# UNIVERSIDADE FEDERAL DE UBERLÂNDIA

# FACULDADE DE MEDICINA VETERINÁRIA

# EFEITO IMUNOMODULADOR DOS ESTEROIDES OVARIANOS NAS RESPOSTAS INFLAMATÓRIAS LOCAL E SISTÊMICA DE VACAS DESAFIADAS COM INFUSÃO INTRAUTERINA DE LIPOPOLISSACARÍDEO

**Layane Queiroz Magalhães**

Médica Veterinária

UBERLÂNDIA – MG – BRASIL

Setembro - 2022

**UNIVERSIDADE FEDERAL DE UBERLÂNDIA**

**FACULDADE DE MEDICINA VETERINÁRIA**

**EFEITO IMUNOMODULADOR DOS ESTEROIDES OVARIANOS NAS RESPOSTAS INFLAMATÓRIAS LOCAL E SISTÊMICA DE VACAS DESAFIADAS COM INFUSÃO INTRAUTERINA DE LIPOPOLISSACARÍDEO**

**Layane Queiroz Magalhães**

**Orientador: Prof. Dr. João Paulo Elsen Saut**
**Coorientador: Prof. Dr. Marcelo José Barbosa Silva**

Tese apresentada à Faculdade de Medicina Veterinária – UFU, como parte das exigências para a obtenção de título de doutora em Ciências Veterinárias (Saúde Animal).

UBERLÂNDIA – MG – BRASIL

**DADOS CURRICULARES DO AUTOR**

**Layane Queiroz Magalhães –** nascida na cidade de Patos de Minas, estado de Minas Gerais, aos quinze dias do mês de julho de um mil novecentos e oitenta e nove. Ingressou no curso de Medicina Veterinária da Universidade Federal de Viçosa (UFV) em março do ano de 2009, tendo concluído o curso em março de 2014. Em fevereiro de 2014 foi aprovada no Programa de Residência Uniprofissional em Medicina Veterinária da Universidade Federal de Uberlândia (UFU), na área de Clínica Médica e Cirúrgica de Grandes Animais, concluindo em março de 2016. Neste mesmo mês, após a aprovação, ingressou no curso de Mestrado pelo Programa de Pós-Graduação em Ciências Veterinárias da UFU, na subárea de Clínica Médica e Investigação Etiológica, sendo bolsista pela Comissão de Aperfeiçoamento de Pessoal do Nível Superior (CAPES) durante todo o período do curso, em que trabalhou com doenças respiratórias dos bovinos (DRB), buscando identificar a prevalência e a importância econômica desta doença nos rebanhos de bovinos de corte no Brasil, além de empenhar no entendimento dos principais fatores de risco e estudo de alguns métodos preventivos para as DRB. Foi docente no Centro Universitário de Patos de Minas (Unipam, 2016-2020) e Médica Veterinária responsável do Setor de Grandes Animais do Centro Clínico Veterinário (Unipam, 2018-2020), onde se dedicou à rotina de clínica médica e cirúrgica de grandes animais. Em março de 2018, ingressou no curso de Doutorado pelo PPGCV-UFU, na subárea de Clínica Médica, sendo bolsista pela Comissão de Aperfeiçoamento de Pessoal do Nível Superior (CAPES) no primeiro semestre de curso. Ao longo do doutorado trabalhou com doenças uterinas no pós-parto de vacas e imunidade uterina, buscando melhor entendimento do efeito dos hormônios esteroides ovarianos sobre a imunidade inata uterina. Tem se dedicado à pesquisa clínica (2020-atual), estando à frente da área de estudos clínicos da PEC AGRO Saúde Animal (2020-2022) e, atualmente, em seu novo centro de pesquisas Lume Saúde Animal (2022-atual).

*"Mas na profissão, além de amar tem de saber.*

*E o saber leva tempo pra crescer."*

*Rubem Alves*

# AGRADECIMENTOS

Agradeço a Deus, por me permitir trilhar esse caminho ao lado de pessoas sábias e acolhedoras.

Meus pais, Alda e Alaerson, que estiveram ao meu lado em todas as decisões e, com amor, permitiram-me ir sempre além.

Meu esposo, Gabriel, por sonharmos juntos, por acreditar em mim, por me encorajar e oferecer o maior amor do mundo.

Meu orientador, Prof. Dr. João Paulo Elsen Saut, pela mentoria, que foi além deste projeto e da Universidade, permitindo tornar-me uma melhor Médica Veterinária com seu exemplo de dedicação e entusiasmo.

Meu coorientador, Prof. Marcelo Silva e, ao Laboratório de Imunoparasitologia, pela disponibilidade, por todo auxílio ao longo do desenvolvimento deste projeto.

Professores do Setor de Grandes Animais, Prof. Dr. Diego Delfiol e Prof. Dr. Geison Nogueira, que participaram de perto de todo esse caminho e contribuíram diretamente com este projeto.

Colegas do LASGRAN, Sara Barbosa, Mariana Almeida, Nádia Fagundes, e à Amanda Nonato e à Naiara Fagundes, por todo empenho, toda presença na execução laboratorial do projeto. Foi desafiadora, mas, com vocês, fomos além do almejado.

Luisa Carneiro, pelas conversas e delineamentos futuros que ainda faremos. Mas, principalmente, pela revisão final de tudo que está neste arquivo, contribuindo para finalizarmos com excelência.

Meus colegas de HV-UFU e de LASGRAN, Residentes Brenda Fernandes, Fernanda Augusta, Kamila Paim, Tatiane Prado, Jordana Cabral e Pedro Casadei, e colega de residência e mestrado, Bruna Souza. A todos os alunos de graduação que participaram do manejo dos animais ou procedimentos laboratoriais, em especial, William Valadares, Rafaela Ribeiro e Marco Antônio Cunha, muito obrigada! Sem vocês nada disso teria se cumprido.

Colegas de pós-graduação, Amanda Rezende, Paula Alvarenga e Beatryz Fonseca pelos dias de UFU divididos.

# SUMÁRIO

# EFFEITO IMUNOMODULADOR DOS ESTEROIDES OVARIANOS NAS RESPOSTAS INFLAMATÓRIAS LOCAL E SISTÊMICA DE VACAS DESAFIADAS COM INFUSÃO INTRAUTERINA DE LIPOPOLISSACARÍDEO

**RESUMO –** No pós-parto de vacas leiteiras, as infecções uterinas promovem uma inflamação local e sistêmica e interferem na eficiência reprodutiva. Diversas interações sistêmicas geram uma resposta inflamatória prévia aos sinais clínicos das doenças uterinas, o que eleva alguns fatores inflamatórios, como proteínas de fase aguda e citocinas inflamatórias. O objetivo deste estudo foi avaliar o efeito dos esteroides ovarianos, progesterona (P4) e estradiol (E2), na resposta inflamatória sistêmica de vacas desafiadas com infusão intrauterina de lipopolissacarídeo (LPS), e na expressão gênica dos receptores de progesterona (PGR), estrogênio α (ESR1), de interleucina 1β (IL1β), interleucina 6 (IL6) e ligante de quimiocina 5 (CCL5) em vacas Gir ovariectomizadas expostas a esses hormônios. Para isso, foram executados quatro grupos experimentais: Grupo 1: grupo controle (n = 8) – as vacas não receberam administração hormonal antes do dia +1; Grupo 2: 24h - E2 (n = 8) – as vacas receberam E2 24 horas (dia -1) antes do desafio com LPS; Grupo 3: 24h - P4 (n = 8) – as vacas receberam P4 24 horas (dia -1) antes do desafio com LPS; Grupo 4: 14d - P4 (n = 8) – as vacas receberam P4 14 dias (dia -14) antes do desafio com LPS. Quatro vacas (n = 4) foram desafiadas (dia 0) com 20 mL de NaCl 0,9% + 12,5 mg/kg LPS, e quatro vacas (n = 4) foram desafiadas (dia 0) com 20 mL de NaCl 0,9%. No dia -14 (14d - P4), dias 0 e dias +1 de cada etapa experimental foram realizados exame clínico e do trato genital feminino, coleta de sangue para dosagem hormonal (P4 e E2) e amostras endometriais para análise de expressão gênica (ESR1, PGR, IL1β e IL6). Para dosagem hormonal (P4 e E2), as amostras de sangue também foram coletadas no dia -1. Na resposta sistêmica ao LPS, observou-se aumento da haptoglobina (Hp) (grupo controle; 24 h - grupo P4; 14 d - grupo P4) e no dia +1 a Hp do grupo 14 d - P4 foi maior quando comparado aos outros grupos. No dia 0, no grupo 14 d - P4, leucócitos e linfócitos apresentaram maiores valores quando comparados ao grupo controle (P < 0,01). No dia +1, após o desafio com LPS, no grupo 14 d - P4 foi observada redução de linfócitos, eosinófilos e monócitos circulantes (P < 0,05). Também foi observada neutrofilia com desvio à esquerda nos dois tratamentos com P4 (dia +1), além de trombocitopenia e plaquetas mais baixas em relação ao grupo 24 h - E2 (P < 0,05) (dia 0). Uma regulação negativa da expressão gênica de PGR e ESR1 foi verificada quando as vacas foram expostas à P4 por um longo período; a exposição ao E2 não interferiu na expressão de PGR e ESR1. As concentrações de P4 foram capazes de regular a expressão de IL6 no grupo 14d - P4, e quando comparado ao grupo 24h - E2, houve diferença significativa na expressão de IL1β e IL6. A expressão de CCL5 apresentou uma regulação negativa apenas no grupo 24h - E2. Concluiu-se que vacas Gir ovariectomizadas desafiadas com LPS, previamente expostas aos hormônios esteroides, induzem uma resposta inflamatória sistêmica. Além disso, a resposta sistêmica é mais intensa após exposição prolongada prévia ao P4 e menos intensa após exposição ao E2. Além disso, as concentrações de P4 e E2 regulam a expressão dos receptores de esteroides ovarianos (ESR1 e PGR). Uma exposição a longo prazo a P4 regulou positivamente a expressão gênica de IL1β e IL6, enquanto o estradiol regulou negativamente a expressão do gene CCL5. Este

estudo forneceu uma importante informação sobre o efeito dos esteroides ovarianos na resposta inflamatória sistêmica e uterina local de vacas desafiadas com LPS intrauterino, após exposição aos hormônios esteroides ovarianos.

**Palavras-chave**: citocinas pró-inflamatórias, estradiol, hemograma, imunidade, progesterona

# IMMUNOMODULATOR EFFECT OF OVARIAN STEROIDS ON LOCAL AND SYSTEMIC INFLAMMATORY RESPONSES OF COWS CHALLENGED WITH LIPOPOLYSACCHARIDE INTRAUTERINE IFUSION

**ABSTRACT –** In dairy cows postpartum, uterine infections promote a local and systemic inflammation and interfere in reproductive efficiency. Several systemic interactions generate a previous inflammatory response to the clinical signs of uterine diseases, which elevates some inflammatory factors, such as acute phase proteins and inflammatory cytokines. The aim of this study was to evaluate the effect of steroid hormones including progesterone (P4) and estradiol (E2) on systemic inflammatory response of cows challenged with an intrauterine infusion of lipopolysaccharide (LPS), and the response of estrogen receptor α (ESR1), progesterone receptor (PGR), interleukin 1β (IL1β), interleukin 6 (IL6), and chemokine ligand 5 (CCL5) gene expression in ovariectomized Gir cows exposed to these hormones. For this, four experimental groups were executed as: Group 1: control group (n = 8) – the cows did not receive hormonal administration before day +1; Group 2: 24h - E2 (n = 8) – the cows received E2 24 hours (day -1) before LPS challenge; Group 3: 24h - P4 (n = 8) – the cows received P4 24 hours (day -1) before LPS challenge; Group 4: 14d - P4 (n = 8) – the cows received P4 14 days (day -14) before LPS challenge. Four cows (n = 4) were challenged (day 0) with 20 mL of 0.9% NaCl + 12.5 mg/kg LPS, and four cows (n = 4) were challenged (day 0) with 20 mL of 0.9% NaCl. On day 0 and day +1 and day -14, clinical and female genital tract examination, blood sampling for hormonal dosage (P4 and E2) and endometrial samples for gene expression analyses (ESR1, PGR, IL1β and IL6) were performed. For hormonal dosage (P4 and E2), blood samples were collected on day -1. In the systemic response to LPS, an increase in haptoglobin (Hp) (control group; 24 h - P4 group; 14 d - P4 group) was observed, and on day +1 the Hp of 14 d - P4 group was higher when compared to the other groups. On day 0 in the 14 d - P4 group leukocytes and lymphocytes were higher when compared to control group (P < 0.01). On day +1 after LPS-challenge in the 14 d - P4 group a decrease in circulating lymphocytes, eosinophils, and monocytes (P < 0.05) was related. A neutrophilia with left shift in the two treatments with P4 (day +1), in addition to a thrombocytopenia and lower platelets compared to the 24 h - E2 group (P < 0.05) (day 0) were also recorded. A down regulation of PGR and ESR1 gene expression was verified when P4 was exposure for a long period; the exposure to E2 did not interfere with both PGR and ESR1 genes expression. Concentrations of P4 were capable to up-regulate the IL6 gene expression at 14d - P4 exposition, and when compared to the 24h - E2 exposition group, there was a significant difference in the expression of IL1β and IL6. The CCL5 gene expression was down-regulated only by the exposure to 24h - E2 It was concluded that ovariectomized Gir cows challenged with LPS, previously submitted to steroid hormones induce a systemic inflammatory response. Also, the systemic response is more intense after previous prolonged exposure to P4 and less intense after exposure to E2. Also, the concentrations of P4 and E2 regulate the ovarian steroid receptors (ESR1 and PGR) expression. Still, a long-term exposure to P4 up-regulated IL1β and IL6 gene expression while estradiol down-regulated CCL5

gene expression. This study provided an important information relating the effect of ovarian steroids on the systemic and local uterine inflammatory response of cows challenged with intrauterine LPS, after exposed to ovarian steroids hormones.

**CAPÍTULO 1 – CONSIDERAÇÕES GERAIS**

O Brasil é o terceiro maior produtor de leite do mundo, com a maior parte de seu rebanho constituída por animais híbridos (Holandês X Gir), que produziram 80% dos 25,4 bilhões de litros produzidos em 2021, liderada pelo Estado de Minas Gerais com 24,5% da captação de leite no País (EMBRAPA, 2022; LA FALCE, 2015). No segundo semestre de 2019, o mercado brasileiro abriu portas para exportação de leite, iniciando pelo mercado chinês e em negociação com o mercado indiano já para o primeiro semestre de 2020, mostrando a importância da produção e melhoria da produtividade agropecuária do País.

Apesar do aumento de quase 70% na produtividade ao longo dos últimos 50 anos, a pecuária leiteira brasileira ainda passa por alguns desafios como alto custo de produção e baixas taxas de fertilidade. Dentre os fatores que contribuem para esses números estão as doenças uterinas no pós-parto, que elevam o período de serviço, aumentam os custos com medicação e atendimento veterinário, bem como perdas na produção e altas taxas de descarte involuntário (LEBLANC *et al.*, 2002; LEWIS, 1997; SHELDON; OWENS, 2017).

Há crescente busca por alternativas de prevenção e tratamento objetivando reduzir o uso excessivo de antimicrobianos no período pós-parto de vacas leiteiras e auxiliar no índice de cura destas doenças. Além das perdas econômicas supracitadas, há necessidade de garantir melhor qualidade de vida ao animal com um pós-parto saudável, sem dor e sofrimento devido às infecções uterinas, tratamento adequado e maior longevidade dentro da propriedade leiteira.

O pós-parto saudável está diretamente ligado ao período de transição das vacas, momento em que ocorrem algumas alterações metabólicas e hormonais que contribuem para a redução da função imune (LEBLANC, 2012). Alguns estudos realizados *in vivo* sugerem que os esteroides ovarianos, o estradiol (E2) e a progesterona (P4), têm impacto na ocorrência das doenças nesse período, podendo regular o sistema imune (ROWSON; LAMMING; FRY, 1953). Os resultados sugerem que o E2 apresente um efeito protetor, enquanto a P4 tenha um efeito imunossupressor (LEWIS, 2003a, 2004; ROWSON, 1951).

No entanto, em uma pesquisa realizada por Saut *et al.* (2014) demonstrando estes efeitos dos hormônios ovarianos, em cultivos celulares do epitélio e estroma endometrial por meio de explantes endometriais, verificou-se que os resultados não foram parecidos com os encontrados anteriormente na literatura (LEWIS, 2003a, 2004; ROWSON, 1951), ao se avaliar a resposta de alguns mediadores inflamatórios e respectivas expressões gênicas, quando submetidos ao desafio com lipopolissacarídeos e *Escherichia coli*.

A hipótese deste estudo foi que os efeitos inflamatórios sistêmicos e a resposta imune inata uterina, são mais intensos sob a predominância da P4 e reduzidos sob efeito do E2. Consequentemente, o objetivo deste estudo foi avaliar efeito da exposição prévia aos hormônios esteroides ovarianos (P4 e E2) na resposta inflamatória sistêmica e uterina local, após o desafio com a infusão intrauterina com LPS em um modelo experimental utilizando vacas primíparas da raça Gir ovariectomizadas.

## A importância da produção leiteira no Brasil

No contexto em que 800 milhões de pessoas sofrem com a escassez de alimentos, a Organização das Nações Unidas para Alimentação e Agricultura informou sobre a necessidade de aumentar a produção mundial de alimentos em 70% até 2050, devido ao crescimento da população (FAO, 2009).

No Brasil, a maior parte do rebanho é constituída de animais híbridos e, de acordo com a EMBRAPA (2003), o estado de Minas Gerais produz 24,5% da produção leiteira do Brasil, tornando o estado um importante polo da agropecuária nacional.

Neste contexto, é importante ressaltar que a produção leiteira representa uma parcela significativa na composição da dieta alimentar humana e, consequentemente, na segurança alimentar mundial, já que o leite é um alimento nutricionalmente completo, sendo rico em nutrientes essenciais ao crescimento e à manutenção de uma vida saudável (VILELA, 2002).

Apesar dos avanços significativos na indústria de laticínios ao longo dos últimos 50 anos, com aumento de quase 70% na produtividade, a fecundidade dos

animais ainda é um fator a ser enfrentado para melhorar a sustentabilidade e minimizar o impacto ambiental da indústria (HUME; WHITELAW; ARCHIBALD, 2011).

**Doenças uterinas no pós-parto de vacas leiteiras**

A função do útero é muitas vezes comprometida em bovinos pela contaminação bacteriana do lúmen uterino após o parto, havendo persistência das bactérias patogênicas que causam as doenças uterinas. Por isso, pesquisas relativas à progesterona e à resposta imune trazem grandes informações sobre a ocorrência de infecção uterina, que é, em parte, dependente do ambiente endócrino (LEWIS, 2003a). Ademais, algumas pesquisas têm demonstrado que micro-organismos desregulam a função endócrina do trato reprodutivo de vacas (HERATH; LILLY; FISCHER; *et al.*, 2009; SHELDON *et al.*, 2002).

O período pós-parto é o período de maiores alterações no metabolismo de vacas leiteiras, pois 75% das doenças ocorrem nos primeiros 30 DPP. Essa ocorrência pode ser atribuída à redução na função imune que ocorre, aproximadamente, entre duas semanas antes até três semanas após o parto (CHAPINAL *et al.*, 2012; LEBLANC *et al.*, 2006).

Essas doenças afetam cerca de 40% das vacas leiteiras no pós-parto e podem levar à infertilidade destes animais (LEWIS, 1997). Portanto, a definição de critérios rigorosos para o diagnóstico, tratamento e prevenção destas enfermidades no pós-parto de vacas leiteiras continua sendo um desafio (LEFEBVRE; STOCK, 2012).

**O sistema imunológico e a imunidade inata uterina**

Há inúmeras pesquisas relacionadas com infecções e imunidade uterina, destacando-se na literatura mundial as revisões de Cronin *et al.* (2012), Galvão *et al.* (2011) e Swangchan-Uthai *et al.* (2012). Estes trabalhos trouxeram uma contribuição muito grande à ciência, no entanto, esses pesquisadores trabalharam com vacas *B. taurus*, criadas em clima e manejo distintos das condições brasileiras e do Estado de

Minas Gerais, condições que possuem relação direta com os desafios e a resposta imunológica dos rebanhos.

O sistema imune no trato reprodutivo das fêmeas é regulado pelas mudanças hormonais, alinhando as funções reprodutivas às condições para migração espermática, fertilização, implantação e manutenção da gestação (WIRA *et al.*, 2014). O potencial para a regulação da imunidade inata pelo sistema endócrino é uma característica do endométrio que talvez seja distante da maioria dos outros tecidos do corpo (SHELDON; OWENS; TURNER, 2017). Em particular, os esteroides ovarianos, E2 e P4, modulam a susceptibilidade à infecção e a resposta inflamatória aos micro-organismos (BEAGLEY; GOCKEL, 2003; WIRA *et al.*, 2005).

O sistema imune dos mamíferos é composto pela imunidade inata e pela imunidade adquirida (AKIRA; UEMATSU; TAKEUCHI, 2006). O sistema imune inato é a primeira linha de defesa do hospedeiro contra patógenos e representa uma resposta rápida e estereotipada a um número grande, mas limitado, de estímulos (AKIRA; UEMATSU; TAKEUCHI, 2006; CRUVINEL *et al.*, 2010). É composto por barreiras físicas, químicas e biológicas, células especializadas e moléculas solúveis, presentes em todos os indivíduos, independentemente de contato prévio com imunógenos ou agentes agressores, e não se altera qualitativa ou quantitativamente após o contato (CRUVINEL *et al.*, 2010).

As principais células efetoras da imunidade inata são: macrófagos, neutrófilos, células dendríticas e células *Natural Killer* (NK), sendo que fagocitose, liberação de mediadores inflamatórios, ativação de proteínas do sistema complemento, bem como síntese de proteínas de fase aguda, citocinas e quimiocinas são as principais atividades da imunidade inata (CRUVINEL *et al.*, 2010). Já a imunidade adquirida está envolvida na eliminação de patógenos na fase tardia da infecção, bem como na geração de memória imunológica (AKIRA; UEMATSU; TAKEUCHI, 2006).

A resposta imune inata uterina é responsável pela eliminação das bactérias, regulação da inflamação e manutenção da saúde endometrial (HERATH *et al.*, 2006). Quando há alterações no ambiente sistêmico, incluindo as variações hormonais do pós-parto, podem ocorrer ativações de outros sistemas, como o complemento, por volta de 45-55 dias pós-parto (RALIOU *et al.*, 2019), coincidindo com a ocorrência de casos de endometrites citológicas nesse período. Vacas com esse quadro inflamatório

também possuem atividade de granulócitos e macrófagos comprometida e essa disfunção celular se associa a esse quadro inflamatório crônico instalado (BRODZKI *et al.*, 2014).

Os neutrófilos, uma das principais células do sistema imune inato, estão na linha de frente contra as infecções e foram identificados em um grande número durante a fase secretória (CRITCHLEY *et al.*, 1999; SALAMONSEN; WOOLLEY, 1999). Já em casos de infecção uterina por *T. pyogenes*, os infiltrados de neutrófilos no endométrio apresentam-se em grande quantidade (HEPPELMANN *et al.*, 2016), sendo altamente sensíveis às variações de quimiocinas no ambiente uterino, sobretudo IL8 (WIRA *et al.*, 2005).

**Citocinas pró-inflamatórias e quimiocinas**

As citocinas e as quimiocinas são proteínas moduladoras da inflamação sintetizadas e secretadas pelas células do sistema imunológico, capazes de controlar a resposta imune (TIZARD, 2014) e classificadas de acordo com a natureza dessa resposta (KOH *et al.*, 2018). A produção dessas proteínas ocorre quando as células sentinelas – macrófagos, células dendríticas e mastócitos – são ativadas por padrões moleculares associados a patógenos (PAMPs) ou padrões moleculares associados a lesões (DAMPs) (TAKEUCHI; AKIRA, 2010; TIZARD, 2014). Citocinas e quimiocinas aumentam o número de seus receptores nos leucócitos e modulam a resposta pró-inflamatória (WIRA *et al.*, 2005). Essas proteínas são capazes de aumentar a permeabilidade do endotélio vascular, liberar peptídeos antimicrobianos e proteínas de fase aguda, além de regularem a resposta inflamatória (SHELDON; OWENS; TURNER, 2017).

As células endometriais de bovinos possuem receptores de reconhecimento de padrões (PRR), como os tipo *toll* (TLR) capazes de ativar e levar à produção de citocinas (CARNEIRO; CRONIN; SHELDON, 2016; TURNER *et al.*, 2014). Em alguns casos, o aumento da expressão desses receptores, como TLR4, TRL6, TLR10, de fator nuclear KappaB1 (NF-κB1), interleucinas IL1, IL6, IL8, IL12 e interferon estão relacionados à intensidade da inflamação endometrial pós-parto (CHAPWANYA *et al.*, 2009). De acordo com Herath *et al.* (2009), na primeira semana pós-parto há

expressão de genes que codificam mediadores pró-inflamatórios como interleucinas IL1A, IL1β, IL6 e óxido nítrico sintase 2 (NOS2). Além disso, estes autores verificaram que animais inférteis têm maior expressão de IL1A e IL1β e, que vacas podem manter a fertilidade limitando a resposta inflamatória contra infecções bacterianas uterinas (HERATH; LILLY; SANTOS; *et al.*, 2009). No trabalho de Loyi *et al.* (2015) houve maior expressão de citocinas e PRR em vacas com endometrite clínica, sendo maior a reposta na fase lútea (14 vezes), mas as vacas com endometrite subclínica também apresentaram valores maiores (seis vezes) na expressão de citocinas e PRR na mesma fase.

As três principais citocinas pró-inflamatórias são: fator de necrose tumoral α (TNF-α), produzido no início do processo, seguido da produção de interleucina 1 (IL1) e interleucina 6 (IL6); concomitante, quimiocinas como CCL5, CXCL8, e enzimas (oxido nítrico sintase 2 – NOS2, por exemplo) também são produzidos pelas células sentinelas (GALVÃO *et al.*, 2011; HERATH; LILLY; SANTOS; *et al.*, 2009; TIZARD, 2014). Tendo em vista a participação dessas citocinas na resposta imune, Ghasemi *et al.* (2012) observaram que vacas com endometrite citológica apresentaram valores maiores de TNF-α, IL6 e IL8 do que vacas normais.

A interleucina 1β (IL1β) é produzida por macrófagos quando seus receptores CD14 e TLR4 são ativados e, junto ao TNF-α, atua sobre as células no local para amplificar a inflamação estimulando os linfócitos B, o que aumenta a expressão de outros fatores, como quimiocinas e células inflamatórias, gerando uma resposta rápida e potente (WIRA *et al.*, 2014). Neutrófilos e monócitos, por exemplo, são ativados pela IL1β e tornam a fagocitose mais eficiente (SICA *et al.*, 1990).

Além dessa ação local, a IL1β tem ação sistêmica, levando ao comportamento doentio e à produção de proteínas de fase aguda (DINARELLO, 1994; TIZARD, 2014). No trabalho de Wira *et al.* (2014) foi observado que o estradiol inibiu a resposta inflamatória mediada por IL1β, reduzindo esses sinais sistêmicos. Acerca disso, Fischer *et al.* (2010) observou maior expressão de IL1β em vacas doentes, sendo que nas vacas com endometrite subclínica a expressão de IL1β foi cinco vezes maior do que em vacas saudáveis e 20 vezes maior em vacas com endometrite clínica. Dessa forma, maiores valores de IL1β têm sido encontrados em vacas com endometrite do

que em vacas saudáveis, podendo estar associado com causas de infertilidade (FISCHER *et al.*, 2010; HERATH; LILLY; SANTOS; *et al.*, 2009; KIM *et al.*, 2014).

A IL6 também é produzida por macrófagos, mas linfócitos T e mastócitos também a sintetizam, além de serem secretadas por celulas endometriais (CRONIN *et al.*, 2012; TURNER *et al.*, 2014), quando estimulados por IL1, TNF-α e endotoxinas bacterianas, participando não apenas da inflamação, mas da imunidade adaptativa, mediando principalmente reação de fase aguda e a resposta entre neutrófilos e macrófagos, que também possuem receptores para IL6 (TIZARD, 2014). Embora, em bovinos, alguns autores não encontraram aumento significativo de IL6 nos casos de endometrite (FISCHER *et al.*, 2010), ela pode estar associada à presença de um maior número de neutrófilos nessas inflamações (GHASEMI *et al.*, 2012). Ademais, a IL6 exerce atividade regulatória da inflamação, pois estimula a produção de IL10, uma citocina anti-inflamatória, porém, a concentração de ambas no período pós-parto é reduzida (KIM *et al.*, 2014). Com relação às variações hormonais, altas concentrações de P4 estão associadas com redução de IL6 (CHENG *et al.*, 2016).

Definindo a progressão da inflamação, as quimiocinas, diferente das citocinas, coordenam a migração das células. A CXCL5, uma quimiocina da família β, produzida por macrófagos e mastócitos, atrai e ativa neutrófilos. Já a CCL5, da família α, é produzida por linfócitos T e macrófagos, atrai monócitos e eosinófilos e ativa basófilos liberando histamina (TIZARD, 2014).

A IL8 (CXCL8) é produzida por monócitos e fibroblastos, quimiotática para neutrófilos e células *natural killer*, toxinas bacterianas e outras citocinas inflamatórias que ativam monócitos e macrófagos a produzirem IL8 (CHAPWANYA *et al.*, 2009; FISCHER *et al.*, 2010; GABLER *et al.*, 2009; LOYI *et al.*, 2015). Esta citocina é também potencialmente angiogênica (STRIETER *et al.*, 1992) e está diretamente ligada à inflamação aguda, agindo no recrutamento e ativação de PMNs, predizendo a infecção da vaca no pós-parto (GHASEMI *et al.*, 2012).

No estudo de Baithalu *et al.* (2017) IL1β, TNF-α e PGFS (PGF sintase) apresentaram maior expressão gênica em vacas saudáveis do que em vacas com alguma infecção uterina, o inverso ocorreu em relação à IL6, IL8, IL10 e PGES (PGE sintase). Já nos estudos *in vitro* realizados por Swangchan-Uthai *et al.* (2012), a indução de reposta imune por LPS aumentou a expressão de PGES e reduziu a

expressão de PGFS. Em relação às mudanças hormonais, a expressão de CXCL5, IL1β e IL8 foi maior próximo à ovulação enquanto IL6, PTGS2 e HP não apresentaram diferença durante o ciclo estral (FISCHER *et al.*, 2010).

Os precursores de prostaglandinas E2 (PGE2), que tem ação pró-inflamatória e imunomoduladora, são também luteotróficos, mas a isoforma PTGS2 é que tem sido encontrada no endométrio de bovinos durante a fase lútea (AROSH *et al.*, 2002). Ao estimular as células epiteliais com LPS, houve maior acúmulo de PGE do que PGF, o que auxilia no entendimento da extensão da fase lútea e infertilidade (HERATH; LILLY; FISCHER; *et al.*, 2009). Porém, no estudo de Swangchan-Uthai *et al.* (2012) em cultura com células epiteliais e estromais, tanto a secreção de $PGE_2$ quanto de $PGF_{2\alpha}$ foram otimizadas na presença de LPS.

A secreção de citocinas, quimiocinas e peptídeos antimicrobianos pelas células epiteliais variam com a exposição hormonal e a localização no trato genital feminino (WIRA *et al.*, 2011). Em mulheres, a ausência do estrógeno após a menopausa resulta em menor proteção contra doenças bacterianas no TGF (WIRA; RODRIGUEZ-GARCIA; PATEL, 2015), mas este hormônio inibe a expressão de IL1α e TNF, reduzindo a inflamação logo antes da ovulação, quando é mais provável a presença do sêmen (WAGNER; JOHNSON, 2012). Há, assim, uma condição não inflamatória entre os epitélios do trato genital feminino inferior e superior que favorece a quimiotaxia do esperma favorecendo a fertilização, a implantação e manutenção da gestação (WIRA; RODRIGUEZ-GARCIA; PATEL, 2015).

**Proteínas de fase aguda**

As proteínas de fase aguda (PFA) têm sua síntese principalmente no fígado e sob o estimulo de interleucinas (IL6 e IL1β) e TNF-α (TIZARD, 2014), podendo ser um excelente marcador inflamatório de algumas doenças de bovinos (MADEN *et al.*, 2012; TÓTHOVÁ *et al.*, 2012).

A principal proteína de fase aguda em ruminantes é a haptoglobina (Hp), cuja função primária é ligar-se à hemoglobina livre e impedir sua oxidação (YANG *et al.*, 2003). Essa proteína tem sua concentração aumentada em doenças crônicas, incluindo as endometrites (TIZARD, 2014). Alguns estudos encontraram altos valores

de Hp tanto na corrente sanguínea quanto nos lavados uterinos de vacas com endometrite (BRODZKI *et al.*, 2015). No entanto, a produção dessa PFA pelas células endometriais não foi confirmada, apenas sabe-se que em altas cargas bacterianas uterinas os valores de Hp aumentam (SHELDON *et al.*, 2001). Além disso, valores aumentados de Hp no período de transição podem ser preditivos de doenças uterinas no pós-parto (DUBUC *et al.*, 2010; GALVÃO *et al.*, 2010). Há muitas mudanças nos valores de Hp no período de transição, encontrando-se valores altos um dia após o parto, enquanto outros autores encontraram valores altos tanto imediatamente antes quanto após o parto (TOTHOVA; NAGY; KOVAC, 2014; UCHIDA; KATOH; TAKAHASHI, 1993).

Outra PFA importante para a saúde dos bovinos é o amiloide sérico A (SAA), uma lipoproteína de alta densidade que altera sua concentração após 24-48 horas do início da infecção. SAA tem capacidade de se ligar ao TLR2 e ser agonista endógeno de TLR4, produzindo, em consequência a isso, algumas citocinas inflamatórias e atraindo linfócitos para o local da inflamação, pois é quimiotático para linfócitos T e também para neutrófilos e monócitos (TIZARD, 2014; TOTHOVA; NAGY; KOVAC, 2014). A principal função do SAA é o transporte reverso de colesterol dos tecidos para o fígado, opsonização e ativação de plaquetas (TOTHOVA; NAGY; KOVAC, 2014), participando ativamente da fase aguda da inflamação.

Diferente dessas proteínas de fase aguda positivas, as proteínas de fase aguda negativas reduzem os níveis durante um processo inflamatório, devido a redução de sua produção pelo fígado e direcionamento desse órgão para outras atividades. Dentre as proteínas de fase aguda negativas, está a albumina, uma importante reserva de aminoácidos, que pode ser requerida quando houver inflamação (TIZARD, 2014).

**O sistema endócrino e a imunidade inata uterina**

Os esteroides ovarianos regulam a atividade das células imunes no TGF, equilibrando o combate às infecções, regulando a resposta imune local e a remodelação tecidual, o que irá permitir a implantação do embrião e a manutenção da futura prenhez (WIRA; FAHEY, 2008). Para que isso ocorra, o endométrio cria um

ambiente em que apenas o trofoblasto seja reconhecido, modulando a resposta inflamatória para manutenção da gestação (CRITCHLEY *et al.*, 2001). Variações nas concentrações destes esteroides regulam funções e mudanças estruturais no endométrio para criar um ambiente para fertilização, implantação e sobrevivência fetal (LEWIS, 2003a; WIRA *et al.*, 2005). Ainda não se conhece todas as vias desses mecanismos e como a P4 leva à imunossupressão (LEWIS, 2004). Porém, sabe-se que o feto não somente evade o sistema imune, mas também provoca uma reposta imune uterina (OTT, 2020), ocorrendo assim, uma resposta anti-inflamatória mais favorável à manutenção da gestação (DESAI; BRINTON, 2019).

A P4 e o E2 modulam a expressão de algumas proteínas, como moléculas de adesão e fatores quimiotáticos, alterando a migração de macrófagos, células dendríticas e linfócitos (WIRA *et al.*, 2005; WIRA; GRANT-TSCHUDY; CRANE-GODREAU, 2005). Diante dessa capacidade de modulação, estes esteroides têm impacto no surgimento de doenças, podendo regular a resposta imune (ROWSON; LAMMING; FRY, 1953). Dessa forma, os resultados de alguns estudos sugerem que o estradiol apresenta um efeito protetor, enquanto a progesterona tem um efeito imunossupressor (LEWIS, 2003a, 2004; MAGALHÃES *et al.*, 2022; ROWSON, 1951).

Durante a fase lútea, onde há regulação das atividades imunes tanto por E2 quanto por P4, há maior limitação da resposta imune aos agentes infecciosos (WIRA; FAHEY, 2008). Essa condição imune, que também favorece a fertilização, implantação e manutenção da gestação, cria uma janela de vulnerabilidade que aumenta as chances de infecção (WIRA; RODRIGUEZ-GARCIA; PATEL, 2015), uma vez que a implantação embrionária é o sucesso de um ambiente criado durante a fase lútea, em que as células epiteliais, fibroblastos e células imunes estão sob ação da P4 (WIRA; RODRIGUEZ-GARCIA; PATEL, 2015).

Por outro lado, durante a fase proliferativa, em que há regeneração do epitélio endometrial coordenada pelo E2, ocorre também intensa angiogênese, contribuindo para a migração dos leucócitos, necessários para que a gestação ocorra (WIRA; RODRIGUEZ-GARCIA; PATEL, 2015). Mas, o número de células imunes só vão de fato aumentar durante a fase lútea tardia, quando também haverá crescimento epitelial glandular e de fibroblastos (KING; CRITCHLEY, 2010; WIRA; RODRIGUEZ-GARCIA; PATEL, 2015).

O E2 atua aumentando a apresentação de antígenos pelas células epiteliais endometriais, (WIRA; ROSSOLL, 1995), sendo menor no início do estro e maior no diestro. Essa ação do E2 sobre as células epiteliais foi elucidada em um estudo com ratas ovariectomizadas, no entanto, no trato genital feminino inferior e nas células estromais dessas ratas, o E2 inibiu a apresentação de antígenos (WIRA; ROSSOLL, 1995). Uma das variações entre as espécies, que justificaria uma maior resposta inflamatória uterina em ratos e bovinos, é o fato de nestas espécies, o sêmen ser depositado diretamente dentro do útero, enquanto em humanos esse depósito ocorre na vagina (WIRA; RODRIGUEZ-GARCIA; PATEL, 2015).

Para que todos os eventos ocorram e permitam a fertilização, a implantação, a sobrevivência fetal ou mesmo uma resposta imune contra algum agente presente no útero, é necessário, primeiramente, a ativação dos receptores de E2 e P4, que têm sua expressão autorregulada pela presença e quantidade destes esteroides, além de terem efeitos sobre a migração e ativação dos leucócitos, já que sua ação nas células epiteliais leva à secreção de citocinas e quimiocinas (WIRA *et al.*, 2005). Dessa forma, os esteroides ovarianos, juntamente a seus receptores nas células do TGF, irão coordenar a resposta imune local. Estes receptores são mais expressos durante a fase folicular e tem sua expressão reduzida na fase lútea (CHAUCHEREAU; SAVOURET; MILGROM, 1992). Os esteroides ovarianos tem regulação cruzada, ou seja, regulam a função um do outro, tendo em vista que o E2 aumenta o número de receptores de progesterona, enquanto a P4 pode reduzir os efeitos do estradiol (WIRA *et al.*, 2005). Além disso, os dois hormônios têm efeitos no local de produção e no transporte de imunoglobulinas no trato reprodutivo (RICHARDSON; KAUSHIC; WIRA, 1993), regulam algumas atividades das células epiteliais uterinas como proliferação ou apoptose (WIRA *et al.*, 2005), controlando os níveis de citocinas e quimiocinas no trato reprodutivo.

As células epiteliais endometriais sofrem mudanças em seu ambiente devido aos esteroides ovarianos, consequentemente, sua função de barreira na imunidade inata também sofre alterações (WIRA *et al.*, 2005). Em geral, para ocorrência de uma resposta é necessário uma alta dose de bactérias para ativação das células epiteliais, mas quaisquer rupturas das junções epiteliais podem levar a infecção uterina (WIRA *et al.*, 2014). Já os macrófagos são estimulados com maior facilidade e produzem

IL1β, citocina que ativará as células epiteliais, aumentando a produção de β-defensinas e modulando a resposta inflamatória (WIRA *et al.*, 2005).

Quando há maiores níveis de P4 circulante, há redução do influxo de células inflamatórias dentro do útero (CRITCHLEY *et al.*, 2001), havendo aumento de susceptibilidade do endométrio a infecções no pós-parto (LEWIS, 2003a; SHELDON *et al.*, 2009). Porém, com os níveis de progesterona basais há uma resistência às infecções uterinas, pois é possível uma resposta imune basal capaz de prevenir infecções (LEWIS, 2003b). Sendo assim, quando as concentrações de P4 aumentam, há maior chance de ocorrência de infecções (ROWSON; LAMMING; FRY, 1953) e, quando a P4 reduz, alguns mediadores inflamatórios como IL1β, IL6, IL8 e TNF-α aumentam, juntamente com o maior influxo de leucócitos (CHALLIS *et al.*, 2009). O aumento da expressão de IL8, por exemplo, leva à quimiotaxia e ativação de neutrófilos (CRITCHLEY *et al.*, 2001) e, quando a P4 é retirada, há aumento da expressão tanto de IL8 quanto de COX-2, além de constrição das artérias espirais levando a anoxia de regiões próximas ao lúmen uterino (CRITCHLEY *et al.*, 2001). Alguns estudos têm sugerido que a progesterona inibe a proliferação de linfócitos e module a função dos neutrófilos, havendo uma maior expressão de citocinas e quimiocinas próximo à ovulação em relação à fase lútea (FISCHER *et al.*, 2010). Uma possível via pela qual a progesterona pode atuar reduzindo a expressão gênica de citocinas pró-inflamatórias é pela sinalização dos fatores de transcrição fator nuclear kappa-beta (NF-κB) e proteino-quinases ativadas por mitógenos (MAPK) (CUI *et al.*, 2020).

Diante da dinâmica de resposta imune local em função das mudanças hormonais, é possível que as bactérias que contaminam o útero durante o parto não levem a uma infecção imediata, mas aproveitem a oportunidade em que a progesterona reduziu a função imune, possibilitando a multiplicação dessas bactérias no lúmen uterino (LEWIS, 2003b). Essa maior susceptibilidade à infecção quando os valores de P4 são elevados foi relatada em diversas espécies e, dentre elas, os bovinos (ROWSON; LAMMING; FRY, 1953; WULSTER-RADCLIFFE; SEALS; LEWIS, 2003).

A infecção uterina ocorre porque o útero não é capaz de controla-la e preveni-la quando está sob efeito da P4, já que este esteroide ovariano é capaz de converter

o útero a um órgão susceptível a infecções (LEWIS, 2003b), tornando-o "mais receptivo" aos antígenos por não haver bloqueio dos PRR (LEWIS, 2003b; WULSTER-RADCLIFFE; SEALS; LEWIS, 2003). Além disso, as células epiteliais, os fibroblastos e as células imunes respondem diretamente aos esteroides ovarianos e indiretamente às citocinas, fatores de crescimento e mediadores inflamatórios que estão envolvidos em diversos eventos fisiológicos do endométrio (WIRA *et al.*, 2014).

O E2, em contrapartida, otimiza a apresentação de antígenos pelas células epiteliais e tem a capacidade de modular as funções dos macrófagos, que expressam seletivamente receptores de E2, mediando atividades não genômicas deste hormônio (WIRA *et al.*, 2005). Nas células epiteliais endometriais, o E2 favorece a apresentação de antígenos, enquanto nas células estromais essa função é inibida pela secreção de TGF-β pelas células epiteliais (WIRA; ROCHE; ROSSOLL, 2002; WIRA; ROSSOLL, 2003), o que varia entre as espécies.

Os leucócitos tem a expressão de moléculas de adesão afetada tanto por E2 quanto por P4 (CHERNYSHOV; VODIANYK; HREKOVA, 2002). Nos macrófagos de camundongos, estrógeno-receptor ativa mitoses, aumenta o AMPc e a concentração de cálcio intracelular, a favor de sua atividade celular (GUO *et al.*, 2002; WIRA *et al.*, 2005) e, durante a fase secretória, em que há altos níveis de E2 e P4, os macrófagos se acumulam no endométrio (ARICI *et al.*, 1999). Já os monócitos, em estudo *in vitro*, quando tratados com E2, reduziram a produção de IL8 induzida por LPS, o que reduz a resposta inflamatória, auxiliando na prevenção do choque séptico (DENTENER *et al.*, 1993; LIEBLER *et al.*, 1994).

*In vitro*, o E2 parece ser primariamente anti-inflamatório (CUNNINGHAM; GILKESON, 2011), mas, em geral, quando há altos níveis de estrógeno, há redução da resposta inflamatória (FAHEY *et al.*, 2008; SCHAEFER *et al.*, 2005). Essa ação anti-inflamatória do E2 inibe a expressão de mediadores pró-inflamatórios pelas células endometriais (WIRA *et al.*, 2014), reduzindo a expressão de TLR4 nos fibroblastos uterinos (HIRATA *et al.*, 2007), aumentando a proliferação de células epiteliais (ZHU; POLLARD, 2007) e a secreção de peptídeos antimicrobianos como SLPI e elafina, estes, por sua vez, reduzem a expressão de TLR3 e 4 via NF-κB (TAGGART *et al.*, 2005). Juntos, esses eventos criam um ambiente anti-inflamatório, reduzindo a secreção de citocinas inflamatórias e, consequentemente, a migração de

células, suspendendo o ambiente inflamatório, associado com infecções (WIRA; RODRIGUEZ-GARCIA; PATEL, 2015).

## REFERÊNCIAS

AKIRA, S; UEMATSU, S; TAKEUCHI, O. Pathogen recognition and innate immunity. **Cell** v. 124, n. 4, p. 783–801 , 2006.0092-8674.

ARICI, A *et al.* Regulation of Monocyte Chemotactic Protein-1 Expression in Human Endometrial Stromal Cells by Estrogen and Progesterone. **Biology of Reproduction** v. 61, n. 1, p. 85–90 , 1 jul. 1999. Disponível em: <https://doi.org/10.1095/biolreprod61.1.85>.

AROSH, J A *et al.* Expression of Cyclooxygenases 1 and 2 and Prostaglandin E Synthase in Bovine Endometrial Tissue During the Estrous Cycle. **Biology of Reproduction** v. 67, n. 1, p. 161–169 , 1 jul. 2002. Disponível em: <https://doi.org/10.1095/biolreprod67.1.161>.

BAITHALU, R. K. *et al.* Transcriptional abundance of antioxidant enzymes in endometrium and their circulating levels in Zebu cows with and without uterine infection. **Animal Reproduction Science** v. 177, p. 79–87 , 2017. Disponível em: <http://dx.doi.org/10.1016/j.anireprosci.2016.12.008>.

BEAGLEY, K W; GOCKEL, C M. Regulation of innate and adaptive immunity by the female sex hormones oestradiol and progesterone. **FEMS Immunology and Medical Microbiology** v. 38, n. 1, p. 13–22 , 2003.

BRODZKI, P. *et al.* Determination of selected parameters for non-specific and specific immunity in cows with subclinical endometritis. **Animal Reproduction Science** v. 148, n. 3–4, p. 109–114 , 2014. Disponível em: <http://dx.doi.org/10.1016/j.anireprosci.2014.06.021>.

BRODZKI, P *et al.* Inflammatory cytokine and acute phase protein concentrations in the peripheral blood and uterine washings of cows with subclinical endometritis in the late postpartum period. **Veterinary Research Communications** v. 39, n. 2, p. 143–149 , 2015.

CARNEIRO, L C; CRONIN, J G; SHELDON, I M. Mechanisms linking bacterial infections of the bovine endometrium to disease and infertility. **Reproductive Biology** v. 16, n. 1, p. 1–7 , 2016.

CHALLIS, J R *et al.* Inflammation and Pregnancy. **Reproductive Sciences** v. 16, n. 2, p. 206–215 , 2009. Disponível em: <https://doi.org/10.1177/1933719108329095>.

CHAPINAL, N. *et al.* Herd-level association of serum metabolites in the transition period with disease, milk production, and early lactation reproductive performance. **Journal of dairy science** v. 95, n. 1, p. 5676–5682 , 2012.

CHAPWANYA, A *et al.* Histopathological and molecular evaluation of Holstein-Friesian cows postpartum: Toward an improved understanding of uterine innate immunity. **Theriogenology** v. 71, n. 9, p. 1396–1407 , 2009.

CHAUCHEREAU, A; SAVOURET, JF; MILGROM, E. Control of Biosynthesis and Post-Transcriptional Modification of the Progesterone Receptor. **Biology of Reproduction** v. 46, n. 2, p. 174–177 , 1 fev. 1992. Disponível em:

<https://doi.org/10.1095/biolreprod46.2.174>.

CHENG, L *et al.* The relationship between progesterone and Th-related cytokines in plasma during early pregnancy in cows. **Frontiers of Agricultural Science and Engineering** v. 3, n. 2, p. 147–152 , 2016.

CHERNYSHOV, V P; VODIANYK, M O; HREKOVA, S P. Effect of female steroid hormones on expression of adhesion molecules by peripheral blood leukocytes. **Fiziolohichnyi zhurnal** v. 48, n. 6, p. 46–53 , 2002.

CRITCHLEY, H O *et al.* The endocrinology of menstruation - a role for the immune system. **Clinical endocrinology** v. 55, n. 6, p. 701–710 , 2001.0300-0664 (Print)r0300-0664 (Linking).

CRITCHLEY, H O D *et al.* Role of Inflammatory Mediators in Human Endometrium during Progesterone Withdrawal and Early Pregnancy1. **The Journal of Clinical Endocrinology & Metabolism** v. 84, n. 1, p. 240–248 , 1999. Disponível em: <https://doi.org/10.1210/jcem.84.1.5380>.

CRONIN, J G *et al.* Toll-Like Receptor 4 and MYD88-Dependent Signaling Mechanisms of the Innate Immune System Are Essential for the Response to Lipopolysaccharide by Epithelial and Stromal Cells of the Bovine Endometrium1. **Biology of Reproduction** v. 86, n. 2 , 2012. Disponível em: <https://academic.oup.com/biolreprod/article-lookup/doi/10.1095/biolreprod.111.092718>.

CRUVINEL, W M *et al.* Sistema Imunitário – Parte I Fundamentos da imunidade inata com ênfase nos mecanismos moleculares e celulares da resposta inflamatória. **Revista Brasileira de Reumatologia** v. 50, n. 4, p. 434–61 , 2010. Disponível em: <https://ead.uftm.edu.br/pluginfile.php/52915/mod_resource/content/1/2010_Imuno01_v50n4a08.pdf>.

CUI, L *et al.* Progesterone inhibits inflammatory response in E.coli- or LPS-Stimulated bovine endometrial epithelial cells by NF-κB and MAPK pathways. **Developmental and Comparative Immunology** v. 105, p. 103568 , 2020. Disponível em: <https://doi.org/10.1016/j.dci.2019.103568>.

CUNNINGHAM, M; GILKESON, G. Estrogen receptors in immunity and autoimmunity. **Clinical Reviews in Allergy and Immunology** v. 40, n. 1, p. 66–73 , 2011.

DENTENER, M A *et al.* Involvement of CD14 in lipopolysaccharide-induced tumor necrosis factor-alpha, IL-6 and IL-8 release by human monocytes and alveolar macrophages. **The Journal of Immunology** v. 150, n. 7, p. 2885–2891 , 1 abr. 1993. Disponível em: <http://www.jimmunol.org/content/150/7/2885.abstract>.

DESAI, M K; BRINTON, R D. Autoimmune Disease in Women: Endocrine Transition and Risk Across the Lifespan. **Frontiers in Endocrinology** v. 10, p. 265 , 2019. Disponível em: <https://www.frontiersin.org/article/10.3389/fendo.2019.00265>.

DINARELLO, C A. The interleukin-1 family: 10 years of discovery. **The FASEB Journal** v. 8, p. 1314–1325 , 1994.

DUBUC, J *et al.* Risk factors for postpartum uterine diseases in dairy cows. **Journal of Dairy Science** v. 93, n. 12, p. 5764–5771 , 2010. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/21094748>.0022-0302.

EMBRAPA. Anuário do leite 2022: Pecuária leiteira de precisão. **Anuário 2022** p. 59 , 2022.

EMBRAPA. *Milk production system;* Disponível em: <https://sistemasdeproducao.cnptia.embrapa.br/FontesHTML/Leite/LeiteZonadaMata Atlantica/expediente.htm>. Acesso em: 15 mar. 2021.

FAHEY, J. V. *et al.* Estradiol selectively regulates innate immune function by polarized human uterine epithelial cells in culture. **Mucosal Immunology** v. 1, n. 4, p. 317–325 , 2008.

FAO. **The State of Food Insecurity in the World 2009**. [S.l: s.n.], 2009. Disponível em: <http://www.fao.org/tempref/docrep/fao/012/i0876e/i0876e_flyer.pdf>.978925106288 3.

FISCHER, C *et al.* Selected pro-inflammatory factor transcripts in bovine endometrial epithelial cells are regulated during the oestrous cycle and elevated in case of subclinical or clinical endometritis. **Reproduction, Fertility and Development** v. 22, n. 5, p. 818–829 , 2010.1031-3613 (Print)r1031-3613 (Linking).

GABLER, C. *et al.* Endometrial expression of selected transcripts involved in prostaglandin synthesis in cows with endometritis. **Theriogenology** v. 71, n. 6, p. 993–1004 , 2009.0093-691X (Print)r0093-691X (Linking).

GALVÃO, K. N. *et al.* Association between uterine disease and indicators of neutrophil and systemic energy status in lactating Holstein cows. **Journal of Dairy Science** v. 93, n. 7, p. 2926–2937 , 2010. Disponível em: <http://dx.doi.org/10.3168/jds.2009-2551>.

GALVÃO, K N *et al.* Association between endometritis and endometrial cytokine expression in postpartum Holstein cows. **Theriogenology** v. 76, n. 2, p. 290–299 , 2011.

GHASEMI, F *et al.* Proinflammatory cytokine gene expression in endometrial cytobrush samples harvested from cows with and without subclinical endometritis. **Theriogenology** v. 78, n. 7, p. 1538–1547 , 2012. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0093691X12003718>.

GUO, Z *et al.* Estradiol-induced nongenomic calcium signaling regulates genotropic signaling in macrophages. **Journal of Biological Chemistry** v. 277, n. 9, p. 7044–7050 , 2002.

HEPPELMANN, M. *et al.* The effect of puerperal uterine disease on histopathologic findings and mRNA expression of proinflammatory cytokines of the endometrium in dairy cows. **Theriogenology** v. 85, n. 7, p. 1348–1356 , 2016. Disponível em: <http://dx.doi.org/10.1016/j.theriogenology.2015.12.022>.

HERATH, S; LILLY, ST; FISCHER, D P; *et al.* Bacterial lipopolysaccharide induces

an endocrine switch from prostaglandin F 2α to prostaglandin E 2 in bovine endometrium. **Endocrinology** v. 150, n. 4, p. 1912–1920 , 2009.

HERATH, S *et al.* Expression and function of toll-like receptor 4 in the endometrial cells of the uterus. **Endocrinology** v. 147, n. 1, p. 562–570 , 2006.0013-7227 (Print).

HERATH, S; LILLY, S T; SANTOS, N R; *et al.* Expression of genes associated with immunity in the endometrium of cattle with disparate postpartum uterine disease and fertility. **Reproductive Biology and Endocrinology** v. 7, n. 1, p. 55 , 2009. Disponível em: <https://doi.org/10.1186/1477-7827-7-55>.

HIRATA, T *et al.* Expression of toll-like receptors 2, 3, 4, and 9 genes in the human endometrium during the menstrual cycle. **Journal of Reproductive Immunology** v. 74, n. 1, p. 53–60 , 2007. Disponível em: <http://www.sciencedirect.com/science/article/pii/S016503780600235X>.

HUME, D A; WHITELAW, C B A; ARCHIBALD, A L. The Future of Animal Production: Improving Productivity and Sustainability. **The Journal of Agricultural Science** v. 149, n. Supplement:S1, p. 9–16 , 2011.1469-5146.

KIM, I H *et al.* Inflammatory cytokine concentrations in uterine flush and serum samples from dairy cows with clinical or subclinical endometritis. **Theriogenology** v. 82, n. 3, p. 427–432 , 2014. Disponível em: <http://dx.doi.org/10.1016/j.theriogenology.2014.04.022>.0093-691X.

KING, A E; CRITCHLEY, H O D. Oestrogen and progesterone regulation of inflammatory processes in the human endometrium. **The Journal of Steroid Biochemistry and Molecular Biology** v. 120, n. 2, p. 116–126 , 2010. Disponível em: <http://www.sciencedirect.com/science/article/pii/S096007601000004X>.

KOH, Y Q *et al.* Regulation of inflammatory mediator expression in bovine endometrial cells: effects of lipopolysaccharide, interleukin 1 beta, and tumor necrosis factor alpha. **Physiological Reports** v. 6, n. 9, p. 1–13 , 2018.

LA FALCE, M. *Teste de progênie da raça girolando será ampliado*. Disponível em: <https://www.embrapa.br/gado-de-leite/busca-de-noticias?p_p_id=buscanoticia_WAR_pcebusca6_1portlet&p_p_lifecycle=0&p_p_state=pop_up&p_p_mode=view&p_p_col_id=column-1&p_p_col_pos=1&p_p_col_count=2&_buscanoticia_WAR_pcebusca6_1portlet_groupId=1355117&_buscan>. Acesso em: 8 set. 2022.

LEBLANC, S. J. Interactions of Metabolism, Inflammation, and Reproductive Tract Health in the Postpartum Period in Dairy Cattle. **Reproduction in Domestic Animals** v. 47, n. SUPPL. 5, p. 18–30 , 2012.1439-0531.

LEBLANC, S J *et al.* Major advances in disease prevention in dairy cattle. **Journal of Dairy Science** v. 89, n. 4, p. 1267–1279 , 2006. Disponível em: <http://dx.doi.org/10.3168/jds.S0022-0302(06)72195-6>.

LEBLANC, S J *et al.* The Effect of Treatment of Clinical Endometritis on Reproductive Performance in Dairy Cows. **Journal of Dairy Science** v. 85, n. 9, p. 2237–2249 , 2002. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0022030202743038>.

LEFEBVRE, R C.; STOCK, A E. Therapeutic Efficiency of Antibiotics and Prostaglandin F2 alpha in Postpartum Dairy Cows with Clinical Endometritis: An Evidence-Based Evaluation. **Veterinary Clinics of North America - Food Animal Practice** v. 28, n. 1, p. 79–96 , 2012. Disponível em: <http://dx.doi.org/10.1016/j.cvfa.2012.01.002>.0749-0720.

LEWIS, G S. Role of ovarian progesterone and potential role of prostaglandin F2alpha and prostaglandin E2 in modulating the uterine response to infectious bacteria in postpartum ewes. **Journal of Animal Science** v. 81, p. 285–293 , 2003a.

LEWIS, G S. Steroidal regulation of uterine immune defenses. **Animal Reproduction Science** v. 82, p. 281–294 , 2004.

LEWIS, G S. Steroidal regulation of uterine resistance to bacterial infection in livestock. **Reproductive Biology and Endocrinology** v. 1, p. 1–8 , 2003b.

LEWIS, G S. Uterine Health and Disorders. **Journal of Dairy Science** v. 80, n. 5, p. 984–994 , 1997. Disponível em: <http://dx.doi.org/10.3168/jds.S0022-0302(97)76024-7>.

LIEBLER, J M *et al.* Production of IL-8 and monocyte chemotactic peptide-1 by peripheral blood monocytes. Disparate responses to phytohemagglutinin and lipopolysaccharide. **The Journal of Immunology** v. 152, n. 1, p. 241–249 , 1 jan. 1994. Disponível em: <http://www.jimmunol.org/content/152/1/241.abstract>.

LOYI, T *et al.* Expression of Pathogen Recognition Receptors and Pro-Inflammatory Cytokine Transcripts in Clinical and Sub-Clinical Endometritis Cows. **Animal Biotechnology** v. 26, n. 3, p. 194–200 , 2015.

MADEN, M *et al.* Acute-Phase Proteins, Oxidative Stress and Enzyme Activities of Blood Serum and Peritoneal Fluid in Cattle with Abomasal Displacement. **Journal of Veterinary Internal Medicine** v. 26, n. 6, p. 1470–1475 , 2012.

MAGALHÃES, L.Q. *et al.* Ovarian steroids modulate the systemic inflammatory response OF COWS challenged with lipopolysaccharide (LPS) intrauterine INFUSION. **Theriogenology** v. 182 , 2022.

OTT, T L. Immunological detection of pregnancy: Evidence for systemic immune modulation during early pregnancy in ruminants. **Theriogenology** , 2020. Disponível em: <https://doi.org/10.1016/j.theriogenology.2020.04.010>.

RALIOU, M *et al.* Subclinical endometritis in dairy cattle is associated with distinct mRNA expression patterns in blood and endometrium. **PLoS ONE** v. 14, n. 8, p. 220–224 , 2019.1111111111.

RICHARDSON, J; KAUSHIC, C; WIRA, C R. Estradiol regulation of secretory component: Expression by rat uterine epithelial cells. **Journal of Steroid Biochemistry and Molecular Biology** v. 47, n. 1–6, p. 143–149 , 1993.

ROWSON, L E. Methods of inducing multiple ovulation. **Journal of Endocrinology** v. 7, n. 3, p. 260–270 , 1951.

ROWSON, L E; LAMMING, G E; FRY, R M. Influence of ovarian hormones on uterine infection. **Nature** v. 171, p. 749–750 , 1953.0028-0836 (Print)0028-0836.

SALAMONSEN, L; WOOLLEY, D E. Menstruation: induction by matrix metalloproteinases and inflammatory cells. **Journal of Reproductive Immunology** v. 44, n. 1, p. 1–27 , 1999. Disponível em: <http://www.sciencedirect.com/science/article/pii/S0165037899000029>.

SAUT, J P.E. *et al.* Ovarian steroids do not affect bovine endometrial cytokine or chemokine responses to Escherichia coli or LPS in vitro. **Reproduction** v. 148, n. 6, p. 593–606 , 2014.1741-7899 (Electronic)r1470-1626 (Linking).

SCHAEFER, T M *et al.* IL-1β-Mediated Proinflammatory Responses Are Inhibited by Estradiol via Down-Regulation of IL-1 Receptor Type I in Uterine Epithelial Cells. **The Journal of Immunology** v. 175, n. 10, p. 6509–6516 , 15 nov. 2005. Disponível em: <http://www.jimmunol.org/content/175/10/6509.abstract>.

SHELDON, I M *et al.* Influence of uterine bacterial contamination after parturition on ovarian dominant follicle selection and follicle growth and function in cattle. **Reproduction** v. 123, n. 6, p. 837–845 , 2002.1470-1626.

SHELDON, I M *et al.* Mechanisms of infertility associated with clinical and subclinical endometritis in high producing dairy cattle. **Reproduction in Domestic Animals** v. 44, n. SUPPL. 3, p. 1–9 , 2009.

SHELDON, I M; OWENS, S E. Postpartum uterine infection and endometritis in dairy cattle. **Animal Reproduction** v. 14, n. 3, p. 622–629 , 2017. Disponível em: <http://www.cbra.org.br/portal/downloads/publicacoes/animalreproduction/issues/download/v14/v14n3/p622-629 (AR1006) AETE.pdf>.

SHELDON, I M; OWENS, S E; TURNER, M L. Innate immunity and the sensing of infection, damage and danger in the female genital tract. **Journal of Reproductive Immunology** v. 119, p. 67–73 , 2017. Disponível em: <http://dx.doi.org/10.1016/j.jri.2016.07.002>.

SHELDON, IM *et al.* Acute phase protein responses to bacterial contamination. **The Veterinary Record** v. 148, n. 6, p. 172–175 , 2001.

SICA, A *et al.* IL-1 transcriptionally activates the neutrophil chemotactic factor/IL-8 gene in endothelial cells. **Immunology** v. 69, n. 4, p. 548–53 , 1990. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/2185985%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC1385627>.

STRIETER, R M *et al.* Interleukin-8. A corneal factor that induces neovascularization. **The American journal of pathology** v. 141, n. 6, p. 1279–1284 , dez. 1992. Disponível em: <https://pubmed.ncbi.nlm.nih.gov/1281615>.

SWANGCHAN-UTHAI, T *et al.* Time Course of Defense Mechanisms in Bovine Endometrium in Response to Lipopolysaccharide1. **Biology of Reproduction** v. 87, n. 6 , 2012.

TAGGART, C C *et al.* Secretory leucoprotease inhibitor binds to NF-κB binding sites in monocytes and inhibits p65 binding. **Journal of Experimental Medicine** v. 202, n. 12, p. 1659–1668 , 13 dez. 2005. Disponível em: <https://doi.org/10.1084/jem.20050768>.

TAKEUCHI, O; AKIRA, S. Pattern Recognition Receptors and Inflammation. **Cell** v. 140, n. 6, p. 805–820 , 2010. Disponível em: <http://dx.doi.org/10.1016/j.cell.2010.01.022>.

TIZARD, I R. Imunidade Inata: Mediadores Próinflamatórios e Antimicrobianos. In: TIZARD, I R (Org.). . **Imunologia Veterinária**. 9. ed. São Paulo-SP: Elsevier, 2014. p. 70–84. 978-85-352-7303-8.

TÓTHOVÁ, C *et al.* Acute phase proteins in relation to various inflammatory diseases of calves. **Comparative Clinical Pathology** v. 21, n. 5, p. 1037–1042 , 2012.

TOTHOVA, C; NAGY, O; KOVAC, G. Acute phase proteins and their use in the diagnosis of diseases in ruminants: A review. **Veterinarni Medicina** v. 59, n. 4, p. 163–180 , 2014.

TURNER, M L. *et al.* Epithelial and stromal cells of bovine endometrium have roles in innate immunity and initiate inflammatory responses to bacterial lipopeptides in vitro via Toll-like receptors TLR2, TLR1, and TLR6. **Endocrinology** v. 155, n. 4, p. 1453–1465 , 2014.0013-7227r1945-7170.

UCHIDA, E; KATOH, N; TAKAHASHI, K. Appearance of haptoglobin in serum from cows at parturition. **The Journal of veterinary medical science** v. 55, n. 5, p. 893–894 , out. 1993.

VILELA, D. A importância econômica, social e nutricional do leite. **Revista Batavo** n. 1975, p. 2001–2002 , 2002. Disponível em: <http://www.nupel.uem.br/importancia.pdf>.

WAGNER, R. D; JOHNSON, S J. Probiotic lactobacillus and estrogen effects on vaginal epithelial gene expression responses to Candida albicans. **Journal of Biomedical Science** v. 19, n. 1, p. 1–8 , 2012.

WIRA, C R.; ROSSOLL, R M. Antigen-presenting cells in the female reproductive tract: influence of sex hormones on antigen presentation in the vagina. **Immunology** v. 84, p. 505–508 , 1995.

WIRA, C R *et al.* Innate and adaptive immunity in female genital tract: Cellular responses and interactions. **Immunological Reviews** v. 206, p. 306–335 , 2005.0105-2896 (Print) 0105-2896 (Linking).

WIRA, C R *et al.* Innate Immunity in the Human Female Reproductive Tract: Endocrine Regulation of Endogenous Antimicrobial Protection Against HIV and Other Sexually Transmitted Infections. **American Journal of Reproductive Immunology** v. 65, n. 3, p. 196–211 , 2011.

WIRA, C R *et al.* Regulation of mucosal immunity in the female reproductive tract: The role of sex hormones in immune protection against sexually transmitted pathogens. **American Journal of Reproductive Immunology** v. 72, n. 2, p. 236–258 , 2014.

WIRA, C R; FAHEY, J V. A new strategy to understand how HIV infects women: identification of a window of vulnerability during the menstrual cycle. **Aids** v. 22, p. 1909–1917 , 2008.

WIRA, C R; GRANT-TSCHUDY, K S; CRANE-GODREAU, M A. Epithelial Cells in the Female Reproductive Tract: a Central Role as Sentinels of Immune Protection. **American Journal of Reproductive Immunology** v. 53, n. 2, p. 65–76 , 2005.

WIRA, C R; ROCHE, M A; ROSSOLL, R M. Antigen presentation by vaginal cells: Role of TGFβ as a mediator of estradiol inhibition of antigen presentation. **Endocrinology** v. 143, n. 8, p. 2872–2879 , 2002.

WIRA, C R; RODRIGUEZ-GARCIA, M; PATEL, M V. The role of sex hormones in immune protection of the female reproductive tract. **Nature Reviews Immunology** v. 15, n. 4, p. 217–230 , 2015.

WIRA, C R; ROSSOLL, R M. Oestradiol regulation of antigen presentation by uterine stromal cells: Role of transforming growth factor-β production by epithelial cells in mediating antigen-presenting cell function. **Immunology** v. 109, p. 398–406 , 2003.

WULSTER-RADCLIFFE, M. C.; SEALS, R. C.; LEWIS, G. S. Progesterone increases susceptibility of gilts to uterine infections after intrauterine inoculation with infectious bacteria. **Journal of Animal Science** v. 81, n. 5, p. 1242–1252 , 2003.0021-8812 (Print)r0021-8812 (Linking).

YANG, F *et al.* Haptoglobin reduces lung injury associated with exposure to blood. **American Journal of Physiology - Lung Cellular and Molecular Physiology** v. 284, n. 2 28-2 , 2003.

ZHU, L; POLLARD, J W. Estradiol-17β regulates mouse uterine epithelial cell proliferation through insulin-like growth factor 1 signaling. **Proceedings of the National Academy of Sciences** v. 104, n. 40, p. 15847 LP – 15851 , 2 out. 2007. Disponível em: <http://www.pnas.org/content/104/40/15847.abstract>.

# OVARIAN STEROIDS MODULATE THE SYSTEMIC INFLAMMATORY RESPONSE OF COWS CHALLENGED WITH LIPOPOLYSACCHARIDE (LPS) INTRAUTERINE INFUSION

**Artigo publicado na Revista Theriogenology**

v. 182, p. 35-44, 2022

**CAPÍTULO 2 – OVARIAN STEROIDS MODULATE THE SYSTEMIC INFLAMMATORY RESPONSE OF COWS CHALLENGED WITH LIPOPOLYSACCHARIDE (LPS) INTRAUTERINE INFUSION**

**ABSTRACT –** Postpartum uterine infections of dairy cows promote a local and systemic inflammation and interfere with reproductive efficiency. The aim of this study was to evaluate the effect of steroid hormones including progesterone (P4) and estradiol (E2) on the systemic inflammatory response of cows after being challenged with an intrauterine infusion of lipopolysaccharide (LPS). For this, a hemogram and serum dosage of haptoglobin (Hp) in eight primiparous Gir cows ovariectomized were performed on day (day 0) and after 24 h (day þ1). Four cows (n ¼ 4) were challenged (day 0) with 20 mL of 0.9% NaCl þ 12.5 mg/kg LPS, and four cows (n ¼ 4) were challenged (day 0) with 20 mL of 0.9% NaCl. For this, the study was divided in four experimental groups as: (1) Control group: without any hormonal treatment before day 0; (2) Group 24 h - E2: 1 mg of estradiol benzoate 24 h before (day-1); (3) Group 24 h - P4: 2.0 g of P4 device 24 h before (day 1); (4) Group 14 d - P4: 2.0 g of P4 device 14 days before (day-14). In the systemic response to LPS, there was an increase in Hp (control group; 24 h - P4 group; 14 d - P4 group), and on day +1 the Hp of 14 d - P4 group was higher when compared to the other groups. On day 0, the 14 d - P4 group had an increase in circulating leukocytes and lymphocytes cells than the control group (P < 0.01). On day +1 after LPS-challenge the 14 d - P4 group showed a decrease in circulating lymphocytes, eosinophils, and monocytes (P < 0.05). A neutrophilia with left shift in the two treatments with P4 (day +1), in addition to a thrombocytopenia and lower platelets compared to the 24 h - E2 group (P < 0.05) (day 0) were recorded. It was concluded that ovariectomized cows challenged with LPS, previously submitted to steroid hormones induce a systemic inflammatory response. Also, the systemic response is more intense after previous prolonged exposure to P4 and less intense after exposure to E2. This study provided important information relating the effect of ovarian steroids on the systemic inflammatory response of cows challenged with intrauterine LPS.

**Keywords**: estradiol, hemogram, immunity, lipopolysaccharide, progesterone, uterus

**INTRODUCTION**

In dairy cattle, the postpartum period is marked by the high incidence of uterine diseases, as 2 – 5 % of retained placenta [1], 25 – 40 % of metritis [1,2], 20 % of clinical

endometritis [1,2], 15 – 40 % of cervicitis at 30 days in milk (DIM) [3] and 10 – 30 % of cytological endometritis between 30 - 60 DIM [3]. Contamination by pathogens in the female genital tract is inherent in parturition, which leads to a high incidence of uterine diseases during the postpartum period of dairy cows [4], associated with other predisposing factors such as dystocia [5–7], retained placenta [6], hypocalcemia [5,8–10], reduction in dry matter intake [11], negative energy balance (NEB) [5,8,10] and an increase in haptoglobin (Hp) values, considered an acute-phase protein (APP) [5,9,12].

The defense against pathogens in the uterine environment is performed initially by the innate [13,14] and adaptative [15] immune systems. Uterine innate immunity is regulated by the endocrine system, with ovarian steroids capable of modulating the local inflammatory response, and the susceptibility to diseases [16–18]. Some authors suggest that estradiol (E2) has a protective effect, while progesterone (P4) has an immunosuppressive effect on the uterine immune response [19–21]. The susceptibility to infection during the P4 predominance phase is well been discussed in cattle for several years [22,23], in this scenario, during postpartum, the uterine immune function is often compromised due to bacterial contamination of the uterine lumen with the persistence of pathogens that cause uterine diseases [19,20].

Ovarian steroid hormones regulate reproduction, embryo implantation and pregnancy maintenance and the immune system develops important effects in each of these moments [24]. These hormones regulate uterine epithelial cell activities such as proliferation or apoptosis [25], that control the gene mRNA expression of cytokines in the reproductive tract, most of which are regulated by the increase of P4 [26], such as interleukin 6 (IL6) which is reduced in high concentrations of P4 [27]. It was suggested that P4 inhibits the proliferation of lymphocytes and modulates the function of neutrophils, with a greater mRNA expression of cytokines close to ovulation in relation to the phase with P4 predominance [28]. In addition, P4 and E2 have effects on the site of production and transport of immunoglobulins in the reproductive tract [29].

Cows diagnosed with clinical mastitis show systemic changes, such as increase in body temperature, Hp [30,31] and serum amyloid A (SAA) concentration [30], negatively affecting milk production and reproductive parameters, such as pregnancy rate, conception rate and days open [32], always taking the importance of

lipopolysaccharide (LPS) interference in the endometrial gene expression of uterine factors and embryonic development [31].

It is noteworthy that in this research area, most studies have been explored in *Bos taurus* and in countries with temperate weathers. In Brazil, most of the herd consists of hybrid animals (*Bos indicus* x *Bos taurus*) [33], especially the Gir breed, since the insertion of Zebu breeds in herds in tropical countries is an alternative for adaptability to the climate in these regions [34,35]. Furthermore, studies with in vivo models that demonstrate the effect of steroid hormones (P4 and E2) exposure on the systemic inflammatory response due to an inflammatory process with an initial focus on the uterus of cows were not found in the literature.

The hypothesis of this study was that inflammatory systemic effects, secondary to a primary uterine inflammatory process, are more intense under P4 and are reduced under E2 effects. Consequently, the objective was to evaluate the previous exposure effect of steroid hormones (P4 and E2) on the systemic inflammatory response after LPS intrauterine (iu) infusion in ovariectomized and primiparous Gir cows.

## MATERIAL AND METHODS

All procedures in this study were conducted according to the Ethical Principles in Animal Experimentation approved by the Committee of Ethics in the Use of Animals (CEUA) of the Federal University of Uberlândia (UFU), under the protocol n.123/15.

### Animals and experiment location

Eight primiparous cows of the Gir breed with an average of 30 months of age, previously evaluated for possible diseases of the female genital tract were used. During the experiment, the cows were housed in two collective pens each having four animals, with available shade, fed with corn silage, mineral salt (with 15% of urea) and water *ad libitum*.

**Surgical procedure – Bilateral ovariectomy**

After 15 days of adaptation, a bilateral flank ovariectomy surgery was performed, as described by Weaver et al. [36]. The procedure was performed in the Surgical Department of the Veterinary Hospital at UFU, where the cows were kept in the right lateral decubitus position under general inhalation anesthesia with isoflurane 2% (BioChimico, Itatiaia - RJ, Brazil).

All surgically manipulated animals were submitted to the postoperative protocol consisting of antibiotic therapy with ceftiofur (Lactofur, Ourofino Saúde Animal, Ribeirão Preto - SP, Brazil), 2.2 mg/kg, im, two times per day during 10 days; flunixin meglumine (Flunixina, Uzinas Chimicas Brasileiras, Jaboticabal -SP, Brazil), 2.2 mg/kg, im, two times per day during three days. The animals were evaluated daily during ten days after the surgery until surgical stitches were removed. The experiment procedure started only after 100 days of the postoperative period.

**Experiment design**

In this study the effect of P4 and E2 on the systemic inflammatory response of Gir cows was evaluated after challenging with an iu infusion of LPS. Consequently, the experiment was developed in four distinct steps (Figure 1) with intervals of approximately 30 days between each step.
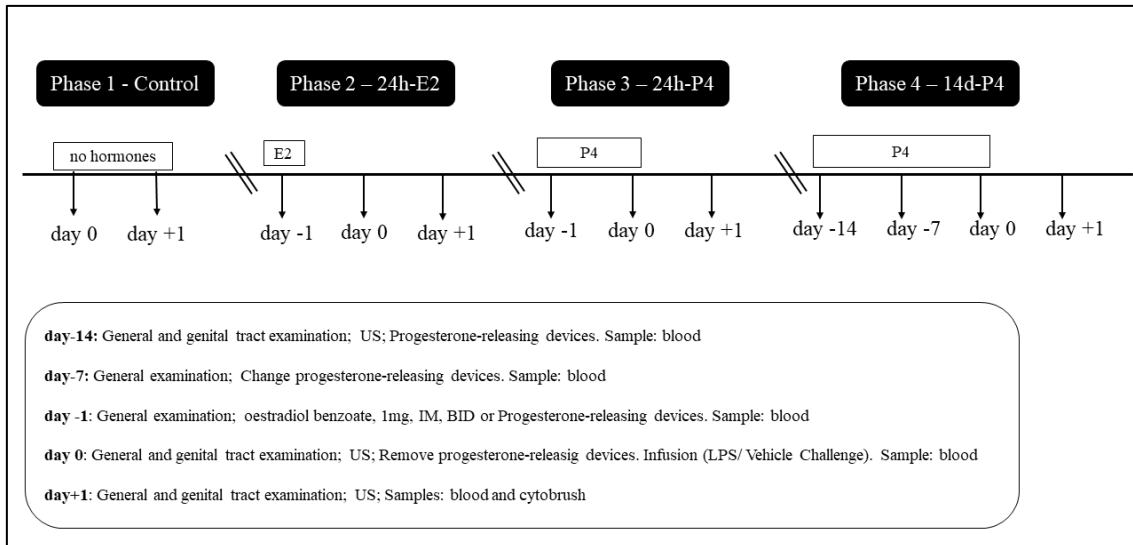
**Fig. 1.** Schematic figure of the procedures performed in the four experimental groups. Eight ovariectomized Gir cows, were exposed to estradiol (E2) or progesterone (P4) and then were challenged with iu administration of lipopolysaccharide (LPS).

In all steps the moment of iu infusion was defined as day zero (D0). The animals were divided into two groups, one group (n = 4/ LPS-challenge) was challenged with an iu infusion of 12.5 ug/kg of LPS from Escherichia coli (Escherichia coli serotype O55: B5 LPS Sigma-Aldrich, Darmstadt - Germany; 5 mg / animal) diluted in 20 mL of 0.9% saline (0.9% NaCl, Eurofarma, Ribeirão Preto-SP, Brazil); the other group (n = 4/vehicle-challenge) was treated with an iu infusion of 20 mL of NaCl saline (0.9%). The respective dose of LPS per animal was sterilely aliquoted in small amount of PBS (5 mg of LPS per 2 mL of PBS), filled in 2 mL Eppendorf type tubes and frozen at – 20 ºC the day before the *in vivo* intrauterine challenge.

The cows were divided by chance each time in the different groups between the four steps of the study. At this time (day 0), physical and female genital tract examination, ultrasound and blood collection were performed to evaluate total blood count, hormonal and Hp parameters.

In addition, day +1 (24 hours after the iu infusion) was defined as the time to evaluate the systemic inflammatory response (in all groups) in which physical and female genital tract examinations, ultrasound, endometrial epithelium and blood samples (to evaluate total blood count, hormonal and Hp measurements) were performed.

Four experimental groups were performed as: (1) control group (n = 8) – in this group the animals did not receive hormonal administration before day 0 (LPS / vehicle challenge); (2) Group 24h - E2 (n = 8) – in this group the cows received E2 (1 mg of estradiol benzoate, Estrogin, Biofarm, Jaboticabal-SP, Brazil, IM) 24 hours (day -1) before day 0 (LPS / vehicle challenge); (3) Group 24h - P4 (n = 8) – in this group the cows received P4 (two intravaginal devices of first use of 1.0 g of P4, Sincrogest, Ourofino Saúde Animal, Ribeirão Preto -SP, Brazil) 24 hours (day -1) before day 0 (LPS / vehicle challenge); (4) Group 14d - P4 (n = 8) – in this group the cows received the previous treatment of 14 days (day -14) with P4 (two intravaginal devices of first use of 1.0 g of P4, Sincrogest, Ourofino Saúde Animal, replaced at seven days) before day 0 (LPS / vehicle challenge).

**Clinical examination and blood sampling**

All procedures were performed in the morning and the animals were properly immobilized. The general clinical evaluation of the animals, body temperature, heart and respiratory rates were performed according to the recommendations by Terra and Reynolds [37]. Blood samples were collected by venipuncture of the median caudal vein, using a vacuum system and in a tube with EDTA K3 (BD Vacutainer, Brazil) for complete blood count (erythrogram and leukogram) [37]. Another blood sample was collected in a tube with a separator gel and clot activator (BD Vacutainer, Brazil) for serum analysis of Hp, P4, and E2. All samples obtained were sent to the Clinical Laboratory Analysis (UFU), where blood count was done immediately.

**Laboratory tests**

The total blood count was performed in an automatic veterinary hematology analyzer pocH-100iV Diff (Sysmex do Brasil, São José do Rio Preto-SP, Brazil) to determine the plasma concentrations of hemoglobin, globular volume, hematimetry, platelet and leukometry. The differential circulating leukocyte count was performed by optical microscopy, in blood extensions stained by May Grünwald Giemsa [38].

For serum analysis of Hp, the technique described by Jones and Mould [39] was used as adapted by Ramos et al. [40]. A serial dilution performed with a standard Hp (human serum measured by the commercial kit Beckman Coulter, Brea, CA, USA) allowed to obtain a curve with a concentration of Hp ranging from 0 to 0.56 g/L. For the elaboration of the standard curve, 180 µL of saline solution was added in the wells A1 and A2, and 100 µL in the following wells until H1 and H2. For the standard curve, 20 µL of standard serum was added under the saline solution in the wells A1 and A2, and then the dilution was performed. Regarding the sample test, 90 µL of saline solution was added in all of the other wells and 10 µL of the sample was added in each well. The samples were diluted twice, one well for the test and one for the control, besides the analyzes were carried out in duplicate. Later, 50 µL of methaemoglobin were added to the test wells and 50 µL of saline solution were added to the control wells of each sample. The plates were incubated in darkroom for 10 minutes. Then, 150 µL of the guaiacol substrate and 50 µL of hydrogen peroxide (0.02 mol/L) were added to all wells. The spectrophotometric reading with a wavelength of 492 nm (ELX 808, Biotek, Vermont, USA) was performed after 10 minutes. The intra and inter coefficients variations were 7.25% and 8.19%, respectively.

Serum dosages of P4 and E2 were performed at the Hormonal Dosage Laboratory from Federal Fluminense University Veterinary School. The measurements of P4 were made by radioimmunoassay technique using a commercial kit (IM1188 - Immunotech, Beckman Coulter, Prague, Czech Republic). The measurements of E2 were performed using the double antigen liquid phase technique (07-138102 - ImmunChem, MP Biomedicals LLC, Oranhgeburg, NY, USA). The sensitivity of the assays and the intra- assay coefficients of variation were 5 pg/mL and 12% for 17 β-estradiol and 0.05 ng/mL and 10% for progesterone, respectively. Samples for setting the curve were evaluated in duplicate and samples of the trial underwent a single evaluation. The Wizard 2 gamma radiation counter was used to obtain the results provided. All concentrations were between the maximum and minimum points of the curve.

**Female genital tract examination**

The female genital tract examination was performed on days 0 and +1, via the inspection of external structures. The evaluation of vaginal secretion was done by Metricheck device (Simcro Datamars, Hamilton, New Zeland) [41] and rectal palpation was performed to evaluate the location, size and consistency of the uterus. In addition, transrectal ultrasonography (US) (DP2200VET, Mindray, Shenzhen, China) was used to collect uterine horn diameter information (obtained by the mean of the perpendicular diameters from the two horns), as well as iu fluid evaluation [42,43].

**Endometrial epithelium sample collection and processing**

Endometrial epithelium samples were obtained by a sterile cervical brush (Labor Import, Joinville - SC, Brazil) adapted and inserted in a universal semen applicator to pass through the cervix by the cytobrush technique [44]. The samples obtained were rolled on microscope slides, and then stained using the Rapid Panotic Kit (Renylab Química e Farmacêutica, Barbacena - MG, Brasil). A total of 100 cells were counted and the percentages of polymorphonuclear (PMN) and epithelial cells was recorded [45,46].

**Statistical Analyses**

Data and graphics were performed by GraphPad Prism 9 statistical software (GraphPad Software, San Diego, CA, USA). Cows were considered as an experimental unit. Data were tabulated initially in Excel spreadsheets and descriptive statistics were presented as mean and standard error of the mean (SEM).

Quantitative variables were subjected to the Kolmogorov-Smirnov test to verify whether or not they had a parametric distribution. Variables with parametric distribution were subjected to analysis of variance (parametric ANOVA) and Bonferroni's Multiple Comparison post-test or Two-way ANOVA with Bonferroni's Multiple Comparison post-test. Variables with non-parametric distribution were analyzed using the Kruskall-Wallis

test (non-parametric ANOVA) and Dunn's Multiple Comparison post-test. Statistical significance was established as P ≤ 0.05.

## RESULTS

### Surgical procedure and clinical examination

No surgical and / or clinical complications were observed in all animals (n = 8) submitted to the surgical procedure and during the trans and postoperative periods. The cows remained clinically healthy during all four steps of the experimental design.

All cows (n = 8) were clinically healthy and had an average weight of 408.7 ± 33 kg. They remained alert with normal ocular and vaginal mucosa. Respiratory rate, heart rate and rectal temperature were in accordance with references values [37].

### Serum levels of progesterone and estradiol

Serum P4 concentration from the control group (n = 8) on day 0 (LPS/vehicle challenge) is described in Fig. 2A. Serum E2 concentrations in the 24h - E2 group (n = 8) were higher on day 0 (LPS / vehicle challenge) compared to the previous time of treatment with E2 (day -1) (Figure 2B). The concentration of P4 in the 24h - P4 group on day 0 was higher (Fig. 2C) compared to day -1 (treatment with P4). In cows from group 14d - P4 (n = 8), P4 concentration on day 0 was higher than on day -14 however, it was reduced compared to day -7 (Fig. 2C).
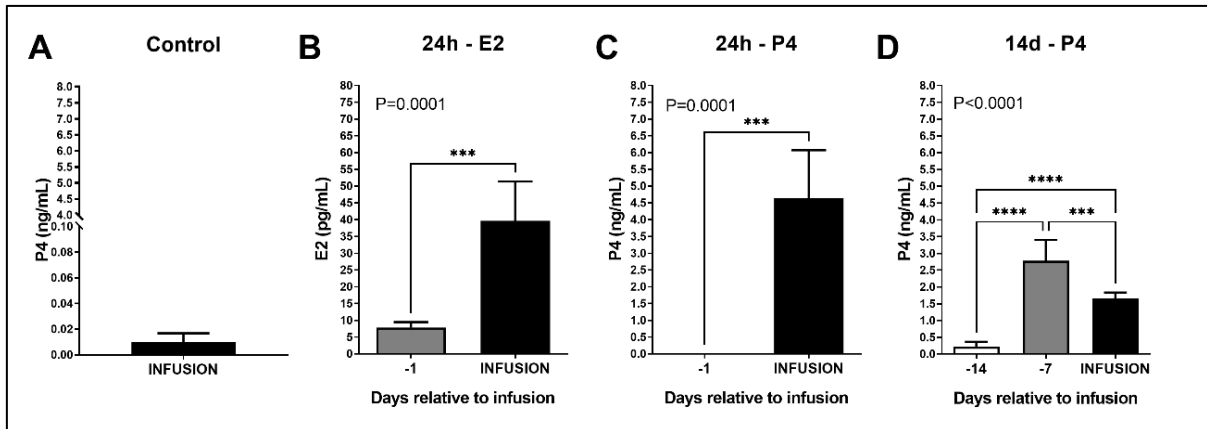
**Fig. 2.** Serum values of progesterone (P4 - ng/mL) in the experimental groups (1) Control, (2) 24h - E2, (3) 24h - P4, and (4) 14d - P4. Each experimental group had eight Gir cows, primiparous and ovariectomized. The animals in the control group did not receive hormonal administration, while the 24h - P4 and 14d - P4 groups were previously treated with 2.0 g of P4 intravaginal devices for 24 hours and 14 days, respectively. The 24h - E2 group was previously treated with 1 mg of estradiol benzoate intramuscular, two times per day. The day of infusion (day 0) was the time of iu infusion of LPS (5 mg / 20 mL 0.9% Nacl) or vehicle (20 mL 0.9% NaCl) and day +1 was considered 24 hours after the infusion. Data were presented as mean + SEM. Fig. 2B-C - Paired T test; Fig. 2D – An One-Way ANOVA and Bonferroni post-test were performed. P value (***, $P < 0.001$; ****, $P < 0.0001$).

**Female genital tract examination**

There were no significant changes in the clinical examination of the female genital tract. In the ultrasound exam, a difference of the diameter of the left horn between the 24h - E2 and 24h - P4 groups on day +1 of the animals challenged with LPS ($P < 0.05$; Table 1) was noted. The ultrasound characteristics of the iu fluid at any time between the four experimental groups were similar ($P > 0.05$; Table 1).

**Tab. 1.** Transrectal ultrasound evaluation of the female genital tract in the four experimental groups: (1) Control; (2) 24h - E2; (3) 24h - P4; and (4) 14d - P4, on day 0 and 24 hours (day +1) after an LPS / vehicle infusion. The diameter (mm) of the uterine horns was measured by the average of two perpendicular measurements from each horn diameter, in addition to the evaluation of the intrauterine fluid (IUF).

| | DAY | VEHICLE | | | |
| --- | --- | --- | --- | --- | --- |
| | | Control (n = 4) | 24h-E2 (n = 4) | 24h-P4 (n = 4) | 14d-P4 (n = 4) |
| Left horn (mm) | 0 | 10.7+0.7 | 12.9+0.6 | 11.7+2.0 | 12.2+1.4 |
| | +1 | 14.3+3.0 | 14.1+2.2 | 15.8+1.8 | 12.6+0.7 |
| Right horn (mm) | 0 | 10.7+1.1 | 12.9+1.0 | 12.2+1.3 | 13.0+0.8 |

| | | Control | 24h-E2 | 24h-P4 | 14d-P4 |
|---|---|---|---|---|---|
| | +1 | 12.4+0.5 | 12.4+0.9 | 14.0+0.7 | 12.3+0.9 |
| IUF (0-3) | 0 | 0.25+0.25 | 1.0+0.0 | 0.25+0.25 | 0.25+0.25 |
| | +1 | 0.5+0.5 | 1.0+0.0 | 1.75+0.75 | 0.75+0.5 |
| | | LPS CHALLENGE | | | |
| | | Control (n = 4) | 24h-E2 (n = 4) | 24h-P4 (n = 4) | 14d-P4 (n = 4) |
| Left horn (mm) | 0 | 10.3+0.7 | 11.3+1.4 | 14.4+0.6 | 13.3+1.1 |
| | +1 | 12.4+2.0 [ab] | 11.7+0.6 [a] | 16.4+0.6 [b] | 14.2+0.7 [ab] |
| Right horn (mm) | 0 | 9.6+0.9 | 12.0+1.3 | 13.7+0.7 | 14.6+1.0 |
| | +1 | 14.4+2.3 | 11.3+1.3 | 15.8+1.2 | 15.9+1.0 |
| IUF (0-3) | 0 | 0.0+0.0 | 1.0+0.0 | 1.0+0.4 | 0.25+0.25 |
| | +1 | 1.0+0.4 | 1.0+0.0 | 10.+0.7 | 1.0+0.4 |

**Note:** Each experimental group was composed by eight Gir cows, primiparous and ovariectomized. The animals in the control group did not receive hormonal administration, while the 24h - P4 and 14d - P4 groups were previously treated with 2.0 g of P4 intravaginal devices for 24 hours and 14 days, respectively. The 24h - E2 group was previously treated with 1 mg of estradiol benzoate intramuscular (im), two times *per* day. The day of infusion (day 0) was the time of iu infusion of LPS (5 mg / 20 mL 0.9% Nacl) or vehicle (20 mL 0.9% NaCl) and day +1 was considered 24 hours after the infusion. The IUF was measured according to Griffin and Ginther (1992) and Matheus et al. (2002). Datawere presented as mean + SEM. Different lower-case letters on the line mean difference using the Two-way ANOVA test and Bonferroni post-test ($P < 0.05$)

No difference was observed between iu infusion of LPS ($P = 0.0829$) or vehicle infusion ($P = 0.1514$; Fig. 3) groups related to the iu % PMN evaluation 24h after the iu infusion (day +1).
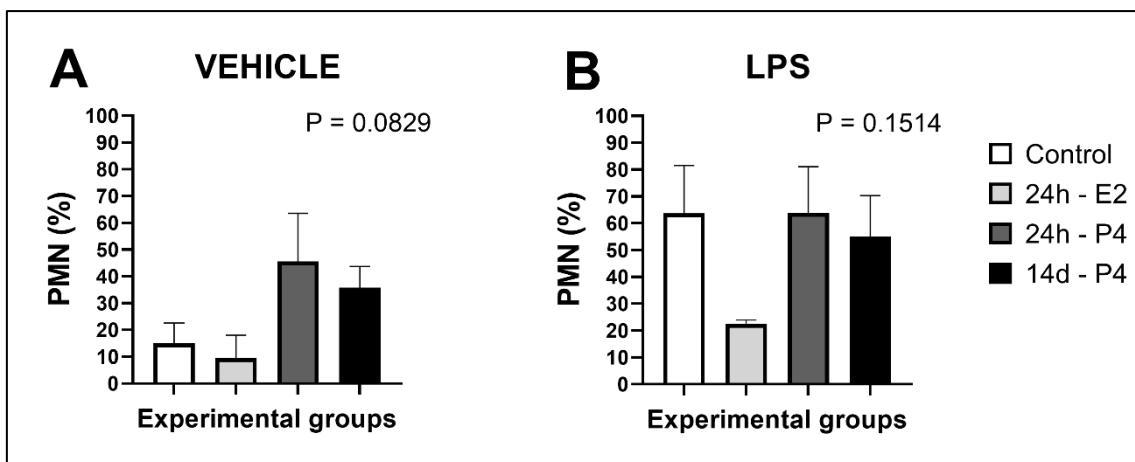


**Fig. 3.** Percentage of polymorphonuclear cells (% PMN) in endometrial cytology evaluation 24 hours after the LPS or vehicle iu infusion of experimental groups: (1) Control; (2) 24h - E2; (3) 24h - P4; and (4) 14d - P4. Each experimental group had eight Gir cows, primiparous and ovariectomized. The animals in the control group did not receive hormonal administration, while the 24h - P4 and 14d - P4 groups were
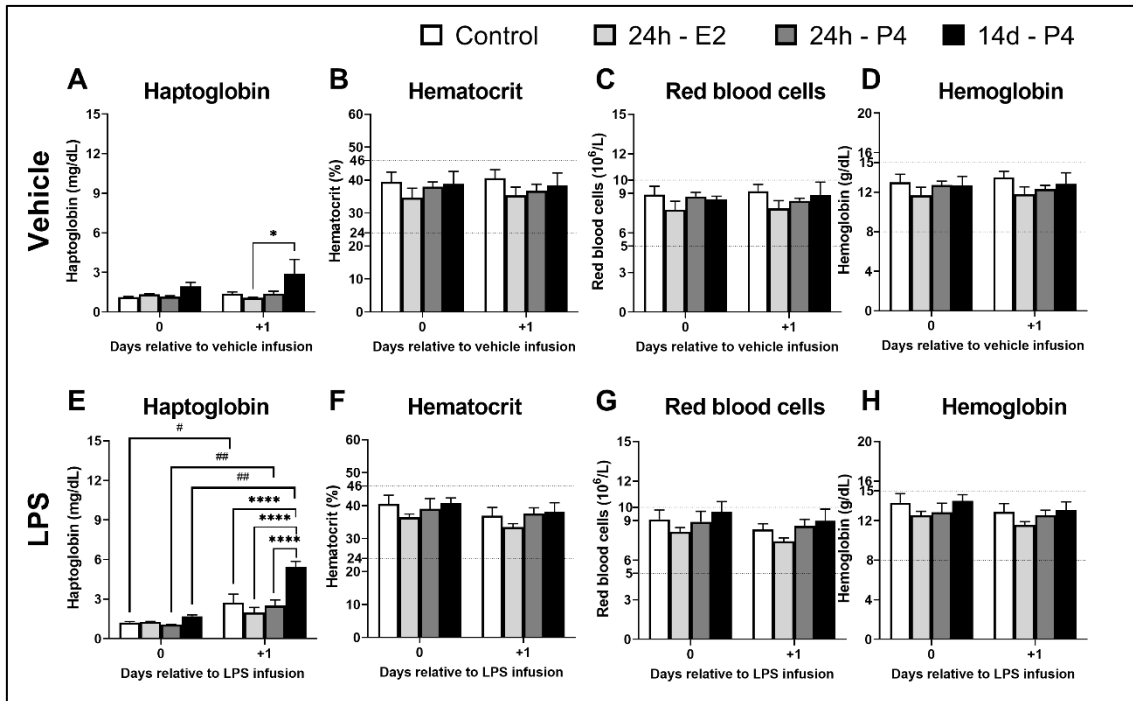
previously treated with 2.0 g of P4 intravaginal devices for 24 hours and 14 days, respectively. The 24h - E2 group was previously treated with 1 mg of estradiol benzoate intramuscular, two times per day. The day of infusion (day 0) was the time of iu infusion of LPS (5 mg / 20 mL 0.9% Nacl) or vehicle (20 mL 0.9% NaCl) and day +1 was considered 24 hours after the infusion. Data were presented as mean + SEM. Kruskal-Wallis test was performed (P < 0.05).

**Serum haptoglobin (Hp) and blood count**

The effect of hormonal treatments (P4 and E2) on the serum Hp concentration and on the complete blood count was performed separately, i.e., according to the iu challenge (Vehicle or LPS).

The serum Hp concentration in cows with iu vehicle challenge was different on day +1 between 14d - P4 (1.82 ± 0.45 mg/dL) and 24h - E2 groups (P < 0.05; Fig. 4A). In animals challenged with LPS, a significant increase in Hp was observed between days 0 (LPS challenge) and + 1 in cows from the control (P < 0.05; Fig. 4E), 24h - P4 (P < 0.01; Fig. 4E) and 14d - P4 (P < 0.005; Fig. 4E) groups, with the exception of the 24h - E2 group. When groups challenged with LPS were compared on day +1, the Hp values of group 14d - P4 were higher than all the other three groups (P < 0.0001; Fig. 4E). The erythrocyte patterns remained within the limits established for cattle [47], with no difference in the moments before (day 0) and after (day +1) of LPS iu infusion or t vehicle iu infusion, nor between the experimental groups (P > 0.05; Fig. 4B-D; Fig. 4 F-H).

**Fig. 4.** Serum Haptoglobin and erythrogram in experimental groups: (1) Control; (2) 24h - E2; (3) 24h - P4; and (4) 14d - P4. Each experimental group had eight Gir cows, primiparous and ovariectomized. The animals in the control group did not receive hormonal administration, while the 24h - P4 and 14d - P4 groups were previously treated with 2.0 g of P4 intravaginal devices for 24 hours and 14 days, respectively. The 24h - E2 group was previously treated with 1 mg of estradiol benzoate intramuscular, two times per day. The day of infusion (day 0) was the time of iu infusion of LPS (5 mg / 20 mL 0.9% Nacl) or vehicle (20 mL 0.9% NaCl) and day +1 was considered 24 hours after the infusion.  Data were presented as mean + SEM. A two-way ANOVA test with Bonferroni post-test were performed. P value (*, $P < 0.05$; ****, $P < 0.0001$; ##, $P < 0.05$; ###, $P < 0.01$). The dashed horizontal lines indicate the minimum and maximum reference values according to Smith (2014).

On day 0 (vehicle / LPS challenge) it was observed that the platelet counts from animals treated with P4 for 14 days (group 14d - P4) were lower than reference values [47] (Fig. 5A-B). Despite the platelet values were below the reference threshold, in the 14d - P4 group, a significant difference was observed only with animals in the 24h - E2 group (day 0), from both vehicle / LPS challenges.
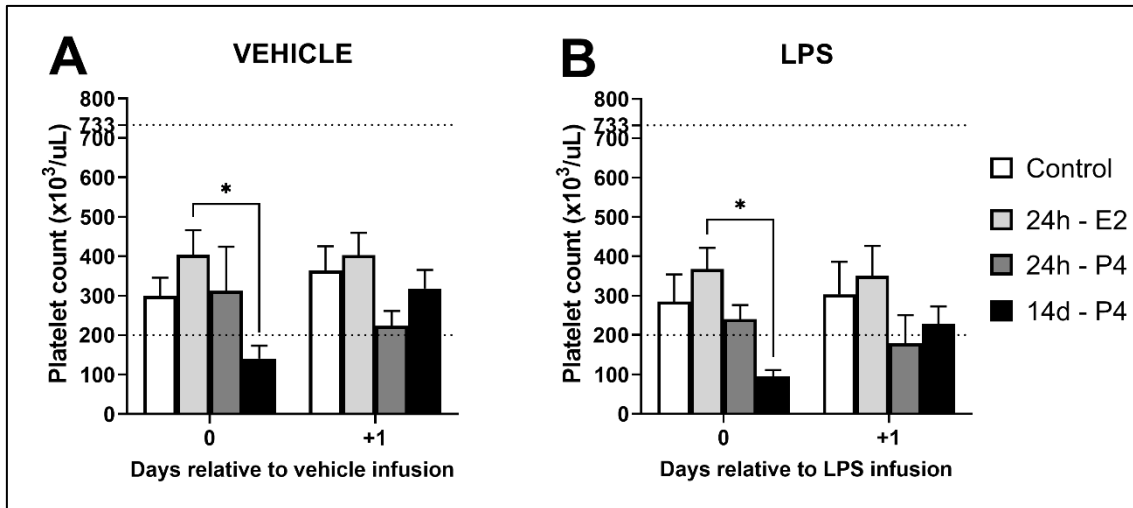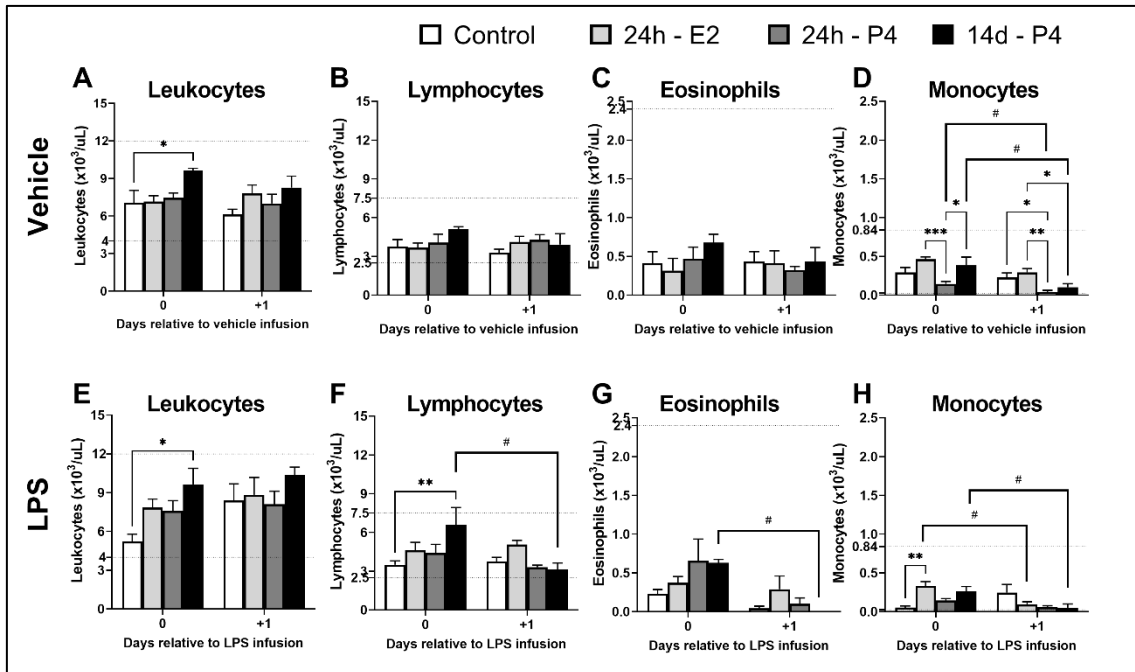
**Fig. 5.** Plaquetogram of the experimental groups: (1) Control; (2) 24h - E2; (3) 24h - P4; and (4) 14d - P4. Each experimental group had eight Gir cows, primiparous and ovariectomized. The animals in the control group did not receive hormonal administration, while the 24h - P4 and 14d - P4 groups were previously treated with 2.0 g of P4 intravaginal devices for 24 hours and 14 days, respectively. The 24h - E2 group was previously treated with 1 mg of estradiol benzoate intramuscular , two times per day. The day of infusion (day 0) was the time of iu infusion of LPS (5 mg / 20 mL 0.9% Nacl) or vehicle (20 mL 0.9% NaCl) and day +1 was considered 24 hours after the infusion. or vehicle (20 mL 0.9% NaCl) and day +1 was considered 24 hours after the infusion.  Data were presented as mean + SEM. A two-way ANOVA test with Bonferroni post-test were performed. P value (*, $P < 0.05$; ****, $P < 0.0001$; ##, $P < 0.05$; ###, $P < 0.01$). The dashed horizontal lines indicate the minimum and maximum reference values according to Smith (2014).

In the leukogram evaluation (Figs. 6-7), with the exception of absolute neutrophils and their fractions (Fig. 7), all other circulating leukocyte parameters remained within the physiological limits for the bovine species [47]. It was observed that on day 0 (vehicle / LPS challenge) the average of the total circulating leukocytes of the cows treated with P4 for 14 days (group 14d - P4) were higher than on those from the control group in both challenges (vehicle / LPS); ($P < 0.01$; Fig. 6A, Fig. 6B). There was a difference in circulating lymphocytes between groups 14d – P4 and control when they were challenged with LPS (Fig. 6F; $P = 0.006$). Still, on day 0, there were significant differences in circulating monocytes between groups 24h – E2 and 24h – P4 (Fig. 6D), groups 24h – P4 and 14d – P4 (Fig. 6D) and control and 24h – E2 groups (Fig. 6H).

**Fig. 6.** Leukogram of experimental groups: (1) Control; (2) 24h - E2; (3) 24h - P4; and (4) 14d - P4. Each experimental group had eight Gir cows, primiparous and ovariectomized. The animals in the control group did not receive hormonal administration, while the 24h - P4 and 14d - P4 groups were previously treated with 2.0 g of P4 intravaginal devices for 24 hours and 14 days, respectively. The 24h - E2 group was previously treated with 1 mg of estradiol benzoate intramuscular, two times per day. The day of infusion (day 0) was the time of iu infusion of LPS (5 mg / 20 mL 0.9% Nacl) or vehicle (20 mL 0.9% NaCl) and day +1 was considered 24 hours after the infusion. or vehicle (20 mL 0.9% NaCl) and day +1 was considered 24 hours after the infusion. Data were presented as mean + SEM. A two-way ANOVA test with Bonferroni post-test were perfomed. P value (*, P < 0.05; ****, P < 0.0001; ##, P < 0.05; ###, P < 0.01;). The dashed horizontal lines indicate the minimum and maximum reference values according to Smith (2014).

On day +1, cows in the 14d - P4 group, challenged with iu LPS infusion, showed a significant reduction in circulating lymphocytes (Fig. 6F; P < 0.05), eosinophils (Fig. 6G; P < 0.05) and monocytes (Fig. 6H; P < 0.05) values. After LPS infusion (day +1), there was a reduction in circulating monocytes in groups 24h - E2 and 14d - P4 in relation to day 0 (P < 0.05; Fig. 6H).

The values of circulating neutrophils in the four groups of cows that received iu vehicle were in accordance to those established for cattle [47]; No difference before (day 0) and after (day +1) nor between the experimental groups was observed (P > 0.05; Fig. 7A-C). However, in animals challenged with iu LPS infusion, a neutrophilia

profile was observed on day +1 in all groups, except for the 24h - E2 group (Fig. 7D). In addition, the groups treated with P4, both 24h and 14 days, on day +1 had a neutrophilia profile with deviation to the left (Fig. 7D-F).



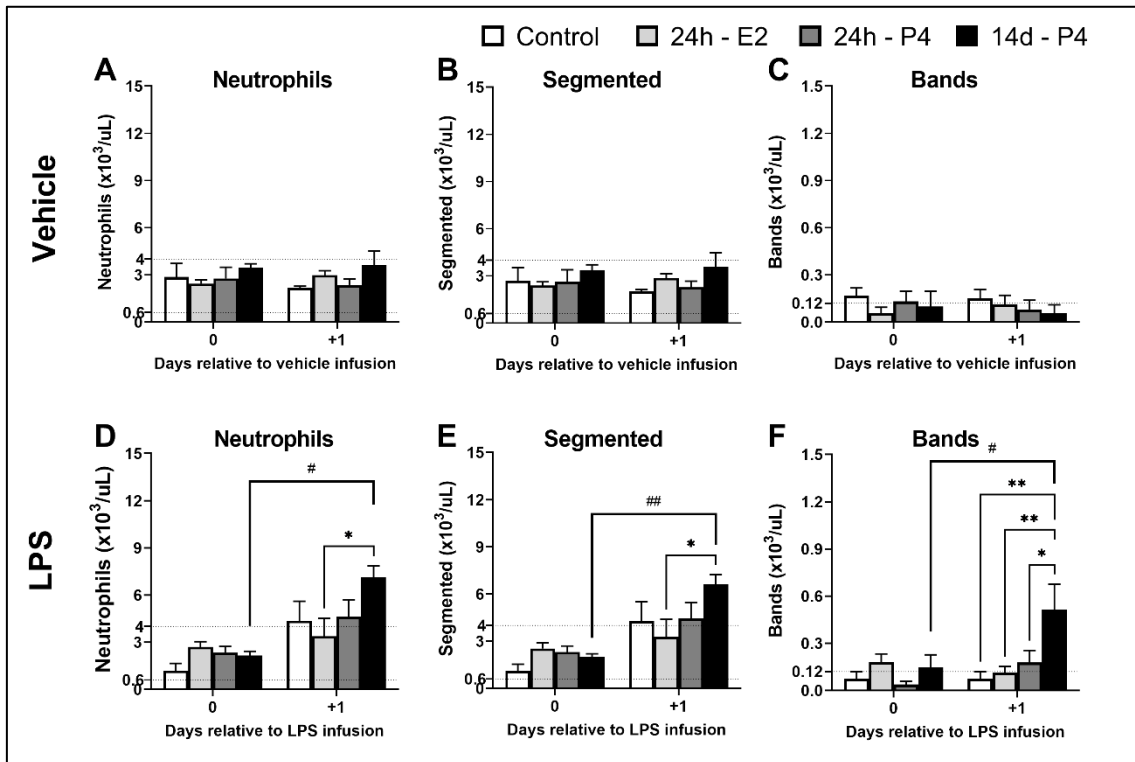**Fig. 7.** Absolut neutrophils count and their fractions in the experimental groups: (1) Control; (2) 24h - E2; (3) 24h - P4; and (4) 14d - P4. Each experimental group had eight Gir cows, primiparous and ovariectomized. The animals in the control group did not receive hormonal administration, while the 24h - P4 and 14d - P4 groups were previously treated with 2.0 g of P4 intravaginal devices for 24 hours and 14 days, respectively. The 24h - E2 group was previously treated with 1 mg of estradiol benzoate intramuscular, two times per day. The day of infusion (day 0) was the time of iu infusion of LPS (5 mg / 20 mL 0.9% NaCl) or vehicle (20 mL 0.9% NaCl) and day +1 was considered 24 hours after the infusion. or vehicle (20 mL 0.9% NaCl) and day +1 was considered 24 hours after the infusion. Data were presented as mean + SEM. A two-way ANOVA test with Bonferroni post-test were performed. P value (*, P < 0.05; ****, P < 0.0001; ##, P < 0.05; ###, P < 0.01). The dashed horizontal lines indicate the minimum and maximum reference values according to Smith (2014).

Total blood neutrophil values were higher in the 14d - P4 group on day +1 compared to the 24h - E2 group (P < 0.01; Fig. 7D), which was also observed in segmented neutrophils (P < 0.05; Fig. 7E) and banded neutrophils (P < 0.01; Fig. 7F)

values. Banded neutrophils values were also higher in the 14d - P4 group when compared to other groups (Control; 24h - P4) on day +1 (P < 0.05; Fig. 7F).

## DISCUSSION

We found that the iu challenge with 12.5 ug/kg of LPS in ovariectomized cows previously submitted to steroid hormones induced a systemic inflammatory response. The systemic response was more intense after the previous prolonged exposure of P4 and less intense in relation to E2 exposure. The LPS challenge after a 14 day exposure to P4 increased Hp, reduced circulating lymphocytes, eosinophils, and monocytes, with a left shift in the neutrophile profile.

The bilateral flank ovariectomy surgical technique [36] worked as expected reducing the serum P4 concentration. It was possible to maintain P4 values below 1 ng/mL before the beginning of hormonal treatments in each experimental group. Also, the treatments gave steroid concentrations similar to physiological levels. A rapid (24h - P4) and prolonged (14d - P4) exposure to P4 using controlled release devices was efficient in increasing the serum concentration of this hormone. According to Rathbone et al. [48] and Rathbone et al. [49], the P4 controlled-release devices keep plasma levels high for up to 12 days; however, for safety and to ensure release, new devices were used in group 14d - P4 after seven days of use, since Shimizu et al. [50], in a study with ovariectomized Holstein cows, used two P4 implants, reaching the plasma levels between 4 and 8 ng/mL.

In this study, the endometrial inflammatory response to the iu challenge with LPS was confirmed by the increase in the endometrial PMNs cells in all experimental groups (> 20 % PMNs). This local endometrial inflammatory response to the iu challenge with LPS was similar to those reported in uterine diseases [51], as well as in the induction of clinical endometritis by *Escherichia coli* and *Trueperella pyogenes* [52]. Increases in endometrial PMNs (%) were not only due to the LPS challenge, as animals that received vehicle also increased endometrial PMNs (%) after 24 hours probably associated with uterine manipulations. Likewise, Lüttgenau et al. [53] diagnosed cases of subclinical endometritis in both groups that received an intrauterine infusion with

PBS or LPS (3 ug/Kg of B.W.), although they manipulated the animals every 6 h and during the first 9 days of the estrous cycle.

In the experimental models of clinical endometritis, in addition to the infusion of bacteria there was also an extra scarification of the endometrium caused by brushes [9,54]. Scarification may be critical for the establishment of the disease, as this exposes the stroma, which is considered a more susceptible layer when compared to the epithelium [55]. Gilbert et al. [56] found that intact endometrium prevents the uptake of 5 ug/Kg *E. coli* endotoxin, but the presence of live pathogenic *E. coli* disrupted endometrial integrity and caused premature luteolysis. However, Lüttgenau et al. [53] induced premature luteolysis by increasing the frequency of iu infusion with repeated doses of 3ug/kg LPS, at 6h intervals (from 12h before and until 9 days after ovulation) without previous endometrial injury.

In the present study, with the intention to reduce as much as possible the variables that could influence the systemic inflammatory response, so we decided to increase the dose of LPS in a single dose of 12.5 ug/kg, to reduce uterine manipulation, in addition to not promote any physical, chemical or biological aggression to the endometrium, so that the experimental groups could become more homogeneous. However, despite a less intense local inflammatory response than that one observed in a natural inflammatory process in the postpartum of dairy cows [4], the present study showed that there was a systemic inflammatory response to the iu challenge with one dose of 12.5 ug/kg of LPS and that it was distinct and dependent on the previous exposure made by E2 and P4. Experimental intrauterine infusion of LPS reaches the peripheral circulation [57], and clinical uterine infections result in an increase in circulating LPS and acute phase proteins (APPs) [58–60].

Our main finding was that LPS challenge after a 14 day exposure to P4 increased Hp, reduced circulating lymphocytes, eosinophils, and monocytes, with a left shift in the neutrophile profile. The Hp is produced by the liver, when stimulated by interleukins (IL6 and IL1β), and is an inflammatory marker of spontaneous uterine diseases in cattle [51,52,61,62]. Piersanti et al. [52] also found that Hp concentration were 3.5x higher 13 days after an iu challenge infusion with *E. coli* and *T. pyogenes*, in cattle subjected to high concentrations of P4.

In relation to hematological changes in inflammatory processes, divergences in the literature were observed. Some studies report leukopenia in cows with clinical metritis [9], while others did not observe hematological changes when clinical endometritis was induced [52]. Subclinical endometritis increases circulating leukocytes, lymphocytes and monocytes when compared to healthy cows [63], although, there was no difference between luteal and non-luteal phases. The immunosuppressive effect of P4 on cattle endometrium has been described since 1953 using an experimental model similar to the one developed in the present study with ovariectomized cows [22], and has been reproduced over the years in the induction of uterine infections in different species, such as cows [64], ewes [65], rats [66,67] and sows [68].

The prolonged exposure to P4 (14 days) before challenge to LPS or 0.9% NaCl increased total circulating leukocytes, lymphocytes and monocytes, and this fact has not been described in the literature. Subandrio et al. [69] and Ahmadi et al. [70] reported that peripheral leukocyte count was not influenced by the reproductive state of cow, only Johnson et al. [71] showed significant changes in the white blood cell count, but none of these changes were sustained over a period longer than 2 days. In the present study, it is believed that the differences observed in circulating lymphocytes and monocytes between the LPS and vehicle groups, even before challenge, are likely due to the reduced number of animals per group (n = 4).

In this study, it was observed changes related to blood neutrophils, with a significant effect on the prolonged exposure of P4 by causing neutrophilia with deviation to the left. In addition, there was a difference in these circulating neutrophils when comparing the prolonged exposure of P4 versus E2, which showed significantly lower values of absolute neutrophils and their fractions. It is established that neutrophils are fast acting phagocytes [72], as they are cells that start protection in response to infectious agents, giving them a fast and efficient response at the infection site [1,4,46].

This study has brought novel information relative to the immune response in Gir cows, the main Zebu breed used in crossbreed genetic programs with Holstein cows in Brazil, considering this crossing as a new breed, called Girolando, since 1996. In this study, we observed and quantified the local and systemic inflammatory response

of Gir cows submitted to the iu challenge with LPS, after being exposed to different hormonal conditions involving ovarian steroids, which reflects the inherent parturition condition, both in relation to bacterial contamination and hormonal variations.

## CONCLUSIONS

This study provides important information related to the effect of steroids hormones on the systemic inflammatory response of cows challenged with intrauterine LPS. The challenge with intrauterine LPS infusion in ovariectomized Gir cows previously submitted to steroid hormones induce a systemic inflammatory response. The systemic response is more intense after the previous prolonged exposure of progesterone and less intense in relation to estradiol exposure.

## Declaration of Interests

None.

## Acknowledgments

# REFERENCES

[1]     Sheldon IM, Williams EJ, Miller ANA, Nash DM, Herath S. Uterine diseases in cattle after parturition. Vet J 2008;176:115–21. doi:10.1016/j.tvjl.2007.12.031.

[2]     Sheldon IM, Price SB, Cronin J, Gilbert RO, Gadsby JE. Mechanisms of infertility associated with clinical and subclinical endometritis in high producing dairy cattle. Reprod Domest Anim 2009;44:1–9. doi:10.1111/j.1439-0531.2009.01465.x.

[3]     LeBlanc SJ. Interactions of Metabolism, Inflammation, and Reproductive Tract Health in the Postpartum Period in Dairy Cattle. Reprod Domest Anim 2012;47:18–30. doi:10.1111/j.1439-0531.2012.02109.x.

[4]     Sheldon IM, Dobson H. Postpartum uterine health in cattle. Anim Reprod Sci 2004;82–83:295–306. doi:10.1016/j.anireprosci.2004.04.006.

[5]     Dubuc J, Duffield TF, Leslie KE, Walton JS, LeBlanc SJ. Risk factors for postpartum uterine diseases in dairy cows. J Dairy Sci 2010;93:5764–71. doi:10.3168/jds.2010-3429.

[6]     Potter TJ, Guitian J, Fishwick J, Gordon PJ, Sheldon IM. Risk factors for clinical endometritis in postpartum dairy cattle. Theriogenology 2010;74:127–34. doi:10.1016/j.theriogenology.2010.01.023.

[7]     Pinedo PJ, Galvão KN, Seabury CM. Innate immune gene variation and differential susceptibility to uterine diseases in Holstein cows. Theriogenology 2013;80:384–90. doi:10.1016/j.theriogenology.2013.04.027.

[8]     Chapinal N, Carson M, Duffield TF, Capel M, Godden S, Overton M, et al. The association of serum metabolites with clinical disease during the transition period. J Dairy Sci 2011;94:4897–903. doi:10.3168/jds.2010-4075.

[9]     Barragan AA, Piñeiro JM, Schuenemann GM, Rajala-Schultz PJ, Sanders DE, Lakritz J, et al. Assessment of daily activity patterns and biomarkers of pain, inflammation, and stress in lactating dairy cows diagnosed with clinical metritis. J Dairy Sci 2018;101:8248–58. doi:10.3168/jds.2018-14510.

[10]    Daibert E, De Alvarenga PB, Rezende AL, Fagundes NS, Krüger BC, Dos Santos RM, et al. Metabolites able to predict uterine diseases in crossbred dairy cows during the transition period. Semin Agrar 2018;39:1037–47. doi:10.5433/1679-0359.2018v39n3p1037.

[11]    Huzzey JM, Veira DM, Weary DM, von Keyserlingk MAG. Prepartum Behavior and Dry Matter Intake Identify Dairy Cows at Risk for Metritis. J Dairy Sci 2007;90:3220–33. doi:10.3168/jds.2006-807.

[12]    Huzzey JM, Duffield TF, LeBlanc SJ, Veira DM, Weary DM, Von Keyserlingk MAG. Short communication: Haptoglobin as an early indicator of metritis. J Dairy Sci 2009;92:621–5. doi:10.3168/jds.2008-1526.

[13]    Turner ML, Cronin JG, Healey GD, Sheldon IM. Epithelial and stromal cells of bovine endometrium have roles in innate immunity and initiate inflammatory responses to bacterial lipopeptides in vitro via Toll-like receptors TLR2, TLR1, and TLR6. Endocrinology 2014;155:1453–65. doi:10.1210/en.2013-1822.

[14]    Carneiro LC, Cronin JG, Sheldon IM. Mechanisms linking bacterial infections of the bovine endometrium to disease and infertility. Reprod Biol 2016;16:1–7. doi:10.1016/j.repbio.2015.12.002.

[15]    Machado VS, De Souza Bicalho ML, De Souza Meira EB, Rossi R, Ribeiro BL, Lima S, et al. Subcutaneous immunization with inactivated bacterial components and purified protein of Escherichia coli, Fusobacterium necrophorumand Trueperella pyogenes prevents puerperal metritis in holstein dairy cows. PLoS One 2014;9:1–11. doi:10.1371/journal.pone.0091734.

[16]    Beagley KW, Gockel CM. Regulation of innate and adaptive immunity by the female sex hormones oestradiol and progesterone. FEMS Immunol Med Microbiol 2003;38:13–22. doi:10.1016/S0928-8244(03)00202-5.

[17]    Wira CR, Grant-Tschudy KS, Crane-Godreau MA. Epithelial Cells in the Female Reproductive Tract: a Central Role as Sentinels of Immune Protection. Am J Reprod Immunol 2005;53:65–76. doi:10.1111/j.1600-0897.2004.00248.x.

[18]    Sheldon IM, Owens SE, Turner ML. Innate immunity and the sensing of infection, damage and danger in the female genital tract. J Reprod Immunol 2017;119:67–73. doi:10.1016/j.jri.2016.07.002.

[19]    Lewis GS. Steroidal regulation of uterine resistance to bacterial infection in livestock. Reprod Biol Endocrinol 2003;1:1–8. doi:10.1186/1477-7827-1-117.

[20]    Cui L, Wang H, Lin J, Wang Y, Dong J, Li J, et al. Progesterone inhibits inflammatory response in E.coli- or LPS-Stimulated bovine endometrial epithelial cells by NF-κB and MAPK pathways. Dev Comp Immunol 2020;105:103568. doi:10.1016/j.dci.2019.103568.

[21]    Madoz L V., Rabaglino MB, Migliorisi AL, Jaureguiberry M, Perez Wallace S, Lorenti N, et al. Association between progesterone concentration and endometrial gene expression in dairy cows. Domest Anim Endocrinol 2021;74:106481. doi:10.1016/j.domaniend.2020.106481.

[22]    Rowson L, Lamming G, Fry R. Influence of ovarian hormones on uterine infection. Nature 1953;171:749–50. doi:10.1038/171749a0.

[23]    Wulster-Radcliffe MC, Seals RC, Lewis GS. Progesterone increases susceptibility of gilts to uterine infections after intrauterine inoculation with infectious bacteria. J Anim Sci 2003;81:1242–52. doi:10.2527/2003.8151242x.

[24]    Wira CR, Fahey J V, Ghosh M, Patel M V, Hickey DK, Ochiel DO. Sex Hormone Regulation of Innate Immunity in the Female Reproductive Tract: The Role of Epithelial Cells in Balancing Reproductive Potential with Protection against Sexually Transmitted Pathogens. Am J Reprod Immunol 2010;63:544–65. doi:10.1111/j.1600-0897.2010.00842.x.

[25]    Wira CR, Fahey J V., Sentman CL, Pioli PA, Shen L. Innate and adaptive immunity in female genital tract: Cellular responses and interactions. Immunol Rev 2005;206:306–35. doi:10.1111/j.0105-2896.2005.00287.x.

[26]    Forde N, Mehta JP, Minten M, Crowe MA, Roche JF, Spencer TE, et al. Effects of low progesterone on the endometrial transcriptome in cattle. Biol Reprod 2012;87:1–11. doi:10.1095/biolreprod.112.103424.

[27]    Cheng L, Xin Y, Liu X, Hu X, Xiang M, Wang D, et al. The relationship between progesterone and Th-related cytokines in plasma during early pregnancy in cows. Front Agric Sci Eng 2016;3:147–52. doi:10.15302/J-FASE-2016099.

[28]    Fischer C, Drillich M, Odau S, Heuwieser W, Einspanier R, Gabler C. Selected pro-inflammatory factor transcripts in bovine endometrial epithelial cells are regulated during the oestrous cycle and elevated in case of subclinical or clinical endometritis. Reprod Fertil Dev 2010;22:818–29. doi:10.1071/RD09120.

[29]    Richardson J, Kaushic C, Wira CR. Estradiol regulation of secretory component: Expression by rat uterine epithelial cells. J Steroid Biochem Mol Biol 1993;47:143–9. doi:10.1016/0960-0760(93)90068-8.

[30]    Turk R, Piras C, Kovačić M, Samardžija M, Ahmed H, De Canio M, et al. Proteomics of inflammatory and oxidative stress response in cows with subclinical and clinical mastitis. J Proteomics 2012;75:4412–28. doi:10.1016/j.jprot.2012.05.021.

[31]    Campos CC, Hartling I, Kaur M, Fernandes ACC, Santos RM, Cerri RLA. Intramammary infusion of lipopolysaccharide promotes inflammation and alters endometrial gene expression in lactating Holstein cows. J Dairy Sci 2018;101:10440–55. doi:10.3168/jds.2018-14393.

[32]    Campos CC, do Prado FL, dos Reis JPJ, Carneiro LC, Silva PRB, de Moraes GF, et al. Effects of clinical mastitis and puerperal diseases on reproductive efficiency of dairy cows. Trop Anim Health Prod 2020;52:3061–8. doi:10.1007/s11250-020-02326-2.

[33]    EMBRAPA. Milk production system; 2003. https://sistemasdeproducao.cnptia.embrapa.br/FontesHTML/Leite/LeiteZonadaMata Atlantica/expediente.htm (accessed March 15, 2021).

[34]    Madalena FE, Teodoro RL, Lemos AM, Monteiro JBN, Barbosa RT. Evaluation of Strategies for Crossbreeding of Dairy Cattle in Brazil. J Dairy Sci 1990;73:1887–901. doi:10.3168/jds.S0022-0302(90)78869-8.

[35]    Freitas AF, Wilcox CJ, Costa CN. Breed Group Effects on Milk Production of Brazilian Crossbred Dairy Cows. J Dairy Sci 1998;81:2306–11. doi:10.3168/jds.S0022-0302(98)75811-4.

[36]    Weaver AD, Atkinson O, Jean GS, Steiner A. Bovine surgery and Lameness. 3th ed. Hoboken: Wiley Blackweel; 2018.

[37]    Terra RL, Reynolds JP. Ruminant history, physical examination, welfare assessment, and records. In: Smith BP, editor. Large Anim. Intern. Med. 5th ed., St. Louis: Elsevier Mosby; 2014, p. 52–63.

[38]    Ferreira Neto JM, Viana ES, Magalhães LM. Patologia clínica veterinária. 2nd ed. Belo Horizonte: Rabelo; 1982.

[39]    Jones GE, Mould DL. Adaptation of the guaiacol (peroxidase) test for haptoglobins to a microtitration plate system. Res Vet Sci 1984;37:87–92. doi:10.1016/s0034-5288(18)31933-7.

[40]    Ramos JS, Klug FSF, Bastos BL, Rocha Filho JTR, Ayres MCC, Guimarães JE. Determinação da concentração sérica de proteínas de fase aguda em equinos por técnicas espectrofotométricas. Pubvet 2018;12:1–7. doi:10.22256/pubvet.v12n1a17.1-7.

[41]    Williams EJ, Fischer DP, Pfeiffer DU, England GCW, Noakes DE, Dobson H, et al. Clinical evaluation of postpartum vaginal mucus reflects uterine bacterial infection and the immune response in cattle. Theriogenology 2005;63:102–17. doi:10.1016/j.theriogenology.2004.03.017.

[42]    Griffin PG, Ginther OJ. Research applications of ultrasonic imaging in reproductive biology. J Anim Sci 1992;70:953–72. doi:10.2527/1992.703953x.

[43]    Mateus L, Lopes Da Costa L, Bernardo F, Robalo Silva J. Influence of puerperal uterine infection on uterine involution and postpartum ovarian activity in dairy cows. Reprod Domest Anim 2002;37:31–5. doi:10.1046/j.1439-0531.2002.00317.x.

[44]    Cardoso B, Oliveira ML, Pugliesi G, Batista EOS, Binelli M. Cytobrush: A tool for sequential evaluation of gene expression in bovine endometrium. Reprod Domest Anim 2017;52:1153–7. doi:10.1111/rda.13037.

[45]    Gilbert RO, Shin ST, Guard CL, Erb HN, Frajblat M. Prevalence of endometritis and its effects on reproductive performance of dairy cows. Theriogenology 2005;64:1879–88. doi:https://doi.org/10.1016/j.theriogenology.2005.04.022.

[46]    Gilbert RO, Santos NR. Dynamics of postpartum endometrial cytology and bacteriology and their relationship to fertility in dairy cows. Theriogenology 2016;85:1367–74. doi:10.1016/j.theriogenology.2015.10.045.

[47]    Smith BP. Large Animal Internal Medicine. 5th ed. St. Louis: Elsevier; 2014.

[48]    Rathbone MJ, Macmillan KL, Bunt CR, Burggraaf S. Conceptual and commercially available intravaginal veterinary drug delivery systems. Adv Drug Deliv Rev 1997;28:363–92. doi:10.1016/S0169-409X(97)00089-6.

[49]    Rathbone MJ, Bunt CR, Ogle CR, Burggraaf S, Macmillan KL, Burke CR, et al. Reengineering of a commercially available bovine intravaginal insert (CIDR insert) containing progesterone. J Control Release 2002;85:105–15. doi:10.1016/S0168-3659(02)00288-2.

[50]    Shimizu T, Krebs S, Bauersachs S, Blum H, Wolf E, Miyamoto A. Actions and interactions of progesterone and estrogen on transcriptome profiles of the bovine endometrium. Physiol Genomics 2010;42 A:290–300. doi:10.1152/physiolgenomics.00107.2010.

[51]    Barragan AA, Lakritz J, Carman MK, Bas S, Hovingh E, Schuenemann GM. Short communication: Assessment of biomarkers of inflammation in the vaginal discharge of postpartum dairy cows diagnosed with clinical metritis. J Dairy Sci 2019;102:7469–75. doi:10.3168/jds.2018-15854.

[52]    Piersanti R, Zimpel R, Molinari PCC, Dickson MJ, Ma Z, Jeong KC, et al. A model of clinical endometritis in Holstein heifers using pathogenic Escherichia coli and Trueperella pyogenes. J Dairy Sci 2019;102:2686–97. doi:10.3168/jds.2018-15595.

[53]    Lüttgenau J, Lingemann B, Wellnitz O, Hankele AK, Schmicke M, Ulbrich SE, et al. Repeated intrauterine infusions of lipopolysaccharide alter gene expression and lifespan of the bovine corpus luteum. J Dairy Sci 2016;99:6639–53. doi:10.3168/jds.2015-10806.

[54]    Nessan GK, King GJ. Sexual behaviour in ovariectomized cows treated with oestradiol benzoate and testosterone propionate. J Reprod Fertil 1981;61:171–8. doi:10.1530/jrf.0.0610171.

[55]    Sheldon IM, Rycroft AN, Dogan B, Craven M, Bromfield JJ, Chandler A, et al. Specific strains of Escherichia coli are pathogenic for the endometrium of cattle and cause pelvic inflammatory disease in cattle and mice. PLoS One 2010;5. doi:10.1371/journal.pone.0009192.

[56]    Gilbert RO, Bosu WTK, Peter AT. The effect of Escherichia coli endotoxin on luteal function in Holstein heifers. Theriogenology 1990;33:645–51. doi:10.1016/0093-691X(90)90541-Z.

[57]    Peter AT, Bosu WT, DeDecker RJ. Suppression of preovulatory luteinizing hormone surges in heifers after intrauterine  infusions of Escherichia coli endotoxin. Am J Vet Res 1989;50:368–73.

[58]    Magata F, Ishida Y, Miyamoto A, Furuoka H, Inokuma H, Shimizu T. Comparison of bacterial endotoxin lipopolysaccharide concentrations in the blood, ovarian follicular fluid and uterine fluid: A clinical case of bovine metritis. J Vet Med Sci 2015;77:81–4. doi:10.1292/jvms.14-0333.

[59]    Mateus L, Lopes da Costa L, Diniz P, Ziecik AJ. Relationship between endotoxin and prostaglandin (PGE2 and PGFM) concentrations and ovarian function in dairy cows with puerperal endometritis. Anim Reprod Sci 2003;76:143–54. doi:10.1016/S0378-4320(02)00248-8.

[60]    Williams EJ, Fischer DP, Noakes DE, England GCW, Rycroft A, Dobson H, et al. The relationship between uterine pathogen growth density and ovarian function in the postpartum dairy cow. Theriogenology 2007;68:549–59. doi:10.1016/j.theriogenology.2007.04.056.

[61]    Brodzki P, Kostro K, Krakowski L, Marczuk J. Inflammatory cytokine and acute phase protein concentrations in the peripheral blood and uterine washings of cows with subclinical endometritis in the late postpartum period. Vet Res Commun 2015;39:143–9. doi:10.1007/s11259-015-9635-4.

[62]    Pohl A, Burfeind O, Heuwieser W. The associations between postpartum serum haptoglobin concentration and metabolic status, calving difficulties, retained fetal membranes, and metritis. J Dairy Sci 2015;98:4544–51. doi:10.3168/jds.2014-9181.

[63]    Raliou M, Dembélé D, Düvel A, Bolifraud P, Aubert J, Mary-Huard T, et al. Subclinical endometritis in dairy cattle is associated with distinct mRNA expression patterns in blood and endometrium. PLoS One 2019;14:220–4. doi:10.1371/journal.pone.0220244.

[64]    Hawk HW, Brinsfield TH, Turner GD, Whitmore GW, Norcross MA. Effect of ovarian status on induced acute inflammatory response in cattle uteri. Am J Vet Res 1964;25:362—366.

[65]    Seals RC, Wulster-Radcliffe MC, Lewis GS. Modulation of the uterine response to infectious bacteria in postpartum ewes. Am J Reprod Immunol 2002;47:57–63. doi:10.1034/j.1600-0897.2002.1o062.x.

[66]    Nishikawa Y, Baba T. Effects of ovarian hormones on manifestation of purulent endometritis in rat uteruses infected with Escherichia coli. Infect Immun 1985;47:311–7. doi:10.1128/iai.47.1.311-317.1985.

[67]    Nishikawa Y, Baba T, Imori T. Effect of the estrous cycle on uterine infection induced by Escherichia coli. Infect Immun 1984;43:678–83. doi:10.1128/iai.43.2.678-683.1984.

[68]    De Winter PJJ, Verdonck M, De Kruif A, Devriese LA, Haesebrouck F. Influence of the Oestrous Cycle on Experimental Intrauterine E. coli Infection in the Sow. J Vet Med Ser A 1994;41:640–4. doi:10.1111/j.1439-0442.1994.tb00131.x.

[69]    Subandrio AL, Sheldon IM, Noakes DE. Peripheral and intrauterine neutrophil function in the cow: The influence of endogenous and exogenous sex steroid hormones. Theriogenology 2000;53:1591–608. doi:10.1016/S0093-691X(00)00300-9.

[70]    Ahmadi MR, Nazifi S, Ghaisari HR. Comparison of hormonal changes of estrous cycle with cytology of cervical mucosa and hematological parameters in dairy heifers. Comp Clin Path 2006;15:94–7. doi:10.1007/s00580-006-0613-7.

[71]    Johnson SK, Johnson AR, Keefer CL, Silcox RW. Blood constituents during the estrous cycle and early pregnancy in dairy cows. Theriogenology 1990;34:701–7.

[72]    Witko-Sarsat V, Rieu P, Descamps-Latscha B, Lesavre P, Halbwachs-Mercarelli L. Neutrophils: molecules, functions and pathophysiological aspects. Lab Investig 2000;80:617–53. doi:https://doi.org/10.1038/labinvest.3780067.

# CAPÍTULO 3 – ASSOCIATION BETWEEN OVARIAN STEROIDS AND PGR, ESR1, IL1B, IL6, AND CCL5 GENE EXPRESSION IN GIR COWS

**Artigo nas normas para publicação na revista
Domestic Animal Endocrinology**

**Association between ovarian steroids and PGR, ESR1, IL1β, IL6, and CCL5 endometrial gene expression in Gir cows**

L. Q. Magalhães[a], A. Nonato[b], L. C. Carneiro[c], M. J. B. Silva[d], J. P. E. Saut[a*]

[a]*Large Animal Health Laboratory, Federal University of Uberlandia (UFU), Uberlandia, MG, Brazil;*
[b] *ABS Brazil;*
[c] *Department of Animal Reproduction, Laboratory of Teaching and Research in Pathology of Reproduction, Center of Biotechnology in Animal Reproduction,*
*School of Veterinary Medicine and Animal Science, University of Sao Paulo (USP), Pirassununga, SP, Brazil;*
[d] *Laboratory of Tumor Biomarkers and Osteoimmunology, Institute of Biomedical Sciences, Federal University of Uberlandia, Uberlândia, Minas Gerais, Brazil.*

*Corresponding author: *Corresponding author: [jpsaut@ufu.br](mailto:jpsaut@ufu.br) (J.P.E. Saut)*

**ABSTRACT –** Several systemic interactions generate a previous inflammatory response to the clinical signs of uterine diseases, which elevates some inflammatory factors, such as acute phase proteins and inflammatory cytokines. The aim of this study was to evaluate the response of estrogen receptor α (ESR1), progesterone receptor (PGR), interleukin 1β (IL1β), interleukin 6 (IL6), and chemokine ligand 5 (CCL5) gene expression in ovariectomized Gir cows exposed to ovarian steroids as progesterone (P4) and estradiol (E2). For this, four experimental groups were executed as: Group 1: control group (n = 8) – the cows did not receive hormonal administration before day +1; Group 2: 24h - E2 (n = 8) – the cows received E2 24 hours (day 0)

before day +1; Group 3: 24h - P4 (n = 8) – the cows received intravaginal devices P4 24 hours (day 0) before day +1; Group 4: 14d - P4 (n = 8) – the cows received P4 before day + 14. On day 0 and day +1 or day +14, clinical and female genital tract examination, blood sampling for hormonal dosage (P4 and E2) and endometrial sampling for gene expression analyses (ESR1, PGR, IL1β and IL6, CCL5) were performed. A down regulation of PGR and ESR1 gene expression was verified when uterus was exposes to P4 for a long period; the exposure to E2 did not interfere with both PGR and ESR1 genes expression. Concentrations of P4 were capable to up-regulate the IL6 gene expression at 14d - P4 exposition, and when compared to the 24h - E2 exposition group, there was a significant difference in the expression of IL1β and IL6. Only the exposure to 24h - E2 was capable to down-regulated CCL5 gene expression. It was concluded that concentrations of P4 and E2 regulate the ovarian steroid receptors (ESR1 and PGR) expression. Still, a long-term exposure to P4 up-regulated IL1β and IL6 gene expression while estradiol down-regulated CCL5 gene expression.

*Keywords: Bos indicus; estradiol; immune response; progesterone; proinflammatory cytokines; uterus*

**INTRODUCTION**

A high incidence of inflammatory diseases in the peripartum of high-yielding dairy cows is related, especially to uterine diseases, which occur in the immediate postpartum period, as retained placenta [1] and metritis [1,2], or later, as clinical [1,2] and cytological endometritis, between 30 and 60 days in milk (DIM) [3], in addition to cervicitis at 30 DIM [3].

There are several systemic interactions that generate a previous inflammatory response to the clinical signs of uterine diseases, which elevates some inflammatory factors, such as acute phase proteins [4] and inflammatory cytokines [5,6]. Among the factors that contribute to the intensification of the inflammatory response in cows, some of them contribute to the appearance of uterine diseases as bacterial contamination during parturition [7]; metabolic [8–11] and endocrine alterations [12–14] that occur

during the transition period (especially when immune function is reduced) [15,16] and the occurrence of some concomitant diseases [17].

Among the inflammatory cytokines that are relevant in uterine diseases, the interleukin 1β (IL1β), is one that is produced by macrophages and amplifies inflammation by activating B lymphocytes [18] and neutrophils [19]. Whereas the cytokine interleukin 6 (IL6), is secreted by endometrial cells [20,21], and they may be associated with the amount of neutrophils in endometritis [22], causing an inflammation activity by stimulating the production of interleukin 10 (IL10) [23]. The chemokine ligand 5 (CCL5), is produced by T lymphocytes and macrophages and its attracts monocytes and eosinophils which activates the basophils cells that will release histamine to sites of action [24].

Recent studies have evaluated the influence of the endocrine on the immune system in the female genital tract [12,14,25] where ovarian steroids are able to regulate certain activities from uterine epithelial cells, controlling the gene expression of some cytokines in the reproductive tract [26]. Different species, including cattle, have demonstrated susceptibility to infection when progesterone (P4) concentrations are high [27,28], suggesting that P4 has an immunosuppressive effect, while estradiol (E2) has a protective effect [29–31].

Some particularities related to greater or lesser resistance to diseases are observed between different breeds of cattle. *Bos indicus* cows have a greater potential for tolerance, however, few studies have demonstrated the genetic mechanisms by which this tolerance is expressed when compared to *Bos taurus*. For example, according to Rezende et al. [32], the incidence of infections such as acute puerperal metritis and clinical endometritis are 14.3% in *Bos taurus* against 11.9% in *Bos indicus*.

To clarify this difference between breeds, Kumar et al. [33], showed the modification in some pathways, with differences in the expression of 6,767 transcripts, involving innate immune response, regulation of the immune response, presentation and processing of antigens, among others. These changes could be related to the lower occurrence of uterine infections in *Bos indicus* cows, showing a certain resistance inherent to these breeds.

In Brazil, the crossing of *Bos indicus* with Bos *taurus* cows (Gir x Holstein) represent most of the dairy herd [34] and 80% of 25,4 bi liters of milk production [35,36].

In Brazilian dairy production, due to the importance of *Bos indicus* animals, especially Gir cows, it is important to better understand the factors that interfere in the uterine immune response of these animals, since they are particular to Brazil, and little explored in studies, given that most of the researches are based on Holstein cows.

In this study, we hypothesized that the uterine inflammatory response is less intense under the effects of P4 and increased under the action of E2. For this, we aimed to evaluate the response of estrogen receptor α (ESR1), progesterone receptor (PGR), IL1β, IL6, and CCL5 gene expression in ovariectomized Gir cows exposed to ovarian steroids as P4 and E2.

## MATERIAL AND METHODS

### Ethics statement

In this study, all procedures were developed according to the Ethical Principles in Animal Experimentation approved by the Committee of Ethics in the Use of Animals (CEUA) of the Federal University of Uberlândia (UFU), under the protocol n.123/15.

### Animals, experiment location and surgical procedure

A total of eight Gir (*Bos indicus*) cows, from a commercial dairy farm located in Uberlândia, Minas Gerais, Brazil, with an average of 30 months of age, were used in this study. They were previously evaluated for female genital tract diseases. The animals selected were transferred to Federal University of Uberlândia (UFU) to two collective pens (n=4/each pen), and they were fed with corn silage, mineral salt (with 15% of urea) and water *ad libitum*. An available shade was also offered for both pens.

After animal adaptation in the respectively pens (15 days), a bilateral flank ovariectomy surgery was performed, as described by Weaver et al. (2018). Surgical procedure was performed in the Surgical Department of the Veterinary Hospital (UFU). They were daily monitored during ten days after the surgery until stitches were removed. The experiment procedure started only after 100 days of the postoperative period as previously described by Magalhães et al. [38].

**Experiment groups**

       The animals were randomly separated in the groups. The experiment was performed in four steps, with intervals of approximately 30 days between each step. In all steps the moment of steroidal hormone administration was defined as day zero (D0). At this time (day 0), physical and female genital tract examinations, and blood samples (to evaluate hormonal measurements) were performed. In addition, day +1 (24 hours after the E2 or P4 exposure, or no hormone exposure) or day +14 (14 days after the P4 exposure) was defined as the time to evaluate the local uterine inflammatory response (in all groups) in which physical and female genital tract examinations, endometrial epithelium, and blood samples (to evaluate hormonal measurements) were performed. In both moments the local uterine inflammatory response (in all groups) in which physical and female genital tract examinations, endometrial epithelium, and blood samples (to evaluate hormonal measurements) was evaluated.
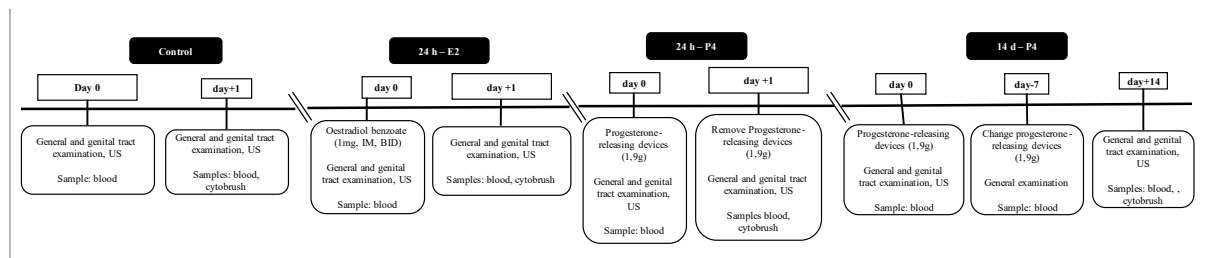


**Figure 1** - Schematic figure of the procedures performed in the four experimental groups. Eight ovariectomized Gir cows, were exposed to estradiol (E2) or progesterone (P4) and then evaluated the endometrial inflammatory response.

       For research development, four experimental groups were executed as: Group 1: control group (n = 8) –the cows did not receive hormonal administration before day +1; Group 2: 24h - E2 (n = 8) –the cows received E2 (1 mg of estradiol benzoate, Estrogin, Biofarm, Jaboticabal-SP, Brazil, IM) 24 hours (day 0) before the proposed evaluation (day +1); Group 3: 24h - P4 (n = 8) – in this group the cows received P4 (two intravaginal devices of first use of 1.0 g of P4, Sincrogest, Ourofino Saúde Animal, Ribeirão Preto -SP, Brazil) 24 hours (day 0) before the proposed evaluation (day +1); Group 4: 14d - P4 (n = 8) – in this group the cows received the previous treatment of

14 days with P4 (two intravaginal devices of first use of 1.0 g of P4, Sincrogest, Ourofino Saúde Animal, replaced at seven days) before the proposed evaluation (day + 14).

**Clinical and female genital tract examination**

For general clinical evaluation of the animals, the body temperature, heart and respiratory rates were measured according to the recommendations by Terra and Reynolds [39].

The genital tract examination was executed on day 0 (all groups), day +1 (group 24h – E2, and group 24h - P4) and, day +14 (group 14d – P4), via the inspection of external structures. The evaluation of vaginal secretion was done by Metricheck device (Simcro Datamars, Hamilton, New Zeland) [40], rectal palpation and transrectal ultrasonography (US) (DP2200VET, Mindray, Shenzhen, China) was used to collect uterine horn diameter information (obtained by the mean of the perpendicular diameters from the two horns), as well as iu fluid evaluation, evaluate the location, size and consistency of the uterus.

**Blood and endometrial samples**

To obtain blood samples, a venipuncture of the median caudal vein in the animals were performed, for serum analysis of P4 and E2 that were realized at the Hormonal Dosage Laboratory from Federal Fluminense University Veterinary School. The measurements of P4 were executed by radioimmunoassay technique using a commercial kit (IM1188 - Immunotech, Beckman Coulter, Prague, Czech Republic), and for E2 a double antigen liquid phase technique (07-138102 - ImmunChem, MP Biomedicals LLC, Oranhgeburg, NY, USA) was used.

The sensitivity of the assays and the intra- assay coefficients of variation were 5 pg/mL and 12% for E2 and 0.05 ng/mL and 10% for P4, respectively. Samples for setting the curve were evaluated in duplicate and samples of the trial underwent a single evaluation. The Wizard 2 gamma radiation counter was used to obtain the

results provided. All concentrations were between the maximum and minimum points of the curve.

Endometrial epithelium samples were obtained by a sterile cervical brush (Labor Import, Joinville - SC, Brazil) adapted and inserted in a universal semen applicator to pass through the cervix by the cytobrush technique [41]. The samples obtained were placed in microtubes containing 500 µL of later RNA Solution Invitrogen® (Thermo Fisher Scientific, Waltham, MA USA), sent under refrigeration to the laboratory and placed in an ultrafreezer (-80ºC) for further analysis of the mRNA expression of the genes selected.

**Gene expression analyses**

Before the RNA extraction procedure, the cytobrush samples were washed in D-PBS (Dulbeco's phosphate buffered saline, Gibco, Thermo Fisher Science, Waltham, MA USA) to remove the RNA later and transferred to another microtube. The RNA extraction from the endometrial cytobrush sample was performed using a Maxwell® 16 Instrument (Promega, Madison, WI, USA) with the extraction kit Maxwell® RSC simplyRNA Cells Kit (AS1390, Promega, Madison, WI, USA). All steps followed the manufacturer's recommendation.

A total of 200 µL of homogenization solution were added to each cryogenic tube containing a cytobrush, then vortexed to recover the cytobrush endometrial secretion. After that, 200 µL of lysis buffer were added and the tubes were vortexed again. The supernatants were collected and placed in the cartridges (disposable rack available in the kit). The cartridges were prepared using all reagents and subsequently inserted into the Maxwell® 16 Instrument for RNA extraction, after which the total RNA was quantified on a Quantus™ Fluorometer using the QuantiFluor® RNA System kit (E3310, Promega, Madison, WI, USA) according to the manufacturer's recommendations. All RNAs were stored in an ultrafreezer at -80°C until reverse transcription.

Reverse transcription (RT) was performed using the QuantiNova Reverse Transcription Kit (205413, Qiagen, Hilden, Germany). All steps were executed according to the manufacturer's recommendations. Initially, 2 µL of Mix qDNA removal

were previously prepared with 3 μL of water and 10 μL of the respective sample and this mixture placed in the thermocycler (6333, Eppendorf, Hamburg, Germany) for the first incubation. Meanwhile, RT Mix was prepared to be mixed in the sample tubes and incubated in a thermocycler again for RT enzyme denaturation. Finally, the cDNA of each sample was quantified on a Quantus™ Fluorometer using the QuantiFluor™ ssDNA System kit (E3190, Promega, Madison, WI, USA), and stored in a freezer at – 20 °C.

Quantification of genes that encode the pro-inflammatory mediators such IL1β, IL6, CCL5, and ESR1 and PGR receptors were performed with an StepOne Plus (Applied Biosystems, Foster, CA, USA), using the threshold cycle (Ct) comparative method (Livak and Schmittgen, 2001). The Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) gene was used as a control. An MicroAmp™ Optical 96-Well Reaction Plate with Barcode (4306737, Applied Biosystems, Foster, CA, USA) was used and the amplifications were performed in duplicate. The reaction conditions were determined previously by standardized cycles of 95°C for 2 minutes, 40 cycles of 95°C for 5 seconds and 60°C for 10 seconds. For the melting curve the conditions were 95°C for 15 seconds, 60°C for 1 minute and 95°C for 15 seconds.

For the reaction, a QuantiNova™ SYBR Green PCR Kit (208056, Qiagen, Hilden, Germany) was used. All steps were according to the manufacturer's recommendation. The proportions of the reagents used in the mix for each sample were: 5 μL of sybr green PCR Master MIX, 1.0 μL of QN ROX Reference Dye, 0.2 μL of forward primer, 0.2 μL of reverse primer, 1.6 μL of RNAse-free water and 2.0 μL of cDNA at a concentration of 35 ng/μL. The primers used to amplify IL1β, IL6, CCL5, ESR1 and PGR are shown in Table 1. The endogenous gene selected was GAPDH, as it is expressed in uterine tissue (Wathes et al., 2009).

**Table 1** – Primers and probes used for analysis of gene expression in qPCR

| Gene | Primers/ probes (5'-3') | Access number Gene Bank* |
|------|--------------------------|--------------------------|
| GAPDH | *Forward:* GGTCACCAGGGCTGCTTTTA<br>*Reverse:* TTCCCGTTCTCTGCCTTGAC | NM_001034034.2 |
| IL1 β | *Forward:* ACGAGTTTCTGTGTGACGCA<br>*Reverse:* TGCAGAACACCACTTCTCGG | NM_174093.1 |
| IL6 | *Forward:* GGGCTCCCATGATTGTGGTA<br>*Reverse*: GTGTGCCCAGTGGACAGGTT | NM_173923.2 |

| CCL5 | *Forward:*CATGGCAGCAGTTGTCTTTATCA<br>*Reverse:* CTCTCGCACCCACTTCTTCTCT | NM_175827 |
|---|---|---|
| PGR | *Forward*: TCCCCCCACTGATCAACTTG<br>*Reverse*: TCCGAAAACCTGGCAGTGA | AJ557823.1 |
| ESR1 | *Forward*: CAGGCACATGAGCAACAAAG<br>*Reverse*: TCCAGCAGCAGGTCGTAGAG | NM_001001443.1 |

Note: *https://www.ncbi.nlm.nih.gov/gene/; GAPDH – glyceraldehyde-3-phosphate dehydrogenase; IL1β – interleukin 1β; IL6 - interleukin 6; CCL5 – chemokines ligand 5; PGR – progesterone receptor; ESR1 – estrogen receptor 1.

**Statistical analyses**

Graphics and data available were executed by GraphPad Prism 9 statistical software (GraphPad Software, San Diego, CA, USA). Cows were considered as an experimental unit. Data were tabulated initially in Excel spreadsheets and descriptive statistics were presented as mean and standard error of the mean (SEM).

Variables considered as quantitative (gene expression) were subjected to the Kolmogorov-Smirnov test to verify whether or not they had a parametric distribution, those with parametric distribution were subjected to analysis of variance (parametric ANOVA) and Tukey's Multiple Comparison post-test (IL6, PGR, ESR1); and those with non-parametric distribution were analyzed using the Kruskall-Wallis test (non-parametric ANOVA) and Dunn's Multiple Comparison post-test (ILβ, CCL5). Statistical significance was considered as $P \leq 0.05$.

**RESULTS**

The cows remained clinically healthy during all four steps of the experimental design. All cows (n = 8) were clinically healthy and had an average weight of 408.7 ± 33 kg. No significant changes in the clinical examination of the female genital tract were observed.

**Serum levels of progesterone and estradiol**

Serum P4 and E2 concentrations are described in table 2. Serum E2 concentrations in the 24h - E2 group (n = 8) were higher on day +1 compared to the

previous time of collection (day 0). In the 24h - P4 group the concentration on day +1 was higher compared to day 0 (treatment with P4). The group 14d - P4 (n = 8) presented P4 concentration on day +14, higher than on day 0 however, it was reduced compared to day +7.

**Table 2.** Serum concentration of progesterone and estradiol according to groups (1) Control, (2) 24h - E2, (3) 24h - P4, and (4) 14d - P4.

| Groups | Days after hormone administration | | | | |
| --- | --- | --- | --- | --- | --- |
| | Day 0 | Day +1 | Day +7 | Day +14 | *P value* |
| Progesterone (ng/mL) | | | | | |
| **(1) Control** | 0.01 ± 0.02 | - | - | - | - |
| **(3) 24h – P4** | 0.00 ± 0.00[a] | 4.64 ± 1.43[b] | - | - | 0.0001 |
| **(4) 14d – P4** | 0.22 ± 0.41[a] | - | 2.78 ± 0.62[b] | 1.66 ± 0.49[c] | <0.0001 |
| 17 β- estradiol (pg/mL) | | | | | |
| **(2) 24h – E2** | 7.86 ± 4.59[a] | 39.64 ± 11.73[b] | | | 0.0001 |

Note: The control (1) group did not receive hormonal administration, while the 24h - P4 (3) and 14d - P4 (4) groups were previously treated with 2.0 g of P4 intravaginal devices for 24 hours and 14 days, respectively. The 24h - E2 (2) group was previously treated with 1 mg of estradiol benzoate i.m. two times *per* day. Data were presented as mean + SD. Paired T test (groups 2 and 3). One-Way ANOVA and Tukey's Multiple Comparison post-test (group 4).

**Steroidal hormones receptors and inflammatory mediators gene expression**

The gene expression of PGR is described in Figure 2A. The P4 pre-treatment groups (24h - P4 and 14d - P4) had lower PGR gene expression (P = 0.001) when compared to the control group. Regarding ESR1 gene expression (Fig. 2B), the difference observed was only in the expression of the 14d - P4 group when compared to the control group (P = 0.0459).
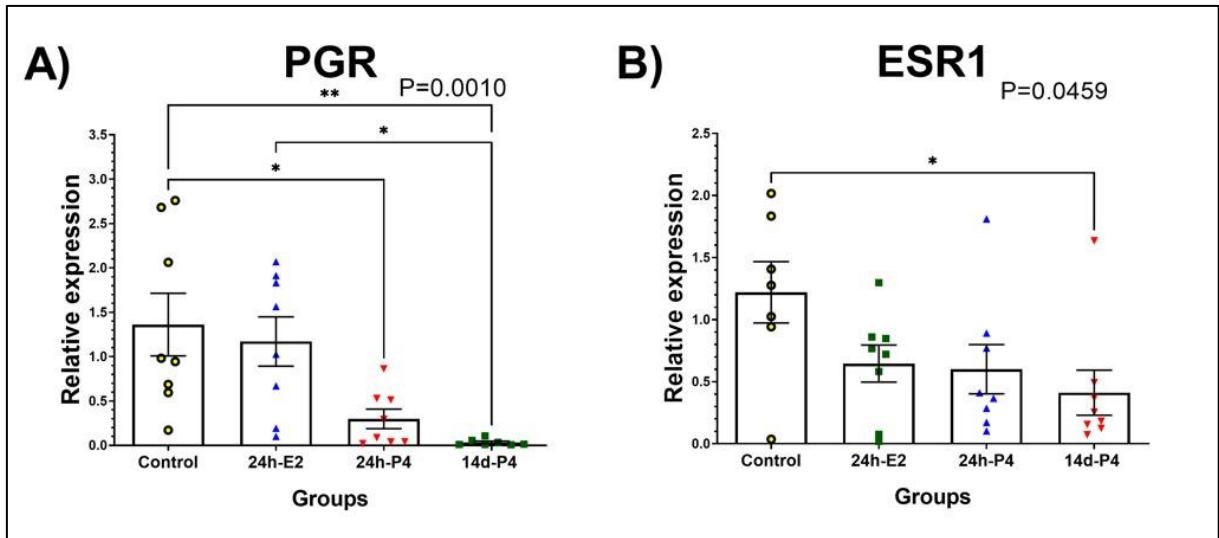
**Figure 2** – Relative gene expression of progesterone and estradiol receptors in endometrial epithelial cells under E2 and P4 treatments, progesterone (PGR) and estradiol 1 (ESR1) of the experimental groups: (1) control; (2) 24h - E2; (3) 24 - P4 e (4) 14d - P4. Each experimental group had eight Gir cows, primiparous and ovariectomized. The animals in the control group did not receive hormonal administration, while the 24h - P4 and 14d - P4 groups were previously treated with 2.0 g of P4 intravaginal devices for 24 hours and 14 days, respectively. The 24h - E2 group was previously treated with 1 mg of estradiol benzoate intramuscular, two times *per* day. Data were presented as mean + SEM. Fig. 2A; 2B – An One-Way ANOVA and Tukey's Multiple Comparison post-test were performed. P value (*, *P < 0.05*; **, *P < 0.01*).

The local response of the endometrium to P4 and E2 is shown in Figure 3 and was evaluated through gene expression of the inflammatory mediators as IL1β (Fig. 3A), IL6 (Fig. 3B) and CCL5 (Fig. 3C). IL1β gene expression (Fig. 3A) was higher (P = 0.0031) in the group exposed to P4 for a long period (14d - P4), when compared to the group exposed to estradiol (24h - E2). IL6 (Fig. 3B) had higher expression (P = 0.0001) in the group treated with P4 for a long period (14d - P4), when compared to the other groups. The chemokine CCL5 (Fig. 3C) had lower expression (P=0.0120) in the 24h - E2 treatment, when compared to the control group.
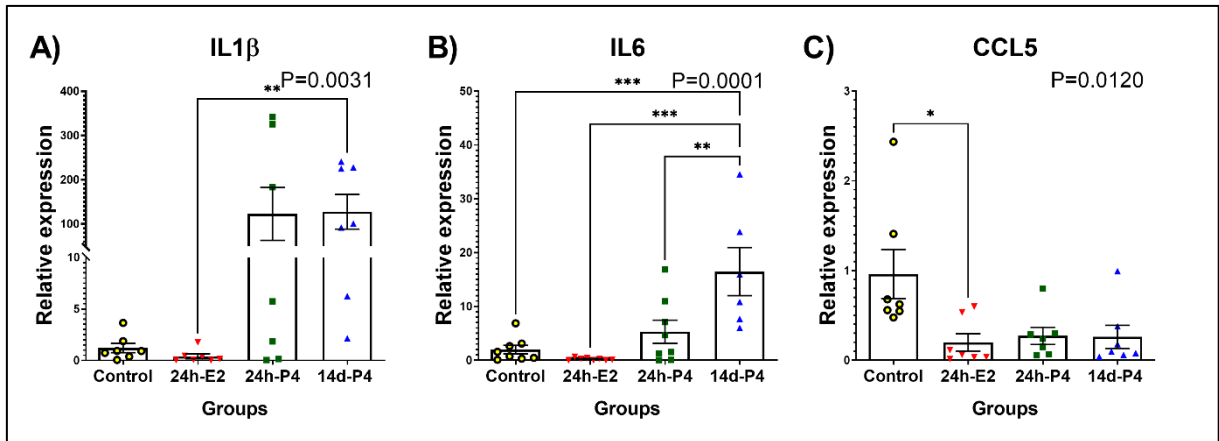
**Figure 3** – Uterine endometrial epithelial cells cytokines IL1β and IL6 and chemokine CCL5 gene expression of the experimental groups: (1) control; (2) 24h - E2; (3) 24h - P4 e (4) 14d - P4. Each experimental group had eight Gir cows, primiparous and ovariectomized. The animals in the control group did not receive hormonal administration, while the 24h - P4 and 14d - P4 groups were previously treated with 2.0 g of P4 intravaginal devices for 24 hours and 14 days, respectively. The 24h - E2 group was previously treated with 1 mg of estradiol benzoate intramuscular, two times *per* day. Data were presented as mean + SEM. Fig. 3A, 3C - Kruskall-Wallis test and Dunn's Multiple Comparison post-test; Fig. 3B - One-way ANOVA and Tukey's Multiple Comparison post-test. P value (*, *P < 0.05*; **, *P < 0.01*; ***, *P < 0.001*).

## DISCUSSION

The effect of ovarian hormones on endometrial immune response and uterine infections is still very controversial, however, some associations have been described. In the present study, when evaluating the effect of these hormones *in vivo* in previously ovariectomized Gir cows, a more effective action was verified in the down regulation of PGR and ESR1 gene expression when there was a long exposure to P4, also with 24h of P4 exposure, a down regulation in PGR gene expression was observed.

Exposure to E2 did not interfere with both PGR and ESR1 genes expression. Regarding the measured cytokines, exposure to P4 promoted greater interference with up-regulation of IL6 gene expression at 14d - P4 exposition, and when compared to the 24h - E2 exposition group, there was a significant difference in the expression of IL1β and IL6. When CCL5 gene expression was evaluated, it was observed that only the exposure to 24h - E2 was capable to down-regulated this gene.

The surgical procedure performed to reduce and standardize the effect of ovarian hormones, was the left flank bilateral ovariectomy surgical technique [37], and it worked as expected reducing the serum P4 concentration [38].

In this study, the long exposure to P4 reduced the gene expression of both PGR and ESR1 receptors on epitelial endometrial cells. These results agree with those described by Spencer and Bazer [42] and Spencer et al. [43] in ewes, and also, are in accordance to Okumu et al. [44], that observed the same effect in cattle. They affirmed that P4 inhibits the expression of its own receptors, and also, are capable to block estrogen receptor during the mid-luteal stage of the estrous cycle. Still, P4 is able to modulate the effects of E2, reducing its action on ESR1 [26], consequently, when there is a predominance of P4, there would be less expression of the two receptors [45]. In addition to the receptor expression pattern, ovarian steroids alter the environment of endometrial epithelial cells and, consequently, their mechanical barrier function in innate immunity also changes, modifying cellular pathways for cytokines and chemokines secretions [26].

In this study, after 24h of induction with E2, it was not possible to demonstrate that estrogen modify the expression of ESR1 receptor as well as those for PGR, as observed in ovariectomized ewes by Wathes et al.  [46]. Also, in *ex vivo* bovine endometrial cultures, ovarian steroids receptors (ESR1 and PGR) expression was regulated by progesterone and estradiol [25].

This experiment showed that primiparous Gir cows exposure to P4, up-regulated the expression of IL1B and IL6, different from Madoz et al. [14], who associated Holstein dairy cows with low and not with high physiological P4 concentration, which was evaluated on days 4 and 9 of the estrus cycle, with up-regulation of genes involved in the immune system and inflammatory response.

Fischer et al. [47], collected cows endometrial samples from a slaughterhouse and classified the estrous cycle according to utero-ovarian morphology characteristics (without hormonal measurement) to evaluate the expression of cytokines in each estrous stage. They found an increase in CCL5, IL1β and IL8 gene expression in the groups around the ovulation (pre- and post-ovulatory phase) in relation to the luteal phase. In contrast, they found significant differences in IL6 mRNA expression between the different stages of cows' estrous cycle.

The treatment with 24h - E2 exposure did not alter the IL1β and IL6 cytokines expression when compared to the control group, however, when compared to P4 treatment, increased IL1β and IL6 cytokines expression and decreased CCL5 gene expression when compared to control group.

The effect of E2 on endometrial immune response and uterine infections is still controversial, as some studies relate that during the follicular phase of the estrus stage, when estradiol concentrations are high, the endometrium is more resistant to infection [29,30]. On the other hand, Galvão et al. [48] observed a higher estradiol concentration on the day of calving for cows that developed postpartum metritis and concluded that greater plasma estradiol levels at calving in cows that had metritis could be another contributing factor to their immunosuppression. According to Wyle and Kent [49] and Goff and Horst [50], the plasma estradiol on the day of calving is also thought to contribute to the overall immunosuppression described in cows around calving.

Conferring to Cunningham and Gilkeson [51], studies exploring estradiol *in vitro* showed a primarily anti-inflammatory action, still, high levels of estradiol reduced the inflammatory response (Fahey et al., 2008; Schaefer et al., 2005), as it inhibits the expression of pro-inflammatory mediators by endometrial cells (Wira et al., 2014), increasing epithelial cells proliferation (Zhu; Pollard, 2007) and secretion of antimicrobial peptides (Taggart et al., 2005). Together, these events create an anti-inflammatory environment, reducing the secretion of inflammatory cytokines and, consequently, cell migration, suspending the inflammatory environment associated with infections (Wira; Rodriguez-Garcia; Patel, 2015).

It is suggested that these differences observed in cytokines expression could be related to the experimental model used, or a characteristic of Gir cows, since they are animals that present greater resistance to diseases compared to European cows, due to better adaptability to tropical climate, since the genotype-environment interaction is very important when considering production models, where these animals will be part of Brazilian climatic conditions [57,58].

**CONCLUSIONS**

It is concluded that in ovariectomized Gir cows, the concentrations of progesterone in long exposition regulate the ovarian steroid receptors (ESR1 and PGR) expression. Still, long-term exposure to progesterone up-regulated IL1β and IL6 gene expression, while estradiol down-regulated CCL5 gene expression.

## Declaration of interests

None.

## Author contributions

**L.Q. Magalhães**: Investigation, Methodology, Writing e original draft. **A. Nonato**: Investigation, Methodology. **L.C. Carneiro**: Investigation, Methodology, Writing e review & editing. **M.J.B. Silva**: Investigation, Methodology. **J.P.E. Saut**: Conceptualization, Funding acquisition, Project administration, Writing e review & editing.

## Acknowledgements

**REFERENCES**

[1]     Sheldon IM, Williams EJ, Miller ANA, Nash DM, Herath S. Uterine diseases in cattle after parturition. Vet J 2008;176:115–21. doi:10.1016/j.tvjl.2007.12.031.

[2]     Sheldon IM, Cronin J, Goetze L, Donofrio G, Schuberth HJ. Defining Postpartum Uterine Disease and the Mechanisms of Infection and Immunity in the Female Reproductive Tract in Cattle. Biol Reprod 2009;81:1025–32. doi:10.1095/biolreprod.109.077370.

[3]     Leblanc SJ. Interactions of Metabolism, Inflammation, and Reproductive Tract Health in the Postpartum Period in Dairy Cattle. Reprod Domest Anim 2012;47:18–30. doi:10.1111/j.1439-0531.2012.02109.x.

[4]     Huzzey JM, Duffield TF, LeBlanc SJ, Veira DM, Weary DM, Von Keyserlingk MAG. Short communication: Haptoglobin as an early indicator of metritis. J Dairy Sci 2009;92:621–5. doi:10.3168/jds.2008-1526.

[5]     Cheng L, Xin Y, Liu X, Hu X, Xiang M, Wang D, et al. The relationship between progesterone and Th-related cytokines in plasma during early pregnancy in cows. Front Agric Sci Eng 2016;3:147–52. doi:10.15302/J-FASE-2016099.

[6]     Forde N, Mehta JP, Minten M, Crowe MA, Roche JF, Spencer TE, et al. Effects of low progesterone on the endometrial transcriptome in cattle. Biol Reprod 2012;87:1–11. doi:10.1095/biolreprod.112.103424.

[7]     Sheldon IM, Dobson H. Postpartum uterine health in cattle. Anim Reprod Sci 2004;82–83:295–306. doi:10.1016/j.anireprosci.2004.04.006.

[8]     Dubuc J, Duffield TF, Leslie KE, Walton JS, LeBlanc SJ. Risk factors for postpartum uterine diseases in dairy cows. J Dairy Sci 2010;93:5764–71. doi:10.3168/jds.2010-3429.

[9]     Chapinal N, Carson M, Duffield TF, Capel M, Godden S, Overton M, et al. The association of serum metabolites with clinical disease during the transition period. J Dairy Sci 2011;94:4897–903. doi:10.3168/jds.2010-4075.

[10]    Barragan AA, Piñeiro JM, Schuenemann GM, Rajala-Schultz PJ, Sanders DE, Lakritz J, et al. Assessment of daily activity patterns and biomarkers of pain, inflammation, and stress in lactating dairy cows diagnosed with clinical metritis. J Dairy Sci 2018;101:8248–58. doi:10.3168/jds.2018-14510.

[11]    Daibert E, De Alvarenga PB, Rezende AL, Fagundes NS, Krüger BC, Dos Santos RM, et al. Metabolites able to predict uterine diseases in crossbred dairy cows during the transition period. Semin Agrar 2018;39:1037–47. doi:10.5433/1679-0359.2018v39n3p1037.

[12]    Cui L, Wang H, Lin J, Wang Y, Dong J, Li J, et al. Progesterone inhibits inflammatory response in E.coli- or LPS-Stimulated bovine endometrial epithelial cells by NF-κB and MAPK pathways. Dev Comp Immunol 2020;105:103568. doi:10.1016/j.dci.2019.103568.

[13] Lewis GS. Steroidal regulation of uterine resistance to bacterial infection in livestock. Reprod Biol Endocrinol 2003;1:1–8. doi:10.1186/1477-7827-1-117.

[14] Madoz L V., Rabaglino MB, Migliorisi AL, Jaureguiberry M, Perez Wallace S, Lorenti N, et al. Association between progesterone concentration and endometrial gene expression in dairy cows. Domest Anim Endocrinol 2021;74:106481. doi:10.1016/j.domaniend.2020.106481.

[15] Chapinal N, Leblanc SJ, Carson ME, Leslie KE, Godden S, Capel M, et al. Herd-level association of serum metabolites in the transition period with disease, milk production, and early lactation reproductive performance. J Dairy Sci 2012;95:5676–82.

[16] LeBlanc SJ, Lissemore KD, Kelton DF, Duffield TF, Leslie KE. Major advances in disease prevention in dairy cattle. J Dairy Sci 2006;89:1267–79. doi:10.3168/jds.S0022-0302(06)72195-6.

[17] Campos CC, do Prado FL, dos Reis JPJ, Carneiro LC, Silva PRB, de Moraes GF, et al. Effects of clinical mastitis and puerperal diseases on reproductive efficiency of dairy cows. Trop Anim Health Prod 2020;52:3061–8. doi:10.1007/s11250-020-02326-2.

[18] Wira CR, Fahey J V, Rodriguez-Garcia M, Shen Z, Patel M V. Regulation of mucosal immunity in the female reproductive tract: The role of sex hormones in immune protection against sexually transmitted pathogens. Am J Reprod Immunol 2014;72:236–58. doi:10.1111/aji.12252.

[19] Sica A, Matsushima K, Van Damme J, Wang JM, Polentarutti N, Dejana E, et al. IL-1 transcriptionally activates the neutrophil chemotactic factor/IL-8 gene in endothelial cells. Immunology 1990;69:548–53.

[20] Cronin JG, Turner ML, Goetze L, Bryant CE, Sheldon IM. Toll-Like Receptor 4 and MYD88-Dependent Signaling Mechanisms of the Innate Immune System Are Essential for the Response to Lipopolysaccharide by Epithelial and Stromal Cells of the Bovine Endometrium1. Biol Reprod 2012;86. doi:10.1095/biolreprod.111.092718.

[21] Turner ML, Cronin JG, Healey GD, Sheldon IM. Epithelial and stromal cells of bovine endometrium have roles in innate immunity and initiate inflammatory responses to bacterial lipopeptides in vitro via Toll-like receptors TLR2, TLR1, and TLR6. Endocrinology 2014;155:1453–65. doi:10.1210/en.2013-1822.

[22] Ghasemi F, Gonzalez-Cano P, Griebel PJ, Palmer C. Proinflammatory cytokine gene expression in endometrial cytobrush samples harvested from cows with and without subclinical endometritis. Theriogenology 2012;78:1538–47. doi:https://doi.org/10.1016/j.theriogenology.2012.06.022.

[23] Kim IH, Kang HG, Jeong JK, Hur TY, Jung YH. Inflammatory cytokine concentrations in uterine flush and serum samples from dairy cows with clinical or subclinical endometritis. Theriogenology 2014;82:427–32. doi:10.1016/j.theriogenology.2014.04.022.

[24] Tizard IR. Imunidade Inata: Mediadores Próinflamatórios e Antimicrobianos. In:

Tizard IR, editor. Imunol. Veterinária. 9th ed., São Paulo-SP: Elsevier; 2014, p. 70–84.

[25] Saut JPE, Healey GD, Borges AM, Sheldon IM. Ovarian steroids do not affect bovine endometrial cytokine or chemokine responses to Escherichia coli or LPS in vitro. Reproduction 2014;148:593–606. doi:10.1530/REP-14-0230.

[26] Wira CR, Fahey J V., Sentman CL, Pioli PA, Shen L. Innate and adaptive immunity in female genital tract: Cellular responses and interactions. Immunol Rev 2005;206:306–35. doi:10.1111/j.0105-2896.2005.00287.x.

[27] Rowson LE, Lamming GE, Fry RM. Influence of ovarian hormones on uterine infection. Nature 1953;171:749–50. doi:10.1038/171749a0.

[28] Wulster-Radcliffe MC, Seals RC, Lewis GS. Progesterone increases susceptibility of gilts to uterine infections after intrauterine inoculation with infectious bacteria. J Anim Sci 2003;81:1242–52. doi:10.2527/2003.8151242x.

[29] Lewis GS. Steroidal regulation of uterine immune defenses. Anim Reprod Sci 2004;82:281–94. doi:10.1016/j.anireprosci.2004.04.026.

[30] Lewis GS. Role of ovarian progesterone and potential role of prostaglandin F2alpha and prostaglandin E2 in modulating the uterine response to infectious bacteria in postpartum ewes. J Anim Sci 2003;81:285–93.

[31] Rowson LE. Methods of inducing multiple ovulation. J Endocrinol 1951;7:260–70.

[32] Rezende EV, Campos CC, Moraes GF, Santos RM. Factors related to uterine score and its influence on pregnancy per artificial insemination in crossbred dairy cows. Livest Sci 2020;241:104231. doi:10.1016/j.livsci.2020.104231.

[33] Kumar A, Gaur GK, Panigrahi M, N. MA V, Priya B, Gupta JP, et al. Comparative gene expression profile in circulating PBMCs of Bos indicus and crossbred cattle to understand disease tolerance mechanism. Anim Biotechnol 2022;0:1–9. doi:10.1080/10495398.2022.2043883.

[34] Facó O, Lôbo RNB, Martins Filho R, Moura AAA. Análise do desempenho produtivo de diversos grupos genéticos Holandês-Gir no Brasil. Rev Bras Zootec 2002;31:1944–52.

[35] La Falce M. Teste de progênie da raça girolando será ampliado. EMBRAPA GADO LEITE 2015. https://www.embrapa.br/gado-de-leite/busca-de-noticias?p_p_id=buscanoticia_WAR_pcebusca6_1portlet&p_p_lifecycle=0&p_p_state=pop_up&p_p_mode=view&p_p_col_id=column-1&p_p_col_pos=1&p_p_col_count=2&_buscanoticia_WAR_pcebusca6_1portlet_groupId=1355117&_buscan (accessed September 8, 2022).

[36] Embrapa. Anuário do leite 2022: Pecuária leiteira de precisão. Anuário 2022 2022:59.

[37] Weaver AD, Atkinson O, Jean GS, Steiner A. Bovine surgery and Lameness. 3th ed. Hoboken: Wiley Blackweel; 2018.

[38]  Magalhães LQ, Barbosa SPF, Fagundes NS, Almeida MO, Carneiro LC, Brandão FZ, et al. Ovarian steroids modulate the systemic inflammatory response OF COWS challenged with lipopolysaccharide (LPS) intrauterine INFUSION. Theriogenology 2022;182. doi:10.1016/j.theriogenology.2022.01.027.

[39]  Terra RL, Reynolds JP. Ruminant history, physical examination, welfare assessment, and records. In: Smith BP, editor. Large Anim. Intern. Med. 5th ed., St. Louis: Elsevier Mosby; 2014, p. 52–63.

[40]  Williams EJ, Fischer DP, Pfeiffer DU, England GCW, Noakes DE, Dobson H, et al. Clinical evaluation of postpartum vaginal mucus reflects uterine bacterial infection and the immune response in cattle. Theriogenology 2005;63:102–17. doi:10.1016/j.theriogenology.2004.03.017.

[41]  Cardoso B, Oliveira ML, Pugliesi G, Batista EOS, Binelli M. Cytobrush: A tool for sequential evaluation of gene expression in bovine endometrium. Reprod Domest Anim 2017;52:1153–7. doi:10.1111/rda.13037.

[42]  Spencer TE, Bazer FW. Temporal and Spatial Alterations in Uterine Estrogen Receptor and Progesterone Receptor Gene Expression During the Estrous Cycle and Early Pregnancy in the Ewe1. Biol Reprod 1995;53:1527–43. doi:10.1095/biolreprod53.6.1527.

[43]  Spencer TE, Becker WC, George P, Mirando MA, Ogle TF, Bazer FW. Ovine Interferon-т Regulates Expression of Endometrial Receptors for Estrogen and Oxytocin but not Progesterone1. Biol Reprod 1995;53:732–45. doi:10.1095/biolreprod53.3.732.

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

[45]  Chauchereau A, Savouret J, Milgrom E. Control of Biosynthesis and Post-Transcriptional Modification of the Progesterone Receptor. Biol Reprod 1992;46:174–7. doi:10.1095/biolreprod46.2.174.

[46]  Wathes DC, Mann GE, Payne JH, Riley PR, Stevenson KR, Lamming GE. Regulation of oxytocin, oestradiol and progesterone receptor concentrations in different uterine regions by oestradiol, progesterone and oxytocin in ovariectomized ewes. J Endocrinol 1996;151:375–93. doi:10.1677/joe.0.1510375.

[47]  Fischer C, Drillich M, Odau S, Heuwieser W, Einspanier R, Gabler C. Selected pro-inflammatory factor transcripts in bovine endometrial epithelial cells are regulated during the oestrous cycle and elevated in case of subclinical or clinical endometritis. Reprod Fertil Dev 2010;22:818–29. doi:10.1071/RD09120.

[48]  Galvão KN, Flaminio MJBF, Brittin SB, Sper R, Fraga M, Caixeta L, et al. Association between uterine disease and indicators of neutrophil and systemic energy status in lactating Holstein cows. J Dairy Sci 2010;93:2926–37.

doi:10.3168/jds.2009-2551.

[49]  Wyle FA, Kent JR. Immunosuppression by sex steroid hormones. The effect upon PHA- and PPD-stimulated lymphocytes. Clin Exp Immunol 1977;27:407–15.

[50]  Goff JP, Horst RL. Physiological Changes at Parturition and Their Relationship to Metabolic Disorders. J Dairy Sci 1997;80:1260–8. doi:10.3168/jds.S0022-0302(97)76055-7.

[51]  Cunningham M, Gilkeson G. Estrogen receptors in immunity and autoimmunity. Clin Rev Allergy Immunol 2011;40:66–73. doi:10.1007/s12016-010-8203-5.

[52]  Fahey J V., Wright JA, Shen L, Smith JM, Ghosh M, Rossoll RM, et al. Estradiol selectively regulates innate immune function by polarized human uterine epithelial cells in culture. Mucosal Immunol 2008;1:317–25. doi:10.1038/mi.2008.20.

[53]  Schaefer TM, Wright JA, Pioli PA, Wira CR. IL-1β-Mediated Proinflammatory Responses Are Inhibited by Estradiol via Down-Regulation of IL-1 Receptor Type I in Uterine Epithelial Cells. J Immunol 2005;175:6509–16. doi:10.4049/jimmunol.175.10.6509.

[54]  Zhu L, Pollard JW. Estradiol-17β regulates mouse uterine epithelial cell proliferation through insulin-like growth factor 1 signaling. Proc Natl Acad Sci 2007;104:15847 LP – 15851. doi:10.1073/pnas.0705749104.

[55]  Taggart CC, Cryan SA, Weldon S, Gibbons A, Greene CM, Kelly E, et al. Secretory leucoprotease inhibitor binds to NF-κB binding sites in monocytes and inhibits p65 binding. J Exp Med 2005;202:1659–68. doi:10.1084/jem.20050768.

[56]  Wira CR, Rodriguez-Garcia M, Patel M V. The role of sex hormones in immune protection of the female reproductive tract. Nat Rev Immunol 2015;15:217–30. doi:10.1038/nri3819.

[57]  Madalena FE, Teodoro RL, Lemos AM, Monteiro JBN, Barbosa RT. Evaluation of Strategies for Crossbreeding of Dairy Cattle in Brazil. J Dairy Sci 1990;73:1887–901. doi:10.3168/jds.S0022-0302(90)78869-8.

[58]  Freitas AF, Wilcox CJ, Costa CN. Breed Group Effects on Milk Production of Brazilian Crossbred Dairy Cows. J Dairy Sci 1998;81:2306–11. doi:10.3168/jds.S0022-0302(98)75811-4.