Early embryonic development of bovine oocytes challenged with LPS *in vitro* or *in vivo*

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Abstract

The aim of this study was to evaluate the effect of exposing bovine oocytes to lipopolysaccharides (LPS) *in vivo* and *in vitro* on early embryo development. In experiment 1, cumulus oocyte complexes (COCs, $n=700/\text{group}$) were challenged with 0, 0.1, 1.0 or 5.0 µg/mL of LPS during *in vitro* maturation (IVM). Later, *in vitro* fertilization (IVF) and *in vitro* culture (IVC) were performed. In experiment 2, COCs ($n=200/\text{group}$) matured and *in vitro* fertilized without LPS were subjected to IVC with the same doses of LPS from experiment 1. In experiment 3, heifers received two injections of saline solution ($n=8$) or 0.5 µg/kg of LPS ($n=8$) 24 h apart, and 3 days later, COCs were recovered and submitted to IVM, IVF, and IVC. In experiments 1 and 3, the expression of *TLR4*, *TNF*, *AREG* and *EREG* genes in cumulus cells was evaluated. Exposure to 1 and 5 µg/mL of LPS during IVM decreased nuclear maturation (39.4 and 39.6%, respectively) compared with control (63.6%, $P<0.05$). Despite that, no effect on cleavage and blastocyst rates were observed. Exposure to LPS during IVC did not affect embryonic development. *In vivo* exposure to LPS decreased the *in vitro* cleavage rate (54.3 vs 70.2%, $P=0.032$), but cleaved embryos developed normally. Number of cells per embryo and gene expression were not affected by the LPS challenge in any experiment. In conclusion, although *in vitro* exposure to LPS did not affect early embryo development, *in vivo* LPS exposure reduced cleavage rate.

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Introduction

In dairy cattle, approximately 75% of diseases occur in the first month after calving (LeBlanc *et al.* 2006). Studies suggest that 20–50% of dairy cows develop mastitis, 40% develop metritis and 20% endometritis (Opsomer *et al.* 2000, Sheldon *et al.* 2009, Magata *et al.* 2014a). The occurrence of infectious diseases has a negative impact on fertility (Sheldon *et al.* 2004, 2009). Cows with uterine or mammary infections have decreased follicular growth and steroid hormone concentrations, prolonged luteal phase, and interruption of ovarian activity (Sheldon *et al.* 2002, 2009, Lavon *et al.* 2011a, b, Krause *et al.* 2014).

One of the main agents involved in uterine and mammary diseases is the gram-negative bacteria *Escherichia coli* (Sheldon *et al.* 2002). The external membrane of *E. Coli* is composed of lipopolysaccharides (LPS) – endotoxins that act as potent stimulators of the host inflammatory response (Mateus *et al.* 2003, Kohchi *et al.* 2006). The main mechanism for LPS recognition is

the Toll-like receptor 4 (TLR4) found in immune cells such as neutrophils and macrophages (Moresco *et al.* 2011). TLR4 in conjunction with its co-receptors recognizes the LPS and activates intracellular signaling cascades, initiating the inflammatory response (Takeuchi & Akira 2010). During infections, the LPS released by bacteria can enter the bloodstream and can be found at sites far from the infected tissue. In this sense, the presence of LPS has already been detected in blood plasma (Mateus *et al.* 2003) and in follicular fluid (Herath *et al.* 2007) of cows with metritis or endometritis, and in the plasma and milk of cows with infectious mastitis (Hakogi *et al.* 1989).

Although the ovarian follicle lacks immune cells (Bromfield & Sheldon 2011), granulosa cells from the follicle wall can modulate the inflammatory response, both *in vitro* and *in vivo*, through activation of the TLR4 pathway (Herath *et al.* 2007, Bromfield & Sheldon 2011, Magata *et al.* 2014a, Campos *et al.* 2017). In addition, the exposure of granulosa cells to LPS *in vitro* alters the expression pattern and the concentration of

pro-inflammatory cytokines (Price *et al.* 2013). Besides this, LPS can impair reproductive function through several mechanisms, and exposure to the LPS in cattle causes reduction in a number of primordial follicles in the ovarian cortex *ex vivo* (Bromfield & Sheldon 2013); changes in the steroid hormone concentration *in vitro* (Herath *et al.* 2007) and *in vivo* (Magata *et al.* 2014a); alteration in the expression of genes associated with steroidogenesis in follicular cells *in vitro* (Magata *et al.* 2014b) and *in vivo* (Magata *et al.* 2014a, Campos *et al.* 2017); decrease in ovulation rate *in vivo* (Williams *et al.* 2008); extension of the luteal phase *in vivo* (Suzuki *et al.* 2001, Lutgenau *et al.* 2016); increase of reactive oxygen species and apoptotic genes in oocytes *in vitro* (Zhao *et al.* 2017), increase methylation rate in oocytes *in vitro* (Zhao *et al.* 2017), reduction of the meiotic maturation rate of oocytes *in vitro* (Bromfield & Sheldon 2011, Magata & Shimizu 2017, Zhao *et al.* 2017), lower number of trophoblastic cells in blastocysts *in vitro* (Magata & Shimizu 2017), and lower embryonic development *in vitro* (Soto *et al.* 2003, Magata & Shimizu 2017, Zhao *et al.* 2017).

Exposure to LPS causes a systemic inflammatory response, increasing body temperature and altering the serum concentration of acute phase proteins and cytokines (Waldron *et al.* 2003, Campos *et al.* 2017); however, a local response at the cumulus oocyte levels is also suggested (Bromfield & Sheldon 2011, Magata & Shimizu 2017). Nevertheless, the mechanisms by which LPS negatively affects fertility in cattle, especially oocytes and embryos, and the involvement of local and systemic factors, are not yet completely understood. Thus, the objective of this study was to compare the exposure of bovine oocytes to LPS *in vitro* and *in vivo* on gene expression, oocyte maturation and early embryonic development and quality. Additionally, we aimed to measure the effects of exposure to LPS during embryo development on embryo development and quality.

Materials and methods

This study was divided into three experiments, aiming to evaluate the effects of LPS on (1) *in vitro* oocyte maturation, (2) *in vitro* embryo culture, and (3) *in vivo* oocytes. All procedures performed in this experiment were approved by the Animal Ethics and Experimentation Committee of the Federal University of Pelotas, Pelotas, RS, Brazil (Protocol 9364-2018).

Culture medium used in the embryos production *in vitro*

In vitro oocyte maturation (IVM) was carried out in TCM199B medium (Gibco®) supplemented with 0.2 mM sodium pyruvate, 0.1 µL FSH, 75 µL/mL amikacin, 0.1 µL/mL estradiol and 10% fetal bovine serum (FBS). *In vitro* fertilization (IVF) was performed in the FIV-TALP medium supplemented with 6 mg/mL bovine serum albumin (BSA), 0.2 mM sodium

pyruvate, 30 µg/mL heparin, 20 µM penicillamine, 10 µM hypotaurine, 1 µM epinephrine and 75 µg/mL amikacin. *In vitro* culture (IVC) was done in a SOFaa medium supplemented with 2.7 mM myo-inositol, 0.2 mM sodium pyruvate, 5 mg BSA and 75 µg/mL amikacin (IVC1). On day 3 (D3) of embryo culture, the medium was replaced by fresh IVC1 medium supplemented with 2.5% FBS (IVC2). On day 5 (D5), the medium was replaced by IVC2 medium supplemented with 1 µg/mL glucose (IVC3).

Experiment 1 – Effect of LPS during IVM

Treatments

To evaluate the effect of LPS on IVM, oocytes were matured with 0 (control); 0.1, 1.0, and 5.0 µg/mL of LPS. Concentrations were based on intrafollicular concentration of LPS during *in vivo* infectious processes such as subclinical endometritis (0–0.04 µg/mL) and clinical endometritis (0.043–0.875 µg/mL) as reported by Herath *et al.* (2009). For this, 1 mg of *E. coli* LPS (O111: B4, Sigma) was diluted in 1 mL of saline (0.9% NaCl), according to the manufacturer's instructions. After that, serial dilutions were performed in the IVM medium in order to obtain concentrated aliquots. After dilution, the aliquots were stored at –20°C until further use.

Selection and maturation of oocytes *in vitro* with LPS

Cumulus oocyte complexes (COCs) were obtained by aspiration of follicles with a diameter of 2–8 mm from bovine ovaries obtained from local slaughterhouses. Grades I, II and III COCs were selected (de Loos *et al.* 1991) and randomly distributed into four previously described groups ($n=50$ COCs/group). The maturation occurred at 39°C with 5% CO₂ for 22 h.

Evaluation of nuclear maturation of LPS-challenged oocytes during IVM

After IVM, 50 COCs/group in three replicates ($n=150$ COCs/group) were denuded from the cumulus cells using hyaluronidase (1 mg/mL, Sigma Aldrich®) and successive pipetting. The denuded oocytes were fixed for 30 min (0.5% glutaraldehyde in PBS), stained with 1% of Hoechst 33342 (Sigma Aldrich®) in PBS for 15 min, washed in PBS for 5 min and transferred with 10 µL PBS:glycerol (1:1) plus 0.5 µL/mL of Hoechst to microscopy slides. The oocytes were evaluated under a fluorescence microscope (Eclipse 80i, Nikon®) using 330–385 nm filters. The oocytes showing extrusion of the first polar body (metaphase II) were considered mature.

Analysis of gene expression in cumulus cells of oocytes challenged with LPS during IVM

After IVM, approximately 40 COCs/group in three replicates were denuded by successive pipetting. The denuded oocytes were removed and the IVM medium containing the cumulus cells was transferred to microtubes and centrifuged at 1500 g for 5 min. Afterward, the supernatant was discarded and 100 µL of TRIzol (Invitrogen) was added. The samples were homogenized and stored at –70°C until RNA extraction.

The total RNA of the cumulus cells was extracted using TRIzol reagent, according to the manufacturer's recommendations. The RNA concentration was measured in a spectrophotometer (Nanodrop Lite, Thermo Fischer Scientific Inc.) and purity was verified through the 260/280 nm absorbance ratio. Reverse transcription was conducted using all the extracted RNA (13 µL) in the reaction volume of 20 µL, using a commercial kit (iScript Synthesis kit, BioRad®). The samples were incubated at 25°C for 5 min, 42°C for 20 min and 95°C for 1 min. Afterward, 40 µL of ultrapure water was added to dilute the cDNA.

Real-time PCR was conducted using GoTaq (GoTaq® qPCR Master Mix, Promega) in the volume of 15 µL in a StepOnePlus thermal cycler (Applied Biosystems). Each reaction was performed in duplicate, using 4 µL of total cDNA, 5 µL of GoTaq, 0.15 µL of DYE, 0.75 µL of each primer (5 µM) and 4.35 µL of ultrapure water. For each assay, 45 cycles were performed (95°C for 15 s and 60°C for 1 min) and at the end of each reaction a dissociation curve (melting) was performed to check the amplification of a single PCR product.

The *H2A histone type 1-C (HIST1H2AC)* and 18S ribosomal RNA (*RN18S1*) genes were used as endogenous control and the target genes associated with the inflammatory response: *TLR4* and *TNF*; the expansion of the cumulus cells and resumption of meiosis: amphiregulin (*AREG*) and epiregulin (*EREG*) were evaluated. The primer sequences are described in Table 1. Relative expression was calculated using equation $2^{-\Delta\Delta C_T}$ as described by Rincón et al. (2018), using the geometric mean of gene expression of *HIST1H2AC* and *RN18S1* as endogenous control.

Fertilization and IVC of oocytes challenged with LPS

After IVM, approximately 50 COCs/group were transferred to the IVF medium. Sperm selection was performed using mini Percoll density gradient (Parrish et al. 1995), and insemination was conducted with a concentration of 1×10^6 sperm/mL. The COCs were incubated with the sperm for 20 h at 39°C and 5% of CO₂. The day of insemination was considered as day 0 (D0) of the embryo culture.

After IVF, the presumptive zygotes were stripped from the cumulus cells by successive pipetting and transferred to the IVC1 medium under mineral oil. The embryos were cultivated at 39°C with 5% CO₂ for 7 days. On D3, the cleavage rate (cleavage/inseminated COCs) was evaluated and 70% of the culture medium was replaced by CIV2. In D5, 70% of the

culture medium was replaced by IVC3. On day 7 (D7), the blastocyst rate (blastocyst/cleaved) was evaluated. To evaluate the cleavage rate and blastocyst rate, ten replicates were performed, totaling 430 inseminated COCs/group.

Total cell count in embryos from oocytes challenged with LPS during MIV

On D7, embryos from three replicates were fixed in PBS plus 0.5% glutaraldehyde for 30 min and then stained (PBS supplemented with 1 mg/mL PVA and 5 µg/mL Hoechst) for 15 min, washed in PBS for 5 min and transferred to slides containing PBS-glycerol (1:1) with 0.5 µg/mL Hoechst. The total cell count was performed identifying and counting the nuclei in each of embryo under a fluorescence microscope (Eclipse 80i; Nikon®) using 330–385 nm filters. A total of 30 embryos/group were evaluated.

Experiment 2 – Effect of LPS during in vitro embryo culture

Treatments

To evaluate the effect of LPS during embryo culture, embryos were exposed to concentrations of 0, 0.1, 1.0 and 5.0 µg/mL of LPS during the 7 days of IVC. After the initial dilution of LPS (O111: B4, Sigma®) in saline solution (0.9% NaCl), serial dilutions were performed on the IVC1, IVC2 and IVC3 media. After dilution, the aliquots were stored at –20°C until further use.

Selection, maturation and fertilization of oocytes

COCs were recovered and selected as described in experiment 1 and randomly assigned to four groups. Approximately 50 COCs/group were matured *in vitro* without addition of LPS in the medium. After 22 h, IVF was performed.

Culture of challenged embryos with LPS in vitro and embryo cell count

Twenty-four hours after IVF, the presumptive zygotes were stripped of cumulus cells and transferred to the CIV1 medium containing: 0 (control), 0.1, 1.0 or 5.0 µg/mL of LPS. These concentrations were maintained during the 7 days of embryo culture. On D3, the cleavage rate was evaluated and 70% of

Table 1 Genes and primer sequences analyzed in this study.

Gene	Sequence 5' → 3'	Access number	Reference
<i>HIST1H2AC</i>	F: GAGGAGCTGAACAAGCTGTG R: TTGTGGTGGCTCTCAGTCTTC	NM_001205596.1	Bettegowda et al. (2006)
<i>RN18S1</i>	F: CCTTCCGCGAGGATCCATTG R: CGCTCCCAAGATCCAACCTAC	NR_036642.1	Rovani et al. (2017)
<i>TLR4</i>	F: CTTGCGTACAGGTTGTCTCTAA R: CTGGGAAGCTGGAGAAGTTATG	NM_174198.6	Campos et al. (2017)
<i>TNF</i>	F: AGCACAGAAAGCATGATCCG R: CTGATGAGAGGGAGGCCATT	NM_173966.3	Campos et al. (2017)
<i>AREG</i>	F: CTTTCGTCTCTGCCATGACCTT R: CGTTCTTCAGCGACACCTTCA	NM_001099092.1	Rincón et al. (2018)
<i>EREG</i>	F: TCACCGCGAGAAGGATGGAG R: GTACTGAAGACCGACGAGC	XM_002688367.3	Rincón et al. (2018)

the culture medium was replaced with IVC2 medium. On D5, 70% of the culture medium was replaced with IVC3 medium and in D7 the blastocyst rate was evaluated. A total of four replicates were performed, totaling 200 inseminated COCs/group. Additionally, three replicates were carried out to evaluate embryo cell count (30 embryos/group), as described previously.

Experiment 3 – Effect of *in vivo* challenge with LPS on *in vitro* embryo development

Treatment and animals

Sixteen healthy heifers (*Bos taurus taurus*), approximately 14 months old, managed in an intensive confined system and receiving a total mix diet were used in this study. Fourteen days before the beginning of the synchronization protocol 25 mg of PGF2 α (i.m., Lutalyse®; Zoetis, São Paulo, Brazil) was injected in all heifers. On day zero of the protocol (D0), heifers received an intravaginal progesterone-releasing device (1 g, CIDR®, Zoetis®), 2 mg of estradiol benzoate (Gonadiol, Zoetis®) i.m. and 25 mg of PGF2 α (Lutalyse®, Zoetis) i.m. The intravaginal device was removed on D5 (Cavaliere et al. 2018).

The heifers were randomly assigned to two groups: LPS group ($n=8$), which received two intravenous applications of 0.5 $\mu\text{g/kg}$ of body weight of LPS (Sigma Aldrich®) diluted in 2 mL of saline solution (0.9% of NaCl) 24 h apart; and control group ($n=8$), which received two applications of 2 mL of saline solution (0.9% of NaCl) at the same interval. The first application of LPS was performed on day 1 (D1) of the follicular wave synchronization protocol (Fig. 1). The rectal temperature was measured with a digital thermometer at 0, 4, 24, 28 and 48 h after the first LPS challenge.

The dose and interval of LPS was chosen according to the lowest dose of LPS that demonstrated to generate an inflammatory response in cattle previously (Waldron et al. 2003, Fernandes et al. 2019).

Collection of ovaries and *in vitro* embryo production

On D5 of the synchronization protocol the heifers were slaughtered, ovaries were collected, identified and transported to the laboratory in saline solution (NaCl 0.9%) supplemented with antibiotics at 30°C.

At the laboratory, COCs were aspirated from follicles with a diameter of 2–8 mm from each pair of ovaries. The weight of the ovaries, total COCs recovered and the number of viable COCs (grades I, II and III according to de Loos et al. (1991)) of each heifer was recorded and the viable COCs were used for IVM. The number of viable oocytes/heifer and the percentage of viable oocytes in each group was evaluated. The procedures for IVM and IVF were performed as previously described, in separate drops for each heifer. After IVF, the COCs were transferred to drops of washing medium and denuded by successive pipetting. The washing medium containing the cumulus cells was transferred to microtubes and centrifuged at 1500 g for 5 min. Afterward, the supernatant was discarded and 100 μL of TRIzol (Invitrogen®) was added. The samples were homogenized and stored at -70°C until the RNA extraction. The denuded oocytes were transferred to IVC1 medium and continued the IVC during 7 days, as previously described. The

Figure 1 Follicular wave synchronization protocol and treatments used in experiment 3. The heifers of the control group received two applications of saline solution (NaCl 0.9%) intravenously, with a 24-h interval, and the LPS group received two applications of 0.5 $\mu\text{g/kg}$ body weight of LPS intravenously with the same interval.

cleavage rate was evaluated on D3 and the blastocyst rate on D7. Embryo cell count was performed in D7 embryos from all groups ($n=35$) as also previously described.

The relative expression of the *TLR4*, *TNF*, *AREG* and *EREG* genes in cumulus cells was analyzed by real-time PCR, as previously described.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software Inc.). Nuclear maturation rate from experiments 1 and 2, and cleavage and blastocyst rates of experiment 2 were analyzed by the chi-square test. Cleavage rate, blastocyst rate, cell count per embryo and gene expression from experiment 1 were analyzed using ANOVA. The cell count per embryo and gene expression from experiment 2 were analyzed by *t*-test. The temperature was analyzed by the two-way ANOVA test. In addition, the GLM procedure was applied to observe the linear, quadratic and/or cubic effect of the LPS dose on the cleavage and blastocyst rate from experiments 1 and 2 (SAS). Values of $P<0.05$ were considered as significant.

Results

Experiment 1

The nuclear maturation rate of oocytes challenged with 1 and 5 $\mu\text{g/mL}$ of LPS during IVM was lower (39.4 and 39.6%, respectively) than in the control group (63.6%, $P<0.05$, Fig. 2A). However, the nuclear maturation rate of the 0.1 $\mu\text{g/mL}$ LPS group (55.7%) was not different from control ($P>0.05$, Fig. 2A).

The cleavage rate of LPS-challenged oocytes during IVM was not different among groups, being 72.6, 72.7, 68.6 and 71.5% ($P>0.05$) for the 0, 0.1, 1 and 5 $\mu\text{g/mL}$ LPS groups, respectively (Fig. 2B). Likewise, the blastocyst rate (40.1, 33.9, 41.8 and 40.8%) was not different between the 0, 0.1, 1 and 5 $\mu\text{g/mL}$ LPS groups, respectively ($P>0.05$, Fig. 3A). In addition, no linear or quadratic effect was observed on cleavage or blastocyst rate. The number of cells per embryo was 64.2 ± 4.1 cells/embryo in the control group and 64.5 ± 4.7 , 63.1 ± 4.0 and 65.0 ± 4.7 cells/embryo for groups challenged with 0.1, 1 and 5 μg LPS during IVM, respectively ($P>0.05$, Fig. 3B). The relative expression of *TLR4*, *TNF*, *AREG* and *EREG* in cumulus cells was similar among groups ($P>0.05$, Fig. 4).

Figure 2 Nuclear maturation rate (A) and cleavage rate (B) of lipopolysaccharide-challenged bovine oocytes (LPS) during oocyte *in vitro* maturation (IVM). Oocytes were matured for 22 h with 0 (control), 0.1, 1, or 5 µg/mL LPS. The maturation rate was analyzed by the chi-square test and the cleavage rate by the ANOVA test. Different letters indicate statistical difference ($P < 0.05$).

Experiment 2

The cleavage (85.7, 81.0, 74.7 and 82.3%) and blastocyst rates (36.4, 34.4, 32.2 and 35.2%) from zygotes challenged with 0, 0.1, 1 and 5 µg/mL LPS during IVC was not different among groups ($P > 0.05$, Fig. 5A and B, respectively). In addition, the number of cells per embryo on D7 was not different among groups ($P > 0.05$), being 58.5 ± 4.9 cells/embryo in the control group and 54.0 ± 3.9 , 54.8 ± 4.6 and 59.1 ± 4.1 cells/embryo in the groups challenged with 0.1, 1 and 5 µg/mL LPS during IVC (Fig. 5C).

Experiment 3

Heifers injected with LPS had higher rectal temperature 4 h after each challenge ($P < 0.05$, Fig. 6). At 4 h, temperature in the LPS group was $39.9 \pm 0.19^\circ\text{C}$ and at 28 h it was $40.2 \pm 0.4^\circ\text{C}$, compared to the control group

Figure 3 Blastocyst rate (A) and number of nuclei/embryo (B) challenged with lipopolysaccharide (LPS) during oocyte *in vitro* maturation (IVM). Oocytes were matured for 22 h with 0 (control), 0.1, 1, or 5 µg/mL LPS. Statistical analysis was performed using the ANOVA test.

that had temperatures of $39.1 \pm 0.1^\circ\text{C}$ ($P < 0.05$) and $39.2 \pm 0.1^\circ\text{C}$ ($P < 0.05$), respectively. At 0 h (38.6 ± 0.1 and $38.7 \pm 0.1^\circ\text{C}$), 24 h (38.6 ± 0.1 and $38.8 \pm 0.2^\circ\text{C}$) and 48 h (38.7 ± 0.1 and $38.9 \pm 0.1^\circ\text{C}$), the temperatures were not different between LPS and control groups, respectively ($P > 0.05$).

There was no difference between groups in ovarian weight, total oocytes aspirated/heifer, viable oocytes/heifer and proportion of viable oocytes/group ($P > 0.05$, Table 2). Cleavage rate of oocytes from heifers challenged with LPS was lower (54.3%, 38/70) than for control group (70.2%, 73/104, $P = 0.032$, Fig. 7A). The blastocyst rate was not different between LPS (31.6%, 12/38) and control groups (31.5%, 23/73, $P > 0.05$, Fig. 7B). Similarly, the number of cells per embryo was not different between groups ($P > 0.05$), being 48.5 ± 4.9 for control group and 50.7 ± 8.9 cells/embryo for LPS group (Fig. 7C).

The relative expression of *TLR4*, *TNF*, *AREG* and *EREG* genes in cumulus cells from heifers challenged

Figure 4 Relative expression of the *TLR4* (A), *TNF* (B), *AREG* (C) and *EREG* (D) genes in oocyte cumulus cells challenged with lipopolysaccharides (LPS) during oocyte *in vitro* maturation (IVM). Oocytes were matured for 22 h with 0 (control), 0.1, 1, or 5 µg/mL LPS. The analysis was performed using the ANOVA test.

or not with LPS did not differ between groups ($P > 0.05$, Fig. 8).

Discussion

In the present study, we evaluated the effects of LPS exposure over *in vitro* and *in vivo* bovine oocyte maturation and early embryo development. In our experimental conditions, exposure of oocytes to LPS during *in vitro* maturation affected IVM rate but did not affect cleavage nor blastocyst rates. Embryonic development was also not affected when cultured in the presence of LPS. However, the cleavage rate of oocytes obtained from heifers challenged with LPS was reduced. Therefore, exposure of bovine oocytes to LPS *in vivo* produced stronger effects on oocyte quality, as cleavage rate was reduced after *in vivo* challenge, whereas after the *in vitro* challenge only nuclear maturation rate was reduced, suggesting a systemic involvement.

The results of the *in vitro* experiment demonstrated that concentrations of 1 and 5 µg/mL of LPS during IVM decreased by ~24% the number of oocytes that were able to resume meiosis and reach nuclear maturation (metaphase II). Magata and Shimizu (2017) observed that LPS during IVM negatively affected nuclear maturation starting at smaller doses of LPS, 0.01, 0.1, 1.0 and 10 µg/mL. However, Bromfield and Sheldon (2011) and Zhao et al. (2017) observed that 1 µg/mL of LPS during IVM did not decrease the nuclear maturation rate, although a reduction was observed at 10 µg/mL. No clear differences among studies was observed that could explain the variability of the results. Bromfield and Sheldon (2011) used serum free media; however,

Figure 5 Cleavage rate (A), blastocyst rate (B) and number of nuclei/embryo (C) challenged with lipopolysaccharide (LPS) during *in vitro* culture (IVC). The zygotes were cultured for 7 days with 0 (control), 0.1, 1, or 5 µg/mL of LPS. The data were analyzed by the ANOVA test.

Zhao et al. (2017) also used FBS similar to our IVM protocol. LPS is able to delay the progression of the cellular cycle and interrupt the meiotic spindle formation (Zhao et al. 2017), therefore impairing maturation.

Figure 6 Rectal temperature of heifers challenged or not with lipopolysaccharide (LPS) at 0 and 24 h, analyzed by the two-way ANOVA test. The heifers of the control group received two applications of saline solution (NaCl 0.9%) intravenously, with a 24-h interval, the LPS group received two applications of 0.5 µg/kg body weight of LPS intravenously with the same interval.

Despite the effect on nuclear maturation, there was no reduction in initial embryonic development. Groups were similar regarding cleavage and blastocyst rates, and number of cells per embryo. Magata and Shimizu (2017) also observed that LPS during IVM did not affect the cleavage rate; however, they found that LPS decreased the blastocyst rate, even at the smallest doses. They attributed this effect to the disturbance of oocyte competence at nuclear (inhibiting progression to metaphase II) and cytoplasmic (affecting mitochondrial status) maturation levels. Others have observed that cleavage and blastocyst rates of parthenogenetic-activated bovine oocytes are reduced at the dose of 10 µg/mL (Zhao *et al.* 2019). However, in our study, although we observed an effect on the maturation rate, the cleavage and blastocyst rates were not affected, which led to the next steps of this study, for experiments during embryo culture, as well, during the *in vivo* development.

Additionally, others showed that oocytes exposed to LPS during IVM presented increased reactive oxygen species, increased expression of *caspase 3* and *BAX* (pro-apoptotic genes) and decreased expression of *BCL2* and *XIAP* (anti-apoptotic genes) (Zhao *et al.* 2017).

Table 2 Parameters evaluated in ovaries of heifers challenged with LPS and controls.

Parameter	Control	LPS	P value
Ovarian weight (g)	9.76 ± 1.4	8.73 ± 1.0	0.56
Total oocytes/heifer	20.13 ± 4.6	15.88 ± 3.0	0.45
Viable oocytes/heifer	13.5 ± 3.6	9.75 ± 2.1	0.39
Proportion of viable oocytes/group	67.1% (108/161)	61.4% (78/127)	0.76

Values expressed as mean ± standard error.

Figure 7 Cleavage rate (A), blastocyst rate (B) and number of nuclei/embryo (C) of oocytes from heifers challenged with lipopolysaccharide (LPS; $n=8$) or control ($n=8$). The heifers of the control group received two applications of saline solution (NaCl 0.9%) intravenously, with a 24-h interval, the LPS group received two applications of 0.5 µg/kg body weight of LPS intravenously with the same interval. The cleavage rate and blastocyst rate were analyzed by the chi-square test and the number of nuclei/embryo was analyzed by the *t*-test. Different letters indicate statistical difference ($P < 0.05$).

Since LPS during IVM did not affect embryonic development, we used the same concentrations of LPS to challenge zygotes during the 7 days of embryo culture, to evaluate whether LPS could have any effect after fertilization. We found that 0.1, 1 or 5 µg/mL of LPS during zygote culture did not affect the initial embryonic development, as the blastocyst rate and number of cells per embryo were not affected by the LPS treatment. To the best of our knowledge, this is the first study to

Figure 8 Relative expression of the *TLR4* (A), *TNF* (B), *AREG* (C) and *EREG* (D) genes in oocyte cumulus cells from heifers challenged with lipopolysaccharide (LPS) and controls. The heifers of the control group received two applications of saline solution (NaCl 0.9%) intravenously, with a 24-h interval, and the LPS group received two applications of 0.5 µg/kg body weight of LPS intravenously with the same interval. The data were analyzed by the *t*-test.

evaluate the direct effects of LPS exposure during the first 7 days after fertilization. This lack of effect may be due to the fact that after fertilization until the activation of the embryo genome, which occurs in the stage between 8 and 16 cells in the bovine species, development is coordinated by the transcripts stored during oocyte maturation (Mamo *et al.* 2011). Thus, if maturation occurred properly, LPS is not able to affect early embryonic development. It is suggested that cumulus cells have the ability to respond to LPS with a cascade of inflammatory reactions (Magata & Shimizu 2017) due to the presence of TLR4 receptors, but it has not been reported if embryos during the pre-implantation phase have the intrinsic capacity to respond to these stimuli, which may explain our current findings and the lack of effect of LPS on the embryo. This is an interesting finding as the LPS can be also found in the uterine fluid in sick cows (Magata *et al.* 2015) and can potentially impair development; however, our study suggests that this may not be a direct effect on the embryo.

Due to this controversy with the direct and indirect effects of LPS and its ability to affect fertility, in the third experiment two applications of intravenous LPS were performed on heifers during the development of a follicular wave. As expected (Waldron *et al.* 2003, Carroll *et al.* 2009, Campos *et al.* 2017), the results of the *in vivo* experiment demonstrated that the dose of LPS used was able to generate a systemic inflammatory response, supported by the increase in body temperature 4 h after each challenge with LPS. In a previous study, we found that a single application of intravenous LPS, in addition to generating a systemic inflammatory response, was able to alter the expression of genes associated to the steroidogenic and inflammatory cascade in the granulosa cells of the dominant follicle (Campos *et al.* 2017). In addition, the LPS altered the serum and follicular activity of acute phase proteins (Campos *et al.* 2017). To our knowledge this is the first study to evaluate the effect of infusions of LPS *in vivo* on the oocyte and initial embryo development *in vitro*. Although systemic alterations triggered by LPS have been observed, ovarian weight, number of total and morphologically viable oocytes aspirated per heifer were not affected by the LPS challenge. Previous studies showed that LPS is able to have direct and indirect effects at ovarian follicle level. In this regard, LPS can reduce the number of primordial follicles in mice, associated to hyperactivation of these follicles and increased atresia (Bromfield & Sheldon 2013), which could impair the number of small follicles for aspiration. In addition, LPS can reduce steroidogenesis in the ovary (Herath *et al.* 2007, Campos *et al.* 2017, Magata & Shimizu 2017), which could in turn affect the development and maturation of the oocyte. This indicates that, unlike the *in vitro* effects, which are restricted to the interaction of LPS with cumulus cells, the *in vivo* effects may

derive from various mechanisms of LPS interaction with the body.

In this sense, the challenge with intravenous LPS reduced the cleavage rate by 16%. In the *in vitro* experiment, we observed that LPS negatively affected nuclear maturation. Additionally, other studies demonstrated that LPS can interrupt meiotic spindle formation, disturb the mitochondria organization and function, increase oxidative stress and modify the methylation pattern in the oocyte (Magata & Shimizu 2017, Zhao *et al.* 2017). These direct effects, added to the systemic inflammatory response may explain the stronger effect and the observed decrease on cleavage rate. Interestingly, we observed that LPS affected cleavage rate only *in vivo*. This suggests that the effects of LPS not merely occur through direct interaction with cumulus cells, but also have the involvement of systemic mechanisms that will rather compromise the maturation and cleavage process. As the systemic inflammatory response is highly complex and mediated by numerous factors, more detailed studies are needed to understand which of these factors may be specifically responsible for decreasing oocyte viability and compromising fertility. Despite this, the blastocyst rate of oocytes from heifers challenged *in vivo* with LPS was not altered. Suggesting that those oocytes that cleaved and passed this challenge imposed by the systemic inflammatory process have normal developmental capacity. It is important to highlight that the time between the LPS challenge (at D1 and D2) and the day of oocyte retrieval (D5) was of 72 h. The animals no longer presented signals of systemic inflammatory response and this may also influence the results observed. Chronic exposure and closer to moment of oocyte retrieval may impair even more the cleavage rate.

During oogenesis, the somatic cells surrounding the oocyte proliferate and differentiate into theca, granulosa and cumulus cells, forming the COCs (van den Hurk & Zhao 2005). These cells play an important role in acquiring oocyte competence, allowing the transfer of nutrients, hormones, amino acids, growth factors, and signals that stimulate or inhibit meiosis (van den Hurk & Zhao 2005). In cattle, the LPS is capable of generating an inflammatory response in theca cells (Magata *et al.* 2014b) and granulosa cells *in vitro* (Herath *et al.* 2007, Bromfield & Sheldon 2011) and granulosa cells *in vivo* (Campos *et al.* 2017), and with this can affect oocyte development. However, to our knowledge, there is no evidence of direct effects of LPS on cumulus cells. Thus, one of the possible routes of action of the LPS would be through the cumulus cells, compromising the oocyte competence and subsequent embryonic development. To evaluate this, we measured the expression of genes in the cumulus cells: *TLR4*, the receptor responsible for the recognition of LPS and activation of the inflammatory response (Takeuchi & Akira 2010); *TNF*-, one of main

pro-inflammatory cytokines (Takeuchi & Akira 2010) and; *AREG* and *EREG*, which are regulators of cumulus cell expansion and oocyte maturation (Friedman & Halaas 1998, Watkins 2004). However, we found no effect of LPS *in vivo* or *in vitro* on the expression of these genes, indicating that the concentrations and time of exposure to LPS used in this study were not able to generate a detectable inflammatory response and compromise cumulus cells functionality. In previous studies, a reduction of *TLR4* and *TNF* expression was observed in the preovulatory follicle mural granulosa cells of LPS *in vivo*-challenged cows (Campos *et al.* 2017), indicating that the effect may not be direct from LPS but rather from the systemic response generated by LPS. However, we should also consider that the response from mural granulosa cells and cumulus cells may be different and explain this finding. Additionally, others showed that oocytes exposed to LPS during IVF presented increased reactive oxygen species, increased expression of *caspase 3* and *BAX* (pro-apoptotic genes) and decreased expression of *BCL2* and *XIAP* (anti-apoptotic genes) (Zhao *et al.* 2017), which could be a reflection of an increased pro-inflammatory state. However, in the mentioned study the dose of 10 µg/mL was used.

Nevertheless, factors such as exposure time, route of administration and dosage of LPS may make it difficult to interpret the direct or indirect effects of this endotoxin. Some studies use higher doses of LPS to better understand its effects in fertility. Moreover, some studies used doses that exceed the concentrations of LPS found during infectious processes *in vivo*. For example, the concentrations of LPS that we used *in vitro* was based on those reported by follicular fluid from cows with subclinical (0–0.04 µg/mL) or clinical endometritis (0.043–0.875 µg/mL) (Herath *et al.* 2007). However, others found that cows diagnosed with clinical metritis had much lower follicular concentrations of LPS ranging from 0.0001 to 0.001 µg/mL (Magata *et al.* 2015). Studies *in vivo* have used doses ranging from 0.5 to 10 µg/kg of body weight of LPS (Bidne *et al.* 2018). In contrast, we used 0.5 µg/kg of body weight of LPS, the lowest dose of LPS that has been shown to generate an inflammatory response in cattle (Waldron *et al.* 2003). Furthermore, Trayhurn and Wood (2005) suggest special attention in the interpretation of serum and follicular LPS measurement results, due to the differences and limitations of the laboratory tests. For example, some tests measure the biological activity of endotoxin, and most do not differentiate free LPS from LPS bound to inflammatory mediators, such as CD14 and lipopolysaccharide-binding protein (LBP). This makes it even more difficult to experimentally simulate the events that take place *in vivo*.

In conclusion, the exposure of bovine oocytes to LPS *in vivo* produced stronger effects on oocyte quality, as cleavage rate was reduced after *in vivo* challenge,

whereas after the *in vitro* challenge only nuclear maturation rate was reduced. This suggests that systemic inflammatory response increases the magnitude of the effect of LPS on oocyte quality. Likewise, the exposure of zygotes to LPS during IVF did not affect embryonic development, suggesting no direct effects of LPS on embryo development are present. Thus, the direct or systemic effects of LPS at oocyte and embryo level are not the same and still unclear, and further studies are needed to better understand how LPS may affect bovine fertility.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

J A A R, B M, P C G and L M C P performed the *in vitro* production of embryos. A A B and A S M assisted in the field experiment and laboratory analysis. M N C and R G M contributed to the discussion of results. J P, J A A R and G A F performed the analysis of gene expression. A S designed the study and did the final review.

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