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Tese

Ações da Prostaglandina F2α e Paraoxonase-1 na ovulação e prenhez de bovinos

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Tese aprovada como requisito parcial para obtenção do grau de Doutora em Ciências, Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas.

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“Para ter algo que você nunca teve, é preciso fazer algo que você nunca fez”
Chico Xavier

Resumo

CASTRO, Natália Ávila de. **Ações da Prostaglandina F2α e Paraoxonase-1 na ovulação e prenhez de bovinos.** 2019. 97f. Tese (Doutorado em Ciências) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2019.

A ovulação é um dos processos reprodutivos mais críticos para a fêmea, por envolver uma série de alterações que afetam o equilíbrio homeostático. A inflamação, por sua vez, é definida como uma resposta adaptativa de um tecido a um estímulo nocivo, que tem por finalidade reestabelecer a homeostase. Neste sentido, a ovulação é considerada um processo inflamatório, por envolver uma série de mediadores inflamatórios. A prostaglandina F2α (PGF) e a paraoxonase-1 (PON1), que são importantes mediadores inflamatórios, já demonstraram ter funções importantes no processo de ovulação e na resposta das fêmeas a biotécnicas reprodutivas. Assim, objetivou-se identificar os efeitos e possíveis mecanismos de ação da PGF na ovulação e avaliar potenciais associações entre a PON1 e a fertilidade em bovinos. No primeiro estudo se buscou identificar se a PGF seria capaz de estimular o eixo hipotalâmico-hipofisário no processo periovulatório. Os resultados demonstraram que, isoladamente a PGF não induz qualquer alteração nos níveis de LH, sugerindo que ela não atua em nível central. Com base nesses resultados, um estudo foi conduzido para avaliar o efeito local da PGF na ovulação. Após a injeção intrafolicular de PGF, todas as vacas ovularam em um intervalo de 24 horas, entretanto, o mesmo ocorreu com as vacas que receberam placebo. Em paralelo, um estudo foi conduzido para avaliar a relação da PON1 com taxa de ovulação, crescimento folicular e prenhez por inseminação artificial em vacas de corte *Bos indicus*. Os resultados indicaram que a atividade sérica de PON1 não afetou os parâmetros avaliados. Finalmente, outro estudo foi conduzido no intuito de caracterizar polimorfismos de nucleotídeo único (SNPs) na região promotora do gene da *PON1* bovina e relacionar com a atividade sérica de PON1 em vacas *B. indicus*. Oito SNPs foram identificados, entretanto não houve associação de nenhum SNP com atividade enzimática de PON1.Coletivamente, os estudos contribuíram para o entendimento acerca das relações de dois mediadores inflamatórios com parâmetros de fertilidade em fêmeas bovinas. Entretanto, os resultados obtidos demonstram que ainda existem lacunas a serem preenchidas no intuito de identificar o mecanismo de ação da PGF na ovulação. Além disso, sugere-se que, embora a PON1 já tenha demonstrado ser um bom biomarcador de fertilidade em vacas leiteiras *B. taurus*, o mesmo não se aplica à vacas de corte *B. indicus*. Dessa forma, estudos complementares são necessários para esclarecer o mecanismo de ação destes mediadores.

Palavras-chave: prostaglandina F2α; paraoxonase-1; polimorfismo; ovulação; prenhez

Abstract

CASTRO, Natália Ávila de. **Actions of Prostaglandin F2α and Paraoxonase-1 on ovulation and pregnancy of cattle.** 2019. 97f. Thesis (Doctor degree in Sciences) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de Pelotas, Pelotas, 2019.

Ovulation is one of the most critical reproductive processes for the female involving a series of changes that affect the homeostatic balance. Inflammation, in turn, is defined as an adaptive response of a tissue to a harmful stimulus, which aims to reestablish homeostasis. In this sense, ovulation is considered an inflammatory process, as it involves a series of inflammatory mediators. Prostaglandin F2 (PGF) and paraoxonase-1 (PON1), which are important inflammatory mediators, have been shown to play important roles in the ovulation process and in the response of females to reproductive biotechniques. Thus, we aimed to identify the effects and possible mechanisms of action of PGF on ovulation and to evaluate potential associations between PON1 and fertility in cattle. In the first study we aimed to identify whether PGF would be able to stimulate the hypothalamic-pituitary axis in the periovulatory process. The results demonstrated that PGF does not induce any change in LH levels, suggesting that it does not act at the central level. Based on these results, another study was conducted to evaluate the local effect of PGF on ovulation. After intrafollicular injection of PGF, all cows ovulated within 24 hours, however, the same occurred in cows receiving placebo. In parallel, a study was conducted to evaluate the association among PON1 activity and ovulation rate, follicular growth and pregnancy by artificial insemination in *Bos indicus* beef cows. The results indicated that serum PON1 activity did not affect the evaluated parameters. Finally, another study was conducted to characterize single nucleotide polymorphisms (SNPs) in the promoter region of bovine PON1 gene and to correlate with serum PON1 activity in *B. indicus* cows. Eight SNPs were identified, however there was no association of any SNPs and PON1 enzymatic activity. Collectively, these studies contributed to the understanding of the relationships of inflammatory mediators with fertility parameters in bovine females. However, our results shown that there are still gaps to be filled in order to identify the mechanism of action of PGF in ovulation. In addition, it is suggested that although PON1 has already been shown to be a good fertility biomarker in *B. taurus* dairy cows, the same can not be applied for *B. indicus* dairy cows. Thus, complementary studies are necessary to clarify the mechanism of action of these two mediators.

Keywords: prostaglandin F2α; paraoxonase-1; polimorfism, ovulation; pregnancy

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Lista de Abreviaturas e Siglas

µg	Microgramas
ADAMTS	*A disintegrin and metalloproteinase with thrombospondin motifs*
AKR	*Aldo-Keto redutase*
BE	Benzoato de estradiol
CIDR	Implante liberador de progesterona
CL	Corpo lúteo
COX	Cicloxigenase
ECC	Escore de condição corporal
eCG	Gonadotrofina coriônica eqüina
EDTA	Ácido etilenodiamino tetra-acético
E2	Estradiol
FD	Folículo dominante
FPO	Folículo pré-ovulatório
FSH	Hormônio folículo-estimulante
g/L	Gramas por litro
GABA	Ácido gama-aminobutírico
GnRH	Hormônio liberador de gonadotrofinas
h	Horas
HDL	Lipoproteína de alta densidade
i.m.	Intramuscular
I.V.	Intravenoso
IATF	Inseminação artificial em tempo fixo
IU	Unidade internacional
Kg	Quilogramas

LH	Hormônio luteinizante
MAPK	*Mitogen-Activated Protein Kinase*
mg	Miligramas
mg/dL	Miligramas por decilitros
mg/Kg	Miligramas por quilogramas
MHz	Mega-hertz
min	Minutos
mm	Milímetros
MPF	maturation promoter fator
n	Número
ng/mL	Nanogramas por mililitro
OPU	Punção de oócitos
P4	Progesterona
PGE	Prostaglandina E2
PGF	Prostaglandina F2alfa
PON1	Paraoxonase-1
PTGS	prostaglandin-endoperoxide synthase
r.p.m	Rotações por minuto
SEM	Erro Padrão da Média
SNP	Polimorfismo de Nucleotídeo Único
SOV	Superovulação
US	Ultrassom
3β-HSD	3beta-hydroxysteroid

Sumário

1 Introdução

Em um país cuja produção de *commodities* gera uma renda anual de 79 bilhões de dólares (FAO, 2017), a produção e exportação de carne bovina se destaca. Com um efetivo de aproximadamente 215 milhões de cabeças de gado (IBGE, 2017), o Brasil é o segundo maior exportador mundial de carne bovina (FAO, 2017). Estes números demonstram a importância da atividade pecuária para o incremento do PIB nacional. Entretanto, considerando a população bovina do Brasil em comparação com a dos Estados Unidos que, com um efetivo bovino menor que a metade do brasileiro (FAO, 2017), é o maior produtor de carne do mundo (~12 milhões de toneladas), percebe-se que ainda há muito que melhorar em termos de produtividade. Em um cenário mundial em que as discussões acerca dos danos ambientais causados pela pecuária extensiva, assim como por outras atividades agropecuárias tem recebido cada vez mais destaque por parte de pessoas e instituições em torno de todo o globo, aumentar a produtividade dos rebanhos se torna ainda mais relevante.

Neste sentido, diversos estudos têm sido conduzidos no intuito contribuir para melhorar a produtividade dos nossos rebanhos por meio de seleção genética, manejos nutricionais, sanitários e reprodutivos (GRUMMER e CARROLL, 1991; LAMB et al., 2010; BANOS et al., 2013; BARCELLOS et al. 2014; SALEHI et al., 2016), sendo que esses três fatores devem estar associados. Em relação à reprodução bovina, nos últimos 30 anos muitos estudos acerca dos mecanismos fisiológicos e formas de controlar desenvolvimento folicular e ovulação (GINTHER et al, 1989; BOLT et al., 1990; MACMILLAN e BURKE, 1996; BRIDGES et al., 1999; GARCIA e SALAHEDDINE, 2001; MARTINEZ et al., 2005; CAVALIERI, 2018) têm sido feitos para que as lacunas no conhecimento ainda existentes sejam preenchidas e os índices reprodutivos sejam melhorados. Neste sentido, um período crítico e que ainda não é totalmente compreendido é o período periovulatório, envolvendo uma série de eventos e interações metabólicas e hormonais que devem ocorrer em determinada ordem e intensidade para que a ovulação seja concluída com sucesso.

A ovulação é considerada em processo inflamatório (ESPEY, 1980), que envolve inúmeros componentes, dentre os quais estão as prostaglandinas (PGs), especialmente a F2α (PGF) e a E2 (PGE) (BRIDGES e FORTUNE, 2007) que são mediadores inflamatórios originados a partir da ação das enzimas cicloxigenases 1 e 2. Além das PGs, outro fator pró-inflamatório que parece ter alguma ação sobre o oócito e afeta a fertilidade em bovinos leiteiros é a paraoxonase-1 (PON1; SCHNEIDER et al., 2013; KRAUSE et al., 2014; SILVEIRA et al., 2019). Estes dois fatores pró-inflamatórios foram o tema de estudo desta tese e serão abordados ao longo deste documento.

Período periovulatório e fatores pró-inflamatórios

A ovulação é considerada um dos processos reprodutivos mais críticos para a fêmea (FORTUNE et al., 2009) por envolver uma série de alterações que afetam o equilíbrio homeostático. A inflamação, por sua vez, é definida como uma resposta adaptativa de um tecido a um estímulo nocivo, que tem por finalidade reestabelecer a homeostase (MEDZHITOV, 2008). Entretanto, sabe-se que os processos inflamatórios não necessariamente estão associados a situações patológicas, sendo que a ovulação, que tem função reprodutiva crucial, é definida como um processo inflamatório (ESPEY, 1980). Esta definição se deve ao fato de fatores pró-inflamatórios, como os eicosanóides, apresentarem funções ovarianas importantes, embora ainda não totalmente esclarecidas, em resposta ao pico de hormônio luteinizante (LH), no processo que antecede a ovulação (ESPEY, 1980; BRIDGES e FORTUNE, 2007, DUFFY et al., 2018).

Após o estabelecimento da dominância folicular, o crescimento do folículo dominante, que antes era dependente do hormônio folículo-estimulante (FSH) passa a ser mediado pelo LH, cuja secreção hipofisária ocorre na forma de pulsos em resposta à liberação também pulsátil do hormônio liberador de gonadotrofinas (GnRH), pelo núcleo arqueado do hipotálamo (EVERET, 1994).O folículo dominante então aumenta a sua capacidade esteroidogênica, e quando a produção de estradiol folicular atinge seu nível máximo, ocorre a indução do pico de GnRH, que estimula o pico de LH e por sua vez, resulta na indução de uma série de alterações foliculares e oocitárias, até que a ovulação seja concluída. Neste sentido, define-se por período periovulatório o intervalo entre o estímulo do GnRH até logo após a ovulação, sendo este intervalo de cerca de 30 horas (BRIDGES et al., 2006).

Os eventos que ocorrem neste período são bastante complexos e ainda não foram totalmente elucidados, mas sabe-se que logo após o pico de LH, ocorre a ativação de fatores no fluido folicular que, associados à ação das células da granulosa, influenciam no processo de maturação final do oócito (RODRIGUEZ e FARIN, 2004) para que este adquira a capacidade de ser fertilizado. Este processo de maturação oocitária ocorre em nível citoplasmático e nuclear, sendo que o primeiro se caracteriza pelo aumento na atividade de cinases, como o fator promotor de maturação (MPF, do inglês *maturation promoter factor*) e a proteína quinase ativada por mitógenos (MAPK, do inglês *Mitogen-Activated Protein Kinase*), que parecem ter funções primordiais na regulação do processo de maturação nuclear oocitária (MURRAY e KIRSCHNER, 1989; KUBELKA et al., 2000). No núcleo do oócito, o pico de LH induz o reinício da meiose (EPPIG, 1991) e a quebra da vesícula germinativa (MERMILLOD et al., 1999), entre outras modificações. Em nível folicular, o pico de LH ativa simultaneamente diversas vias de sinalização intracelular, que resultam na produção, pelas células da teca e granulosa, de mediadores ovulatórios responsáveis por controlar a transcrição de genes que codificam fatores de crescimento (STOUFFER et al., 2007), moduladores da atividade vascular, quimiocinas, citocinas e proteases (DUFFY et al., 2018).

Sabe-se que produção intrafolicular de hormônios esteróides é fundamental para o sucesso da ovulação. Nesse sentido, os hormônios esteroides, como a progesterona (P4) e o estradiol (E2), assim como as prostaglandinas que são conhecidamente mediadores de respostas inflamatórias (STOUFFER et al., 2007), também possuem funções primordiais na regulação do processo ovulatório (DUFFY et al., 2018). Uma importante alteração que ocorre em resposta ao aumento na concentração de esteroides é um aumento na vascularização do folículo pré-ovulatório (FORD, 1982), o que resulta, entre outras alterações, na infiltração de leucócitos para folículo ovulatório, que atuam na remoção de células danificadas e na angiogênese (DUFFY et al., 2018). A microvascularização das células da granulosa contribui na inibição na síntese da enzima citocromo P450c17 (CYP17), responsável pela conversão de P4 em androstenediona, que será posteriormente convertida em testosterona (HALL, 1986). Em consequência disso, ocorre um acúmulo de P4 intrafolicular, que induz diversas alterações, como a síntese de colagenases pelas células da teca interna. As colagenases são responsáveis pela proteólise da parede folicular (CURRY e OSTEEN, 2003) e formação do estigma ovariano (MURDOH e

MCDONNEL, 2002) por onde o oócito será liberado no momento da ovulação. Estes mediadores são essenciais em processos inflamatórios e parecem igualmente necessários na cascata ovulatória.

Embora nem todas as alterações pré-ovulatórias estejam esclarecidas, sabe-se que alguns fatores pró-inflamatórios têm funções essenciais no processo ovulatório. Logo após o pico de LH, as células da granulosa e da teca do folículo pré-ovulatório secretam uma variedade de moléculas pró-inflamatórias, como as prostaglandinas F2α (PANG et al., 1981) e prostaglandina E2 (MURDOCH e MYERS, 1983), os leucotrienos (ESPEY et al., 1989), os tromboxanos (WILKEN et al., 1990) e a histamina (ORIOWO et al., 1987), que se acumulam no fluido folicular e exercem funções específicas no processo ovulatório. Outra alteração decorrente da proximidade da ovulação é o acúmulo de gotículas lipídicas contendo colesterol, precursor dos hormônios esteroides, nas células da granulosa (DUFFY et al., 2018). O pico de LH induz o aumento na expressão de enzimas como a citocromo P450scc, a proteína regulatória esteoidogênica aguda (STAR, do inglês *Steroidogenic acute regulatory protein*) e a 3 beta-hydroxysteroid dehydrogenase (HSD3B1), que estão envolvidas nas primeiras etapas da esteroidogênese (KING e LAVOI, 2012). Isto resulta na síntese de P4 (XU et al., 2011), a qual se caracteriza por atuar tanto como regulador chave na ovulação e luteinização. No folículo pré-ovulatório, a P4 parece regular a expressão de genes de proteases, como *a disintegrin and metalloproteinase with thrombospondin motifs* (ADAMTS), que auxiliam na expansão das células do cumulus (RUSSEL et al., 2003), na ruptura da parede folicular no momento da ovulação (ROBKER et al., 2000) e no remodelamento folicular (ESPEY et al., 2000). Importante mencionar também que o aumento nas concentrações intrafoliculares de E2 resulta em um aumento no fluxo sanguíneo e, consequentemente, em um aumento da pressão intrafolicular, que representa um fator desencadeador da ovulação.

Em resumo, muitos são os componentes e complexos são os eventos que participam da cascata ovulatória, impossibilitanto a descrição detalhada de todos esses eventos neste documento. Entretanto, participação de mediadores da resposta inflamatória em diversos tecidos caracteriza a própria ovulação como um processo inflamatório.

Ação da prostaglandina F2α na ovulação

As prostaglandinas (PGs) são eicosanoides derivados do ácido aracdônico, que após ser convertido em prostaglandina H2 (PGH2) pelas enzimas *prostaglandin-endoperoxide synthase* (PTGS, anteriormente chamadas de COX) 1 e 2, dá origem aos tromboxanos e a algumas prostaglandinas, incluindo a PGF e a PGE2 (HANNA e HAFEZ, 2018). Sobre o corpo lúteo, a PGF possui uma potente ação vasoconstritora, sendo que em bovinos a aplicação de PGF exógena é amplamente utilizada em programas de controle do ciclo estral devido a sua ação luteolítica (TSAI e WILTBANK, 1997), causando a regressão morfológica e funcional do corpo lúteo (CL). Este evento é essencial para que o folículo dominante adquira capacidade ovulatória, entretanto, sabe-se que as PGs estão associadas à diversos eventos reprodutivos (WEEMS et al., 2006; RICCIOTTI e FITZGERALD, 2011), incluindo a ovulação em diversas espécies (MURDOCH et al., 1993; DAVIES et al., 2006; PFEIFER et al., 2009, NEGLIA et al., 2012; LEONARDI et al., 2012; PFEIFER et al., 2014; DUFFY et al., 2018).

No processo de luteólise, a PGF é liberada em forma de quatro a oito pulsos discretos, induzidos pela ocitocina e liberados pelas células endometriais (MANN et al., 2006), sendo que a luteólise parece ser prejudicada na ausência de um folículo dominante (ARAÚJO et al., 2009). Por outro lado, quando é feito tratamento exógeno com PGF, sabe-se que ela é capaz de atuar sobre o CL independente da presença ou não de folículo dominante. Da mesma forma, foi demonstrado que a PGF exógena aplicada em novilhas pré-púberes (sem CL) induziu a primeira ovulação, através de um mecanismo independente da luteólise (LEONARDI et al., 2012).

Em relação ao papel das PGs na cascata ovulatória, nem todos eventos foram esclarecidos. Sabe-se que as células da teca e granulosa expressam principalmente a PTGS2 e em menor extensão a PTGS1 (DUFFY e STOUFFER, 2001), sendo que logo após o pico de LH, ocorre um rápido aumento na expressão de PTGS2 nessas células (RICHARDS, 1997). Além disso, foi demonstrado que o pico de LH induz um aumento nos níveis intrafoliculares de PGE e PGF (SIROIS e DORE, 1997), o que pode ser devido a um aumento na expressão de enzimas da família *Aldo-Keto reductase* (AKR) 1C, que são capazes de sintetizar PGF a partir de PGH2 ou de PGE em resposta ao pico de LH (DUFFY et al., 2018). Após o tratamento com GnRH em bovinos foram demonstrados aumentos transitórios na expressão de receptores de PGF e PGE nas células da teca e da granulosa (BRIDGES e FORTUNE, 2007), bem como nos níveis intrafoliculares dessas PGs 24 h após o tratamento com GnRH

(BRIDGES et al., 2006), indicando a sua importância na cascata ovulatória. Em um estudo *in vitro*, também foi demonstrado que as células foliculares produzem PGs em resposta ao pico de LH (ALGIRE et al., 1992). No entanto, a indometacina não inibiu o pico de LH (ARMSTRONG et al., 1975), indicando que as PGs devem atuar em sinergismo com o LH para completar o processo ovulatório.

Embora o papel das PGs ainda não esteja totalmente esclarecido, sabe-se que as PGE e PGF têm funções diferentes de acordo com a espécie animal. Em camundongos (NAOR et al., 2007) e primatas (DUFFY et al., 2018), a PGE parece ser a principal envolvida no estímulo à ovulação, enquanto que a PGF possui ação inibitória (NAOR et al., 2007), ao contrário do que parece ocorrer em bovinos (PFEIFER et al., 2014; PFEIFER et al., 2016).Neste sentido, os primeiros relatos indicando a PGF no processo ovulatório foram feitos a partir da década de 70, quando se relacionou a injeção de PGF ao aumento na liberação de LH em ovelhas (CARLSON et al., 1973), camundongos (RATNER et al., 1974) e vacas pós-parto (RANDEL et al., 1988).Como mecanismo de ação, Weems et al. (2006) sugeriram que a PGF exerce um efeito direto sobre a hipófise anterior para aumentar a capacidade de resposta da hipófise, e aumentar a liberação de LH (RANDEL et al., 1996). Entretanto, os efeitos da PGF sobre a secreção de LH são controversos, pois embora a sua administração cause ovulação em vacas e ovelhas em anestro (CRUZ et al., 1997; DAVIES et al., 2006), a relação com o aumento na liberação de LH é citada apenas em vacas (RANDEL et al., 1996). Assim, alguns estudos indicam que a PGF pode atuar também localmente, no ovário, para induzir a ovulação. De acordo com Murdoch et al. (1993), a PG secretada pelo folículo pré-ovulatório está intimamente ligada com o processo ovulatório. Como se sabe, a PGE e a PGF são produzidas pelas células da granulosa (BRIDGES e FORTUNE, 2007), agindo diretamente no folículo dominante.

Independente do mecanismo de ação, a capacidade de análogos de PGF induzirem ovulação em bovinos já foi demonstrada. Atualmente, existem alguns análogos de PGF disponíveis no mercado, que são especialmente utilizados em programas de controle do ciclo estral, no intuito de causar a luteólise. O Cloprostenol e o d-Cloprostenol são dois exemplos de análogos sintéticos bastante eficazes em doses menores do que as usadas com Dinoprost, que é o análogo natural, que apresenta meia-vida mais curta, sendo mais rapidamente metabolizado (BOURNE et al., 1981), em comparação com os demais.

Ao avaliar o efeito do d-Cloprostenol na indução de ovulação em protocolos de sincronização de ovulações, foi demonstrado taxas de ovulação e de prenhez semelhantes às alcançadas com o uso de ésteres de estradiol (PFEIFER et al., 2014; PFEIFER et al., 2016), tanto em bovinos de corte (PFEIFER et al., 2014), como em vacas leiteiras (PFEIFER et al., 2016). Da mesma forma, foi demonstrado uma alta taxa de sincronia nas ovulações após tratamento com PGF em vacas submetidas a protocolos a base de GnRH (CASTRO et al., 2018), permitindo que seja possível programar com segurança as inseminações no momento adequado. Estes resultados demonstram que a PGF pode induzir a ovulação de forma sincronizada, entretanto, ainda não está claro se a aplicação de PGF exógena induz a ovulação por um mecanismo local no ovário ou se atua em nível hipotalâmico em bovinos.

Paraoxonase-1, inflamação e fertilidade

A PON1 é uma enzima produzida no fígado (FERRE et al., 2002) e encontrada no sangue ligada à lipoproteína de alta densidade (HDL; DRAGANOV et al., 2000). Uma importante função atribuída a PON1 é a sua capacidade de evitar a oxidação e peroxidação da lipoproteína de baixa densidade (LDL), assim como de outras membranas celulares (DRAGANOV et al., 2005; DEAKIN et al., 2011), protegendo dos danos oxidativos. Esta enzima faz parte de um grupo de três tipos de PON (PON1, PON2 e PON3). Assim como a PON1, a PON3 é sintetizada no fígado e se liga ao HDL, apresentando a mesma função antioxidante. Apesar disso, as concentrações plasmáticas de PON3 são consideravelmente menores do que as de PON1 (DRAGANOV et al., 2000). A PON2 é menos estudada do que as demais, mas também desempenha função antioxidante e previne a peroxidação do LDL, por outro lado, é uma proteína exclusivamente intracelular (NG et al., 2001).

Por essas ações, as paraoxonases, mas especialmente a PON1, tem sido bastante estudada em humanos, em que a atividade desta enzima está relacionada com a prevenção de doenças cardiovasculares (KOWALSKA et al., 2015). Em bovinos, por ser caracterizada como uma proteína de fase aguda negativa, cuja atividade sérica reduz em resposta às citocinas liberadas durante a inflamação (BIONAZ et al., 2007), a PON1 tem sido usada como um biomarcador para diagnóstico de doenças, como as do trato uterino em vacas pós-parto (SCHNEIDER et al., 2013; KRAUSE et al., 2014, CAMPOS et al., 2017). Por outro lado, já foi demonstrada uma relação entre maiores níveis séricos de PON1 com parâmetros de fertilidade em vacas

leiteiras (KRAUSE et al., 2014; RINCÓN et al., 2016; SILVEIRA et al., 2019), assim como em humanos (BROWNE et al., 2008).

Tanto em humanos (BROWNE et al., 2008), como em bovinos (SCHNEIDER et al., 2013, CAMPOS et al., 2017) existe uma correlação positiva entre os níveis séricos e intrafoliculares de PON1, indicando que as variações sistêmicas são refletidas em nível folicular. Em mulheres submetidas a fertilização *in vitro*, o desenvolvimento embrionário e o número de células embrionárias foram superiores naquelas com maior atividade sérica e intrafolicular de PON1 (BROWNE et al., 2008). Além disso, foi recentemente demonstrado que a adição de PON1 no meio de maturação de oócitos *in vitro* melhorou o desenvolvimento embrionário inicial em bovinos (RINCÓN et al., 2016). Estes dados indicam que a PON1 pode desempenhar um papel de proteção antioxidante do óocito, se refletindo no desenvolvimento embrionário inicial subsequente (FUJIMOTO et al., 2010).

Estudos *in vivo* também demonstraram a importância da PON na reprodução animal. Em vacas de leite, a atividade de PON1 foi maior em folículos saudáveis em comparação com folículos atrésicos (SCHNEIDER et al., 2013). Neste sentido, sabe-se que folículos ovarianos de vacas que, após o parto, são afetadas por doença do trato uterino apresentam um atraso no crescimento e uma redução nas concentrações de E2 (SHELDON et al., 2002). Corroborando com esses relatos, quando uma resposta inflamatória aguda foi induzida pela injeção sistêmica de LPS, houve uma redução na expressão de STAR (CAMPOS et al., 2017), que é um importante regulador da esteroidogênese aguda, pelas células da granulosa do folículo dominante, bem como uma redução na atividade sérica e intrafolicular de PON1 (CAMPOS et al., 2017). Além disso, sugere-se que a atividade de PON1 é estimulada pelo E2 (AHMAD e SCOTT, 2010) e que folículos em crescimento possuem maior concentração de E2 e PON1 (SCHNEIDER et al., 2013). Desta maneira, se hipotetiza que a PON1 pode ser um importante mediador da resposta inflamatória sobre a capacidade esteroidogênica do folículo.

Em vacas leiteiras, níveis de PON1 abaixo de 80 U/mL indicam a ocorrência de infecção uterina (SCHNEIDER et al., 2013). Nesse sentido, foi demonstrado que maiores níveis séricos de PON1 em vacas leiteiras sete dias após o parto foi relacionado com ovulação mais precoce no período pós-parto (KRAUSE et al., 2014). Além disso, a PON1 parece ser um bom indicador de susceptibilidade a doenças do pós-parto, uma vez que em vacas que apresentaram metrite pós-parto, a atividade

sérica de PON1já estava reduzida sete dias antes do parto (SCHNEIDER et al. 2013). Apesar disso, em vacas zebuínas com cerca de 60 dias pós-parto não foi detectado efeito dos níveis séricos de PON-1 na prenhez após inseminação artificial em tempo-fixo (CASTRO et al., 2018). Estes dados sugerem que os efeitos da PON1 sobre fertilidade são variáveis dependendo da condição metabólica e inflamatória do animal.

Dessa forma, é preciso considerar as diferenças nas exigências de vacas leiteiras em comparação com as raças destinadas ao corte, especialmente quando se compara *Bos taurus* com *Bos indicus* (BÓ et al., 2003; SARTORI et al., 2010). No Brasil, a principal raça leiteira é a Holandês pura ou presente no cruzamento Girolando, enquanto que a principal raça de corte é a Nelore. Devido à produção leiteira, vacas leiteiras são mais susceptíveis à doenças do pós-parto, sendo que mais de40% das vacas leiteiras em diferentes níveis de produção, raças e sistemas de manejo desenvolvem doenças metabólicas ou infecciosas nos primeiros meses de lactação (SANTOS et al., 2010). Além disso, aproximadamente 80% das doenças pós-parto ocorrem durante as primeiras 3 semanas pós-parto (RIBEIRO et al., 2016).

Os efeitos negativos das doenças inflamatórias sobre o desempenho reprodutivo em vacas leiteiras já são conhecidos (SANTOS et al., 2010; RIBEIRO et al., 2016). Estudos nos quais o processo inflamatório foi induzido pela injeção de LPS no trato reprodutivo ou na glândula mamária resultaram em uma redução na secreção de LH (PETER et al., 1989; LAVON et al., 2008), essencial no desencadeamento da ovulação. Além disso, mesmo que ocorra a ovulação, vacas acometidas por algum processo inflamatório, seja do trato reprodutivo ou não, no período pós-parto, sofrem uma redução nas taxas de gestação e de parto, bem como um aumento nas perdas gestacionais (RIBEIRO et al., 2016). O desenvolvimento embrionário inicial é afetado e há uma redução na expressão de mRNA para genes estimulados por interferon *tau* durante o período de reconhecimento da gestação nas vacas acometidas por doenças inflamatórias (RIBEIRO et al., 2016). Sabe-se que no processo inflamatório, as citocinas pró-inflamatórias produzidas no tecido infectado atuam em nível cerebral para induzir sintomas comuns de doença (DANTZER e KELLEY, 2007). Assim, é sugerido que esses mediadores inflamatórios afetam o eixo hipotálamo-hipófise-gonadal, prejudicando amplamente os eventos fisiológicos de controle do ciclo estral, a qualidade do oócito e manutenção da gestação (RIBEIRO et al., 2016).

Além de avaliar a atividade de PON1 no intuito identificar animais com menor predisposição a doenças no pré-parto, ou seja, mais propensas a terem sucesso na

reprodução, é possível selecionar animais que serão destinados à reprodução com base em uma análise genética de PON1, considerando determinados polimorfismos na região promotora do gene de PON1. Em termos moleculares, o gene da PON1 bovino está localizado no cromossomo quatro e tem um tamanho aproximado de 33.000 pares de bases (pb). A presença de polimorfismos de nucleotídeos únicos (SNPs) na região promotora do gene da PON1 bovina já foi identificada em vacas da raça Holandês, sendo que seis dos sete SNPs caracterizados foram associados à atividade sérica da PON1 (SILVEIRA et al., 2015). Além disso, foi demonstrado que determinados alelos foram associados à menor atividade sérica de PON1 (SILVEIRA et al., 2015), o que pode indicar maior susceptibilidade a doenças ou infertilidade uma pressão de seleção contra esses alelos específicos. Em particular, o SNP presente na posição -221, que é identificado como um local para a transcrição de moduladores de resposta de fase aguda (WEDEL e ZIEGLER-HEITBROCK, 1995), foi fortemente associado à atividade sérica da PON1 e com as demais posições de SNPs caracterizados em vacas Holandês (SILVEIRA et al., 2015). Além disso, o genótipo associado à maior atividade plasmática de PON1 nos locais de SNP -221 e -392 foi associado a um menor intervalo entre parto e concepção (SILVEIRA et al., 2019). Apesar de todas estas evidências da importância da PON1 em vacas leiteiras, existem poucos estudos avaliando a atividade de PON1 em vacas de corte, assim como a ocorrência e localização de SNPs na região promotora do gene PON1 em bovinos de corte *B. indicus*.

Devido às condições climáticas do RS, as raças taurinas, que são mais precoces (SANTOS et al., 2010), são amplamente criadas neste estado, tanto para produção de leite, como para corte. Entretanto, de forma geral, a principal raça de corte criada no Brasil é o Nelore, devido à sua maior resistência a doenças parasitárias. Dessa forma, é importante investigar se alterações, como no caso da relação da atividade de PON1 com parâmetros de fertilidade detectadas em vacas *B. taurus* (KRAUSE et al., 2014; SILVEIRA et al., 2019) podem ser extrapoladas para *B. indicus*.

2 Objetivos

2.1 Objetivo Geral

Identificar os efeitos e possíveis mecanismos de ação da PGF na ovulação de bovinos e avaliar potenciais associações entre a PON1 e a fertilidade em bovinos.

2.2 Objetivos Específicos

1) Avaliar o efeito da PGF em diferentes momentos na ovulação em bovinos;

2) Avaliar se a PGF é capaz de induzir ovulação sob condições de altos níveis séricos de progesterona em bovinos;

3) Avaliar os níveis plasmáticos de LH após tratamento com PGF em vacas ovariectomizadas;

4) Determinar a expressão de receptor de PGF em células luteotróficas na hipófise de ovinos e bovinos;

5) Avaliar o efeito da injeção intrafolicular da PGF na ovulação do folículo dominante bovino;

6) Caracterizar a atividade sérica de PON1 durante o período periovulação em bovinos;

7) Avaliar a associação entre a atividade sérica de PON1, o diâmetro do folículo dominante e a prenhez por IA em vacas pós-parto submetidas a IATF;

8) Identificar polimorfismos de nucleotídeo único (SNP) na região promotora do gene da PON1 em vacas Nelore;

9) Genotipar o SNP da posição -221 com atividade de PON1 em vacas Nelore.

3 Artigos

3.1 Artigo 1

Does Prostaglandin F2α affect the hypothalamic-pituitary axis in cattle?

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Does Prostaglandin F2α affect the hypothalamic-pituitary axis in cattle?

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ABSTRACT

Prostaglandin F2alpha (PGF) is able to induce ovulation in cattle. The objective of this study was to determine if the PGF action is associated to the release of luteinizing hormone (LH) in cattle. In Experiment 1 cows were subjected to an estradiol-high progesterone hormonal protocol (natural CL + 2 CIDR devices), maintained throughout the experiment. On Day 9 of the protocol, cows were randomly separated into two experimental groups to receive 150 μg of d-Cloprostenol (PG Group, n = 12) or 2 mL of NaCl 0.9% (Control Group, n = 11). No cow ovulated in Control and PG groups, but there was an increase in the blood flow of dominant follicle after treatment in the PG Group (P = 0.03). In Experiment 2, cows with a natural CL (diestrus phase) were subjected to an estradiol-low progesterone hormonal protocol (twice-used CIDR device). On Day 9 of the protocol, cows were randomly separated into two experimental groups to receive 4 injections, once a day, 150 μg of d-Cloprostenol (4xPG Group, n = 9) or 2 mL of NaCL 0.9% (Control Group, n = 9). CIDRs were removed on Day 13. No cow ovulated in Control and 4xPG groups during low progesterone treatment. However, after CIDR removal, the ovulations were detected 54.6 ± 3.5 h in Control group and 42.6±6.5 h after CIDR removal in 4xPG group (P=0.12). In Experiment 3, ovariectomized cows were allocated to three experimental groups to receive hourly three i.m. injections of 300μg of PGF analog (PG Group, n = 5), 100μg of lecirelin (GnRH Group, n = 5) or 2 mL of PBS (Control Group, n = 4). Serum LH concentrations were analyzed until 36 hours after treatments. The LH concentration was higher (P <0.0001) in cows from the GnRH group than in the PG and Control groups. The LH secretion pattern did not differ between the PGF or Control groups (P > 0.05). In Experiment 4, cow pituitaries were collected to determine the presence of PGF receptors (FP) in gonadotrope cells, by immunohistochemistry and immunofluorescence. No colocalization between gonadotropes and FP receptor was detected in any slide analyzed. In conclusion, PGF was not able to induce ovulation in cows with high or low plasma progesterone concentration. Additionally, PGF alone was not able to induce LH release at the time of evaluation, therefore suggesting PGF has no central role in the hypothalamic-pituitary axis to induce ovulation.

Keywords: Cow, Ovary, Pituitary, Prostaglandin, Reproduction.

Introduction

Prostaglandin F2alpha (PGF) analogs are usually used to induce luteolysis in estrous synchronization programs in cattle (Pursley et al., 1995; Weems et al., 2006; Colazo & Mapletoft, 2014). However, PGF is also able to hasten and synchronize ovulation in cattle (Pfeifer et al., 2014; 2016; Castro et al., 2018) by a mechanism independent of its luteolytic action (Leonardi et al., 2012). Previously, we observed that *d*-Cloprostenol (PGF analog) has a similar ovulatory effect to that of estradiol benzoate in timed artificial insemination (TAI) protocols, resulting in similar ovulation and pregnancy rates in dairy and beef cows (Castro et al., 2018, Pfeifer et al., 2014, 2016, 2018). Despite this, the mechanism of action of PGF to induce ovulation in cattle is still unknown.

During the natural estrous cycle, for the ovarian dominant follicle to reach ovulatory capacity, luteolysis and a consequent decrease of the endogenous progesterone levels are necessary (Colazo & Mapletoft, 2014). However, in a controlled estrous cycle program, it is known that GnRH is able to induce ovulation even in the presence of progesterone (Colazo et al., 2008), because it stimulates pituitary LH release (Halasz et al., 1989). Furthermore, it was shown that estradiol is able to induce LH surge in ovariectomized cows under high progesterone levels (Martinez et al., 2007). On the other hand, in our previous studies, PGF was tested as an ovulatory inducer when progesterone levels were very low, therefore not much is known about its possible central hypothalamic effects and its ability to trigger LH release.

It was shown that the administration of PGF associated with GnRH can induce an increase in plasma LH levels in postpartum cows, suggesting that PGF increases the hypothalamic responsiveness to GnRH, increasing LH release (Randel et al., 1996). However, it is not known whether PGF alone is able to induce an LH surge resulting in ovulation. In addition, the presence of PGF and PGE receptors has been demonstrated in mice pituitary gland (Naor et al., 2007), however in mice, PGE stimulates LH secretion while PGF inhibits it (Murdoch et al., 1993). Therefore, identifying the presence of PGF pituitary receptors (FP receptor) in cattle would be an important indication of the role of PGF on the LH release.

Based on these considerations, our hypothesis is that i.m. injection of PGF has a role at the hypothalamic-pituitary level to induce ovulation in cattle. Therefore, the aims of this study were to evaluate: 1) the effect of PGF administered in different moments and doses; 2) whether PGF is able to induce ovulation in cows with high and low serum progesterone concentrations; 3) the pattern of LH release after PGF treatment; and 4) the presence of FP receptor in pituitary luteotropic cells.

Materials and Methods

Experiment 1. Effect of a single PGF dose in a high progesterone environment

Crossbred cows (*Bos taurus* x *Bos indicus*; *n* = 23) between 3 and 6 years old, parity between 2 and 4, body condition score (BCS) between 2.5 and 3.5 (1 = cachectic, 5 = obese) were used. Cows were maintained in a *Brachiaria brizantha* pasture, with free access to water and mineral supplement. Prior to the experiment, all cows were examined by transretal ultrasound (SIUI CTS-900 equipped with 5 MHZ linear probe, Guangdong, China) twice, with an interval of 11d between exams, to confirm that none had uterine infection as well as to evaluate the ovarian activity. All cows presented a corpus luteum (CL) and/or a dominant follicle (DF) and were included in the study.

Cows were submitted to a pre-synchronization treatment as shown in Fig. 1A. On Day 0, corresponding with approximately 6 to 7 d after ovulationcows were treated with two progesterone-releasing intravaginal implants devices (1.9 g progesterone each, CIDR®, São Paulo, Brazil), which result in approximately 6-9 ng/mL of serum progesterone, when associated with endogenous progesterone from CL (Dias et al., 2014),plus 2 mg of EB i.m.. On Day 8, cows received 300 IU of eCG (Novormon®, São Paulo, Brazil), i.m., and 24h later were randomly separated into two experimental groups to receive: 1) 150 μg of d-Cloprostenol (PG Group, n = 12), or 2) 2 mL of NaCl (Control Group, n = 11). All cows were evaluated by transrectal ultrasonography, every 12 hours from Day 6 until ovulation, or five days after the treatments in the absence of ovulation. In addition, color Doppler ultrasonography (Mindray® equipped with 5 MHZ linear probe) was used to evaluate blood flow in the DF. Color gain settings were kept constant for all ultrasonic assessments. Images of the follicles were obtained from ultrasonic assessments with maximum color intensity, focusing on the widest diameter of the follicle. When consistent Doppler signals were detected in the follicular wall, it was considered that the follicle had detectable blood flow. The intensity of follicular vascularization was expressed subjectively as the percentage of peri-follicular circumference enriched with Color Doppler signals as described by Ginther (2007).

Experiment 2. Effect of multiple PGF doses in a low progesterone environment

Crossbred cows (*B. taurus* x *B. indicus*; *n* = 18) were given PGF im twice, 11 d apart. Ten days after the second PGF (Day 0), corresponding with approximately 5 to 8 d after ovulation, all cows received a hormonal treatment (Fig. 1B). On Day 0, cows were treated with a twice-used CIDR device (CIDR®, São Paulo, Brazil), which result in approximately 1.2

ng/mL of progesterone in the absence of CL (Pereira et al., 2017), 2 mg of EB i.m. and 150 μg of d-Cloprostenol to induce luteolysis. On Day 6, cows received 150 μg of a PGFanalog (d-Cloprostenol, Croniben®, Curitiba, Brazil), i.m., and 48 hours later, 300 IU of eCG (Novormon®, São Paulo, Brazil), i.m., were given.On Day 9, cows were randomly separated into two experimental groups to receive: 1) 4 injections, once a day, of 150 μl of d-Cloprostenol (4xPG Group, n = 9), or 2) 4 injections of 2 mL of NaCl (Control Group, n = 9). CIDRs were removed on Day 13. All cows were evaluated by transrectal ultrasonography, every 12 hours from Day 9 until ovulation, or five days after CIDR removal in the absence of ovulation, in order to determine the ovulation time and rate.

Experiment 3. Pattern of circulating LH release after PGF treatment

This study was performed on the same experimental farm as experiments 1 and 2. Ovariectomized Girolando cows (n = 14) were randomly allocated into three groups to receive one of three i.m. injection (Hour 0, 1 and 2) of one of the following treatments: 300μg of d-Cloprostenol (PGF analog; Croniben®, Curitiba, Brazil, PG Group, n = 5), 100μg of lecirelin (GnRH analogue, Gestran plus®, Tecnopec, São Paulo, Brazil; GnRH Group, n = 5) or 2 mL of PBS (Control Group, n = 4). To determine the serum concentration of LH, blood samples were collected from all cows, as follows: Once 2 h before the injections, hourly from the moment of injections (Hour 0) to hour 6, and once every 6h from hour 6 to 36 after injections. Serum LH concentrations were analyzed by radioimmunoassay, adapted from Bolt & Rollins (1983) and Bolt et al. (1990). The intra-assay CVs were 4.9 and 7.4% for low- and high- reference samples, whereas interassay CVs were 21.9% and 7.2% for low- and high-reference samples.

Experiment 4. Expression of FP receptor in pituitary gonadotrope cells

Cow pituitaries (n=3) obtained from a local slaughterhouse were used for this study. Soon after collection, pituitaries were fixed for 24h in formaldehyde. Pituitaries were embedded in paraffin, cut into 20um sections and mounted on slides using standard procedures. For the immunohistochemistry, slides containing pituitary sections were submerged in xylene (10 min) and after in 100%, 95% and 70% ethanol (10, 5 and 5 min, respectively). Sections were washed in distilled water and PBS for 5 min. Slides were then submerged in sodium citrate buffer 0.01M pH 6.0 in the microwave for about 10 minutes. After that the slides were submerged in PBS plus BSA 1%, as a blocking agent, for 45 min. After blocking, sections were incubated

(overnight, 4ºC) in 200µl of a mix containing the two primary antibodies; lutropin β (C-6) monoclonal antibody (Santa Cruz Biothecnology, Inc.) at 1:100 dilution, to identify LH cells, and FP receptor polyclonal antibody (Cayman Chemical Company) at 1:100 dilution to identify FP receptors. After 24 h, the sections were washed in PBS again and incubated in a dark room with Donkey anti-mouse Alexa Fluor®488 (green, Abcam®) secondary antibody to stain LH cells. For staining FP receptors, Biotin goat anti-rabbit (Sigma-Aldrich®) at 1:200 dilution was used to reduce non-specific signals, and after 90 min, sections were washed, and incubated in ST Alexa 546 (red, Abcam®) at 1:2000 dilution, to stain the FP receptors. Furthermore, cell nucleus was counterstained with DAPI.

To analyze the immunofluorescent colocalization of PF and LH receptors, the bright-field images were captured using an Olympus (Center Valley, PA) Provis microscope fitted with a Kodak (Rochester, NY) DCS 330 digital camera. Colocalization of FP receptor and LH was examined by merge across each slide, and all luteotropic cells were counted to determine the number of gonadotropes (green), number of cells with FP receptor (red), and number of colocalization between gonadotropes and FP.

Statistical analysis

All statistical analyses were performed using SAS 9.0 (SAS, Cary, NC, USA). Continuous variables (diameter of dominant follicle, time of ovulation and concentration of LH) were analyzed by One-way ANOVA, and means were compared between groups by post-hoc Tukey test. Analyses involving repeated measures over time were compared by analysis of variance for repeated measures using the MIXED procedure to evaluate the main effects of treatment, time (sampling period), and their interaction (treatment vs. time). When the interaction was significant, means were compared among treatments using the Tukey post-hoc test adjustment to account for multiple comparisons in the model. Ovulation rate was analyzed by the Chi-square test. Differences between groups were considered significant when the P value was less than or equal to 0.05.

Results

Experiment 1

No cow ovulated in Control and PG groups when exposed to high progesterone concentrations. Follicular blood flow score was not different between groups ($P > 0.05$) from

day 6 to 13. However, there was an increase in the blood flow of dominant follicle after treatment in the PG Group (P = 0.03). Follicular growth and follicular blood flow score are shown in Fig 2A and 2B, respectively.

Experiment 2

No cow ovulated in Control and 4xPG groups during low progesterone treatment. However, after CIDR removal, all cows (9/9) from Control group ovulated and 78% (7/9) of the cows treated with PGF ovulated. Average time to ovulation from CIDR removal was 54.6 ± 3.5 h in Control and 42.6 ± 6.5 h in PG group (P=0.12).

Experiment 3

Serum LH concentrations after saline, GnRH or PGF injection are shown in Fig. 3. Prior to the injections (Hour 0), LH concentration was similar between groups (P = 0.97; 4.48 ± 0.25 ng/mL). However, 1 hour after the first injections, the LH concentration was higher (P <0.0001) in cows from GnRH group (33.59 ± 13.24 ng/mL) compared to the PG and Control groups (5.23 ± 0.50 and 4.22 ± 0,47 ng/mL, respectively). This difference persisted up to 4 hours after the first injections (P<0.05). After this period, LH concentrations were similar (P > 0.05) among groups. The LH secretion pattern was not different between PG and Control groups (P > 0.05), maintaining between 3 and 8 ng/mL throughout the study.

Experiment 4

No colocalization between gonadotropes and FP receptor was detected in any of the samples analyzed (Fig. 4).

Discussion

The results of this study suggest that PGF is not able to act in the hypothalamic-pituitary level to stimulate the LH release and ovulation in cattle. Although it is known that in association with GnRH, PGF is able to increase LH secretion in postpartum cows (Randel et al., 1996), our results demonstrated that intramuscular injection of PGF without any pretreatment (Exp. 3) or in the presence of progesterone, even at low levels (Exp. 1 and 2), is not able to activate pituitary secretion of LH and induce ovulation. In addition, the absence of colocalization between FP receptors and bovine luteotropic cells (Exp. 4) are further evidence that PGF does not act at the central level to control ovulation in cattle.

PGF analogs are the most commonly used commercial products in estrous cycle control protocols (Lamb et al., 2010), besides being widely used in the treatment of reproductive tract disorders (Weems et al., 2006). Thus, the understanding about the mechanism of action of the PGs is essential for the development of more efficient techniques and methods for the control of reproductive cycles in cows. In this sense, it is known that commercial analogs of PGF promote induction of ovulation in sheep (Davies et al., 2006) and cattle submitted to different ovulation synchronization protocols (Pfeifer et al., 2012; 2014; 2016; 2018; Castro et al., 2018), and that the effect on ovulation is independent of the luteolytic effect of PGF, since it is able to induce ovulation in prepubertal heifers (Leonardi et al., 2012). Although the exact mechanism by which this occurs has not yet been elucidated, the results of our study clearly indicate that PGF does not act at the hypothalamic-pituitary stimulus to induce the release of LH and ovulation, as is the case for GnRH (Bolt et al., 1990) which induce a LH surge even in the presence of progesterone (Martinez et al., 2007).

The importance of prostaglandins in the cascade of processes that precede ovulation has been demonstrated in several species (Evans et al., 1983; Dhaliwal et al., 1991; Murdoch et al., 1993; Duffy & Stouffer et al., 2001; Bridges & Fortune, 2007). However, most of the available reports involving the action of endogenous PGF on periovulatory events in cattle are focused in its local action, directly in the dominant follicle (Bridges & Fortune, 2007, Fortune et al., 2009, Willis et al., 2017). In response to GnRH, there is an increase in the PGF and PGE receptors expression in theca and granulosa cells of preovulatory follicles in cattle (Bridges and Fortune, 2007). In addition, the pre-ovulatory LH surge induces cyclooxygenase-2 activity, which converts arachidonic acid to PGH2, precursor of prostaglandins E2 and F2alpha (Sirois, 1994). During the periovulatory period there is a regulation of PGE and PGF receptors expression in theca and granulosa cells (Bridges and Fortune, 2007), and the presence of these prostaglandins stimulates the activation of proteases in the granulosa (Fortune et al., 2009) and theca interna cells (Willis et al., 2017), promoting follicular rupture and extracellular matrix remodeling (Richards et al., 2005; Shozu et al., 2005). These reports demonstrated the importance of endogenous PGF in ovulation. In our study, although PGF was not able to induce ovulation in cows with high progesterone levels, it apparently improved follicular vascularization, suggesting a local action on the preovulatory follicle at the level of follicular wall or cell matrix. Initially, we tested the effect of PGF in a high progesterone environment, and since PGF did not induce ovulation, we attempted to test multiple PGF injections in a low P4 environment. Therefore, it was clear that even in low P4 concentrations, PGF is not able to induce ovulation.

In rhesus monkeys, LH induced an increase in PGE and PGF concentrations in follicular fluid about 10 hours prior to ovulatory follicle rupture (Duffy & Stouffer, 2001), suggesting that PGs may modulate the effects of LH increase in regulation of ovarian tissue remodeling, which is essential for periovulatory processes, such as follicular rupture, ovulation and follicular luteinization. These results, associated with our findings, suggest that PGF could have a local effect in the pre-ovulatory follicle. The presence of progesterone, even at low levels, as expected in experiment 2, blocked the ovulatory action of PGF; however, after progesterone source removal, 57% (4/7) of cows in PG Group ovulated until 36 hours, while in the Control Group, only 22% (2/9) ovulated during this period (P = 0.15).

In previous studies by our research group, in which we evaluated the ability of PGF to anticipate ovulation, progesterone sources were removed prior to PGF treatment to induce ovulation (Leonardi et al., 2012; Pfeifer et al., 2014, Pfeifer et al., 2016, Castro et al., 2018), as is performed in conventional hormonal protocols. However, in the current study, in order to evaluate possible mechanisms of action of PGF, we maintained the source of progesterone. This aimed to determine if, under a pituitary gonadotrophin release block, PGF would still be able to induce ovulation, however our results demonstrated that this is not possible. Likewise, PGF was not able to direct stimulate release of LH in ovariectomized cows. Thus, the effect of PGF on LH secretion suggested by some authors before (Randel et al., 1996; Weems et al., 2006) was not confirmed in our current study using PGF alone.

In postpartum cows the injection of PGF in association with GnRH resulted in an increase in LH secretion (Randel et al., 1996), suggesting a direct effect of PGF on the anterior pituitary (Weems et al. al., 2006), increasing responsiveness to GnRH (Randel et al., 1996). However, the results of our study demonstrate that PGF, by itself, is not able to act at the pituitary level to stimulate the LH surge and to cause ovulation in cattle, which was proven by the maintenance of low levels of LH, even after three injections of PGF. This finding is further supported by the absence of colocalization between PGF receptors and luteotropic cells in bovine pituitary. Although only three animas were used for this experiment, the data obtained, together with the absence of LH realse upon PGF stimulation, indicate that these cells are indeed not able to respond to changes in PGF levels directly.

In contrast to our study, prostaglandins appear to act at the hypothalamic-pituitary level in rodents (Armstrong and Grinwich, 1972, Orczyk and Behrman, 1972, Sato et al., 1974 Naor et al., 2007). The presence of PGF and prostaglandin E_2 (PGE_2) receptors has been demonstrated in rat gonadotrophic cells (Naor et al., 2007). In mice, PGE_2 released by astrocytes in the hypothalamus appears to stimulate GnRH secretion (Clasadonte et al., 2011) and

administration of exogenous PGE_2 resulted in increased concentration and pulse amplitude of LH in rats (Matsuwaki et al., 2017) and induction of ovulation in prepubertal mice (Andrade et al., 2017). On the other hand, administration of indomethacin, a potent inhibitor of PG synthesis, inhibited LH secretion and ovulation in immature rats (Armstrong and Grinwich, 1972). However, in small rodents, only PGE_2 appears to have a stimulatory action, whereas PGF inhibits the expression of GnRH receptors and LH secretion in rats (Naor et al., 2007), demonstrating an important difference in the action of PGF in different species, since the ovulatory action of PGF in cattle and sheep has already been established (Davies et al., 2006, Leonardi et al., 2012, Pfeifer et al., 2014, 2016, Castro et al., 2018). Despite these findings we cannot rule out that prostaglandin may have an indirect effect on LH secretion trough stimulation of local mechanisms in the pre-ovulatory follicle. As expected, treatment with GnRH induced a sudden increase in LH level. However, even after three injections of PGF there was no change in LH secretion in ovariectomized cows. The action of GnRH on the release of LH is well known (Bolt et al., 1990), and occurs even in the presence of progesterone (Bolt et al., 1990).

Differently from observed in cows of our experiments, exogenous PGF induced ovulation in anestrous ewes under the effect of medroxyprogesterone (Davies et al., 2006), suggesting a direct effect on the follicle, since circulating progesterone concentrations did not change and ovulation was not preceded by a LH or FSH surge. On the other hand, in our study, although the progesterone levels were not measured in experiments 1 and 2, previous studies have already validated that it should be high in Exp. 1 (>5 ng/mL, Dias et al., 2014) by the presence of two CIDRs plus CL and low in Exp. 2 (<2 ng/mL, Pereira et al., 2017) by the presence of only one CIDR used in cows without CL. Despite this, in neither of the two experiments PGF was able of inducing ovulation during progestogen exposure. It is known serum progesterone concentrations around 2ng/mL are able to prevent the LH release needed for ovulation of the dominant follicle in cattle (Kojima et al., 2003). It is possible therefore, that even the low progesterone environment is able to prevent the PGF stimulus on ovulation.

In summary, although it is known that PGF can induce ovulation in cattle (Pfeifer et al., 2014, 2016) by a mechanism of action independent of luteolysis (Leonardi et al., 2012), our results shown that prostaglandin can not induce ovulation under any serum progesterone level. Furthermore, the maintenance of basal LH levels after treatment with PGF in ovariectomized cows and the absence of colocalization between PGF receptors and gonadotropes in bovine pituitary indicate that PGF is not able to directly stimulate the preovulatory increase of gonadotrophins. In this sense, together, our results suggest that exogenous PGF do not have an

effect at the hypothalamic-pituitarylevel in cows and further studies are necessary to clarify its mechanism of action.

Declaration of interest

The authors declare no conflict of interest.

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Figures

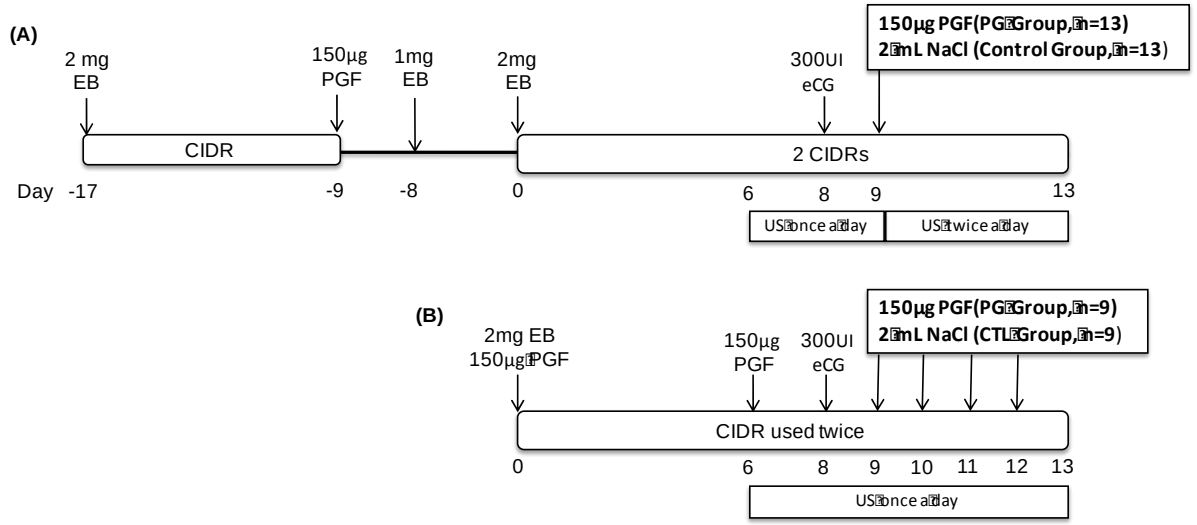

Fig. 1. Ovulation synchronization protocol used in cows from (A) Experiment 1 and (B) Experiment 2. Abbreviations: EB: estradiol benzoate, PGF: prostaglandin F2α, ECP: estradiol cypionate, eCG: equine chorionic gonadotropin, US: ultrasound examination.

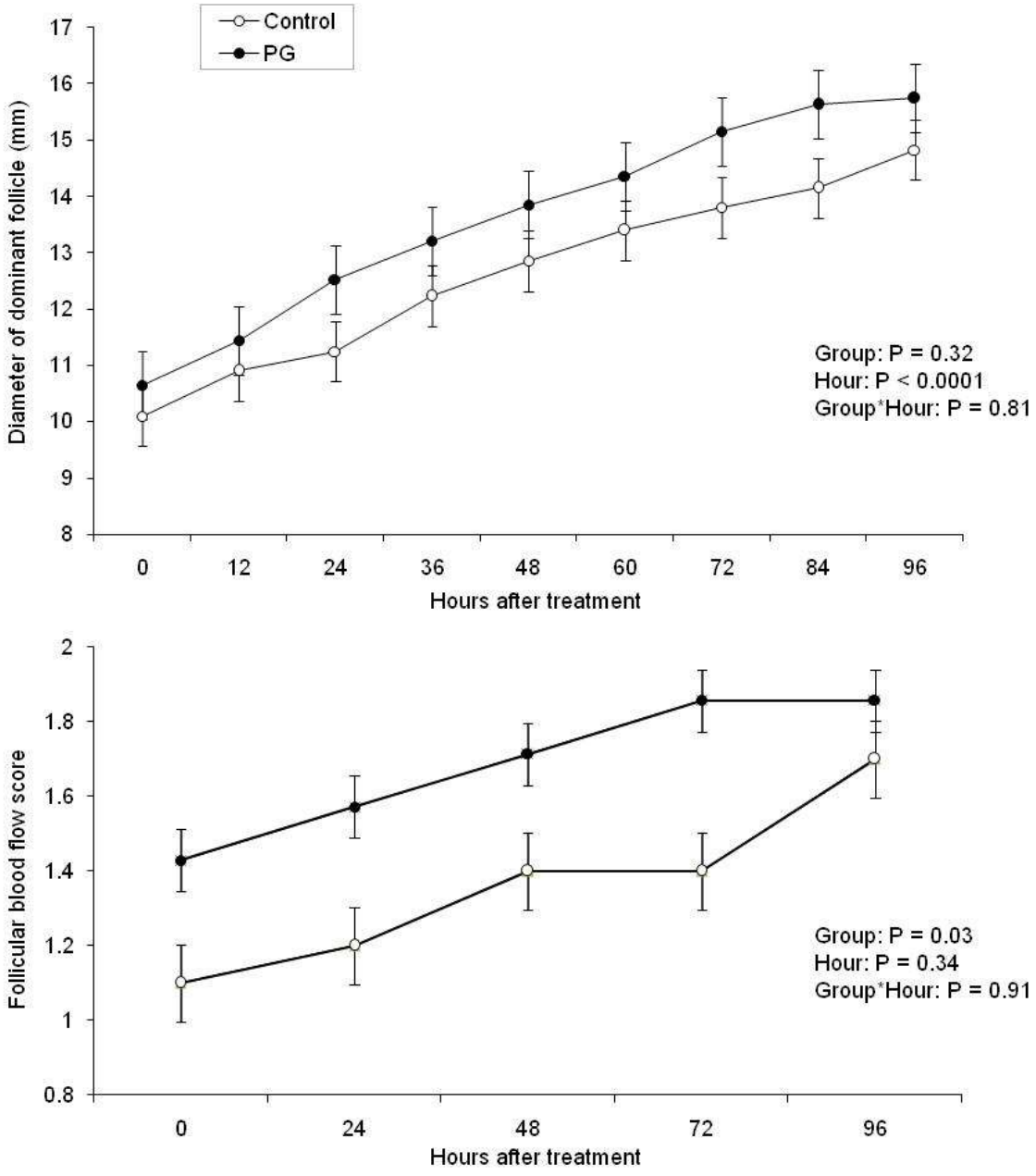

Fig. 2. (A) Diameter (Mean ± SEM) of the dominant follicle and (B) blood flow score in cows treated with 150µg of PGF analog (PG Group, n = 12) or NaCL (Control Group, n = 11) in Experiment 1.

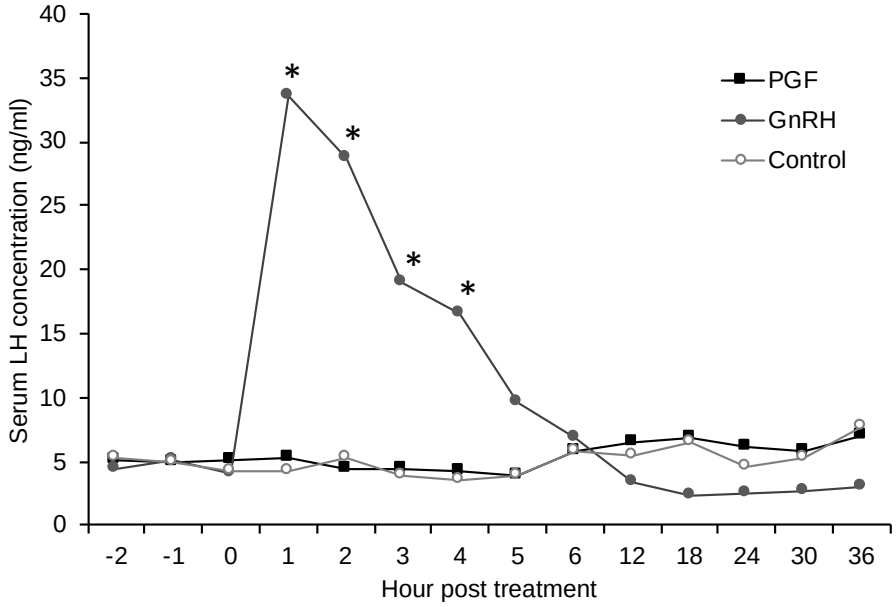

Fig. 3. Means of serum LH concentrations after injections of d-Cloprostenol (PG Group, n = 5), Lecilerin (GnRH Group, n = 5) or Saline (Control Group, n = 4) in ovariectomized cows from Experiment 3.

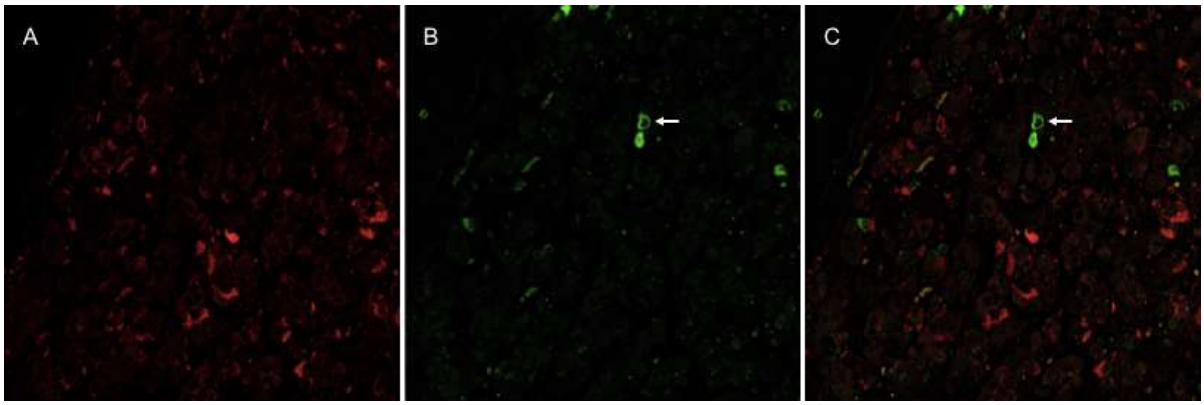

Fig. 4. Gonadotropes cells stained for (A) FP receptor (red) with polyclonal antibody; (B) LH cells (green), stained with lutropin β (C-6) monoclonal antibody; and (C) merged. Sections were incubated in Biotin goat anti-rabbit (red) and Donkey anti-mouse Alexa Fluor®488 (green), used for FP receptor and LH cells, respectively. To stain FP receptor, sections were incubated in ST Alexa 546 (red). Arrow: gonadotrope without FP receptor co-staining.

3.2 Artigo 2

Effect of serum paraoxonase-1 (PON1) activity on follicular development and pregnancy rate in cattle

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Effect of serum paraoxonase-1 (PON1) activity on follicular development and pregnancy rate in cattle

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ABSTRACT

Paraoxonase-1 (PON1) activity has been associated with improvement in ovarian function in early postpartum dairy cows and improved *in vitro* embryo development. The aim of the current study was to evaluate the potential association among PON1 activity and follicular growth, diameter of the preovulatory follicle and pregnancy per artificial insemination (AI) service in cattle. In Experiment 1, cows (n = 33) were subjected to an estradiol-progesterone based protocol to control time of ovulation. Starting on Day 8 of the protocol, follicular growth and serum PON1 activity were monitored. Cows were separated according to the occurrence of ovulation into two groups: Ovulatory (Ov; n = 22) and Anovulatory (Anov; n = 11). The serum activity of PON1 was not different between Ov and Anov cows (P = 0.94). In addition, using a regression model there was no effect of serum PON1 activity on the diameter of dominant follicle (r^2 = 0.00; P = 0.99). In Experiment 2, cows (n = 193)were submitted to the same hormonal protocol as in Experiment 1. On the day of the timed artificial insemination (TAI), the diameter of dominant follicle was evaluated and blood samples were collected for analysis of PON1 activity. According to the serum PON1 activity, cows were divided into three groups: Low (< 70 U/mL), Medium (70-90 U/mL) or High (> 90 U/mL) PON1 activity. The overall pregnancy rate was 62.7% (121/193), with no difference among PON1 activity groups. Additionally, using a regression model there was no effect of serum PON1 activity on the diameter of the preovulatory follicle (r^2 = 0.03; P = 0.65) and pregnancy rate (r^2=0.005; P=0.94). The results of this study indicate that there is no effect of serum PON1 activity on the diameter of preovulatory follicle or establishment of pregnancy in cows submitted to time of ovulation synchronization protocols.

Keywords: Ovulation, *Bos indicus*, Estrus synchronization, Pregnancy, PON1

1. Introduction

Advances in the understanding of the physiological mechanisms involved in ovarian follicular growth and establishment of pregnancy in domestic animals allowed the development of protocols for synchronization of estrus, specifically designed for cattle (Bó and Baruselli et al., 2014). It was, therefore, possible to improve pregnancy per AI in cows subjected to fixed-timed artificial insemination (FTAI) in the last few years. Several factors affect the fertility of cows, however, such as the diameter of the ovulatory follicle (Perry et al., 2005), duration of proestrus (Bridges et al., 2010; Dadarwal et al., 2013), metabolic status (Ponsart et al., 2014) and health of the reproductive tract (Burke et al., 2010, Schneider et al., 2013a).

Paraoxonase-1 (PON1) activity has been associated with ovulation, fertility and uterine health in cows (Schneider et al., 2013b; Krause et al., 2014; Rincón et al., 2016). Paraoxonase-1 is an enzyme synthesized in the liver, which is released into the bloodstream, circulating associated with high-density lipoprotein (HDL; Draganov et al., 2005). Antioxidant properties of HDL that are a consequence of PON1 being a component of HDL inhibit the oxidation of low-density lipoprotein (LDL) and cell membranes, among other actions (Deakin et al., 2011). It is, therefore, suggested that PON1 functions to protect the oocyte from oxidative stress, because in humans, serum and intrafollicular PON1 activity was associated with improved embryo development after *in vitro* fertilization (Browne et al., 2008). Similar results were reported in cattle when recombinant PON1 was added during *in vitro* oocyte maturation, resulting in improved early embryonic development (Rincón et al., 2016). Importantly, there is a positive correlation between serum and intrafollicular concentrations of PON1 in both humans and cattle (Browne et al., 2008; Schneider et al., 2013a; Campos et al., 2017). Intrafollicular PON1 activity originates from the serum because it is not present in granulosa cells (Schneider et al., 2013a).

Considering all these previous studies, the associations between serum PON1 activity and follicular growth in cattle has not been studied. Previous research in dairy cows during the early postpartum period suggests that the overall inflammatory status affects the time of the first postpartum ovulation (Krause et al., 2014; Cheong et al., 2017). In a study with postpartum dairy cows, those with greater PON1 activity at 7 days postpartum had the ovulation earlier than cows with less PON1 activity at 7 days postpartum (Krause et al., 2014). Moreover, ovulation is one of the most significant inflammatory processes in female reproduction. This process disrupts homeostasis and involves changes that are deleterious to other physiological processes (Fortune et al., 2009). Thus, the ovulatory process has been compared to an inflammatory reaction (Espey, 1994). Although, the synthesis of inflammatory mediators has been characterized during the ovulatory period (Duffy and Stouffer, 2001; Sirois et al., 2004), the association of these events and synthesis of PON has not been studied. In addition, there are no studies investigating the effect of serum PON1activity on pregnancy rate in cows submitted to FTAI which may be an important assessment because the follicle diameter and ovulation rate are important predictors of pregnancy outcomes when FTAI is utilized for breeding. Based on these considerations, the hypotheses for the present studies were: 1) serum PON1 activity will be increased in the peri-ovulatory period and 2) serum PON1 activity will be greater in cows with ovulations and that become pregnant after use FTAI as compared with cows that are anovulatory. The aims of this study were: 1) to characterize serum PON1 activity during the peri-ovulatory period and 2) to evaluate the potential association between serum PON1 activity, follicle diameter and pregnancy per AI (P/AI) in postpartum cows submitted to FTAI.

2. Material and methods

The Committee for Ethics in Animal Experimentation from the Brazilian Agricultural Research Corporation (Embrapa – Rondônia) approved all procedures performed in this experiment (Number F.02/2014).

2.1. Experiment 1

This study was performed at the experimental farm of the Brazilian Agricultural Research Corporation (Embrapa, Porto Velho, RO, Brazil, 08°48'12"S, 63°50'56" W). Crossbred cows (*Bos Taurus-Bos indicus*; *n* = 33; 18 lactating and 13 non-lactating cows), between 3 and 6 years old, parity between 2 and 4, body condition score (BCS) between 2.5 and 3.5 (1 = cachectic, 5 = obese) were used. Lactating cows were an average 137.7 ± 10.7 days postpartum when the experiment was initiated. All cows were maintained in a *Brachiaria brizantha* pasture, with free access to water and mineral supplement. Prior to the experiment, all cows were examined to confirm that none had uterine or mammary infection. Cows were submitted to a synchronization of time-of-ovulation protocol as shown in Fig. 1. At Day 0, 2 mg of estradiol benzoate (EB, Bioestrogen®, Curitiba, Brazil) i.m. was given and a progesterone-releasing intravaginal implant (1.9 g progesterone, CIDR®, São Paulo, Brazil) was inserted. On Day 8, the progesterone inserts were removed and cows received 300 IU of eCG (Novormon®, São Paulo, Brazil), 150 μg of a $PGF_{2\alpha}$ analog (d-Cloprostenol, Croniben®, Curitiba, Brazil), and 1 mg ECP (E.C.P.®, São Paulo, Brazil).

Starting at the time of CIDR removal, blood samples were collected from all cows and ovarian evaluation was performed by trans-rectal ultrasonography (SIUI CTS-900, equipped with a 5 MHz linear probe, Guangdong, China) to determine the occurrence and timing of ovulation, as well as the diameter of the preovulatory follicle. Ultrasonography were performed every 12 hours after CIDR removal until the time when ovulation had occurred was detected.

For cows that did not have ovulations, ultrasonic examinations were performed up to 5 days after CIDR removal. Based on the occurrence of ovulation, cows were separated into two groups: Ovulatory (Ov; *n* = 22) and Anovulatory (Anov; *n* = 11). To test the effect of PON1 activity on timing of ovulation, cows that had ovulations were also separated into two groups: cows with ovulations by 60h after CIDR removal (*n* = 14) and cows with ovulations after 60h from the time of CIDR removal (range 72-84h, *n* = 8).

2.2. Experiment 2

This study was conducted at two commercial farms in the state of Rondônia, Brazil. All animals were maintained on *Brachiaria brizantha* pasture, with free access to water and mineral supplement. Nelore cows (53 primiparous and 140 multiparous), 69.8 ± 11.4 d postpartum, were submitted to the same time-of-ovulation synchronization protocol described for Experiment 1 (Fig. 1). On Day 10 of the protocol, however, cows were evaluated by trans-rectal ultrasonography (SIUI CTS-900, probe linear with 5MHZ, Gangdong, China) to confirm that none had uterine abnormalities and to measure the diameter of preovulatory follicle and immediately after that, FTAI was performed. Eight cows did not respond to the estrous synchronization protocol (did not have preovulatory follicle at Day 10) and were excluded from further analysis.

At the time of FTAI, blood samples were collected from all cows to determine PON1activity. Cows were divided into thirds according to the serum PON1 activity at the time of TAI, as follows: Low (< 70 U / mL), Medium (70-90 U / mL) and High (> 90 U / mL) PON1 activity. Pregnancy was diagnosed via trans-rectal ultrasonography 30 days after the FTAI. Furthermore, cows were separated according the diameter of dominant follicle to evaluate PON1 activity and pregnancy per AI in cows with dominant follicle ≤13 (*n* = 84) and >13mm (*n* = 109) on Day 10 (Day of FTAI).

2.3. Blood collection and PON1 analysis

In both experiments, blood samples were collected from the coccygeal vein, in vacuum tubes, without anticoagulant. Immediately after collection, samples were refrigerated at 4 °C, then centrifuged (3000 x *g* for 15 minutes) and stored at -20ºC. In Experiment 1, samples were collected every 12 hours, from the time of CIDR removal to the time of ovulation detection, or in the absence of ovulation, up to 5 days after CIDR removal. In Experiment 2, blood samples were collected at the time of FTAI.

The serum activity of PON1 of all samples was determined by spectrophotometry, as described by Browne et al. (2007) and validated in cattle (Schneider et al., 2013; Krause et al., 2014; Campos et al., 2017). Briefly, arylesterase activity was measured by the phenol formation rate by monitoring the increase in absorbance at 270 nm and 25°C. The working reagent consisted of 20 mmol/L Tris/HCl, pH 8.0, containing 1 mmol/L of $CaCl_2$ and 4 mmol/L phenyl acetate as substrate. The samples were diluted 1:3 in a 20 mmol/L Tris/HCl buffer and were added to the working reagent and the change in absorbance was recorded for 60 seconds. The activity was expressed in U/mL, based on the phenol extinction coefficient. The inter- and intra-assay coefficient of variation in were6.1% and 11.2%, respectively.

2.4. Statistical analysis

All statistical analyses were performed using SAS University Edition (SAS, Cary, NC, USA). For Experiment 1, PON1 activity and follicle diameter were evaluated by repeated measures ANOVA to evaluate the effect of lactation (lactating or non-lactating cows), time, ovulatory status and its interaction. The category (lactating or non-lactating cows) did not have a significant effect on PON1 when included in the model and was, therefore, excluded from the final statistical model. Cows were also separated between those ovulating by 60 h after CIDR

removal and those having ovulations after 60 h. For Experiment 2,cows were divided in thirds according to serum PON1 activity and PON1 activity and follicle diameter were evaluated between each third by One-way ANOVA. The same procedure was performed to compare cows with follicles ≤ 13mm and > 13 mm. The pregnancy rate was assessed by Chi-square test between groups. In addition to that, logistic regression was performed to evaluate the relationship between follicle diameter, PON-1 activity and its interactions on the probability of pregnancy. Pearson's correlation analysis was performed to evaluate the correlation between PON1 activity and follicle diameter in both experiments. The differences between groups were considered significant when the *P* value was less than or equal to 0.05.

3. **Results**

3.1. Experiment 1

All cows developed dominant follicles (> 8 mm) at the end of the hormonal treatment. There was no association between serum PON1 activity and the diameter of preovulatory follicle (r^2= 0.00; *P* = 0.99). Ovulation occurred 66 ±1.7 h after CIDR removal for all cows having ovulations. In both groups, follicular diameter increased with time (*P*<0.001, Fig. 2A), and cows having ovulations had a greater follicular diameter than anovulatory cows (*P* = 0.01, Fig. 2A), as expected. The serum PON1 activity was not different between cows of the Ov and Anov groups (*P* = 0.94. Fig. 2B). Overall, there was an effect of time on serum PON1 activity, with less activity observed at 60 h when compared with 0, 24, 36 and 48 h (*P*<0.05). There was no difference in PON1 activity between cows that had ovulations by 60or after 60 h from the time of CIDR removal (77.31 ± 8.40 and 86.33 ± 12.9 at 48 h; *P*=0.55 and 71.83 ± 5.32 and 79.84 ± 10.2 at 60 h post CIDR removal; *P* = 0.45).

3.2. Experiment 2

Data for serum PON1 activity at the time of FTAI in Nelore cows are shown in Table 1. There was no effect of parity (P = 0.11) or preovulatory follicle diameter (P= 0.19) on serum PON1 activity. Overall, the pregnancy per AI (P/AI) was 62.7% (121/193), and there was no difference between the Low, Medium or High PON1 activity groups (P>0.05).

The results indicate no effect of PON1 activity on the probability of pregnancy in postpartum cows. Additionally, there was no correlation between PON1 activity and diameter of preovulatory follicle (r^2 = 0.03; P = 0.65). The logistic regression analysis indicated that there was no effect of PON1 activity (P = 0.94) and diameter of preovulatory follicle (P = 0.15) individually, as well as of its interaction (P>0.05) on the probability of pregnancy. When dividing cows based on the ovulatory response, no difference for PON1 activity and pregnancy per AI according the diameter of dominant follicle (≤ 13mm and > 13 mm) on Day 10 was detected (Table 2).

4. Discussion

The positive effect of PON1 on *in vitro* fertilized oocytes has been established in cattle (Rincón et al., 2016) and humans (Browne et al., 2008). To the best of our knowledge the present study was the first to evaluate the association between serum PON1 activity, follicular growth and pregnancy rate of cows submitted to FTAI. The results from the present study lead to rejection of the hypotheses that PON-1 activity is associated with follicle diameter and time of ovulation. There was, however, no effect of PON-1 activity on the time of ovulation and probability of pregnancy.

The analysis of serum PON1 activity and follicular growth needed to occur because there is a high correlation between its serum and intrafollicular activity in cattle (Schneider et al., 2013b; Campos et al., 2017) and humans (Browne et al., 2008). Evaluating the PON1 serum

activity can be a good indicator of its intrafollicular activity, as validated for the preovulatory follicle of cows (Schneider et al., 2013b; Campos et al., 2017). Although ovulation is an acute inflammatory process (Espey, 1994), as demonstrated in Experiment 1 of the present research, it was not associated with changes in serum PON1 activity even when there was a lesser follicular growth in anovulatory cows. There was, however, an overall decrease in serum PON1 activity 60 h after CIDR removal, when most cows had ovulations, and this could be a reflection of the associated inflammatory process. The evaluation of serum PON1 activity is widely used in cattle studies as an acute phase protein marker that has reduced activity during inflammatory processes (Schneider et al., 2013; Krause et al., 2014; Campos et al., 2017). In a previous study, dairy cows with greater serum PON1 activity in the early postpartum period had ovulations earlier than cows with less PON1 activity (Krause et al., 2014). Also, an elevated systemic inflammation during the early postpartum period was negatively associated with the ovulatory status of the first dominant follicle postpartum in dairy cows (Cheong et al., 2017). Importantly, in a study by Cheong et al. (2017), haptoglobin, another acute phase protein, was negatively associated with occurrence of ovulation, however, there was no association of PON1 serum activity and occurrence of ovulation. These results suggest, therefore, that PON1 is not a reliable marker of ovulatory status or follicle growth in cows.

The finding that no difference was detected in serum PON1 activity of cows that had ovulations and those that were anovulatory in the present study indicates that PON1 may not be a determinant of the ovulatory response of late postpartum cows. A reduction in amount of PON1 activity is indicative of a reproductive tract infection and its consequent systemic inflammatory response, which is reflected in a delay in the resumption of ovarian activity in postpartum dairy cows (Krause et al., 2014; Cheong et al., 2017). In the present study, however, in healthy cows and cows that were later in the postpartum period as compared with those used in the previous research, the lesser concentrations of PON1 activity was not associated with

ovulation failure. In addition, PON1 activity was greater in functional follicles of cattle compared with atretic follicles (Schneider et al., 2013b), and this likely resulted because amount of PON1 activity increases as estradiol concentrations increase (Ahmad and Scott, 2010). In the present study, it was hypothesized that ovulatory cows, with increased rates of follicular growth, would have greater serum PON1 activity as observed in early postpartum dairy cows in previous research. Results of the present study, however, suggest there is no association between PON1 activity and follicle growth. Serum PON1 activity can be regarded, therefore, as a marker of postpartum inflammatory conditions and not ovulatory status. This inflammatory status may have an effect on the ovulatory process as suggested by others (Krause et al., 2014; Cheong et al., 2017), however, PON1 activity *per se* is not directly associated with the ovulatory process during the later postpartum period when uterine and other diseases are less prevalent.

Serum PON1 activity at the time of the FTAI was not associated with pregnancy rate in the present study, and even cows with PON1 activity of <70 U/mL had acceptable pregnancy rates (greater than 60%). For women undergoing an IVF procedure, embryo development and cell number were greater in those with greater serum and intrafollicular PON1 activity (Browne et al., 2008). These data indicate that PON1, as well as other HDL-binding proteins within the follicular fluid, may have an antioxidant protective role for the human oocyte, reflected in subsequent improved early embryonic development (Fujimoto et al., 2010). This was further expanded in a recent study in which different doses of human recombinant PON1 were added in the oocyte maturation medium during the IVF procedure for cattle and a dose-dependent improvement in embryo development rate was reported (Rincón et al., 2016). This again indicates PON1 can improve *in vitro* fertility and oocyte quality. Based on these previous findings, it was expected before conducting the present study, that cows with greater serum PON1 activity would have greater pregnancy rates. Data from the present study, however, indicated that there is no *in vivo* association between PON1 activity and pregnancy rate of cows

submitted to FTAI. It is not possible, therefore, to propose the use of serum PON1 as an *in vivo* marker for fertility in cattle and other factors may have a more important role in this process.

Cows in the second experiment of the present research were all more than 45 days postpartum and had an adequate body condition score (most of them > 3.0). For this reason, in all PON1 groups, cows responded to the estrous synchronization protocol by developing large follicles and had pregnancy/AI rates of about 60%. Previously, it was found that PON1 activity of less than 80 U/mL was indicative of early postpartum uterine infection (Schneider et al. 2013a). Later postpartum these cows had, however, had recovered from the early postpartum decrease in PON1 activity and had concentrations similar to healthy cows (Schneider et al. 2013a). The observations of previous and current studies, therefore, indicate that PON1 activity may be more important *in vitro*, where there is a greater amount of oxidative stress (Agarwal et al., 2005) than *in vivo* situations. During the earlier postpartum period there are suggestions that oxidative stress is increased (Bionaz et al, 2007) and, therefore, may explain why positive effects of greater PON1 concentrations on ovulation are observed only during this period.

In conclusion, the results of the present study indicate no relationship between PON1 activity and follicular growth, ovulatory status or pregnancy rate in cows submitted to ovulation synchronization. There results, therefore, suggest that PON1 it is not a good predictor of ovulation or fertility *in vivo* for cattle.

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Tables

Table 1
Paraoxonase-1 (PON1) activity (U/mL± SEM), diameter of the preovulatory follicle (mm ± SEM) and pregnancy rate in postpartum Nelore cows with Low, Medium or High PON1 activity at the time of fixed time artificial insemination

Parameter	PON1 activity			*P*-value
	Low	Medium	High	
Paraoxonase-1 (U/mL)	55.78 ± 1.19[a]	79.33 ± 0.76[b]	110.52 ± 1.91[c]	< 0.01
Diameter of preovulatory follicle (mm)	13.22 ± 0.23	13.25 ± 0.32	12.87 ± 0.26	0.55
Pregnancy per AI	65.7% (44/67)	64.40% (38/59)	58.2% (39/67)	> 0.05

Table 2
Paraoxonase-1 (PON1) activity (U/mL ± SEM) and pregnancy per AI in cows with a dominant follicle ≤13 or >13mm of diameter on Day 10 (FTAI day)

Parameter	≤13mm	>13mm	P-value
Diameter of dominant follicle (mm ± SEM)	11.13±0.16	14.48±0.12	<0.0001
Paraoxonase-1 (U/mL)	81.20±3.00	82.65±2.30	0.69
Pregnancy per AI	55.9% (47/84)	67.9% (74/109)	0.10

Figures

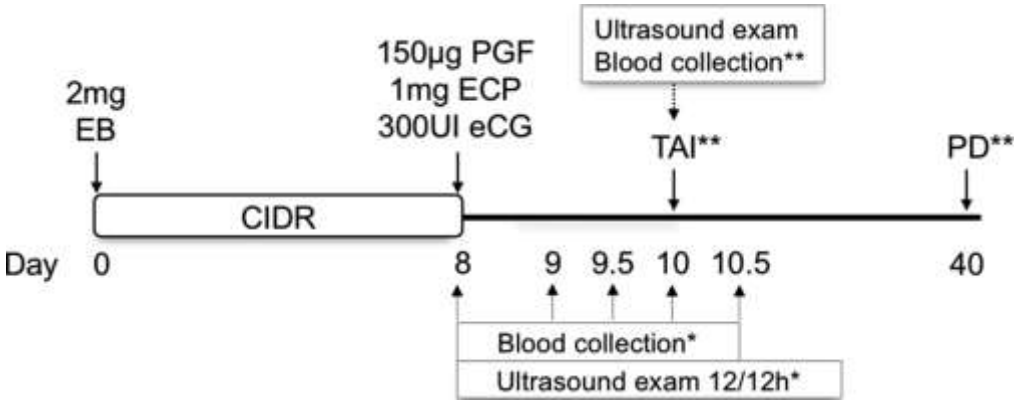

Fig. 1. Ovulation synchronization protocol used in cows from Experiments1* and 2**. Abbreviations: EB: estradiol benzoate, PGF: prostaglandin F2α, ECP: estradiol cypionate, eCG: equine chorionic gonadotropin, TAI: timed artificial insemination, PD: pregnancy diagnosis; *Only in Exp. 1, **Only in Exp. 2

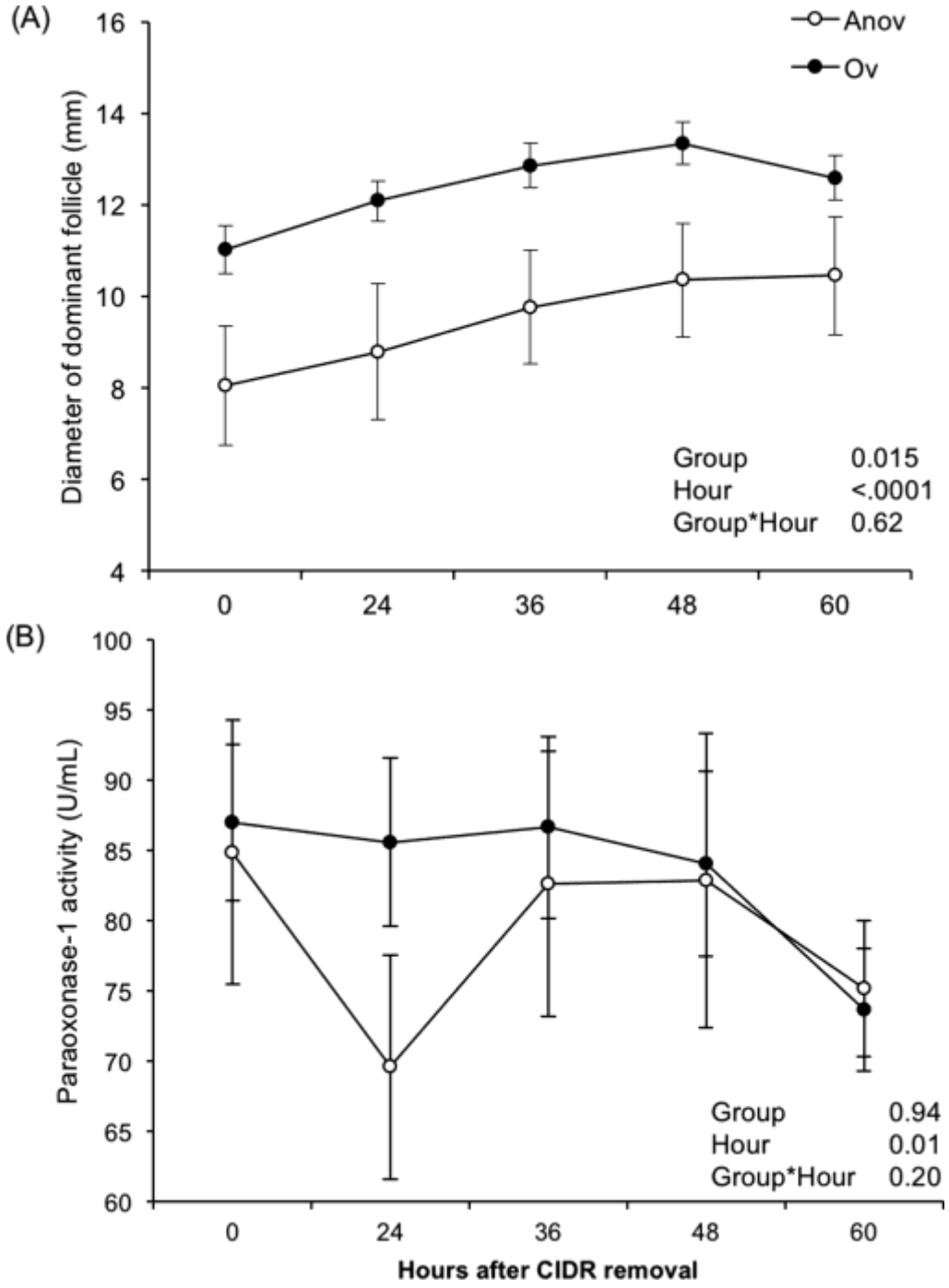

Fig. 2. (A) Diameter of the dominant follicle and (B) serum paraoxonase-1 activity from 0 to 60 h after progesterone device removal for Ovulatory (Ov, *n*= 22) and Anovulatory (Anov, *n* = 11) cows.

3.3 Artigo 3

Single nucleotide polymorphisms in the promoter region of the bovine paraoxonase 1 gene in *Bos indicus* cows

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Short Communication

Single nucleotide polymorphisms in the promoter region of the paraoxonase 1 gene in *Bos indicus* cows

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Abstract

The aims of the present study were to characterize single nucleotide polymorphisms (SNPs) in the promoter region of the paraoxonase 1 (PON1) gene and to determine the relationship between SNPs and PON1 serum activity in *Bos indicus* cows. Samples from Nelore cows were used for genetic sequencing (n=17) and for genotyping of the SNP-221 and PON1 activity analysis (n=52). Eight SNPs were identified in the promoter region of PON1. There was no association between any SNP position and PON1 activity. Our results suggest that SNPs in the PON1 promoter does not affect serum PON1 activity in *B. indicus* cows.

Keywords: Cattle; Polymorphism; PON1

The paraoxonase 1 (PON1) is an enzyme synthetized in the liver and associated with high-density lipoproteins (HDL; Ceron et al., 2014), acting as a protection factor against the oxidation of low density lipoprotein (LDL) and cell membranes (LDL; Aviram, 1999). PON1 reduces its activity during inflammatory processes (Bionaz et al., 2007), and in cattleisconsidered a reliablebiomarker of uterinehealth, especially in postpartum dairy cows (Schneider et al., 2013). In addition, dairy cows with higher levels of PON1 are more likely to resume ovulation soon after calving (Krause et al., 2014). Moreover, the inclusion of PON1 in the medium increase the *in vitro* embryo production in cattle (Rincón et al., 2016).

Recently, seven single nucleotide polymorphisms (SNPs) were described in the promoterregion of the bovine PON1 gene, and the SNP present at the -221 position was strongly associated with serum PON1 activity (Silveira et al., 2015). In addition, the SNP located at position -221 was identified as a site for transcription of acute phase response modulators (Wedel &Ziegler-Heitbrock, 1995), which may be involved in the pathogenesis of postpartum uterine diseases. In Holstein cows, the genotypes associated with higher plasma PON1 activity in SNP locations -221 and -392 were also associated with a reduced calving to conception interval (Silveira et al., 2019). However, to date there are no studies evaluating the occurrence and location of SNPs in the promoter region of the PON1 gene in *Bos indicus* cattle, which is the main breed of cattle raised in Brazil.

Based on these considerations, the aims of this study were to characterize SNPs in the promoter region of the bovine PON1 gene and to evaluate the genotypic distribution of the PON1 -221 SNP, and its association to PON1 activity in Nelore cows.

Blood samples from 52 suckled Nelore cows subjected to a fixed-timed artificial insemination (FTAI) protocol were used. On the day of the insemination, blood samples were collected for PON1 activity analysis and DNA extraction. Determination of serum PON1

arylesterase activity (U/mL) was performedaccording to the method described by Browne et al. (2007).

The promoter region of the bovine PON1 gene (828 bp) was amplified by the polymerase chain reaction (PCR) technique with specific primers (Forward: 5′-CGGTAATCCCTGAAGAATGC-3′ and Reverse: 5′-GCACTTCCTACCCTGCTTTG-3), as described by Silveira et al. (2015).

The appropriately amplified PCR products from 17 samples were purified and then submitted to sequencing (HELIXXA) to determine the occurrence and location of SNPs. The obtained nucleotide sequences were aligned through the BioEdit software (Ibis Bioscienses), using the bovine (*Bos taurus*) PON1 sequence published in NCBI (number: AC_000161.1) as reference for the alignment. The positions of the SNPs were determined based on the data previously studied in Holstein cows (Silveira et al., 2015). Also, alignment of the sequences obtained with the reference sequence of PON1 gene of ovine, swine, bison and alpaca was performed.

In order to characterize the distribution of genotypes and to correlate with the serum PON1 activity, genotyping of the SNP-221 was performed in all samples (n=52), through enzymatic digestion by specific restriction polymorphism using the BseLI enzyme according to the described and validated by Silveira et al. (2019). The enzyme was incubated with the PCR product at 37 °C for 12 hours and genotyping was determined after 2h of running the DNA fragments by 2.5% agarose gel electrophoresis. Visualization of the amplified and digested gene segments was done in ultraviolet light. Statistical analysis was performed using GraphPad software and means were compared by *t* test. Differences were considered significant when $P \leq 0.05$.

Sequencing indicated the presence of eight SNPs, located at positions -105, -111, -130, -221, -267, -392, -440 and -455inthe promoter region of the bovine PON1 gene of Nelore cows (Table 1). The positions of the SNPs were numbered relative to the first nucleotide of the published mRNA sequence (NM_001046269.2; Silveira et al., 2015). The nucleotide sequences of the PON1 gene from swine, alpaca and bison can not be aligned with the sequence of the Nelore cows promoter region ofPON1 gene from this study, perhaps because it was not possible to identify the gene promoter region in those species. However, alignment with the *Ovis aries* sequence identified a 91% homology with *B. indicus* cows in this study. The sheep sequence was more similar to the *B. indicus* sequence than to *B. taurus*.

There was no association between any of the identified SNPs and PON1 activity. When investigating the occurrence of haplotype, based on the presence of homozygous alleles for every SNP, 70% of cows presented the combination G-C-A-G-A-A-T-T as homozygous at all SNP positions. The PON1 serum activity of cows that had or did not have this profile was 98.9±8.4 U/mLand 79.5±13.9 U/mL, respectively (P=0.24).In genotyping the SNP-221 in a larger sample population, the prevalence of genotypesAA,AG and GG was 5.8% (3/52), 28.8% (15/52) and 65.4% (34/52), respectively, and also not associated to serum PON1 activity.

Of all eight SNPs characterized in the promoter region of the PON1 gene, three were also previously described in Holstein cows (-105, -221 and -392; Silveira et al., 2015; 2019) and were associated with serum activity of PON1 in that breed (Silveira et al., 2015; 2019). However, different from what was observed in Holstein cows (Silveira et al., 2019) as well as in humans (Brophy et al., 2001), none of the polymorphism was associated with serum PON1 activity in Nelore cows from our study. Previously, we observed thatPON1 activity was not associated with ovulation and pregnancy in *B. indicus* cows (Castro et al., 2018), in contrast with what was observed in *B. taurus* (Schneider et al., 2013, Silveira et al., 2019). These results may be associated to the metabolic and endocrine differences between *B. taurus* and *B. indicus*

breeds, as described elsewhere (for review see Sartori et al., 2016). It is important to mention that cows from this study were all more than 45 days postpartum, healthy and had a body condition score > 3.0. This condition may have affected, PON1 activity, which was relatively high (> 70 U/mL) for all cows. Furthermore, we recognized the limitations regarding the number of cows used in this study and only one sample per cow was used. However, this was an initial screening study and larger population and/or more samples per animal should be collected in order to confirm the effect of this SNP on PON1 activity.

The genotyping of the SNP location -221 was performed because this SNP has a strong linkage with all the other SNPs characterized in the promoter region of the PON1 gene in Holstein cows (Silveira et al., 2015; 2019). Furthermore, the allele A was associated with PON1 activity and reproductive performance in Holstein cows (Silveira et al., 2019). The A allele was the most prevalent (~ 80%, AA + AG) among *B. taurus* cows (Silveira et al., 2019).In contrast, the A allele was present in only 34.6% (AA + AG) of *B. indicus* cows in our study, while 65% of the cows had a GG genotype. In the SNP positions -105 and -392, which was also identified in *B. taurus* (Silveira et al., 2015), the highest prevalence was of GG and AA genotype (88% for both) for *B. indicus*, while in *B. taurus*, the proportion was inverse, with 85 % of cows had AA genotype at position -105 and at position -392, AA, AC, and CC genotypes was about 25%, 40% and 36%, respectively (Silveira et al., 2015).Although it was not possible to associate the genotype with reproductive performance in the present study, these differences indicate a selection derivation of the zebu cattle in relation to the european cattle. We observed that in sheep the reported allele is G, which is more similar to what we observed in *B. indicus* cows, suggesting that a selection in *B. taurus* or more specifically in the Holstein breed. However, more studies are necessary to further explore this.

In summary, eight SNPs were identified in the promoter region of the bovine PON1 gene in *B. indicus* cows. However, no association among SNPs and PON1 activity was detected,

suggesting that PON1 does not appear to be a significant genetic marker for use in *B. indicus* cows. Important differences in the prevalence of specific alleles were observed in comparison to previous studies with *B. taurus* cows.

Conflict of interest statement

The authors declare no conflict of interest.

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Table 1

Single nucleotide polymorphisms (SNPs) identified in the promoter region of the bovine paraoxonase 1 (PON1) gene in Nelore postpartum cows.

SNP position	Genotypes / PON1 activity*			*P*-Value
-105	AA	AG	GG	AA+AG vs GG
	5.9% (1/17)	5.9% (1/17)	88% (15/17)	
	43.9	116.7	95 ± 7.2	0.54
-111	CC	CT	TT	CC vs TT
	70.6% (12/17)	0% (0/17)	71.4% (5/17)	
	99 ± 8.1	--	79.5 ± 12.5	0.24
-130	AA	AG	GG	AA vs GG
	70.6% (12/17)	0% (0/17)	29.4% (5/17)	
	99 ± 8.1	--	79.5 ± 12.5	0.24
-221	AA	AG	GG	AA+AG vs GG
	5.9% (1/17)	5.9% (1/17)	88% (15/17)	
	43.9	116.7	95 ± 7,2	0.54
-267	AA	AG	GG	AA vs GG
	70.6% (12/17)	0% (0/17)	29.4% (5/17)	
	99 ± 8.1	--	79.5 ± 12.5	0.24
-392	AA	AC	CC	AA vs AC
	88% (15/17)	11,8% (2/17)	0% (0/17)	
	95 ± 7,2	80.3 ± 25.7	--	0.54
-440	CC	CT	TT	CC+CT vs TT
	23.5% (4/17)	5.9% (1/17)	70.6% (12/17)	
	72.8 ± 13.7	106.43	99 ± 8.1	0.24
-455	CC	CT	TT	CC vs TT
	29.4% (5/17)	0% (0/17)	70.6% (12/17)	
	79.5 ± 12.5	--	99 ± 8.1	0.24

*PON1 activity was evaluated on day of timed artificial insemination and is expressed in mean of U/mL ± Standard error.

4 Projeto em andamento

Este experimento está em andamento e, portanto, serão apresentados dados preliminares.

Todas etapas do experimento foram aprovadas pela Comissão de Ética em Experimentação Animal da Universidade Federal de Pelotas, sob o código 23110.048165/2018-58.

Efeito da injeção intrafolicular de Prostaglandina F2αsobre a ovulação em bovinos

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Introdução

Em programas de controle do ciclo estral, a aplicação de prostaglandina F2α(PGF) exógena é amplamente utilizada devido a sua ação luteolítica, essencial para que o folículo dominante adquira capacidade ovulatória. Entretanto, estudos já demonstraram que a aplicação de análogos de PGF é capaz de induzir a ovulação em bovinos de corte (PFEIFER et al., 2014) e de leite (PFEIFER et al., 2016; CASTRO et al., 2018). Embora o mecanismo de ação da PGF ainda não tenha sido esclarecido, sugere-se que o mesmo ocorre independente da luteólise, uma vez que induziu a primeira ovulação em novilhas pré-púberes (PFEIFER et al., 2009, LEONARDI et al., 2012).

Endogenamente, sabe-se que as prostaglandinas atuam localmente no processo de ovulação, sendo demonstrado um aumento na expressão de receptores de PGF em células da teca e da granulosa de folículos periovulatórios bovinos coletados após tratamento com GnRH (BRIDGES e FORTUNE, 2007).

Resultados ainda não publicados de nosso grupo demonstraram que a PGF não é capaz de induzir um aumento na secreção hipofisária de LH, descartando uma ação a nível central. Assim, hipotetizamos que a PGF exógena atue por um mecanismo direto no folículo ovariano. Assim, o objetivo deste estudo foi avaliar o efeito da injeção intrafolicular de PGF na ovulação do folículo dominante em bovinos.

Material e Métodos

Para este estudo, 21 vacas da raça Jersey pertencentes ao campo experimental da Embrapa Terras Baixas (Capão do Leão, RS, Brasil) foram utilizadas. Os animais foram mantidos em pastagem com livre acesso a água. O desenho experimento está ilustrado na fig. 1. No Dia 0 do experimento, as vacas foram avaliadas por ultrassonografia transretal (Aquila Pro®, Esaote, São Paulo, SP, Brasil) para avaliação do trato uterino e a todas que não apresentarem alteração patológica no útero e/ou ovários foram utilizadas no experimento. As vacas foram submetidas a anestesia epidural baixa (Bravet®), limpeza da região perianal e vulvar com água e sabão e em seguida foi feita aspiração de todos os folículos ovarianos ≥ 5mm, através de punção folicular guiada por ultrassonografia transvaginal. Além disso, as vacas receberam um implante liberador de progesterona (Primer®) e injeção intramuscular contendo 2 mg de benzoato de estradiol (BE; Ric-BE®) visando sincronizar a emergência da nova onda folicular (Dia 0). No Dia 7, todas as vacas reberam 500μg de Cloprostenol (análogo de PGF; Estron®) para remover a fonte endógena de progesterona, através da luteólise e no Dia 8 os implantes foram removidos e as vacas pintadas com tinta para detecção de cio. Doze horas após a remoção dos implantes de progesterona, as vacas com folículo dominante (FD) com diâmetro ≥10 mm que não foram detectadas em cio foram separadas em dois grupos, de forma pareada para que os dois grupos tivessem a média similar de diâmetro folicular. Assim, cada um dos grupos recebeu injeções intrafoliculares contendo um dos seguintes tratamentos: 1) PBS (Grupo Controle, n = 8) ou 2) PGF purificada (Cayman Chemical®; Grupo PGF, n= 8) na concentração final de 100ng/mL de fluido folicular. As doses foram calculadas com base em dados de pesquisa anterior, em que foram aspirados folículos pré-ovulatórios após tratamento com PGF intramuscular (BRIDGES et al., 2007).

Do Dia 5 ao Dia 8, as vacas foram avaliadas por ultrassonografia transretal a cada 24 horas para monitorar a completa lise do corpo lúteo e crescimento do FD. A partir do Dia 8 até a detecção da ovulação o FD que recebeu a injeção contendo os

tratamentos experimentais foi monitorado por ultrassonografia a cada 12 horas. A ovulação foi definida como o desaparecimento do FD que recebeu o tratamento intrafolicular e o diâmetro do folículo pré-ovulatório foi determinado de forma retrospectiva, através da avaliação do diâmetro detectado na última avaliação antes da ovulação. O momento da ovulação foi caracterizado pelo momento da última visualização do folículo pré-ovulatório (FPO) acrescido de seis horas.

Nos dias 5 e 12 após a ovulação de cada vaca, o diâmetro do CL foi avaliado por ultrassom e o sangue foi coletado da veia coccígea, através de sistema de vacutainer, para análise de progesterona circulatória.

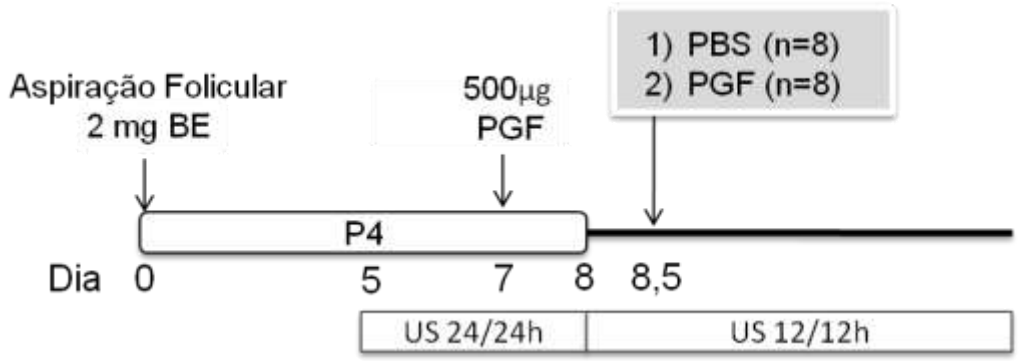

Figura 1. Protocolo de sincronização de ovulação utilizado nas vacas do experimento. Abreviações: BE, benzoato de estradiol; P4, implante intravaginal de progesterona; PGF, análogo de prostaglandina F2α; US, ultrassonografia transretal.

Diluições e injeções intrafoliculares

Para as injeções do Grupo PGF, a PGF purificada foi inicialmente diluída em etanol a 100% para a obtenção de uma solução estoque na concentração de 500μg/mL. Essa solução foi então diluída em PBS para a obtenção de uma solução estoque em PBS a 25μg/mL de PGF. A partir desta solução foi feita outra diluição em PBS para que a solução final de injeção atingisse a concentração de 1μg/mL, que era 10 vezes superior à concentração desejada no fluido folicular. Para o Grupo Controle, o PBS foi diluído em álcool para que se obtivesse a solução de injeção na mesma concentração de etanol presente no Grupo PGF (0,2%). O volume injetado foi calculado com base no volume de fluido folicular estimado através da equação de regressão V=-685,1+120D, onde V corresponde ao volume folicular estimado e D ao diâmetro do folículo a ser injetado, segundo estudo de Ferreira et al. (2007).

Previamente às injeções intrafoliculares, foi feita anestesia epidural através da injeção de lidocaína a 2%, e a região perianal e vulvar foram higienizadas com água, detergente neutro, álcool e solução de iodopolividona. As injeções intrafoliculares foram guiadas por ultrassonografia transvaginal (probe convexa, 7,5 mHz) em metodologia adaptada do método descrito por Ferreira et al. (2007). Um sistema contendo a pobre e uma agulha 20G (0.8mm x 40mm; Becton, Dickinson and Company, USA) foi utilizada para perfurar a parede vaginal, peritônio e estroma ovariano até atingir o folículo e então a agulha 25G 3 ½ (BD, USA) foi inserida no interior do folículo dominante para a injeção dos tratamentos (PGF ou PBS). Imediatamente após a injeção, o sistema foi removido.

Análises estatísticas – em andamento

Todas as análises estatísticas serão realizadas através do programa estatístico GraphPad. O momento da ovulação, o diâmetro do folículo dominante no momento da injeção intrafolicular e o diâmetro do folículo pré-ovulatório foram analisados por teste T, sendo as médias comparadas entre os grupos através do teste de Tukey. Os diâmetros do corpo lúteo nos dias 5 e 12 após a ovulação serão avaliados da mesma forma que as demais análises. Os valores são apresentados na forma de Média ± Desvio Padrão. Valores de $P < 0,05$ serão considerados estatisticamente significativos.

Resultados parciais

Do total de 21 vacas sincronizadas, cinco foram excluídas do experimento. Uma foi excluída por apresentar lesão vulvar e outras quatro por não responderem adequadamente ao protocolo de sincronização de ovulações, apresentando FD menor que 10mm no dia em que as injeções intrafoliculares foram feitas (Dia 8,5). Dessa forma, 16 vacas permaneceram no estudo, sendo divididas igualmente entre os dois grupos.

No momento das injeções intrafoliculares, o diâmetro dos FDs dos Grupos Controle e PGF foram semelhantes (12,4 ± 1,3 mm e 12,2 ± 1,2 mm, respectivamente, P = 0,8). Todas as vacas ovularam em um intervalo de 66 a 78 horas após as injeções intrafoliculares, em ambos os grupos. Também não houve diferença no diâmetro do FPO (13,7 ± 1,2 mm e 14,1 ± 0.8 mm, P = 0,5) e no momento da ovulação (66 ± 6,4 h

e 63 ± 8,5 h após as injeções intrafoliculares, P = 0,4) para os grupos Controle e PGF, respectivamente. Os dados de diâmetro do CL e progesterona ainda serão analisados.

Agradecimentos

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5 Considerações Finais

Os dados apresentados neste estudo envolvem duas linhas de pesquisa relacionadas com os mediadores inflamatórios PGF e PON1 e parâmetros de fertilidades em bovinos.

Em relação à PGF, é possível concluir que, embora estudos anteriores de nosso grupo de pesquisa tenham demonstrado que a PGF induz ovulação em bovinos, o seu mecanismo de ação parece não ser em nível hipotalâmico-hipofisário, como ficou demonstrado no artigo 1, em que o LH circulatório manteve seus níveis basais após a administração de PGF, ao contrário do que ocorreu após o tratamento com GnRH. Além disso, não foram detectados receptores de PGF nos gonadotrofos de hipófises bovinas. Coletivamente, esses resultados demonstram que a PGF não é capaz de estimular o eixo hipotalâmico-hipofisário diretamente para induzir o pico pré-ovulatório de LH.

No artigo 2, em que pesquisamos a relação da atividade de PON1 com crescimento folicular, ovulação e prenhez em vacas de corte submetidas à sincronização de cio, nossos resultados demonstraram que a PON1 não é um bom preditor de ovulação e fertilidade *in vivo*. Ao sequenciar a região promotora do gene da PON1 em vacas da raça Nelore, foi possível caracterizar oito SNPs. Entretanto, não foi detectada associação de nenhum dos SNPs com atividade de PON1, sugerindo que a PON1 não é um bom marcador genético para ser usado em *B. indicus*, ao contrário do que ocorre em vacas *B. taurus* leiteiras.

Em relação aos dados do experimento em andamento, aparentemente não houve diferença na ovulação após injeção intrafolicular de PGF ou PBS em folículos maiores de 10mm, sugerindo a ausência de um efeito local na dosagem utilizada e no momento em que o tratamento foi feito.

Em conjunto, os nossos estudos contribuíram para o entendimento acerca das relações de dois mediadores inflamatórios com parâmetros de fertilidade em fêmeas bovinas. Entretanto, os resultados obtidos demonstram que ainda existem lacunas a serem preenchidas no intuito de identificar o mecanismo de ação da PGF na ovulação.

Além disso, sugere-se que, embora a PON1 já tenha demonstrado ser um bom biomarcador de fertilidade em vacas leiteiras *B. taurus*, o mesmo não se aplica à vacas de corte *B. indicus*. Dessa forma, estudos complementares são necessários para esclarecer o mecanismo de ação destes mediadores.

Finalmente, é importante considerar a relevância dos estudos conduzidos durante o doutorado para a formação acadêmica e profissional desta autora. Além dos experimentos apresentados nesta tese, o doutorado permitiu que vários outros estudos em reprodução animal fossem conduzidos em colaboração com colegas de Porto Velho/RO, Pelotas/RS e Saskatoon/Canadá. Além de contribuir com a comunidade científica, a condução desses estudos, permitiu a doutoranda que novos conhecimentos e habilidades fossem adquiridos em diferentes subáreas envolvidas na reprodução de fêmeas. A absorção de novos conhecimentos é de grande relevância para que possamos dar continuidade nas pesquisas iniciadas, utilizando-nos do senso crítico necessário para colaborar de forma efetiva para o desenvolvimento de pesquisas de qualidade e relevância para esta área de estudo.

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Anexos

Documento da Comissão de Ética e Experimentação Animal

UNIVERSIDADE FEDERAL DE PELOTAS

PARECER Nº **127/2018/CEEA/REITORIA**
PROCESSO Nº 23110.048165/2018-58

Certificado

Certificamos que a proposta intitulada "**Efeito da injeção intrafolicular de Prostaglandina F2 alfa na ovulação de bovinos**" processo número 23110.048165/2018-58, de responsabilidade de Augusto Schneider - que envolve a produção, manutenção ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto humanos), para fins de pesquisa científica (ou ensino) – encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e recebeu parecer **FAVORÁVEL** a sua complementação pela Comissão de Ética em Experimentação Animal, em reunião de 10/12/2018.

Finalidade	(X) Pesquisa ( ) Ensino	
Vigência da autorização	15/12/2018 a 01/12/2019	
Espécie/linhagem/raça	Bovino/Jersey	
Nº de animais	22	
Idade	15 a 48 meses	
Sexo	Fêmeas	
Origem	Embrapa Clima Temperado – Capão do Leão/RS	

Código para cadastro **CEEA 48165-2018**

M.V. Dra. Anelize de Oliveira Campello Felix

Presidente da CEEA

Documento assinado eletronicamente por **ANELIZE DE OLIVEIRA CAMPELLO FELIX, Médico Veterinário,** em 14/12/2018, às 11:44, conforme horário oficial de Brasília, com fundamento no art. 6°, § 1°, do Decreto nº 8.539, de 8 de outubro de 2015.

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