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INDICADORES DE SAÚDE UTERINA PÓS-PARTO E FERTILIDADE EM VACAS DE CORTE

JÉSSICA DE SOUZA ANDRADE

Porto Velho – RO

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Tese de doutorado apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Biodiversidade e Biotecnologia - Rede BIONORTE, na Fundação Oswaldo Cruz de Rondônia, como requisito parcial para obtenção do Título de Doutora em Biodiversidade e Biotecnologia.

Orientador: Prof. Dr. Luiz Francisco Machado Pfeifer.

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ANDRADE, Jéssica de Souza. **Indicadores de saúde uterina pós-parto e fertilidade em vacas de corte.** 2022. 136f. Tese (Doutorado em Biodiversidade e Biotecnologia) – Programa de Pós-Graduação em Biodiversidade e Biotecnologia - Rede BIONORTE da Fundação Oswaldo Cruz, Porto Velho, 2022.

RESUMO

O intervalo entre partos em vacas de corte é diretamente afetado pela duração do puerpério. Dessa forma, o adequado restabelecimento da função uterina é fundamental para que vacas de corte lactantes apresentem adequada fertilidade quando submetidas a protocolos de inseminação artificial em tempo fixo (IATF). Neste contexto, a caracterização da inflamação uterina no período pós-parto recente é importante para melhor entender o processo de reparação endometrial. Portanto, o objetivo geral deste estudo foi caracterizar a saúde uterina e a fertilidade pós-parto em vacas de corte. O primeiro estudo foi conduzido para avaliar o efeito da saúde uterina na fertilidade de vacas de corte no pós-parto. Os resultados deste estudo mostraram que vacas que apresentam maior proporção de células PMN apresentaram menor fertilidade e maior expressão das citocinas pró-inflamatórias IL-1β e IL-8 no útero pós-parto. O segundo estudo teve como objetivo comparar a dinâmica uterina e o metabolismo pós-parto de vacas de corte primíparas e multíparas. Os resultados indicaram que vacas multíparas apresentam menor resposta inflamatória uterina associada a uma menor expressão relativa de IL-8 no período pós-parto e maior probabilidade de estarem sadias já aos 35 dias pós-parto. Em geral, os resultados obtidos neste estudo demostram que vacas que apresentaram distúrbios uterinos tiveram uma pior performance reprodutiva. Entretanto, existem lacunas a serem preenchidas no intuito de identificar o impacto da saúde uterina na fertilidade de vacas de corte no pós-parto.

Palavras-chave: Bovinos; Puerpério; Endométrio; Citocinas; Prenhez.

ANDRADE, Jéssica de Souza. **Indicators of postpartum uterine health and fertility in beef cows**. 2022. 136f. Thesis (Doctorate in Biodiversity and Biotechnology) – Programa de Pós-Graduação em Biodiversidade e Biotecnologia - Rede BIONORTE da Fundação Oswaldo Cruz, Porto Velho, 2022.

ABSTRACT

The calving interval in beef cows is directly affected by the duration of the puerperium. Thus, adequate restoration of uterine function is essential for lactating beef cows to present adequate fertility when submitted to fixed-time artificial insemination (FTAI) protocols. In this context, the characterization of uterine inflammation in the recent postpartum period is important to better understand the endometrial repair process. In this context, the characterization of uterine inflammation in the recent postpartum period is important to better understand the endometrial repair process. The first study was conducted to evaluate the effect of uterine health on fertility in postpartum beef cows. The results of this study showed that cows with a higher proportion of PMN cells had lower fertility and higher expression of the pro-inflammatory cytokines IL-1β and IL-8 in the postpartum uterus. The second study aimed to compare uterine dynamics and postpartum metabolism of primiparous and multiparous beef cows. The results indicated that multiparous cows have a lower uterine inflammatory response associated with a lower relative expression of IL-8 in the postpartum period and a greater probability of being healthy at 35 days postpartum. In general, the results obtained in this study demonstrate that cows that presented uterine disorders had a worse reproductive performance. However, there are gaps to be filled in order to identify the impact of uterine health on the fertility of postpartum beef cows.

Keywords: Cattle; Puerperium; Endometrium; Cytokines; Pregnancy.

LISTA DE FIGURAS

Revisão Bibliográfica

Figura 1 - Categorias bacterianas isoladas do lúmen uterino de bovinos de acordo com seu potencial patogênico. 24

Figura 2 - Classificação de muco vaginal: pontuação 0 = muco claro ou translúcido; pontuação 1 = muco contendo manchas de pus branco ou esbranquiçado; pontuação 2 = descarga contendo ≤50% de exsudato mucopurulento branco ou esbranquiçado; e 3 = ≥50% de exsudato purulento, geralmente branco ou amarelo, mas ocasionalmente sanguíneo. 29

Capítulo I

Figure 1 - Venn diagram showing the number of cows according to the proportion of polymorphonuclear cells (PMN) and purulent vaginal discharge (PVD). Squares within the diagram demonstrate the number of cows in each group. ……65

Figure 2 - Relationship between proportion of PMN cells in the uterine endometrium and the probability of pregnancy in the postpartum period in Nellore cows submitted to the TAI protocol (*P* = 0.002). ……...…… 65

Figure 3 - Relationship between days postpartum and the probability of pregnancy in the postpartum period in Nellore cows submitted to the TAI protocol (*P* = 0.1). …….……66

Figure 4 - Relationship between proportion of PMN cells in the uterine endometrium and Days postpartum in Nelore cows subjected to TAI protocol. 66

Figure 5 - Receiver Operating Characteristic (ROC) curve for determining the relationship between proportion of PMN in the uterus and pregnancy status in beef cows; Analysis of the ROC indicated that the use of a cut-off point of more than 4.75% of PMN was 42.9% specific and 82.8% sensitive; Area under the curve = 0.68, P < 0.001. ……..….67

Figure 6 - Relative abundance of mRNA transcript in uterine endometrial tissues of beef cows categorized into groups according to uterine health in the postpartum period. ^{ab} indicates means differ by $P \leq 0.1$, ^{ABC} by $P \leq 0.05$. ……..... 68

Capítulo II

Figure 1 - Evolution of body weight (A), subcutaneous fat thickness (B, internal angle of the rump (°C) and serum glucose concentration (D), βHB (E), albumin (F) and haptoglobin (G) (mean ± SEM) during the transition period in primiparous (n=15) and multiparous (n=9) beef cows from -20 to 42 days postpartum. Statistical analysis was performed for the pre and postpartum periods for the effects of category, days from calving and the interaction between category and days from calving. ….....…….....…..…..91

Figure 2 - Endometrial area (A) and uterine diameter (B) of primiparous (n=15) and multiparous (n=9) (mean ± SEM) beef cows from 7 to 42 days postpartum. Statistical analysis was performed for the pre and postpartum periods for the effects of category, days from calving and the interaction between category and days from calving. ……..….. 92

Figure 3 - Purulent vaginal discharge (A) and proportion of PMN (B) of primiparous (n=15) and multiparous (n=9) beef cows (mean ± SEM) from 7 to 42 days postpartum. Statistical analysis was performed for the pre and postpartum periods for the effects of category, days from calving and the interaction between category and days from calving. ……...…… 92

Figure 4 - Relative abundance of pro-inflammatory cytokine transcripts for tumor necrosis factor alpha (A; *TNFA*), interleukin 1 beta (; *IL1β*), interleukin 6 (C; *IL6*) and interleukin 8 (D; *IL8*) and anti-inflammatory interleukin 10 (E; *IL10*) mRNA in endometrial tissues of primiparous and multiparous beef cows at 7 to 42 days postpartum. Statistical analysis was performed for the pre and postpartum periods for the effects of category, days from calving and the interaction between category and days from calving. .....……..... 93

Figure 5 - Relative abundance of gene transcripts for regulation of cell proliferation progesterone receptor (A; *PGR*), estrogen receptor 1 (B; *ESR1*), estrogen receptor 2 (C; *ESR2*) Patched 2a (D; *PTCH2*), epidermal growth factor receptor (E; E*GFR*) heparin-binding EGF-like growth factor (F; *HB-EGF*), and basement membrane component (G; *COL4A1*) and

Integrin beta 3 (H; *ITGB3*) mRNA in endometrial tissues of primiparous and multiparous beef cows from 7 to 42 days postpartum. Statistical analysis was performed for the pre and postpartum periods for the effects of category, days from calving and the interaction between category and days from calving. …….....……... 94

LISTA DE TABELAS

Capítulo I

Table 1 - Primer sequences for quantitative real-time polymerase chain reaction (qRT-PCR) amplification of mRNA. ……..……..... 69

Table 2 - Fertility and metabolic response of cows according to the experimental groups…….... 69

Capítulo II

Table 1 - Primer sequences for quantitative real-time polymerase chain reaction (qRT-PCR) amplification of mRNA. .....… 95

Table 2 - Uterine, ovarian and fertility responses in primiparous and multiparous beef cows at different postpartum days. 96

LISTA DE ABREVIATURAS E SIGLAS

ACTB - Actin β

AI - Artificial insemination

AIG - Ângulo interno da garupa

AMPs - Peptídeos antimicrobianos

BEN - Balanço energético negativo

βHB – β-hidroxibutirato

CA - Califórnia

cDNA – DNA complementar

CL – Corpo lúteo

CLRs - Receptores de lectina tipo C

CMS – Consumo de matéria seca

COL4A1 - Collagen, type IV, α 1

CT – Cycle threshold ou Cq

DAMPs - Padrões Moleculares Associados a Danos

dL – Decilitros

DPP – Dias pós-parto

DVP – Descarga vaginal purulenta

DVP+ – Descarga vaginal purulenta positiva

EC – Endometrite clínica

ECC - Escore de condição corporal

eCG – Gonadotropina coriônica equina

ECP – Cipionato de estradiol

EGFR - Epidermal growth factor receptor

EGS - Espessura de gordura subcutânea

Embrapa - Empresa Brasileira de Pesquisa Agropecuária

ESC – Endometrite subclínica

ESR1 – Estrogen receptor 1

ESR2 - Estrogen receptor 2

E2 - Estradiol

FTAI – Fixed-time artificial insemination

FPO - Folículo pré-ovulatório

g – Grama

GAPDH - Gliceraldeído-3-fosfato desidrogenase

GM-CSF - Fator estimulador de colônia de granulócitos-macrófagos

HB-EGF - Heparin binding EGF-like growth factor

HE - Healthy

IA – Inseminação Artificial

IATF – Inseminação artificial em tempo fixo

IFNγ - Interferon-gama

IgA – Imunoglobulina A

IgE – Imunoglobulina E

IgG - Imunoglobulina G

IgM - Imunoglobulina M

i.m. – Intramuscular

IL-1β – Interleucina 1 beta

IL- 4 – Interleucina 4

IL-6 – Interleucina 6

IL-8 – Interleucina 8

IL-10 – Interleucina 10

IL-17 – Interleucina 17

IL-22 – Interleucina 22

IL-33 – Interleucina 33

ITGB3 - Integrin, beta 3

Kg – Quilograma

LAP - Peptídeo antimicrobiano lingual

LPS - Lipopolissacarídeo

LTA - Ácido lipoteicóico

MAPA – Ministério da Agricultura, Pecuária e Abastecimento

MHC - Complexo principal de histocompatibilidade

mg - Miligrama

MHz – Megahertz

min. - Minutos

mL – Mililitro

mm – Milímetros

mmol - Milimol

MO – Missouri

MUC1 – Mucina 1

NLRs - Receptores semelhantes ao domínio de oligomerização ligante de nucleotídeos

^{o}C – Graus Celsius

PAMPs - Padrões Moleculares Associados a Patógenos

$PGF_{2\alpha}$ – Prostaglandina $F_{2\alpha}$

PGR - Progesterone receptor

PMN - Polimorfonucleares

PP – Pós-parto

PPP – Período pós-parto

PRRs- Receptores de reconhecimento de padrões

P/IA - Pregnancy per artificial insemination

PC – Peso corporal

PPP – Período pós-parto

PVD - Purulent vaginal discharge

PVD+ - Positive Purulent Vaginal Discharge

PTCH2 - Patched 2ª

P4 - Progesterona

qRT-PCR - Quantitative real-time polymerase chain reaction

RNA – Ácido ribonucleico

RNAm - Ácido ribonucleico mensageiro

RO – Rondônia

RP – Retenção de placenta

RT – Transcrição reversa

SCE - Subclinical Endometritis

SCEP - Subclinical Endometritis + purulent vaginal discharge

SP – São Paulo

TAI – Time artificial insemination

TAP - peptídeo antimicrobiano traqueal

TcR - receptores de células T αβ e γδT

TGF - Fator de crescimento de transformação

TLR - Receptores Toll-like

TNFα – Fator de necrose tumoral alfa

µL – Microlitro

µM - Micromolar

USA – United States of America

USDA – United States Department of Agriculture

xg - Força centrífuga relativa

SUMÁRIO

1 INTRODUÇÃO 17
1.1. OBJETIVO GERAL 19
1.2 OBJETIVOS ESPECIFICOS 19
2 REVISÃO BIBLIOGRÁFICA 21
2.1 PERÍODO PÓS-PARTO 21
2.1.1 Patógenos uterinos após o parto 22
2.1.2 Microbiota uterina e vaginal 25
2.1.3 Doenças uterinas no pós-parto 27
2.1.4 Imunologia do útero bovino 30
2.1.5. Balanço energético negativo e a saúde reprodutiva 33
2.2 RECEPTIVIDADE UTERINA 37
CAPÍTULO I 42
CAPÍTULO II 70
CONCLUSÕES 97
REFERÊNCIAS 98
ANEXOS 118

1 INTRODUÇÃO

O Brasil possui o segundo maior rebanho bovino e o maior rebanho comercial mundial, com estimativa que o rebanho brasileiro atingirá 276 milhões de cabeças ano final de 2022 (USDA, 2022). O Ministério da Agricultura, Pecuária e Abastecimento (MAPA) relata que até julho de 2022 o Valor Bruto de Produção (VBP) da pecuária bovina no Brasil já foi de R$ 152,4 bilhões, ficando atrás apenas da soja com R$ 350,5 bilhões e algodão de R$ 157,7 bilhões no mesmo período. Adicionalmente, em 2020, o Brasil foi o maior exportador mundial de carne bovina, com 2,2 milhões de toneladas e 14,4% do mercado internacional (EMBRAPA, 2021). Entretanto, apesar do grande volume de produção e da grande extensão de terras disponíveis para a atividade pecuária, o rebanho brasileiro é caracterizado por baixa produtividade. Ainda, levando em consideração que o efetivo bovino do Brasil é o dobro que a dos Estados Unidos, este é o maior produtor de carne bovina do mundo, com estimativa de aproximadamente 9,5 milhões de toneladas em 2022 (USDA, 2022).

Dentro deste contexto, destaca-se a região Norte, com mais de 36 milhões de cabeças de bovinos e como a segunda região com maior rebanho do Brasil (ANUALPEC, 2021). Especificamente, o estado de Rondônia se revela em sétimo lugar com o maior rebanho em nível nacional, possuindo um total de ~9 milhões de cabeças, sendo ~1 milhões de cabeças para aptidão leite e ~8 milhões para aptidão corte em 2021 (ANUALPEC, 2021). A região Norte é a maior produtora de leite e a segunda maior produtora de carne bovina, ficando atrás somente do Pará. O rebanho rondoniense produziu cerca de 588,4 milhões de litros de leite e, aproximadamente, 552,1 mil toneladas de carne em 2021, sendo responsável por 60,7% e 29,5% da produção de leite e carne, respectivamente, na região Norte nesse mesmo período. Além de ser o estado com a sexta maior produção de carne bovina do país, é o terceiro maior exportador de carne do Brasil (ANUALPEC, 2021).

O aumento da fertilidade de vacas mantidas em sistema extensivo contribui substancialmente para tornar o sistema de produção de carne mais eficiente e sustentável, uma vez que ganhos de produtividade influenciam diretamente na mitigação de gases do efeito estufa (como o metano e o dióxido de carbono), que estão intimamente ligados aos danos ambientais e ao aquecimento global. Desta forma, o aumento do desempenho reprodutivo do rebanho de cria pode representar um caminho seguro para que a produção de carne brasileira esteja alinhada com as demandas globais por sistemas de produção mais sustentáveis. Neste sentido, estudos estão sendo realizados no intuito de contribuir positivamente para os índices de produtividade do rebanho nacional por meio da associação da seleção genética, manejo nutricional, sanitário

e reprodutivo (GRUMMER e CAROLL, 1991; BARCELOS *et al.*, 2014; SALEHI *et al.*, 2016; MANN *et al.*, 2018).

Um dos fatores que resultam em um baixo desempenho reprodutivo do rebanho bovino é o longo intervalo de partos, decorrente do anestro prolongado no pós-parto (DODE *et al.*, 1989), dessa forma, prolongar a vida útil de uma vaca está diretamente relacionado aos eventos que ocorrem no período pós-parto. O tempo de gestação pode variar de 280-290 dias dependendo do grupo genético da vaca e do sexo da progênie. Como o período de gestação apresenta uma pequena variação, o principal fator determinante do intervalo de partos é o primeiro cio pós-parto (CERDÓTES *et al.*, 2004). Para que as fêmeas possam atingir a máxima eficiência dentro de um sistema de produção, é necessário que a vaca conceba em até 85 dias após o parto, possibilitando um intervalo de partos de 365 dias (CROWE, 2008). Analisando que, para conseguir uma taxa de concepção satisfatória, é necessário intervalo de 60 dias do parto ao primeiro cio, restando apenas 25 dias para a vaca ficar prenhe a fim de alcançar um bezerro/vaca/ano.

Neste sentindo, durante o puerpério, ocorrem alterações no perfil inflamatório e transcriptômico que devem ser muito bem orquestradas para que o endométrio retome as atividades biológicas culminando com o preparo do útero para a concepção. No momento do parto, a abertura das barreiras anatômicas constituídas pela vulva, vagina e cérvix possibilita a invasão de bactérias do ambiente externo para o útero. O ambiente uterino é comprometido quando ocorrem alterações nos mecanismos de defesa locais e consequente persistência de bactérias patogênicas resultando no estabelecimento de diferentes quadros de infecção uterina (SHELDON et al., 2009). A presença de patógenos no útero provoca inflamação e lesões histológicas do endométrio ocasionando o retardamento da involução uterina (BONNETT, et al., 1991; WILLIAMS et al., 2005). Para eliminar esses patógenos, o animal depende de um eficiente mecanismo de defesa uterina. Desta forma, uma das primeiras respostas uterinas para eliminar a infecção pós-parto é a ativação da imunidade inata do endométrio por meio da capacidade de células epiteliais tem de reconhecer os Padrões Moleculares Associados a Patógenos (PAMPs) e os Padrões Moleculares Associados a Danos (DAMPs) por meio dos receptores de reconhecimento de padrões (PRRs), bem como a migração intensa de células polimorfonucleares (PMN) e outros leucócitos de defesa para o útero (SHELDON et al., 2006; DADARWAL et al., 2017). Além disso, ocorre alteração do funcionamento molecular em resposta à inflamação uterina neste período. Já foram observadas mudanças no padrão de expressão de transcritos de genes para os receptores Toll-like nas células epiteliais do endométrio bovino (TLR; 1-7 e 9) e no estroma endometrial (TRL 1-4, 6,7 e 9) (DAVIES et

al., 2008). Além do mais, citocinas (interleucinas (IL)-1, IL-6 e fator de necrose tumoral (TNF)) (KOLLS et al., 2008; HERATH et al., 2009; DADARWAL et al., 2017) e componentes do sistema complemento já foram detectados em distintos locais do trato uterino e no muco cervical que são capazes de modular a secreção de peptídeos antimicrobianos (KANIA et al., 2001; DRAB et al., 2014). No útero de vacas sadias a resolução da resposta inflamatória é identificada por meio da redução de PMN, principalmente de neutrófilos à medida que a involução uterina acontece (CHAPWANYA et al., 2012). Em animais com atraso na involução uterina, dependendo da proporção de PMN no lúmen uterino podemos determinar a gravidade da inflamação uterina e classificar o quadro como: endometrite clínica e a endometrite subclínica (BARLUND et al., 2008). Diante deste contexto, é necessário estudar os eventos e identificar os componentes envolvidos na resposta imune de mecanismo de reparação tecidual que definem o restabelecimento da condição uterina pós-parto, pois o ambiente uterino pós-parto saudável é fator determinante de uma boa eficiência reprodutiva.

Adicionalmente, nos últimos anos muitos estudos voltados para a reprodução de fêmeas bovinas acerca dos mecanismos fisiológicos do útero (CALL, 1989; WAGNER & HANSEL, 1969; SHELDON, 2004; GALVÃO *et al.*, 2011; FOLEY *et al.*, 2012; GHASEMI *et al.*, 2012; MAREY *et al.*, 2016; DADARWAL *et al.*, 2017; PFEIFER *et al.*, 2018) e a ciclicidade ovariana (OPSOMER *et al.*, 2000; SHELDON, 2004; GILBERT, *et al.*, 2005; KRAUSE *et al.*, 2014; PFEIFER *et al.*, 2018) têm sido realizados para que lacunas do conhecimento em torno deste assunto sejam preenchidas e que a performance reprodutiva sejam melhorados. Neste sentido, um período importante e crucial e que ainda não é totalmente compreendido é o período pós-parto, pois envolve uma série concomitante de eventos, interações hormonais, metabólicas, imunológicas e transcricionais para que a próxima prenhez seja estabelecida com sucesso. Estes eventos e interações que ocorrem em fêmeas bovinas de corte no período pós-parto foram o tema de estudo desta tese e serão descritos no decorrer deste documento. Perante a isso, nossa hipótese geral é vacas de corte que apresentam uma inadequada condição uterina no período pós-parto possuem um menor desempenho reprodutivo. Desta forma, este estudo teve como:

1.1. OBJETIVO GERAL

Caracterizar a saúde uterina pós-parto e a fertilidade em vacas de corte.

1.2 OBJETIVOS ESPECIFICOS

Os objetivos específicos dessa tese são:

Avaliar:

1. As condições uterinas relacionadas à taxa de concepção;
2. O efeito da presença das células polimorfonucleares no tecido endometrial uterino e da descarga vaginal na taxa de concepção;
3. O processo de involução morfológica do útero;
4. A relação da inflamação do tecido endometrial com o retorno à ciclicidade ovariana;
5. O padrão de expressão gênica de citocinas pró e anti-inflamatórias e de genes para reparo e proliferação celular do endométrio, de vacas de corte no período pós-parto.

2 REVISÃO BIBLIOGRÁFICA

2.1 PERÍODO PÓS-PARTO

O período pós-parto é o período compreendido entre o parto até a completa involução uterina, geralmente entre 25 e 47 dias após o parto (GIER e MARION, 1968; CALL 1989; SHELDON, 2004). Durante esse período, o trato reprodutivo bovino passa por pelo menos quatro eventos dinâmicos para que a próxima prenhez seja estabelecida com sucesso: a involução do útero, a reparação do endométrio, a retomada da atividade cíclica ovariana e a depuração bacteriana do trato reprodutivo (SHELDON, 2004).

A involução uterina é o termo designado para descrever redução física sofrida no útero e na cérvix após o parto. Esse evento ocorre devido às fortes contrações musculares uterinas, necrose e descamação das carúnculas uterinas, alterações na matriz extracelular e reparação do endométrio (GIER e MARION, 1968). O monitoramento da involução uterina pode ser realizado por palpação retal e/ou ultrassonografia transretal por meio de avaliação repetida do tamanho do útero (OKANO e TOMIZUKA, 1987; SHELDON *et al.*, 2000; SHELDON *et al.*, 2003). Aproximadamente duas semanas após o parto, todo o trato genital é palpável pelo reto em animais sadios; apesar do corno previamente gravídico ainda possa ser identificado por ser mais extenso e largo que o corno não gravídico. Esta diferença é perceptível até quatro semanas após o parto, podendo estar completa em seis semanas (OKANO e TOMIZUKA, 1987; RISCO *et al.*, 1994; SHELDON *et al.*, 2017). Ainda, diversos estudos relatam que o tempo para a conclusão da involução uterina (CALL, 1989; SHELDON, 2004), entretanto, na prática, é difícil estimar. Fatores como o parto distócico, hipocalcemia, retenção de membranas fetais e placenta e inflamações das camadas uterinas, como a metrite e endometrite, podem retardar a conclusão da involução e, consequentemente, causar futura subfertilidade (FONSECA *et al.*, 1983; RISCO *et al.*, 1994). Alguns estudos relatam o efeito da categoria animal na involução uterina (ZAIN *et al.*, 1995; GONZALEZ SANCHEZ *et al.*, 1999; ZHANG *et al.*, 2010), como já observado em vacas de leite que a involução uterina ocorre mais rápido em vacas primíparas do que em multíparas (BAEZ *et al.*, 2016; SHARMA *et al.*, 2018). Entretanto, alguns relatos demostram que a involução uterina não é afetada pela ordem de paridade (MIETTINEN, 1990; NOGUEIRA *et al.*, 1993).

Em vacas sadias, a reparação do endométrio geralmente está superficialmente completa aos 25 dias pós-parto, mas as camadas mais profundas não são totalmente reparadas até 6 a 8 semanas (CALL, 1989; DADARWAL *et al.*, 2017; SHELDON, 2004). Durante este período, o

endométrio é remodelado para sua condição normal devido às lesões sofridas durante o parto (GIER e MARION, 1968; WAGNER e HANSEL, 1969). Posteriormente a saída do alanto-córion, as carúnculas sofrem necrose por volta dos 12 dias após o parto, contribuindo para a formação do lóquio juntamente com os restos dos fluídos fetais, debris celulares e do sangue derivado da ruptura do umbigo. Nos primeiros 2 a 3 dias após o parto, o útero bovino contém cerca de 1 a 2 litros de lóquio e desaparece por volta dos 14 a 18 dias. Geralmente, o lóquio de vacas sadias é um líquido viscoso no tom de amarelo a marrom sem presença de odor desagradável. Em caso de atraso da involução uterina, o lóquio apresenta coloração escura, secreção purulenta e odor fétido (SHELDON, 2004).

Se os eventos de reparação do tecido uterino estão atrasados, o endométrio está apresentando um processo inflamatório e/ou infeccioso. Se a inflamação for prolongada e não tratada, resulta em um estado patológico denominado endometrite, que consequentemente pode resultar em disfunção ovariana e da fertilidade (PETER e BOSU, 1988; OPSOMER, *et al.*, 2000; SHELDON *et al.*, 2002; DAHIYA *et al.*, 2018).

2.1.1 Patógenos uterinos após o parto

No decorrer do parto, a abertura das barreiras anatômicas constituídas pela vulva, vagina e cérvix possibilita a colonização de microrganismos do ambiente externo, como os presentes nas fezes e pele do animal, para o lúmen uterino (SHELDON e DOBSON, 2004; FÖLDI *et al.*, 2006; MARTINS e BORGES, 2011; APPIAH *et al.*, 2020). As alterações ocorridas no endométrio e o lóquio formado após o parto contribuem para uma acelerada multiplicação bacteriana (AZAWI, 2008).

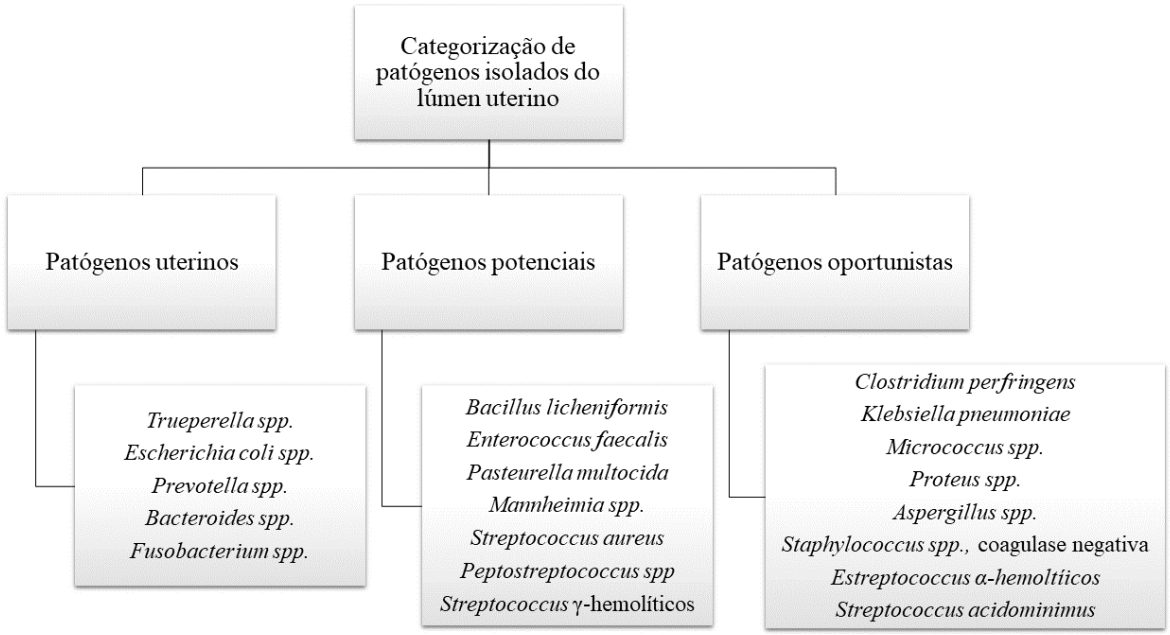
Geralmente, a contaminação uterina está presente em 80-100% das vacas nas primeiras semanas pós-parto (SHELDON *et al.*, 2008). A doença clínica pode ser relatada em até 40% dos animais (MARKUSFELD, 1987; SHELDON *et al.*, 2008) chegando a 21% apresentando sinais sistêmicos, como pirexia (BENZAQUEN *et al.*, 2007). A presença de patógenos no útero provoca inflamação e lesões histológicas do endométrio ocasionando o retardamento da involução uterina (BONNETT, *et al.*, 1991; WILLIAMS *et al.*, 2005). Existe uma ampla gama de bactérias aeróbias, anaeróbias, gram-negativas, gram-positivas podem ser isoladas do lúmen do útero pós-parto (SHELDON & DOBSON, 2004; AZAWI, 2008). Bactérias aeróbicas frequentemente isoladas no útero de vacas sadias no pós-parto são a *Escherichia coli*, *Streptococcus spp*, *Staphylococcus spp* e *Arcanobacterium pyogenes*; enquanto as anaeróbicas mais isoladas são a *Trueperella pyogenes*, *Fusobacterium necrophorum*, *Provetella*

melaninogenica e *Bacteroides spp*. (BRETZLAFF, 1987, BONDURANT, 1999; WILLIAMS *et al.*, 2005; AZAWI, 2008; SHELDON *et al*., 2009; BICALHO *et al.*, 2016), que, ainda, podem agir sinergicamente para aumentar a probabilidade e a gravidade da patologia uterina (RUDER *et al.*, 1981; BONNETT *et al.*, 1991; LEWIS, 1997; WILLIANS et a., 2005; SHELDON *et al.*, 2004).

Relato com vacas Nelore primíparas recém-paridas encontraram sinergismo em todas as amostras de material uterino, destacando *S. aureus* com *S. intermedius*; *E. coli*, *P. aeruginosa*, *T. pyogenes* e *S. schenckii*; *S. aureus* com *Streptococcus spp*.; *E. coli*, *P. mirabilis*, *T. pyogenes*, *C. albicans* e *S. schenckii*; *S. aureus* com *E. coli*, *T. pyogenese* e *C. albicans* (RODRIGUES FARIAS, *et al.*, 2016). Estudo recente utilizando técnica MALDI-TOF MS observou que vacas leiteiras de rebanhos brasileiros com endometrite clínica (EC) tiveram uma taxa de isolamento para *Trueperella pyogenes* maior do que vacas em com endometrite subclínica (ESC) e sadias, sendo em vacas com ESC essa bactéria não foi isolada; além disso, algumas bactérias, como *Prevotella heparinolytica*, *Porphyromonas levii*, *Fusobacterium necrophorum*, *Bacteroides pyogenes* e *Helcococcus spp*., foram identificadas apenas em vacas EC (PAIANO *et al.*, 2022). Em análises metagenômicas de sequências do gene 16S rRNA para avaliar a microbiota amostras endometrial em vacas de leite observaram a nível de filo, a abundância relativa de *Bacteroidetes* e *Fusobacteria*, e a nível de gênero, de *Trueperella* foi maior em vacas EC do que em vacas sadias ou com ESC. *Trueperella pyogenes* foi o gênero de bactéria mais isolada em vacas com EC, e uma ampla variedade de crescimento bacteriano foi observado em vacas sadias e SCE (PASCOTTINI *et al*., 2020).

As bactérias que comumente contaminam o lúmen uterino de vacas leiteiras no pós-parto recente podem ser categorizadas de acordo com sua patogenicidade potencial, como: patógenos uterinos reconhecidos (associados a lesões endometriais uterinas), patógenos potenciais (frequentemente isolados do lúmen uterino bovino e em casos de endometrite, mas não comumente associados a lesões uterina) e patógenos oportunistas (isolados transitoriamente do lúmen uterino e não associados à endometrite) (WILLIAMS *et al.*, 2005; GALVÃO, 2018), conforme mostrado na Figura 1.

Figura 1 - Categorias bacterianas isoladas do lúmen uterino de bovinos de acordo com seu potencial patogênico.



Fonte: (Adaptado de WILLIAMS et al., 2005 e APPIAH et al., 2020).

A infecção do útero por patógenos bacterianos (como a *E. coli*) parece contribuir para uma infecção por outras bactérias ou vírus (DOHMEN *et al.*, 2000; WILLIAMS *et al.*, 2007; DONOFRIO *et al.*, 2008). O herpesvírus bovino 4 (BoHV- 4) é comumente considerado o patógeno viral associado à doença uterina no período pós-parto em fêmeas bovinas (THIRY *et al.*, 1992; SHELDON *et al.*, 2009). O BoHV-4 pode estabelecer infecções persistentes que estão ocultas em vacas, especialmente em uma linhagem celular de macrófagos bovinos BoMac (DONOFRIO e VAN SANTEM, 2001), além de possuir tropismo por células endometriais, proliferar-se rapidamente, induzindo a morte de células epiteliais ou estromais uterinas (DONOFRIO *et al*., 2007). Neste sentido, o estabelecimento das condições infecciosas e inflamatórias que resultam em doenças no trato reprodutivo da fêmea bovina depende do mecanismo imune inato e adquirido que limitam a invasão e propagação de agentes infecciosos, bem como os fatores associados à patogenicidade dos microrganismos, como a carga bacteriana, virulência e o sinergismo entre as espécies (DADARWAL *et al*., 2017).

2.1.2 Microbiota uterina e vaginal

Por muito tempo prevalecia a visão tradicional de que o útero era estéril fora do período pós-parto e posteriormente contaminado por bactérias não específicas oriundos de animais e do ambiente após o parto (SHELDON *et al.*, 2008; SHELDON *et al.*, 2009). No entanto, evidencias atuais de estudos utilizando sondas fluorescentes para bactérias e do sequenciamento do gene RNA ribossômico 16S, que há um microbioma esparso no útero, mesmo durante a prenhez (KARSTRUP *et al.*, 2017; MOORE *et al.*, 2017) e que microrganismos comensais se adaptam ao endométrio e podem influenciar as células uterinas presentes no microambiente (APPIAH *et al.*, 2020).

Da mesma maneira, estudos mostraram uma continuidade da microbiota ao longo o sistema reprodutivo do bovino consistindo de diferentes nichos do trato reprodutivo de gado leiteiro e de corte e variando em comunidades ou suas populações (SANTOS e BICALHO, 2012; CHEN *et al.*, 2017; KARSTRUP *et al.*, 2017; MOORE *et al.*, 2017; NESENGANI *et al.*, 2017; *et al.*, 2018; GALVÃO *et al.*, 2019; GIANNATTASIO-FERRAZ *et al.*, 2019; MIRANDA-CASOLUENGO *et al.*, 2019; APPIAH *et al.*, 2020). No entanto, a microflora do útero é menos numerosa do que a do intestino ou da vagina em relação a composição microbiana, e somente uma pequena parte dessa carga bacteriana coopera para a doença uterina no pós-parto (SHELDON *et al.*, 2019; APPIAH *et al.*, 2020; ADNANE e CHAPWANYA, 2022). A atribuição taxonômica mostrou que os filos bacterianos uterinos dominantes foram *Fusobacteria*, *Bacteroidetes*, *Proteobacteria*, *Firmicutes* e *Tenericute*s. Sequências de *Spirochaetes*, *Synergistetes* e *Actinobacteria* também estão presentes, mas com menos frequência (SANTOS e BICALHO, 2012).

Em estudo avaliando o efeito da paridade sobre a dinâmica do microbioma uterino em vacas leiteiras no pós-parto observou a nível de filo que a abundância relativa de Actinobactérias foi maior em vacas primíparas do que em multíparas e, a nível de gênero, houve menor abundância relativa de *Bifidobacterium*, *Lactobacillus*, *Neisseriaceae*, *Paracoccus*, *Staphylococcus* e *Streptococcus* e maior abundância relativa de *Bacillus* e *Fusobacterium* em vacas primíparas do que multíparas (PASCOTTINI *et al.*, 2021). Além disso, atualmente sabe-se que durante o período gestacional o útero também possui uma microflora com uma carga bactérina não muito alta (KARSTRUP *et al.*, 2017; MOORE *et al.*, 2017), tendo como bactérias sempre isoladas *F. necrophorum*, *Porphyromonas levii* e *T. pyogenes*, localizadas no tecido endometrial ou no estroma caruncular, mas sua presença estava desassociada à inflamação, indicando uma possível tolerância aos microrganismos durante a prenhez (KARSTRUP *et al.*,

2017). Em estudos com novilhas virgens e vacas prenhes, bactérias associadas a doenças uterinas também se encontraram presentes, sendo elas: *Trueperella spp*., *Acinetobacter spp*., *Fusobacteria spp*., *Proteus spp*., *Prevotella spp.* e *Peptostreptococcus spp.* (MOORE *et al.*, 2017). Além disso, as alterações na diversidade bacteriana no microbioma do trato reprodutivo em vacas com doenças reprodutivas também indicaram uma associação entre disbiose e saúde reprodutiva bovina (ONG *et al.*, 2021; ADNANE e CHAPWANYA, 2022).

Os filos *Firmicutes*, *Bacteroidetes* e *Proteobacteria* já foram especificamente identificados na microbiota vaginal de bovinos da raça Nelore, Holandês e Fleckvieh (LAGUARDIA-NASCIMENTO *et al.*, 2015; NESENGANI *et al.*, 2017). Além disso, já foi observado uma relação entre a microbiota vaginal de bovinos Nelore e Gir, sendo encontrada principalmente as espécies bacterianas *Aeribacillus*, *Bacillus*, *Clostridium*, *Bacteroides* e Ruminococcus e para os gêneros fúngicos *Mycosphaerella, Cladosporium*, *Methanobrevibacter* e *Archaea* (GIANNATTASIO-FERRAZ *et al.*, 2019). Em outro estudo utilizando somente vacas Nelore, as análises da microbiota vaginal não observaram uma variação clara nas investigações em nível de filo (os mais abundantes foram *Firmicutes*, B*acteroidetes* e *Proteobacteria*) e gênero (especialmente *Aeribacillus*, *Bacteroides*, *Clostridium* e *Ruminoccocus*) entre novilhas e vacas nem entre animais prenhes e não prenhes. Além disso, a microbiota vaginal nestes animais aparentemente não apresentou uma influência hormonal, tanto na puberdade quanto na prenhez (LAGUARDIA-NASCIMENTO *et al.*, 2015). No entanto, em estudo com a microflora do trato reprodutivo bovino de vacas de corte foi observada uma diferença significativa moderada na estrutura da comunidade microbiana vaginal entre a fase lútea e a fase folicular, sendo que a quantidade de *Streptococcus spp*. na fase lútea foi significativamente maior do que na fase folicular, enquanto o número de *Lactobacillus spp*. na fase folicular foi significativamente maior do que na fase lútea (WANG *et al.*, 2019). Além disso, uma mudança na diversidade dessas das comunidades microbianas também contribui para uma adequada fertilidade em bovinos (AULT *et al.*, 2019; SERRANO *et al.*, 2019).

Observações levantadas por Appiah *et al.* (2020) e Dardawal *et al.* (2017) apontaram sobre as diferenças na composição microbiana pode ser devido aos métodos utilizados, aos efeitos da raça e genótipo e localização geográfica. Já foi relatado o efeito da fazenda sobre a diferença no microbioma uterino de vacas sadias e com doença uterina (GÄRTNER, *et al.*, 2015). Deste modo, os resultados destes relatos devem ser cuidadosamente interpretados para reproduzir informações errôneas sobre a verdadeira diversidade da microbiota do trato genital bovino.

2.1.3 Doenças uterinas no pós-parto

As patologias do útero no pós-parto possuem impacto deletérios à saúde reprodutiva, resultando em subfertilidade e infertilidade (SHELDON e OWENS, 2017; PFEIFER *et al.*, 2018; ANDRADE *et al.*, 2021). O aumento da infecção ou inflamação no corpo uterino e no oviduto aparentemente perturba o minucioso equilíbrio do sistema imunológico necessários para a fertilização (MAREY *et al.*, 2016; CHASTANT *et al.*, 2019). Além do mais, as respostas imune frente à infecção podem influenciar diversos processos reprodutivos tais como a dinâmica folicular ovariana e a esteroidogênese (SPICER e ALPIZAR, 1994; MAGATA *et al.*, 2014; SHELDON *et al.*, 2002), a ovulação (SUZUKI *et al.*, 2001; SHELDON *et al.*, 2002; LAVON *et al.*, 2008), a qualidade e competência oocitária, com aumento das taxas de parada meiótica e falha na quebra da vesícula germinativa (ROTH *et al.*, 2013; BROMFIELD e SHELDON, 2011; SHELDON e OWENS, 2017), inadequada função do corpo lúteo (CL) e de concentrações de progesterona (P4) (RIBEIRO *et al.*, 2016; CHASTANT *et al.*, 2019), a fertilização (RIBEIRO *et al.*, 2016; RIBEIRO e CARVALHO, 2017), o desenvolvimento embrionário e placentário (HILL e GILBERT, 2008; HANSEN *et al.*, 2004; LUCY *et al.*, 2016), a manutenção da gestação (EDELHOFF *et al.*, 2020; MOLINA-COTO *et al.*, 2020), interações materno embrionárias (GUO *et al.*, 2019), entre outros mecanismos reprodutivos.

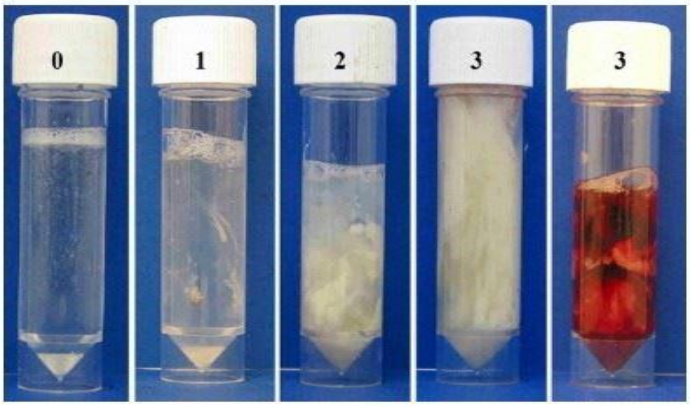
A retenção de placenta (RP) é conhecida pela deficiência da eliminação das membranas fetais após o nascimento do feto dentro de 24 horas após o parto, sendo visíveis na vulva ou identificadas na vagina ou útero (KELTON *et al.*, 1998; LEBLANC, 2008). Um período de 6 horas é requerido para o descolamento dos anexos fetais de modo fisiológico e considerado patológico quando superior a 12 horas após o parto (VAN WERVEN *et al.*, 1992). As causas associadas à RP incluem partos gemelares, distocia, auxílio ao parto, natimortalidade, parto induzido com prostaglandina $F_{2\alpha}$ ($PGF_{2\alpha}$) e glicocorticoides, aborto, hipocalcemia, aumento da idade, efeitos sazonais e manejo inadequado (SANDALS *et al.*, 1979; CORREA *et al.*, 1993; HORTA, 1994; HAN e KIN, 2005; GROHN e RAJALA-SCHULTZ, 2000; LEBLANC, 2008). O balanço energético negativo (BEN) pré e pós-parto interfere na resposta imune, no que pode resultar também em um fator para a RP (GOFF e HORST, 1997), mas também indica o fato de que o desenvolvimento da RP é multifatorial. O principal fator da patogênese da RP é da deficiência na ruptura imediata entre cotilédones e carúnculas após a expulsão do feto, mediada por uma falha do sistema imune em degradar de forma correta os placentomas (LEBLANC, 2008). Além do mais, a contratilidade uterina para efetuar expulsão da placenta desempenha

pouco ou nenhum papel no caso de RP (FRAZER, 2005). Com o decorrer dos dias após o parto, a placenta retida começa a autólise, tornando-se um meio favorável para possível infecção bacteriana, com isso, o tecido fica friável, com coloração amarelo-castanha e odor fétido (GRUNERT *et al.*, 2005). Como resultado, a RP não só aumenta o risco de doenças uterinas, como a metrite e endometrite, como também outras doenças metabólicas, como a cetose (LEBLANC, 2008).

A metrite puerperal é uma doença sistêmica aguda ocasionada pela infecção uterina que acomete todas as camadas do útero (endométrio, submucosa, muscular e serosa). A metrite ocorre dentro de 21 DPP; no entanto, sua versão mais grave ocorre nos primeiros 10 DPP e é designada como metrite puerperal aguda (SHELDON *et al.*, 2006). A metrite é caracterizada pelo aumento do volume uterino, secreção aquosa e coloração vermelho-amarronzado ou mucopurulenta, que muitas vezes tem um odor fétido (DRILLICH *et al.*, 2001; SHELDON *et al.*, 2006). A metrite puerperal pode ser classificada em três diferentes categorias pelos sinais de saúde. Na metrite grau 1 percebe-se atraso na involução uterina devido ao útero com tamanho aumentado, presença de descarga vaginal purulenta (DVP), porém, sem sintomatologia sistêmica. A metrite de grau 2 é caracterizada pelo aumento anormal do útero, DVP e por sinais adicionais de doença sistêmica, como diminuição da produção de leite, apatia, toxemia e febre >39,5°C. A metrite de grau 3 é a forma mais severa da metrite puerperal aguda, na qual a fêmea exibe um quadro de toxemia no qual observa-se decúbito, depressão, anorexia, desidratação grave, taquicardia, taquipneia, extremidades frias, sendo necessária uma intervenção veterinária rápida e intensivista (SHELDON *et al.*, 2006; SHELDON *et al.*, 2009).

A endometrite clínica é definida pela inflamação do endométrio caracterizada por uma descarga vaginal purulenta associada à infecção bacteriana uterina, detectável na vagina 21 dias ou mais após parto, ou corrimento mucopurulento detectável na vagina após 26 dias pós-parto e não está associada a alterações sistêmicas (SHELDON *et al.*, 2006; LEBLANC, 2008), e está frequentemente relacionada ao atraso tanto na involução uterina quanto na concepção (SHELDON e NOAKES, 1998). Além disso, um sistema de classificação fundamentado no caráter da descarga vaginal (Figura 2) é utilizado para avaliar vacas com endometrite clínica (WILLIAMS *et al.*, 2005). Na histologia, a endometrite é caracterizada por ruptura do epitélio endometrial, migração de células inflamatórias e acúmulo de linfócitos, congestão vascular e edema estromal (BONNETT *et al.*, 1991, BONDURANT, 1999; LEBLANC, 2008).

Figura 2 - Classificação de muco vaginal: pontuação 0 = muco claro ou translúcido; pontuação 1 = muco contendo manchas de pus branco ou esbranquiçado; pontuação 2 = descarga contendo ≤50% de exsudato mucopurulento branco ou esbranquiçado; e 3 = ≥50% de exsudato purulento, geralmente branco ou amarelo, mas ocasionalmente sanguíneo.



Fonte: (SHELDON *et al*., 2006).

A endometrite subclínica é caracterizada por uma proporção aumentada de células polimorfonucleares (PMN) no endométrio com ausência de sinais de endometrite clínica (GILBERT *et al.*, 2005). A utilização da citologia para diagnosticar endometrite subclínica em condições de campo não é prática, embora o uso da técnica de cytobrush possa ser mais simples do que a lavagem uterina (KASIMANICKAM *et al.*, 2005). Diversos estudos têm demonstrado um limiar para a proporção de células PMN (principalmente neutrófilos) para diagnosticar a endometrite, podendo variar entre pontos de corte de ≤4,75% de PMN (ANDRADE *et al.*, 2021) ou 5,5% das células (SANTOS *et al.*, 2009); além de >10% de PMN em 34 a 47 DPP ou >18% de PMN em 20 a 33 DPP (KASIMANICKAM *et al.*, 2004).

É importante ressaltar os efeitos negativos da endometrite clínica e da subclínica no desempenho reprodutivo de vacas de corte. A endometrite subclínica pode estar relacionada a deficiência na resolução da infecção bacteriana uterina e na regeneração tecidual do útero após o parto (SHELDON e OWENS, 2017). A probabilidade das vacas de corte acometidas pela endometrite clínica e subclínica se tornarem gestantes foi de aproximadamente 40% menor quando comparadas ao desempenho das vacas sadias (ANDRADE *et al.*, 2021). Desta forma, a eficiência reprodutiva de vacas de corte diagnosticadas com endometrite é afetada devido à baixa taxa de concepção ao primeiro serviço e aumento do intervalo parto-concepção, o que impacta diretamente na rentabilidade da bovinocultura de corte.

2.1.4 Imunologia do útero bovino

Durante o período de transição, a vaca passa por alterações endócrinas, metabólicas e imunológicas importantes para o parto e início da lactação (DRACKLEY, 1999). Essas alterações associadas à redução da ingestão de matéria seca (CMS) induzem ao estado de balanço energético negativo e imunossupressão (ROCHE *et al.*, 2009; LEBLANC, 2012). Desta forma, as vacas ficam mais suscetíveis a patógenos e predispostas a desenvolver infecções uterinas durante o período periparto (DADARWAL *et al.*, 2017).

Após o parto, o trato reprodutivo bovino possui uma gama de defesas para limpar os debris celulares da placenta e responder a contaminação microbiana. A abertura das barreiras anatômicas constituídas pela vulva, vagina e cérvix possibilita a invasão de bactérias do ambiente externo para o útero durante o parto. Para combater e eliminar a contaminação proveniente do parto, o animal depende de um eficiente mecanismo de defesa uterina. Desta forma, uma das primeiras respostas uterinas para eliminar a infecção pós-parto é o recrutamento intenso de PMN e outros leucócitos para o útero e, juntamente com as células epiteliais endometriais, formam os principais atores na defesa imunológica do útero (DAVIES *et al.*, 2008; SHELDON *et al*., 2009; LEBLANC *et al.*, 2011; LEBLANC, 2012 SHELDON *et al.*, 2006; DADARWAL *et al.*, 2017; SHELDON e OWENS, 2017).

A manutenção da integridade da barreira epitelial uterina é de extrema importância para o processo de involução uterina. Como parte do processo de involução do útero as carúnculas uterinas começam a sofrer necrose logo após o parto e, entre os 12 a 14 DPP, as vilosidades carunculares são descamadas por completo (WAGNER e HANSEL, 1969). A ausência da integridade da barreira epitelial que protege as carúnculas nas primeiras duas semanas após o parto contribui para o aparecimento de infecções e doenças uterinas. Deste modo, as bordas das regiões carunculares são as primeiras a apresentarem regeneração do epitélio uterino e, até os 30 DPP, o epitélio já se sobrepõe por completo cada carúncula (WAGNER e HANSEL, 1969).

As células epiteliais uterinas e uma extensa gama de células imunes possuem a capacidade de reconhecer moléculas associadas a patógenos (PAMPs) e os padrões moleculares associados a danos (DAMPs) por meio dos receptores de reconhecimento de padrões (PRRs). Já foram observadas mudanças no padrão de expressão de transcritos de genes para os receptores Toll-like nas células epiteliais do endométrio bovino (TLR; 1-7 e 9) e no estroma endometrial (TRL 1-4, 6,7 e 9) (DAVIES *et al.*, 2008). Os receptores TLR1, TLR2 e TLR6 reconhecem lipídios bacterianos, como ácido lipoteicóico (LTA), enquanto TLR3, TLR7, TLR8 e TLR9 reconhecem ácidos nucleicos, geralmente de vírus, embora TLR9 também

reconheça DNA bacteriano. O lipopolissacarídeo (LPS) de bactérias Gram-negativas como E. coli é ligado à proteína de ligação ao LPS e reconhecido por TLR4 em complexo com CD14 e LY96 (MD2). TLR5 liga-se à flagelina e TLR9 também reconhece DNA bacteriano (BEUTLHER, 2004; AKIRA *et al.*, 2006; DAVIES *et al.*, 2008; SHELDON *et al.*, 2009). As funções do TRL10 ainda não foram totalmente elucidadas, porém já se sabe de suas propriedades anti-inflamatórias (FORE *et al.*, 2020). Diversos outros PRRs também podem existir no epitélio do trato genital, como os receptores semelhantes ao domínio de oligomerização ligante de nucleotídeos (NLRs, do inglês NOD-like receptors), os receptores semelhantes ao gene I induzíveis por ácido retinóico (RIG-I) (RLRs) e os receptores de lectina tipo C (CLRs) também podem existir na região genital (TURNER *et al.*, 2012; OGUEJIOFOR *et al.*, 2015; DADARWAL *et al.*, 2017). A ativação de PRRs inicia cascatas de sinalização resultando na síntese e produção de citocinas e quimiocinas pró-inflamatórias, que mobilizam e ativam células imunes (BEUTLHER, 2004; AKIRA *et al.*, 2006; SHELDON *et al.*, 2009; TURNER *et al.*, 2012). Além disso, componentes do sistema complemento já foram observados no útero e muco vaginal após contaminação com *Tritrichomonas foetus* (KANIA *et al*., 2001)

Os peptídeos antimicrobianos (PAM ou AMPs, do inglês Antimicrobial peptides), são um componente do sistema imunológico e a família das β-defensinas são essencialmente importantes para a imunidade uterina. O epitélio celular bovino expressa peptídeo antimicrobiano lingual (LAP), peptídeo antimicrobiano traqueal (TAP), β-defensinas de neutrófilos bovinos (BNBD4, DEFB5) e β-defensinas bovinas (BBD19, BBD123 e BBD124) (CORMICAN *et al.*, 2008; DAVIES *et al.*, 2008). Estudo *in vitro* com células epiteliais endometriais tratadas com LPS observaram a expressão aumentada de LAP, TAP, BNBD4 e DEFB5 (DAVIES *et al.*, 2008). A mucina 1 (MUC1) é uma proteína transmembrana glicosilada de células epiteliais que também pode possuir função na defesa contra patógenos uterinos em mamíferos (BRAYMAN *et al.*, 2004), que possui expressão aumentada quando as células epiteliais foram tratadas com LPS (DAVIES *et al.*, 2008).

As células epiteliais do endométrio bovino podem atuar como células imunes sensores e efetores, bem como reguladores de células imunes por meio da secreção de citocinas. Além disso, é comum dos 17 a 24 DPP o recrutamento de neutrófilos e o aumento da secreção de citocinas em vacas sadias (GLABER *et al.*, 2010). A resposta imune inata às infecções causada por microrganismos é organizada por citocinas pró-inflamatórias e quimiocinas (Fator de necrose tumoral (TNF)α, Interleucina (IL)-1β, IL-6 e IL-8) (FOLEY *et al.*, 2012; FISCHER *et al.*, 2010; DADARWAL *et al.*, 2017). A IL-8 possui atividade quimiotática para alguns leucócitos, principalmente neutrófilos (MACKINTOSH *et al.*, 2013) e fator estimulador de

colônia de granulócitos-macrófagos (GM-CSF) é essencial para a maturação dos macrófagos (DE MORAES, *et al.*, 1999). A IL-6 secretada no endométrio bovino também podem exercer função de recrutamento e em etapas posteriores à involução uterina podem estimular a migração de monócitos ou macrófagos (HURST *et al.*, 2001; HEALY *et al.*, 2015). As citocinas de células epiteliais em diferentes mucosas, como IL-1, IL17 e IL-22, são capazes de modular a secreção de peptídeos antimicrobianos (KOLLS *et al.*, 2008). Por outro lado, a resolução da inflamação também pode ser suportada pela secreção de IL-10 e TGF-β (DADARWAL *et al.*, 2017).

Alguns relatos já observaram que vacas com doença uterina no pós-parto apresentaram aumento nas concentrações de neutrófilos com capacidade fagocitária reduzida em relação a vacas com útero sadio, podendo ir além da terceira semana pós-parto (MATEUS *et al.*, 2002; BRODZKI *et al.*, 2014). Macrófagos e monócitos também possuem capacidade fagocítica, de apresentação de antígenos (por meio do complexo principal de histocompatibilidade (MHC) II) e de regulação da inflamação uterina (BRODZKI *et al.*, 2014). O predomínio de macrófagos com fenótipo pró-inflamatório ativados por Interferon-gama (IFNγ) e LPS no início do processo de involução uterina tem por finalidade a fagocitose. Após a eliminação da contaminação bacteriana, citocinas IL-4 e IL-33 ativam macrófagos com fenótipo anti-inflamatório que ainda podem estar predominantemente presentes após os estágios da involução uterina e promover a regeneração tecidual (DADARWAL *et al.*, 2017). Além disso, macrófagos com fenótipo anti-inflamatório já foram observados durante a gestação de vacas que promovem funções de regulação inflamatória e reparo tecidual (OLIVEIRA *et al.*, 2010). Outro leucócito encontrado no trato genital bovino foram os mastócitos que apresentam o MHC II (EREN *et al.*, 2010). No início do pós-parto, estes mastócitos sofrem ativação e degranulação (liberação de histamina, bem como de citocinas IL-6, IL-8, TNFα e IFNγ) inicialmente por meio prostaglandinas, PAMPS e leucotrienos (THEOHARIDES *et al.*, 2012). O dano tecidual ocorrido no útero pós-parto responde com degranulação contendo histamina desencadeada por IgE e pela regulação positiva da expressão do gene do receptor de histamina 2 (WANG *et al.*, 2016)

A participação de linfócitos B no trato genital bovino são observados por meio do encontro de imunoglobulinas (Ig) que desempenham papel de opsonização bacteriana, promovendo a ação fagocítica dos macrófagos e neutrófilos. IgA, IgE e IgG já foram identificadas no útero (COBEIL *et al.*, 1974; BUTT *et al.*, 1993; CORBEIL *et al.*, 2005; WATSON *et al.*, 1990) e muco cervico-vaginal (COBEIL *et al.*, 1974; MURILA e SAUNDERS, 1994; DHALIWALY *et al.*, 1996; AGNEW *et al.*, 2008). Adicionalmente, nas regiões perivasculares do estroma uterino bovino foram observadas células T CD4+ (células T

auxiliares) e células T CD8+ (células T citotóxicas) que expressam receptores de células T αβ e γδT (TcR) (OLIVEIRA *et al.*, 2013). Células T γδTcR também são abundantes nos tecidos da mucosa bovina (OLIVEIRA *et al.*, 2013) e estão envolvidas na regulação da resposta imune devido expressarem citocinas pro-inflamatórias (IFNγ e TNFα) e anti-inflamatórias (IL-10 e TGF-β) (FOX *et al.*, 1998).

Fica evidente que a associação e comunicação dos leucócitos, células epiteliais e seus mecanismos imunológicos são essenciais para manter a integridade da barreira da mucosa do trato genital tubular bovino. Alterações nos mecanismos de defesa imune cooperam para a permanência do agente infeccioso e do processo inflamatório, favorecendo o estabelecimento de patologias e modificações no ambiente do trato reprodutivo da fêmea bovina.

2.1.5. Balanço energético negativo e a saúde reprodutiva

O estado nutricional é um importante fator que influencia a atividade reprodutiva da fêmea bovina. Nesse sentido, o periparto ou período de transição em vacas (principalmente as leiteiras) é comumente definido como as três semanas pré-parto e três semanas pós-parto, é um período crítico em que ocorrem alterações hormonais, metabólicas e físicas que levam à um desequilíbrio entre a demanda de energia para a produção de leite e a disponibilidade de energia adquirida na alimentação (DRACKLEY, 1999). As necessidades nutricionais do feto atingem altos níveis nas três semanas antes do parto; entretanto, e o consumo de matéria seca (CMS) diminui em 10-30% (BELL, 1995). No entanto, apesar desses eventos serem necessários para o desenvolvimento fetal e produção de leite, fazem com que a vaca entre em BEN (ou seja, o aporte nutricional da dieta é inferior às suas necessidades metabólicas) que é evidenciado principalmente pela perda de peso e queda no ECC logo após o parto (ESPOSITO *et al*., 2014). Em vista disso, o periparto representa um ponto crítico da criação das fêmeas bovinas e deve receber atenção especial para que o restabelecimento da atividade ovariana e de uma nova prenhez ocorra de maneira mais precoce o possível após o parto.

O aumento das demandas de glicose do útero gravídico durante o final da gestação e das demandas ainda maiores das glândulas mamárias em lactação exigem grandes ajustes na produção e utilização de glicose (BELL e BAUMAN, 1997). Estudo com vacas Nelore, não observaram efeito da categoria animal sob a glicemia no período periparto e pós-parto, no entanto, vacas nulíparas apresentaram um maior nível de glicose sanguínea também ao parto (FERREIRA et al., 2021). Entretanto, Janovick et al., (2011) relatou que a paridade afetou a

glicemia, na qual vacas primíparas apresentaram maior concentração de glicose no pré-parto comparado com vacas multíparas e, ao parto, todos os animais também apresentaram um pico glicêmico. Em vacas leiteiras a demanda de glicose pela glândula mamária para a síntese de lactose no início da lactação leva a uma queda drástica no nível sanguíneo de insulina circulante juntamente com uma maior sensibilização de agentes lipolíticos (BELL, 1995; BUTLER et al., 2003). A redução dos níveis de insulina causa o desacoplamento do eixo GH-IGF devido a uma regulação negativa da expressão do receptor do GH hepático (GHR), especialmente o GH 1A (BUTLER et al., 2003). Com isso, existe uma redução do fator de crescimento semelhante a insulina I (IGF-I, do inglês insulin-like growth factor I) circulante devido sua síntese ser em resposta a ativação do GHR pelo hormônio do crescimento (GH, do inglês growth hormone) (LUCY et al., 2001). Desse modo, a queda dos níveis de IGF-I eleva as concentrações circulantes de GH 1A (LUCY et al., 2001; BUTLER et al., 2003). Essa elevação das concentrações séricas de GH contribui para a produção de leite devido a estimulação da lipólise e aumento da disponibilidade de glicose para a síntese de leite pela glândula mamária (BELL, 1995). Em vacas de corte também já foi observada a redução do nível sérico de IGF-I após o parto (SPICER et al., 2002). Além disso, Schneider et al., (2010) observaram que as alterações nas concentrações de IGF-I durante o período pós-parto não estavam associadas as mudanças na expressão hepática de transcritos para GHR, GHR 1A e IGF-I em vacas de corte. Adicionalmente, devido as baixas concentrações de IGF-I circulante, não somente a resposta pró-inflamatória modulada por esse hormônio é diminuída, mas também está relacionada ao retorno da primeira ovulação após o parto (KAWASHIMA et al., 2007; O'CONNOR et al., 2008) e a sobrevivência embrionária (VELAZQUEZ et al., 2008). Além disso, o IGF-I atua em diversas etapas da foliculogênese (MAGALHÃES et al., 2012).

A leptina é outro hormônio metabólico sintetizado principalmente em adipócitos de importante influência ao BEN, pois exerce influência no consumo voluntário de alimentos e também pode contribuir para a resistência à insulina (RI) em ruminantes no periparto (BLACHE et al., 2000; INGVARTSEN e BOISCLAIR, 2001). A baixa concentração plasmática de leptina no final da gestação está correlacionada com o ECC e podem permanecer baixas no pós-parto mesmo com a melhora do balanço energético (WATHES et al., 2007).

No período final da gestação e início da lactação as necessidades nutricionais são maiores e se intensificam. A mobilização de tecido adiposo das reservas corporais provoca a liberação de ácidos graxos de cadeia longa, principalmente os ácidos graxos não esterificados (NEFA, do ingles, *non-esterified fatty acids*), e o acúmulo destes no fígado (SMITH *et al*., 1997). Desta forma, a concentração sanguínea de NEFA demostra a intensidade da mobilização

do tecido adiposo (PULLEN *et al*., 1989). No fígado, os NEFA podem ser oxidados em: a) dióxido de carbono, para fornecer energia para o fígado, b) parcialmente oxidados para produzir corpos cetônicos ou acetato que são transportados para uso em outras partes do corpo e c) esterificados em triacilgliceróis que se acumulam no fígado, atingindo o pico de concentração em 7-13 dias após o parto e diminuindo gradualmente (BAUMAN e CURRIE, 1980; GRUMMER, 1995; WATHES *et al*., 2007). O β-hidroxibutirato (βHB) é um dos corpos cetônico de maior concentração no sangue e é utilizado como um indicador de oxidação de ácidos graxos (WATHES *et al*., 2007). Estudos anteriores em vacas de leite já mostraram que maiores concentrações de βHB ocorrem na primeira semana pós-parto (DOEPEL *et al*., 2002, BERTONI *et al*., 2008, LIEN *et al*., 2010, CINCOVIC *et al*., 2012) e também encontraram um aumento de βHB no dia seguinte ao parto, mas seu maior valor foi observado no décimo dia pós-parto (VAZQUEZ-AÑON *et al*., 1994). LeBlanc *et al*. (2005) e Shin *et al*. (2015) observaram aumento do βHB uma semana antes do parto, refletindo a mobilização precoce das reservas corporais. Embora este estudo mostre que o βHB não é alterado pela categoria animal, estudos anteriores mostram que a má adaptação das vacas ao BEN e altas concentrações de βHB estão associadas a um declínio na função das células PMN (KEHRLI *et al*., 1989; GILBERT *et al*., 1993; HAMMON *et al*., 2006) com possíveis implicações para infecções sistêmicas pós-parto (KLUCINSKI *et al*., 1988). Andrade et al., (2021) observaram que não houve diferença na concentração média de βHB entre vacas de corte multíparas sadias e com DVP+ e endometrite subclínica. Ainda, em vacas de corte multíparas a concentração de βHB aos 45 e 60 DPP não diferitam de vacas com 20 e 30 DPP (PFEIFER *et al.*, 2018). Adicionalmente, estudos relataram que a exposição de PMNs a níveis elevados de βHB reduziu a liberação de espécies reativas de oxigênio dos PMN (HOEBEN *et al.*,1997) e capacidade de quimiotaxia é prejudicada (HOLTENIUS *et al*., 2004) e concluíram que o βHB pode, em parte, ser responsável por maior suscetibilidade a infecções locais e sistêmicas durante o período pós-parto e indicativas de melhor desempenho reprodutivo em vacas de corte (MULLINIKS *et al*., 2013).

Como já demonstrado acima, atualmente sustenta-se uma ideia crescente de que a inflamação não é apenas uma reação à lesão ou doença, mas também pode ser um efeito da doença metabólica. Postula-se que as demandas metabólicas associadas ao período final da gestação, parto e início da lactação aumentam a produção de espécies reativas de oxigênio (ROS) pelos leucócitos (SORDILLO, 2005). No período de transição, os níveis circulantes de IgG e IgM diminuem (HERR *et al.*, 2011), a função dos leucócitos é prejudicada (HOEBEN *et al.*, 2000) e há neutrofilia e eosinopenia (MEGLIA *et al.*, 2005). Além disso, vacas não

saudáveis têm menor atividade de paraoxanase (PON) (BIONAZ *et al.*, 2007). Isso pode ser devido à mobilização de gordura e deposição de triglicerídeos nas células do fígado, que causam danos e disfunção hepática (TURK *et al.*, 2004), redução do colesterol total no sangue e lipoproteínas de alta densidade (HDL) (TURK *et al.*, 2005) para qual a PON está ligada no sangue, associada a um aumento do estresse oxidativo (TURK *et al.*, 2004; TURK *et al.*, 2005) ou uma combinação destes. Genes imunes inflamatórios são regulados positivamente (WATHES *et al.*, 2009), enquanto genes envolvidos nas respostas imunes adquiridas são regulados negativamente em vacas com BEN grave (MOYES *et al.*, 2010). As citocinas pró-inflamatórias IL-1β, IL-6 e TNFα estimulam o fígado a produzir proteínas de fase aguda, incluindo haptoglobina, amiloide A sérica e paraoxonase (BANKS *et al.*, 1995; FEINGOLD *et al.*, 1998; CECILIANI *et al.*, 2012). Além disso, a mobilização do tecido adiposo libera glicerol e citocinas pro-inflamatórias (TNFα, IL-6 e MCP-1 (do inglês, *monocyte chemoatractant protein*)) que interrompe a sinalização intracelular da insulina, contribuindo para a RI, resultando em uma liberação exacerbada NEFA do tecido adiposo (TILG e MOSCHEN, 2008; SHI *et al.*, 2019; KOSTER e OPSOMER, 2013). O NEFA contribui diretamente para uma maior inflamação, pois liga-se e ativa TLR4, um receptor principal para LPS, e ativa NFkB (do inglês, *fator nuclear kappa B*) e levando à síntese de TNFα e IL-1 e IL-8. O aumento das concentrações circulantes de NEFA pode ativar o inflamassoma, resultando na produção de IL-1β e diminuição da tolerância à glicose e sensibilidade à insulina (OSBORN e OLEFSKY, 2012) e pode prejudicar as células β pancreáticas, levando à diminuição da secreção de insulina (KAHN *et al.*, 2006). Concentrações séricas elevadas de NEFA alteram a capacidade de proliferação dos linfócitos ou de secretar IgM e IFN em resposta à estimulação policlonal (LACETERA *et al.*, 2004), e explosão oxidativa e viabilidade de PMNs (SCALIA *et al.*, 2004). Alguns estudos indicam que os NEFAs plasmáticos possam ser marcadores indicativos de imunidade periparturiente prejudicada e aumento do risco de infecções (MOYES *et al.*, 2009; ESPOSITO *et al.*, 2014).

Para o retorno da atividade cíclica ovariana, a glicose, insulina NEFA e IGF-1 enviam sinais para induzir a secreção de LH, que parece ser um fator determinante na progressão para a primeira ovulação pós-parto (LEBLANC, 2012). À medida que vacas de corte com anestro anovulatório prolongado estão próximas da primeira ovulação após o parto, aumenta frequência de pulso de LH (STAGG *et al.*, 1998; CROWE, 2008). Além disso, o aumento das concentrações de IGF-I associada a uma diminuição linear nas concentrações de GH já foi observada antes da primeira ovulação (STAGG *et al.*, 1998). Portanto, vacas de corte pós-parto necessitam de secreção suficiente de estradiol para induzir um pico de LH que geralmente sofre

influência da amamentação e do estado nutricional, além do aumento das concentrações de IGF-I para colaborar na maturação e o crescimento do folículo dominante (STAGG *et al.*, 1998; CROWE, 2008). A infecção e inflamação que acometem o trato reprodutivo, principalmente o útero, contribuem negativamente para a função dos folículos dominantes iniciais, a probabilidade de ovulação e a luteólise precoce com função e tempo de vida do CL curta (SHELDON *et al.*, 2002; WILLIAMS *et al.*, 2007).

Desta forma, acredita-se que a inflamação no trato reprodutivo pode ser acentuada pela inflamação sistêmica e, inversamente, como também pode colaborar para o aumento da inflamação sistêmica em vacas no período de transição. Além disso, a resposta imunológica e reprodutiva no periparto pode estar relacionada com a extensão das alterações metabólicas e hormonais.

2.2 RECEPTIVIDADE UTERINA

Nos últimos anos, pesquisas se concentraram nos componentes celulares e moleculares da inflamação e receptividade uterina pós-parto e progrediram muito na compreensão no entendimento das doenças uterinas e fertilidade em vacas. Para isso, o uso de tecnologias de sequenciamento de alto rendimento forneceram pistas sobre as tendências globais na expressão gênica que podem oferecer uma abordagem prognóstica para rastrear e selecionar animais receptores com características endometriais que favorecem a receptividade (BINELLI et al., 2015). Além disso, a utilização da citologia uterina (usando lavagem uterina de baixo volume ou a técnica de *cytobrush*) permite diferenciar casos de endometrite clínica e subclínica, bem como identificar a ocorrência de doença uterina verdadeira, (ANDRADE *et al.*, 2021) de condições que podem ou não envolver o útero, como cervicite ou descarga vaginal purulenta. Adicionalmente, a amostragem repetida do endométrio bovino usando a técnica do *cytobrush* é segura e fornece amostras representativas para o estudo de expressão gênica do endométrio superficial *in vivo*, com a grande vantagem de não interferir na fertilidade imediata (CARDOSO *et al.*, 2017).

O tecido endometrial uterino é um tecido complexo, composto por células epiteliais luminais, células epiteliais superficiais e glandulares profundas, bem como células estromais semelhantes a fibroblastos. Esses diferentes tipos celulares desempenham papéis fundamentais na condução do processo de alongamento do concepto, via secreções endometriais (FORDE e LONERGAN, 2012). Além disso, alterações espaciais e temporais no transcriptoma endometrial são necessárias para estabelecer a receptividade uterina à implantação (SPENCER

e BAZER, 1995). Desta forma, durante cada ciclo estral, o útero se reorganiza para atender as demandas do concepto em desenvolvimento. Nesse sentido, a concentração de estradiol (E2) no proestro é importante para a modulação do útero para a fase lutea subsequente (MESQUITA *et al*., 2014; MESQUITA *et al*., 2015; MANN e LAMMING, 2000; BRIDGES *et al*., 2012). Esta influência do E2 no endométrio é importante para a indução de receptores endometriais P4 (evitando a luteólise prematura) (LAMMING e MANN, 1995; ROBINSON *et al*., 2001), sendo a P4 o principal hormônio responsável pela preparação do endométrio para implantação embrionária e manutenção da gestação (BAZER *et al*., 2008). Essas mudanças são críticas para a probabilidade de ocorrência de uma gestação bem-sucedida em bovinos, pois, acredita-se que grande parte da mortalidade ocorre por falhas durante o período de reconhecimento materno da gestação.

Em ruminantes, a principal proteína que regula o reconhecimento materno durante o início da gestação é o interferon tau (IFN-τ) (SPENCER *et al*., 2007). Aos dias 15 a 17 após estro, a luteólise deve ser prevenida pela liberação de IFN-τ pelo concepto (BINELLI et al., 2001). A produção insuficiente de IFN-τ pelo concepto causa perda embrionária devido à incapacidade de inibir a luteólise (MANN e LAMMING, 2001). O bloqueio da luteólise ocorre pela inibição da expressão dos receptores de estradiol (ESR1) e do receptor de ocitocina (OXTR) no endométrio, inibindo os pulsos endometriais de PGF2α (SPENCER *et al*., 1995; ROCHA et al., 2021). Considera-se que a capacidade do concepto em produzir suficiente IFN-τ depende de estímulos uterinos (BINELLI *et al*., 2017), pois nos primeiros 7 dias após estro, a P4 regula positivamente a expressão de alguns genes relacionados com a produção de histotrofo e metabolismo energético (FOLEY *et al*., 2009), favorecendo o desenvolvimento embrionário (GARRETT *et al*., 1988). Sponchiado *et al*. (2017) observaram em vacas Nelore prenhes a ocorrência de uma interação entre o embrião e endométrio no dia 7 após IA, com mudanças na expressão de genes em localizações específicas (regiões mais craniais do corno uterino ipsilateral ao CL, onde o embrião estava localizado) no endométrio que é de grande importância para o desenvolvimento e sobrevivência do embrião. Doze genes foram expressos de forma diferente entre vacas prenhes e não prenhes, sendo eles: genes clássicos estimulados por IFN-τ (ISG15, MX1, MX2 e OAS1Y), genes de biossíntese de prostaglandinas (PTGES, HPGD e AKR1C4), transportador de água (AQP4) e um transportador de soluto (SLC1A4) (SPONCHIADO *et al*. 2017).

Embora o caminho desde a concepção até uma gestação saudável englobe uma infinidade de processos celulares que ocorrem desde os primeiros eventos que precedem a fertilização, aceita-se que o resultado geral da fertilidade está sob grande influência do ambiente

uterino (MESQUITA *et al*., 2015). Impactos na fertilidade podem ocorrer por meio de modificações da expressão dos receptores de E2 e P4 (ESR e PGR, respectivamente) no útero e oviduto que alteram funções moduladas por estes esteroides, como a produção de secreções (BAZER e SLAYDEN, 2008; BAZER *et al*., 2009; GONELLA-DIAZZA, *et al*., 2015; BINELLI *et al*., 2017). Desta forma, as inadequações no meio periovulatório dos receptores e concentração de esteroides sexuais levam a diferentes perfis de expressão gênica do oviduto e do útero, alterar as funções ovidutais e endometriais e afetar a sobrevivência e o desenvolvimento do embrião e o estabelecimento da gestação na fêmea bovina (OKUMU *et al*., 2010; GONELLA-DIAZZA *et al*., 2015). Animais com folículos maiores e apresentando um CL subsequente maior apresentaram um maior enriquecimento na expressão de genes relacionados as vias de remodelação da MEC (GONELLA-DIAZZA *et al*., 2015).

A receptividade endometrial já foi associada a um aumento nas vias de remodelação da matriz extracelular (MEC) (BAUERSACHS *et al*., 2006; FOLEY *et al*., 2012; GONELLA-DIAZZA *et al*., 2015; SÁ FILHO *et al*., 2017). Vacas de corte aos 30 DPP apresentam enriquecimento das vias de remodelação do MEC, bem como uma via associada ao câncer que se destaca pela capacidade proliferativa do endométrio (FOLEY *et al*., 2012). Essas vias estão associadas à regeneração do tecido danificado e se assemelham aos de tumores altamente malignos devido à regulação positiva de genes envolvidos na proliferação celular em ambos os casos (CHANG *et al*., 2004). Esta mudança temporal de transcritos no tecido endometrial caracterizada por resposta imune pró-inflamatória para um perfil regenerativo do tecido demonstra uma indução da resposta imune imediata, no entanto, transitória com o intuito de eliminar patógenos e reduzir a inflamação associada dentro de um período de 2 semanas em vacas sadias (FOLEY *et al*., 2012).

Uma grande parte de assinaturas transcriptômicas relacionadas à implantação e interações maternas embrionárias são significativamente afetadas pelo LPS (GUO *et al*., 2019) e que muitos transcritos importantes relacionados ao sistema imunológico e vias de sinalização em células estromais endometriais bovinas são induzidos por LPS (DING *et al*., 2020). Desvios na elevação de citocinas pró-inflamatórias no ambiente uterino durante o período de pré-implantação podem danificar as células endometriais, perturbar as interações maternas embrionárias e desencadear danos no DNA das células embrionárias (GUO *et al*., 2019) e resultar em falhas de implantação (JAISWAL *et al*., 2000). Embora o sistema imunológico do trato reprodutivo esteja geralmente associado à resposta a doenças uterinas no pós-parto, o sistema imunológico desempenha um importante papel nas funções reprodutivas normais e o desenvolvimento e manutenção da prenhez (POOLE *et al*., 2021). As células se comunicam por

meio de citocinas presentes no trato reprodutivo para então regular o ambiente imune local e estimular a resposta adequada ao feto semi-alogênico antes e durante a prenhez por meio da limitação dos processos inflamatórios para conduzir uma resposta do endométrio materno para a tolerância imunológica (OTT, 2019). Em bovinos, o trato reprodutivo passa por resolução do processo inflamatório por meio da diminuição na abundância de transcritos de citocinas pró-inflamatórias, como interleucina IL-1β e IL-6, e um aumento de citocinas regulatórias como TGF-β, IL-10, IL-27, G-LCR e da remodelação uterina, como LIF, IL-6, IL-17 (OLIVEIRA *et al*., 2013; ROBERTSON e MOLDENHAUER, 2014; HEPPELMANN *et al*., 2015). Além disso, em bovinos, as citocinas anti-inflamatórias IL-10 e TGF-β aumentam durante o início da gestação e placentação, respectivamente (OLIVEIRA e HANSEN, 2008; OLIVEIRA e HANSEN, 2009; VASUDEVAN *et al*., 2017).

Em estudo utilizando análise transcriptômica de tecido endometrial (e que nos orientou pela busca de primers a serem utilizados para avaliar o reparo e receptividade endometrial das vacas utilizadas no capítulo 2 deste estudo) de vacas de corte em anestro suplementadas com eCG foram observadas uma maior expressão de transcritos para ESR2, HB-EGF, ITGB3 e diminuição da expressão de ESR1, sugerindo o estabelecimento de uma fase de transição, de proliferativa para secretora. Além disso, vacas que receberam tratamento com cipionato de estradiol (ECP) apresentaram uma maior abundância de genes associados à proliferação PTCH2 e COL4A1, enquanto a expressão de EGFR foi regulada negativamente (SÁ FILHO *et al*., 2017). O PTCH2 é um receptor transmembrana da família de genes patched, sendo componente da via de sinalização Hedgehog, funcionando como uma proteína para supressão tumoral (TOFTGÅRD, 2000). COL4A1 (cadeia alfa 1 do colágeno tipo 4) é um componente das membranas basais (KÜHN, 1995). No endométrio, sugere-se que COL4A1 está associado remodelação do útero durante a prenhez para implantação do concepto e placentação (BIASE *et al*., 2016; NEUPANE *et al*., 2017). A família do fator de crescimento epidérmico (EGF, sendo o HB-EGF pertencente à essa família) estimula o crescimento celular, proliferação e diferenciação em vários tecidos por ligação ao seu receptor EGFR (ou ErbB/HER) (TAKATSU *et al*., 2018). Em estudos utilizando tecido endometrial para ensaios imuno-histoquímicos demonstrou que a expressão de EGFR na fase folicular e lútea em células epiteliais glandulares luminais e superficiais, apresentaram maior imunorreações para EGFR do que em células epiteliais glandulares profundo e conjuntivo e estroma (SAĞSÖZ *et al*., 2012). Além disso, foi demonstrado que HB-EGF também é expresso no endométrio uterino (YOO *et al*., 1997; TAKATSU e ACOSTA, 2015) e participa de diversos processos fisiológicos, como a sinalização molecular entre embriões e útero no processo de implantação (XIE *et al*., 2007).

Desta forma, o tecido endometrial receptivo vem sendo caracterizado pela expressão de genes responsáveis pela resposta do endométrio ao embrião até a ocorrência da sua implantação. A avaliação dos padrões de expressão gênica durante o período puerperal permite o desenvolvimento marcadores moleculares associados com a receptividade endometrial, bem como revelar informações importantes sobre a resposta imune associadas ao processo de reparação da mucosa uterina no pós-parto para determinar tomadas de decisões em rebanhos de bovinos e outros ruminantes.

CAPÍTULO I

Saúde uterina e fertilidade de vacas de corte Nelore pós-parto com inseminação artificial em tempo fixo criadas no bioma Amazônia

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Uterine health and fertility of timed AI postpartum Nelore beef cows

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Abstract

This study evaluated the effect of the uterine health on the fertility of postpartum beef cows. Multiparous lactating Nelore cows (*Bos indicus*; n = 155) were submitted to TAI protocols. Cows were given 2 mg of estradiol benzoate i.m. and received an intravaginal progesterone-releasing device (CIDR®) to synchronize follicular wave emergence on Day 0. The CIDR was removed and cows were given 150 μg of d-Cloprostenol i.m. (Croniben®), 1 mg of ECP im (E.C.P.®), and 300 IU of eCG (Novormon®) i.m. on Day 8. All cows were TAI 48 h after CIDR removal. At Day 0, before the beginning of the protocol, cows were subjected to evaluation of purulent vaginal discharge (PVD) and collection of uterine tissue using the cytobrush technique and stored in a microtube with 1 mL of RNAlater®. Slides for polymorphonuclear (PMN) cells counting were prepared before the same cytobrush was used for RNA extraction and analysis of IL-1β, IL-6, IL-8, TNFα, GAPDH, and βactin gene expression. Cows were separated into 4 categories according to the proportion of PMN and PVD: 1) Healthy (HE; n = 87), cows with ≤ 4.75% PMN and PVD of 0; 2) Positive Purulent Vaginal Discharge (PVD+; n = 24), cows with ≤ 4.75% PMN and PVD ≥ 1; 3) Subclinical Endometritis (SCE; n = 29), cows with > 4.75% PMN and PVD of 0, and, 4) Subclinical Endometritis + PVD+ (SCEP; n = 15), cows with > 4.75% PMN and PVD ≥ 1. Cows in HE had a greater (P<0.05) pregnancy per AI (P/IA) than cows in SCE and SCEP groups. However, P/AI in cows the PVD+ was not different in comparison to the HE and SCE groups (P > 0.05). Cows from the SCE and SCEP group had a higher (P< 0.001) proportion of PMN cells in the uterus than the HE and PVD+ groups. Relative expression of IL-6 and TNFα did not change among groups (P > 0.05). In contrast, expression of IL-8 was greater (P = 0.02) in SCEP than the HE and SCE. The relative expression IL-1β tended to be greater (P = 0.08) in SCEP than in PVD+, but the cows in the HE and SCE groups were not different from the other groups. Our results demonstrate that postpartum beef cows

with a higher proportion of PMN cells have lower fertility and higher expression of pro-inflammatory cytokines IL-1β and IL-8 in the uterus.

Keywords: calving, cytobrush, puerperium, cytokines and PMN cells.

1. Introduction

In the puerperium, the bovine uterus undergoes involution - comprised of the process involving reduction of the size of the uterus, uterine muscle contractions, regeneration and reestablishment of the role of the endometrial tissue. Involution ensures that the uterus returns to a state of physiological function, becoming receptive to a new conceptus (Gier and Marion, 1968; Foley et al., 2012). However, at calving, the physical barriers constituted by the cervix, vagina and vulva are compromised, allowing the invasion of bacteria from the external environment to the genital tract, as well as from skin and feces (Elliot et al., 1968, Gier and Marion, 1968; Sheldon and Dobson, 2004). Modifications in the local defense mechanisms of the uterus and the consequent persistence of pathogens, mainly associated with *Trueperella pyogenes*, *Escherichia coli*, *Fusobacterium necrophorum e Prevotella melaninogenicus* (Bicalho et al., 2016; Williams et al., 2005), can result in uterine infections imparing fertility and reducing herd performance (Sheldon et al., 2009).

In the uterus of healthy cows, the resolution of the inflammatory response is identified through the reduction of polymorphonuclear cells (PMN, mainly neutrophils) as uterine involution occurs (Chapwanya et al., 2012; Hart, 2002). In cows with delayed uterine involution, depending on the proportion of PMN in the uterine lumen, it is possible to determine the severity of uterine inflammation and classify the condition as: clinical endometritis, with purulent and mucopurulent exudate; and subclinical endometritis, without uterine discharge (Barlund et al., 2008). In addition, associated with the infiltration of PMN cells into the uterus of dairy and beef cows during the puerperium, numerous pro-inflammatory cytokines, including interleukin (IL) -1β, IL-6 and IL-8 and tumor necrosis factor-α (TNF-α) are synthesized (Galvão et al., 2011; Ghasemi et al., 2012; Pfeifer et al., 2018), resulting in a significant reduction in reproductive performance (Kasimanickam et al., 2004; Ricci et al., 2017; Sheldon et al., 2009).

Still, in countries with tropical climates, there is a lack of information about the effect of uterine inflammatory status in postpartum beef cows (Pfeifer et al., 2018).

Considering the need for intensive breeding in tropical cattle production systems, the fixed-time artificial insemination (FTAI) protocols based on estradiol and progesterone allow the farmers to inseminate cows early in the post-partum period (Baruselli et al., 2012). Considering that the pregnancy length of zebu cows is 290 days, to produce one calf per year, the interval between parturition and the next conception must not exceed 75 days (Rossa et al., 2009). *B. indicus* suckled cows kept under tropical conditions can take long periods to re-establish their cyclicity, resulting in extended calving-conception interval (Ruiz-Cortez and Olivera-Angel, 1999). This can be due a high incidence of postpartum anestrous, which negatively affects reproductive performance. In this scenario, the use of estradiol-progesterone TAI based protocols can be beneficial in anestrous *B. indicus* cows after calving (Baruselli et al., 2004). In a attempted to dramatically reduce the the calving-conception interval in anestrus *B. indicus* cows, differently from conventional TAI protocols, which start 50-70 DPP, farmers have been including cows in TAI programs as early as 20 days after calving in tropical beef operations (Carvalho et al., 2020). Therefore, establishing a correct diagnosis of the uterine condition of postpartum beef cows can potentially select cows that are more likely to become pregnant early in the breeding season and the ones that need a greater interval from parturition before they enter in a TAI program.

Based on these considerations, the objective of the present study was to evaluate the relationship between uterine health and fertility in Nelore postpartum beef cows. The hypothesis tested was that early postpartum beef cows subjected to TAI protocols with a higher proportion of polymorphonuclear cells and vaginal discharge have greater proinflammatory cytokines genes expression in the endometrial tissue and lower pregnancy per AI (P/AI).

2. Materials and methods

The Committee for Ethics in Animal Experimentation from Embrapa approved all of the procedures performed in the experiment described in this manuscript (Protocol 03/2017).

2.1. Animals, hormonal treatments and sampling

Multiparous lactating Nelore cows (*Bos indicus*; n = 155), 4 to 10 years old, from two comercial beef farms in Rondônia – Brazil, were used in this study. All cows were maintained on *Brachiaria brizantha* pasture and given mineralized-salt and free access to water. The TAI protocols were initiated between 20 and 60 days postpartum (DPP). Cows were given 2 mg of estradiol benzoate (Bioestrogen®, Biogénesis-Bagó, Curitiba, Brazil) intramuscular (i.m.) and received an intravaginal progesterone-releasing device (1.9 g progesterone, CIDR®, Pfizer Animal Health, São Paulo, Brazil) to synchronize follicular wave emergence on Day 0. The CIDR was removed and cows were given 150 μg D-Cloprostenol i.m. (PGF2α-analogue; Croniben® , Biogénesis-Bagó, Curitiba, Brazil), 1 mg of ECP im (E.C.P.®, Pfizer, Cravinhos, Brazil), and 300 IU of eCG (Novormon®, Syntex, Buenos Aires, Argentina) i.m. on Day 8. All cows were TAI 48 h after CIDR removal. Semen from two Nellore bulls at each farm was used for TAI and was equally distributed among treatments. The AI procedures were performed by one experienced technician at each farm. Serum β-hydroxybutyrate (BHB) concentrations were measured on Day 0 with a hand meter device (TD - 4235®; Ketovet, MG, Brazil).

2.2. Experimental design, groups and uterine health evaluation

The cows were examined by vaginoscopy to evaluate and characterize the presence of vaginal discharge (VD) (Pleticha et al., 2009) on Day 0 of the TAI protocol. For vaginoscopic examination, the vulva was cleaned with a paper towel, and a sanitized vaginal speculum was introduced into the vaginal canal. Using a light source, the cervix and the vaginal canal were inspected, and the presence of secretion was classified and registered. Vaginal discharge was graded on a scale from 0 to 3 (0 = mucus, 1 = mucus with flecks of pus, 2 = ≥50% purulent exudate, 3 = hemorrhagic and/or purulent exudate), as adapted from others (Williams et al., 2005; Sheldon et al., 2006). Cows with positive purulent vaginal discharge (PVD+) were defined as cows having a vaginal discharge score of ≥ 1.

The same subset of cows from vaginoscopy were selected to collection of samples from endometrium. For these purposes, an adapted cytological brush was coupled to the tip of a conventional AI equipment, covered by a disposable AI sheath and protected by a sanitary sheath, as described elsewhere (Cardoso et al., 2017). The apparatus was inserted via cervix and the cytobrush was rotated to collect cells from uterine body. Immediately after the sampling, the cytobrush was uncoupled from the apparatus and was gently rolled onto a clean microscope slide using only half of its entire circumference, to ensure that a suitable quantity of cellular material remained on the untouched surface for gene expression analysis. Slides were air-dried and packaged for transport to the laboratory. The cytobrush was then placed in a 2-mL cryotube filled with 1 mL of RNA later buffer and stored at −80 °C for later processing. In the laboratory, the slides were stained using a commercial kit (Quick Panoptic®, Laborclin, Pinhais, Brazil). Two hundred cells per slide were counted in an optical microscope (100 X magnification), including PMN, mononuclear and epithelial cells to calculate the proportion of PMN cells.

The cows were categorized in four groups according to the PMN cells proportions and vaginal discharge, as follows: 1) Healthy (HE; n = 87), cows with ≤ 4.75% PMN and PVD of

0; 2) Positive Purulent Vaginal Discharge (PVD+; n = 24), cows with ≤ 4.75% PMN and PVD ≥ 1; 3) Subclinical Endometritis (SCE; n = 29), cows with > 4.75% PMN and PVD of 0, and; 4) Subclinical Endometritis + PVD+ (SCEP; n = 15), cows with > 4.75% PMN and PVD ≥ 1. The Venn diagram shows how animals were distributed among groups (Figure 1).

2.3. Ultrasound examinations

Ultrasonic examination (SIUI CTS-900, linear probe with 5 MHZ, Guangdong, China) was performed 30 d post-TAI to assess pregnancy status. Visualization of the embryonic vesicle and detection of the embryo were the positive criteria for determining pregnancy.

2.4. RNA isolation and cDNA synthesis

Total RNA was extracted from 70 cytobrush samples (42, 15, 7 and 6 samples from HE, PVD+, SCE, SCEP, respectively). Cells in tubes were pelleted with centrifugation at 2000 x g at 4 °C for 10 min. Supernatant (600 μL) was discarded and 600 μL of 1% β-mercaptoethanol was added before total RNA was extracted according the commercial kit protocol (PureLink® RNA Mini Kit, Thermo Fisher Scientific, Carlsbad, CA). Purity and quantity of RNA was assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The RNA was treated with DNase I (Invitrogen, Thermo Fisher Scientific, USA) before reverse transcription to remove possible genomic DNA contamination.

For cDNA synthesis, the Anchored Oligo primer (dT) 23 (Sigma-Aldrich, USA) was incubated with the treated total RNA at 70 °C for 10 min, as described by the manufacturer. Then the reverse transcription (RT) reaction was performed using the HighCapacity cDNA kit (Applied Biosystems, USA) according to the protocol described by the manufacturer, in a final

volume of 20 μL/ reaction, using the following conditions: 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 min. The synthesized cDNA was subsequently quantified by spectrophotometry (NanoDrop 2000 spectrophotometer; Thermo Fisher Scientific, USA) to assess the RT reaction efficiency, and was stored at −80 °C until quantitation assessments were conducted.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to assess relative abundance of cytokine mRNAs in the cytobrush samples and was performed by an operator blinded to the cytology results. The Perfecta SsoFast EvaGreen® Supermix™ Kit (Bio-Rad Laboratories Inc., USA) was used according to the manufacturer's instructions to perform qRT-PCR analysis of relative abundance of IL-1β, IL-6, IL-8, TNFα, GAPDH, and βactin mRNA. Briefly, 10 μL of Perfecta SsoFast EvaGreen® master mix for iQ (2X), 1 μL of the appropriate primer set (Table 1) at 10 μM, 250 ng/μL of cDNA template, and 4 μL of water were added for a final reaction volume of 20 μL. The reaction was performed in the Bio-Rad CFX96™ detection system (Bio-Rad Laboratories Inc., Singapore) using the following program: 1 cycle at 95 °C for 5 s, 39 cycles of 95 °C for 5 s, 60 °C for 20 s, and a melting curve standardized from a variable annealing temperature ramp from 65 to 95 °C with an increase of 0.5 °C every 5 s. Samples were amplified in duplicate and a melt curve was completed after each PCR reaction to ensure fluorescence quantification was specific to a single PCR product. Both no-template and no-reverse transcriptase controls were utilized to verify DNA-free status of the negative control samples. The changes in gene expression were calculated by the 2ΔΔCt method (Livak and Schmittgen, 2001) using the selected references genes (βactin and GAPDH). The first control sample was expressed as 1.0 by this equation, and all other samples were calculated in relation to this value. Afterward, the results in the control (Healthy) were averaged, and all

other outputs were divided by the mean value of the relative abundance in the control group to yield the fold change of the genes of interest compared to the control group (Masternak et al., 2005).

2.6. Statistical analyses

Statistical analysis was performed using SAS Student's T-test for the proportion of PMN cells, days postpartum and concentration of BHBA. The Mann-Whitney test was used for analysis of relative abundances of mRNA. Pregnancy per AI was analyzed using the Chi-square test. The relationship between the proportion of PMN cells in the uterus and days postpartum on the probability of pregnancy was determined. A logistic regression curve was generated using the coefficients calculated from the interactive data analyses of SAS. Optimal cut-off points for determining the relationship between proportion of PMN in the uterus and pregnancy was calculated using receiver operating characteristic (ROC) curve for cows with less than 30 DPP and cows with more than 30 DPP. As for both periods the cut-off values for proportion of PMN in the uterus was the same (4.75%), we decided to run a single ROC curve in this study. Specificity (Sp), sensitivity (Se), area under the curve (AUC) and P-value of the ROC test are reported. The relationship between the proportion of PMN cells in the uterine endometrium and DPP was determined by polynomial regression. Differences among groups were considered statistically significant when P value ≤ 0.05. Tendency was considered when P value was > 0.05 and ≤ 0.1.

3. Results

Fertility responses are summarized in Table 2. Cows in HE had a greater ($P < 0.05$) pregnancy per AI than cows in SCE and SCEP. However, P/AI in cows PVD+ was not diferent in comparison to HE and SCE cows (Table 2).

Blood concentrations of BHB are also depicted in Table 2 and were not different ($P = 0.88$) among groups. Regarding DPP, we observe that cows in SCEP group had less DPP at the moment of TAI ($P < 0.001$; Table 2). Similarly, cows with more DPP were classified as Healthy and had a higher P/AI.

Disregarding the specific groups, 25% of the cows were PVD+ and 28.3% had PMN >4.75%. In cows, in which, PVD+ was not observed, 25% had SCE due to a PMN proportion > 4.75%. Still, in cows that presented PVD+, 38.4% had proportion of PMN > 4.75%, characterizing cows with clinical and cytological endometritis (Figure 1).

The data for the correlation between proportion of PMN cells in the uterus and the probability of pregnancy is depicted in Figure 2. The correlation between days postpartum and probability of pregnancy is depicted in Figure 3. Overall we observed that the longer is the interval from calving to cytological uterine sampling, the lower is the proportion of PMN cells in the uterus (Figure 4).

The proportion of PMN cells in the uterus is shown in Table 2. Cows from the SCE and SCEP groups had a higher ($P < 0.001$) proportion of PMN cells in the uterus than the HE and PVD+. Regardless of days postpartum, cows with lower proportion of PMN cells in the uterus were 8.11 - 9.9 times more likely to become pregnant than cows with higher proportion of PMN cells. From the ROC curves for determining the relationship between proportion of PMN in the uterus and pregnancy status in beef cows, the cut-off for PMN percentage was 4.75% (Sp 42.9%, Se 82.8% with AUC 0.68; $P < 0.001$; Figure 5).

The relative abundance of proinflammatory cytokine IL-1β, IL-6, IL-8, and TNFα mRNA is depicted in Figure 6. There was no difference in the control (βactin and GAPDH) Ct values between groups (P = 0.50; 26.7, 25.7, 28.4 and 26.4 in cows from HE, PVD+, SCE and SCEP, respectively). The consistency of abundance of βactin and GAPDH mRNA confirms the suitability as reference genes. Relative abundance of mRNA transcripts for IL-6 and TNFα did not change among groups (P > 0.05). In contrast, relative abundance of the mRNA transcripts for IL-8 was greater (P = 0.02) in SCEP than the HE and SCE, but not from PVD+ cows. The relative abundance of mRNA transcripts for IL-1β tended to be greater (P = 0.08) in SCEP than in PVD+, but the cows in the HE and SCE group were not different from other groups.

3. Discussion

The results of the current study supported the hypothesis that postpartum beef cows subjected to TAI protocols with a higher proportion of PMN cells and with PVD have greater proinflammatory cytokines gene expression in the endometrial tissue and lower P/AI. The group of cows submitted to TAI protocols with a higher proportion of PMN cells and vaginal discharge (SCEP group) had lower P/AI and a greater relative abundance of mRNA transcripts for IL-1β and IL-8 in the uterine endometrium. Importantly, cows that had a lower proportion of uterine PMN and vaginal discharge had a higher P/AI. Although, the relationship between uterine health and fertility have been described in dairy (Barlund et al., 2008; Galvão et al., 2011; Gilbert et al., 2005; Kasimanickam et al., 2004) and beef cows (Ricci et al., 2015; 2017; Pfeifer et al., 2018), to the best of our knowledge, this is the first report to characterize the fertility in postpartum Nelore beef cows with different uterine conditions.

Cows enrolled in this study were evaluated according to absence or presence of PVD and the proportion of uterine PMN cells (greater or less than 4.75%) according to the threshold established by the ROC curve. Similarly, several authors established a similar PMN cut-off

value (5%) to determine SCE (Gilbert et al., 2005; Santos et al., 2009; Fischer et al., 2010; Gabler et al., 2009; Plöntzke et al., 2010; Ribeiro et al., 2013; Madoz et al., 2013; Pfeifer et al., 2018). Therefore, we separated the cows in 4 different groups according to the uterine condition. As observed, cows with shorter calving TAI interval, had compromised uterine condition at the beginning of the TAI protocol, and were less likely to become pregnant. Although the number of cows with uterine inflammation was lower in comparison to healthy cows, this resulted in a negative impact on the reproductive performance of the beef herd. Overall, it was clear that pregnancy reduce strongly as uterine PMN increases. However, DPP did not significantly correlate with pregnancy and the association between DPP and PMN was more variable. Further, the DPP were similar between HE and SCE cows, however, PMN was increased and pregnancy decreased in SCE cows. This suggests overall that the effect of PMN on pregnancy is a stronger predictor of fertility than DPP.

In the present study, we categorized the cows into four groups according to the classification of vaginal discharge (clinical diagnosis) and endometrial cytology to assess the relationship between uterine health and fertility. Interestingly, there is little information on the simultaneous use of clinical and cytological criteria for the diagnosis of endometritis in beef cows (Barlund et al., 2008; Dubuc et al., 2011). Dubuc et al., (2011) was among the first to classify endometritis into clinical, cytological and a combination of clinical and cytological based on the type of vaginal discharge in dairy cows. Additionally, Gobikrushanth et al., (2016) observed that clinical and cytological endometritis had a negative impact on first service conception rate and the subsequent services of TAI protocols in dairy cows. The combination of different diagnostic methods is more efficient in identifying cows with inflammatory uterine diseases due to the discrepancies between the techniques (De Boer et al., 2014). Therefore, we used the combination of vaginal discharge and endometrial cytology to better separate the categories and obtain a more reliable result of the uterine inflammatory status.

Studies have set different PMN cut-off values for different postpartum period (Barlund et al., 2008; Galvão et al., 2011; Kasimanickam et al., 2004). Conventionally, PMN cut-off values increase as time postpartum decrease (Wagener et al., 2017). However, in the present study, as the probability of pregnancy did not increase as time postpartum increase, therefore we set a single PMN cut-off value (4.75 %) to determine SCE. Moreover, when cows were divided into two groups to determine PMN cut-off values, cows with less than 30 DPP and cows with more than 30 DPP had the same cut-off value (4.75% of PMN). Ricci et al. (2015), also set a single PMN cut-off value (8%) for piedmontese beef cows sampled from 28 to 68 DPP. Although the differences in the threshold to determine SCE, both studies detected similar proportion of cows with SCE, 31% (Ricci et al., 2015) and 28.4% (present study). These considerations led us to infer that the genetic group (*B. indicus* vs. *B. taurus*), management, and environment affect the postpartum uterine health, fertility of TAI protocols, and consequently, calving interval. However, more studies comparing postpartum uterine health between *B. taurus* and *B. indicus* cows are necessary.

Our results showed that the proportion of PMN cells decreases as the interval from parturition increases. Thus, a healthier uterus is associated to improved first service conception rate. In the SCEP group, cows had a shorter interval from calving to the moment oF TAI, i.e. less time to recover the uterine environment, therefore, the proportion of PMN was higher and P/AI lower compared to the other groups. Previous studies have also observed the disappearance of cytological inflammation in most cows as the interval from parturition increases, which reflects a decrease in the prevalence of endometritis (Plöntzke et al., 2010; Dubuc et al., 2011; Barański et al., 2013) and increase the likelyhood of pregnancy (Santos et al., 2009; Pfeifer et al., 2018) This finding may suggest that an adequate voluntary waiting period (VWP) may be sufficient to resolve uterine inflammation ad recover fertility. Although VWP is a term not commonly used for beef cows, our previous results demonstrated the

determination of a VWP for beef cows is a very useful tool for farmers to decide the best moment to start a TAI program after calving (Pfeifer et al., 2018). Beef cows subjected to TAI >35 DPP had higher P/AI and lower proportion of PMN in the uterus.

Cows that resume ovarian activity earlier after calving resolve uterine inflammation more quickly than those cows in which postpartum ovulation has been delayed. (Sheldon et al. 2002; Williams et al. 2007; Krause et al., 2014). In addition, dairy cows that ovulate early in the postpartum period have a reduced incidence of endometritis and an increased pregnancy rate (Galvão et al., 2011). Although, in the present study, the ovarian status was not accessed at the beginning of the TAI protocol, in the early postpartum, almost all *B. indicus* cows raised in tropical conditions are in anestrus. The seasonality of pasture production in extensive beef operations may impair fertility of cows during the first postpartum AI. Almost all cows calving during the dry season have limited pasture availability and quality in Amazone Biome. In this scenario, at the beginning of the TAI protocol, the proportion of cows in anestrus was presumably higher in cows in the SCEP group because these cows had less DPP compared to the other groups.

The cytobrush technique was appropriate to collection of endometrial epithelial cells for cytology and to obtain RNA of quantity and quality for the analysis of the gene expression of the pro-inflammatory cytokines IL-1β, IL-6, IL-8, and TNFα (Cardoso et al., 2017; Ghasemi et al., 2012). The results of the present study confirm that pro-inflammatory cytokines have a role in the migration of PMN and in the infiltration of the endometrium after infection (Fischer et al. 2010). We observed significantly higher levels of IL-1β and IL-8 in cows from the SCEP group, which also had a higher PMN count and lower P/AI. These results were similar to the only report for uterine health from *B. indicus* beef cows (Pfeifer et al., 2018). The association between expression of IL-1β, IL-8 and PMN in uterine samples from cows with endometritis was also observed by others (Fischer et al., 2010; Ghasemi et al., 2012). Galvão et al., (2011)

reported a significant increase in IL-8 expression in cows diagnosed with subclinical endometritis using uterine lavage. Thus, when inflammation is established in the uterus, IL-8 expression levels can accurately reflect the uterine inflammatory status (Ghasemi et al., 2012). Similarly, Fischer et al., (2010) suggested that IL-1β and IL-8 may represent potential marker genes for the detection of cows with endometritis and for monitoring new therapeutic approaches. Therefore, the increased inflammatory mediators present in uterus during puerperium can be detrimental for fertility in cows subjected to TAI as observed before (Pfeifer et al., 2018).

In conclusion, the results of this study showed that P/AI is higher in cows with lower proportion of PMN cells in the uterus. Cows that had a shorter time to recover from from calving to TAI, had more uterine inflammation (SCE and SCEP), with a higher proportion of PMN and higher IL-1β and IL-8 expression in the uterine endometrium. Consequently, this group of cows had lower P/AI in response to the TAI protocol. In addition, our study suggest that in herds with a high prevalence of endometritis cows can be given a longer interval from calving to TAI, which will result in higher P/AI.

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Declaration of interest

The authors declare no conflicts of interest.

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Figures:

Figure 1. Venn diagram showing the number of cows according to the proportion of polymorphonuclear cells (PMN) and purulent vaginal discharge (PVD). Squares within the diagram demonstrate the number of cows in each group.

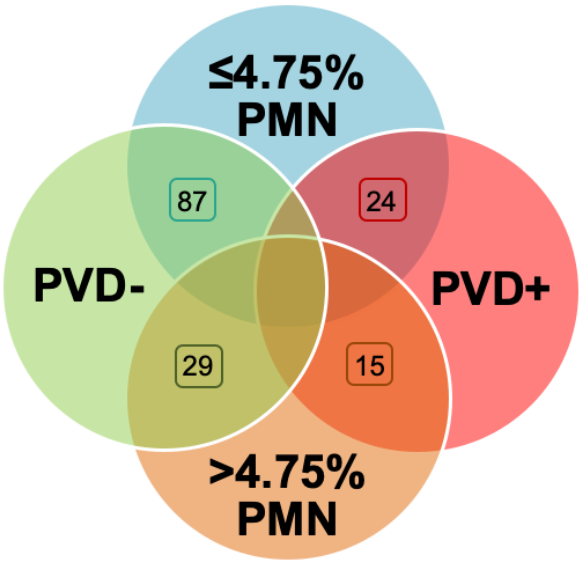

Figure 2. Relationship between proportion of PMN cells in the uterine endometrium and the probability of pregnancy in the postpartum period in Nellore cows submitted to the TAI protocol (*P* = 0.002).

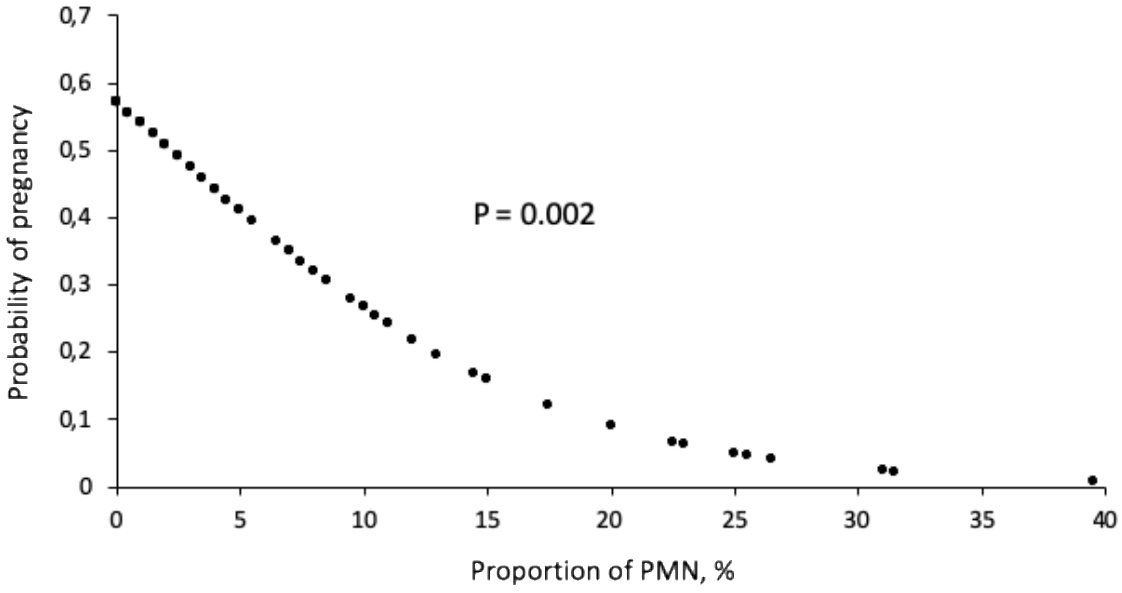

Figure 3. Relationship between days postpartum and the probability of pregnancy in the postpartum period in Nellore cows submitted to the TAI protocol (*P* = 0.1).

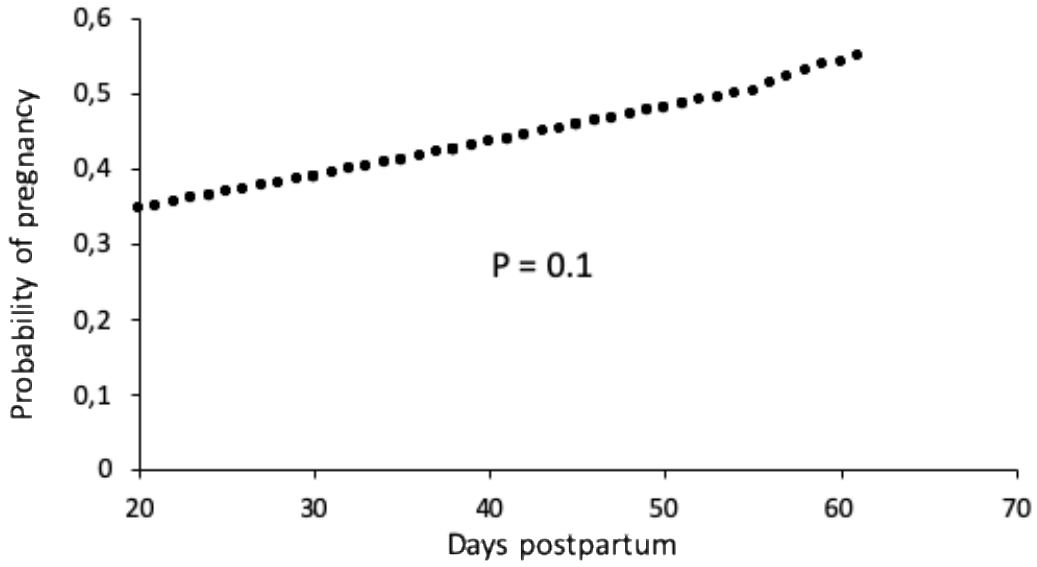

Figure 4. Relationship between proportion of PMN cells in the uterine endometrium and Days postpartum in Nelore cows subjected to TAI protocol.

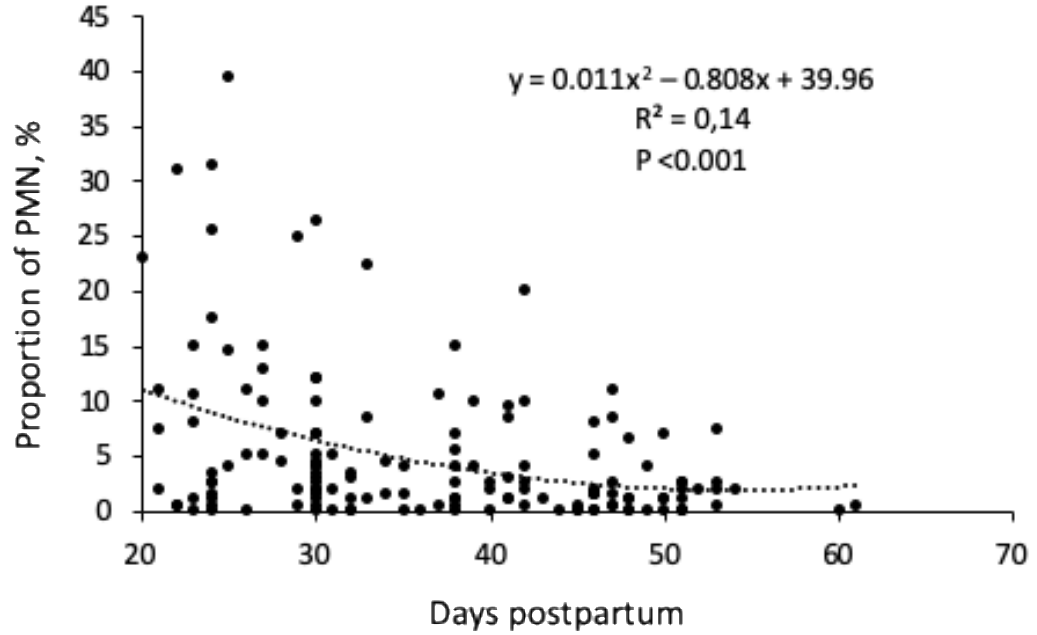

Figure 5. Receiver Operating Characteristic (ROC) curve for determining the relationship between proportion of PMN in the uterus and pregnancy status in beef cows; Analysis of the ROC indicated that the use of a cut-off point of more than 4.75% of PMN was 42.9% specific and 82.8% sensitive; Area under the curve = 0.68, $P < 0.001$.

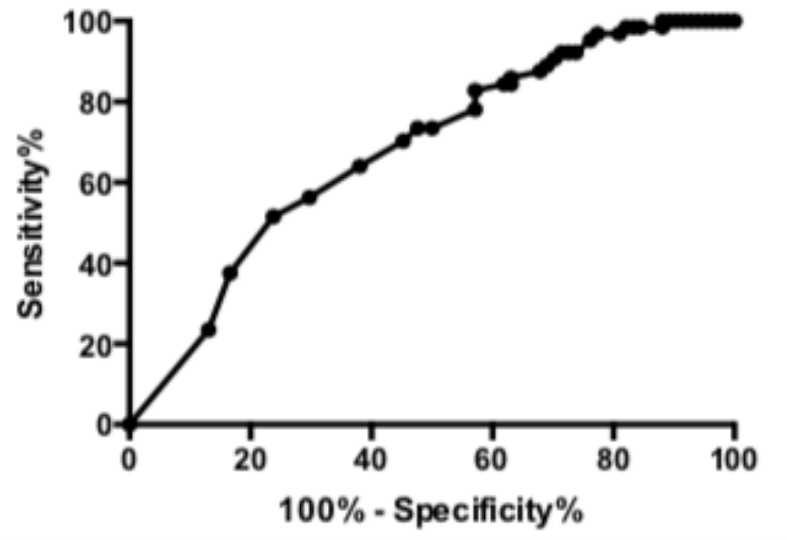

Figure 6. Relative abundance of mRNA transcript in uterine endometrial tissues of beef cows categorized into groups according to uterine health in the postpartum period. [ab] indicates means differ by $P \leq 0.1$, [ABC] by $P \leq 0.05$.

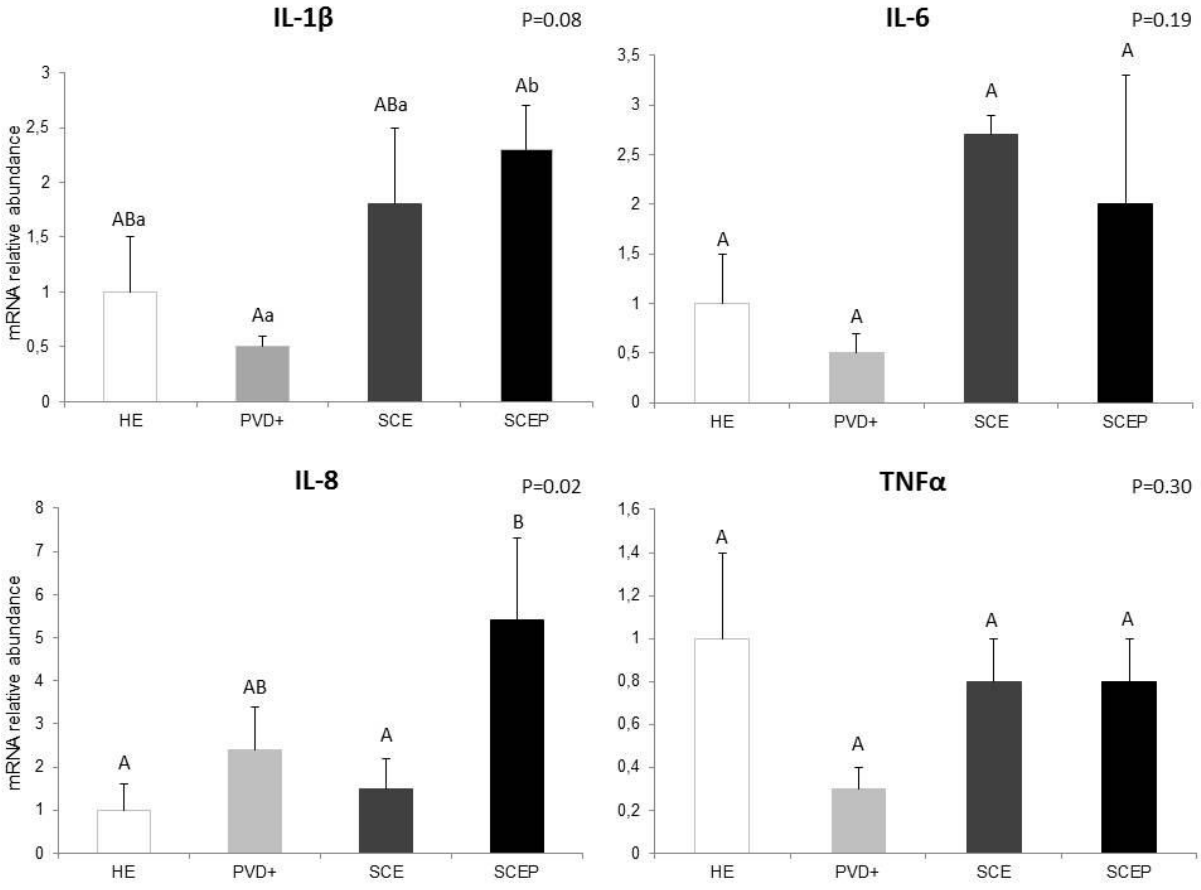

Table 1. Primer sequences for quantitative real-time polymerase chain reaction (qRT-PCR) amplification of mRNA

Gene	Primer direction	Primer sequence (5' - 3')
GAPDH	forward	ACACTGAGGACCAGGTTG
	reverse	TGGTCGTTGAGGGCAATG
β-actin	forward	AGGCATCCTGACCCTCAAGTA
	reverse	GCTCGTTGTAGAAGGTGTGGT
IL-1β	forward	CAAGGAGAGGAAAGAGACA
	reverse	TGAGAAGTGCTGATGTACCA
IL-6	forward	CCAGGAACGAAAGAGAGC
	reverse	CAGAAGTCATCACCAGGAG
IL-8	forward	CAAGAGCCAGAAGAAACCTGAC
	reverse	AGTGTGGCCCACTCTCAATAAC
TNF-α	forward	CTCTTCTGCCTGCTGCACTTC
	reverse	CCATGAGGGCATTGGCATACG

Table 2. Fertility and metabolic response of cows according to the experimental groups.

Parameters	Groups				P-Value
	Healthy	PVD+	Subclinical Endometritis (SCE)	Subclinical Endometritis + PVD+ (SCEP)	
Pregnancy per AI[1]	54.0% (47/87)^{A}	37.5% (9/24)^{AB}	27.5% (8/29)^{B}	13.3% (2/15)^{C}	<0.05
Proportion of PMN[2] cells (%)	1.7 ± 0.1^{A}	1.6 ± 0.3^{A}	13.8 ± 1.5^{B}	16.9 ± 3.3^{C}	<0.0001
BHBA[3] (mmol/L)	0.9 ± 0.04	0.9 ± 0.07	1.0 ± 0.06	0.9 ± 0.07	0.88
DPP at TAI[4]	40.3 ± 1.2^{A}	34.7 ± 2.3^{A}	35.1 ± 1.6^{A}	26.9 ± 1.8^{B}	<0.0001

^{AB}Within a row, means without a common superscript differed (P < 0.05).
[1]Artificial insemination
[2]Polymorphonuclear cells.
[3]β-hydroxybutyrate
[4]Days postpartum at the moment of TAI

CAPÍTULO II

Uterine health and energy metabolism of primiparous and multiparous beef cows during the transition period

Uterine health and energy metabolism of primiparous and multiparous beef cows during the transition period

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Abstract

The objective of this study was to compare the uterine dynamics and postpartum energetic metabolism of primiparous and multiparous beef cows. For this purpose, 24 Nellore cows (*Bos indicus*) were allocated by parity order into primiparous (n = 15) and multiparous (n = 9) cows. These cows were evaluated at 20 and 10 days prepartum and weekly from parturition to 42 days postpartum (DPP). During this period, body weight (BW), internal angle of the rump (IAR), body condition score, subcutaneous fat thickness (SFT), and serum concentration of glucose and β-hydroxybutyrate (βHB) were evaluated. During the postpartum period, the cows were evaluated for purulent vaginal discharge (PVD). Uterine tissue was collected using the cytobrush technique to assess the proportion of polymorphonuclear (PMN) cells in the uterus and the abundance of interleukin 1 beta (*IL1β*), *IL6*, *IL8*, tumor necrosis factor alpha (*TNF*A), progesterone receptor (*PGR*), estrogen receptor 1 (*ESR1*), *ESR2*, integrin beta 3 (*ITGB3*), patched 2 (*PTCH2*), epidermal growth factor receptor (*EGFR*), and heparin-binding EGF-like growth factor (*HB-EGF*) transcripts. At the end of the evaluation, the cows were subjected to an estradiol-progesterone-based timed artificial insemination (TAI) protocol. The animal category ($P > 0.05$) did not affect BW, IAR, SFT, βHB, glucose serum concentration, PVD, and proportion of PMN cells in the uterus, as well as pregnancy per artificial insemination. However, primiparous cows had higher ($P = 0.002$) proportion of PMN cells than multiparous cows at 35 DPP (6 ± 1.1% vs. 0.6 ± 0.2%). Similarly, all multiparous cows were considered healthy at 35 DPP, whereas only 40% (6/15) primiparous cows were healthy ($P = 0.003$). Primiparous cows had higher ($P < 0.04$) relative abundance of *IL8* and tended to have lower ($P = 0.1$) relative abundance of *IL1B* transcripts than multiparous cows. Nevertheless, the animal category did not significantly affect the relative abundance of the other gene transcripts analyzed in this study. Overall, the findings indicated that animal category does not significantly affect energy metabolism, and that multiparous cows have lower uterine inflammatory response associated with lower relative expression of *IL8* in the postpartum period and higher likelihood of being healthy at 35 DPP than primiparous cows.

Keywords: Animal category; cattle; *Bos indicus*; Postpartum; Uterus; Metabolism.

1. Introduction

Reproductive efficiency and the establishment and maintenance of pregnancy depend on the uterine environment in all mammalian species (ATKINS *et al.*, 2013). Reproductive performance directly affects the productivity and profitability of both dairy and beef cattle production systems (BARUSELLI *et al.*, 2012). Uterine diseases affect 15–70% of dairy cows in the first 60 days postpartum and are associated with delayed resumption of ovarian cyclicity, prolonged calving interval, decreased conception rates, and consequently, increased culling rates (LEBLANC *et al.*, 2002; GILBERT *et al.*, 2005, LEBLANC, 2008; SHELDON *et al.*, 2009; OLIVEIRA FILHO *et al.*, 2022). In beef cows, reproductive efficiency after calving is related to delayed resumption of ovarian cyclicity, negative energy balance (NEB), and calf suckling (LAMB *et al.*, 1999; CROWE *et al.*, 2014; OLIVEIRA FILHO *et al.*, 2022). However, the uterine environment is known to exert only limited effects on subsequent reproductive performance (SANTOS *et al.*, 2009).

During the puerperium, changes in the endometrial inflammatory profile must be well-coordinated for adequate resumption of uterine activity. During this process, endometrium undergoes extensive remodeling and structural changes aimed at providing an environment propitious for subsequent conception (ZAIN *et al*., 1995; ZHANG *et al*., 2010). The expression of certain genes is crucial for complete uterine function and conception. The spatiotemporal distribution and function of heparin-binding epidermal growth factor *(HBEGF*), collagen type IV alpha 1 (*COL4A1*), integrin beta 3 (*ITGB3*), patch 2 (*PTCH2*), the receptors of estradiol 1 and 2 (*ESR1* and *ESR2*), progesterone (*PGR*), and epidermal growth factor (*EGFR*/*ERbB1*) in the uterus of cows has been associated with postpartum uterine remodeling and the generation of uterine environment conducive for new pregnancies (SIMÓN *et al.*, 2000; GUZELOGLU-KAYISLI *et al.*, 2009; BAZER *et al.*, 2010; SHIMIZU *et al.*, 2010; GONELLA-DIAZA *et al.*, 2015; TAKATSU; ACOSTA, 2015; SÁ FILHO *et al.*, 2017). Furthermore, during postpartum endometrial repair, the innate immune response to infections caused by pathogens is partially mediated by pro-inflammatory cytokines and chemokines such as tumor necrosis factor alpha (*TNFA*), interleukin 1 beta (*IL1β*), *IL6*, and *IL8* (FOLEY *et al.*, 2012; FISCHER *et al.*, 2010; DADARWAL *et al.*, 2017). Resolution of inflammation is also be supported by secretion anti-inflammatory cytokines such as *IL10* and transforming growth factor beta (*TGFβ*) (DADARWAL *et al.*, 2017).

Recent studies conducted with postpartum multiparous Nellore cows have shown that approximately 25% cows subjected to timed artificial insemination (TAI) have purulent vaginal discharge (PVD) and/or subclinical endometritis (SCE), conditions that lead to lower pregnancy

per AI (P/AI) (PFEIFER *et al.*, 2018; ANDRADE *et al.*, 2021). In contrast, Oliveira Filho *et al.* (2022) have observed that only 6% Nellore cows had ≥5% polymorphonuclear (PMN) cells between 28 and 40 days postpartum (DPP). In addition to uterine health, NEB severity in the postpartum period (PPP), may be responsible for conception failure and the reduced fertility rates of TAI protocols. Accordingly, several studies have reported that primiparous cows have lower P/AI than multiparous cows (SA FILHO *et al.*, 2009; GRILLO *et al.*, 2015; PFEIFER *et al.*, 2017). Some studies have associated decreased fertility with increased postpartum metabolic challenge in primiparous cows (WILTBANK, 1970; RANDEL, 1990; FERREIRA *et al.*, 2021). Although several studies have characterized the energy balance and postpartum uterine health of dairy cows (GILBERT *et al.*, 1993; HAMMON *et al.*, 2006; DUBUC, J. *et al.*, 2010; GALVÃO *et al.*, 2010; CHEONG *et al.*, 2011; STER *et al.*, 2012; LEBLANC 2012; WITTROCK *et al.*, 2012; KRAUSE *et al.*, 2014), few have assessed the interaction between metabolism, uterine repair dynamics, and expression pattern of cytokines and genes associated with uterine receptivity during the PPP in beef cows raised in an extensive grazing system and under tropical conditions. Furthermore, to the best of our knowledge, studies comparing the postpartum uterine dynamics of primiparous and multiparous *Bos indicus* cows are lacking.

Considering the above, we hypothesized that primiparous cows have more pronounced NEB and longer postpartum anestrus than multiparous beef cows raised in an extensive grazing system. Hence, in this study, we aimed to evaluate the relationship between parity and uterine health, metabolism, resumption of ovarian cyclicity, and fertility of *B. indicus* beef cows in the pre- and postpartum periods. This study will help the understanding of the puerperium dynamics that may support reproductive management strategies to increase the fertility of postpartum *B. indicus* beef cows.

2. Material and methods

2.1 Animals

The Committee for Ethics in Animal Experimentation from Brazilian Agricultural Research Corporation (Embrapa) approved all the procedures performed in the experiment described in this manuscript (Protocol 03/2017).

This study was performed at the experimental farm of Embrapa (Porto Velho, RO, Brazil, 08°48′12″S, 63°50′56″W). Thirty Nellore (*Bos indicus*) cows were selected for this study. The animals were kept in *Urochloa brizantha* cv. Xaraes pasture, with free access to water and mineral supplementation. Cows were grouped according to calving order into

primiparous (n = 15) and multiparous (n = 9). Cows that had retained placenta (RP) and/or other pathology during the experimental period were excluded from the study.

2.2 Blood collection and metabolic assessments

Cows were evaluated on days -20, -10, before calving and on days 7, 14, 21, 28, 35, and 42 postpartum, the cows were evaluated for body weight (BW), internal angle of the rump (IAR), subcutaneous fat thickness (SFT) glucose and β-hydroxybutyrate (βHB). For the evaluation of the IAR, a goniometer was used to measure the angle between the two sides of the rump. The goniometer device was placed on the rump, one rod to each side of the rump, between the sacral bone and the first coccygeal vertebrae.

Ultrasonography (Mindray M5; equipped with a 5 MHz linear transducer, Shenzhen, China) was used to measure the SFT. After immobilization of the animal, the evaluation site was cleaned with a brush with nylon bristles and vegetable oil was applied to increase the contact surface between the transducer and the animal's skin. Therefore, the transducer was positioned linearly between the coxal tuberosity and the iliac tuberosity and slightly moved until obtaining an adequate image that allowed the visualization of the upper limit of the Biceps femoris, as described by Ayres *et al.* (2009).

Blood was collected by coccygeal venipuncture using vacuum tubes without anticoagulant (13 × 75 mm, 5 mL VacuTube®, Zhejiang, China). Peripheral concentrations of βHB and glucose were measured with a manual meter (TD - 4235®; Ketovet, MG, Brazil; Accu-Chek® Active, Roche, SP, Brazil, respectively), according to the manufacturer's instructions.

Albumin and haptoglobin were measured in serum samples by colorimetric method in an automated biochemical analyzer (LabMax Plenno, Labtest, Brazil), using commercial kits following the protocol established by the manufacturer (Labtest Diagnóstica, Brazil; aptec diagnostic nv, Belgium and Diasys Diagnostic System, USA).

2.4 Gynecological and reproductive assessments

Cows were examined by vaginoscopy to assess and characterize the presence of vaginal discharge on days 7, 14, 21, 28, 35, and 42 postpartum (PP). For the vaginoscopic examination, the vulva was wiped with a paper towel and a sanitized vaginal speculum was introduced into the vaginal canal. Using an external light source, the uterine cervix and vaginal canal were inspected and the presence of discharge was graded on a scale of 0 to 3 (0 = mucus, 1 = mucus

with flecks of pus, 2 = ≥50% exudate purulent, 3 = hemorrhagic and/or purulent exudate), as adapted from others (WILLIAMS *et al.* 2005). Cows with positive purulent vaginal discharge (PVD+) were defined as cows having a vaginal discharge score of ≥ 1 (PLETICHA *et al.*, 2009).

After the evaluation, cows were submitted to collection of endometrial tissue for cytological analysis of the inflammatory condition of the uterus and gene expression. For these purposes, an adapted cytology brush was coupled to the tip of a conventional artificial insemination (AI), covered by a disposable AI sheath and protected by a sanitary sheath, as described by Cardoso *et al.*, 2017. The apparatus was inserted via cervix and the cytobrush was rotated to collect cells from the uterine body. Immediately after sampling, the cytobrush was uncoupled from the apparatus and was gently rolled onto a clean microscope slide using only half of its entire circumference, to ensure that a suitable quantity of cellular material remained on the untouched surface for gene transcription analysis. Slides were air-dried and packaged for transport to the laboratory. The cytobrush was then placed in a 2-mL cryotube filled with 1 mL of RNA later buffer and stored at − 80◦C for later processing and analysis of the relative abundance of mRNA of the genes described in Table 1. In the laboratory, the slides were stained using a commercial kit (Quick Panoptic®, Laborclin, Pinhais, Brazil). Two hundred cells per slide were counted in an optical microscope (100 X magnification), including polymorphonuclear cells (PMN), mononuclear and epithelial cells to calculate the proportion of PMN cells.

Cows were considered healthy when the proportion of PMN in the uterus was ≤ 4.75% and the PVD assessment was equal to 0 on day 35 (ANDRADE *et al.*, 2021).

The diameter of the uterine horns and the endometrial area of the uterine horns were obtained through cross-sectional images by proper positioning of the 5 MHz linear transducer in B-mode. Measurements were collected from frozen images of the uterine horns approximately 10 cm from the bifurcation point of the uterus. Diameter was defined as the vertical dimension from serosa to serosa through the center of the organ (adapted from CANADAS *et al.*, 2019). For the endometrial area, the measurement of the diameter of the endometrium of the uterine horns (left and right) was performed and the average of the two diameters was used to calculate the endometrial area from the formula $A = \pi.R^2$, where the radius (R) was determined as the mean of the endometrial diameters (adapted from ALMEIDA *et al.*, 2015).

Ultrasonic examination (SIUI CTS-900, linear probe with 5 MHZ, Guangdong, China) of the ovaries was performed on the same days of the cytobrush collections. The ovaries were evaluated to assess the presence of a corpus luteum (CL). Therefore, first

postpartum ovulation was determined by the first postpartum detection of the CL on ultrasound assessments of the ovaries (Adapted from MESQUITA *et al.*, 2014).

2.5 Reproductive management

After the end of the metabolic, gynecological and reproductive evaluations, the cows were submitted to a fixed-time artificial insemination protocol (FTAI). Cows were given 2 mg of estradiol benzoate (Bioestrogen®, Biogénesis-Bagó, Curitiba, Brazil) intramuscular (i.m.) and received an intravaginal progesterone-releasing device (1.9 g progesterone, CIDR®, Pfizer Animal Health, São Paulo, Brazil) to synchronize follicular wave emergence on Day 0 (D0). The CIDR was removed and cows were given 150 μg D-Cloprostenol i.m. (PGF2α analogue; Croniben®, Biogénesis-Bagó, Curitiba, Brazil), 1 mg of ECP im (E.C.P.®, Pfizer, Cravinhos, Brazil), and 300 IU of eCG (Novormon®, Syntex, Buenos Aires, Argentina) i.m. on Day 8 (D8). All cows were TAI 48 h after CIDR removal (D10).

Ultrasonic examination (SIUI CTS-900, linear probe with 5 MHZ, Guangdong, China) was performed 30 d post-TAI to assess pregnancy status. Visualization of the embryonic vesicle and detection of the embryo were the positive criteria for determining pregnancy.

2.6 RNA isolation and cDNA synthesis

For the extraction of total RNA, samples of endometrial tissue collected by cytobrush were used. Endometrial and PMN cells present in the cytology brush that were stored in the cryovials containing RNAlater® were sedimented by centrifugation at 2000 x g at 4°C for 10 min. The supernatant (600 μL) was discarded and 600 μL of 1% β-mercaptoethanol was added before total RNA was extracted according to the commercial kit protocol (PureLink® RNA Mini Kit, Thermo Fisher Scientific, Carlsbad, CA).

The purity and quantity analysis of total RNA was evaluated using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

cDNA synthesis was performed by reverse transcription using the high throughput cDNA reverse transcription kit (Applied Biosystems, USA) according to the manufacturer's instructions. The RNA was treated with DNase I (Invitrogen, Thermo Fisher Scientific, USA) before reverse transcription to remove possible contamination from the genomic DNA. Anchored Oligo primer (dT) 23 (Sigma-Aldrich, USA) was incubated with the treated total RNA at 70 °C for 10 min as described by the manufacturer. The reverse transcription (RT) reaction was performed using the HighCapacity cDNA kit (Applied Biosystems, USA)

according to the protocol described by the manufacturer, in a final volume of 20 μL/ reaction, using the following conditions: 25 ^{0}C for 10 min, 37 ^{0}C for 120 min and 85 ^{0}C for 5 min. The synthesized cDNA was subsequently quantified by spectrophotometry (NanoDrop 2000 spectrophotometer; Thermo Fisher Scientific, USA) to assess the RT reaction efficiency, and was stored at − 80◦C until quantitation assays were conducted.

2.7 Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative real-time polymerase chain reaction (qRT-PCR) was used to assess the relative abundance of mRNAs in the cytobrush samples and was performed by an operator blinded to the cytology results. The Perfecta SsoFast EvaGreen® Supermix™ Kit (Bio-Rad Laboratories Inc., USA) was used according to the manufacturer's instructions to perform qRT-PCR analysis of the relative mRNA abundance of the genes described in Table 1. Briefly, 10 μL of Perfecta SsoFast EvaGreen® Master Mix for iQ (2X), 1 μL of the appropriate primer set (Table 1) at 10 μM, 250 ng/μL of cDNA template, and 4 μL of water were added for a final reaction volume of 20 μL. The reaction was performed in the Bio-Rad CFX96™ detection system (Bio-Rad Laboratories Inc., Singapore) using the following program: 1 cycle at 95◦C for 5 s, 39 cycles of 95◦C for 5 s, 60◦C for 20 s, and a melting curve standardized from a variable annealing temperature ramp from 65 to 95◦C with an increase of 0.5◦C every 5 s. Samples were amplified in duplicate and a melting curve was completed after each PCR reaction to ensure fluorescence quantification was specific to a single PCR product. Both no-template and no-reverse transcriptase controls were utilized to verify DNA-free status of the negative control samples. The changes in gene transcription were calculated by the 2ΔΔCt method (LIVAK and SCHMITTGEN, 2001) using the selected reference genes (βactin and GAPDH). Gene expression values of healthy multiparous cows (PVD = 0 and PMN ≤ 4.75%) at 35 DPP were used as a reference for all genes evaluated in this study because the uterus was within the expected standards for the bovine female have adequate fertility. The gene expression value for each gene from the reference samples was expressed as 1.0, and all other samples were calculated in relation to this value and then averaged. Then, all other values of the genes evaluated in the different DPP for multiparous and primiparous cow were divided by the mean value of the relative abundance of the reference value. In this way, it was possible to produce the fold change of the genes of interest compared to the reference value (MASTERNAK *et al.*, 2005).

2.8 Statistical analyses

All statistical analyzes were performed using SAS 9.0 software (SAS Institute Inc., Cary, NC, USA). Variables for repeated measures (BW, βHB, glucose, BCS, PMN, vaginal discharge, IAR, SFT, endometrial area, uterine diameter and relative abundances of mRNA) were evaluated using an analysis of variance (ANOVA) with the MIXED procedure to test the main day effects, category and their interactions. The proportion of PMN cells and the day of first ovulation was analyzed using a one-way ANOVA. Pregnancy by artificial insemination (AI) and analysis of the proportion of healthy cows at 35 DPP were analyzed using the chi-square test. In all tests, the significance level was set at $P < 0.05$, and P values between 0.05 and 0.1 were considered as a trend.

3. Results

Of the 30 Nellore cows (18 primiparous and 12 multiparous) that were initially included in the study, 6 cows (3 primiparous and 3 multiparous) presented retained placenta. These cows were excluded from the study.

The data on energy metabolism are shown in Figure 1. The effect of time (days) was observed on all the variables analyzed ($P < 0.05$). Significant differences in body weight (BW) and subcutaneous fat thickness (SFT) were not detected between the parity during the study period ($P > 0.05$; Figure 1A and 1B). Significant differences in IAR were found by parity and time interaction ($P < 0.05$; Figure 1C). No differences in serum glucose ($P = 0.19$; Figure 1D), βHB ($P = 0.89$; Figure 1E), albumin ($P = 0.30$; Figure 1F) and haptoglobin ($P = 0.30$; Figure 1G) concentrations were observed between multiparous and primiparous cows.

Endometrial area and uterine diameter differed significantly by parity and time interaction ($P < 0.05$; Figure 2). Multiparous cows had larger uterine diameter and endometrial area than primiparous cows. Furthermore, endometrial diameter and area decreased significantly with increase in postpartum interval.

The proportion of uterine PMN cells and PVD during PPP is shown in Figure 3. No differences were observed in the proportion of PMN cells ($P = 0.40$) or in PVD positivity ($P = 0.31$) between multiparous and primiparous cows throughout the experiment. However, the proportion of PMN cells and PVD positivity decreased significantly ($P < 0.05$) with increase in days postpartum (Figure 3). The proportion of healthy multiparous cows at 35 DPP was higher ($P = 0.003$) than that of primiparous cows (Table 2). Thus, multiparous cows showed lower ($P = 0.002$) proportion of PMN cells than primiparous cows at 35 DPP (Table 2). In addition, a

higher proportion of multiparous cows had PMN cells <4.75% at 35 DPP (P = 0.03) and tended (P = 0.08) to have a higher proportion of PVD = 0 than primiparous cows (Table 2).

The ovulation response is shown in Table 2. From parturition to 42 DPP, multiparous cows tended (P = 0.08) to have higher chances of ovulation than primiparous cows. Only 2 (13%) primiparous cows ovulated between calving and the beginning of the TAI protocol. No differences in mean interval from calving to first ovulation (P = 0.4) and in P/AI (P = 0.28) were observed between the categories.

Data on the relative abundance of *IL1B*, *IL6*, *IL8*, *TNFA*, and *IL10* mRNA are shown in Figure 5, while those on the relative abundance of *PGR*, *ESR1*, *ESR2*, *ITGB3*, *PTCH2*, *EGFR*, *HB-EGF*, and *COL4A1* mRNA are show in Figure 6. Neither time (day) nor parity or their interaction affected *IL6*, *IL10*, *ESR2*, *PTCH2*, *EGFR*, *COL4A1*, and *ITGB3* expression in uterine tissue (P > 0.04; Figures 4C, 4E, 5C, 5D, 5E, 5G, and 5H, respectively). However, primiparous cows had higher (P < 0.04) relative expression of *IL8* than multiparous cows (Figure 4D). The relative abundance of *IL1B* transcripts tended to be lower (P = 0.1; Figure 4B) in multiparous cows than in primiparous cows. In addition, significant differences in *TNFA*, *PGR*, and *HB-EGF* levels were found over time (days) and when considering the interaction between time (days) and categories (P < 0.05; Figures 4A, 5A, and 5F, respectively). *ESR1* level varied significantly as a function of time (days) (P = 0.0002; Figure 5B).

4. Discussion

In the present study, we compared energy metabolism, vaginal discharge, proportion of PMN cells in the uterus, gene expression for uterine receptivity, pro- and anti-inflammatory cytokine levels, and resumption of ovarian activity and fertility between primiparous and multiparous beef cows between 20 days before and 42 days after parturition. The hypothesis of this study was partially supported. Although no differences in energy status (BW, SFT, glucose and βHB), vaginal discharge, and proportion of PMN cells in the uterus were detected during the study period, the proportion of healthy cows at 35 DPP was lower in primiparous, while the proportion of PMN cells was higher in multiparous. Furthermore, primiparous cows had higher relative expression of *IL8* in the uterus than multiparous cows. Although the relationship between uterine health, metabolism, resumption of ovarian cyclicity, and fertility has already been well documented in dairy cows (WATHES *et al.*, 2007; GALVÃO *et al.*, 2011; LEBLANC, 2012; KASIMANICKAM *et al.*, 2013; KRAUSE *et al.*, 2014; DE OLIVEIRA MARQUES *et al.*, 2015; GRILLO *et al.*, 2015), to the best of our knowledge, this is the first

study to characterize the relationship of these variables with parity order in *B. indicus* beef cows raised under tropical conditions.

BW, SFT, and βHB did not differ significantly between the groups, varying only between days postpartum. In addition, previous studies have already reported that IAR is strongly related to BCS (PFEIFER *et al.*, 2017) and positively related to SFT in Nellore cows (PFEIFER and NEVES, 2018). Although multiparous cows had lower IAR than primiparous cows during the study period, the mean SFT and βHB did not differ between the groups. In contrast, Pfeifer *et al.* (2017) observed that primiparous beef cows had lower BCS, which was associated with lower IAR, than multiparous cows.

Glucose concentrations did not differ between the two categories, showing only small changes in their basal levels throughout the pre- and postpartum period. However, at parturition, the concentration of glucose increased, as was evident from the increase in glucagon, epinephrine, and cortisol levels, which are known to induce glycogenolysis and thereby increase the blood glucose level in dairy (VAZQUEZ-ANON *et al.*, 1994; GRUMMER, 1995) and beef (FERREIRA *et al.*, 2021) cows. However, these results did not translate into severe changes in energy metabolism.

Considering the entire period of evaluation in the present study, no significant differences in PVD and the proportion of PMN cells in the uterus were observed between the categories. However, these variables decreased over time after calving. In agreement with these results, the proportion of PMN cells in the uterus has been shown to decrease over time after calving (DPP) in dairy (PRUNNER *et al.*, 2014; TANAI *et al.*, 2020) and beef (SANTOS *et al.*, 2009; PFEIFER *et al.*, 2018; ANDRADE *et al.*, 2021) cows. In this study, we considered that cows with ≥4.75% PMN cells in uterine cytology and PVD = 0 had SCE at 35 DPP, as established by Andrade *et al.* (2021). We observed that at 35 DPP, 100% multiparous cows were healthy, whereas only 40% primiparous cows were healthy. Similarly, a cutoff point of 35 DPP has already been established for the inclusion of multiparous cows in TAI programs (PFEIFER *et al.*, 2018) because of their adequate fertility and low proportion of PMN cells in the uterus. Thus, the reduction in cytological inflammation in most multiparous cows with increase in the days postpartum reflects a decrease in the prevalence of endometritis, complete uterine involution, and consequently, an increased likelihood of pregnancy. Similarly, data from the present study demonstrated that multiparous cows have only 0.6% PMN cells in the uterus. However, for primiparous beef cows, this voluntary waiting period should perhaps be reconsidered because the mean proportion of PMN cells at 35 DPP in this category was 6%. Although the results of this study include uterine health data from primiparous cows, further studies should be performed with more animals to establish the relationship between the

calving-TAI interval and fertility in primiparous Nellore cows raised in extensive production systems.

In this study, we observed that a higher proportion of multiparous cows were considered healthy at 35 DPP. Furthermore, multiparous cows tended to ovulate more than primiparous cows in the study period. Studies have already shown that the immune response to uterine infections is affected by ovarian steroids (LEWIS, 1997; LEWIS, 2004). Therefore, the resumption of ovarian cyclicity and the consequent increase in estradiol concentrations aids the uterus to eliminate pathogens early, thereby decreasing the proportion of PMN cells in the uterus (SUBANDRIO *et al.*, 2000; DOREY *et al.*, 2011). These events may contribute to the high proportion of primiparous cows with SCE and low proportion of ovulations in the recent postpartum period.

Although some studies have shown that the postpartum uterine involution interval increases with the number of parturitions (ZAIN *et al.*, 1995; GONZALEZ SANCHEZ *et al.*, 1999; ZHANG *et al.*, 2010), others have suggested that cow uterine involution is not affected by the parity order (MIETTINEN, 1990; NOGUEIRA *et al.*, 1993). Our study demonstrated that the diameter of the uterine horns and endometrial area postpartum decreased more rapidly in primiparous than in multiparous cows. However, the faster reduction of uterine size observed in the primiparous cows was not associated with uterine health, as by 35 DPP, the primiparous cows had higher proportion of PMN cells in the uterus than the multiparous cows.

The trend in *IL1B* expression and the increase in *IL8* expression in the uterine tissue of primiparous cows may be associated with the increase in the proportion of PMN cells and the number of cows with endometritis in this category. This contradicts the results of Galvão *et al.* (2011), who reported that primiparous dairy cows expressed less *IL1β*, *IL8*, and *IL6* than multiparous cows during the postpartum period. From 21 days before parturition to 21 days after parturition, multiparous *B. indicus* cows had higher TNFα serum concentration than primiparous cows, albeit without significant differences in IL-8 expression between the categories (WANKHADE *et al.*, 2022). Although this study aimed to characterize differences between animal categories, the findings clearly demonstrated that the intensity of the inflammatory response in the immediate postpartum period was associated with high expression of pro-inflammatory cytokines, which correlated with higher proportion of PMN cells and PVD. Kasimanickam *et al.* (2013) proposed that the loss in body condition and the resulting postpartum metabolic and endocrine changes also prolonged the uterine inflammatory process.

The expression of genes related to endometrial repair and proliferative activity did not differ between the parity. The relative expression of *ESR1* and *PTCH2*, which increased around the days of first ovulation postpartum (21 to 35 DPP), did not differ between the two categories.

Studies have reported that gene expression in cattle endometrial tissue changes temporally according to the phase of the estrous cycle and is mainly orchestrated by the concentrations of E2, P4, and their receptors (FORDE et al., 2009; OKUMU et al., 2010; FORDE et al., 2011; MESQUITA et al., 2014; MESQUITA et al., 2015; SÁ FILHO et al., 2017). Moreover, enrichment of differentially expressed genes associated with tissue repair and proliferative activity at 30 DPP was observed by FOLEY et al. (2012). Therefore, the elevation in E2 concentration in proestrus is important for uterine modulation (MANN; LAMMING, 2000; MESQUITA et al., 2014; SÁ FILHO et al., 2017) and induction of P4 receptor synthesis in the endometrium (LAMMING et al., 1995; ROBINSON et al., 2001), which will prevent early luteolysis during the luteal phase and short cycles in beef cows (KIEBORZ-LOOS et al., 2003). Sá Filho et al. (2017) observed that uterine biopsies from anestrus cows supplemented with equine chorionic gonadotropin (eCG), which increases steroidogenesis in the ovary,showed higher expression of *ESR2* transcripts than that of *ESR1* transcripts. These changes on the estradiol receptors transcripts evidenced the establishment of a proliferative-to-secretory transitional phase. In addition, beef cows supplemented with estradiol cypionate showed increased expression of proliferation-related candidate genes, *PTCH2* and *COL4A1*, whereas *EGFR* expression was suppressed. However, our results showed that the relative expression levels of *EGFR, COL4A1*, and *ITGB3* transcripts increased only at 7 DPP, and that the expression of these genes was suppressed over the remaining study days, whereas the expression of *PTCH2* was high at 7, 35, and 42 DPP. We also observed that the relative expression levels of the *ITGB3* and *EGFR* did not differ between the two categories but varied over time (days). The expression of *HB-EGF* transcripts only differed over time (days). These results showed that transcripts of inflammatory cytokines and genes related to endometrial proliferative repair and activity are temporally expressed in the bovine endometrium during the postpartum period, with pro-inflammatory cytokine expression peaking mainly in the first weeks after parturition. This is unlike the expression dynamics of genes related to cell repair and proliferation in the uterus postpartum; for example, *PGR, ESR1*, and *PTCH2* expression increased after 28 DPP. To the best of our knowledge, this is the first study to compare endometrial proliferative activity between multiparous and primiparous postpartum beef cows. Evaluation of the expression patterns of these genes revealed important information regarding the repair process in the postpartum uterine mucosa, which may help in understanding the underlying molecular processes and in defining reproductive management strategies of postpartum beef cows.

In conclusion, the results from this study demonstrades that primiparous and multiparous beef cows display equivalent energy mobilization during the pre- and postpartum

periods. Multiparous cows have lower relative abundance of *IL8* transcripts and tend to express less *IL1B* in the uterine endometrium than primiparous cows. Moreover, the proportion of primiparous cows with healthy uterine status at 35 DPP and that ovulated until 42 DPP were lower than those of multiparous cows. Nevertheless, further field studies with higher number of animals should be performed to elucidate the impact of uterine health on the fertility of primiparous and multiparous cows raised in the Amazon Biome.

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Figures

Figure 1 - Evolution of body weight (A), subcutaneous fat thickness (B, internal angle of the rump (°C) and serum glucose concentration (D), βHB (E), albumin (F) and haptoglobin (G) (mean ± SEM) during the transition period in primiparous (n=15) and multiparous (n=9) beef cows from -20 to 42 days postpartum. Statistical analysis was performed for the pre and postpartum periods for the effects of category, days from calving and the interaction between category and days from calving.

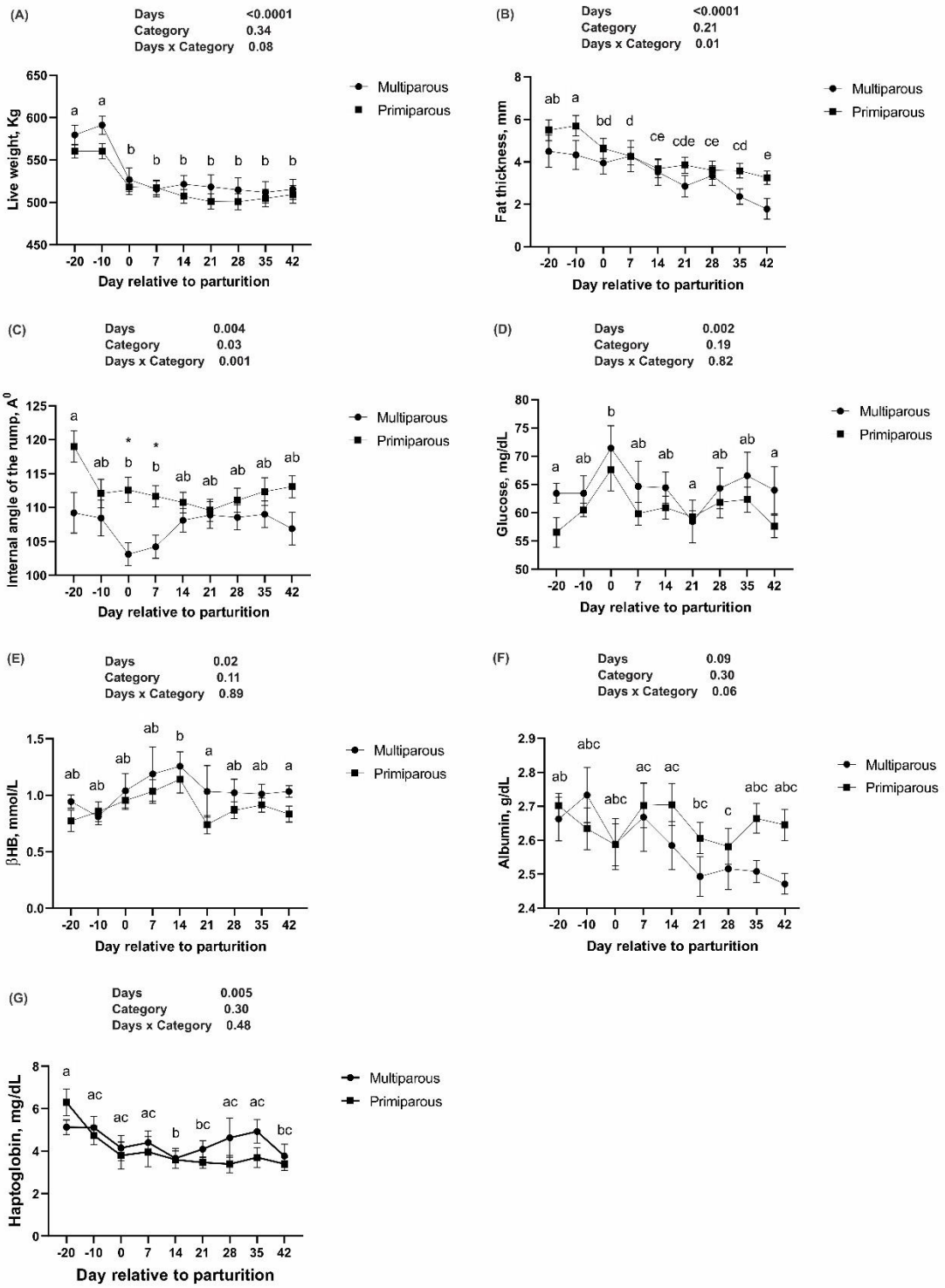

Figure 2 - Endometrial area (A) and uterine diameter (B) of primiparous (n=15) and multiparous (n=9) (mean ± SEM) beef cows from 7 to 42 days postpartum. Statistical analysis was performed for the pre and postpartum periods for the effects of category, days from calving and the interaction between category and days from calving.

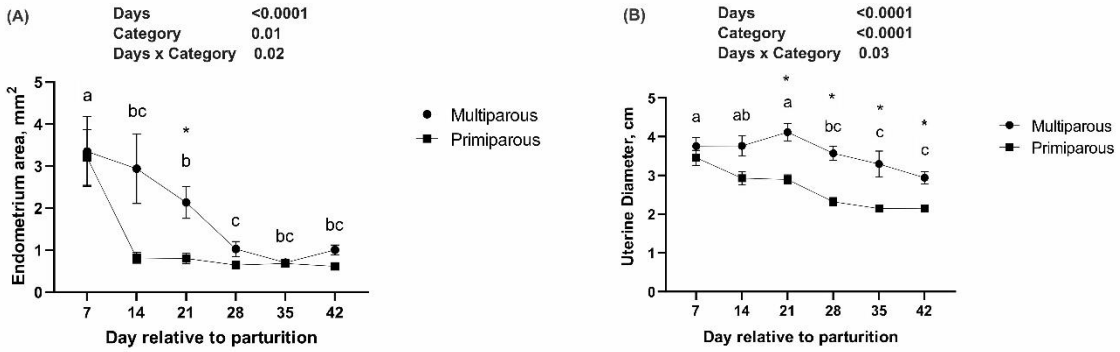

Figure 3 - Purulent vaginal discharge (A) and proportion of PMN (B) of primiparous (n=15) and multiparous (n=9) beef cows (mean ± SEM) from 7 to 42 days postpartum. Statistical analysis was performed for the pre and postpartum periods for the effects of category, days from calving and the interaction between category and days from calving.

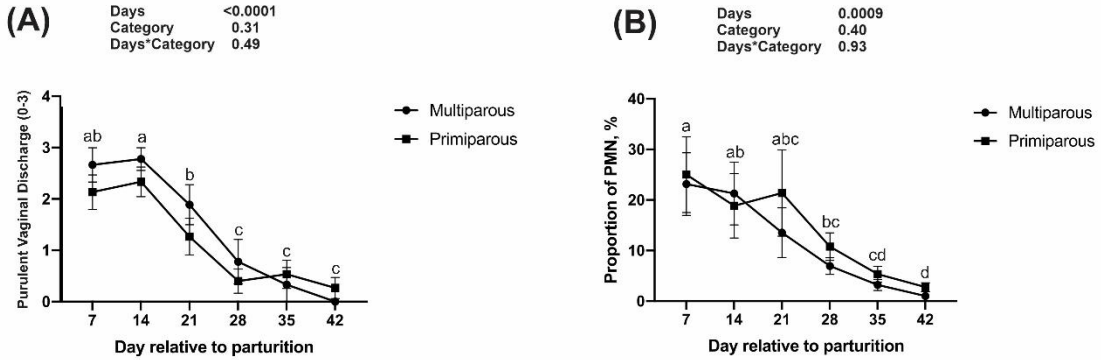

Figure 4 - Relative abundance of pro-inflammatory cytokine transcripts for tumor necrosis factor alpha (A; *TNFA*), interleukin 1 beta (B; *IL1β*), interleukin 6 (C; *IL6*) and interleukin 8 (D; *IL8*) and anti-inflammatory interleukin 10 (E; *IL10*) mRNA in endometrial tissues of primiparous and multiparous beef cows at 7 to 42 days postpartum. Statistical analysis was performed for the pre and postpartum periods for the effects of category, days from calving and the interaction between category and days from calving.

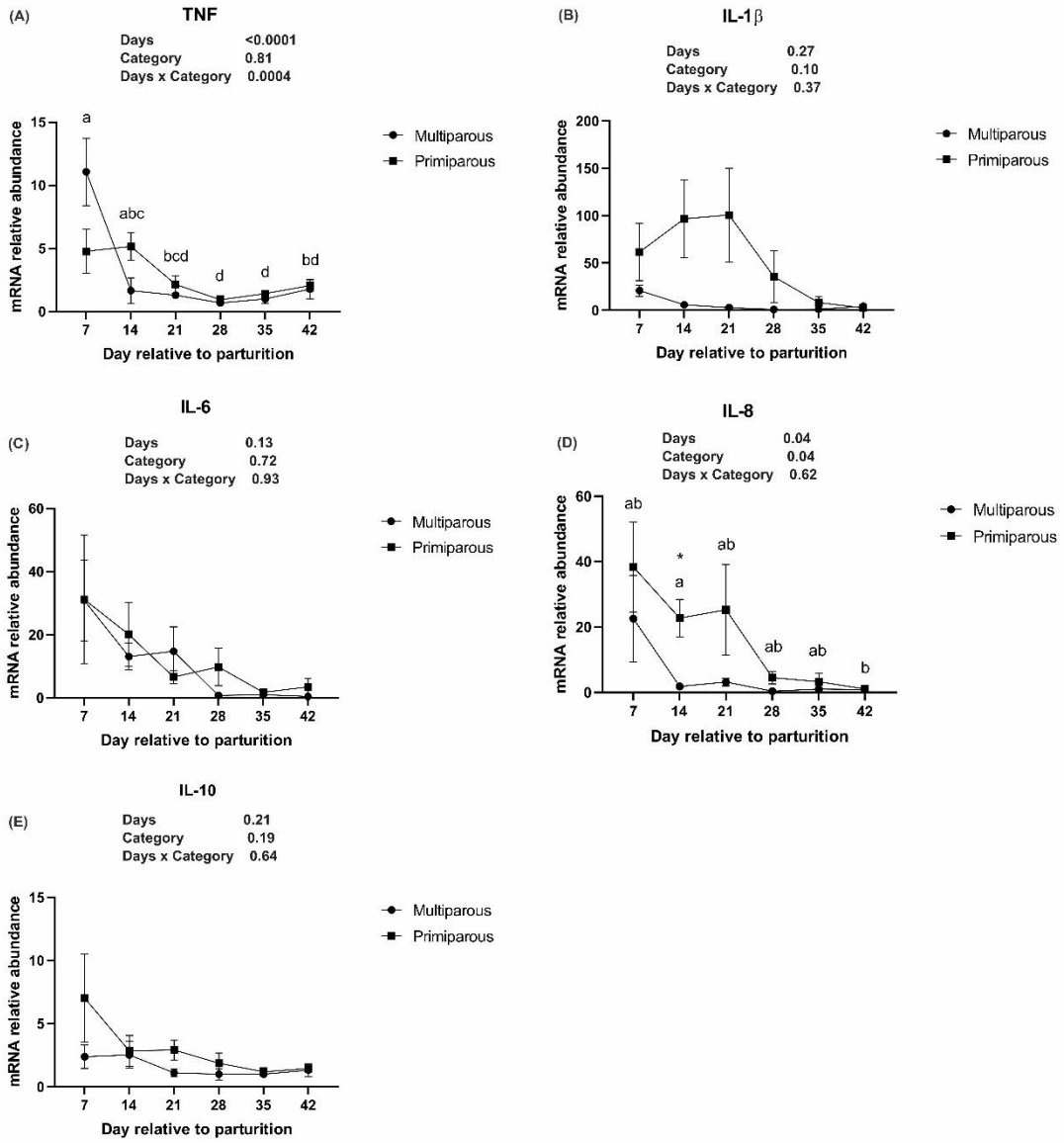

Figure 5 - Relative abundance of gene transcripts for regulation of cell proliferation progesterone receptor (A; *PGR*), estrogen receptor 1 (B; *ESR1*), estrogen receptor 2 (C; *ESR2*) Patched 2a (D; *PTCH2*), epidermal growth factor receptor (E; E*GFR*) heparin-binding EGF-like growth factor (F; HB-EGF), and basement membrane component (G; *COL4A1*) and Integrin beta 3 (H; *ITGB3*) mRNA in endometrial tissues of primiparous and multiparous beef cows from 7 to 42 days postpartum. Statistical analysis was performed for the pre and postpartum periods for the effects of category, days from calving and the interaction between category and days from calving.

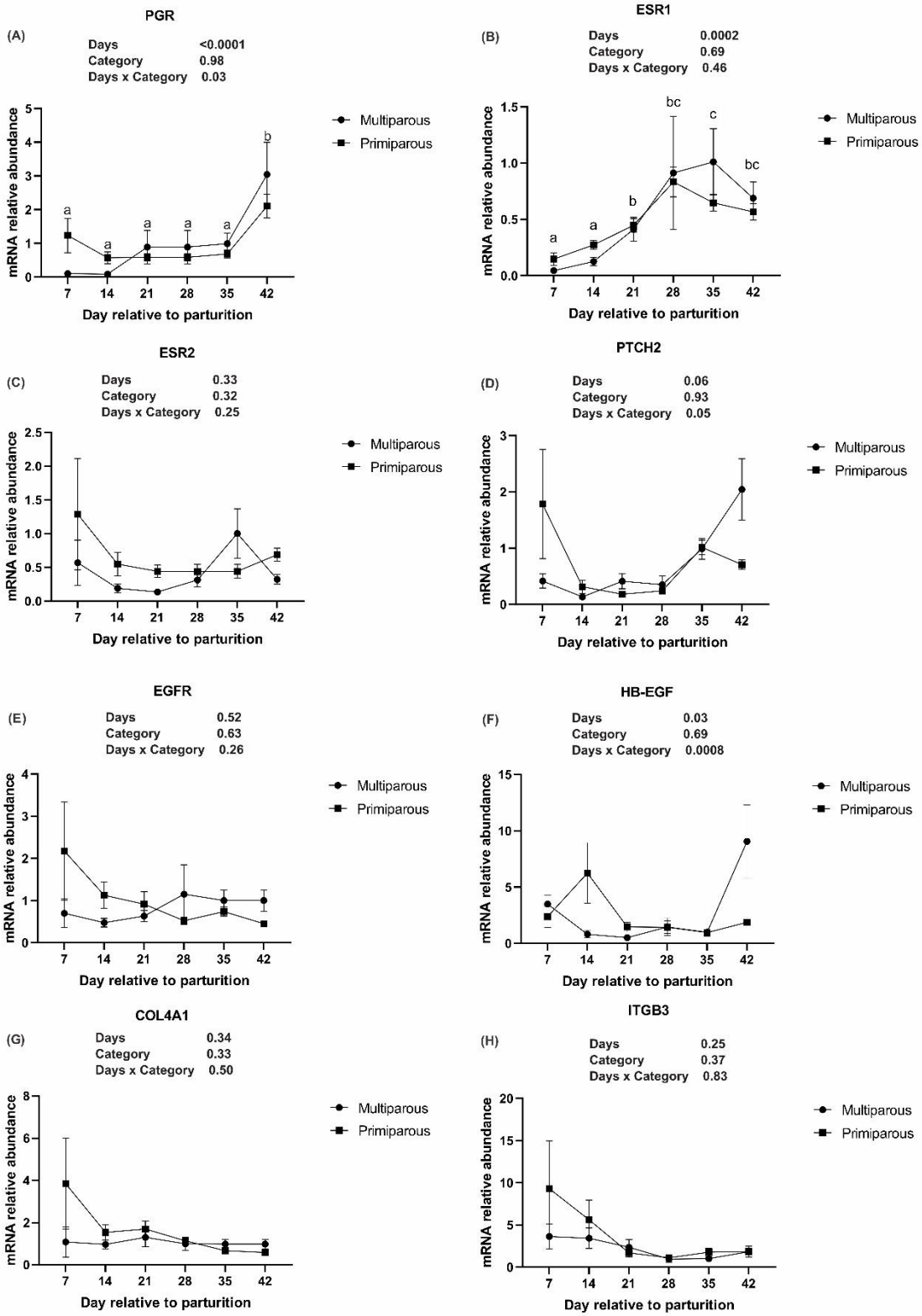

Table 1 - Primer sequences for quantitative real-time polymerase chain reaction (qRT-PCR) amplification of mRNA.

Gene Name	Gene ID	Sequence Forward	Sequence Reverse	Function
Interleucin 1 Beta	*IL1β*	CAAGGAGAGGAAAGAGACA	TGAGAAGTGCTGATGTACCA	Cytokine
Interleucin 6	*IL6*	CCAGGAACGAAAGAGAGC	CAGAAGTCATCACCAGGAG	Cytokine
Interleucin 8	*IL8*	CAAGAGCCAGAAGAAACCTGAC	AGTGTGGCCCACTCTCAATAAC	Cytokine
Interleucin 10	*IL10*	AGAACCACGGGCCTGACATAGCTCACTGAAGACTCTCTTCACCTT	TTCTGCCCTGCGAAAACAAGAGCAA	Cytokine
Factor growth tumoral alpha	*TNFA*	TCTTCTCAAGCCTCAAGTAACAAGTCCATGAGGGCATTGGCATAC	AGCCCACGTTGTAGCCGACATCAACTC	Cytokine
Progesterone receptor	*PGR*	GCCGCAGGTCTACCAGCCCTA	GTTATGCTGTCCTTCCATTGCCCTT	Regulation of cell proliferation in the uterus
Estrogen receptor 1	*ESR1*	CAGGCACATGAGCAACAAAG	TCCAGCAGCAGGTCGTAGAG	Regulation of cell proliferation in the uterus
Estrogen receptor 2	*ESR2*	TCACGTCAGGCACGCCAGTAAC	CACCAGGTTGCGCTCAGACCC	Regulation of cell proliferation in the uterus
Integrin, beta 3	*ITGB3*	GGGAGAGTGCTATGGTTAGA	CTTCACAAGACACCCAAGAG	Cell proliferation and integrity of the endometrial mucosa
Patched 2[a]	*PTCH2*	CATCCTGCTGCTGTGTACTT	ATCGCCAGGACCAGTACTAT	Cell proliferation Embryogenesis
Epidermal growth factor receptor	*EGFR*	ATGCTCTATGACCCTACCAC	TTCCGTTACAAACTTTGCCA	Cell growth factor
Heparin binding EGF-like growth factor	*HB-EGF*	CATCCACGGAGAATGCAAATAC	CAGCAGACAGACGGATGATAG	Cell growth factor
Collagen, type IV, α 1	*COL4A1*	CACGGCTACTCTTTGCTCTAC	GAAGGGCATGGTACTGAACTT	Basement membrane component
Glyceraldehyde-3-Phosphate Dehydrogenase	*GAPDH*	ACACTGAGGACCAGGTTG	TGGTCGTTGAGGGCAATG	Reference genes
Actin β	*ACTB*	AGGCATCCTGACCCTCAAGTA	GCTCGTTGTAGAAGGTGTGGT	Reference genes

Table 2 - Uterine, ovarian and fertility responses in primiparous and multiparous beef cows at different postpartum days.

Item	Category		P-value
	Primiparous	Multiparous	
Proportion of PMN[a] cells at 35 DPP[b] (%)	6 ± 1.1%	0.6 ± 0.2%	0.002
Proportion of healthy cows at 35 DPP (%)	40.0% (6/15)	100% (9/9)	0.003
Proportion of cows with PMN ≤ 4,75% at 35 DPP (%)	53.3% (8/15)	100% (9/9)	0.03
Proportion of with PVD[c] = 0 at 35 DPP	73.34% (11/15)	100% (9/9)	0.08
Proportion of ovulated cows, % (n/total)[d]	13.3 (2/15)	44.4% (4/9)	0.08
Parturition interval - first ovulation (days)	28 ± 7.0	33.2 ± 1.7	0.4

[a]Polymorphonuclear
[b] Postpartum days [c]Purulent vaginal discharge
[d]Cows ovulated until the beginning of the FTAI protocol (around 42 DPP)

CONCLUSÕES

Os dados apresentados neste estudo abrangeram a saúde uterina pós-parto e parâmetros de fertilidades em bovinos em vacas de corte. Em um primeiro momento foi avaliada a relação entre a saúde uterina e a fertilidade em vacas de corte. Os resultados deste estudo mostraram que a proporção de prenhez é maior em vacas com menor proporção de células polimorfonucleares no útero e vacas que tinham um menor intervalo entre o parto e a IATF, apresentaram mais inflamação uterina, com maior proporção de células polimorfonucleares e maior expressão relativa de transcritos para *IL-1β* e *IL-8*. Consequentemente, este grupo de vacas apresentou menor prenhez na IATF P/IA em resposta ao protocolo IATF. Em um segundo momento foi avaliada o a relação da categoria animal na saúde uterina, metabolismo energético, o retorno à ciclicidade e fertilidade no período pré- e pós-parto. Neste estudo observou-se que vacas de corte primíparas e multíparas tiveram equivalente mobilização de energia durante o pré e pós-parto e que vacas multíparas ovularam mais cedo e tiveram melhor saúde uterina do que vacas primíparas. Além disso, uma maior proporção de vacas multíparas apresentou condição uterina sadia aos 35 dias pós-parto. Este conjunto de resultados evidencia a importância da condição uterina para o desempenho reprodutivo em programas de IATF de vacas de corte pós-parto criadas em regiões tropicais sob o sistema intensivo, sendo a condução de mais estudos com abordagens que envolvam maior número de animais contribuirão para elucidar o impacto da saúde uterina na fertilidade, principalmente em vacas primíparas e multíparas.

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ANEXOS

ANEXO A – Comprovante de publicação de artigo.

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Uterine health and fertility of timed AI postpartum Nelore beef cows raised in the Amazon biome

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HIGHLIGHTS

- Early postpartum beef cows subjected to TAI protocols with a higher proportion of polymorphonuclear cells have lower pregnancy per AI (P/AI).
- Postpartum beef cows subjected to TAI protocols with a higher discharge have greater proinflammatory cytokines genes expression in the endometrial tissue.
- The longer is the interval from calving to cytological uterine sampling, the lower is the proportion of PMN cells in the uterus.

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PMN cells

ABSTRACT

This study evaluated the effect of uterine health on the fertility of postpartum beef cows. Multiparous lactating Nelore cows (*Bos indicus*; n = 155) were subjected to a timed artificial insemination (TAI) protocol. Cows were included in a progesterone-estradiol based TAI protocol. On Day 0, before the beginning of the protocol, cows were subjected to evaluation of purulent vaginal discharge (PVD) and collection of uterine tissue using the cytobrush technique. Slides for polymorphonuclear (PMN) cells counting were prepared before the same cytobrush was used for RNA extraction and analysis of IL-1β, IL-6, IL-8, TNFα, GAPDH, and βactin gene transcription. Cows were separated into 4 categories according to the proportion of PMN and PVD: 1) Healthy (HE; n = 87), cows with ≤ 4.75% PMN and PVD of 0; 2) Positive Purulent Vaginal Discharge (PVD+; n = 24), cows with ≤ 4.75% PMN and PVD ≥ 1; 3) Subclinical Endometritis (SCE; n = 29), cows with > 4.75% PMN and PVD of 0, and; 4) Subclinical Endometritis + PVD+ (SCEP; n = 15), cows with > 4.75% PMN and PVD ≥ 1. Cows in the HE group had a greater ($P < 0.05$) pregnancy per AI (P/IA) than cows in SCE and SCEP groups. However, P/AI in cows from the PVD+ group was not different in comparison to HE and SCE groups ($P > 0.05$). Cows from the SCE and SCEP groups had a higher ($P < 0.001$) proportion of PMN cells in the uterus than HE and PVD+ groups. Relative transcription of IL-6 and TNFα did not change among groups ($P > 0.05$). In contrast, transcription of IL-8 was greater ($P = 0.02$) in SCEP than HE and SCE groups. The relative transcription IL-1β was greater ($P < 0.05$) in SCEP than in PVD+, but the cows in the HE and SCE groups were not different from the other groups ($P = 0.08$). Our results demonstrate that postpartum beef cows, raised in the Amazon biome, with a higher proportion of PMN cells have lower fertility. Moreover, ~ 40% of the postpartum cows included in TAI programs early postpartum had suboptimal uterine conditions (subclinical endometritis, purulent vaginal discharge, or both).

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ANEXO B – Diretrizes (guidelines/guide of authors) da revista Livestock Science para formatação de artigos e autorização da publicação em tese acadêmica (item Submission declaration and verification).

ELSEVIER

LIVESTOCK SCIENCE
An International Journal

AUTHOR INFORMATION PACK

TABLE OF CONTENTS

- **Description** p.1
- **Audience** p.1
- **Impact Factor** p.1
- **Abstracting and Indexing** p.2
- **Editorial Board** p.2
- **Guide for Authors** p.4

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DESCRIPTION

Livestock Science promotes the sound development of the **livestock sector** by publishing original, peer-reviewed research and review articles covering all aspects of the broad field of animal production and animal science. The journal welcomes submissions on the avant-garde areas of **animal genetics**, **breeding**, **growth**, **reproduction**, **nutrition**, **physiology**, and **behaviour** in addition to **genetic resources**, **welfare**, **ethics**, **health**, **management** and **production systems**. The high-quality content of this journal reflects the truly international nature of this broad area of research. Submissions focusing on diagnosis, disease treatments and epidemiology are not welcomed and works entirely based on either laboratory work or laboratory animals are only rarely considered. Papers presenting reviews and meta-analyses must ensure that they provide new insights to our readers. When the novelty of the research presented (including meta-analyses) is due to the methods used, authors are encouraged to classify their works as either Short Communications or Technical Notes.

AUDIENCE

Animal Scientists, Animal Breeders.

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2. Review Articles
3. Short Communications
4. Position Papers
5. Technical Notes
6. Book Reviews

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The repeated writing of chemical formulae in the text is to be avoided where reasonably possible; instead, the name of the compound should be given in full. Exceptions may be made in the case of a very long name occurring very frequently or in the case of a compound being described as the end product of a gravimetric determination (e.g. phosphate as P_2O_5).

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Strunk Jr., W., White, E.B., 2000. The Elements of Style, fourth ed. Longman, New York.
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Mettam, G.R., Adams, L.B., 2009. How to prepare an electronic version of your article, in: Jones, B.S., Smith , R.Z. (Eds.), Introduction to the Electronic Age. E-Publishing Inc., New York, pp. 281–304.
Reference to a website:

Cancer Research UK, 1975. Cancer statistics reports for the UK. http://www.cancerresearchuk.org/aboutcancer/statistics/cancerstatsreport/ (accessed 13 March 2003).
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Coon, E., Berndt, M., Jan, A., Svyatsky, D., Atchley, A., Kikinzon, E., Harp, D., Manzini, G., Shelef, E., Lipnikov, K., Garimella, R., Xu, C., Moulton, D., Karra, S., Painter, S., Jafarov, E., & Molins, S., 2020. Advanced Terrestrial Simulator (ATS) v0.88 (Version 0.88). Zenodo. https://doi.org/10.5281/zenodo.3727209.

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