http://dx.doi.org/10.1590/1678-4162-12848

Arq. Bras. Med. Vet. Zootec., v.75, n.3, p.381-390, 2023

Lipopolysaccharide effects on activation and apoptosis of primordial ovarian follicles in heifers

[*Efeitos de lipopolissacarídeos na ativação e apoptose de folículos ovarianos primordiais em novilhas*]

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ABSTRACT

The objective of the present study was to evaluate the effect of lipopolysaccharide (LPS) administration on activation and apoptosis of primordial follicles. There was no difference in the total number of follicles as well as in the different types of follicles. Furthermore, the LPS challenge didn't modulate the expression of genes related with ovarian reserve (HAM), oocyte survival (Survivin), activation rate (Pten, KIT, KITL1, KITL2, AKT1, SIRT1), and follicular abnormalities. Therefore, the LPS exposure with 24h interval had no effect on activation rate and primordial follicles abnormalities, and also had no effect on expression of anti-apoptotic genes and genes related with ovarian reserve, oocyte survival, activation rate, and primordial follicles abnormalities.

Keywords: endotoxin, follicle, inflammation, oocyte, reproduction

RESUMO

O objetivo do presente estudo foi avaliar o efeito da administração de lipopolissacarídeo (LPS) na ativação e a apoptose de folículos primordiais. Dez novilhas saudáveis (Bos taurus taurus), com idade média de 14 meses, alojadas em sistema de confinamento e alimentadas com TMR, foram utilizadas neste experimento. Os animais foram distribuídos aleatoriamente em dois grupos: grupo LPS (LPS; n = 5), que recebeu duas injeções intravenosas de 0,5µg/kg de peso corporal de lipopolissacarídeo (Sigma Aldrich®) diluído em 2mL de solução salina (0,9% de NaCl), com intervalo de 24h; e grupo controle (CTR; n = 5), que recebeu duas injeções intravenosas de 2mL de solução salina (0,9% de NaCl), com intervalo de 24h. A primeira injeção de LPS foi realizada no d 1, e no d 5 os animais foram abatidos, os ovários foram pesados e as amostras dos ovários foram coletadas para avaliação histológica e molecular. Não houve diferença no número total de folículos, bem como nos diferentes tipos de folículos. Além disso, o desafio com LPS não modulou a expressão de genes relacionados à reserva ovariana (HAM), à sobrevivência oocitária (Survivin), à taxa de ativação (Pten, KIT, KITL1, KITL2, AKT1, SIRT1) e às anormalidades foliculares. Portanto, a exposição ao LPS com intervalo de 24h não teve efeito sobre a taxa de ativação e as anormalidades dos folículos primordiais, bem como não teve efeito sobre a expressão de genes antiapoptóticos e de genes relacionados com a reserva ovariana, a sobrevivência oocitária, a taxa de ativação e as anormalidades dos folículos primordiais.

Palavras-chave: endotoxina, folículo, inflamação, oócito, reprodução

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Submitted: August 22, 2022. Accepted: January 11, 2023.

INTRODUCTION

The ovarian lifespan is determined by the number of oocytes at birth, as well as by the rate at which these oocytes will be depleted (Morita *et al*., 2000). The programmed cell death (apoptosis) is the main mechanism responsible for the age-related oocyte exhaustion and the relation between pro-survival and pro-apoptotic molecules is key for the follicles life cycle (Morita *et al*., 2000). Studies have shown the relation between ovarian reserve and fertility (Evans *et al*., 2010, 2012; Ireland *et al*., 2011). There is evidence that low follicular reserve is associated with impaired fertility, reducing the conception rate and increasing calving-conception interval (Evans *et al*., 2010;).

Bromfield and Sheldon (2013) have shown that follicle reserve quality is influenced by inflammatory and infectious events. Regarding the dairy industry, calves are often exposed to cases of diarrhea and bronchopneumonia; and adult animals are exposed to mastitis, endometritis, and acidosis (Seegers *et al.*, 2003; Gilbert *et al*., 2005; Haimerl and Heuwieser, 2014; Pederzolli *et al*., 2018). In the beef industry, the major challenges for adult animals are bronchopneumonia and acidosis (Härtel *et al*., 2004; Pederzolli *et al*., 2018). These diseases are caused by bacteria, mainly Gram negative, which release lipopolysaccharide (LPS), one of the constituents of their outer membrane. LPS has the capacity to migrate to the blood, where it is recognized by membrane Toll-like receptor 4 (TLR4) initiating an inflammatory response with the production of cytokines (Eckel and Ametaj, 2016). Moreover, LPS can be transported to peripheral tissues such as the ovaries, inducing a localized inflammation and altering the development and endocrine function of antral follicles (Bromfield and Sheldon, 2011; Lavon *et al*., 2011).

It is thought that endotoxins and inflammatory processes can lead to chronic effects, since the animals present impairment of the reproduction performance up to 60-80 days after the disease occurrence (Sheldon *et al*., 2009; Hertl *et al*., 2010; Hudson *et al*., 2012). Bromfield and Sheldon (2013) demonstrated in an in vitro study that exposure of the bovine cortex to LPS increases the follicles' activation rate. In this same work the authors evaluated the effects of LPS in vivo in rats, demonstrating a 3 time increase in the apoptosis of primary follicles and reduction of 1.2 times in the pool of primary follicles in animals exposed to LPS, which could lead to an early depletion of the follicular reserve.

However, there are few in vivo studies conducted in cattle to evaluate the effects of LPS on the ovarian reserve. Thus, the hypothesis of our study is that exposing cows to LPS would create a greater activation and greater apoptosis rate in primary follicles. The objective of the present study was to assess the effect of LPS *in vivo* on the activation and apoptosis of primordial follicles and other follicle types in cows.

MATERIALS AND METHODS

The experiment was approved by the Animal Ethics and Experimentation Committee of the Federal University of Pelotas (no. 9364). Ten healthy heifers (*Bos taurus taurus*), averaging 14 months old, housed in a confinement system, and fed a TMR were used in this experiment. All heifers were submitted to the same synchronization protocol. Estrous cycles were pre synchronized with administration of 25mg of prostaglandin (PGF2α) (i.m., Lutalyse®; Zoetis, São Paulo, Brasil) fourteen days prior to the synchronization protocol. On day zero (D0), heifers received a controlled internal drug-release insert impregnated with progesterone (1.9 g, CIDR®, Zoetis®), 2mg of estradiol benzoate (Gonadiol, Zoetis®) i.m., and 25mg of PGF2α (Lutalyse®, Zoetis) I.M. The CIDR was removed on D5 (Cavalieri *et al*., 2018). Heifers were randomly assigned into two groups: LPS group (n = 5) which received two intravenous injections of 0.5µg/kg of BW of LPS (Sigma Aldrich®) diluted in 2 mL of saline solution (0,9% de NaCl) with 24h interval; and Control group (n = 5) which received two intravenous injections of 2mL of saline solution (0.9% de NaCl) with 24h interval. LPS dose and administration interval were chosen according to the lowest dose that induced an inflammatory response in bovine previously (Waldron *et al*., 2003; Fernandes *et al*., 2019). The first LPS injection was performed on D1, and heifers were slaughtered on D5, when ovaries were weighted and samples from the ovaries were collected. For histological evaluations ovary samples were fixed in formalin. For gene expression, samples

were transferred to cryogenic tubes and following homogenization in 0.5mL of trizol (Invitrogen®, Carlsbad, CA, USA) samples were stored in liquid nitrogen.

Ovaries were removed from formalin, dehydrated in alcohol solution, cleared with xylene, and embedded in Paraplast Plus® (Sigma Chemical Company®, St. Louis, MO, EUA). Embedded ovaries were then sectioned using a 5 µm microtome (RM2245, Leica Biosystems, San Diego, CA, USA) and samples were collected every 120 histological sections, with cuts being made in the entirety of the ovary, according to methodology done by Driancourt *et al.,* 1985. Microscope slides were dried in an oven at 56°C for 24 hours and stained with hematoxylin and eosin. Subsequently, ovary images were captured at 10 x magnification by a camera attached to the microscope (Nikon Eclipse E200, Nikon Corporation, Japan) using the Motic Image Plus 2.0 software (Motic®, Hong Kong, China).

To determine the population of follicles in each category, the correction factor used was the described by (Gougeon and Chainy, 1987) in the following formula Nt= No x St x Ts ⁄ So x Do. Where Nt: Estimated total number of follicles of each category; No: Number of follicles observed in the ovary; St: Total number of cuts made in the ovary; Ts: Thickness of the cut; So: Total number of cuts observed and Do: Mean core diameter do oocyte. To determine the diameter of the core of the oocyte, a horizontal and a vertical measurement was made.

Preantral follicles were classified according to the developmental stage as primordial (one layer of flattened granulosa cells around the oocyte), transition (flattened and cuboidal granulosa cells around the oocyte), primary (one layer of cuboidal granulosa cells around the oocyte), or secondary (two or more layers of cuboidal granulosa cells around the oocyte) (Hulshof *et al*., 1994). Follicle degeneration was characterized by one or more of the following aspects: condensed oocyte nucleus, shrunken oocyte, pyknotic bodies in the granulosa cells, low cellular density, widespread disintegration of the granulosa cells (Driancourt *et al.,* 1985, Silva-Santos *et al*., 2011). Only follicles in which the oocyte nucleus was visible in each histological section were counted, to avoid counting the same follicle repeatedly.

Total RNA from the ovarian tissue was extracted using Trizol reagent (Invitrogen®) following the manufacturer's instructions. The RNA concentration was measured with a spectrophotometer (Nanodrop Lite, Thermo Fischer Scientific Inc., USA) and purity was assessed through the ratio of absorbance at 260/280nm. Reverse transcription was conducted with 1 pg of total RNA in a reaction volume of 20µL, using a commercial kit (iScript Synthesis kit, BIORAD®, Hercules, CA, USA) following the manufacturer's instructions. A thermal cycler was used following these cycling parameters: 5°C for 5 min, 42°C for 20 min, and 95°C for 1 min.

The real-time polymerase chain reaction (PCR) was performed in a volume of 15 µL containing GoTaq reagent (GoTaq® qPCR Master Mix, Promega, Madison, WI, USA) in a thermal cycler StepOnePlus (Applied Biosystems, Foster City, CA, USA). Each reaction was performed in duplicates containing 4 µL of cDNA (20 ng), 5µL of GoTaq, 0.75µL of each primer (5µM), and 4,5µL of ultrapure water. Forty-five cycles were conducted (95°C for 15 seg and 60 °C for 1 min) and the last of each reaction was performed in a dissociation curve (Melting) to verify the amplification of a single PCR product.

The genes H2A clustered histone 6 (H2AC6), 18S ribosomal RNA (RN18S1), ubiquitously expressed prefoldin like chaperone (UXT), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as endogenous reference and the target genes associated with primordial follicles activation were phosphatase and tensin homolog (PTEN), phosphoinositide-3-kinase regulatory subunit 1 (PIK3R1), proto-oncogene, receptor tyrosine kinase (KIT), KIT ligand (KITLG), KIT ligand 2 (KITL2), sirtuin 1 (SIRT1) AKT serine/threonine kinase 1 (AKT1) anti-Mullerian hormone (AMH), mechanistic target of mammalian target of rapamycin (MTOR), and survivin (SURVIVIN) were. The primers sequences are described in Table 1.

Table 1. Genes evaluated from ovary samples of heifers challenged or not with LPS

Gene	Primer sequence 5′ → 3′	NCBI Code	Reference
H2AC6	F: GAGGAGCTGAACAAGCTGTTG R: TTGTGGTGGCTCTCAGTCTTC	NM_001205596.1	[55]
RN18S1	F: CCTTCCGCGAGGATCCATTG R: CGCTCCCAAGATCCAACTAC	NR_036642.1	[56]
UXT	F: TGTGGCCCTTGGATGGATGGTT R: GGTTGTCGCTGAGCTGAGCTG	NM_001037471.2	[57]
GAPDH	F: GATTGTCAGCAATGCCTCCT R: GGTCATAAGTCCCTCCACGA	NM_001034034.2	[56]
KIT	F: ATCATGAAGACCTGCTGGGATGCT R: GGGCTGCAGTTTGCTAAGTTGGAA	NM_001166484.1	[58]
KITL1	F: GTGTGATTTCCTCAACATCAAGTCC R: TGCTACTGCTGTCATTCCTAAGGG	NM_174375.2	[58]
KITL2	F: AAGGGAAGGCCTCAAATTCCATTGAAGA R: AGCAAACCCGATCACAAGAGA	NM_174375.2	[58]
AMH	F: ACACCGGCAAGCTCCTCAT R: CACCATGTTTGGGACGTGG	NM_173890.1	[59]
MTOR	F: TCCTTGTCACGAGGCAACAA R: GGCGTATCAATTCTTGCAATGA	XM_002694043.6	This study
AKT1	F: GATTCTTCGCCAGCATCGTG R: GGCCGTGAACTCCTCATCAA	NM_173986.2	[60]
PTEN	F: GCCACAAAGTGCCTCGTTTACC R: AGAAGGCAACTCTGCCAAACAC	NM_001319898.1	[61]
SIRT1	F: CAACGGTTTCCATTCGTGTG R: GTTCGAGGATCTGTGCCAAT	NM_001192980.3	[62]
PIK3R1	F: ACACAGCTGACGGGACCTTT R: CCATATTTCCCATCTCGGTGA	NM_174575.1	[61]

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The coefficient of variation was less than 5% for all the primer pairs used. Relative expression from real-time PCR was calculated from the equation 2A-B/2C-D (where A = Cycle Threshold (Ct) number for the gene of interest in the first control sample; B = Ct number for the gene of interest in the analyzed sample; C = Ct number for the housekeeping gene (geometric mean of genes H2AC6, RN18S1, UXT e GAPDH) in the first control sample; and D = Ct number for housekeeping gene in the analyzed sample). The first control was expressed as 1.00 by this equation, and all other samples were calculated in relation to this value. Afterward, the results in the control group (N-AL) were averaged, and all other outputs were divided by the mean value of the relative expression in the control group to yield the fold change of the genes of interest expression compared to the control group (Masternak *et al*., 2005).

Data relative to histological analysis and gene expression were analyzed with a t-test in the GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA, USA). Values of $p < 0.05$ were considered significant. The abnormal, primordial, transition, primary follicles and total follicles did not present a normal distribution and were transformed into log10.

RESULTS

There was no difference in the total follicular population (Control: 70939±5662; LPS: 95890±35024; P = 0,78), as well as in the different follicle stages (Table 2).

Besides, the frequency of healthy follicles was 51.52% in the control group and 40.62% in the LPS group (P=0.74).

There was no difference in the expression of anti-apoptotic genes and genes related to ovarian reserve, oocyte survival, activation rate, and primordial follicles abnormalities (Figure 1; P>0,05).

Table 2. Total follicles according to phase from heifers challenged or not with LPS

Group / Phase	Control	LPS	P-value
Primordial	15872± 3.225 (15.83%)	16557± 6395 (16.07%)	0.92
Transition	23916 ± 3786 (30.92%)	21285 ± 4461 (18.05%)	0.67
Primary	4369 ± 1476 (3.70%)	6235 ± 1498 (5.53%)	0.40
Secondary	1416± 441 (1.07%)	1292 ± 406 (0.96%)	0.84
Abnormal primordial	7308 ± 1874(10.02%)	15042±6939 (18.51%)	0.31
Abnormal transition	13236± 2339 (33.30%)	22245 ± 13755 (28.12%)	0.90
Abnormal primary	3422 ± 977 (3.92%)	11067± 6426 (10.72%)	0.16
Abnormal secondary	1400 ± 206 (1.24%)	2167 ± 595 (2.01%)	0.25

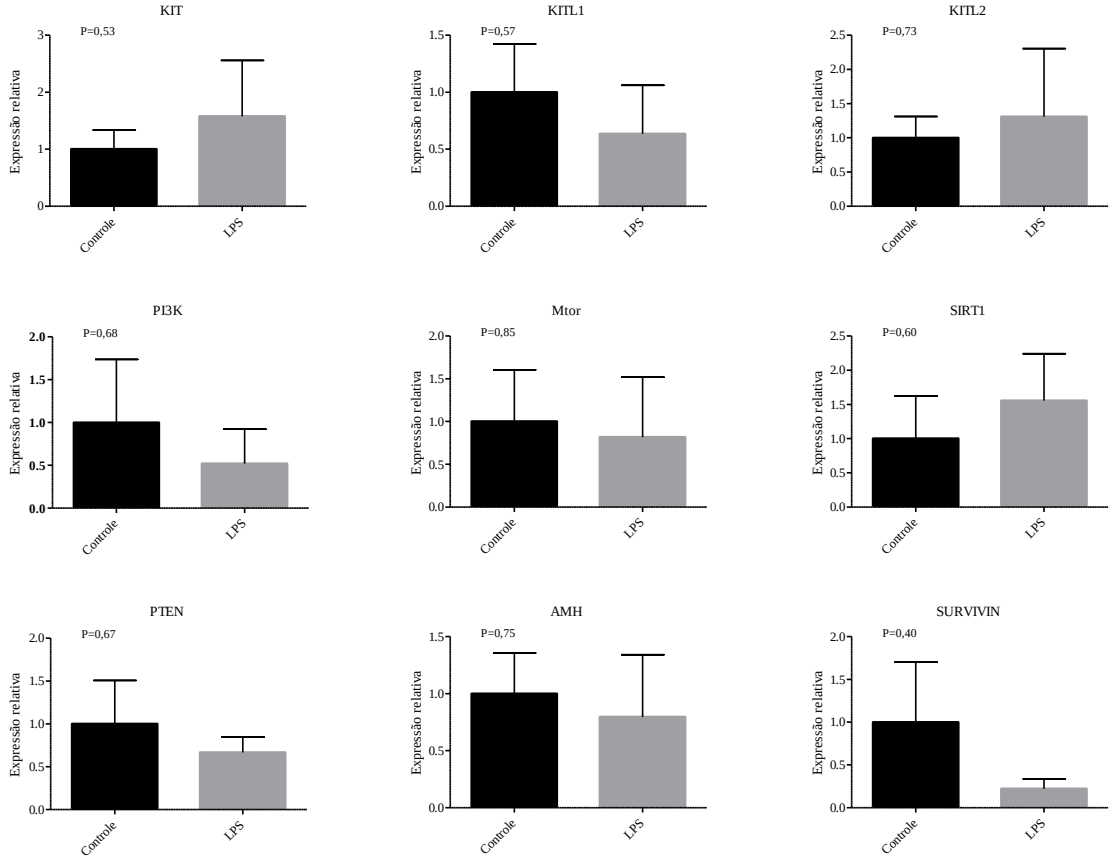

Figure 1. Gene expression related to follicular activation, ovarian reserve and anti-apoptotic in heifers which were challenged with LPS or not.

DISCUSSION

The binding of pathogen-associated molecular patterns (PAMPs) or LPS to toll-like receptors (TLRs) stimulates the production of cytokines such as interleukin-1 α and β (IL1-α and β), interleukin-6 (IL6), interleukin-8 (IL-8) and tumor necrosis factor alpha (TNF-α) in the blood (Beutler, 2009; Takeuchi and Akira, 2010) and also locally in the ovary, which seem to act by increasing the rate of atresia and follicular activation (Bromfield and Sheldon, 2013; Passos *et al*., 2016). Based on this hypothesis, we analyzed genes involved in the process of primordial follicle activation (KIT, KITL1, KITL2, AKT1, SIRT1) and inhibition of activation (PTEN) (, Chen *et al*., 2020, Zhao *et al*., 2021), as well as a gene linked to ovarian reserve (HAM) and an anti-apoptotic gene (Survivin) (Chen *et al*., 2020).

In our study, the challenge with LPS did not alter the expression of genes related to abnormality and follicular activation, nor did it change the percentage of atretic follicles, and the rate of follicular activation evaluated histologically. The results of HAM expression corroborate the results found in the histological evaluation, since this hormone is secreted from granulosa cells of the ovarian follicles and has been studied as an endocrine marker capable of estimating the ovarian reserve (Ireland and Mossa, 2018).

The activation of primordial follicles is morphologically characterized by the transition from flattened to cuboidal granulosa cells, independent of gonadotropins and involved in pathways such as PI3K-AKT-mTORC (Adhikari *et al*., 2010; Li *et al*., 2010, Maidarti *et al*., 2020). This process requires accumulation of phosphatidylinositol-triphosphate (PIP3), which causes PI3K stimulation and promotes Akt phosphorylation, resulting in the induction of oocyte growth and meiotic maturation (Hoshino *et al*., 2004; Kalous *et al*., 2006; Wang *et al*., 2016). The stimulation of mTORC promotes protein synthesis, lipid and nucleotide biogenesis (Guo and Yu, 2019), and the super activation of the PI3K/Akt/mTOR signaling pathway has been linked to premature activation of primordial follicles, leading to early follicular atresia (McLaughlin and Sobinoff *et al*., 2011). Growth factors KIT, KITL1 and KTL2 also participate in the activation pathway, which stimulate the AKT/PI3K pathway (Jones and Pepling, 2013; Cavalcante *et al*., 2016. On the other hand, the PTEN protein, which is a lipid phosphatase, acts by inhibiting the activation of this pathway), by transforming PIP3 into PIP2, keeping the follicles inactive and reducing cell proliferation (Wang *et al*., 2016, Takeuchi *et al*., 2019, Maidarti *et al.*, 2019). The specific deletion of PTEN from oocytes in the primordial stage in mice, allows the accumulation of PIP3 to occur, which triggers the global activation of all these follicles (Reddy *et al*., 2008).

Differently from our results, Bromfield and Sheldon (2013) have observed that an in vitro bovine ovarian cortex culture containing 10µg/mL of LPS for 6 days, induced a reduced expression of PTEN, associated with greater follicular activation. In the same study, there was a higher percentage of follicles that transitioned from primordial to the primary phase (56% higher in the group exposed to LPS when compared to the control group), also there was a greater number of follicles in the group challenged with LPS that transitioned from the primary phase to the secondary phase (17% higher).

Similarly, LPS exposure caused a reduction in follicular reserve in mice (Wu *et al*., 2011; Sominsky *et al*., 2012). Fuller *et al.* (2017), mice exposed to LPS 3 or 5 days after birth, generated a reduction in the number of primordial follicles in animals exposed to LPS on day 5 and a greater proportion of activated primordial follicles in both animals exposed on day 3 and day 5.

In addition to follicular activation, previous studies have shown higher occurrence of apoptosis in animals exposed to LPS. Bromfield and Sheldon (2013) have conducted an in vivo challenge with LPS in mice and have found an increase in follicular atresia, going from 3% to 9.8%, mediated by the inflammatory response, since TLR4 knockout mice did not show this increase. Follicular atresia is mediated by ligands such as TNF-α and interferon or by the mitochondrial pathway in which members of the Bcl-2 family play an important role (Hussein *et al.*, 2003, 2005). It has been demonstrated that TNF-α can induce a decrease in the number of primordial oocytes and follicles by inducing apoptosis (Morrison, L.J.; Marcinkiewicz, J.L, 2002, Silva *et al.*, 2020).

The difference in our results, considering studies already carried out in vitro with ovaries of cattle and mice and in vivo with mice, is probably due to the type of LPS exposure and the dose used. In in vitro studies there is constant exposure of follicles to LPS and inflammatory cytokines, and in addition, the doses used are higher than those that occur naturally, since in in vivo studies, the organism uses mechanisms to eliminate these endotoxins, such as the detoxification that occurs in the liver (*Jirillo et al*., 2002). The dose recommended in our study was based on previous research (Waldron *et al*., 2003) that showed that 0.5µg/kg can promote the activation of defense cells and a systemic response.

Moreover, bovines usually have a large individual variation in the total follicular population (Ericksson, 1966; Silva-Santos, 2011), which was also observed in our study, and

it is another factor that interferes in the results. Silva-Santos (2011) has demonstrated that animals from 0 to 24 months had a variation from 0 to 700,000 germ cells. In addition, the total number of follicles remains similar until around 4-6 months, progressively decreasing after this period (Silva-Santos, 2011). This variation is already widely reported and related to the lower or higher performance of animals in reproductive biotechnology protocols (Ireland, J., Mossa, F., 2018), and several studies seek to find markers that indirectly indicate the germ cell population.

In dairy and beef cattle, exposures to bacteria are common throughout the life of the animals, and from our study we observed that acute exposures with short duration do not influence the rate of activation and follicular abnormality. However, it is worth mentioning that in infectious and metabolic conditions, exposure to these endotoxins occurs for a longer period, varying according to the beginning of the treatment. Therefore, further studies are needed to understand the damage caused by LPS and inflammatory cytokines in the bovine ovarian reserve.

CONCLUSION

The exposure of beef heifers to LPS in a 24 hour interval was not capable of altering the primordial follicles rate of activation and abnormality, as well as the expression of anti-apoptotic genes and genes related to the activation and inhibition of primordial follicles.

ACKNOWLEDGEMENTS

The authors would like to thank the Coordination for the Improvement of Higher Education Personnel (CAPES, Brasilia, Brazil) and the National Council for Scientific and Technological Development (CNPq, Brasilia, Brazil) for the financial support. Also, we would like to thank the Espinilho Slaughterhouse for providing the animals used in this study.

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