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Tese

Prostaglandinas E2 e F2alfa no processo ovulatório de fêmeas bovinas

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Resumo

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O pico da gonadotrofina ovulatória desencadeia a ativação de cascatas de sinalização intracelular, como a via das prostaglandinas (PGs), especialmente a $PGE2_2$ e a $PGF2A_2\alpha$. Essas moléculas desempenham funções essenciais na ovulação e na manutenção da função luteal em bovinos, o que evidencia a potencial aplicação em biotécnicas reprodutivas associadas aos protocolos hormonais de sincronização do estro para inseminação artificial em tempo fixo.. Nesse contexto, têm sido explorados modelos de estudo de inibição da síntese de PG e, consequentemente, da ovulação, utilizando anti-inflamatórios não esteroidais, como a flunixina meglumina (FM), que atuam sobre a cicloxigenase, enzima chave nesse processo. Com base nessas premissas, o presente trabalho teve como objetivos: avaliar a capacidade das PGs de induzir a ovulação mesmo na ausência do estímulo gonadotrófico; estabelecer modelos de bloqueio ovulatório utilizando FM por vias intramuscular (IM) e intraovariana (IO); verificar se as PGs conseguem reverter a inibição da ovulação causada pela FM quando coadministradas em diferentes momentos; e identificar os mecanismos envolvidos nesse bloqueio, com ênfase na sinalização intercelular mediada por vesículas extracelulares. Foram conduzidos sete experimentos com fêmeas B. taurus cíclicas, não lactantes, cujo desenvolvimento folicular foi sincronizado com progesterona, benzoato de estradiol, cloprostenol sódico e análogos do hormônio liberador de gonadotrofinas (GnRH). No Experimento 1, avaliou-se o efeito da injeção intrafolicular (IIF) de PGE2, isolada ou associada à PGF2A, sobre o momento da ovulação e a produção de progesterona. Os Experimentos 2, 3 e 4 testaram a aplicação de FM em uma ou duas doses, pelas vias IM e IO. Os Experimentos 5 e 6 investigaram se a coadministração IF de PGs poderia reverter o bloqueio ovulatório provocado pela FM aplicada 16 ou 22 horas após o GnRH. O Experimento 7 caracterizou as vesículas extracelulares presentes no fluido folicular de animais tratados com FM, seu perfil de microRNAs e as rotas funcionais moduladas por eles. A ovulação foi monitorada por ultrassonografia, e a função luteal, por concentrações séricas de progesterona. No Experimento 1, a IIF de PGE2 ou PGE2+PGF2A não antecipou a ovulação (mediana de 66 h vs. 42 h no controle), embora a PGE2 tenha aumentado significativamente os níveis de progesterona ($P = 0,0014$). As administrações IM (75–80% de ovulação) e IO (100% de ovulação) de FM não suprimiram a ovulação de maneira consistente. Por outro lado, a IIF de FM realizada 22 horas após o GnRH impediu a ovulação em todos os animais tratados. A tentativa de reverter o bloqueio ovulatório pela coadministração de PGs às 16 horas não foi eficaz (FM: 40%; FM+PGE2: 57%; FM+PGE2+PGF2A: 25%; $P = 0,12$); entretanto, quando aplicadas às 22 horas, a PGE2 restabeleceu parcialmente a

ovulação (44%; P = 0,04), coincidindo com o período de maior liberação fisiológica de PGs. A análise molecular revelou quatro miRNAs (bta-miR-584, bta-miR-369-5p, bta-miR-218 e bta-miR-411c-5p) envolvidos na modulação de vias relacionadas à ruptura folicular e ao metabolismo das PGs, incluindo regulação do citoesqueleto de actina, interação matriz extracelular–receptor, migração transendotelial de leucócitos, contração de músculo liso vascular, biossíntese de arginina, degradação de lisina, proteólise mediada por ubiquitina e ferroptose. Portanto, as PGs exógenas não foram capazes de antecipar a ovulação, embora a PGE2 tenha demonstrado um efeito luteotrófico marcante. A FM, quando administrada por via sistêmica ou intraovariana, não promoveu inibição consistente da ovulação; contudo, sua aplicação intrafolicular mostrou-se altamente eficaz quando realizada pouco antes do pico natural de PGs. Além disso, a capacidade das PGs de reverter parcialmente a inibição induzida pela FM reforça a importância crítica e temporalmente sensível dessas moléculas no processo de ruptura folicular em bovinos, evidenciada também pela alteração do perfil de miRNAs presentes em vesículas extracelulares.

Palavras-chave: anti-inflamatório não esteroidal; flunixina meglumina; inibição da ovulação; miRNA; reversão do bloqueio ovulatório

Abstract

LAZZARI, Jéssica. P**rostaglandins E2 and F2α in the ovulatory process of female cattle.** 2026. 74f. Thesis (Doctor degree in Sciences) - Programa de Pós-Graduação em Veterinária, Faculdade de Veterinária, Universidade Federal de

The ovulatory gonadotropin surge triggers the activation of intracellular signaling cascades, including the prostaglandin (PG) pathway, particularly PGE2 and PGF2A. These molecules play essential roles in ovulation and maintenance of luteal function in cattle, highlighting their potential application in reproductive biotechnologies associated with estrus synchronization protocols for timing artificial insemination. In this context, experimental models have explored inhibition of PG synthesis, and consequently ovulation, using nonsteroidal anti-inflammatory drugs such as flunixin meglumine (FM), which act on cyclooxygenase, a key enzyme in this process. Based on these premises, the objectives of the present study were: to evaluate the ability of PGs to induce ovulation in the absence of a gonadotropic stimulus; to establish ovulatory blockade models using FM administered via intramuscular (IM) and intraovarian (IO) routes; to determine whether PGs can reverse FM-induced ovulation inhibition when co-administered at different time points; and to identify the mechanisms involved in this blockade, with emphasis on intercellular signaling mediated by extracellular vesicles. Seven experiments were conducted using cyclic, non-lactating B. taurus females whose follicular development was synchronized with progesterone, estradiol benzoate, sodium cloprostenol, and gonadotropin-releasing hormone (GnRH) analogs. In Experiment 1, the effect of intrafollicular (IF) administration of PGE2, alone or combined with PGF2A, on the timing of ovulation and progesterone production was evaluated. Experiments 2, 3, and 4 tested FM administration in one or two doses via IM and IO routes. Experiments 5 and 6 investigated whether intrafollicular co-administration of PGs could reverse FM-induced ovulatory blockade when FM was applied 16 or 22 hours after GnRH. Experiment 7 characterized extracellular vesicles present in the follicular fluid of FM-treated animals, their microRNA profiles, and the functional pathways they modulate. Ovulation was monitored by ultrasonography, and luteal function was assessed by serum progesterone concentrations. In Experiment 1, IF administration of PGE2 or PGE2+PGF2A did not advance ovulation (median 66 h vs. 42 h in controls), although PGE2 significantly increased progesterone levels ($P = 0.0014$). IM (75–80% ovulation) and IO (100% ovulation) administration of FM did not consistently suppress ovulation. In contrast, intrafollicular FM administered 22 hours after GnRH prevented ovulation in all treated animals. Attempts to reverse ovulatory blockade by co-administering PGs at 16 hours were not effective (FM: 40%; FM+PGE2: 57%; FM+PGE2+PGF2A: 25%; $P = 0.12$). However, when applied at 22 hours, PGE2 partially restored ovulation (44%; $P = 0.04$), coinciding with the period of peak physiological PG release. Molecular analysis identified four miRNAs (bta-miR-584, bta-miR-369-5p, bta-miR-218, and bta-miR-411c-5p) involved in modulating pathways related to follicular rupture and PG

metabolism, including regulation of the actin cytoskeleton, extracellular matrix–receptor interaction, leukocyte transendothelial migration, vascular smooth muscle contraction, arginine biosynthesis, lysine degradation, ubiquitin-mediated proteolysis, and ferroptosis. Therefore, exogenous PGs could not advance ovulation, although PGE_2 demonstrated a marked luteotropic effect. Systemic or intraovarian FM administration did not consistently inhibit ovulation; however, its intrafollicular application was highly effective when performed shortly before the natural PG peak. Moreover, the ability of PGs to partially reverse FM-induced inhibition reinforces the critical and temporally sensitive role of these molecules in the process of follicular rupture in cattle, further supported by alterations in the miRNA profile of extracellular vesicles.

Keywords: flunixin meglumine; miRNA; nonsteroidal anti-inflammatory drug; ovulation inhibition; reversal of ovulatory blockade.

Lista de Figuras

Lista de Abreviaturas e Siglas

AINE	Anti-inflamatório não esteroidal
BE	Benzoato de Estradiol
CL	Corpo lúteo
COX	Cicloxigenase
COX1	Cicloxigenase-1
COX2	Cicloxigenase-2
CS	Cloprostenol Sódico
D0	Dia zero
D10	Dia dez
D7	Dia sete
D8	Dia oito
D9	Dia nove
EB	Estradiol Benzoate
EV	Vesícula Extracelular
EVs	Vesículas Extracelulares
FF	Fluido Folicular
FFs	Fluidos Foliculares
FM	Flunixina Meglumina
FSH	Hormônio Folículo-Estimulante
GnRH	Hormônio Liberador de Gonadotrofinas
IF	Intrafolicular
IIF	Injeção Intrafolicular
IM	Intramuscular
IO	Intraovariana
IV	Intravenosa

IVD	Intravaginal device
LH	Hormônio Luteinizante
miRNA	micro RNA
mRNA	RNA mensageiro
NSAID	Non-Steroidal Anti-Inflammatory Drug
P4	Progesterona
PG	Prostaglandina
PGE2	Prostaglandina E_2
PGF2A	Prostaglandina $F_2\alpha$
PGs	Prostaglandinas
RNA	Ácido Ribonucleico
RNAm	RNA mensageiro

Sumário

1 Introdução

A bovinocultura brasileira contabilizou, em 2024, 35,7 bilhões de litros de leite e o abate de 39,27 milhões de cabeças, conferindo ao país a posição em nível mundial de terceiro maior produtor de leite e segundo maior produtor de carne (IBGE, 2025). Dessa forma, para atender à demanda, é necessária a implementação de biotécnicas reprodutivas, como a inseminação artificial e a transferência de embriões, que maximizem o desempenho reprodutivo. Em 2023, 16,6 milhões de fêmeas bovinas foram inseminadas e estima-se a movimentação de 800.000 a 900.000 embriões (ASBIA, 2024). Para atingir esses patamares, a manipulação do ciclo estral é uma ferramenta fundamental.

Ao final dos protocolos hormonais de sincronização da onda folicular são utilizados indutores de ovulação, representados principalmente pelos ésteres de estradiol e pelos análogos do GnRH (Bó et al., 2016a). O GnRH liga-se à adenohipófise, induz um pico do LH e, por conseguinte, desencadeia a ovulação entre 24 e 30 horas pós-GnRH (Bó et al., 2016a). Apesar do avanço nos protocolos hormonais para aprimorar a sincronia na emergência de uma nova onda folicular e no momento da ovulação, este ainda é um gargalo a ser estudado. Por isso, têm-se buscado novas estratégias farmacológicas relacionadas aos indutores de ovulação.

O pico pré-ovulatório de LH desencadeia a ativação de cascatas de sinalização intracelular, como a via das PG, que culminam na expansão do cúmulo, ruptura folicular, luteinização, produção de P4 e manutenção do CL (Duffy, 2015). As PGE2 e PGF2A são os principais mediadores envolvidos nesses processos e são sintetizadas a partir do ácido araquidônico presente nos fosfolipídios de membrana (Duffy, 2015). Ao chegarem à corrente sanguínea, são rapidamente inativadas nos pulmões. Dentre as enzimas que catalisam esse processo, a COX2, também conhecida como PG G/H sintase-2, é a principal enzima limitante, enquanto sua isoforma COX-1 está presente no ovário, mas não é fundamental nessa cascata (Duffy, 2015).

Os eventos de aumento da expressão da COX2 e, posteriormente, do pico de PG no FF ocorrem em diferentes espécies (Berisha et al., 2019; Duffy; Stouffer, 2001;

Espey; Norris; Saphire, 1986; Liu et al., 1997; Murdoch; Dunn, 1983), sendo todos LH-dependentes. Em equinos, a hCG induz aumento da expressão da COX2 entre 24 e 30 horas pós-hCG e o pico de PG entre 33 e 36 horas pós-hCG (Cuervo-Arango Lecina; Domingo Ortíz, 2011). Em bovinos, o pico de COX2 ocorre a partir de 18 horas pós-GnRH, enquanto o pico de PG e de seus receptores ocorre em 24 horas pós-GnRH (Berisha et al., 2019; Liu et al., 1997; Markosyan; Duffy, 2009). Ainda, o pico pré-ovulatório dos prostanoides no FF de bovinos apresenta concentrações de 484,21 ng de PGE2/mL e 101,01 ng de PGF2A/mL, demonstrando a importância da PGE2 para a espécie (Bridges; Komar; Fortune, 2006).

O potencial ovulatório de PG exógenas administradas por meio de IIF já foi avaliado em equinos e bovinos. A PGE2, associada ou não à PGF2A, foi capaz de antecipar a ovulação e promover a produção de P4 (Aguilar et al., 2018; Gaber et al., 2024; Martínez-Boví; Cuervo-Arango, 2016). Em contrapartida, a PGF2A isoladamente inibiu ou não afetou a ovulação (De Moraes et al., 2021a; Grippo et al., 2020). Dessa forma, fica evidente a lacuna de conhecimento em relação aos mecanismos de ação da PGE2 no período pré ovulatório. Assim, torna-se necessário averiguar e compreender melhor a capacidade de regulação da ovulação mediada por PG.

Os principais modelos de estudo de PG envolvem o bloqueio de sua síntese por meio de AINEs, que impedem a ação da COX2 em diferentes espécies (Ainsworth et al., 1979; Cuervo-Arango, 2011; Espey; Norris; Saphire, 1986; Murdoch; Dunn, 1983; Tsafriri; Koch; Lindner, 1973; Vernunft et al., 2022). Inicialmente, a indometacina predominava como agente inibitório, mas, com o passar do tempo, deu lugar à FM, fármaco amplamente utilizado na clínica de animais de grande porte e recentemente explorado em biotécnicas reprodutivas por antagonizar a ação luteolítica (Barkhori-Mehni et al., 2018).

Além da variação de princípios ativos, diferentes vias de aplicação já foram testadas, com ação sistêmica (IM, IV, SC) ou local. O aperfeiçoamento dos modelos e técnicas levou à preferência por modelos locais, uma vez que o foco dos estudos é apenas a ação ovariana. Em bovinos, a ovulação pode ser totalmente bloqueada pela IIF de 338 µM de FM ou 70 µM de indometacina administradas 16 horas pós-GnRH (Vernunft et al., 2022), antes do aumento da expressão da COX2 (Bridges; Komar; Fortune, 2006; Liu; Sirois, 1998). Contudo, visto que a análise do conteúdo do FF pode contribuir para melhor compreensão do processo ovulatório, a IIF não é a via mais

adequada, pois uma segunda perfuração do folículo resultaria em uma amostra contaminada com sangue, prejudicando as análises. Assim, como alternativa local, é possível o bloqueio da ovulação com a administração de indometacina pela via IO (De Silva; Reeves, 1985) e, como alternativa sistêmica, múltiplas doses de FM (Cuervo-Arango Lecina; Domingo Ortíz, 2011; Pugliesi et al., 2012).

A continuidade das investigações a partir desses modelos inclui a averiguação da capacidade das PG exógenas em restabelecer a ovulação, fenômeno já relatado em roedores, primatas, suínos, equinos e bovinos (De Moraes et al., 2021a; Downey; Ainsworth, 1980; Kim; Harris; Duffy, 2014; Martínez-Boví; Cuervo-Arango, 2016; Orczyk; Behrman, 1972). Nesses estudos, a administração de PGE2, associada ou não à PGF2A, foi realizada no intervalo correspondente ao pico endógeno de PG e por diferentes vias (predominantemente sistêmicas). Em primatas, demonstrou-se viável o modelo de coadministração, no qual a IIF de indometacina juntamente com PGE2 foi capaz de restabelecer a ovulação (Duffy; Stouffer, 2002). Já em éguas, o efeito inibitório da FM IM foi revertido com a IIF de PGE2 associada à PGF2A (PGE2+PGF2A) (Martínez-Boví; Cuervo-Arango, 2016).

Averiguada a capacidade das PG de regular a ovulação, é necessário compreender melhor de que forma esses processos são mediados. Nos últimos anos, a sinalização intercelular por meio das VE tem ganhado destaque. Essas vesículas são nanopartículas secretadas no ambiente extracelular e carreiam moléculas bioativas (Valadi et al., 2007). Dentre essas moléculas, destacam-se os miRNA, RNAs não codificantes (~22 nucleotídeos) e reguladores pós-transcricionais (Cai et al., 2009). Em bovinos, já foi demonstrado que alterações fisiológicas na expressão de miRNAs regulam o desenvolvimento folicular e o estabelecimento da gestação (Mazzarella et al., 2021; Navakanitworakul et al., 2016). A partir da identificação dos fragmentos diferencialmente expressos, conduzem-se análises de bioinformática que permitem traçar quais rotas funcionais são influenciadas. Assim, possibilita revelar os mecanismos regulatórios envolvidos na ovulação e abrir caminhos para estratégias reprodutivas mais precisas e eficientes.

Portanto, esta tese é composta pelo artigo de revisão, que será submetido a *Reproduction in Domestic Animals* e pelo artigo científico publicado na *Theriogenology* composto por sete experimentos que foram conduzidos com os objetivos gerais de avaliar o potencial ovulatório das PGs na ausência de gonadotrofina ovulatória, estabelecer modelos de estudo de bloqueio de ovulação com a administração da FM

pelas vias paranteral e IO, avaliar a capacidade das PGs em neutralizar o efeito inibitório da FM sobre a ovulação quando administradas conjuntamente em diferentes momentos e compreender os mecanismos regulatórios associados ao bloqueio ovulatório induzido por FM, com ênfase na comunicação intercelular mediada por vesículas extracelulares.

Os objetivos específicos incluem: avaliar o efeito da administração de PGE2, isolada ou associada à PGF2A, sobre a indução da ovulação e a função luteal; determinar se uma ou duas doses de FM IM são capazes de bloquear a ovulação; avaliar se a administração IO de FM impede a ovulação; verificar se a PGE2, isolada ou associada à PGF2A, restaura a ovulação previamente bloqueada pela FM quando coadministrada 16h pós-GnRH; verificar se a coadministração de PGE2 e FM, 22h pós-GnRH é capaz de reverter o bloqueio ovulatório; caracterizar as alterações no perfil de miRNAs de VE do FF em animais tratados FM e determinar as rotas funcionais moduladas por esses miRNAs.

2 Artigos

2.1 Artigo de revisão

Controle farmacológico da ovulação em espécies domésticas: papel das prostaglandinas e seus inibidores

Jéssica Lazzari, Fabiane Pereira de Moraes, Rafael Gianella Mondadori

Será submetido à revista Reproduction in Domestic Animals

Controle farmacológico da ovulação em espécies domésticas: papel das prostaglandinas e seus inibidores

Jéssica Lazzari, Fabiane Pereira de Moraes, Rafael Gianella Mondadori

Resumo

A cascata ovulatória é regulada pelas prostaglandinas (PG), especialmente a prostaglandina E2 (PGE) e a prostaglandina F2A (PGF) em diferentes espécies e, por isso, tem sido alvo de estudos como estratégia para indução da ovulação. A PGE2 é capaz de antecipar a ovulação e possui efeito luteotróficos, enquanto a PGF2A possui efeito lutelítico. Para estudar esses mediadores, tem sido utilizados anti-inflamatórios não-esteroidais, fármacos inibidores da cicloxigenase 2, reduzindo a síntese de PG. A indometacina e a flunixina meglumina diminuem a concentração de PG no fluído folicular, causando a inibição da ovulação com efeito dose dependente, vias e momento de aplicação, sem comprometer a formação ou a funcionalidade do corpo lúteo. Esses fatores afetam igualmente o restabelecimento da ovulação por PG exógenas, possivelmente devido à variação da concentração alcançada no folículo. Portanto, essa revisão abordará o papel das prostaglandinas no processo ovulatório, a partir do bloqueio farmacológico de sua síntese em espécies domésticas.

Palavras-chave: anti-inflamatórios não-esteroidais, inibição da cicloxigenase, inibição da ovulação, prostaglandina E, bloqueio ovulatório

1. INTRODUÇÃO

As biotécnicas reprodutivas maximizam o desempenho dos rebanhos e para isso, na bovinocultura, a manipulação do ciclo estral é fundamental. Ao final dos protocolos de sincronização da onda folicular são utilizados indutores de ovulação, representados principalmente pelos ésteres de estradiol e pelos análogos do Hormônio Liberador de gonadotrofinas (GnRH, Bó et al., 2016). O GnRH liga-se à gonadotrofos, induz um pico do hormônio luteinizante (LH) e, por conseguinte, a ovulação entre 24 e 30 horas após a aplicação de GnRH (Bó et al., 2016b). Apesar do aprimoramento dos protocolos hormonais para sincronização da emergência de uma nova onda folicular e do momento da ovulação, ainda é possível observar uma dispersão no momento da ovulação (Sales et al., 2025). Assim sendo, vários estudos tem buscado novas estratégias farmacológicas relacionadas aos indutores de ovulação (Barbosa et al., 2022; Garcia-Ispierto; Llobera-Balcells; López-Gatius, 2021).

O pico pré-ovulatório de LH desencadeia a ativação de cascatas de sinalização intracelular, como a via das prostaglandinas (PG), que culminam na expansão do cumulus, ruptura folicular, luteinização, produção de progesterona (P4) e manutenção do corpo lúteo (CL, Duffy, 2015). As prostaglandinas E2 (PGE) e F2A (PGF) são importantes mediadores envolvidos nesses processos e são sintetizadas a partir do ácido araquidônico presente nos fosfolipídios de membrana (Duffy, 2015). Dentre as enzimas que catalisam esse processo, a cicloxigenase 2 (COX2), também conhecida como prostaglandina G/H sintase-2, é a principal enzima limitante, enquanto sua isoforma cicloxigenase 1 (COX-1), apesar de estar presente no ovário, não é fundamental nessa cascata (Duffy, 2015).

Os eventos de aumento da expressão da COX2 e, posteriormente, do pico de PG no fluido folicular (FF) já foram descritos em diferentes espécies (Berisha et al., 2019; Duffy; Stouffer, 2001; Espey; Norris; Saphire, 1986; Liu et al., 1997; Murdoch; Dunn, 1983) sendo todos LH-dependentes. Em equinos, a gonadotrofina coriônica humana (hCG) induz aumento da expressão da COX2 entre 24 e 30 horas após a sua aplicação, enquanto o pico de PG ocorre entre 33 e 36 horas pós-hCG (Cuervo-Arango Lecina; Domingo Ortíz, 2011). Em bovinos, o pico de COX2 ocorre a partir de 18 horas pós-GnRH, enquanto o pico de PG e de seus receptores ocorre em 24 horas pós-GnRH im (Berisha et al., 2019; Liu et al., 1997; Markosyan; Duffy, 2009). Ainda, o pico pré-ovulatório desses prostanoides no FF de bovinos apresenta concentrações

de 484,21 ng de PGE2/mL e 101,01 ng de PGF2A/mL, demonstrando a importância da PGE2 para a espécie (Bridges; Komar; Fortune, 2006).

O potencial ovulatório de PG exógenas administradas por meio de injeções intrafoliculares (IIF) já foi avaliado em equinos e bovinos. A PGE2, associada ou não à PGF2A, foi capaz de antecipar a ovulação de equinos (Aguilar et al., 2018; Gaber et al., 2024; Martínez-Boví; Cuervo-Arango, 2016), enquanto o mesmo efeito não foi observado por nosso grupo em bovinos (Lazzari et al., 2025). Em fêmeas púberes IIF de PGF2A isoladamente apresentou diferentes efeitos sobre a ovulação (De Moraes et al., 2021b; Grippo et al., 2020; Leonardi et al., 2012), porém em novilhas pré-púberes, a aplicação de PGF2A por via intramuscular (IM) induziu a ovulação (Leonardi et al., 2012). Independente da espécie, a PGE2 possui efeito luteotrófico, ao promover a produção de P4 (Aguilar et al., 2018; Gaber et al., 2024; Lazzari et al., 2025; Martínez-Boví; Cuervo-Arango, 2016).

Os principais modelos de estudo de PG envolvem o bloqueio de sua síntese por meio de anti-inflamatórios não esteroidais (AINEs), que impedem a ação da COX2 em diferentes espécies (Ainsworth et al., 1979; Cuervo-Arango, 2011; Espey; Norris; Saphire, 1986; Murdoch; Dunn, 1983; Tsafriri; Koch; Lindner, 1973; Vernunft et al., 2022). Esses modelos permitem isolar o efeito das PG, considerando que outros fatores também estão envolvidos na cascata da ovulação. Nos estudos iniciais, a indometacina predominava como agente inibitório, mas, com o passar do tempo, a flunixina meglumina (FM) passou a ser mais utilizado. Pelo seu amplo uso na clínica de animais de grande porte, recentemente passou a ser explorado em biotécnicas reprodutivas por antagonizar a ação luteolítica (Barkhori-Mehni et al., 2018).

Além da variação de princípios ativos, diferentes vias de aplicação já foram testadas, com ação sistêmica (IM, intravenosa — IV e subcutânea — SC) ou local (injeção intrafolicular – IIF e intraovariana – IO). O aperfeiçoamento dos modelos e técnicas levou à preferência por modelos locais, uma vez que o foco dos estudos é apenas a ação ovariana. Em bovinos, a dinâmica da ovulação foi afetada pela IIF de 338 µM de FM, bem como é possível obter o bloqueio total da ovulação com a aplicação de 70 µM de indometacina (Vernunft et al., 2022). Em ambos os casos, a IIF foi realizada 16 horas pós-GnRH, ou seja, antes do aumento da expressão da COX (Bridges; Komar; Fortune, 2006; Liu; Sirois, 1998). Outra estratégia eficiente é a IIF de 50 µg de FM/mL FF 22h pós-GnRH (Lazzari et al., 2025), anterior ao pico pré-ovulatório de PG (Berisha et al., 2019). Contudo, visto que a análise do conteúdo do

FF poderia contribuir para melhor compreensão do processo ovulatório, a IIF não é a via mais adequada, pois uma segunda perfuração do folículo resultaria em uma amostra contaminada com sangue, prejudicando as análises. Assim, como alternativa local, é possível o bloqueio da ovulação com a administração de indometacina pela via intraovariana (IO) (De Silva; Reeves, 1985) e, como alternativa sistêmica, múltiplas doses de FM (Cuervo-Arango Lecina; Domingo Ortíz, 2011; Pugliesi et al., 2012).

A continuidade das investigações a partir desses modelos inclui a averiguação da capacidade das PG exógenas em restabelecer a ovulação, fenômeno já relatado em roedores, primatas, suínos, equinos e bovinos (De Moraes et al., 2021b; Downey; Ainsworth, 1980; Kim; Harris; Duffy, 2014; Lazzari et al., 2025; Martínez-Boví; Cuervo-Arango, 2016; Orczyk; Behrman, 1972). Nesses estudos, a administração de PGE2, associada ou não à PGF2A, foi realizada no intervalo correspondente ao pico endógeno de PG e por diferentes vias (predominantemente sistêmicas). Em primatas, foi demonstrada a viabilidade do modelo de coadministração, no qual a IIF de indometacina juntamente com PGE2 foi capaz de restabelecer a ovulação (Duffy; Stouffer, 2002). Na mesma linha, mas em bovinos com FM, a coadministração às 16h pós-GnRH não foi eficiente, mas às 22h pós-GnRH a ovulação foi reestabelecida (Lazzari et al., 2025). Já em éguas, o efeito inibitório da FM IM foi revertido com a IIF de PGE2 associada à PGF2A (PGE2+PGF2A) (Martínez-Boví; Cuervo-Arango, 2016).

Considerando a visão geral descrita até aqui, esta revisão sintetiza (1) bloqueio ovulatório com AINE/inibição de COX, (2) estratégias e limitações do restabelecimento da ovulação com PG exógenas e (3) evidências sobre indução da ovulação com PG e destacando variações experimentais (dose, via, tempo, molécula) e diferenças entre espécies, com ênfase em bovinos.

2. AINE NO BLOQUEIO DA OVULAÇÃO

A utilização de AINE para estudar PG na ovulação é uma importante ferramenta pois essas drogas reduzem a ação da PG competindo com o ácido araquidônico pelo sítio ativo da COX (Duffy, 2015). Os AINE podem ser seletivos para COX-2, como o NS-398 (altamente seletivo) e o meloxicam, porém, a maioria dos experimentos que buscam avaliar a capacidade dos AINE de bloquear a ovulação foram realizados utilizando a indometacina e FM, que são inibidores não seletivos, ligando-se as duas isoformas da COX (Vernunft et al., 2022).

A posologia recomendada da FM indica 2,2 mg/kg a cada 24 h ou 1,1 mg/kg a cada 12h, pelas vias intravenosa (IV), intramuscular (IM) ou subcutânea (SC) (Kissell et al., 2012). A flunixina é metabolizada em 5-hidroxi FLU no fígado e excretada na urina e nas fezes, podendo ser identificada em baixas proporções no leite (Kissell et al., 2012). A aplicação parenteral de FM (1,1 mg/kg no intervalo de 12h), gerou um pico plasmático médio de 8,8 µg/ml para a via SC; 2,2 ± 0,96 µg/ml para via IM (com tempo para concentração máxima de 0,25 a 0,5h pós injeção) e 1,33 ± 0,65 µg/ml para via SC (com tempo para concentração máxima de 0,25 a 2h). Vinte e quatro horas após a segunda aplicação, a flunixina não foi identificada no grupo IV e em metade dos animais dos demais grupos (Kissell et al., 2012). Conforme está descrito nos parágrafos abaixo, a literatura sobre a inibição da ovulação com FM e indometacina, em diferentes espécies (roedores, coelhos, ovinos, suínos, equinos, bovinos), demonstra uma extensa variação nas doses, momentos de aplicação em relação ao momento da ovulação e vias de administração (IIF, IM, IV, SC, IO, intraperitoneal e infusão intrauterina).

Quarenta e seis horas após a estimulação do desenvolvimento folicular com Gonadotrofina Coriônica equina (eCG) (ovulação 72h pós injeção), ratas pré-púberes receberam 7 mg/kg de indometacina SC sendo observado o bloqueio completo da ovulação (Orczyk; Behrman, 1972). Já, em fêmeas cíclicas no proestro, a indometacina SC na dose de 10mg/ranimal foi eficaz no bloqueio de 83 – 95% das ovulações (Tsafriri; Koch; Lindner, 1973).

Em coelhas, 10mg/Kg de indometacina IV, aplicada 8h após estímulo com hCG (2h antes da ovulação prevista) provocou inibição total da ovulação com diminuição da concentração das PG por até 7h pós-indometacina. Quando a aplicação foi realizada 9h e 10h a ovulação foi inibida em 60% e 31% dos animais, respectivamente. A aplicação mais próxima ao momento da ovulação, apesar de não afetar o momento da ovulação em relação ao controle, foi capaz de reduzir a concentração de PG. Os autores sugerem que na hora anterior a ovulação, a produção de PG é dispensada, sem prejudicar a ruptura folicular (Espey; Norris; Saphire, 1986).

Em ovelhas a IIF de 100 µg de indometacina, 12h após a detecção do início do estro (próximo do pico de LH), também foi capaz de bloquear completamente a ovulação, reduzindo concentração de PGF no FF em 7X. A luteinização e a produção de P4 não foi afetada pelo tratamento (Murdoch; Dunn, 1983). De forma similar, em leitoas pré-púberes com ovulação sincronizada por eCG e hCG (ovulação 42h pós-

hCG), a indometacina (IM; 650 mg 12h pós-hCG e 325 mg 24, 36 e 48h pós-hCG ou 650 mg - 20 e 32h pós-hCG) bloqueou completamente a ovulação. O tratamento provocou a queda dos níveis de PGE2 e PGF2A no FF que se mantiveram baixos até 120h pós-hCG e, tanto a luteinização e produção de P4, não foram afetadas pelo AINE (Ainsworth et al., 1979).

Em éguas tratadas com 2 mg de FM/kg IV a cada 12h (dose recomendada, 1,1 mg/kg, até 2x/dia) a partir da indução da ovulação com hCG, houve a inibição da ovulação em 83% dos animais (Cuervo-Arango & Domingo-Ortiz, 2011). Em outro estudo, foi utilizado 1,7 mg de FM/kg IV no momento do aumento da expressão de COX (24h pós-hCG) e no momento pico de PGs (36h pós-hCG), também levando a bloqueio da ovulação em 80% dos animais (Cuervo-Arango, 2011).

Em vacas, a IIF do inibidor seletivo de COX-2 NS-398, 24 horas antes do horário previsto da ovulação foi capaz de bloquear a ovulação, reduzindo a concentração de PGE2 no FF. Apesar disso, oito dias após a IIF, os folículos luteinizados produziam P4 com concentração compatível a um CL fisiológico (P4>2,7ng/mL) (Peters et al., 2004). Quando aplicada pela mesma via, a indometacina provocou efeitos similares com bloqueio da ovulação, redução da concentração de PGE2 no FF e formação de folículos luteinizados (LAPP et al., 2020). De Silva e Reeves (1985), avaliando diferentes vias de aplicação (IM, intrauterina e IO) da indometacina observaram que apenas a via IO (16h após o início do estro) foi capaz de bloquear a ovulação. No entanto, em experimento conduzido por nosso grupo, a aplicação de FM IO não foi capaz de bloquear a ovulação (Lazzari et al., 2025). Por outro lado, repetidas doses de FM IM (2,5 mg/kg - 4, 8, 16, 24, 32 e 40h após o início do estro) são capazes de bloquear a ovulação de bovinos, provocando redução na concentração e número de pulsos de metabólitos de PGF. De forma similar ao descrito anteriormente os folículos anovulatórios luteinizaram, indicando que a inibição das PG bloqueou a ovulação sem afetar a formação das células lúteas (Pugliesi et al., 2012).

Avaliando diferentes AINE via IIF, Vernunft et al. (2022) obtiveram bloqueio total da ovulação utilizando 70 µM de indometacina e 338 µM de FM. A aplicação de 35 µM de indometacina e 1725 µM de meloxicam inibiram a ovulação de 75% dos animais, enquanto 60 µM NS-398 não alterou a ovulação. Apesar de algumas dosagens não interferirem no momento da ovulação, foi observado redução na concentração de PGE2 no FF (Vernunft et al., 2022). Em nossos achados foi possível observar que a IIF de 338 µM de FM, 16h após a indução da ovulação com GnRH (lecirelina),

provocou o bloqueio da ovulação em 66,6% dos animais, porém, quando a mesma dose foi aplicada 22h pós-GnRH, próximo ao pico de PG, o bloqueio foi completo (Lazzari et al., 2025), mostrando a importância de observar o momento da aplicação do tratamento ao avaliar estudos dessa natureza. Assim, em conjunto esses dados demonstram que, em diferentes espécies, os AINEs são capazes de bloquear a ovulação e reduzir da concentração de PG no FF, sem comprometer a posterior luteinização e a produção de P4.

3. PGs NO RESTABELECIMENTO DA OVULAÇÃO

Considerando que a inibição da COX por AINE reduz a síntese de PG no folículo pré-ovulatório e pode bloquear a ruptura folicular, é importante avaliar se a reposição de PG é capaz de restabelecer a ovulação quando esta foi farmacologicamente bloqueada. Nesse sentido, em ratas pré-púberes tanto a PGE2 (0,25 mg/rato) quanto a PGF2A (0,25 mg/rato) SC foram capazes de reestabelecer a ovulação (bloqueada por 7 mg/kg de indometacina SC) em 66,6% dos animais (Orczyk; Behrman, 1972). De forma similar, em ratas cíclicas, a PGE via intraperitoneal (1mg/rata) também reestabeleceu a ovulação bloqueada por 10mg/rata SC (Tsafriri; Koch; Lindner, 1973). Por outro lado, Espey et al. (1992) ao avaliarem a aplicação conjunta de PGE2 e PGF2A bem como o fracionamento da dose das PG, não obtiveram sucesso em reverter o efeito inibitório da ovulação obtido por indometacina. Considerando que a utilização PGE2 ou PGF2A não foi capaz de recuperar os níveis fisiológicos das PG, os autores apontam que a quantidade de PG disponível nos folículos ovarianos foi insuficiente para mimetizar a elevação pré-ovulatória, em virtude da rápida metabolização pulmonar.

Em camundongos fêmeas com deleção do gene da COX-2, a PGE2 e não a PGF2A foi capaz de reverter o bloqueio da ovulação. A PGE2 também foi capaz de manter a organização estrutural da expansão do *cumulus oophorus* (CO). Assim, vias mediadas pela PGE2 são as principais vias envolvidas na expansão do CO e na ovulação. Esse estudo também demonstrou que as PG não afetaram a morfologia ovariana nem a produção de P4 (Davis et al., 1999). De forma similar ao observado em roedores, em primatas, a PGF2A (6 aplicações de SC 3mg/animal) não foi capaz de reestabelecer a ovulação bloqueada por indometacina (Wallach et al., 1975). Em outro estudo, a indometacina e PGE2 foram injetadas simultaneamente

(coadministração) no folículo de macacas, sendo possível corroborar a hipótese de que a PGE2 é capaz de prevenir a falha ovulatória, sendo essencial para a liberação do oócito em primatas (Duffy; Stouffer, 2002; Kim; Harris; Duffy, 2014). Avaliando a coadministração de AINE e PG em vacas, nosso grupo observou que quando a IIF foi realizada 16h pós-GnRH, o momento e a taxa de ovulação não foram alteradas, porém, quando o tratamento foi realizado 22h pós-GnRH, a PGE2 reestabeleceu parcialmente (44%) a ovulação (Lazzari et al., 2025).

Em leitoas pré-púberes com ovulação bloqueada por indometacina 600mg de indometacina/animal IM 24h pós-hCG (provável ovulação 42h pós-hCG) a ovulação foi parcialmente restaurada com 10mg PGF2A 38h pós-hCG e 5 mg de PGF2A 32, 36 e 40h pós-hCG. Foi observado que quanto mais próximo ao momento da provável ovulação, maior o percentual de folículos ovulados. Por outro lado, a aplicação de PGE em diferentes momentos não foi capaz de restaurar a ovulação nessa espécie. (Downey; Ainsworth, 1980). Esses resultados demonstram que, em suínos, o papel da PGF em restaurar a ovulação parece ser mais importante que o da PGE2, provavelmente porque a proporção de PGE2:PGF2A no fluído folicular dessa espécie é de 1:1, enquanto em roedores e bovinos é de 5:1.

Em equinos, a aplicação seriada de PGF2A IV (coincidindo com o pico de PG pré ovulatório) não foi capaz de reverter o bloqueio da ovulação causado por FM, provavelmente por não haver nível mínimo de PGF2A no FF (Cuervo-Arango, 2012). Por outro lado a IIF 500 µg PGE2 + 125 µg PGF2A (análogos sintéticos) foi capaz de promover a ovulação das éguas (Martínez-Boví; Cuervo-Arango, 2016).

Em vacas, nosso grupo observou que, em animais tratados com FM IM previamente ao aumento da expressão de COX no folículo pré ovulatório, a utilização de PGF IM reduziu a vascularização da parede folicular (De Moraes et al., 2021b). Também observamos que a IIF de PGE2, próxima ao pico de PG no folículo ovulatório (22h após GnRH), foi capaz de reverter o bloqueio ovulatório obtido com FM (Lazzari et al., 2025). Assim, os resultados demonstram que, de forma geral, a PGE2 é a principal mediadora do processo ovulatório em roedores, primatas e bovinos, diferente do observado em suínos onde a PGF parece exercer papel relativamente mais relevante. Fica também evidente que variações de espécie, protocolo e momento de intervenção explicam grande parte das inconsistências observadas entre estudos.

4. PGs NA ANTECIPAÇÃO DA OVULAÇÃO

O pico da gonadotrofina pré-ovulatória é fundamental para que ocorra a síntese de PG e ovulação (Duffy, 2015). Assim, para avaliar a capacidade das PGs em induzir a ruptura folicular na ausência de LH, faz necessário que as intervenções experimentais sejam realizadas antes do pico endógeno dessa gonadotrofina que em equinos ocorre progressivamente até o final do estro (Ginther et al., 2008) e em bovinos 3 a 5 horas após o início do estro (Roelofs et al., 2004) . Os estudo conduzidos em éguas por Martínez-Boví; Cuervo-Arango (2016b) e Aguilar et al. (2018) avaliaram o efeito da IIF dos análogos sintéticos das PG (500 µg PGE2 + 125 µg PG2AF; dinoprostone e dinoprost, respectivamente), em uma concentração 500 vezes maior que os níveis fisiológicos. O intervalo IIF-ovulação foi menor para o grupo tratamento (20 ± 5,9h vs.72 ± 10,7h); sendo que quatro fêmeas ovularam até 12h e outras duas em 24 e 48h. A concentração de P4 no grupo tratado foi maior as 48 e 72h pós-IIF, porém essa diferença não persistiu aos nove dias pós IIF. O desenvolvimento do CL foi morfologicamente semelhante (Martínez-Boví; Cuervo-Arango, 2016b)entre os grupos. Corroborando esses achados Aguilar et al. (2018) observaram que cinco de seis folículos monitorados ovularam até 12h pós IIF e o intervalo punção-ovulação foi menor para o grupo tratado (18h X 68h e 90h para os controles). Ainda em éguas, quando foi realizada a IIF de 750 µg de análogo PGF, 83% dos animais não ovularam e a única ovulação ocorreu 96h pós-IIF. Houve atraso no aumento de P4, que no grupo controle iniciou em 24h, enquanto no grupo PGF em 96h (Grippo et al., 2020). Na comparação entre a IIF de 500 µg PGE2 e 125 µg PGF2A, as ovulações ocorreram apenas no grupo PGE (91%). O grupo PG apresentou menor intervalo IIF-ovulação (24h X 77 ± 9h grupo controle) e maior produção de P4 (Gaber et al., 2024), o que era esperado uma vez que a PGE2 possui efeito luteotrófico enquanto a PGF2A ação luteolítica (Kim et al., 2001).

Já em vacas, nosso grupo avaliou a IIF de 100 ng de PGF2A/mL (concentração fisiológica) de volume folicular, 12h após a remoção da P4, utilizada para prévia sincronização da onda folicular. Foi possível observar que todas as vacas ovularam entre 54 e 78h pós-IIF e não houve diferença no intervalo IIF-ovulação (Controle: 66 ± 6,4; PGF: 63 ± 8,5 h) (De Moraes et al., 2021b). Posteriormente, comparamos a IIF de 500ng/mL PGE2 (concentração fisiológica) e associação entre PGE2+PGF2A, não sendo observada alteração no momento da ovulação, porém a produção de P4 foi superior no grupo PGE2 (Lazzari et al., 2025). Assim, tanto em equinos como em

bovinos, a IIF de PGF2A não foi capaz de antecipar a ovulação, enquanto a PGE2 demonstrou antecipar a ovulação em equinos (utilização de análogo sintético e concentração superior) e promover efeito luteinizante nas duas espécies.

5. CONCLUSÃO

As PGs exercem papel fundamental na regulação do processo periovulatório e a supressão da síntese por meio de AINE é capaz de impedir a ruptura folicular, sem comprometer a formação e funcionalidade do corpo lúteo. Tanto para o bloqueio quanto para o restabelecimento da ovulação, os resultados obtidos com diferentes fármacos, doses, vias e momentos de aplicação indicam que o sucesso depende da concentração no local de ação.

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2.2 Artigo científico

Time-dependent intrafollicular COX inhibition with prostaglandin: Effects on bovine ovulation and follicular fluid extracellular vesicles miRNAs

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Time-dependent intrafollicular COX inhibition with prostaglandin: Effects on bovine ovulation and follicular fluid extracellular vesicles miRNAs

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Abstract

Prostaglandins (PGs), specifically prostaglandin E2 (PGE2) and prostaglandin F2α (PGF2α), are critical regulators of the periovulatory cascade in cattle. While their roles are established, their precise, time-sensitive application for ovulation induction and rescue remains under investigation. We evaluated whether exogenous PGE2 and PGF2α, alone or combined, modulate ovulation and whether flunixin meglumine (FM), a nonsteroidal anti-inflammatory drug and COX inhibitor, reliably generates ovulation-block models. Experiments were conducted in cyclic Bos taurus cows. Estrous cycles were synchronized using intravaginal progesterone (P4) device (IVD), estradiol benzoate, cloprostenol sodium, and GnRH analogs. In Experiment 1, the effects of intrafollicular (IFI) of PGE2 alone or in combination with PGF2α on ovulation timing and luteal P4 production were evaluated. Experiments 2–4 examined FM-induced ovulation blockade via intramuscular (IM), intraovarian (IO), or IFI administration. Experiments 5–6 examined whether IFI prostaglandins reverse FM-induced blockade at defined periovulatory windows (16 h and 22 h post-GnRH). Experiment 7 evaluated extracellular vesicles (EV) from follicular fluid (FF) after systemic FM, including miRNA profiling. Ovulation timing was monitored by ultrasonography and luteal function by serum P4. In Experiment 1, IFI PGE2 or PGE2+PGF2α did not advance ovulation (median 66 h for PG groups vs. 42 h for control), but PGE2 increased serum P4 ($P = 0.0014$). Systemic IM FM (≈75–80% ovulation) and IO FM (100% ovulation) did not consistently block ovulation (Experiments 2–4), whereas IFI FM at 22 h post-GnRH inhibited ovulation in all treated cows (Experiment 6). Co-administration of prostaglandins at 16 h did not significantly reverse FM blockade (Experiment 5), but PGE2 partially restored ovulation at 22 h (44% ovulated; $P = 0.04$), closer to the physiological PG peak (Experiment 6). Four miRNAs (bta-miR-584, bta-miR-369-5p, bta-miR-218, bta-miR-411c-5p) were downregulated in FF EV after FM, which were mapped to eight pathways pertinent to follicular rupture and PG-linked mechanisms. In conclusion, exogenous prostaglandins did not hasten ovulation, although PGE2 exerted a luteotropic effect. IFI FM administered near the late periovulatory window effectively blocked follicular rupture, and timely IFI PGE2 partially rescued FM-inhibited ovulation. Systemic FM also modulated FF EV miRNAs, suggesting pathway-level mechanisms underlying ovulatory failure.

Keywords: cyclooxygenase inhibition, follicular fluid extracellular vesicles, GnRH-timed ovulation, intrafollicular injection, ovulation timing, periovulatory regulation

1.INTRODUCTION

Prostaglandins (PGs) are fundamental to reproductive physiology across species, regulating ovulation, fertilization, corpus luteum formation, and luteolysis [1]. The luteinizing hormone (LH) surge increases cyclooxygenase (COX) expression, followed by a rise in prostaglandin E2 (PGE2) and prostaglandin F2α (PGF2α), which are involved in ovulatory mechanisms [2,3]. In cattle, the periovulatory prostanoid peak occurs near the time of follicular rupture, approximately 24 h after GnRH administration or estrus onset [2–4]. Within bovine follicular fluid (FF), reported peak concentrations of PGE2 and PGF2α are 484.21 ng/mL and 101.01 ng/mL, respectively, underscoring the predominance and potential role of PGE2 in follicular physiology [2].

The ovulatory potential of PGs has been demonstrated in mares, where intrafollicular injection (IFI) of PGE2—alone or combined with PGF2α—induces ovulation [5–7]. By contrast, IFI of PGF2α alone failed to induce ovulation in mares [6,8] and in cattle [9]. When administered via intramuscular (IM), PGF2α promoted ovulation in prepubertal heifers [10]. Altogether, these data support a role for PGE2 and PGF2α in ovulatory control and suggest their potential use in strategies to manipulate the timing of ovulation in cattle. Consequently, three knowledge gaps must be addressed: (1) establish a robust model to block ovulation by inhibiting endogenous PGs; (2) determine whether exogenous PGs can rescue ovulation when endogenous synthesis is inhibited; and (3) elucidate how PG signaling modulates biological pathways via extracellular vesicle (EV)–mediated communication in FF.

For ovulation inhibition, nonsteroidal anti-inflammatory drugs (NSAIDs) have been used to block COX activity and reduce PG synthesis in multiple species [11–13]. Flunixin meglumine (FM) is a widely used nonselective COX inhibitor that targets both COX-1 and COX-2 isoforms in clinical settings and has been associated with and is associated with the prevention of luteolysis [14,15] including its use in reproductive biotechniques [16]. In cattle, ovulation can be blocked by IFI administration of 338 μM FM or 70 μM indomethacin at 16 h post-GnRH [17], a time preceding the rise in COX expression (from ~18 h post-GnRH) [4,18]. Although FF analysis would enhance mechanistic insight into the ovulatory cascade, repeated follicular puncture risks blood contamination and compromises downstream assays. Therefore, routes that avoid multiple follicular punctures are preferable for this purpose. Systemically, multiple FM doses can block ovulation in mares and cows [15,19], though prolonged NSAID exposure poses adverse effects that limit use. Intraovarian (IO) delivery of indomethacin was also effective [20], albeit achieved by laparotomy—an approach impractical for routine applications but not invalidating the route, given modern ultrasound-guided techniques such as IFI.

The potential of PGs to restore ovulation has been tested in single-puncture models in mares and nonhuman primates. In mares, an IM FM blockade was reversed by IFI co-administration of PGE2 with PGF2α [21]. In primates, IFI co-administration of indomethacin and PGE2 reinstated ovulation [22]. These results validate the co-administration approach while avoiding multiple punctures near ovulation, which increases the risk of inadvertent follicular rupture. Because this co-administration model has not been fully evaluated in cattle, it is critical to determine the optimal timing: favoring maximal NSAID action (e.g., 16 h post-GnRH) or aligning with the physiological prostaglandin peak (24 h post-GnRH). Consistent with the importance of timing, ovulation was blocked in rabbits when

indomethacin was administered 2 h before ovulation [12], suggesting greater NSAID efficacy closer to follicular rupture.

Intercellular signaling via EV has gained prominence as a mechanism of local regulation. EV are nanoparticles secreted to the extracellular space that carry bioactive cargo, including nucleic acids [23]. In the follicle, EV support autocrine/paracrine communication between granulosa cells (GC), theca cells, and the oocyte. In cattle and mice, cumulus–oocyte complexes exposed to EV isolated from bovine FF exhibited cumulus expansion and upregulation of expansion-related genes, including COX [24]. EV cargo includes microRNAs (miRNAs), non-coding ~22-nucleotides RNAs that post-transcriptionally regulate gene expression [25]. In cattle, miRNA abundance changes across follicular development, modulating pathways linked to follicular growth and oocyte maturation [26]. EV are also key mediators of maternal–embryonic communication in the oviduct during early development [27]. Thus, profiling miRNAs in FF EV under defined periovulatory perturbations provides a window into molecular mechanisms governing the ovulatory cascade.

Based on these premises, experiments were conducted to: (1) evaluate the ovulatory potential of PGE2 alone or combined with PGF2α and the effects on luteal function; (2) test whether reduced-dose FM administered IM or IO can block ovulation; (3) assess whether PGs can restore FM-induced ovulatory blockade when co-administered; and (4) identify FF EV miRNAs that modulate biological pathways in FM-treated cows. The hypotheses were: (1) PGE2 alone or combined with PGF2α induces ovulation and increases progesterone (P4); (2) one or two IM doses of FM block ovulation; (3) IO FM prevents ovulation; (4) PGE2 alone or with PGF2α restores FM-blocked ovulation when co-administered 16 h post-GnRH; (5) PGE2 alone or with PGF2α restores FM-blocked ovulation when co-administered 22 h post-GnRH; and (6) the FF EV miRNA profile is differentially modulated in FM-treated cows.

2. MATERIALS AND METHODS

All procedures were approved by the Animal Ethics Committee of the Federal University of Pelotas (No. 288/2015 and 213/2023—CEUA/UFPel) and conducted at the university's experimental station and at EMBRAPA. Cyclic, non-lactating Bos taurus cows with body condition scores of 2.5–3.5 (1–5 scale) were used. Before each experiment, ovarian activity and uterine condition were evaluated by ultrasonography.

2.1 Experiment 1: Does IFI PGE2 or PGE2+PGF2α at 12 h after IVD removal induce ovulation and promote progesterone production?

To test whether prostaglandins induce ovulation before the endogenous LH surge, 24 cows underwent estrous synchronization (Figure 1A). On Day 0 (D0; random cycle stage), cows received an intravaginal device (IVD) containing 1 g P4 (Primer, Agener União, São Paulo, SP, Brazil), 2 mg estradiol benzoate intramuscularly (IM) (RIC-BE, Agener União), 0.52 mg cloprostenol sodium (CS) IM (Estron, Agener União), and 25 μg of lecirelin IM (TEC Relin, Agener União). On D7, all animals received a new injection of 0.52 mg CS IM. On D9, the IVD was removed and the sacrocaudal region was marked with specific paint for estrus detection. Follicular dynamics were evaluated every 24 h from D7 to D9 by ultrasonography (Domed DM10v PRO, 7.5 MHz probe). On the third evaluation day, cows with a dominant follicle >10 mm and with no estrus manifestation were enrolled; follicular diameters were

balanced across groups. Treatments (all intrafollicular injection, IFI) were: Control (PBS with 0.2% ethanol, prostaglandin diluent), PGE2 (500 ng/mL in follicular volume), and PGE2+PGF2α (500 ng/mL PGE2 + 100 ng/mL PGF2α) [2]. IFI was performed 12 h after IVD removal, coinciding with the end of hypothalamic blockade by P4 [28].

Immediately before IFI, epidural anesthesia was performed (5 mL, 2% lidocaine) and the perianal and vulvar regions were aseptically prepared. The preovulatory follicle (POF) diameter was recorded to calculate follicular volume [29]. The injected volume corresponded to 10% of follicular volume. To achieve the desired intrafollicular (IF) concentrations (484.21 ng/mL for PGE2 and 101.01 ng/mL for PGF2α), solutions were prepared at 10× the target concentration [9]. The PGE2 stock was prepared by adding 2 mL ethanol to a 10 mg PGE2 vial (Cayman Chemical, item 14010; 5 mg/mL), then diluting 10 µL stock in 10 mL PBS (5 µg/mL). The PGF2α stock was prepared by adding 2.5 mL ethanol to a 5 mg PGF2α vial (Cayman Chemical, item 16010; 2 mg/mL), then mixing 20 µL PGE2 stock and 10 µL PGF2α stock in 20 mL PBS to yield 5 µg/mL PGE2 and 1 µg/mL PGF2α. IFI was performed under transvaginal ultrasound guidance (SonoScape E2V Pro, convex probe, 5 MHz) using a double-needle system [9].

Ovulation was monitored every 12 h by ultrasonography (Domed DM10v PRO, 7.5 MHz) from D10 to D14. Luteal function was assessed by serum P4 on Days 7 and 14 post-IFI by chemiluminescence in a commercial laboratory (ADVIA Centaur Systems Progesterone Kit; Siemens; Ref. 01586287; sensitivity 0.21 ng/mL), with intra- and inter-assay CVs <10%. Experimental units' exclusions: estrus at IFI (n = 2), follicular fluid leakage after IFI (n = 2), no ovulation (n = 2), and no CL formation (n = 4). Final group sizes: Control, n = 4; PGE2, n = 6; PGE2+PGF2α, n = 4.

2.2 Experiment 2: Does IM FM at 17 h after GnRH block ovulation?

To validate an IM NSAID ovulation blockade model, 27 cows underwent estrous synchronization (Figure 2A), similar to Experiment 1, with the following differences: no CS on D0; addition of 10.5 µg buserelin (Gonaxal, Biogénesis Bagó, Curitiba, PR, Brazil) on D0; CS on D9 concurrent with IVD removal; and 10.5 µg buserelin IM as ovulation inducer. Follicular dynamics were evaluated every 24 h from D8 to D10; inclusion criteria matched Experiment 1. On D10 (day of GnRH treatment), cows were allocated by follicular diameter to Control (n = 15; no additional treatment) or FM (n = 12; 2.2 mg/kg FM, IM [29]; Flumedin, Jofadel, Varginha, MG, Brazil) at 17 h post-GnRH, prior to the increase in PGs [4]. All animals received identical handling, including sham injections to minimize potential stress differences. Ovulation was monitored every 24 h (SonoScape A5V, 7.5 MHz) form D11 to D14.

2.3 Experiment 3: Can two IM FM doses at 16 h and 26 h after GnRH block ovulation?

Based on experiment 2 results, to further validate an IM NSAID model, 10 cows underwent estrous synchronization (Figure 3A), similar to Experiment 1, with the following differences: no CS or GnRH on D0; CS on D8; and 10.5 µg buserelin IM as ovulation inducer. Follicular dynamics followed prior protocols. On D10, cows were assigned by dominant follicle (DF) diameter to Control (n = 5; no additional treatment) or FM (n = 5; 1.1 mg/kg FM IM at 16 h and again at 26 h post-GnRH; Flumedin), within the recommended therapeutic range [30]. This split dosing (total 2.2 mg/kg) was chosen to achieve sustained plasma levels during the

periovulatory window. All animals received identical handling, including sham injections to minimize potential stress differences. Ovulation timing was monitored as in Experiment 1.

2.4 Experiment 4: Does IO FM injection at 16 h after GnRH block ovulation?

To test whether IO FM blocks ovulation, 18 cows underwent estrous synchronization (Figure 4A) as in Experiment 3, except the ovulation inducer was 25 μg of lecirelin (GnRH analog) IM (TEC Relin, Agener União). Follicular dynamics followed prior protocols. On D10, cows were allocated by DF diameter (n = 6/group) to: Control (IFI saline), FM-IF (10 μg FM IFI), or FM-IO (10 mg FM IO). Injections were performed at 16 h post-GnRH [17]. The 10 μg IFI dose was selected to achieve an intrafollicular concentration (~338 μM, based on the 0.2 mL injection volume), as established in a previous study [17]. The 10 mg IO dose was achieved using the same injection volume (0.2 mL) as IFI. For IFI, a 1:1000 dilution of Banamine (50 mg/mL, MSD, Rahway, NJ, USA) was used; for IO, undiluted Banamine was injected into the ovarian cortex in a region without antral follicles or CL [31]. All injections were transvaginal and ultrasound-guided [9] and standardized at 0.2 mL [17]. Ovulation was monitored as in Experiment 1.

2.5 Experiment 5: Does IFI PGE2, with or without PGF2α, at 16 h after GnRH reverse FM-induced ovulation blockade?

To evaluate whether PGE2 or PGE2+PGF2α reverse FM-induced blockade when co-administered, 16 cows underwent the same procedures as Experiment 4 (Figure 5A). On D10, cows were allocated by DF diameter to: FM (n = 5; IFI 50 μg/mL FM), FM+PGE2 (n = 7; IFI 50 μg/mL FM + 500 ng/mL PGE2), or FM+PGE2+PGF2α (n = 4; IFI 50 μg/mL FM + 500 ng/mL PGE2 + 100 ng/mL PGF2α). IFIs were performed 16 h post-GnRH [17], the time previously identified as ideal for FM injection.

The higher FM concentration (50 μg/mL) was based on Experiment 4, in which the suggested dose [17] did not fully block ovulation. FM was prepared by adding 121 μL Banamine (50 mg/mL) to 20 mL saline (FM solution). For FM+PGE2, 20 μL PGE2 stock was added to 20 mL FM solution; for FM+PGE2+PGF2α, 20 μL PGE2 stock plus 10 μL PGF2α stock were added to 20 mL FM solution. Ovulation was monitored as in Experiment 1.

2.6 Experiment 6: Does IFI PGE2 at 22 h after GnRH reverse FM-induced ovulation blockade?

Because the efficacy of COX inhibition is highly time-dependent, to assess whether PGE2 reverses FM-induced blockade at a later periovulatory window, 18 cows underwent the same procedures as Experiment 4 (Figure 6A). On D10, cows were allocated by DF diameter to: FM (n = 9; IFI 50 μg/mL FM) or FM+PGE2 (n = 9; IFI 50 μg/mL FM + 500 ng/mL PGE2). IFIs were performed 22 h post-GnRH, i.e., closer to the prostaglandin peak and a time at which indomethacin can block ovulation [12]. Solutions were prepared as in Experiment 5. Ovulation was monitored by ultrasonography (Domed DM10v PRO, 7.5 MHz) at 6, 12, 24, 36, and 96 h after IFI.

2.7 Experiment 7: Does FM alter the levels of miRNAs present in FF EV?

Although a complete ovulation blockade model without follicular puncture was not feasible, systemic FM decreases PGF concentration [9] and can impact the follicular microenvironment; therefore, FF analysis was pursued. Fourteen cows underwent estrous synchronization (Figure 7A) as in Experiment 5, except IVD removal was advanced to D8. Follicular dynamics followed Experiment 1. On D9 (day of GnRH treatment), cows were allocated by DF diameter to Control (n = 7; no additional treatment) or FM (n = 7; 2.2 mg/kg FM IM; Banamine, 50 mg/mL, MSD) at 16 h post-GnRH. All animals received identical handling, including sham injections to minimize potential stress differences.

Follicular aspiration was performed 8 h after FM (i.e., 24 h post-GnRH) using transvaginal ultrasound guidance (Mindray M5, convex probe, 5 MHz). Immediately before FF collection, epidural anesthesia (5 mL, 2% lidocaine) and asepsis of the perianal and vulvar regions were performed. FF was transferred to a cell culture plate to verify and, if necessary, remove the cumulus oocyte complex, then transferred to a 2 mL microtube for serial centrifugations to isolate EV and GC [27].

To isolate the EV from the FF, the FF underwent a series of centrifugation steps to remove live cells, cellular debris, and large vesicles, respectively: 300 x g for 10 min, 2000 x g for 10 min, and 16,500 x g for 30 min. The supernatant was stored at −80°C until further processing. For EV isolation, the FF supernatant was filtered through a 0.20 µm sterile syringe filter (PES membrane; Corning, United States of America) to remove residual large EV. Subsequently, the filtered FF was centrifuged twice at 119,700 x g for 70 min at 4°C using an ultracentrifuge (Optima XE-90 Ultracentrifuge; rotor 70 Ti; Beckman Coulter, United States of America). The supernatant was discarded, and the resulting EV pellets were resuspended in 50 µL of phosphate-buffered saline (PBS) (1 × Ca2+/Mg2+ free PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4) for further analysis. After isolation, the EV samples were divided into two sets: One set was analyzed for EV size and concentration (40 µL), and the other set was diluted in QUIAzol Lysis Reagent, (QIAGEN; 10 µL) and stored at−80°C for RNA analysis.

Nanoparticle tracking analysis (NTA; NanoSight NS300; NTA 3.4 Build 3.1.45; Malvern) was used to determine EV size and concentration. For NTA, samples were diluted at a ratio of 1:100 in 1 × Ca2+/Mg2+ free PBS before analysis, and each sample was analyzed by capturing three to five 30-s videos were recorded at 38.5 °C (camera level 10; threshold 4). Data on particle size and concentration were processed using NanoSight NTA 3.4 Analytical Software (NTA 3.4 Build 3.4.003; Malvern, United Kingdom).

Total FF-EV RNA was extracted (QUIAzol Lysis Reagent, QIAGEN) per manufacturer instructions. Before RNA precipitation, 1.33 µL of GlycoBlue coprecipitant (Thermo Fisher Scientific) was added to the aqueous phase, as described by [32] RNA concentration and purity were assessed by spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific; absorbance ratio 260/280). After extraction, RNA samples were stored at −80 °C until further analysis. The relative levels of 382 bovine miRNAs were determined by quantitative reverse-transcription PCR (qRT-PCR) from 150 ng total RNA. A Poli-A tail was first added to the RNA and the samples were processed as described by Ferst et al. [33]. Differentially expressed miRNAs were used for target prediction (TargetScan), followed by pathway analysis using DAVID Bioinformatics Resources 6.8 (NIAID/NIH) [34].

2.8 Statistical analysis

Analyses were performed in JMP Pro 18.0.2 (SAS Institute Inc., Cary, NC, USA). Follicular diameter and P4 concentration were tested for normality (Shapiro–Wilk) and homoscedasticity (Levene). Group comparisons used analysis of variance (ANOVA) followed by Tukey's test. The miRNA expression levels were compared between groups using Student's t-test. Ovulation rates were analyzed with Fisher's exact test. Given limited number of ovulations in some groups, ovulation timing was described. Results are presented as mean ± standard error or median with interquartile ranges. Statistical significance was set at $P \leq 0.05$; trends were considered for $0.051 \leq P \leq 0.10$.

3. RESULTS

3.1 Experiment 1: Does IFI PGE2 or PGE2+PGF2α at 12 h after IVD removal induce ovulation and promote progesterone production?

Follicular diameter on the IFI day was similar among groups (Control: 13.66 ± 0.63 mm; PGE2: 12.43 ± 0.52 mm; PGE2+PGF2α: 12.48 ± 0.63 mm; P = 0.31). As shown in Figure 1B, median ovulation time did not differ (Control: 42 h [24–84]; PGE2: 66 h [36–72]; PGE2+PGF2α: 66 h [24–84]). In contrast, serum P4 concentrations were higher in the PGE2 group (treatment effect, P = 0.0014), with a day effect (P = 0.0001) and no interaction (P = 0.43). At Day 7, values (mean ± SE) were: Control, 2.34 ± 1.18 ng/mL; PGE2, 4.29 ± 0.96 ng/mL; PGE2+PGF2α, 2.23 ± 1.18 ng/mL; at Day 14: Control, 8.81 ± 1.18 ng/mL; PGE2, 12.21 ± 0.96 ng/mL; PGE2+PGF2α, 8.67 ± 1.18 ng/mL (Figure 1C). The results indicate that, although prostaglandins did not induce ovulation, PGE2 was able to increase P4 production by the CL.

3.2 Experiment 2: Does IM FM at 17 h after GnRH block ovulation?

Follicular diameter on the assessment day was similar between groups (Control: 12.99 ± 0.41 mm; FM: 12.75 ± 0.45 mm; P = 0.70). All control cows (15/15) ovulated within 42 h post GnRH. In the FM group, 9/12 (75%) ovulated within 42 h; two (16,67%) ovulated later (between 42 and 114 h), and one (8,33%) developed an anovulatory structure (Figure 2B). Therefore, IM FM was unable to block ovulation when administered 17 hours after GnRH.

3.3 Experiment 3: Can two IM FM doses at 16 h and 26 h after GnRH block ovulation?

Follicular diameter on the assessment day was similar (Control: 14.78 ± 1.04 mm; FM: 14.76 ± 1.04 mm; P = 0.98). All control cows (5/5) and 80% (4/5) of FM cows ovulated within 40 h post GnRH (Figure 3B), and did not differ (P > 0.05). One FM cow (1/5; 20%) developed an anovulatory structure that persisted until Day 7 post GnRH.

3.4 Experiment 4: Does IO FM at 16 h after GnRH block ovulation?

Follicular diameter on the IFI day was similar among groups (Control: 13.81 ± 1.30 mm; FM IFI: 13.77 ± 1.30 mm; FM IO: 13.57 ± 1.30 mm; P = 0.99). The IO injection of FM was not able to block ovulation, as all control (6/6) and FM IO (6/6) cows ovulated within 34

h post GnRH. In FM IFI, two cows ovulated by 34 h, and the rest of the cows (66.6%) remained anovulatory up to 72 h (Figure 4B).

3.5 Experiment 5: Does IFI PGE2, with or without PGF2α, at 16 h after GnRH reverse FM induced ovulation blockade?

Follicular diameter on the IFI day was similar (FM: 15.52 ± 1.61 mm; FM+PGE2: 14.40 ± 1.12 mm; FM+PGE2+PGF2α: 15.37 ± 0.80 mm; P = 0.78). As shown in Figure 5B, ovulation rate did not differ among groups (FM: 40%; FM+PGE2: 57%; FM+PGE2+PGF2α: 25%; P = 0.12), indicating that prostaglandins were unable to reverse the FM induced ovulation blockade at this timepoint. Mean ovulation time was 34 h for FM and 48 ± 11.48 h for FM+PGE2; the single ovulation in FM+PGE2+PGF2α occurred at 42 h post GnRH (Figure 5C).

3.6 Experiment 6: Does IFI PGE2 at 22 h after GnRH reverse FM induced ovulation blockade?

Follicular diameter on the IFI day was similar (FM: 14.81 ± 1.21 mm; FM+PGE2: 14.84 ± 0.73 mm; P = 0.98). Ovulation was blocked in all FM cows (9/9), whereas 44% (4/9) of FM+PGE2 cows ovulated (P = 0.04; Figure 6B), showing the ability of PGE2 to reverse FM-induced ovulation blockade when administered 22 h after GnRH. Among the ovulated cows, two ovulations occurred by 27 h post GnRH, one by 32 h, and one between 46 and 96 h (Figure 6C).

3.7 Experiment 7: Does FM alter the levels of miRNAs present in FF EV?

Follicular diameter on aspiration day was similar (Control: 12.66 ± 0.34 mm; FM: 13.13 ± 0.59 mm; P = 0.50). Total EV concentration and size did not differ between groups (concentration, × 108 particles/mL: FM, 28.4 ± 4.6 vs. Control, 22.9 ± 4.9; P = 0.40; size: FM, 183.2 ± 7.2 nm vs. Control, 195.8 ± 5.2 nm; P = 0.20). Of 333 miRNAs detected in both groups, four were downregulated by FM (bta miR 584, bta miR 369 5p, bta miR 218, bta miR 411c 5p; Figure 7B). Then, although there were no miRNAs expressed exclusively in one group, systemic FM was able to modulate the expression of these molecules. Predicted pathways modulated by these four miRNAs and pertinent to the ovulatory process included actin cytoskeleton regulation (P = 0.003), extracellular matrix receptor interaction (P = 0.006), leukocyte transendothelial migration (P = 0.054), vascular smooth muscle contraction (P = 0.038), arginine biosynthesis (P = 0.052), lysine degradation (P = 0.008), ubiquitin mediated proteolysis (P = 0.018), and ferroptosis (P = 0.011) (Figure 7C).

4. DISCUSSION

The results indicate that, under the conditions tested, intrafollicular prostaglandins (PGE2 and PGF2α) did not advance ovulation timing; one or two IM doses of FM, or a single IO) dose, did not block ovulation; PGE2, with or without PGF2α, failed to reverse FM-induced ovulatory inhibition when co-administered at 16 h post-GnRH; IFI FM administered at 22 h post-GnRH effectively inhibited ovulation; IFI PGE2 restored ovulation when co-administered with FM at 22 h post-GnRH; and four FF EV miRNAs presented lower levels in FM-treated cows, mapping to eight biological pathways implicated in follicular rupture and

prostaglandin-linked mechanisms, suggesting an increased activity of these pathways in FM treated animals.

To systematically evaluate the role of COX inhibition and prostaglandin signaling in bovine ovulation, this study tested three administration routes for FM: IM for systemic exposure, IO for targeted ovarian delivery, and IFI for direct follicular intervention. Additionally, we examined three molecule combinations: prostaglandins (PG; PGE2 alone or with PGF2α) to mimic endogenous signaling, FM alone to induce COX blockade, and FM+PG co-administration to assess reversal potential. These approaches were timed relative to the GnRH-induced LH surge (16 h or 22 h post-GnRH), allowing dissection of route-, dose-, and timing-dependent effects on ovulation dynamics.

Hypothesis: PGE2 alone or combined with PGF2α is capable of inducing ovulation and progesterone production. The LH surge upregulates COX-2 expression and increases prostaglandin synthesis [2,18]. To test whether prostaglandins can drive ovulation independently of the endogenous LH effect, we elevated IF prostaglandins 12 h after IVD removal, when P4 is basal and the hypothalamic–pituitary–gonadal axis is unblocked [28]. In our study, IFI PGE2, alone or with PGF2α, did not advance ovulation, likely due to insufficient receptor availability and signaling readiness in GC at that time point, as receptor expression/activity increase closer to ovulation [35]. In mares, IFI PGE2 or PGE2+PGF2α administered at early/mid-estrus (before the LH peak) advanced follicular rupture [5–7]. However, those equine studies used potent analogs with longer half-life and ≈500× physiological concentrations, whereas our protocol mimicked bovine follicular physiology.

As expected, PGE2 showed a luteotropic effect whereas PGF2α is luteolytic [36]. Although Martínez-Boví and Cuervo-Arango [7] observed advanced ovulation in mares with PGE2+PGF2α, they did not evaluate PGE2 alone; later work with IFI PGF2α alone impaired ovulation and produced anovulatory follicles in 5/6 mares [8]. In that study, P4 exceeded 1 ng/mL only from 96 h post-IFI (physiologically expected within 12–24 h), and on Day 10 mean P4 was 5.11 ng/mL, indicating initial luteal impairment by PGF2α was followed by partial recovery [8]. By contrast, our data show that the luteotropic effect of pre-ovulatory PGE2 persisted through Day 14. Cross-species evidence supports a PGE2 protective role: at the onset of luteolysis in pigs, PGF2α and PGE2 pulses rise concurrently and PGE2 preserves corpus luteum function [37]; in sheep, ovarian-artery infusion of PGF2α reduced P4, whereas PGE2+PGF2α maintained P4 [38]. Thus, in mares, pigs, sheep, and cattle, PGE2 can antagonize the luteolytic action of PGF2α, maintaining P4 at control-like levels.

Hypothesis: One or two IM doses of FM block ovulation. A single IM dose of FM did not block ovulation, despite documented reductions in PGF2α with systemic FM [9], likely reflecting insufficient timing/exposure. Splitting the dose into two administrations following therapeutic recommendations also failed. In mares, 1.1 mg/kg IV once daily for two days did not delay or inhibit ovulation [39]. Consistent ovulation blockade with systemic FM has been reported only with multiple daily doses in cattle [15] and mares [40], suggesting at least four daily administrations for reliable effects [41]. Therefore, NSAID-mediated ovulation inhibition via the IM route at therapeutic concentrations proved insufficient for ovulation blockade, likely due to systemic dilution and inadequate peri-follicular exposure despite documented reductions in circulating PGF2α [9]. In contrast, local IFI FM effectively blocked ovulation at 22 h post-GnRH [Exp. 6], highlighting the superiority of direct follicular delivery for site-specific COX-2 inhibition.

Hypothesis: IO administration of FM prevents ovulation. This is the first ultrasound-guided IO NSAID study in cattle. The route is viable for other agents [31,42], but here IO FM at 16 h did not block ovulation, whereas IFI at the same time produced anovulatory structures in most cows. Ovary manipulation in the presence of a large preovulatory follicle hindered precise parenchymal placement without jeopardizing the follicle. Although injection was performed as close as possible to the follicle, drug diffusion to the follicle cannot be assumed, as we did not quantify FM in FF or plasma. It is likely that the IO dose (~1000× the IFI dose used by Vernunft et al. [17]) still fell short, possibly because diffusion to the follicle was suboptimal. IO indomethacin delivered via laparotomy achieved complete blockade [20], underscoring that while higher nominal concentrations enhance ovarian exposure, precise intrafollicular targeting is required for blockade efficacy, unlike the broader but diluted IM route.

Hypothesis: PGE2 alone or with PGF2α restores ovulation blocked by FM when co-administered 16 h after GnRH. To avoid multiple follicular punctures while testing ovulation block restoration, NSAID and prostaglandins were co-administered IF at 16 h post-GnRH, a timepoint previously effective for IFI of indomethacin or FM in cattle [17,43]. Based on Experiment 4 results, the FM concentration was increased fivefold. Despite a partial blockade, ovulation was not prevented in all animals. If prostaglandins were able to restore ovulation at this window, most FM+PGE2 and FM+PGE2+PGF2α cows would have ovulated relative to FM alone, which did not occur. As in Experiment 1, the possible explanation for the inability to reverse the blockage is mistiming: at 16 h, prostaglandin receptor expression/activity remain below the threshold necessary for a robust ovulatory response [44], limiting the capacity of exogenous PGE2 (± PGF2α) to overcome COX inhibition.

Hypothesis: PGE2 restores ovulation blocked by FM when co-administered 22 h after GnRH. To our knowledge, IF co-administration of prostaglandins and NSAIDs in the periovulatory period has been documented only in primates [22], where PGE2 prevented ovulatory failure. Complementing this timing-sensitive effect, studies in rabbit showed that indomethacin given 1 h before ovulation reduced PGE2 and PGF2α to just over half within 10 min and to roughly one quarter by 60 min, yet ovulation still occurred in 41.6% of animals; when administered 2 h before ovulation, however, ovulation was completely blocked [12]. Given the strong time dependence of COX inhibition, we therefore repeated the Experiment 5 (IFI 16h after GnRH) at a later window, performing IFI at 22 h post-GnRH, i.e., closer to the expected time of ovulation (reviewed by [45]).

This is the first description in cattle of co-administration of FM and PGE2 shortly before ovulation. To mitigate rupture risk from late manipulation, the number of groups was reduced, and no procedural ruptures occurred. Mirroring rabbit studies with indomethacin [12], IFI FM 2 h before the endogenous PG peak achieved complete blockade. Although we induced a PGE2 peak nearer to the receptor-upregulation window [44], rescue was partial (4/9). Among the ovulated FM+PGE2 cows, two ovulations occurred by 27 h post-GnRH (close to the expected timing [45]), one by 32 h, and one delayed between 46 and 96 h, indicating variable efficacy in reversing the FM-induced blockade. This heterogeneity may stem from PGE2's short half-life [46]. and the relatively low physiological dose used, which allowed prompt reversal in some animals (aligning with receptor upregulation near ovulation [35]) but insufficient sustained signaling in others, leading to prolonged delays. In contrast to high-dose analogs in mares that fully reversed inhibition without notable delays [21], our bovine model highlights the challenges of mimicking endogenous PG dynamics for complete restoration.[46][21][5–8].

Altogether, the data support a late periovulatory window in which local COX inhibition reliably blocks rupture and timely PGE2 can partially restore it.

Hypothesis: The FF EV miRNA profile is differentially modulated in FM-treated animals. This work provides the first characterization of the FF-EV miRNA profile in NSAID-treated cows. COX inhibition was associated with a coordinated reduction of four EV-associated miRNAs—bta-miR-584, bta-miR-369-5p, bta-miR-218, and bta-miR-411c-5p. Target prediction and pathway enrichment predicted biological programs important to the ovulatory cascade. Because miRNAs typically repress gene expression post-transcriptionally [25] the downregulation observed here is an indicative that the predicted transcripts would be available to become proteins and influence these pathways; nevertheless, enrichment alone does not establish directionality or causality and should be interpreted as hypothesis-generating.

The enrichment for actin cytoskeleton regulation and ECM–receptor interaction aligns with the mechanical requirements of ovulation—cumulus expansion, cellular protrusiveness, and localized weakening of the follicular apex [47] and with known PGE2-responsive cytoskeletal rearrangement (RHO/ROCK-dependent). In fish, pharmacological inhibition of the RHO/ROCK axis suppresses ovulation [48]. These cytoskeletal/ECM pathways also mediate apex remodeling via proteolytic enzymes and leukocyte infiltration; indomethacin reduces the expression/activity of such proteases in GC [35,49]. Consistent with Experiments 2 and 4, systemic or IO FM administered at earlier windows decreased prostaglandins but did not fully disrupt these downstream effector mechanisms, whereas complete blockade was achieved only with IFI FM at 22 h—highlighting the time and site dependence of COX inhibition.

Enrichment in vascular smooth muscle contraction suggests a shift toward vasoconstrictive tone, whereas arginine biosynthesis points to potential nitric oxide (NO)-linked compensation. Physiologically, PGE2 promotes vasodilation, increases flow and permeability, and facilitates leukocyte trafficking [1,50]. Although arginine can augment NO production and ovarian perfusion [51] and is implicated in oocyte maturation and periovulatory events [52], the net vascular effect under COX inhibition may favor constriction or limit NO bioactivity, attenuating perfusion-dependent components of rupture (e.g., pressure dynamics and endothelial activation).

Additional enrichment in lysine degradation, ubiquitin-mediated proteolysis, and ferroptosis suggests stress-related programs with potential to impair ovulation. Rapid proteome turnover via the ubiquitin–proteasome system [47] is compatible with the swift signaling transitions around the LH surge; its modulation by EV-miRNAs could fine-tune receptor and transducer abundance. Ferroptosis—a non-apoptotic, iron-dependent death modality driven by lipid peroxidation—may be favored when COX-2 inhibition leads to arachidonic acid accumulation [53], and inactive COX-2 is itself targeted for ubiquitin–proteasome degradation [54,55]. These signatures offer a coherent pathway-level context linking COX inhibition to impaired rupture while revealing compensatory responses that, when exposure and timing are suboptimal, can still permit ovulation to proceed.

Taken together, the downregulation of bta-miR-584, bta-miR-369-5p, bta-miR-218, and bta-miR-411c-5p supports a working model in which COX inhibition attenuates prostaglandin signaling and concurrently reshapes EV cargo, derepressing gene networks across granulosa, theca, cumulus, and endothelial compartments. The resulting reprogramming of cytoskeletal and matrix dynamics, vasomotor control, immune cell trafficking, metabolic/proteostatic balance, and redox-sensitive cell fate could plausibly delay or blunt follicle wall rupture. Although follicular fluid contains molecules originating from both local and systemic sources,

evidence indicates that FF EV–associated miRNAs reflect the local follicular environment and participate in intercellular communication across follicular cell types [56]. Based on this, we suggest that the miRNAs identified in our FF EV reflect local COX-inhibition–driven modulation of follicular signaling. These interpretations remain provisional because they rely on in silico prediction and pathway enrichment.

5. CONCLUSION

PGE2, whether administered alone or with PGF2α, did not advance ovulation when delivered 12 h after removal of the P4 source. Consistent with its known pharmacology, PGE2 exerted a luteotropic effect, whereas PGF2α counteracted this effect on luteal function. Among ovulation-block models using the NSAI FM, systemic (parenteral) and intraovarian routes—and IF administration at earlier periovulatory windows—were insufficient to prevent follicular rupture. By contrast, IFI FM given approximately 2 h before the endogenous prostaglandin peak (22 h post-GnRH) consistently blocked ovulation, underscoring the critical time dependence and local exposure required for effective COX inhibition.

When attempting to overcome the ovulatory blockade, co-administration of prostaglandins with FM at 16 h post-GnRH failed to restore ovulation, whereas timely IFI PGE2 at 22 h partially reversed the FM-induced blockade. Systemic FM was also associated with downregulation of four FF-EV-associated miRNAs in follicular fluid (bta-miR-584, bta-miR-369-5p, bta-miR-218, bta-miR-411c-5p), with pathway enrichment implicating cytoskeletal/ECM remodeling, leukocyte trafficking, vasomotor control, amino-acid metabolism, protein turnover, and ferroptosis—processes integral to follicular wall weakening and rupture.

Together, these findings position prostaglandins as essential and time-sensitive regulators of bovine ovulation: local COX inhibition immediately before the late periovulatory window reliably prevents rupture, and targeted prostaglandin delivery at that window can partially re-establish ovulation while modulating luteal P4. The EV–miRNA signatures provide a mechanistic context for how COX inhibition reshapes the follicular microenvironment and suggest candidate biomarkers and pathways for precision modulation of ovulatory timing. These results support further studies to optimize prostaglandin-based rescue strategies and to validate EV-miRNA readouts against oocyte competence and fertility outcomes.

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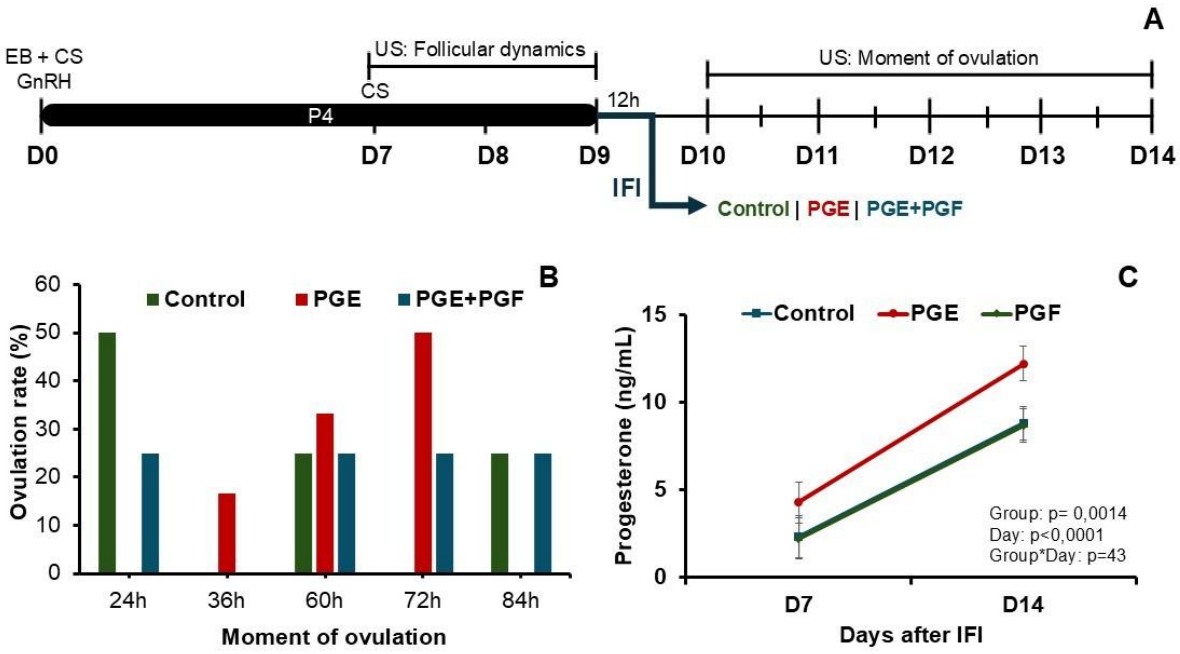

Figure 1. Experiment 1 — Does intrafollicular PGE or PGE+PGF administered 12 h after IVD removal induce ovulation and promote progesterone production? Experimental design: (A) Fourteen cows underwent estrous synchronization. On Day 0 (D0), they received an intravaginal device (IVD) containing 1 g progesterone (P4), 2 mg estradiol benzoate (EB) intramuscularly (IM), 0.52 mg cloprostenol sodium (CS) IM, and 25 µg lecirelin (GnRH) IM. On D7, cows received 0.52 mg CS, and on D9, the IVD was removed. Follicular dynamics were monitored daily by ultrasonography (US). Twelve hours after IVD removal, cows received an intrafollicular injection (IFI) of buffered saline (Control; n = 4), 500 ng/mL of PGE in the follicular volume (PGE; n = 6), or 500 ng/mL of PGE plus 100 ng/mL of PGF in the follicular volume (PGE+PGF; n = 4). Ovulation was monitored every 12 h by US. Results: (B) Ovulation rate (%) over time (h) from IFI in Control, PGE, and PGE+PGF groups. (C) Serum progesterone concentration (ng/mL) at 7 and 14 days after IFI in Control, PGE, and PGE+PGF groups (mean ± standard error).

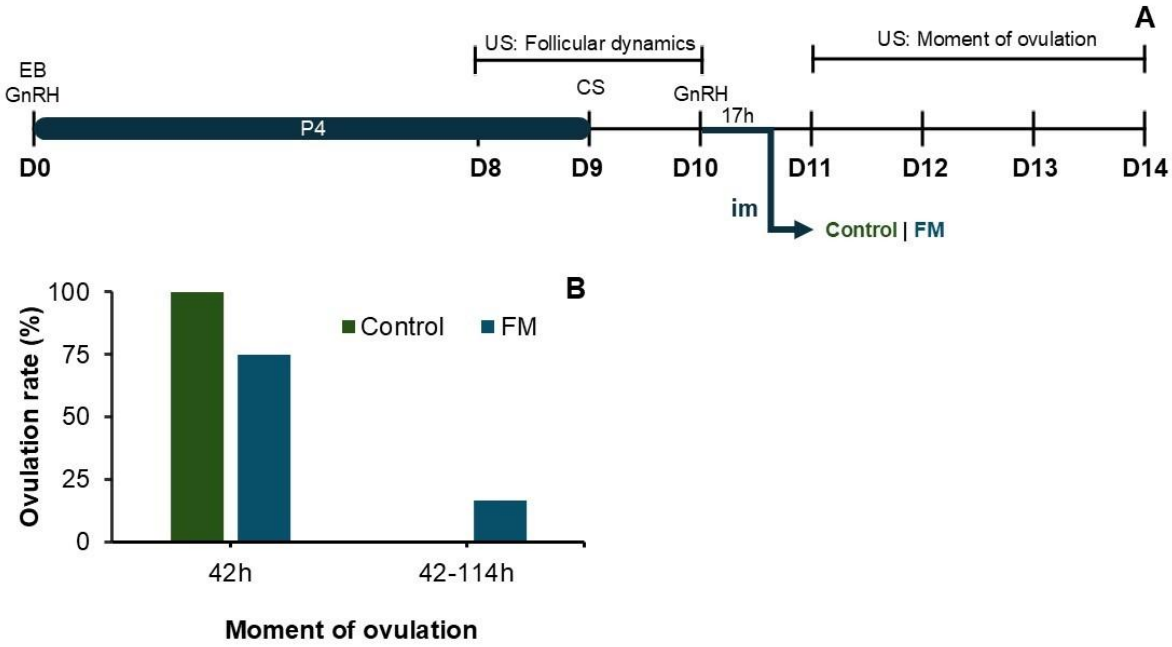

Figure 2. Experiment 2 — Does intramuscular (IM) flunixin meglumine (FM) administered 17 h after GnRH block ovulation? Experimental design: (A) Twenty-seven cows underwent estrous synchronization. On Day 0 (D0), they received an intravaginal device (IVD) containing 1 g progesterone (P4), 2 mg estradiol benzoate (EB) IM, and 10.5 µg buserelin IM. On D9, cows received 0.52 mg CS and the IVD was removed. Twenty hours after IVD removal, they received 10.5 µg buserelin. Follicular dynamics were monitored daily by ultrasonography (US) from D8 to D10. Seventeen hours post GnRH, 2.2 mg/kg flunixin meglumine (FM) was administered IM in the FM group (n = 12), and no additional treatment was performed in the Control group (n = 15). Ovulation was monitored daily by US. Results: (B) Ovulation rate (%) over time (h) from GnRH administration in Control and FM groups.

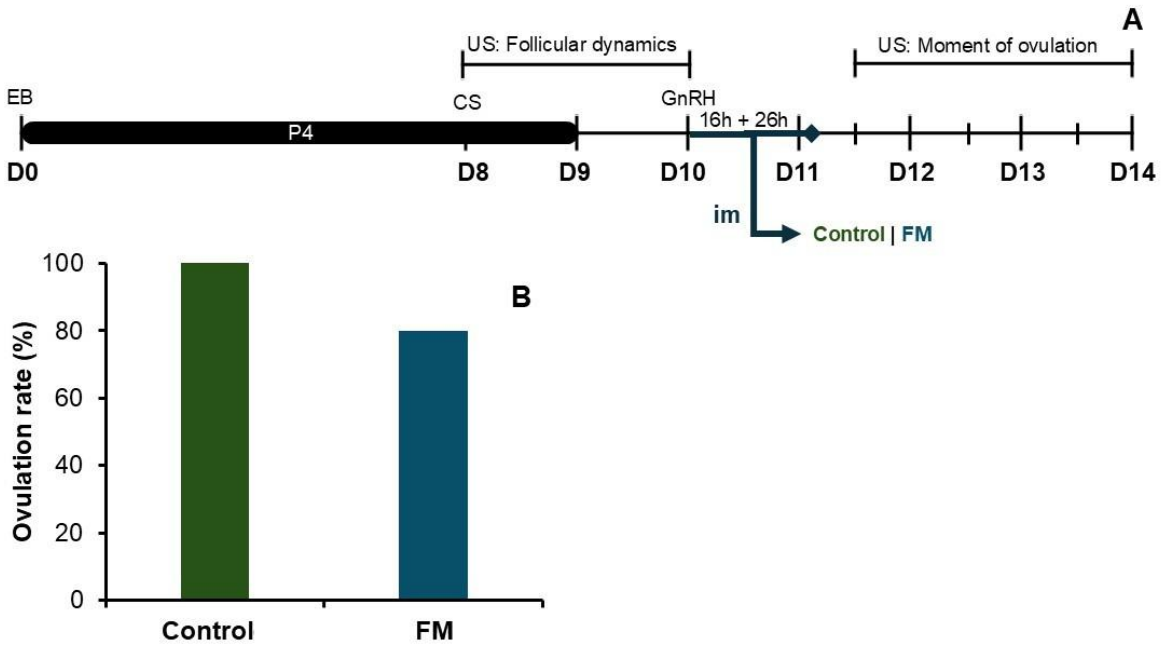

Figure 3. Experiment 3 — Is it possible to block ovulation using two intramuscular FM administrations at 16 h and 26 h post GnRH? Experimental design: (A) Ten cows underwent estrous synchronization. On Day 0 (D0), they received an intravaginal device (IVD) containing 1 g progesterone (P4) and 2 mg estradiol benzoate (EB) IM. On D8, they received 0.52 mg CS and on D9, the IVD was removed. Twenty hours after IVD removal, they received 10.5 µg buserelin. Follicular dynamics were monitored daily by ultrasonography (US) from D8 to D10. At 16 h and 26 h post GnRH, the FM group (n = 5) received 1.1 mg/kg flunixin meglumine (FM) IM at each time point, and no additional treatment was performed in the Control group (n = 5). Ovulation was monitored every 12 h by US. Results: (B) Ovulation rate (%) at 40 h post GnRH, the only time point at which follicular rupture was identified, in Control and FM groups.

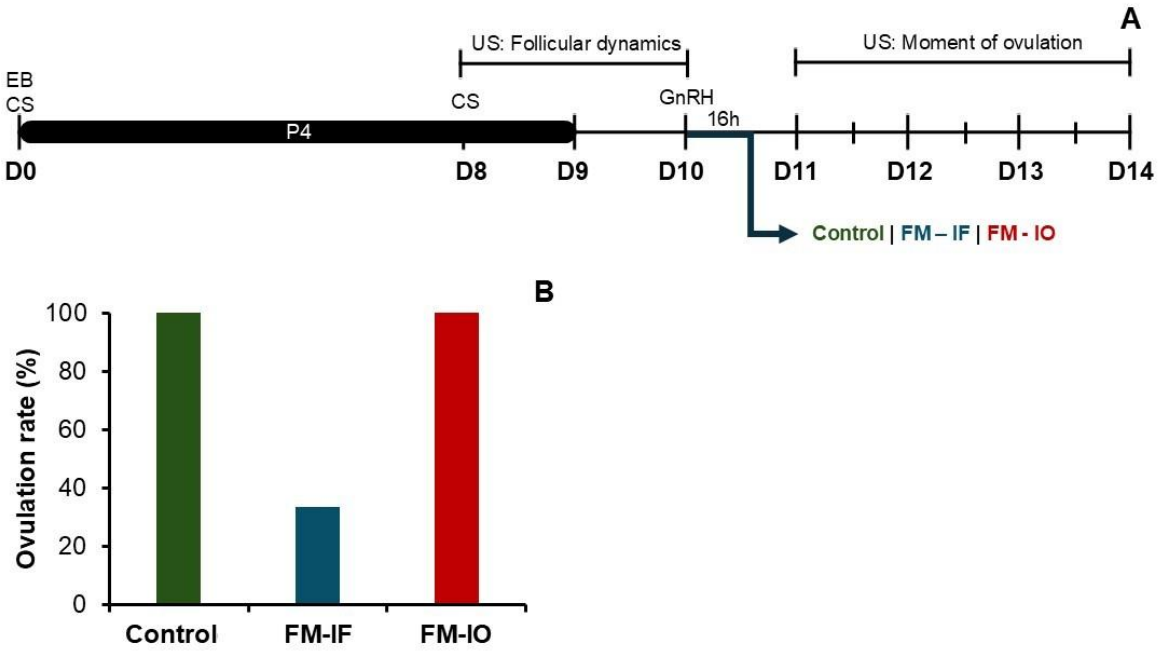

Figure 4. Experiment 4 — Does intraovarian FM injection at 16 h post GnRH block ovulation? Experimental design: (A) Eighteen cows underwent estrous synchronization. On Day 0 (D0), they received an intravaginal device (IVD) containing 1 g progesterone (P4), 2 mg estradiol benzoate (EB) IM, and 0.52 mg cloprostenol sodium (CS). On D8, they received 0.52 mg CS, and on D9, the IVD was removed. Twenty hours after IVD removal, they received 25 µg lecirelin (GnRH) IM. Follicular dynamics were monitored daily by ultrasonography (US) from D8 to D10. At 16 h post GnRH, cows received an intrafollicular injection (IFI) of saline in the Control group (n = 6), IFI injection of 10 µg flunixin meglumine (FM) in the FM-IF group (n = 6), or intraovarian (IO) injection of 10 mg FM in the FM-IO group (n = 6). Ovulation was monitored every 12 h by US. Results: (B) Ovulation rate (%) at 34 h post GnRH, the only time point at which follicular rupture was identified, in Control, FM-IF, and FM-IO groups.

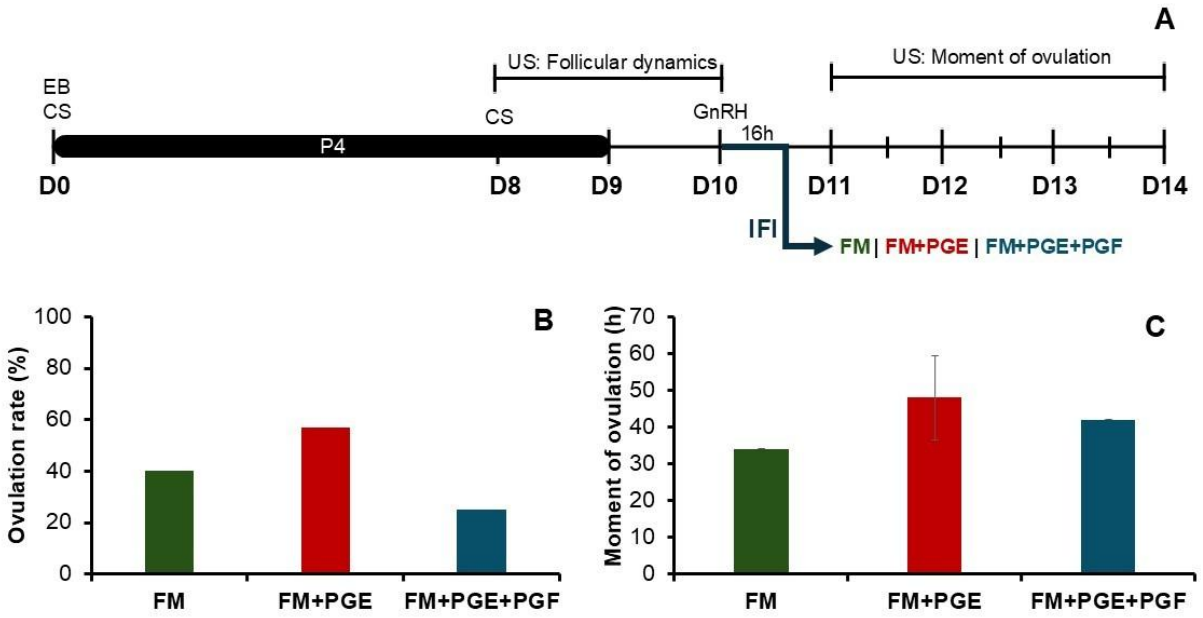

Figure 5. Experiment 5 — Does IFI PGE, alone or combined with PGF, at 16 h post GnRH reverse FM-induced ovulation blockade? Experimental design: (A) Sixteen cows underwent estrous synchronization. On Day 0 (D0), they received an intravaginal device (IVD) containing 1 g progesterone (P4), 2 mg estradiol benzoate (EB) IM, and 0.52 mg cloprostenol sodium (CS). On D8, they received 0.52 mg CS, and on D9, the IVD was removed. Twenty hours after IVD removal, they received 25 µg lecirelin (GnRH) IM. Follicular dynamics were monitored daily by ultrasonography (US) from D8 to D10. At 16 h post GnRH, cows received: intrafollicular injection (IFI) of 50 µg/mL flunixin meglumine (FM) in the FM group (n = 5); IFI 50 µg/mL FM plus 500 ng/mL PGE in the FM+PGE group (n = 7); or IFI 50 µg/mL FM, 500 ng/mL PGE, and 100 ng/mL PGF in the FM+PGE+PGF group (n = 4). Ovulation was monitored every 12 h by US. Results: (B) Ovulation rate (%) in FM, FM+PGE, and FM+PGE+PGF groups; (C) Ovulation timing (h) in FM, FM+PGE, and FM+PGE+PGF groups (mean ± standard error).

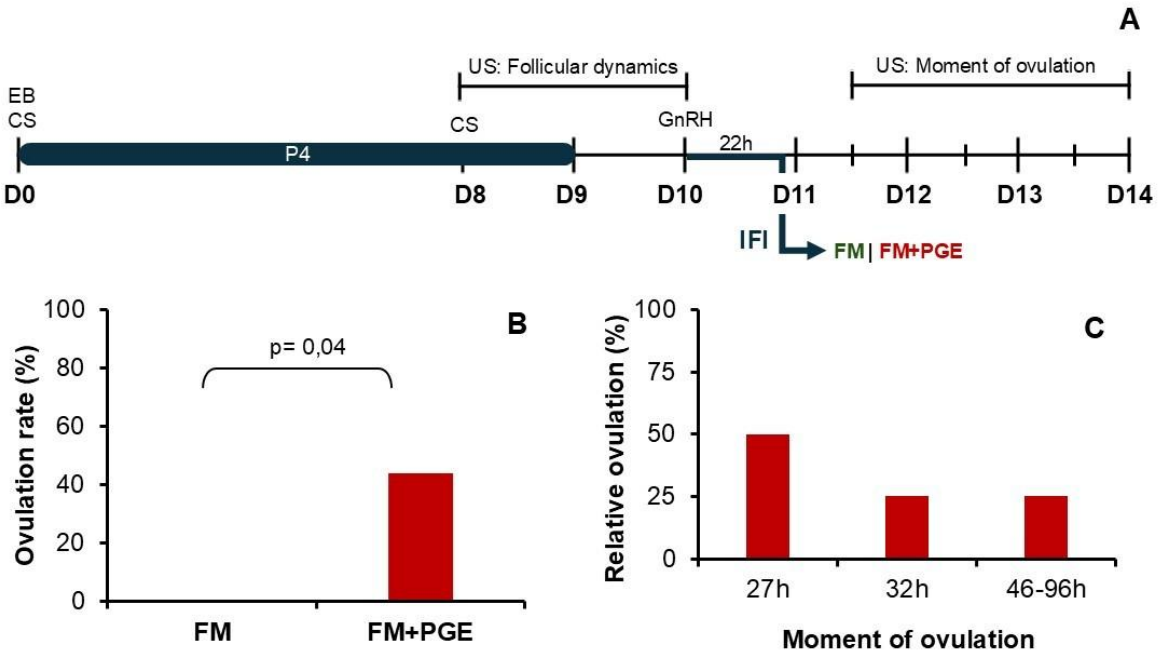

Figure 6. Experiment 6 — Does IFI PGE at 22 h post GnRH reverse FM-induced ovulation blockade? Experimental design: (A) Eighteen cows underwent estrous synchronization. On Day 0 (D0), they received an intravaginal device (IVD) containing 1 g progesterone (P4), 2 mg estradiol benzoate (EB) IM, and 0.52 mg cloprostenol sodium (CS). On D8, they received 0.52 mg CS, and on D9, the IVD was removed. Twenty hours after IVD removal, they received 25 µg lecirelin (GnRH) IM. Follicular dynamics were monitored daily by ultrasonography (US) from D8 to D10. At 22 h post GnRH, cows received: intrafollicular injection (IFI) of 50 µg/mL flunixin meglumine (FM) in the FM group (n = 9); or IFI 50 µg/mL FM plus 500 ng/mL PGE in the FM+PGE group (n = 9). Ovulation was monitored every 12 h by US. Results: (B) Ovulation rate (%) in FM and FM+PGE groups. (C) Ovulation rate (%) over time (h) in the FM+PGE group.

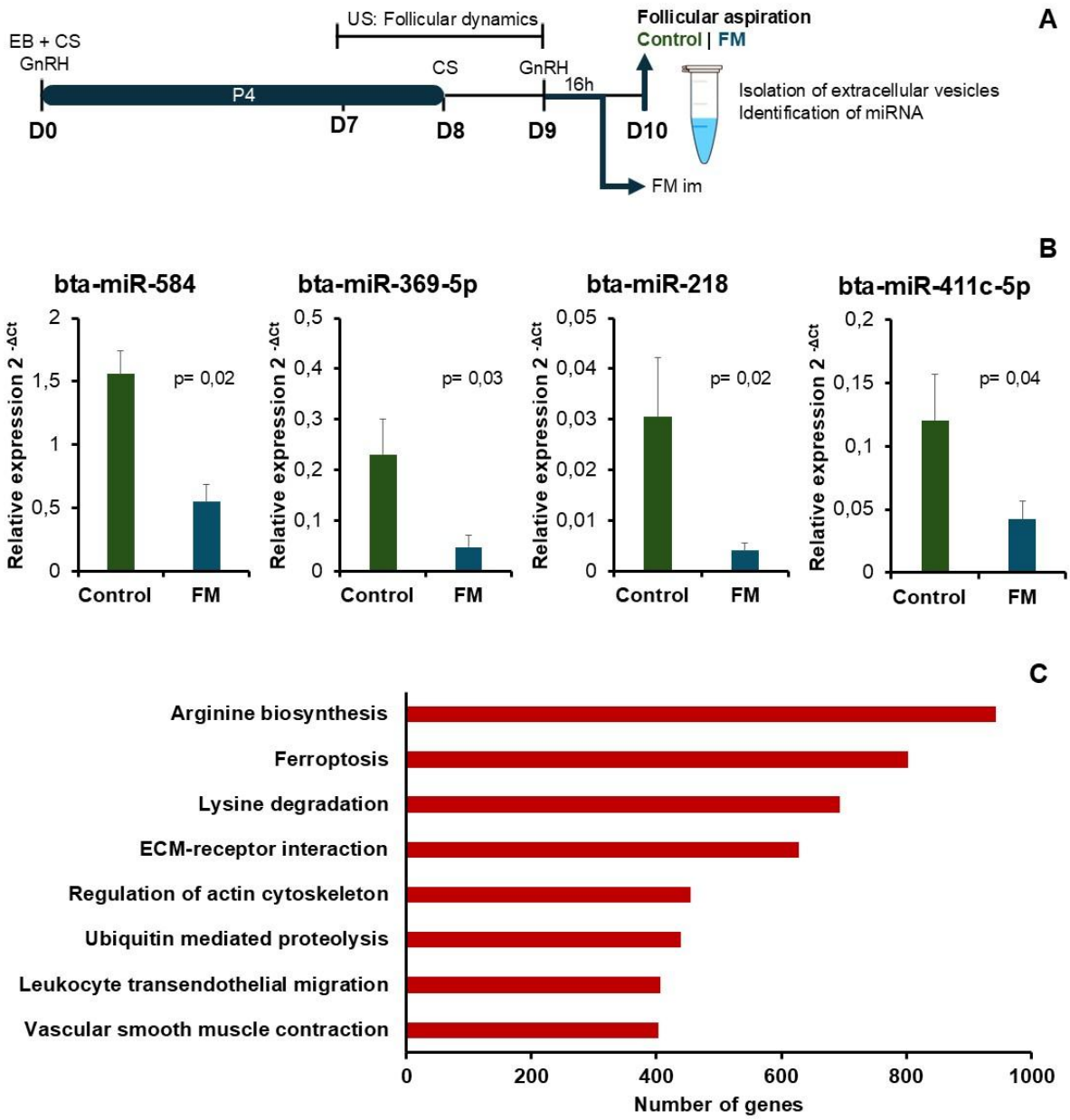

Figure 7. Experiment 7 — Does FM alter the modulation of miRNAs present in EVs from FF? Experimental design: (A) Fourteen cows underwent estrous synchronization. On Day 0 (D0), they received an intravaginal device (IVD) containing 1 g progesterone (P4), 2 mg estradiol benzoate (EB) IM, 0.52 mg cloprostenol sodium (CS) IM, and 25 µg lecirelin (GnRH) IM. On D8, they received 0.52 mg CS and had the IVD removed. Twenty hours after IVD removal, they received 25 µg lecirelin (GnRH) IM. Follicular dynamics were monitored daily by ultrasonography (US) from D7 to D9. At 16 h post GnRH, the FM group (n = 7) received 2.2 mg/kg flunixin meglumine (FM) IM, and no additional treatment was performed in the Control group (n = 7). At 24 h post GnRH, US-guided follicular aspiration was performed to isolate extracellular vesicles, identify differentially expressed miRNAs, and predict regulated

biological pathways. Results: (B) Relative expression of differentially expressed miRNAs between Control and FM groups: bta-miR-584, bta-miR-369-5p, bta-miR-218, and bta-miR-411c-5p. (C) Predicted biological pathways negatively regulated by FM treatment, with total number of modulated genes.

4 Considerações Finais

O papel das prostaglandinas, em especial PGE2, no processo de ruptura folicular e na manutenção da função lútea foi reforçado com os resultados. As prostaglandinas exógenas não demonstraram possuir capacidade de antecipar a ovulação quando aplicadas anteriormente ao pico fisiológico da gonadotrofina ovulatória, refutando a primeira hipótese. Contudo, a PGE2 exerceu um efeito luteotrófico marcante, pois promoveu o aumento nos níveis séricos de progesterona e antagonizou a ação luteolítica da PGF2A.

O estabelecimento de modelos de estudo com o uso anti-inflamatório não esteroidal FM, quando administrado por vias sistêmica ou intraovariana, não foi possível, uma vez que a inibição da ovulação foi inconsistente ou nula. Além disso, a injeção intrafolicular de FM 16h pós GnRH, mesmo em uma concentração mais elevada, não foi capaz de atingir 100% de bloqueio da ovulação conforme apontado na literatura. O resultado foi obtido na aplicação das 22h pós-GnRH.

No estudo do reestabelecimento da ovulação no formato de coadministração, às 16h pós-GnRH, momento anterior ao aumento da expressão da cicloxigenase, não foi eficaz em reverter da anovulação, contrapondo nossa hipótese. Já quando aplicado próximo do pico das prostaglandinas, o reestabelecimento parcial da ovulação foi possível, o que reforça a janela de ação crítica dessas moléculas no mecanismo de ruptura folicular em bovinos.

O tratamento com FM alterou o perfil de miRNAs transportados em vesículas extracelulares no fluido folicular. A diferença na expressão de bta-miR-584, bta-miR-369-5p, bta-miR-218 e bta-miR-411c-5p e a modulação de vias biológicas relacionados a ruptura folicular e do metabolismo das prostaglandinas. essenciais para a ovulação, contribuem para o entendimento dos mecanismos moleculares do bloqueio ovulatório.

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Anexos

Anexo A - Documento da Comissão de Ética e Experimentação Animal

PARECER Nº **213/2023/CEUA/REITORIA**
PROCESSO Nº 23110.029007/2023-66

Certificado

Certificamos que a proposta intitulada **"Estratégias inovadoras para o controle do ciclo estral e ovulação em fêmeas bovinas"**, registrada com o n ° **23110.029007/2023-66,** sob a responsabilidade de **Rafael Gianella Mondadori** - 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 execução pela Comissão de Ética no Uso de Animais da Universidade Federal de Pelotas.

Finalidade	(x) Pesquisa ( ) Ensino
Vigência da autorização	Início: 15/12/2023 Término: 31/12/2026
Espécie/linhagem/raça	*Bos taurus* / Sem raça definida
N° de animais	**2.222**
Idade	1 a 10 anos
Sexo	Fêmeas

Origem	Centro Agropecuário da Palma da UFPel (CAP-UFPel), Capão do Leão/RS. Central de Embriões Biotec - Capela Salete, Protásio Alves - RS, 95345-000, Brasil. Fazenda Posto Branco Corredor da Reúna - Batovi, São Gabriel - RS, 97300-148, Brasil. Fazenda Nossa Senhora da Conceição – Capão dos Patos, Bom Jesus - RS, 95290-000, Brasil. Fazenda Santo Isidoro- Estrada Caraguatá Km 2,4, Coxilha - RS, 99145-000, Brasil.

Código para cadastro nº **CEUA 029007/2023-66**

Priscila Marques Moura de Leon

Coordenadora da CEUA

Documento assinado eletronicamente por **PRISCILA MARQUES MOURA DE LEON**, **Professor do Magistério Superior**, em 08/12/2023, às 11:09, conforme horário oficial de Brasília, com fundamento no art. 4º, § 3º, do Decreto nº 10.543, de 13 de novembro de 2020.

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