composition and (2) embryo survival in beef cattle.

5.3 MATERIALS AND METHODS

5.3.1 Animals

Non-lactating, cycling, multiparous Nelore cows (*Bos taurus indicus*, average body weight 593.80 ± 12.07 kg) were maintained under grazing conditions, supplemented with chopped sugarcane, concentrate and minerals to fulfill their maintenance requirements and received water *ad libitum*. The experiment was conducted in the Southern Hemisphere tropics at the Pirassununga Campus of the University of São Paulo, Brazil. All experimental procedures involving animals complied with the Ethics and Animal Handling Committee of the School of Veterinary Medicine and Animal Science of the University of São Paulo (CEUA-FMVZ/USP, n° 9585220316).

This study comprises two experiments. In the Exp. 1, we aimed to determine the effect of uterine flushings performed over diestrus on the uterine luminal protein content at d 7.5 post-estrus. In the Exp. 2, the aim was to determine the impact of uterine flushing on the establishment of pregnancy in embryo recipient cows. The experimental designs are illustrated in Fig. 1.

5.3.2 Experimental designs

In the Exp. 1, follicular growth was synchronized using an intravaginal P4-releasing device (1 g; Sincrogest®, Ourofino Saude Animal, Cravinhos, SP, Brazil), an intramuscular administration of estradiol benzoate (2 mg; Sincrodiol®, Ourofino Saude Animal) and prostaglandin F2α analogue ($PGF_{2\alpha}$; 500 µg of sodium cloprostenol; Sincrocio®, Ourofino Saude Animal). Eight d later, the P4-devices were removed; cows received another injection of $PGF_{2\alpha}$ analogue and an Estrotect™ (Western Point Inc., Apple Valley, MN) heat detector patch. Cows were checked for signs of estrus twice a d between 36 and 96 h after P4-releasing device withdrawal. Cows observed in standing estrus and/or presenting an activated heat detector patch were considered in estrus (D0 of the study; *n* = 44). Animals were submitted

randomly to collection of uterine luminal flushings (ULF) on D1; D4; D7; D1, 4 and 7; or to remain not-collected, to compose the 5 experimental groups: D1-ULF (*n* = 9), D4-ULF (*n* = 9), D7-ULF (*n* = 9), D1+D4+D7-ULF (*n* = 9) and Control (*n* = 8). On D7.5, ULF were collected from all groups for determination of total protein concentration and abundance of albumin. Control group was used to assess the perturbations promoted by the ULF collections on these endpoints.

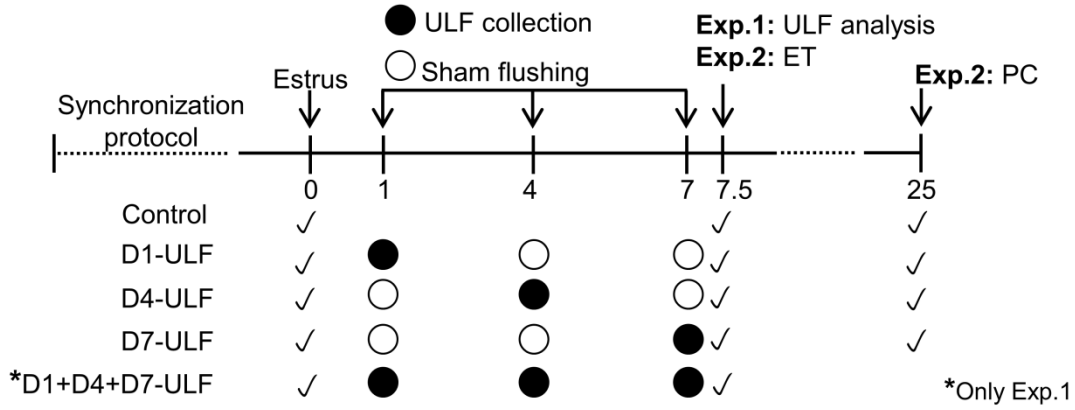

Figure 1. Experimental designs of the two experiments performed in this study. Collection of uterine luminal flushings (ULF) was performed during diestrus to determine: the effect on the uterine luminal protein content at D7.5 post-estrus (Exp. 1) or on pregnancy establishment (Exp. 2). Accordingly, follicle growth of cycling, non-suckled, multiparous Nelore cows was synchronized and estrus was detected (D0). Uterine horns were flushed non-surgically with 30 mL of DMPBS thrice (Exp. 1, 90 mL total) or once (Exp. 2) and ULF was collected. In Exp. 1, animals were submitted randomly to collection of ULF on D1; D4; D7; D1, 4 and 7; or to remain not-collected, to compose the 5 experimental groups: D1-ULF, D4-ULF, D7-ULF, D1+D4+D7-ULF and Control. On D7.5, ULF were collected from all groups for determination of total protein concentration and abundance of albumin. On Exp. 2, the same experimental groups were present, with the exception of D1+D4+D7-ULF group*. On D7.5, three in vitro-produced embryos were transferred non-surgically to the uterine horn ipsilateral to the ovary containing a CL and pregnancy was checked (PC) on D25 by transrectal ultrasonography. In both experiments, a sham ULF collection (all procedures except delivering DMPBS to the uterus) was performed in each cow, in each experimental d when no ULF collection was scheduled.

In the Exp. 2, estrous cycles were synchronized using a slightly modified version of the protocol described above. An injection of GnRH analogue (10 μg of buserelin acetate; Sincrofort®, Ourofino Saude Animal) was administered intramuscularly at P4-device insertion, followed by its removal 7 d later and by an administration of $PGF_{2\alpha}$ analogue 24h earlier. Cows were checked for signs of estrus as described previously. Animals observed in standing estrus and/or with an activated Estrotect patch (*n* = 64) were assigned randomly to one of four groups: (i) Control (*n* = 16), (ii) D1-ULF (*n* = 15), (iii) D4-ULF (*n* = 17), (iv) D7-ULF (*n* = 16).

5.3.3 Ultrasound examinations

Transrectal ultrasonography (Mindray M5, Shenzen, China; equipped with multifrequency linear transducer set to 7.5 MHz) in B-mode was performed to check ovulation of pre-ovulatory follicle on D1 and confirm the side of corpus luteum (CL) on D4 and D7. In Exp.1, diameter of pre-ovulatory follicle and CL area were recorded for analysis. In addition, blood flow of measured CLs was examined using ultrasound Color Doppler-mode, to confirm CL functionality, as defined by Pugliesi et al. [23].

5.3.4 Quantification of P4 concentrations

In Exp. 1, blood samples were collected on D7 for determination of serum P4 concentrations. Serum was harvested on D7 by centrifugation of blood at 2,700 x g for 15 min at 4°C. Progesterone was assayed using a solid-phase RIA kit (Immuchem^{TM} Double Antibody Progesterone Kit; Cat. 07-170105, MP Biomedicals, NY, USA). The sensitivity of the assay was 0.1 ng/mL. The intra-assay coefficient of variation (CV) for quality control samples was 1.15% (low standard) and 0.01% (high standard).

5.3.5 ULF collection

Caudal epidural anesthesia was performed with 2% lidocaine solution (4 mL; 80 mg; Lidovet®, Bravet, Engenho Novo, RJ, Brazil) immediately before ULF collection. Uterine horns were flushed non-surgically, always flushing the horn ipsilateral to ovary containing the CL first and flushing the contralateral horn subsequently, at different d post-estrus, as described in the experimental design. ULF was collected using a similar method described previously [12]. Accordingly, a sterile silicone Foley catheter (2 vials, 20 mL cuff, 18” or 20 diameter; Rusch®, Teleflex, US) was fixed onto a stylette. Guided by rectal palpation, the catheter was

passed through the cervix into the body of the uterus and directed to the target uterine horn. The catheter was positioned and fixed at the horn's bifurcation by inflating the cuff. The distal end of the uterine horn adjacent to the uterotubal junction was manually occluded through rectal palpation. Then, the uterine horn was slowly filled with 30 mL of Dulbecco's modified phosphate buffered saline (DMPBS, Nutricell, Campinas, SP, Brazil) at 37°C using a sterile 60-mL catheter tip syringe (SR, Manaus, AM, Brazil). After infusion of DMPBS, the ULF was immediately recovered by aspiration with a syringe only while a steady flow could be achieved. In Exp.1, for each uterine horn, this procedure was repeated three times without removing the catheter. ULF recovered from each uterine horn was placed in 50-mL conical tubes to record volume and visual aspect. ULFs were classified subjectively as bloody when they contained any traces of blood or presented a pinkish coloration, as opposed to a translucent aspect (Fig. 2a). Total volume recovered and visual aspect of ULFs were used to characterize the pooled three ULF collected from each cow. In the groups D1-ULF, D4-ULF and D7-ULF, a sham flushing (all procedures except delivering DMPBS to the uterus) was performed on each experimental d when no collection was scheduled. For example, group D1-ULF was subjected to sham flushing on D4 and D7. On D7.5, each uterine horn of each animal was submitted to a single ULF collection with 30 mL of DMPBS and the ULF was used for protein analysis. Volume and visual aspect of ULF-D7.5 were recorded. In addition, to evaluate the impact of three sequential collections on ULF composition, on D7.5, only in the Control group, two additional collections were performed. Volume and aspect of each ULF collected (ULF1, ULF2 and ULF3) were recorded individually for analysis. On D7.5, ULFs were transferred to light-safe 50-mL conical tubes and kept on ice until processed in the laboratory. Within 15 min of flushing, ULFs were clarified by centrifugation (1,000 x g for 10 min at 4 ºC), supernatants were transferred to cryotubes and stored at −20 °C for subsequent analyzes.

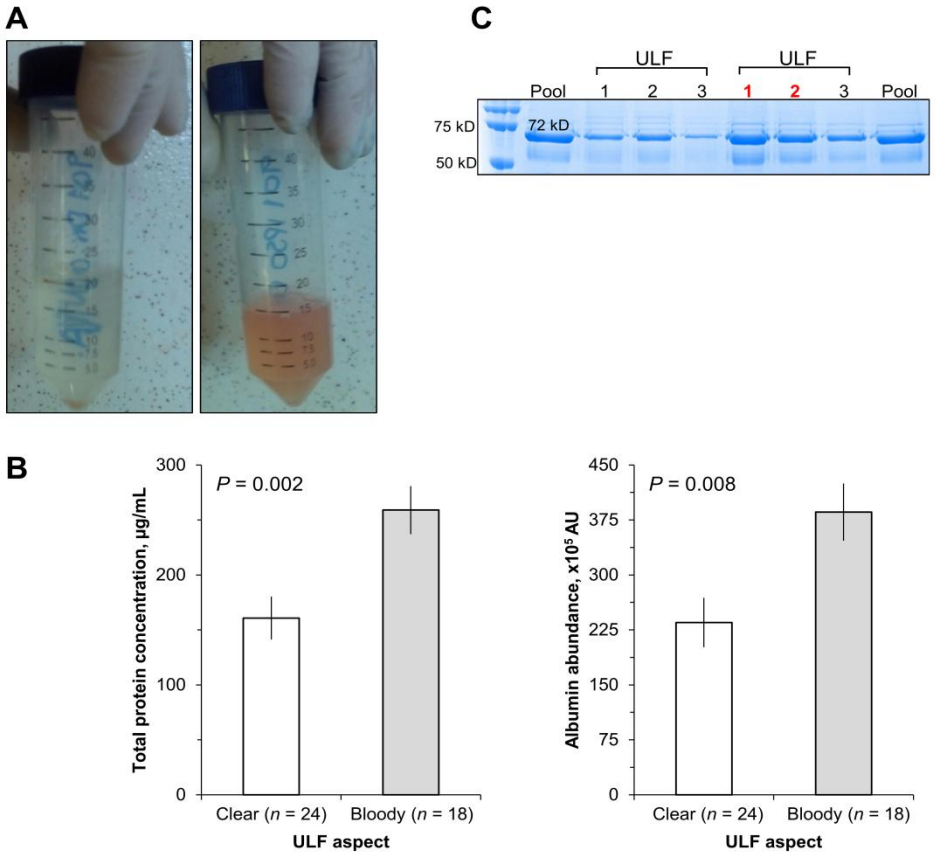

Figure 2. Effect of ULF classified as clear or bloody on total protein concentration and albumin abundance. On D7.5 (D0: estrus), each uterine horn of beef cows (*n* = 7) were flushed thrice with 30 mL of PBS and the resulting uterine luminal flushings were collected (ULF1, ULF2 and ULF3), visually classified as clear or bloody (Panel A) and stored for analysis. Quantification of total protein concentrations and determination of albumin abundance was performed in each ULF (Panel B). Panel C: Representation of the three successive ULF classified as clear (black bold marked) or bloody (red bold marked) in the polyacrylamide gel stained with Coomassie-Blue for albumin determination. Pool: Mixture of all collected ULF. AU: Arbitrary units. Values are presented as LSMEANS±SEM.

Collection of ULF in the Exp. 2 was performed as described, but with minor modifications. Specifically, the uterine horns were flushed only once with 30 mL of DMPBS, instead three times as in Exp. 1.

5.3.6 Embryo Production and Embryo Transfer procedures

On D7.5, all animals from Exp. 2 were submitted to embryo transfer. Cumulus oocyte complexes used to produce embryos were aspirated from ovaries collected in a local slaughterhouse. Embryos were produced according a standard protocol for *in*

vitro embryo production [24]. Recipients received a caudal epidural anesthesia immediately before the procedure of embryo transfer. Three *in vitro*-produced, grade 1 blastocysts were transcervically placed in the middle of the cranial third of the uterine horn ipsilateral to the ovary containing a CL, using standard nonsurgical techniques. By transferring three embryos per recipient cows, we expected to minimize the random effect of a single incompetent embryo to influence pregnancy establishment [25].

On D25, pregnancy status was based on visualization of the embryo proper and heartbeat by transrectal B-mode ultrasonography under optimal conditions, as described previously [26, 27]. All cows received a luteolytic dose of $PGF_{2\alpha}$ after pregnancy diagnosis.

5.3.7 Total Protein quantification assay

The protein content in the ULFs collected on D7.5 was determined by a colorimetric assay using a Micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) according to manufacturers' instructions. The micro-assays were conducted in 96-well plates. The total protein concentration in each sample was determined in triplicates and calculated using the standard curve supplied on the kit. The standard curve was based in bovine serum albumin diluted in DMPBS and ranged from 25 to 2000 µg/mL. The colorimetric absorbances were measured by a spectrophotometer (Multiskan MS Primary EIA, Thermo-Fisher) adjusted at 570nm wavelength.

5.3.8 Determination of albumin abundance

Albumin content was determined in ULF samples by Comassie-Blue staining. ULF samples (18 µL) from D7.5 were diluted in 2x Laemmili buffer and denatured at 95 ºC for 5 min. Proteins were separated in 12% SDS-Polyacrylamide gel

electrophoresis at 160 V for 120 min. The resulting polyacrylamide gels were fixed by submerging in 50% MeOH, 10% HoAC and 40% distilled water (v/v) solution for 30 min. Fixed gels were stained in 0.25% (w/v) Coomassie Brilliant Blue R-250 (Bio-Rad laboratories) solution for 2 h and then, cleared overnight in a destaining solution (5% MeOH, 7.5% HoAC, 87.5% distilled water). Stained polyacrylamide gels were exposed to ChemiDoc MP Imaging System (Bio-Rad) apparatus and band intensities were quantified by densitometry using Image-Lab software version 4.01 (Bio-Rad). Protein molecular weights were estimated according to a molecular weight standard (Precision Plus Protein^{TM} Dual Color Standards, Bio-Rad laboratories) that was run in each gel. The identification of the albumin band was based on molecular weight of bovine serum albumin (~67 kDa). Only ULF samples collected from the ipsilateral uterine horn were used for determining the abundance of albumin on D7.5. The effect of three sequential flushings on the abundance of albumin was determined on ULF collected from the Control animals on D7.5 from ipsi- and contralateral uterine horns.

5.3.9 Statistical analyses

Dataset of Exp.1 (i.e. total protein concentration and abundance of albumin) was analyzed by ANOVA using MIXED procedure of SAS (SAS Inst. Inc., Cary, NC, USA) version 9.3. Model included fixed effect of experimental group, horn (ipsi- and contralateral) and their interaction. Assumptions of normality of residues and homogeneity of variances were checked by influencing diagnostics outputs from the MIXED and F-max test, respectively. Data that did not follow the assumptions was transformed by the natural logarithmic or square root before analyses. When necessary, comparisons were performed contrasting D-ULF groups versus the Control group, using the DIFF command incorporating the Dunnett test.

Aiming to determine the effect of sequential ULF collections on the protein and albumin quantity of collected ULFs, three-sequential ULFs collected on D7.5 were analyzed by split-plot design using MIXED procedures. Model included fixed effect of ULF collection order (ULF1, ULF2 or ULF3), ULF visual aspect, horn and all their interactions. The random effect of cows nested within combination of flushing aspect and uterine horn side was used as an error term to test the main plot effect. The type

of variance–covariance structure used was chosen based on smaller magnitude of the corrected Akaike's information criterion (AICC). When the effect of a categorical variable was significant, the LSD post-hoc test was used to compare means.

The variable, volume on D1, D4 and D7 from groups D1-ULF, D4-ULF and D7-ULF, respectively, was analyzed using MIXED procedure. Similarly, binomial variable, proportion of ULF with blood from each group was analyzed using GLIMMIX procedure. Model included fixed effect of group, horn and their interaction. For the D1+D4+D7-ULF group, variables volume and proportion of ULF with blood on D1, D4 and D7 were analyzed as split-plot design, including fixed effect of d, horn and their interaction. The random effect of cows nested within uterine horn side was used as error term. On D7.5, those variables (volume and visual aspect) were analyzed including fixed effect of group, horn and their interaction. In addition, volume of three-sequential ULFs collected on D7.5 from Control group was analyzed as split-plot design. Model included fixed effect of flushing order, horn and their interaction.

In Exp. 2, the analyzes of frequency of pregnant cows were performed with FREQ procedure using Chi-square distribution. Orthogonal contrast comparison was used to assess differences between groups (Control vs. ULF-D1, ULF-D4 vs. ULF-D7 and Control & ULF-D1 vs. ULF-D4 & ULF-D7).

Continuous variables are reported as Least Square Means ± Standard error of the mean (LSMEANS ± SEM) and binomial variables as means.

5.4 RESULTS

5.4.1 Experiment 1: Ovarian characteristics

Mean ± SEM of size of pre-ovulatory follicle (13.42 ± 0.27 mm), and CL area (2.42 ± 0.37 cm^2) and serum P4 concentrations on D7 (3.29 ± 0.24) were similar among groups ($P > 0.10$; data not shown). In all cows, a functional CL was detected on D4 and D7 at ultrasound scan, according to criteria described previously [23].

5.4.2 Experiment 1: Effect of same day, sequential ULF collections on ULF composition

For this analysis, only data from the sequential collections of ULF on D7.5 from the Control group were used (Fig. 2). We expected that ULF collection would have the effect of reducing total protein concentration and abundance of albumin progressively from ULF1 to ULF3. However, these endpoints were not affected by sequential collections among ULF1, ULF2 and ULF3 ($P > 0.1$). Irrespective of order of collection and side of uterine horn of the collected ULF, compared to the ULF classified as clear, those classified as bloody presented greater total protein concentration (bloody: 258.95 ± 21.97 vs. clear: 160.80 ± 19.55 μg/mL) and abundance of albumin (bloody: 385.78 ± 38.96 vs. clear: 235.06 ± 33.74 arbitrary units; $P < 0.01$).

5.4.3 Experiment 1: Effect of different days, sequential ULF collections on ULF composition

For this analysis, we used ULF samples collected on D1, D4 and D7, from group D1+D4+D7-ULF only. There was an effect of d ($P \leq 0.05$) on the volume of recovered ULF, regardless of uterine horn side flushed. Volume of recovered ULF on D4 (81.8 ± 1.71 mL) was lower than that on D7 (87.7 ± 1.77 mL), but similar to D1 (85.3 ± 1.77 mL). Proportion of ULF classified as bloody for the D1+D4+D7-ULF group was not affected ($P > 0.1$) by d nor uterine horn side (D1: 29% [5/17]; D4: 29% [5/17] and D7: 11.1% [2/18]).

5.4.4 Experiment 1: Effect of different days, single ULF collections on same day ULF composition

Initially, we analyzed volume recovered and visual aspect of ULFs collected on D1, D4 or D7. Because blood flow to the uterus decreases from metestrus to early diestrus, we expected that frequency of ULF containing blood would also decrease from D1 to D7. There was no effect of group (D1-ULF, D4-ULF and D7-ULF) on the proportion of ULF classified as bloody (46.3% [25/54], *P* > *0.1*), neither on total volume of recovered ULF (87.7 ± 0.76 mL, *P* > 0.1), regardless of uterine horn side collected.

5.4.5 Experiment 1: Effect of single or multiple days ULF collections on subsequent ULF composition

Next, we analyzed ULF samples collected on D7.5 from animals submitted to UFL collections conducted on D1, D4, D7 or D1, D4 and D7. Proportion of ULFs classified as bloody (47.8% [43/90]) and volume collected (26.1 ± 0.53 mL) were all similar among groups (*P* > 0.1), regardless of uterine horn side sampled. Despite these similar gross characteristics among groups, total protein concentration in the ULF-D7.5 was affected (*P* = 0.01) by group, regardless of the uterine horn side sampled (Fig. 3). The D7-ULF had 1.9-fold higher (*P* < 0.01) total protein concentration in the ULF-D7.5 compared to the Control group. Compared to the Control group, total protein concentrations in ULF-D7.5 of groups D1-ULF, D4-ULF and D1+D4+D7-ULF increased numerically 1.3, 1.5 and 1.4 fold, respectively, but concentrations were not significantly different than the Control group. Consistently, the abundance of albumin (that was only analyzed in the ULF-D7.5 collected from the ipsilateral uterine horn), there was an effect of group (*P* = 0.02) on the abundance of albumin measured in the ULF-D7.5 (Fig. 4). The albumin abundance in the D4-ULF and D7-ULF groups were respectively, 2.0 and 2.1-fold greater compared to the Control group (*P* = 0.06), while that of the D1+D4+D7-ULF group was 2.2-fold greater (*P* = 0.03) than the abundance verified in the Control group. Changes in total protein and albumin abundances were caused by the ULF collection, not by the manipulations associated with the ULF collection. Specifically, compared to Control, the ULF-D1 group had no significant alterations in total protein and albumin abundances, despite of sham flushing procedures performed on D4 and D7.

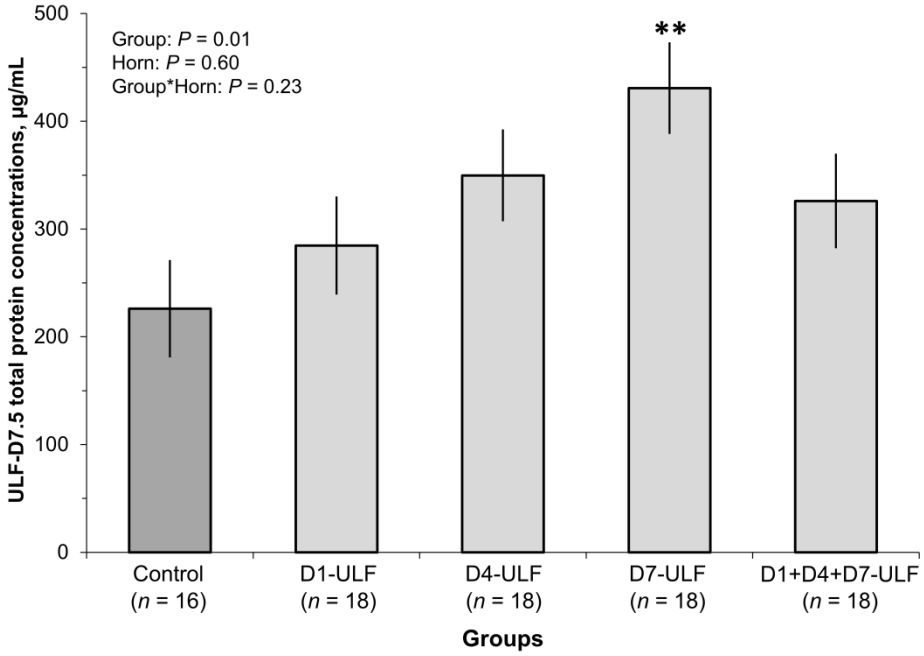

Figure 3. Effect of previous ULF collections on total protein concentration in the ULF-D7.5. Uterine horns of beef cows detected in estrus (D0) were flushed non-surgically thrice with 30 mL of DMPBS (90 mL) and the resulting uterine luminal flushing (ULF) was collected. Animals were assigned randomly to be submitted to ULF collections on D1; D4; D7; D1+D4+D7; or to remain not-collected, composing 5 experimental groups. On D7.5, ULF were collected from all groups for quantification of total protein concentrations. Control group was used to assess the perturbations promoted by the ULF collections on the total protein concentrations. Values are presented as LSMEANS ± SEM. Main effects of group, uterine horn (Horn) and their interaction are indicated. $^{**}P < 0.01$, differed from Control group as determined by Dunnett test.

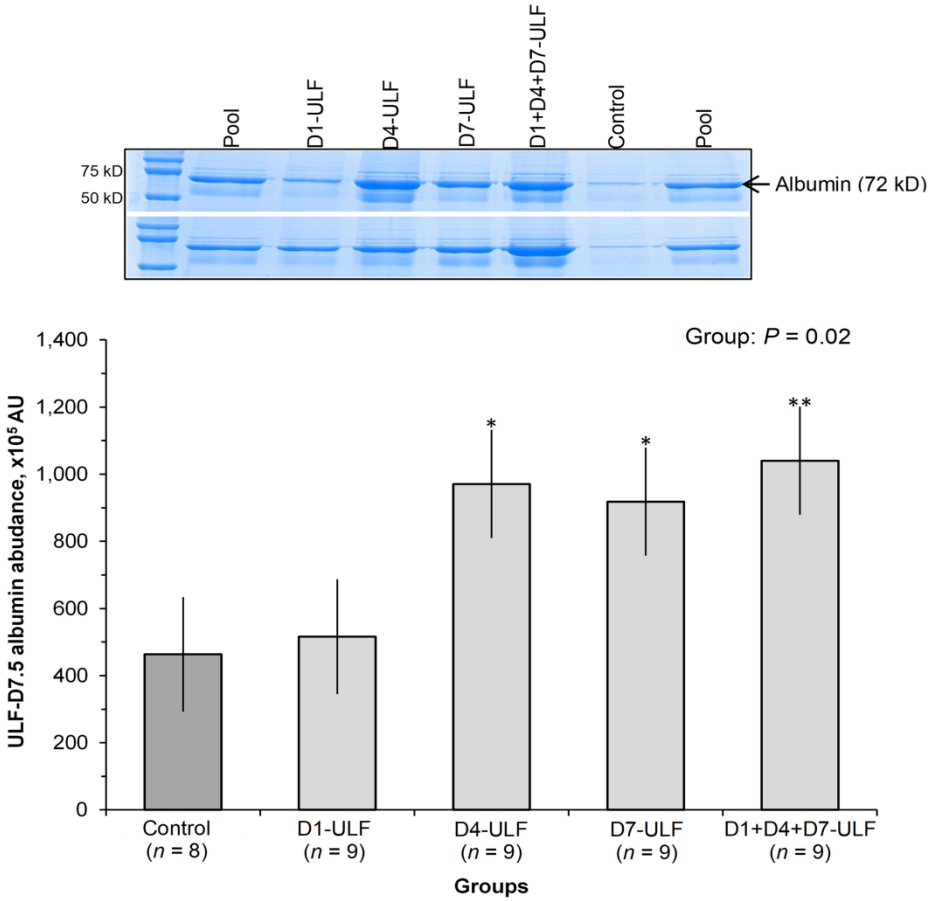

Figure 4. Effect of previous ULF collections on total protein concentration in the ULF-D7.5. Uterine horns of beef cows detected in estrus (D0) were flushed non-surgically thrice with 30 mL of DMPBS (90 mL) and the resulting uterine luminal flushing (ULF) was collected. Animals were assigned randomly to be submitted to ULF collections on D1; D4; D7; D1+D4+D7; or to remain not-collected, composing 5 experimental groups. On D7.5, ULF were collected from all groups and albumin abundance was determined in samples from ipsilateral uterine horn. SDS-PAGE was conducted, polyacrylamide gels were stained with Coomassie-Blue and abundance of albumin was determined by densitometry. Values are presented as LSMEANS±SEM. Main effect of group is indicated (P = 0.02). Differed from Control at **P = 0.03 and *P = 0.06 as determined by Dunnett test. Pool: Mix of all flushing sample. AU: Arbitrary units

5.4.6 Experiment two: The effect of single ULF collections on conception rate of embryo recipients

In this experiment, ULF procedure at selected time points was performed only once instead of thrice, because as verified in Experiment 1, sequential ULF resulted in no further modifications on the composition of subsequent ULFs. Furthermore, ULF collection in all selected days (D1+D4+D7-group) had not cumulative effect on

the ULF-D7.5, so this group was not included in the design. Compared to Controls (62.5%), the conception rate was not affected by ULF collection when it was performed on D1 (60%; P > 0.1). In contrast, conception rate was reduced when the ULF were collected on D4 (29%; P = 0.06) and D7 (37.5%; P = 0.16) compared to the non-flushed Control (62.5%), Fig. 5.

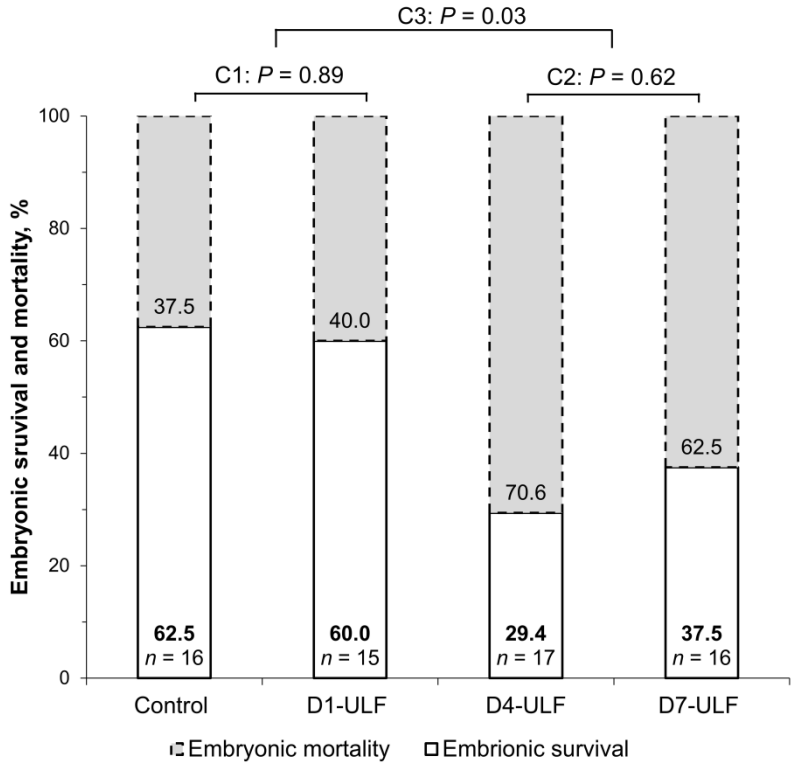

Figure 5. Effect of previous ULF collections on embryonic survival and mortality. Uterine horns of beef cows detected in estrus (D0) were flushed non-surgically with 30 mL of DMPBS and the resulting uterine luminal flushing (ULF) was collected. Animals were randomly assigned to be submitted to ULF collections on D1; D4; D7; or to remain not-collected, composing 4 experimental groups. On D7.5, three *in vitro*-produced high grade embryos were non-surgically transferred to the horn ipsilateral to the ovary containing CL. On D25, embryonic survival was determined based on detection of embryonic vesicle with heartbeat by ultrasound scan. Values are presented as means. Pregnancy rate was affected by group (*P* = 0.06). Orthogonal contrast was used for comparisons between groups (C1: Control vs. ULF-D1; C2: ULF-D4 vs. ULF-D7; C3: Control & ULF-D1 vs. ULF-D4 & ULF-D7).

5.5 DISCUSSION

It is widely accepted that a biochemically well-defined uterine luminal environment (i.e., the histotroph) is required for successful pre-implantation embryo development in cattle. Moreover, developmental needs of the growing embryo/conceptus are expected to change over time. Thus, specific mechanisms must be in place to take in effect the dynamic changes needed to generate and modify the luminal molecular composition. More importantly, because incidence of embryo mortality is disproportionately elevated during this stage of gestation, it is reasonable to assume that mortality is associated with an inadequate environment. However, luminal composition that would support or suppress embryo development has not been determined. The aim of the present study was to disturb the histotroph composition through the removal of ULF to evaluate the dynamics of restoration and the effects of a disturbed uterine environment on the pregnancy outcome. We expected that uterine flushings would remove molecules that are critical for embryo development, generating an impoverished environment that would compromise development. We further anticipated that disturbances that were elicited closer in time to the moment of embryo transfer would be more detrimental, and perhaps fatal, to embryo survival. We found that uterine flushings conducted at early diestrus (D4 and D7) actually increased the total protein content in the ULF samples collected at time of ET (D7.5). Interestingly, one protein that was dramatically increased in response to ULF was albumin, the most abundant protein in serum. This supports the idea that collecting ULF caused influx of blood proteins to the uterine lumen, consequently causing a dramatic change in its composition. Alterations were most obvious in animals whose ULF were performed at 0.5 and 3.5 d before ET, but not in animals whose ULF were performed 6.5 d before ET, indicating that it takes a relatively long time (i.e., at least 4 d) for the uterine environment to be restored after it is disturbed. Pregnancy rates to ET were consistent with the time dynamics of recovery after ULF. Indeed, ULF at time points closer to the ET (i.e. D4-ULF and D7-ULF groups) generated poorer pregnancy. Remarkably, even when ULF was conducted just 12 hours prior to embryo transfer, a proportion of recipients was still able to maintain their pregnancies. This suggests that approximately one third of pregnancies will be maintained to d 25 of gestation even in a severely altered luminal

environment. In contrast, it was also noteworthy that even when three embryos were transferred, approximately one third of the recipients in the control, non-manipulated group were not able to support embryo development. This indicates that there are situations in that the recipient is incompetent to maintain a gestation. Altogether, our data provide original, clear indication that recipients have varying abilities to sustain pregnancies, and embryos have varying resilience to thrive in uterine environments of very distinct quality.

Modifications on the native uterine luminal environment were evidenced by an increase on total protein quantity of ULF sampled at time of ET (D7.5) when uterine flushings were performed 0.5 to 3.5 d earlier. By conducting sequential uterine flushings we expected to deprive this specific microenvironment, decreasing the protein content according to the number of ULF collections performed. However, in fact, the abundance of proteins evaluated on the sequential ULFs sampled was unchanged. Rather than this, alterations promoted on total protein concentrations and abundance of albumin was positively influenced by samples containing blood (Fig. 2). Therefore, the effect of ULF collection on impoverishing the uterine luminal protein was probably disguised by the influx of blood proteins, such as albumin, that alone constitutes 35% to 50% of total serum protein [28]. While albumin is a normal component of uterine fluid in cattle [29], it is found in much lower concentrations in the uterine fluid (0.88 to 0.98 g/dL) than in serum samples (2.11 to 2.36 g/Dl; Alavi-Shoushtari et al. [28]). Contamination by plasma and interstitial fluid is a common issue when uterine flushings are performed *in vivo*, transcervically in cattle [29,31]. Even a ULF sample classified as clear by subjective evaluation contained substantial number of erythrocytes (50,400 to 4,940,000 cells/mL) as determined by hemocytometer counts [29]. Thus, the ULF collection conducted in our study clearly disrupted the native composition of the intrauterine milieu, mainly by contaminating the histotroph with plasma components.

It takes at least 4 d for total protein content and albumin concentrations in the ULF to be restored after ULF collection. To the best of our knowledge, this is the first time that dynamics of ULF re-composition after a designed disturbance was examined in cattle, *in vivo*. Using models that sample a given animal only in a specific, single d, others have shown the dynamic nature of histotroph changes across the estrous cycle [12–17]. The unique luminal uterine milieu seems to be maintained by dynamically active boundaries, collectively referred as blood-uterine

lumen barrier [32]. Accordingly, transport and permeability properties of this barrier are influenced by factors such as steroid hormones. In rats treated with estrogen, the blood-uterine lumen barrier exhibited a selective permeability according to the biochemical nature and molecular weight of radioactive substances injected (i.e., urea, sucrose, insulin or bovine serum albumin) [33]. Furthermore, using an regimen of steroid treatment in rats, at the P4 dominance state, uterine glands showed fluid absorptive ability, while at E2 dominance state, the ability became secretory, rather than absorptive [34,35]. Thus, during the initial diestrus, we speculate that the sex-steroid exposure may play an important role to timely regulate the restoration of the uterine luminal condition. Under the expected condition of early diestrus, i.e. progressive increase of circulating P4 concentrations, at least 4 d was necessary before the uterine luminal condition was restored.

Perturbations on the composition of ULF decreased fertility to ET. Consistent with the degree of alterations measured in the ULF at D7.5 on Experiment 1, pregnancy rates were poorer when ULF collection was conducted at time points closer to D7.5 (Fig. 5). Accordingly, ULF collection performed at D4 or D7 reduced about 50% the pregnancy maintenance to d 25, but fertility was not impacted by collection of ULF performed on D1. Interestingly, collection of ULF conducted just 12 hours prior to embryo transfer, did not completely abolish pregnancy. This indicates that the native uterine milieu is permissive to deviations, although pregnancy establishment is hampered. Probably, disturbed luminal conditions exceeded the limits of tolerance of embryos in a large proportion of recipient submitted to flushing on D4 and D7, increasing the frequency of embryonic losses. Complacency of uterine luminal condition from D4-ULF and D7-ULF to embryonic survival may be also related to the similarity between the proteins found in the uterine luminal fluid and serum. In this regard, early electrophoretic evaluation, revealed that uterine fluid consisted mainly of serum proteins and small amount uterine-specific proteins [36,37]. Despite of gross similarities, more recent proteomic analysis demonstrated that many plasma proteins identified in the uterine fluid had significantly different concentrations than in the plasma [38]. Thus, while suboptimal uterine condition produced by ULF-D4 and ULF-D7 is still compatible with embryonic survival, a well-defined and balanced intrauterine condition is probably required for optimal pregnancy rates. Finally, it is noteworthy, that even when three embryos were transferred, the incidence of embryonic losses in the Control group reached 37.5%,

which is similar to previous reports [6–8]. This supports the concept that despite increasing the odds of embryo survival, poor uterine receptivity limited pregnancy success. This study showed that subtle perturbations of the uterine environment, such as those caused by a single, low-volume collection of ULF, profoundly disturbs intrauterine composition and pregnancy success. However, after at least 4 d of the insult, the uterus has the capacity to recover its composition and the functional capacity to carry post-implantation gestation.

5.6 REFERENCES

1. Spencer TE, Johnson GA, Bazer FW, Burghardt RC. Implantation mechanisms: Insights from the sheep. Reproduction. 2004;128:657–68.

2. Spencer TE, Johnson GA, Bazer FW, Burghardt RC, Palmarini M. Pregnancy recognition and conceptus implantation in domestic ruminants: Roles of progesterone, interferons and endogenous retroviruses. Reprod Fertil Dev. 2007;19:65–78.

3. Gray CA, Burghardt RC, Johnson GA, Bazer FW, Spencer TE. Evidence that absence of endometrial gland secretions in uterine gland knockout ewes compromises conceptus survival and elongation. Reproduction. 2002;124:289–300.

4. Gray CA, Taylor KM, Ramsey WS, Hill JR, Bazer FW, Bartol FF, et al. Endometrial glands are required for preimplantation conceptus elongation and survival. Biol Reprod. 2001;64:1608–13. Available from: http://www.ncbi.nlm.nih.gov/pubmed/11369585

5. Bazer FW, Spencer TE, Johnson GA, Burghardt RC, Wu G. Comparative aspects of implantation. Reproduction. 2009;138:195–209.

6. Diskin MG, Sreenan JM. Fertilization and embryonic mortality rates in beef heifers after artificial insemination. J Reprod Fertil. 1980;59:463–8.

7. Diskin MG, Parr MH, Morris DG. Embryo death in cattle: An update. Reprod Fertil Dev. 2012;24:244–51.

8. Wiltbank MC, Baez GM, Garcia-Guerra A, Toledo MZ, Monteiro PLJ, Melo LF, et al. Pivotal periods for pregnancy loss during the first trimester of gestation in lactating dairy cows. Theriogenology. 2016;86:239–53. Available from: http://dx.doi.org/10.1016/j.theriogenology.2016.04.037

9. Bauersachs S, Ulbrich SE, Gross K, Schmidt SEM, Meyer HHD, Einspanier R, et al. Gene expression profiling of bovine endometrium during the oestrous cycle: detection of molecular pathways involved in functional changes. J Mol Endocrinol. Soc Endocrinology; 2005;34:889–908.

10. Bauersachs S, Mitko K, Ulbrich SE, Blum H, Wolf E. Transcriptome studies of bovine endometrium reveal molecular profiles characteristic for specific stages of estrous cycle and early pregnancy. Exp Clin Endocrinol Diabetes. 2008;116:371–84.

11. Mesquita FS, Ramos RS, Pugliesi G, Andrade SCS, Van Hoeck V, Langbeen A, et al. The Receptive Endometrial Transcriptomic Signature Indicates an Earlier Shift from Proliferation to Metabolism at Early Diestrus in the Cow. Biol Reprod. 2015;93:52–52. Available from: http://www.biolreprod.org/cgi/doi/10.1095/biolreprod.115.129031

12. Faulkner S, Elia G, O'Boyle P, Dunn M, Morris D. Composition of the bovine uterine proteome is associated with stage of cycle and concentration of systemic progesterone. Proteomics. 2013;13:3333–53. Available from: http://doi.wiley.com/10.1002/pmic.201300204

13. Mullen MP, Elia G, Hilliard M, Parr MH, Diskin MG, Evans ACO, et al. Proteomic characterization of histotroph during the preimplantation phase of the estrous cycle in cattle. J Proteome Res. 2012;11:3004–18.

14. Mullen MP, Bazer FW, Wu G, Parr MH, Evans ACO, Crowe MA, et al. Effects of systemic progesterone during the early luteal phase on the availabilities of amino acids and glucose in the bovine uterine lumen. Reprod Fertil Dev. 2014;26:282–92.

15. Forde N, Simintiras CA, Sturmey R, Mamo S, Kelly AK, Spencer TE, et al. Amino acids in the uterine luminal fluid reflects the temporal changes in transporter expression in the endometrium and conceptus during early pregnancy in cattle. PLoS One. 2014;9.

16. França MR, da Silva MIS, Pugliesi G, Van Hoeck V, Binelli M. Evidence of endometrial amino acid metabolism and transport modulation by peri-ovulatory endocrine profiles driving uterine receptivity. J Anim Sci Biotechnol. 2017;8:1–14.

17. Ramos RS, Oliveira ML, Izaguirry AP, Vargas LM, Soares MB, Mesquita FS, et al. The periovulatory endocrine milieu affects the uterine redox environment in beef cows. Reprod Biol Endocrinol. 2015;13:39. Available from: http://www.rbej.com/content/13/1/39

18. Rowson LEA, Lawson RAS, Moor RM, Baker AA. Egg transfer in the cow: synchronization requirements. J Reprod Fertil. 1972;28:427–31. Available from: http://www.reproduction-online.org/content/28/3/427.full.pdf

19. Rowson LE, Moor RM. Embryo transfer in the sheep: the significance of synchronizing oestrus in the donor and recipient animal. J Reprod Fertil. 1966;11:207–12.

20. Randi F, Fernandez-Fuertes B, McDonald M, Forde N, Kelly AK, Amorin HB, et al. Asynchronous embryo transfer as a tool to understand embryo–uterine interaction in cattle: is a large conceptus a good thing? Reprod Fertil Dev. 2016;28:1999–2006. Available from: https://doi.org/10.1071/RD15195

21. Carter F, Forde N, Duffy P, Wade M, Fair T, Crowe MA, et al. Effect of increasing progesterone concentration from Day 3 of pregnancy on subsequent embryo survival and development in beef heifers. Reprod Fertil Dev. 2008;20:368–75.

22. Mann GE, Lamming GE. The Influence of Progesterone During Early Pregnancy in Cattle. Reprod Domest Anim. 1999;34:269–74.

23. Pugliesi G, Miagawa BT, Paiva YN, Franca MR, Silva LA, Binelli M. Conceptus-Induced Changes in the Gene Expression of Blood Immune Cells and the Ultrasound-Accessed Luteal Function in Beef Cattle: How Early Can We Detect Pregnancy? Biol Reprod. 2014;91:95–95. Available from: http://www.biolreprod.org/cgi/doi/10.1095/biolreprod.114.121525

24. Pontes JHF, Melo Sterza FA, Basso AC, Ferreira CR, Sanches B V., Rubin KCP, et al. Ovum pick up, in vitro embryo production, and pregnancy rates from a large-scale commercial program using Nelore cattle (Bos indicus) donors. Theriogenology. 2011;75:1640–6. Available from: http://dx.doi.org/10.1016/j.theriogenology.2010.12.026

25. Berg DK, van Leeuwen J, Beaumont S, Berg M, Pfeffer PL. Embryo loss in cattle between Days 7 and 16 of pregnancy. Theriogenology. 2010;73:250–60.

26. Kastelic JP, Northey DL, Ginther OJ. Spontaneous embryonic death on days 20 to 40 in heifers. Theriogenology. 1991;35:351–63.

27. Kastelic JP, Curran S, Ginther OJ. Accuracy of ultrassonography for pregnancy diagnosis on days 10 to 22 in heifers. Theriogenology. 1989;31:813–20.

28. David Eckersall P. Chapter 5 - Proteins, Proteomics, and the Dysproteinemias A2 - Kaneko, J. Jerry. In: Harvey JW, Bruss MLBT-CB of DA (Sixth E, editors. San Diego: Academic Press; 2008. p. 117–55. Available from: https://www.sciencedirect.com/science/article/pii/B9780123704917000052

29. Guise MB, Gwazdauskas FC. Profiles of Uterine Protein in Flushings and Progesterone in Plasma of Normal and Repeat-Breeding Dairy Cattle. J Dairy Sci. 1987;70:2635–41. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0022030287803338

30. Alavi-Shoushtari SM, Asri-Rezai S, Abshenas J. A study of the uterine protein variations during the estrus cycle in the cow: A comparison with the serum proteins. Anim Reprod Sci. 2006;96:10–20.

31. Velazquez MA, Parrilla I, Van Soom A, Verberckmoes S, Kues W, Niemann H. Sampling techniques for oviductal and uterine luminal fluid in cattle. Theriogenology. 2010;73:758–67. Available from: http://dx.doi.org/10.1016/j.theriogenology.2009.07.004

32. Mcrae AC. The blood-uterine lumen barrier and exchange between extracellular fluids. 1988;857–73.

33. Mcrae AC, Kennedy TG, Grant C, Mcrae AC, Kennedy TG. Evidence for a Permeability Barrier between Blood and Uterine Luminal Fluid in Estrogen-Treated, Immature Rats1. Biol Reprod. 1979;20:919–23. Available from: http://dx.doi.org/10.1095/biolreprod20.4.919

34. Naftalin RJ, Thiagarajah JR, Pedley KC, Pocock VJ, Milligan SR. Progesterone stimulation of fluid absorption by the rat uterine gland. Reproduction. 2002;123:633–8.

35. Salleh N, Baines DL, Naftalin RJ, Milligan SR. The hormonal control of uterine luminal fluid secretion and absorption. J Membr Biol. 2005;206:17–28.

36. Roberts GP, Parker JM. Macromolecular components of the luminal fluid from the bovine uterus. J Reprod Fertil. 1974;40:291–303. Available from: http://www.reproduction-online.org/content/40/2/291.abstract

37. Dixon SN, Gibbons A. Proteins in the uterine secretions of cows. J. Reprod. Fert. 1978; 56: 119-127

38. Faulkner S, Elia G, Mullen MP, O'Boyle P, Dunn MJ, Morris D. A comparison of the bovine uterine and plasma proteome using iTRAQ proteomics. Proteomics. 2012;12:2014–23.

GENERAL CONCLUSIONS

In this thesis, we aimed to clarify how the embryo interacts with the maternal unit to establish pregnancy in a receptive- and non-receptive uterine model. Receptive-uterine status was induced pharmacologically through injection of long-acting P4 (iP4) at early diestrus. This receptive-uterus model was used to answer two main questions: (1) Does supplementary P4 hamper pregnancy rates by increasing the incidence of early luteolysis, and (2) Can exposure of the uterus to the embryo decrease the incidence of early luteolysis? On the other hand by producing a non-receptive uterine model, which was obtained by carrying out uterine luminal flushings collections at early stages of diestrus, we sought to answer a third question: (3) Is a non-receptive uterine status compatible with pregnancy? To address the first two questions, four studies were carried out (chapters 2 to 5). A final study was performed to answer the last question (chapter 6).

Early luteolysis has been considered as the main cause associated to the absence or negative effect on pregnancy rates of cows supplemented with P4 at early diestrus. It has been hypothesized that early P4 supplementation advances uterine events, leading to luteolysis taking place before the maternal recognition of pregnancy, and subsequent embryonic loss. However, our results don't support this paradigm. In the first study, prevention of early luteolysis in cows supplemented with iP4, was not associated with an increment of pregnancy per TAI (P/TAI). Indeed, injection of estradiol cypionate (EC) at the end of the TAI protocol was successful in reducing the incidence of P4-induced early luteolysis (EC: 8.3% vs. NoEC: 26.0%), but P/TAI was not affected. Further analysis revealed that pregnancy outcome was not merely dictated by the incidence of early luteolysis in these animals. Rather than this, we evidenced a scenario where the pre-ovulatory period plays an important role on determining iP4 fertility response. Interestingly, fertility response to iP4 supplementation was modulated according to EC treatment and diameter of dominant follicle at TAI. For animals treated with EC, an iP4-positive effect on fertility (11 to 13% increase) occurred in cows where the dominant follicle was <12.35 mm in diameter. Conversely, for those animals that where not treated with EC, an iP4-

positive effect occurred in a subpopulation of cows that had a dominant follicle diameter of ≥12.35 mm. Collectively, these findings support the idea that the uterus plays an important role in modulating the iP4- luteolytic and embryotrophic effects. Apparently, an optimal uterine exposure to pre-ovulatory estradiol is required to supplementary P4 stimulate fertility. Suboptimal E2 estradiol exposure may result in null effects of iP4 on fertility, whereas overexposure to E2 may result in negative effects of iP4 on fertility. Furthermore, we have evidence for the first time a pre-ovulatory component that drives the response towards early P4 supplementation. The importance of this finding relies on the fact that different hormonal combinations are currently available to synchronize estrus. Because the fertility of iP4 supplemented animals can be affected by the exposure of the uterus to hormones during the pre-ovulatory period, we believe that subtle alterations of the synchronization protocol can impact the net fertility outcome after iP4 supplementation.

The role of uterine exposure to an embryo in P4-induced early luteolysis was studied in the second and third studies of this thesis. To determine whether the embryo induces changes in iP4-luteolytic response, a group of cows were AI and luteolysis was assessed throughout a 21 days period. Additionally, we evaluated different ovarian factors related to the iP4-luteolytic response. In accordance with the findings of our first study, we evidenced that the iP4-luteolytic effect was mainly a consequence of precocious exposure of the uterus to elevated P4 concentrations, which probably led to advances in the uterine luteolytic cascade. We also verified that iP4 impaired luteal development, probably due to the high P4 reducing the luteal LH support. However, this impairment on CL formation seems to affect the iP4-luteolytic effect only when early luteal demise occurs at very early stages of diestrus. Therefore, our results revealed that iP4 supplementation did not produce a classical short cycle (i.e., CL regression within 2 weeks post-estrus), but it accelerated luteolysis onset by one day. Considering the characteristics of the luteolytic phenomenon studied here, where the uterus had a pivotal role, the embryo becomes more relevant in changing the luteolytic stimulus. Nonetheless, the effect of AI on downregulating the luteolytic process was dictated by the establishment of pregnancy. In other words, for pregnancy to be established, the embryo has to be competent and has to interact properly with the iP4-stimulated uterine environment. The dynamics of luteolysis in iP4 supplemented non-pregnant AI cows (i.e. a uterus

that could have had exposure to an embryo) were similar to the iP4-supplemented but not-AI cows. These findings give us an alternative interpretation of study 1. The observed effect of EC reducing early luteolysis incidence, could be related to the effect of EC increasing P/TAI (EC: 51.6% vs. No-EC: 35.0%). In fact, it is well-described in the literature that a uterine environment stimulated by adequate concentrations of pre-ovulatory E2 is more supportive of embryonic survival. Perhaps by indirectly stimulating embryonic survival through the modulation of the uterine environment, EC treatment stimulated the conceptus-triggered antiluteolytic effect.

The findings of the third study further corroborated the hypothesis that the uterus is the central component for the iP4-luteolytic effect. Follicular growth dynamics during diestrus was also involved in iP4-induced early luteolysis, probably through regulation of uterine function. This was evidenced by the fact that cows exhibiting early luteolysis had a smaller dominant follicle from first follicular wave, likely resulting in lesser uterine exposure of E2 at early diestrus. Interestingly, the number of follicular waves during mid- to late diestrus did not affect iP4-induced luteolytic outcome. However, three-wave cycles were positively associated with the conceptus-driven antiluteolytic effect, suggesting that ovarian features during mid- to late diestrus play a role in the iP4-induced embryotrophic responses. Therefore, ovarian factors during early diestrus drive the iP4-induced luteolytic response, and those at mid- to late diestrus drive the P4-embryotropic responses. The fact that the number of follicular waves during diestrus may change the P4-embryotropic response underlines the idea that a more supportive uterine environment favors the ability of the embryo to block the early luteolytic stimulus.

In the fourth study, by using a multiple embryo transfer model (5 embryos per recipient) to minimize the individual embryonic response variability to supplementary P4, we aimed to determine whether a more receptive uterine environment reduces early and late embryonic mortality. The iP4-embryotropic effect was evidenced by an enhanced conceptus signaling, measured as increased *ISG15* expression in peripheral blood mononuclear cells. Despite this clear positive effect, iP4 treatment doubled the incidence of early embryonic mortality (Non-iP4: 22.2% vs. iP4: 44.4%), but did not alter late embryonic mortality (Non-iP4: 22.2% vs. iP4: 18.6%). Intriguingly, even after the transfer of 5 embryos, the proportion of non iP4-supplemented cows that experienced early embryonic mortality remained high, and approached the rate that has been described in the literature (30%). While this points

to the fact that the problem lies within the recipient and that it was already present at the time of transfer, supplementary P4 did not mitigate embryonic losses. It is important to highlight that in this study there was no evidence of early luteolysis. Therefore, the detrimental luteolytic aspect could not serve as an explanation for the absence of positive effects due to iP4 on embryonic survival. This reinforces the hypothesis that P4-luteolytic effect is not necessarily the cause of the non-positive effect of P4 supplementation on fertility. In this study, the absence of a positive effect due to iP4 supplementation on embryo survival seemed to be related to a lack of additional P4 requirements. Indeed, early or late embryonic mortality was not associated to low circulatory P4 concentration. It would seem that iP4 supplementation induced a loss of synchrony between the developmental stimulus required by the embryo and the uterine environment, which hampered embryonic survival. Thus, the benefits of supplementary P4 on fertility may be restricted to the conditions where female fertility is limited. Our first study serves as a clear example, in that experiment iP4 supplementation favored fertility of cows that had small dominant follicles at TAI (<12.35 mm). However, the absence of an expected P4-luteolytic effect was intriguing. This could be due to differences in the estrous synchronization protocol used in this study, versus the one used in the second and third studies (where a clear iP4-luteolytic effect was evidenced). In this third experiment, a single dose of PGF2α analogue was administrated a day before intravaginal P4 device removal, whereas in previous studies PGF2α analogue was injected at the beginning and at the end of the protocol. Because all cows used in the three studies were cyclic, and thus had a CL, the uterine exposure to P4 during estrous synchronization was possibly higher in the third study due to the fact that the CL was not regressed by an initial dose of PGF2α. It is important to point out that the intravaginal P4 implants used in all studies were always new. This speculative assessment, would suggest that alterations during the estrous synchronization protocol can also alter the iP4 response.

The last study of this thesis was carried out to evaluate if a non-receptive uterus can be compatible with pregnancy. The non-receptive status was induced through collection of uterine luminal flushings (ULF) at days 1, 4 and/or 7 post-estrus. The aim of these ULF collections was to deplete the uterine luminal milieu, in order to evaluate the histotroph requirements for the maintenance of pregnancy. Interestingly,

protein content analysis of ULF collected on day 7.5, evidenced that ULF collection procedure led to an increase of total protein concentration. Only day 4 or 7 collection of ULF induced this paradoxical effect observed on day 7.5, which was likely due to an influx of proteins from the blood to the uterine lumen. Albumin, a common serum protein, was about 2.0-fold higher than control when ULF was collected on D4 or D7, but unchanged when ULF was collected on D1. Accordingly, we verified that it takes a relative long time (i.e. at least 4 days) for the uterine environment to be restored after its disruption. Collectively, these findings validated our model for the subsequent trial where three embryos were transferred on D7.5. Conception rates to ET were consistent with the time dynamics of ULF collection. This was evidenced by lower conception rates (about 50% reduction) when ULF collection took place closer to the day of ET (i.e. D4 and D7). However, even when ULF collection was conducted just 12 hours prior to embryo transfer (D7.5), approximately 30% of recipients were still able to maintain pregnancy. This indicates that the native uterine milieu is permissive to deviations, but pregnancy establishment is profoundly affected. Furthermore, even after transferring 3 embryos, about one third of control cows were not able to conceive. This substantial number of recipient cows, who could not conceive, indicates that poor uterine receptivity limited pregnancy success. Collectively, these data underlie the importance of an appropriate intrauterine milieu for pregnancy success in cattle. Identification of cows with poor uterine environment will help to overcome the high rates of embryonic loss in cattle.

In conclusion, our data has shown that the uterus plays a central role in supplementary P4-induced early luteolysis. Although the P4-luteolytic effect has been traditionally considered to be the main factor that blocks the beneficial effects of supplementary P4 on fertility, this negative relationship was not clear in our studies. Because both P4-induced luteolytic and embryotrophic effects are driven by the uterus, alterations in the uterine programming, such as those caused by follicular steroids during the pre-ovulatory period, early diestrus and mid- to late- diestrus; are fundamental on determining the response to P4. Remarkably, we demonstrated for the first time that the pre-ovulatory period dictates P4-luteolytic and P4-embryotropic responses, and, as a result, influences the net fertility outcome to supplementary P4. At early diestrus, further reduction on dominant follicle diameter from first follicular wave, was related to the shortened luteal lifespan. On the other hand, during mid- to late diestrus, three-wave cycles seemed favorable for the effectiveness of the

conceptus-driven antiluteolytic effect. Considering the importance of the pre-ovulatory period on iP4 response, hormonal changes induced by TAI programs may affect the fertility outcome of iP4 supplementation. These findings are schematically illustrated on Figure 1. Furthermore, we have evidenced the central role of uterine environment as causative of embryonic loss (Figure 1). Both, a receptive- and non-receptive uterine status may be associated to higher frequency of embryonic losses. Overall, the variability in fertility rates after P4 supplementation, are in part attributable to the complexity of the uterine programming by steroids. Therefore, identifying certain ovarian conditions or manipulating the steroid composition and availability during the TAI protocol may increase pregnancy success to supplementary P4.

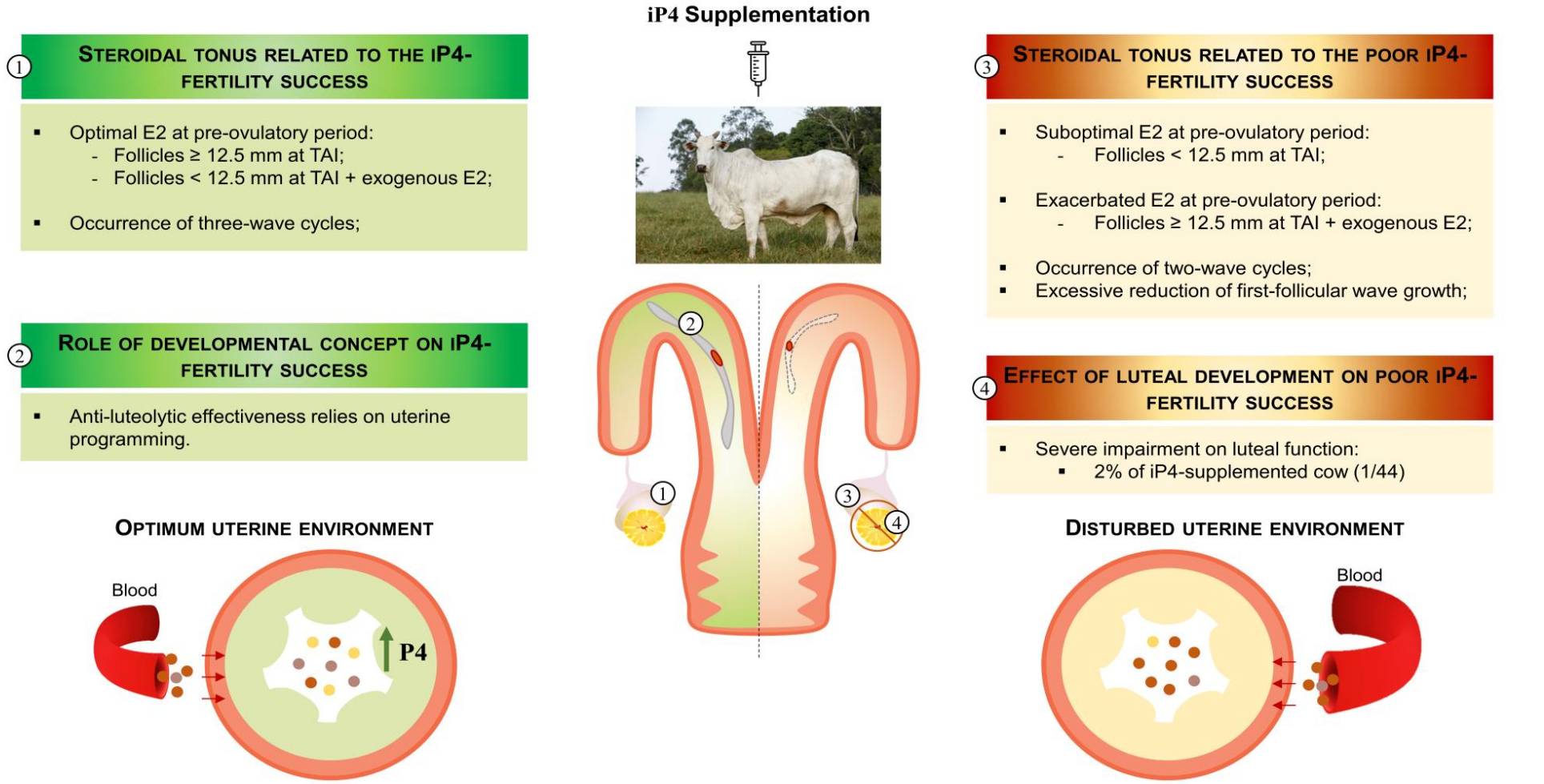

Figure 1. **Summary of main results**. In this thesis, four studies were carried out to evaluate the role of pre-implantational conceptus on preventing P4-luteolytic effects in *Bos indicus* beef cows, due the supplementation with 150 mg of long acting P4 (iP4) on day 3 post-ovulation. In a fifth study, the importance of the uterine luminal milieu on establishment of pregnancy was evaluated. For this, uterine luminal flushings were performed on days 1, 4 and/or 7 post-estrus, aiming to deplete the uterine milieu. We evidenced that the uterus plays a central role on determining the iP4-luteolytic response. Accordingly, (i) administration of estradiol cypionate at preovulatory period prevent P4-luteolytic effect and (ii) further reduction of first follicular wave by supplementary iP4, then probably lower follicular estradiol uterine exposure, was related to the P4-luteolytic effect. Interestingly, supplementation with iP4 hindered luteal development, but only one cow in the entirely second study presented early luteal demise (Day 8), due the luteal cause. About half of iP4-supplemented cows presented early luteolysis, which occurred by day 15 post-ovulation. Thus, the iP4-induced luteolytic process was not so advanced. Despite of this, the role of conceptus on preventing the iP4-luteolytic effect was restricted to the embryonic capability in establish pregnancy. In this sense, three-wave cycles favored the antiluteolytic conceptus signaling. Further, we verified that beneficiary effect of supplementary iP4 on fertility was dependent of uterine exposure to estradiol at preovulatory period. Collectively, those findings indicate that the anti-luteolytic effectiveness of conceptus in an uterine environment stimulated by supplementary P4 relies on uterine programming. Remarkably, the central role of the uterine environment on this process was underlined when 5 *in vitro* produced embryos were transferred to the recipient cows and the embryonic mortality frequency was not ameliorated by supplementary iP4. Rather than, the embryonic survival still driven by the uterine environment conditions. In this sense, disturbance on the composition of the uterine environment by uterine flushings impaired pregnancy when flushings were performed close to the embryo transfer time, but did not abolish embryonic survival. Again highlighting that optimal uterine environment is necessary to establishment of pregnancy, despite of a disturbed uterine environment still be compatible with pregnancy.

APPENDICES AND SUPPLEMENTAL MATERIAL

APPENDIX A

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Impact of estradiol cypionate prior to TAI and progesterone supplementation at initial diestrus on ovarian and fertility responses in beef cows

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ABSTRACT

In cattle, early diestrus progesterone (P4) supplementation modulates endometrial function to exert pro- and anti-pregnancy establishment effects; specifically, P4 stimulates conceptus growth, but also induces early onset of luteolysis. This paradoxical effect is frequently related to the inconsistent fertility outcomes that result from P4 supplementation experiments. Aim was to investigate the impact of exogenous estradiol (E2) treatment at the end of timed fixed AI (TAI) on frequency of early luteolysis and pregnancy of beef cows supplemented with P4. Ovulations (D0 of study) of suckled multiparous (n = 643) and primiparous (n = 193) Nelore cows (*Bos indicus*) were synchronized with an E2/P4-based protocol for TAI and assigned to receive 1.0 mg of estradiol cypionate (CP) or nothing (NoCP) on D−2 and 150 mg of injectable long-acting P4 (iP4) or Placebo (NoiP4) on D4 on a 2 × 2 factorial arrangement. On D15, the iP4 supplementation increased ($P < 0.05$) the frequency of early luteolysis (NoCP + iP4: 26.0%; [13/50] vs. NoCP: 8.0% [4/50]), but CP prevented this effect (CP + iP4: 8.3% [4/48] and CP: 6.4% [3/47]). The CP improved pregnancy/AI (P/AI) of multiparous (CP: 51.6% [165/320] and NoCP: 35.0% [113/323]; $P < 0.001$) and primiparous cows (CP: 40.4% [40/99] and NoCP: 24.5% [23/94], $P < 0.05$), regardless of iP4 treatment. The iP4 supplementation affected P/AI of CP and NoCP treated cows according to follicle size at TAI. For the CP treated cows, the iP4 supplementation improved P/AI of sub-populations of cows with follicles <12.35 mm (42.0% [34/81] vs. 53.1% [34/64]), while for NoCP treated cows, the improvements occurred in subpopulations of cows with follicles ≥12.35 mm (46.1% [35/76] vs. 58.7% [37/63]). In conclusion, strategies associating E2 and P4 supplementation decrease the incidence of early onset of luteolysis and improve P/AI of suckled beef cows with smaller follicles.

1. Introduction

In addition to genetic gains, timed artificial insemination (TAI) programs in beef cattle improves reproductive efficiency because it overcomes challenges associated to long anestrous periods and estrus detection, that delay the time to first service post-partum. Despite of such benefits, cows induced to ovulate dominant follicles smaller than 11.0 mm at TAI present low pregnancy per AI (P/AI) [1–3]. Such poor results are mainly attributable to the insufficient uterine exposure to estradiol (E2) and progesterone (P4) at the pre-ovulatory (proestrus/estrus) and post-ovulatory (diestrus) periods [4,5], respectively. Indeed, aiming to achieve an adequate sequential uterine exposure to E2 and P4, different strategies to stimulate follicle growth in beef cattle submitted to TAI were proposed [6,7]. For example, consistent fertility gains in TAI protocol have been achieved by extending the proestrus period [8,9] or adding exogenous E2 [5,10,11]. An adequate uterine exposure to E2 affects positively the fertilization process [12], reduces the incidence of early luteolysis [13,14], and provides an uterine

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APPENDIX B

APPENDIX C

Elsevier Editorial System(tm) for Domestic Animal Endocrinology

Manuscript Draft

Manuscript Number:

Title: Supplementation with long-acting progesterone in early diestrus in beef cattle: I. Effect of artificial insemination on onset of luteolysis

Article Type: Original Research Paper

Keywords: corpus luteum, pregnancy, embryo, cattle

Corresponding Author: Dr. Mario Binelli,

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Abstract: In beef cattle, progesterone (P4) supplementation in early diestrus advances changes in the endometrial transcriptome causing detrimental (early onset of luteolysis) and stimulatory (accentuated conceptus growth) effects on fertility. Hypothesis was that AI decreases incidence of early luteolysis in cows supplemented with P4 at early diestrus. Non-suckled beef cows received AI 12h after estrus (D0: ovulation) or were not inseminated (no-AI). On D3, the AI cows received 150 mg injectable P4 (AI+iP4; n=23) and no-AI cows received iP4 (iP4; n=21) or saline (Control, n=22). CL development and pregnancy were determined by ultrasonography and blood P4 concentrations by radioimmunoassay. iP4 reduced CL development between D5 and D10. Proportion of cows undergoing luteolysis by D15 in the iP4 group was greater than in the Control group and proportion on the AI+iP4 group was intermediate. Interval to luteolysis was similar between groups iP4 and AI+iP4 but smaller than the Controls. P4 concentrations on D15 of the iP4 group were lower than those of control and AI+iP4 groups. AI only inhibited early luteolysis in cows detected pregnant in the AI+iP4 group. Remaining animals in this group showed frequency of luteolysis by D15 and interval to luteolysis similar to the iP4 group. When cows were partitioned according to day of luteolysis (≤ 15d or > 15d), CL development from D3 to D10 was similar. In conclusion, AI was only able to overcome iP4-induced early luteolysis if pregnancy was established and maintained. Also, iP4 deleterious effects on CL development were not associated with early luteolysis.

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Opposed Reviewers:

APPENDIX D

Elsevier Editorial System(tm) for Domestic Animal Endocrinology

Manuscript Draft

Manuscript Number:

Title: Supplementation with long-acting progesterone in early diestrus in beef cattle: II. Relationships between follicle growth dynamics and luteolysis

Article Type: Original Research Paper

Keywords: embryo, pregnancy, first follicular wave, estradiol

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Abstract: The aims were to characterize follicular growth and timing of luteolysis in cows supplemented with injectable progesterone (iP4) at early diestrus and submitted or not to AI. Non-suckled beef cows detected in estrus were assigned to receive AI or to remain no-AI. Three days after ovulation (D3), AI cows received 150 mg of iP4 (AI+iP4; n=22) and the no-AI cows received iP4 (n=19) or saline (Control, n=19). CL development and pregnancy were determined by ultrasonography (P: pregnant and NP: non-pregnant). Blood P4 concentrations were measured by radioimmunoassay. iP4 decreased growth of first-wave DF (DF1). Daily diameter of DF1 in iP4-treated cows was smaller when luteolysis occurred by day 15 compared to later. iP4 supplementation did not affect day of emergence of subsequent follicular waves, nor the proportion of two- or three-wave cycles. Daily mean diameter of DF2 and DF3 was similar between groups. P4 concentrations between D9 to D19 decreased earliest in iP4, latest in Control and intermediate in NP-AI+iP4 group. Three-wave cycles presented a delayed decrease on plasma P4 than two-wave cycles. On two-wave cycles, P4 concentrations on D15 were lowest in iP4 and NP-AI+iP4 compared to the Control and P-AI+iP4 groups. In three-wave cycles, on D15, P-AI+iP4, NP-AI+iP4 and Controls had greater P4 concentrations than iP4 group. In summary, in iP4-supplemented cows, DF1-estradiol inhibits iP4-induced early luteolysis and three-wave cycles post-AI are more supportive of pregnancy maintenance after iP4. We speculate that such characteristics are critical to define the embryonic ability to inhibit iP4-induced early luteolysis.

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APPENDIX E

Journal of Animal Science and Biotechnology

Exacerbated conceptus signaling does not favor establishment of pregnancy in beef cattle

--Manuscript Draft--

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Abstract:	Background: Inadequate, insufficient production of anti-luteolytic signals by the pre-attachment embryo is considered a major cause of pregnancy failure in cattle. We tested the hypothesis that multiple-embryo transfers (n=5/recipient) and progesterone supplementation amplify anti-luteolytic signaling and reduce embryonic losses in beef cattle. Cows detected in estrus (D0; n=104) were assigned randomly to receive 150 mg of injectable long-acting P4 (iP4) or vehicle (non-iP4) on D4 and transcervical transfer of none or 5 embryos produced in vitro, on D7. Luteal development and time of structural luteolysis was monitored by ultrasound scans. Plasma P4 concentrations were determined on D4, D5 and D7, and daily between D14 and D20. Embryo signaling was monitored by abundance of interferon-stimulated gene 15 (ISG15) in peripheral blood mononuclear cells isolated on D14, D16, D18 and D20. Early embryonic mortality (EEM) was defined as the absence of ISG15 mRNA upregulation over time and/or luteal regression up to D20. Late embryonic mortality (LEM) was defined as the absence of embryonic vesicles with a heartbeat on pregnancy diagnosis at D30 (PD) after observing upregulation of ISG15 mRNA and extension of luteal lifespan. Cows with viable embryonic vesicle(s) were considered pregnant. Results: On D5, iP4-treated cows had P4 concentrations 2.07-fold greater than non-iP4 treated (P<0.001). On D7, P4 concentrations were similar. Pregnant and LEM animals had a progressive increase on the abundance of ISG15 from D14 to D20. iP4-treated cows detected pregnant at PD had 1.53-fold greater abundance of ISG15 mRNA between D14 and D20 than non-iP4 treated cows (P= 0.05). iP4 treatment did not shorten CL lifespan, but hindered CL development. iP4 doubled the frequency of EEM while it did not affect LEM. At PD, embryonic survival was 37.0% vs. 55.6% for iP4-treated vs. control cows. Conclusions: A substantial proportion of cows presented EEM (31%) and LEM (20%) after transferring 5 embryos produced in vitro. This argues that mortality is due to poor uterine receptivity, pre-established at the time of transfer and could not be reversed by supplementary P4. Further, potentiating of embryonic signaling through supplementing P4 and transfer multiple embryos cannot overcome such deficits.
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APPENDIX F

Journal of Animal Science and Biotechnology

Perturbations in the uterine luminal fluid composition are detrimental to pregnancy establishment in cattle

--Manuscript Draft--

Manuscript Number:	
Full Title:	Perturbations in the uterine luminal fluid composition are detrimental to pregnancy establishment in cattle
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Funding Information:	FAPESP (2011/03226-4) — Dr. Mario Binelli FAPESP (2015/26215-9) — Mr. Thiago Martins CAPES (33002010047P6) — Mr. Thiago Martins
Abstract:	Background: A major, unresolved issue is how the uterine microenvironment determines pregnancy success in cattle. Before implantation, conceptus development depends on the uterine secretome (i.e., histotroph). Despite its pivotal role, little is known about the dynamics of histotroph synthesis and changes in composition throughout the early diestrus and the relevance to pregnancy establishment. We hypothesize that disturbances on histotroph composition affect the establishment of pregnancy. Aim was to disturb histotroph composition at early diestrus and verify the effects on: (Experiment 1) timing to restore its composition; and (Experiment 2) conception rate after multiple-embryo transfer. Estrous cycle of multiparous Nelore cows were synchronized and estrus was considered Day 0 (D0) of the experiments. Disturbance was through flushing each uterine horn with 30 mL of DMPBS and collecting the resulting uterine luminal flushing (ULF) on D1; D4; D7; D1+D4+D7. Control group remained not-collected. In Experiment 1, ULF was collected on D7.5 from all animals and used for quantification of total protein concentration and abundance of albumin. In the Experiment 2, three in vitro-produced embryos were transferred to the uterine horn ipsilateral to the ovary containing the CL on D7.5 and pregnancy was checked on D25 by ultrasound. Results: In experiment 1, ULF collection on D4 or D7 increased (1.5 to 2.2-folds) the total protein concentration and albumin abundance. ULF collection on D1 did not alter (P>0.10) these endpoints. In experiment 2, ULF collected on D4 or D7 decreased conception rates to approximately half of that measured in the remaining groups. Conclusions: Subtle perturbations imposed to the native intrauterine milieu, such as those caused by a single, low-volume collection of ULF, profoundly disturbs intrauterine composition and pregnancy success. At least 4 days were necessary for the uterus to recover its composition and the functional capacity to carry post-implantation gestation.
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