

Postpartum subclinical endometritis prevalence and pregnancy outcomes in *Bos indicus* primiparous superprecocious cows subjected to timed artificial insemination

Prevalência de endometrite subclínica pós-parto e prenhez em vacas superprecoces primíparas *Bos indicus* submetidas à inseminação artificial programada

Prevalencia de endometritis subclínica posparto y preñez en vacas superprecoces primíparas *Bos indicus* sometidas a inseminación artificial programada

Karen Lauanne Silva Lacouth

Master in Animal Science

Institution: Universidade Federal do Acre

Address: Rio Branco – Acre, Brazil

E-mail: lacouthveterinaria@gmail.com

Jéssica de Souza Andrade

Postdoctoral in Animal Reproduction

Institution: Empresa Brasileira de Pesquisa Agropecuária (EMBRAPA)

Address: Porto Velho – Rondônia, Brazil

E-mail: jessica_andrade_@hotmail.com

Ingrid Pedraça Barbosa

Master in Animal Science

Institution: Faculdades Integradas Aparício Carvalho (FIMCA)

Address: Porto Velho – Rondônia, Brazil

E-mail: ingridpedraca97@gmail.com

Samira Alves de Souza Silva

Master in Animal Science

Institution: Universidade Federal de Pelotas (UFPel)

Address: Pelotas – Rio Grande do Sul, Brazil

E-mail: samirasouzazootecnista@gmail.com

Renata Reis da Silva

Master in Natural Resources Chemistry

Institution: Universidade Estadual de Londrina (UEL)

Address: Londrina – Paraná, Brazil

E-mail: renata.reis@embrapa.br

Luiz Francisco Machado Pfeifer

Postdoctoral in Animal Breeding and Reproduction

Institution: Universidade Federal de Pelotas (UFPel)

Address: Pelotas – Rio Grande do Sul, Brazil

E-mail: luiz.pfeifer@embrapa.br

RESUMO

O objetivo desse estudo foi avaliar a prevalência de endometrite subclínica (ESC) em vacas primíparas e seu impacto na prenhez. Foram utilizadas 147 vacas Nelore (*Bos indicus*) primíparas superprecoces (PSP), com idade de $26,6 \pm 1,32$ meses, em período pós-parto recente (30 - 60 days). As vacas foram incluídas em um protocolo de IATF a base de estradiol/progesterona/eCG/PGF. No Dia 0 do protocolo de TAI, as vacas passaram por exame citológico do útero pela técnica do citobrush para detecção de casos de ESC. O cytobrush foi usado para avaliar a proporção de células polimorfonucleares (PMN) e para extração de mRNA para análise de expressão gênica dos genes (IL-1 β , IL-8, TNF α , ESR1, ESR2, PGR e PTCH2) pela técnica do qRT-PCR. Vacas com SCE foram consideradas aquelas que apresentaram PMN > 5%. Assim, as vacas foram divididas em 2 grupos: Sadias (n = 85) e SCE (n = 20). Assim, A prevalência de endometrite subclínica em vacas Nelore PSP foi de 19 % (20/105). Não houve diferença na prenhez por IA entre os grupos (P = 0,15). Vacas do grupo SCE apresentou maior (P < 0.05) abundância de mRNA do gene IL-1 β e menor (P < 0.01) expressão dos genes ESR1 e PTCH2. Em conclusão, vacas PSP submetidas a IATF apresentam alta prevalência de ESC, mas a prenhez não foi diferente do que vacas sadias. Vacas com ESC tiveram maior expressão gênica de genes relacionados a inflamação e menor expressão gênica dos genes relacionados à receptividade e integridade do endométrio.

Palavras-chave: gado de corte, fertilização, ovulação, ruminante.

ABSTRACT

This study aimed to evaluate the prevalence of subclinical endometritis (SCE) in primiparous precocious (PP) Nelore cows and its impact on pregnancy outcomes. A total of 147 primiparous nelore (*Bos indicus*) cows, with 26.6 ± 1.32 months of age in the early postpartum period (30-60 days), were enrolled. The cows were subjected to estradiol/progesterone/eCG/PGF-based timed artificial insemination (TAI) protocols. On Day 0 of the TAI protocol, cows underwent uterine cytological examination using the cytobrush technique to detect SCE. The cytobrush was used to assess the proportion of polymorphonuclear cells (PMNs) and for mRNA extraction to analyze gene expression of inflammation-related genes (IL-1 β , IL-8, TNF α) and uterine receptivity genes (ESR1, ESR2, PGR, PTCH2) via qRT-PCR. Cows with >5% PMNs were classified as having SCE. Cows were then divided into two groups: Healthy (n = 85) and SCE (n = 20). The prevalence of SCE in PP Nelore cows was 19% (20/105). No difference in pregnancy per AI was observed between the groups (P = 0.15). However, cows in the SCE group exhibited greater (P < 0.05) mRNA abundance of the IL-1 β gene and lower (P < 0.01) expression of the ESR1 and PTCH2 genes. In conclusion, PP cows subjected to TAI in the early postpartum period show a notable prevalence of SCE, but this did not significantly affect pregnancy rates. Nevertheless, cows with SCE had higher expression of genes related to inflammation and lower expression of genes associated with endometrial function.

Keywords: cattle, fertilization, ovulation, ruminant

RESUMEN

Este estudio evaluó la prevalencia de endometritis subclínica (ESC) en vacas Nelore primíparas precoces (PP) y su impacto en la gestación. Se incluyeron 147 vacas Nelore (*Bos indicus*) primíparas, con una edad de 26.6 ± 1.32 meses en el período posparto temprano (30-60 días). Fueron sometidas a protocolos de inseminación artificial a tiempo fijo (IATF) basados en estradiol/progesterona/eCG/PGF. En el día 0 del protocolo, fueron sometidas a un examen citológico uterino utilizando la técnica del citobrush para detectar ESC. El citobrush se utilizó para evaluar la proporción de células polimorfonucleares (PMN) y para la extracción de ARNm a analizar la expresión de genes relacionados con la inflamación (IL-1 β , IL-8, TNF α) y genes de receptividad uterina (ESR1, ESR2, PGR, PTCH2) mediante qRT-PCR. Las vacas con >5% de PMN fueron clasificadas con ESC. Fueron divididos en dos grupos: Sanas (n = 85) y ESC (n = 20). La prevalencia de ESC en vacas Nelore PP fue del 19% (20/105). No hubo diferencia en la gestación por IA entre los grupos (P = 0.15). Las vacas del grupo ESC mostraron una (P < 0.05) abundancia de ARNm del gen IL-1 β y una menor (P < 0.01) expresión de los genes ESR1 y PTCH2. En conclusión, las vacas PP sometidas a IATF en el período posparto muestran una prevalencia de ESC, pero esto no afectó las tasas de gestación. Las vacas con ESC tuvieron una mayor expresión de genes relacionados con la inflamación y una menor expresión de genes asociados con la función endometrial.

Palabras clave: ganado, fertilización, ovulación, rumiante.

1 INTRODUCTION

Reproductive efficiency directly influences the profitability of beef cattle operations. However, several factors can compromise the reproductive capacity of females, among which uterine infections, such as Subclinical Endometritis (SCE), are particularly noteworthy. The SCE affects uterine health and can significantly impact the efficiency of timed artificial insemination (TAI) protocols (Sheldon *et al.*, 2019). The SCE is characterized by an inflammation of the endometrium, indicated by an increased presence of polymorphonuclear cells (PMNs) in the uterine tissue, without evident clinical signs of infection such as mucopurulent and/or bloody vaginal discharge (Gilbert *et al.*, 2005). While this condition has been extensively studied in dairy cattle (Van Schyndel *et al.*, 2018; Fagundes *et al.*, 2019; Sheldon *et al.*, 2019; Pascottini *et al.*, 2023), research associating uterine condition with reproductive impact remains scarce in beef cattle, especially in primiparous precocious (PP) *Bos indicus* cows. Understanding the prevalence of SCE in this category of females is essential for developing strategies to minimize its negative consequences on herd reproductive efficiency (Kasimanickam *et al.*, 2004).

Previous research by Andrade *et al.* (2023) observed that PP cows exhibit a high proportion of PMNs in the uterus at 35 days postpartum, suggesting that these females may experience delays in the restoration of postpartum uterine functionality. However, it remains unclear whether this level of inflammation affects the fertility of these females when subjected to TAI protocols in the early postpartum period.

A previous study indicated that postpartum uterine inflammation can affect endometrial receptivity and embryonic survival, reducing pregnancy rates and increasing the occurrence of early pregnancy loss (Galvão *et al.*, 2011). Other has demonstrated that cows affected by SCE exhibit alterations in the expression of genes involved in the inflammatory response, such as interleukin 1 beta (IL-1 β), interleukin 8 (IL-8), and tumor necrosis factor alpha (TNF α ; Pfeifer *et al.*, 2018), as well as in the expression of genes related to uterine receptivity, such as estrogen receptors (ESR1 and ESR2) and progesterone receptors (PGR; Andrade *et al.*, 2023). Therefore, it is important to investigate the impact of subclinical inflammation on the expression of these genes and on the fertility of PP Nelore cows subjected to TAI.

Based on these considerations, the present study aims to evaluate the prevalence of subclinical endometritis in PP *Bos indicus* and its impact on pregnancy. The hypotheses of this study are that primiparous precocious cows have a high prevalence of subclinical endometritis and, when subjected to TAI, exhibit lower pregnancy rates compared to healthy cows.

2 METHODOLOGY

All procedures performed in this study were approved by the Animal Use Ethics Committee of the Brazilian Agricultural Research Corporation (Embrapa), protocol number 03/2017. The practices followed adhered to the ethical principles and current regulations for the use of animals in scientific research.

2.1 ANIMALS AND LOCATION

This study was conducted on a commercial farm located in the state of Rondônia, Brazil (10°01'27.4"S 62°56'16.7"W). A total of 147 PP Nelore (*Bos indicus*) cows, with an average age of 26.6 ± 1.32 months, were used. The cows were maintained on *Brachiaria brizantha* pastures

with free access to water and mineral supplementation. Body condition score (BCS) was assessed using the Vetscore tool, which classifies BCS into three categories based on color: Red (RD) for thin animals, Green (GN) for animals with adequate body condition, and Yellow (YW) for obese animals, as described by Pfeifer *et al.* (2017).

2.2 HORMONAL TREATMENTS

The cows included in timed artificial insemination (TAI) protocols were between 30 and 60 days postpartum (DPP). On Day 0 of the protocol, each cow received an intramuscular (i.m.) injection of 2 mg of estradiol benzoate (Bioestrogen®, Biogénesis-Bagó, Curitiba, Brazil) and an intravaginal progesterone device (IPD, 1.9 g progesterone, CIDR®, Pfizer Animal Health, São Paulo, Brazil) was inserted to synchronize follicular wave emergence. On Day 8, the IPD insert was removed, and cows received 150 µg D-Cloprostenol i.m. (PGF2 α -analogue; Croniben®, Biogénesis-Bagó, Curitiba, Brazil), 1 mg of estradiol cypionate (ECP) i.m. (E.C.P.®, Pfizer, Cravinhos, Brazil), and 300 IU of eCG i.m. (Novormon®, Syntex, Buenos Aires, Argentina). On the same day, a tail chalk marker was applied to the sacrococcygeal region to identify cows that displayed estrus. On Day 10, estrus was evaluated at the time of TAI, and was considered to have occurred in cows with > 75% of the tail-head chalk mark removed. The cows were inseminated by an experienced technician using semen from three Nelore bulls that were randomly assigned to the groups.

Pregnancy status was assessed 30 days post-TAI via ultrasonic examination (SIUI CTS-900, linear probe with 5 MHz, Guangdong, China). Visualization of the embryonic vesicle and detection of the embryo were the positive criteria for determining pregnancy.

2.3 UTERINE HEALTH ASSESSMENT AND EXPERIMENTAL DEFINITIONS

To evaluate uterine health, cows underwent a vaginoscopy examination on Day 0 of the TAI protocol to identify cows with positive vaginal discharge (PVD), as described by Pfeifer *et al.* (2018). The presence of discharge was graded on a scale of 0 to 3 (0 = mucus, 1 = mucus with flecks of pus, 2 = $\geq$ 50% purulent exudate, 3 = hemorrhagic and/or purulent exudate). Cows with a vaginal discharge score of $\geq$ 1 were defined as cows with positive purulent vaginal discharge

(PVD+; Pleticha *et al.*, 2009). Only animals with PVD = 0, indicative of clinically normal uterine health, were included in this study. Therefore, 42 cows were detected with positive PVD and were excluded from the study.

Immediately after PVD evaluation, endometrial tissue samples were collected from the remaining 105 cows using the cytobrush technique, as described by Kasimanickam *et al.* (2004). Samples were analyzed for cytology and gene expression. For cytology, samples were fixed on slides with 70% alcohol and stained with a rapid panoptic stain. The proportion of polymorphonuclear cells (PMNs) was evaluated under optical microscopy at 400x magnification. Based on the proportion of PMNs, cows were categorized into two groups: Healthy (n = 85) and SCE (n = 20), for those that presented PMNs > 5%, according to the criteria described by Wagener *et al.* (2017).

For gene expression analysis of endometrial tissue, a representative sample of 28 animals was selected (Healthy = 14; SCE = 14). RNA purity and quantity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). Subsequently, RNA was extracted from these samples and subjected to reverse transcription for the synthesis of complementary DNA (cDNA), using a commercial kit (Invitrogen, Thermo Fisher Scientific, USA). The expression of genes related to inflammation (IL-1 β , IL-8, TNF α) and uterine receptivity (ESR1, ESR2, PGR, PTCH2) was evaluated by qRT-PCR, using β -actin and GAPDH as internal controls, as described by Pfeifer *et al.* (2018) (Table 1). The analysis was conducted using the $2^{-\Delta\Delta C_t}$ method, with healthy cows as the reference group (Livak & Schmittgen, 2001).

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

Gene Name	Gene ID	Sequence Forward	Sequence Reverse
Interleucin 1 Beta	<i>IL1β</i>	CAAGGAGAGGAAAGAGACA	TGAGAAGTGCTGATGTACCA
Interleucin 8	<i>IL8</i>	CAAGAGCCAGAAGAAACCTGAC	AGTGTGGCCCACTCTCAATAAC
Factor growth tumoral alpha	<i>TNFα</i>	TCTTCTCAAGCCTCAAGTAACAAGTC	AGCCCACGTTGTAGCCGACATC
Progesterone receptor	<i>PGR</i>	GCCGCAGGTCTACCAGCCCTA	GTTATGCTGTCCTTCCATTGCCC
Estrogen receptor 1	<i>ESR1</i>	CAGGCACATGAGCAACAAAG	TCCAGCAGCAGGTCGTAGAG
Estrogen receptor 2	<i>ESR2</i>	TCACGTCAGGCACGCCAGTAAC	CACCAGGTTGCGCTCAGACCC
Patched 2 ^a	<i>PTCH</i> 2	CATCCTGCTGCTGTGTACTT	ATCGCCAGGACCAGTACTAT

Glyceraldehyde-3-Phosphate Dehydrogenase	<i>GAPDH</i>	ACACTGAGGACCAGGTTG	TGGTCGTTGAGGGCAATG
Actin β	<i>ACTB</i>	AGGCATCCTGACCCTCAAGTA	GCTCGTTGTAGAAGGTGTGGT

Source: Prepared by the authors

2.4 STATISCAL ANALYSIS

Data were analyzed using SAS 9.0 software (SAS Institute Inc., Cary, NC, USA). Comparisons between the Healthy and SCE groups for binomial variables such as DPP, Vetscore, pregnancy rate, and estrus expression were performed using a generalized linear mixed model (GLMM). The analysis included uterine health (SCE and Healthy), BCS (low and adequate), and their interactions as fixed variables.

The relationship between the proportion of PMN cells and DPP was evaluated using analysis of variance (ANOVA), as well as the expression of genes individually for the groups.

In all tests, the significance level was set at $P < 0.05$, and P-values between 0.05 and 0.1 were considered as a trend.

3 RESULTS AND DISCUSSION

The prevalence of subclinical endometritis (SCE) in PP Nelore cows was 19% (20/105). As expected, the proportion of PMNs in the endometrium was significantly higher ($P < 0.0001$) in cows with SCE compared to healthy cows (Table 2). Furthermore, cows with SCE had significantly lower ($P < 0.0001$) DPP at the time of cytological sampling compared to the healthy group (Table 2).

Table 2. Relationship between body condition, subclinical endometritis, and reproductive parameters in postpartum primiparous cows after TAI.

Parameters	Group		P-Value
	Healthy	ESC	
Proportion of PMN cells, %	1.5	22.8	<0.0001
Days postpartum, d	44.9	35.6	<0.0001
Proportion of cows that showed estrus at TAI, %	45.9 (39/85)	35 (7/20)	0.59
Pregnancy per IA, %	60 (51/85)	50 (10/20)	0.15
Proportion of cows with low BCS* %	12,9 (11/85)	30 (6/20)	0.05

Proportion of cows with adequate BCS%	87,1 (74/85)	70 (14/20)	0.05
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*BCS was evaluated with Vetscore's device.

Source: Prepared by the authors

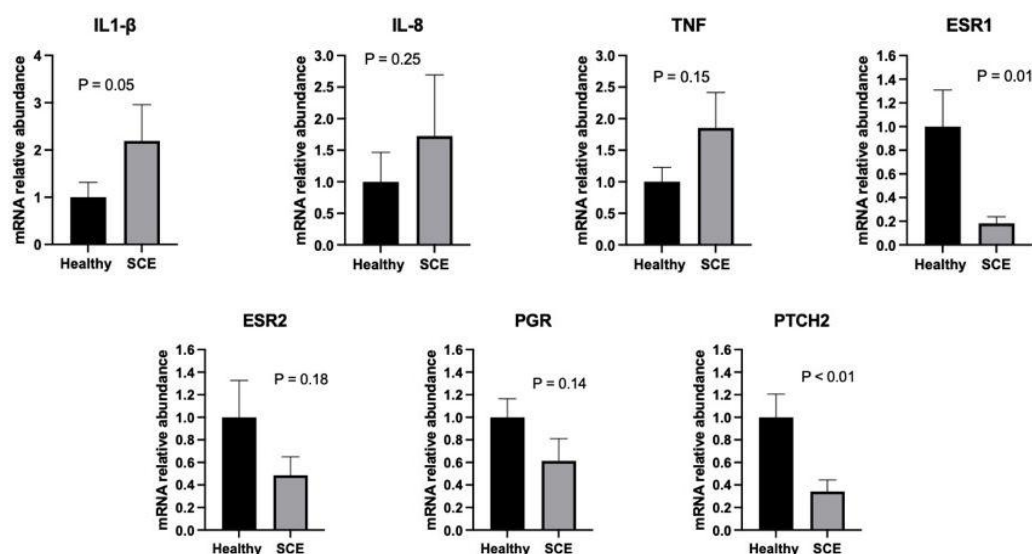
There was no significant difference in the proportion of cows exhibiting estrus between the removal of the IPD and TAI ($P = 0.59$), nor was there a significant difference in pregnancy per AI (P/AI) ($P = 0.15$) between the groups (Table 2).

When evaluating the proportion of cows with SCE according to body condition score (BCS) assessed by Vetscore, cows classified as low BCS had a significantly higher ($P = 0.05$) prevalence of SCE (35%), while cows classified as adequate BCS had a prevalence of SCE of 15.9%.

The relative mRNA abundance for the IL1- β , IL8, TNF α , ESR1, ESR2, PGR, and PTCH2 genes according to uterine health is presented in Figure 1.

Regarding the relative mRNA abundance of inflammation-related genes, cows in the SCE group showed significantly higher ($P < 0.05$) mRNA abundance of the IL-1 β gene and significantly lower ($P < 0.01$) mRNA abundance of the ESR1 and PTCH2 genes, which are related to uterine receptivity. The mRNA abundance of the other genes studied did not differ significantly between the groups (Figure 1).

Figure 1. Relative abundance of pro-inflammatory cytokine transcripts for interleukin 1 beta (IL1 β), interleukin 8 (IL8) and tumor necrosis factor alpha (TNF α) and gene transcripts for regulation of the progesterone receptor of cell proliferation (PGR), estrogen receptor 1 (ESR1), estrogen receptor 2 (ESR2) Patched 2a (PTCH2) mRNA in endometrial tissues of postpartum primiparous precocious beef cows.



Source: Prepared by the authors

This study investigated the prevalence of SCE in postpartum PP Nelore cows and its relationship with pregnancy after TAI. We observed that approximately 20% of the cows enrolled in this study were detected with SCE. As expected, cows with SCE had greater gene expression of inflammation-related genes and lower gene expression of genes related to endometrial receptivity and integrity. To the best of our knowledge, this is the first study to characterize the relationship between uterine health of postpartum primiparous precocious cows and fertility. Although healthy cows had 20% higher pregnancy rates than cows with SCE, our study did not have sufficient statistical power to find a significant difference in this fertility-related variable. Therefore, studies involving more animals of this category would be important to better characterize the fertility of these animals in the postpartum period.

Uterine inflammation is a necessary component of uterine involution (Walker *et al.*, 2015). Although the gene expression of genes associated with inflammation and uterine receptivity has been previously studied (Andrade *et al.*, 2023), in this study, we observed that cows with SCE had increased expression of IL-1 β , while uterine receptivity genes, such as ESR1 and PTCH2, had their expression significantly reduced. However, although these molecular and cellular alterations indicate a uterine environment unfavorable to conception, P/AI was not markedly affected, suggesting that PP cows may possess metabolic and immunological mechanisms that allow these animals to have adequate fertility even in the presence of these adverse uterine conditions. High expression of inflammatory cytokines such as IL-1 β and IL-8 has been identified as one of the main factors that compromise endometrial receptivity and embryonic implantation (Pfeifer *et al.*, 2018; Andrade *et al.*, 2021). This finding enhance that subclinical endometritis is associated with an exacerbated inflammatory response, which may compromise fertility and uterine receptivity (Walker *et al.*, 2015).

Despite the lack of observed difference in fertility between the groups, we observed in the present study that a considerably high number (~20%) of PP cows may reach TAI possibly without completing postpartum uterine recovery. Previously, Andrade *et al.* (2023) studied the uterine repair dynamics of a small number of PP cows and observed that these animals require a longer postpartum time for uterine recovery compared to multiparous cows, corroborating the findings of this study. Ricci *et al.* (2017) demonstrated that subclinical endometritis in beef cows can evolve differently according to the DPP, influencing the severity of uterine inflammation and its impact on fertility. Both cited articles observed that endometrial cytology revealed a higher

proportion of PMNs in the first days after calving, indicating an intense inflammatory process in the initial period of uterine involution. With the advancement of postpartum days, inflammation tends to decrease in cows that can effectively eliminate the infection. However, in cows with persistent uterine inflammation until late postpartum periods, there was a reduction in reproductive efficiency, with higher chances of conception failure and a negative impact on TAI protocols (Pfeifer *et al.*, 2018; Andrade *et al.*, 2021).

Lucy (2019) stated that the transition between physiological and pathological inflammation in the postpartum period can impact the expression of genes involved in the immune response and hormonal signaling, influencing long-term reproductive efficiency.

Another critical factor identified was the influence of BCS on the incidence of SCE. Cows with lower BCS have a higher incidence of SCE. Cows with low BCS were more likely to develop uterine inflammation, which can directly interfere with reproductive results, which corroborates several studies already published (Brandão *et al.*, 2021; Cooke *et al.*, 2021; Lucy, 2019; Sheldon *et al.*, 2019). This phenomenon can be explained by the relationship between negative energy balance and compromised uterine immune response (Dai *et al.*, 2023), which leads to greater persistence of postpartum inflammation and less ability to recover before TAI.

Although most recent studies on uterine health focus on dairy cows, the results are important to reinforce the context of findings in beef cattle. Studies such as those by Sheldon *et al.* (2019) point out that the balance between mechanisms of resistance and tolerance to infection is fundamental for uterine recovery and prevention of endometritis. In addition, recent studies in dairy cattle demonstrate that cows with lower body condition and less metabolic stress have a lower risk of developing uterine inflammation, which reinforces the importance of nutritional and immunological monitoring also in beef cattle (Sheldon *et al.*, 2019; Lucy, 2019).

5 CONCLUSIONS

This pioneering study sheds light on the prevalence of subclinical endometritis (SCE) in primiparous precocious Nelore cows in the postpartum period and its potential implications for fertility after timed artificial insemination (TAI). Our results reveal that a considerable proportion (approximately 20%) of the evaluated PP cows presented with SCE, highlighting the clinical relevance of this condition in intensive production systems. The characterization of gene

expression in cows with SCE revealed an unfavorable profile, marked by greater expression of the pro-inflammatory gene (IL-1 β) and lower expression of genes associated with endometrial receptivity and integrity. This immunological and inflammatory imbalance suggests a uterine environment with a reduced capacity to express full reproductive potential. However, although we observed an important difference (20%) in pregnancy rates between healthy cows and cows with SCE, the absence of statistical significance in this variable highlights the need for future studies with greater statistical power. We believe that an expanded sample size will allow us to elucidate the true impact of SCE on the fertility of PP cows and identify more precise predictive biomarkers.

In summary, this study represents a significant advance in understanding uterine health in postpartum PP Nelore cows and its relationship with fertility. Our findings open new perspectives for the development of early diagnostic strategies and targeted therapeutic interventions, aiming to optimize reproductive performance and production efficiency in beef cattle breeding systems. We believe that this research will stimulate additional investigations into the molecular mechanisms involved in SCE and its impact on fertility, ultimately leading to improved reproductive management practices in this important category of beef cattle.

The results obtained in this research contribute significantly to the understanding of uterine health in super-precocious heifers and may guide more effective reproductive programs and preventive strategies in the health and nutritional management of beef herds.

As a limiting factor of the study, the small number of animals in the ESC group stands out, which may have limited the detection of statistical differences in pregnancy rates. It is recommended that new studies be carried out with a larger number of animals and the inclusion of metabolic and immunological variables to better understand the mechanisms associated with SCE in super-precocious heifers.

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