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Low-dose lipopolysaccharide exposure during oocyte maturation disrupts early bovine embryonic development

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Abstract

Gram-negative bacteria release of lipopolysaccharide (LPS) endotoxin elicits robust immune responses capable of disrupting normal ovarian function contributing to female infertility. However, effects of subclinical or non-detectable infections on oocyte competence and subsequent embryo development remain to be fully elucidated. The aim of this study was to investigate the effects of exposing bovine oocytes to low LPS doses on oocyte and embryo competence. Bovine oocytes were collected from slaughterhouse-derived ovaries and matured with vehicle-control or increasing doses of LPS (0.01, 0.1, and 1 μg/mL) for 21 h. Oocytes (n = 252) were evaluated for nuclear maturation. A set of embryos from LPS-matured oocytes (n = 300) were cultured for 8 d to evaluate day 3 cleavage rates and day 8 blastocyst rates along with blastocyst cell counts. A subset of oocytes (n = 153) was fertilized and cultured for time-lapse image capture and analysis of embryo development. Results demonstrate no significant treatment differences among treatment groups in percent of oocytes at germinal vesicle (GV; $P = 0.90$), germinal vesicle breakdown (GVBD; $P = 0.13$), meiosis I (MI; $P = 0.26$), or metaphase II (MII; $P = 0.44$). Likewise, treatment differences were not observed in cleavage rates ($P = 0.97$), or blastocyst rates ($P = 0.88$) evaluated via traditional microscopy. Treatment with LPS did not affect total blastocyst cell count ($P = 0.68$), as indicated by trophectoderm ($P = 0.83$), and inner cell mass ($P = 0.21$)

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CRediT authorship contribution statement

B. Castro: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, preparation. J.I. Candelaria: Methodology, Investigation, Formal Analysis, Writing – review & editing. M.M. Austin: Investigation. C.B. Shuster: Investigation, Formal analysis, Writing – review & editing. C.A. Gifford: Formal analysis, Writing – review & editing. A.C. Denicol: Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & editing. J.A. Hernandez Gifford: Conceptualization, Methodology, Investigation, Formal analysis, Writing – review & editing, Funding acquisition.

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cell counts. Time-lapse embryo evaluation demonstrated no differences among control or LPS matured oocytes in number of zygotes that did not cleave after fertilization ($P = 0.84$), or those that cleaved but arrested at the 2-cell stage ($P = 0.50$), 4-cell ($P = 0.76$), prior to morula ($P = 0.76$). However, embryos derived from oocytes challenged with 0.1 μg/mL LPS tended to have reduced development to the morula stage compared with vehicle-treated controls ($P = 0.06$). Additionally, the percentage of blastocysts derived from oocytes matured in 0.01 μg/mL LPS tended to decrease compared to vehicle-treated controls (11.38 and 25.45%, respectively; $P = 0.09$). Similarly, the proportion of oocytes that developed to the blastocyst stage was greater in vehicle-treated controls (25.45%) compared with embryos derived from oocytes matured in 0.1 and 1 μg/mL (5.92 and 6.55%, respectively; $P = 0.03$) LPS. These data suggest LPS-matured oocytes that subsequently underwent in vitro fertilization, experienced decreased competence to develop to the blastocyst stage.

Keywords

Blastocyst; Disease; Embryo; In-vitro maturation; Lipopolysaccharide; Oocyte

1. Introduction

Efficiency and profitability of cattle operations rely on optimal reproductive performance. Economic success of the cow-calf system is dependent on cows calving and weaning a calf on an annual basis [1]. Cows and heifers are culled from a herd primarily due to reproductive failure and contributors to compromised fertility include embryo mortality resulting in pregnancy loss, poor quality gametes, ovulation failure, among others [2]. In addition, disease states related to bacterial infection have been implicated in reduced conception rates and infertility [3].

Gram-negative bacterial pathogens such as *Escherichia coli* are often present in clinical states of metritis and mastitis in postpartum cows [4]. However, subclinical or undetectable infections are also capable of disrupting reproductive parameters leading to decreased fertility in cattle [4, 5]. Lipopolysaccharide (LPS) is an endotoxin motif on the outer membrane of gram-negative bacteria recognized to induce immune responses and activate inflammatory pathways [6]. As pathogens proliferate and lyse, LPS is shed and released into circulation where it is bound to its binding protein allowing for travel to locations distant from infection origin [7]. Within the ovary, LPS has the ability to accumulate in follicular fluid resulting in increased pro-inflammatory cytokine production [8, 9], reduced oocyte competence [10], disrupted follicle development [3], and altered hormone production [11]. Lipopolysaccharide quantified in follicles from slaughterhouse-derived bovine ovaries showed deleterious effects in hormone production and gamete quality when LPS was detectable at relatively low concentrations [10, 11]. Importantly, bovine granulosa cells express the receptor complex Toll-like receptor 4 (TLR4), cluster of differentiation 14 (CD14), and myeloid differentiation factor 2 (MD2) necessary for immune responses within the follicle [8, 10].

Disease-related infertility is associated with perturbation of ovarian function including oocyte development within the ovarian follicle. Successful oocyte fertilization and subsequent embryonic development depend on specific oocyte-controlled events and proper nuclear maturation and are reliant on the intra-follicular environment where the oocyte is in direct communication with the follicular fluid and cumulus granulosa cells. Recent data has demonstrated that slaughterhouse ovaries with follicular fluid LPS concentrations above 1.6 EU/mL have increased percentage of abnormal oocytes [10]. Likewise, accumulation of low concentrations of follicular LPS is detrimental to ovarian steroid production [11, 12].

While studies have examined the adverse effects of disease associated with gram negative bacteria or infusion of high LPS doses in vivo and in vitro, the consequence of low-dose LPS exposure comparable to that quantified in undetectable disease states is less clearly understood. The aim of the current study is to investigate the impact of low-dose LPS concentrations that may mimic subclinical or non-detectable disease on bovine meiotic progression and early embryonic development. It is hypothesized that low-dose LPS exposure has the potential to disrupt oocyte competence and subsequent development of the preimplantation embryo.

2. Materials and methods

2.1 Materials

Lyophilized *Escherichia coli* LPS, serotype O55:B5 (1 mg) (Sigma Aldrich, Inc., St. Louis, MO) was reconstituted in 1 mL of filter-sterilized 0.1 M Dulbecco's phosphate-buffered saline (DPBS) (Sigma Aldrich). Dilutions were made using in vitro oocyte maturation media (BO-IVM; IVF Bioscience, Falmouth, England) for 0.01, 0.1, and 1 μg/mL LPS treatments. All media used for oocyte maturation and embryo production were purchased from IVF Bioscience (Falmouth, England) unless stated otherwise.

2.2 Cumulus oocyte complex collection

Oocytes were sourced from slaughterhouse-derived ovaries following techniques previously described [13]. Bovine ovaries were obtained from a local slaughterhouse and transported in warmed saline solution. Upon arrival, ovaries were washed with pre-warmed saline solution containing 1% Penicillin-Streptomycin (Fisher Scientific, Waltham, MA) to remove excess blood and debris. Cumulus oocyte complexes (COCs) were obtained by slicing the surface of the ovarian cortex with a scalpel and rinsing the ovary in a beaker containing 38.5°C oocyte collection media (OCM) composed of Medium-199 with Hank's salts and L-glutamine (Sigma Aldrich), 25 mmol/l HEPES and sodium bicarbonate, 1% fetal bovine serum (Fisher Scientific), 1% glutaMAX (Thermo Fischer Scientific), and 1% heparin (Sigma Aldrich). Cumulus oocyte complexes were filtered through a 100 μm sterile cell strainer (Fisher Scientific) and transferred to a square Petri dish with grid for evaluation under a microscope. The COCs were continuously maintained on a benchtop plate warmer set at 38.5°C.

2.3 In vitro maturation of oocytes

The COCs with at least three layers of compact cumulus cells and uniform cytoplasm were selected and washed three times in 35-mm Petri dishes containing warmed wash media (IVF Bioscience) followed by a wash in warmed oocyte maturation media (Bo-IVM, IVF Bioscience). The COCs were evenly distributed in 4-well plate containing 0 (vehicle-treated control), 0.01, 0.1, or 1 μg/mL LPS in 500 μl of BO-IVM per well with no more than 45 COCs per 500 μl of media. This medium is serum free and supplemented with gonadotropic hormones, gentamycin and low glucose by the manufacturers. The COCs were matured for 21 h at 38.5°C, 5% CO_2 and humidified air.

2.4 Denuding, fixation, and immunofluorescence of oocytes

Following the 21 h maturation period, COCs were placed in warmed hyaluronidase (1 KU/mL) (Sigma Aldrich) diluted in wash buffer (0.1 M DPBS, 0.1% bovine serum albumin, and 0.1% Tween) and vortexed vigorously for 3 min to remove tightly adhered cumulus cells. Oocytes were washed three consecutive times in 50 μl wash buffer drops per treatment. Oocytes were then transferred to 50 μl drops of 4% paraformaldehyde (Electron Microscopy Sciences) in DPBS for 15 min at room temperature. Oocytes were washed and transferred to 50 μl drops of permeabilization buffer composed of 0.25% Triton-X-100 in 0.1% polyvinylpyrrolidone treated DPBS (DPBS-PVP) for 20 min at room temperature. Following permeabilization, oocytes were washed and blocked for 1 h at room temperature in blocking buffer consisting of 0.5% bovine serum albumin in DPBS. Oocytes were removed from blocking buffer and incubated overnight in primary antibody, rabbit anti-non-phosphorylated (active) beta-catenin (Ser33/37/Thr41) in antibody buffer (0.1% Tween, 1% BSA in DPBS) (1:800, Cell Signaling Technologies, Danvers, MA) at 4°C. Oocytes were washed in wash medium and incubated in Texas Red-labeled goat anti-rabbit secondary antibody in antibody buffer (1:2000, Life Technologies, Carlsbad, CA) for 1 h at room temperature. Oocytes were washed following secondary antibody and counterstained at room temperature in Hoechst 33342 diluted in wash buffer (1:1000, Invitrogen, Waltham, MA) for 5 min in the dark and at room temperature for nuclear labeling. Oocytes were mounted on slides over 5 μl of SlowFade Gold antifade reagent (Life Technologies), covered with a coverslip, and kept at 4°C until imaging. Digital images for oocytes were acquired using a Revolve fluorescence microscope using DAPI and Texas Red channels (Discover Echo Inc., San Diego, CA). All images were taken at 565 nm light exposure. Digital images were analyzed using Fiji software (NIH, Bethesda, MD).

2.5 Assessment of oocyte nuclear maturation

Oocytes were analyzed through evaluation of meiotic progression following LPS challenge. Oocytes were categorized as germinal vesicle (GV), germinal vesicle breakdown (GVBD), meiosis I (MI), or metaphase II (MII) following 21 h incubation with or without increasing LPS concentrations. Oocytes were evaluated by three different experienced observers without reference to previously collected data.

2.6 In vitro production and fixation of embryos

An additional set of oocytes utilized for embryo production were matured with increasing concentrations of LPS as described above. Following the 21 h maturation period, expanded COCs were fertilized utilizing the IVF Bioscience bovine IVF protocol (Falmouth, England) as previously described [13]. Expanded COCs were washed and transferred to in vitro fertilization media (BO-IVF, IVF Bioscience) without LPS. Cryopreserved bull sperm from a single sire was used for IVF, sperm was washed and resuspended in BO-SemenPrep media (IVF Bioscience) at a final concentration of 1 x 10^6/mL. Fertilization occurred in pre-equilibrated 4-well plate in 400 μl of Bo-IVF media plus sperm over 18 h at 38.5°C, 5% CO_2, atmospheric O_2, and humidified air and was designated day 0. Following fertilization, presumptive zygotes were washed and vortexed vigorously for 3 min to remove cumulus cells and transferred to a pre-equilibrated 4-well plate containing 500 μl of in vitro culture media (BO-IVC, IVF Bioscience) and 400 μl of mineral oil overlay (IVF Bioscience) per well. Embryos were cultured for 8 d at 38.5°C, 5% O_2, 5% CO_2, and the balance N_2. Cleavage rates were assessed at day 3 of development and blastocyst rates were evaluated at day 8 of development. Following the 8-d culture period, expanded blastocysts were washed three consecutive times in wash buffer and fixed in 4% paraformaldehyde drops for 20 min at room temperature. After fixation, blastocysts were washed and transferred to a permeabilization solution for 30 min at room temperature, washed, and blocked for 1 h at room temperature.

2.7 Embryo immunofluorescence

Embryos were subsequently removed from blocking buffer and incubated overnight with anti-CDX2 at 4°C (used at working concentration provided by manufacturer, BioGenex, Fremont, CA). Embryos were washed in antibody buffer and incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-mouse IgG diluted in antibody buffer (1:2000, Life Technologies) for 1 h at room temperature in the dark. Embryos were washed three consecutive times in wash buffer and placed overnight in rabbit anti-non-phosphorylated (active) beta-catenin (Ser33/37/Thr41) in antibody buffer (1:1000) at 4°C. Following overnight incubation, embryos were washed in antibody buffer and placed in Texas Red-labeled goat anti-rabbit secondary antibody (1:2000) diluted in antibody buffer for 1 h at room temperature in the dark. Embryos were washed in antibody buffer and counterstained with Hoechst 33342 diluted in wash buffer (1:1000) and incubated for 15 min at room temperature in the dark. Three final washes were done with wash buffer and embryos were mounted on a slide over 5 μl of SlowFade Gold antifade reagent and covered with coverslip, embryos were kept at 4°C until imaging.

2.8 Embryo cell count analysis

Individual blastocysts were visualized and imaged using the Revolve fluorescence microscope under DAPI, Texas Red, and FITC channels (Discover Echo Inc., San Diego, CA). Blastomere or cell numbers were evaluated using the cell count function of Fiji software. Total cell number was determined by counting individual nuclei staining with Hoechst 33342. Trophectoderm cells were determined by counting nuclei positive

for CDX2, and inner cell mass (ICM) was determined by subtracting the number of trophectoderm cells from the total cell number.

2.9 Time-lapse embryo development

Bovine embryos for time-lapse experiments were produced by IVF from oocytes matured with increasing concentrations of LPS. All procedures for embryo production followed techniques described above. One to three presumptive zygotes of similar treatment groups were placed in wells and incubated in the Miri multiroom incubator (Esco Medical, Denmark) for 8-d at 38.5°C, 5% O2, 5% CO_2, and the balance N_2. Images of individual wells were captured every 5 min over the course of the 8-d culture period and compiled into a time-lapse video for analysis of embryo development.

2.10 Statistical Analyses

All statistical analyses were performed using SAS (Version 9.3; SAS Institute, Inc., Cary, NC). Oocyte nuclear maturity, embryo development stages, and cell counts were analyzed using the GLM procedure in SAS. Analysis by ANOVA and least square means comparisons between LPS treatments were performed. Statistical significance was denoted by *P*-values ≤ 0.05 and *P*-values > 0.05 and < 0.1 reflect trends.

3. Results

3.1 Low-dose lipopolysaccharide on in vitro maturation

Nuclear maturation was assessed based on meiotic progression. Oocytes were categorized as GV, GVBD, MI, or MII following a 21 h maturation period in the presence or absence of increasing LPS concentrations. Appropriate nuclear maturation was identified as oocytes arrested at MII with complete extrusion of a polar body (Fig. 1A), oocytes arrested at MI demonstrated delayed maturation represented by condensed chromosomes and absence of a polar body (Fig. 1B), while oocytes that failed to mature were maintained at GV or GVBD, primarily represented by intact or partially degraded nuclear envelope and minimal to no chromosomal condensation (Fig. 1C). No treatment differences were observed in the percentage of oocytes at GV ($P = 0.90$), GVBD ($P = 0.13$), MI ($P = 0.26$), or MII ($P = 0.44$) among LPS treatment groups (Fig. 2). Oocytes arrested at GVBD stage was limited to 0.1 and 1 μg/mL LPS treatment groups (Fig. 2).

3.2 IVM exposure to lipopolysaccharide impacts development of the preimplantation embryo

Immunostaining for total cell numbers and specific cell types to determine LPS impacts on cell number evaluated in day 8 blastocysts indicated by DNA staining in blue, trophectoderm cells stained with CDX2 antibody and FITC in green, and cell boundaries stained with non-phosphorylated (active) CTNNB1 (Ser33/37/Thr41) in red for LPS treatments can be observed in Fig. 3. Active non-phosphorylated CTNNB1 was localized primarily within the cell membranes of day 8 blastocysts serving as an indicator of cell perimeter. Additionally, no overt differences were detected in gross embryo evaluation among LPS treatments. Blastocyst cell counts as determined by immunostaining were used to quantify total cell number, trophectoderm (TE) cell number, and inner cell mass (ICM) cell number. Inner

cell mass cell counts were determined by subtracting TE cells from total cell numbers. Lipopolysaccharide exposure did not affect total blastocyst cell numbers at 0.01 ($P = 0.68$), 0.1 ($P = 0.97$), or 1 ($P = 0.61$) μg/mL LPS compared with vehicle-treated controls (n = 163; 5 independent replicates; Fig. 6). Likewise, trophectoderm cell counts ($P = 0.83$) and inner-cell mass cell counts ($P = 0.21$) did not differ among treatments (Fig. 4).

Embryo development was examined via traditional routine microscopy evaluation at days 3 and 8 of culture for cell cleavage and blastocyst development, respectively. Traditional routine microscopy evaluation at day 3 revealed no detectable treatment differences in the percent of embryos that cleaved among LPS treatment groups ($P = 0.97$; n = 300 embryos; 5 independent replicates; Fig. 5). Comparably, microscopic evaluation did not detect treatment differences in the percentage of day 8 LPS-matured embryos that progressed to expanded blastocyst stage ($P = 0.88$; Fig. 5). Evaluation of captured images collected via Miri time-lapse incubator at 72 h following IVF were analyzed as a potential predictive value for embryo viability. Developing embryos were evaluated and separated into those with 4 or fewer cells at 72 h of culture and those with greater than 4 cells at 72 h of culture (Fig. 6). Treatment differences were not observed among embryos produced from LPS-matured oocytes ($P = 0.69$; Fig. 6).

Additionally, embryo development was assessed in real-time over the duration of an 8-d period utilizing sensitive and progressive time-lapse microscopy. Time-lapse video capture allowed for evaluation of individual embryos to determine cleavage rate, stage of arrest, or development to blastocyst stage (Supplementary Video 1 and 2) n = 153; 3 independent replicates). Initially, individual embryos were analyzed solely based on arrest before blastocyst development or successful blastocyst development. Embryos derived from LPS-challenged oocytes with 0.01 ($P = 0.06$), 0.1 ($P = 0.03$), or 1 ($P = 0.03$) μg/mL LPS demonstrated increase in percent of embryos that arrested prior to blastocyst stage when compared with vehicle-treated controls (Fig. 7). Likewise, blastocyst development decreased with LPS challenge during oocyte maturation in those embryos derived from 0.01 ($P = 0.09$), 0.1 ($P = 0.03$), or 1 ($P = 0.03$) μg/mL LPS oocytes (Fig. 7). Embryos were subsequently evaluated to identify distinct stages of developmental arrest. No treatment differences were observed among LPS groups for presumptive zygotes that failed to undergo a cleavage event ($P = 0.84$; Fig. 8). Additionally, oocytes that cleaved but subsequently arrested at the 2-cell stage ($P = 0.50$), 4-cell stage ($P = 0.76$), or those that arrested prior to morula but past 4-cell stage ($P = 0.76$) (n = 153; 3 independent replicates; Fig. 8). Embryos arrested at morula stage were limited to 0, 0.01, and 0.1 μg/mL LPS treatment groups (Fig. 8). Embryos derived from oocytes challenged with 0.1 μg/mL LPS tended to have reduced development to the morula stage compared with vehicle-treated controls ($P = 0.06$; Fig. 8). Additionally, the percentage of blastocysts from oocytes matured in 0.01 μg/mL LPS tended to decrease compared with vehicle-treated controls (11.38 ± 8.74 and 25.45 ± 2.73 %, respectively; $P = 0.09$; Fig. 8). Moreover, the percentage of embryos that develop into blastocysts from oocytes matured in 0.1 and 1 μg/mL LPS decreased compared with vehicle-treated controls (5.92 ± 3.62, 6.55 ± 3.62, and 25.45 ± 2 .73 %, respectively; $P = 0.03$; Fig. 8).

4. Discussion

Cattle operations rely on herd reproductive performance; however, the presence of disease can negatively modulate reproductive parameters and attribute to culling animals from the herd [10]. Infertility in cattle is exasperated by bacterial infections such as clinical mastitis and metritis, prevalent in post-partum cows [11]. Meanwhile, the effects of subclinical or non-detectable bacterial infections on oocyte maturation and development are less clearly elucidated. The Gram-negative bacteria, *Escherichia coli* is a prevalent pathogen in livestock that releases LPS endotoxin in the host's circulation, capable of concentrating in the follicular fluid of developing follicles [8, 12].

The present study investigated the effects of varying low-dose LPS concentrations on oocyte nuclear maturation and subsequent early embryonic development. Experimental conditions for oocyte maturation were designed to mimic subclinical or non-detectable disease states. Exposure of bovine oocytes to low-LPS (0.01 to 1 μg/mL LPS) doses during IVM did not affect the meiotic competence of the oocytes as indicated by comparable meiotic progression among oocytes matured in increasing LPS doses. Similarly, embryos produced from LPS-matured oocytes appeared to have similar cleavage rates at day 3 and blastocyst rates at day 8 when evaluated using traditional methods. This is in contrast to studies indicating oocytes derived from slaughterhouse ovaries tend to have a greater percentage of meiotic failure if sourced from follicles containing higher natural LPS accumulation compared to low LPS containing follicles [10], and reports demonstrating that oocytes challenged with 0.01 and 0.1 μg/mL LPS experience compromised meiotic progression in vitro [14]. However, the results presented are consistent with findings indicating that in vitro bovine oocyte maturation in the presence of LPS as high as 1 and 5 ug/mL did not impact cleavage rates or blastocyst rates [15]. However, meiotic progression, necessary for events leading to ovulation, fertilization, and early zygote cleavage, was compromised when oocytes were matured for 24-h in the presence of 10 ug/mL LPS [9].

It is possible that in vivo accumulation of LPS in the follicular fluid in response to pathogenic stress may result in greater consequences on follicular and oocyte development compared to in vitro LPS exposure. Numerous experimental factors may also contribute to discrepancies across studies related to IVM such as oocyte quality prior to treatment, oocyte selection criteria, maturation and fertilization protocol, time and route of exposure to endotoxin, and LPS batch potency can contribute to variance. Factors such as transportation, time, temperature from slaughterhouse to laboratory, media composition, and hormone supplementation can interfere with IVM of bovine oocytes [16]. Previous reports indicate that supplementation of exogenous FSH in oocyte maturation media does not rescue the percentage of oocytes that experience meiotic progression failure [9]. In the present study, slaughterhouse to laboratory transport time estimated 3 h with an additional 3 h from arrival to IVM. All media used for IVM and in vitro production (IVP) of embryos were purchased from IVF Bioscience with a proprietary ingredient list, and thus, results cannot fully account for the media composition.

Following fertilization, blastomere counts at 72 h can be used as a predictive measure of embryo developmental success [17]. Embryos at a 4-cell stage at 48 h and 8-cell at 72

h are more likely to develop to blastocyst stage, while those with fewer than 5 cells at 72 h of culture will rarely develop into blastocysts [17]. Retrospective analysis of embryo development to evaluate blastomere counts at 72 h of culture did not reveal treatment differences in the number of embryos containing more than 4 cells or those with 4 or fewer cells at 72 h of culture. However, time-lapse embryo evaluation indicated that although nuclear maturation of oocytes was not affected by exposure to low concentrations of LPS, oocytes matured in the presence of LPS were developmentally affected as demonstrated by dose-dependent decrease in percentage of embryos developing to the blastocyst stage. The ability to detect the distinct point of embryonic development failure was only made possible through the evaluation of time-lapse embryo development analysis. Compiled images continuously captured throughout culture period allow for the detection of specific developmental events such as cell division, cleavage stages of embryonic development, stunted growth, or arrest. Evaluation of embryo development using routine microscopic evaluation was unable to tease apart the late embryonic failure in LPS-treated groups. This is likely due to the limitation of this technique which requires embryos to be removed from culture conditions and assessed for cleavage and blastocyst rates at a single time point.

Bovine embryos enter the blastocyst stage at day 7 of gestation, at this stage the embryo is approximately 160 μm in diameter, containing 120 to 160 cells [18, 19]. As they continue to grow, by day 8 to 10 embryos increase in size to roughly 280 μm and contain approximately 200 cells [18]. The adverse effects of LPS have been observed at the blastocyst stage where inner cell mass and trophectoderm cell ratios are negatively impacted in bovine embryos from LPS-matured oocytes in 10 μg/mL LPS, however no ratio effects are detected at lower LPS doses [14]. This is consistent with the present study where results demonstrate that low LPS concentrations do not impair total cell counts as indicated by trophectoderm and inner-cell mass cell numbers.

Time of LPS exposure during in vitro maturation is a tightly controlled process, meanwhile, follicular LPS accumulation is a gradual event affecting oocyte and follicular development temporally, leading to disruption of the estrous cycle [20]. Systemic LPS exposure in rats has been demonstrated to significantly decrease follicular development by increasing percent of atretic antral follicles compared to non-exposed animals [20]. Similarly, rat ovaries exposed to LPS demonstrated decreased ovulation rates marked by the absence of CL compared to non-exposed ovaries [21]. In cattle, LPS decreases primordial follicle count in vitro and induces immune responses within the follicle [9, 22]. Immune activation in research models is often performed using a single high-dose LPS bolus. This is contrary to naturally occurring bacterial endotoxin exposure where effects rely on continuous growth and proliferation of pathogens with subsequent death or lysis resulting in gradual LPS release in the host [23]. Consequently, continuous endotoxin exposure mimicking that of naturally occurring subclinical disease can interfere with the hypothalamic-pituitary-gonadal (HPG) axis, disrupting follicular growth, oocyte development, and subsequent ovulation.

5. Conclusions

In conclusion, although nuclear maturation of oocytes was not affected by low concentrations of LPS, oocytes matured in the presence of the endotoxin that subsequently

undergo in vitro fertilization experience decreased blastocyst percentage. Importantly, these findings are only evident when evaluated retrospectively with the use of time-lapse evaluation compared to routine microscopy. Trophectoderm and inner cell mass cell counts were not affected in embryos developed from LPS-matured oocytes. Nevertheless, oocyte development is impacted by the microenvironment of the follicle, and cumulus-oocyte complexes respond to inflammatory cues as the granulosa cells within the follicle express the receptor complex required to initiate immune responses. Disruption of the intrafollicular environment surrounding the oocyte can have detrimental consequences on ovarian function and these data suggest that even low LPS concentrations that may mimic a subclinical or non-detectable disease state in cattle negatively impact development in the bovine preimplantation embryo. Thus, bacterial infections that may go undetected have the capability to decrease pregnancy success in cattle.

Future work utilizing time-lapse embryo evaluation along with in vivo studies could provide insightful information regarding embryo cell dynamics in response to varying degrees of infection. In the present study, time-lapse evaluation allowed for the identification of distinct stages of embryonic arrest upon oocyte exposure to LPS endotoxin. Continuous and non-invasive time-lapse monitoring demonstrated deleterious effects on embryo development even at low LPS concentrations. Detailed exploration of the LPS-dependent cytotoxic impacts on physiological processes in IVM-derived oocytes and on the developmental competence of resulting IVF-derived embryos could lend insight into biological causes of diminished embryo quality not only in cattle but in a number of mammalian species. This, in turn, might result in biotechnological improvements in the efficiency of strategies including in vitro fertilization (IVF) by either gamete co-incubation [24–26] or intracytoplasmic sperm injection (ICSI) [27–29] and somatic cell nuclear transfer (SCNT)-based cloning [30–33]. Collectively, investigation regarding mechanistic pathways and cellular dynamics involved in decreased oocyte and embryo competence provides a greater understanding of the decreased female fertility in the presence of bacterial disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Oocyte exposure to low-dose endotoxin impacts early embryo development.
- Low-dose LPS did not compromise oocyte nuclear development.
- Subtle differences in embryo development are revealed by time-lapse capture analysis.
- Low-dose LPS-matured oocytes demonstrated decreased development to the blastocyst stage.

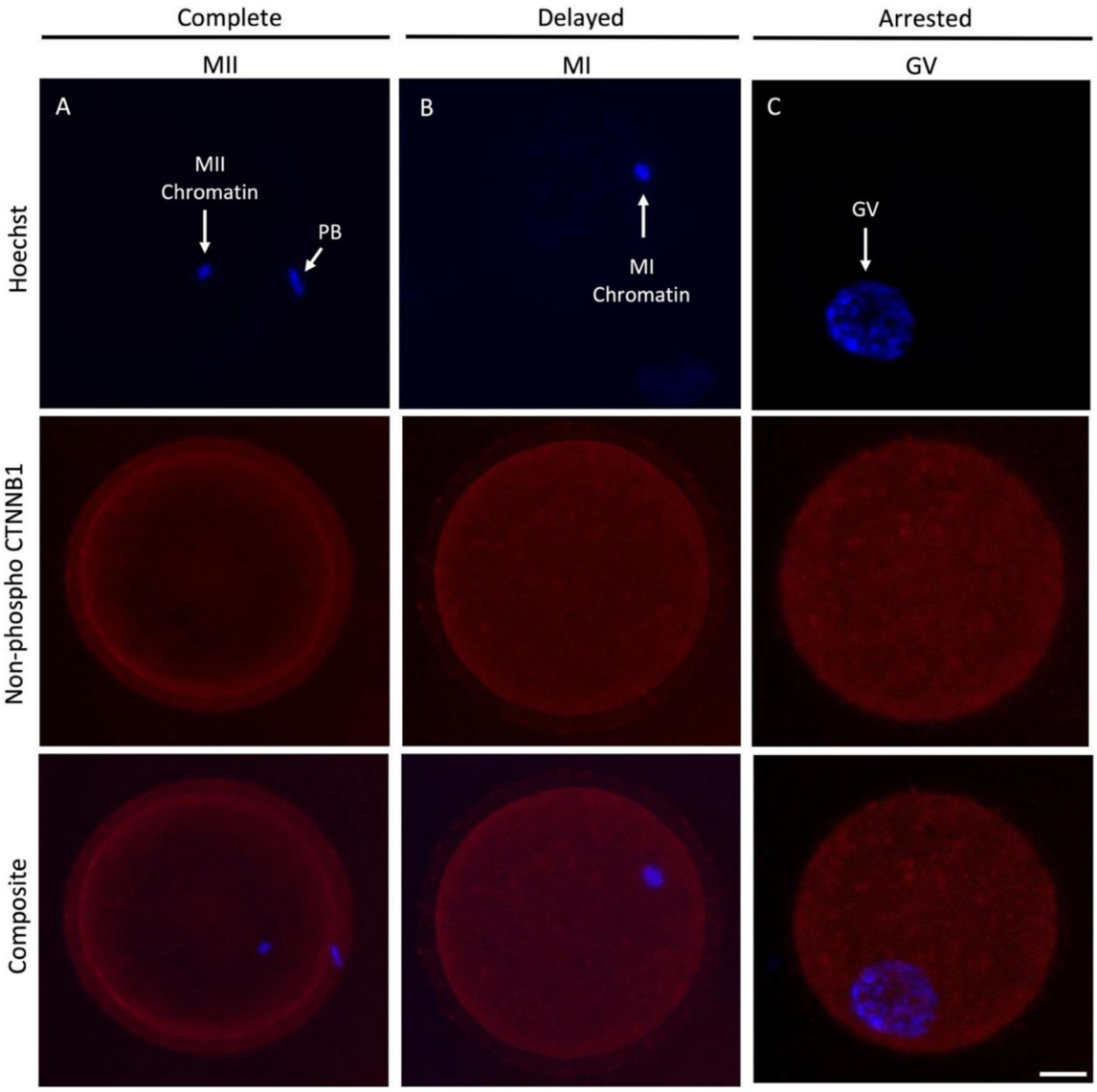

Figure 1.

Representative examples of bovine oocyte meiotic progression following 21 h in vitro maturation. Oocytes were categorized as having (A) complete nuclear maturation arrested at metaphase II (MII) with clear polar body (PB) extrusion, (B) delayed nuclear maturation at meiosis I (MI) with lack of polar body, (C) oocyte arrested at germinal vesicle (GV) stage with evident absence of chromatin condensation. Oocytes were probed for DNA visualization with Hoechst 33342 staining (blue) and immunoreactive non-phosphorylated (active) beta-catenin (CTNNB1) antibody with Texas Red (red) for oocyte boundaries. Bovine oocytes were collected and cultured in maturation media with or without increasing lipopolysaccharide (LPS) concentrations for 21 h, denuded, and fixed. A total of 252 oocytes were evaluated in 3 independent replicates. Scale bar = 20 μm.

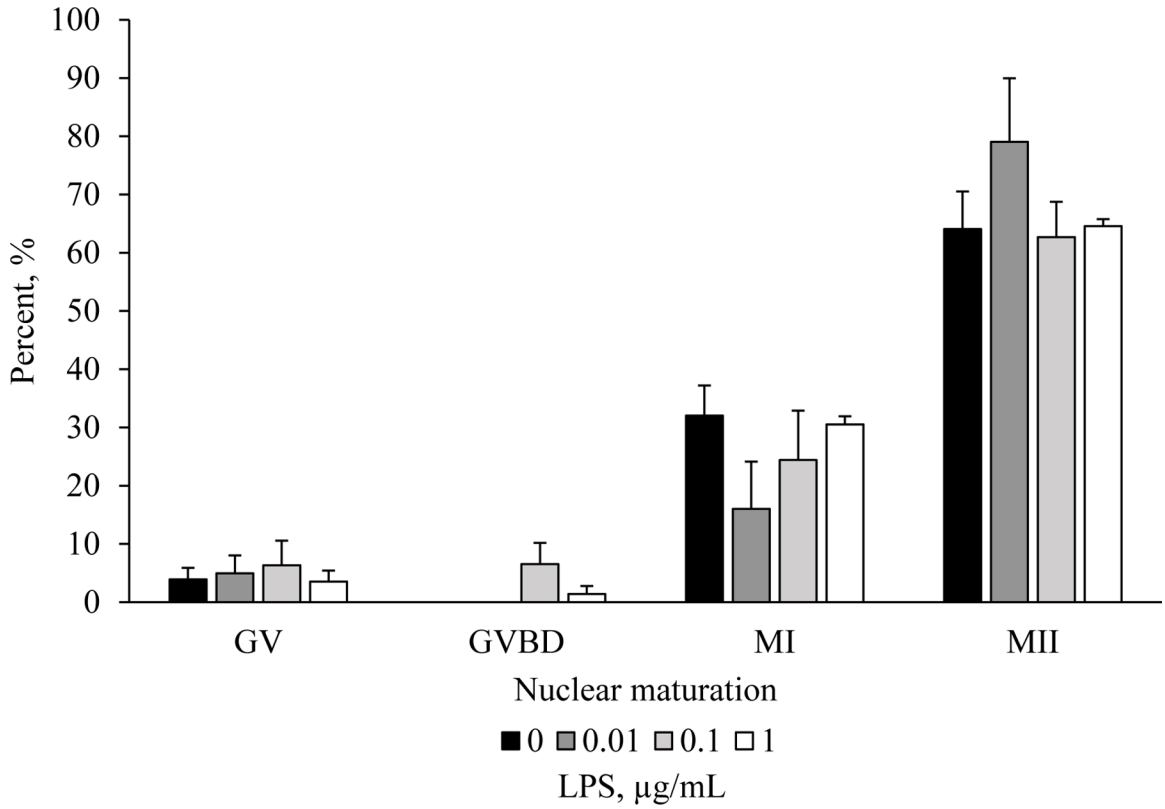

Figure 2.

Bovine oocyte meiotic progression following 21 h in vitro maturation. Nuclear maturation of oocytes matured in 0 (vehicle-treated control), 0.1, 0.1, or 1 µg/mL lipopolysaccharide (LPS) for 21 h. No detectable differences were identified in the percentage of oocytes in germinal vesicle (GV) ($P = 0.90$), germinal vesicle breakdown (GVBD) ($P = 0.13$), meiosis I (MI) ($P = 0.26$), or meiosis II (MII) ($P = 0.44$; n = 252; 3 independent replicates). Statistical analysis was performed using the GLM procedure of SAS. All values are shown as means ± SEM.

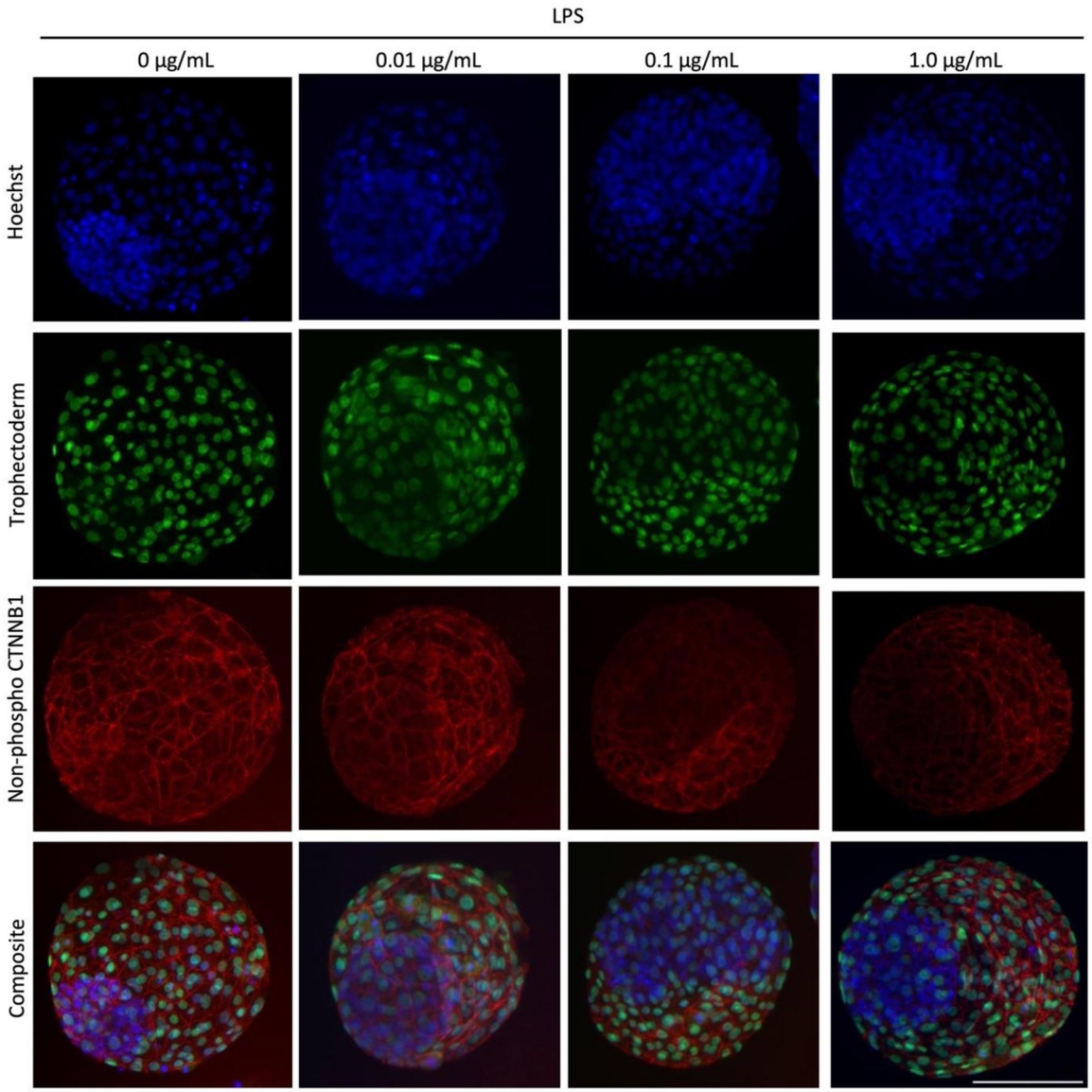

Figure 3.

Representative images of preimplantation development of day 8 bovine blastocysts from oocytes matured in 0, 0.01, 0.1, or 1 µg/mL lipopolysaccharide (LPS). Embryos were assessed for morphology and individual cell counts. Total cells were assessed by staining DNA with Hoechst 33342 (blue). Trophectoderm cells were assessed using CDX2 antibody and FITC (green). Active non-phosphorylated (active) beta-catenin (CTNNB1) antibody with Texas Red (red) was used to evaluate cell boundaries. No differences were observed in gross embryonic morphology among embryos produced from LPS-challenged oocytes with 0, 0.01, 0.1, or 1 µg/mL LPS. A total of 1,126 oocytes were used to produce embryos in 5 independent replicates. Scale bar = 80 µm.

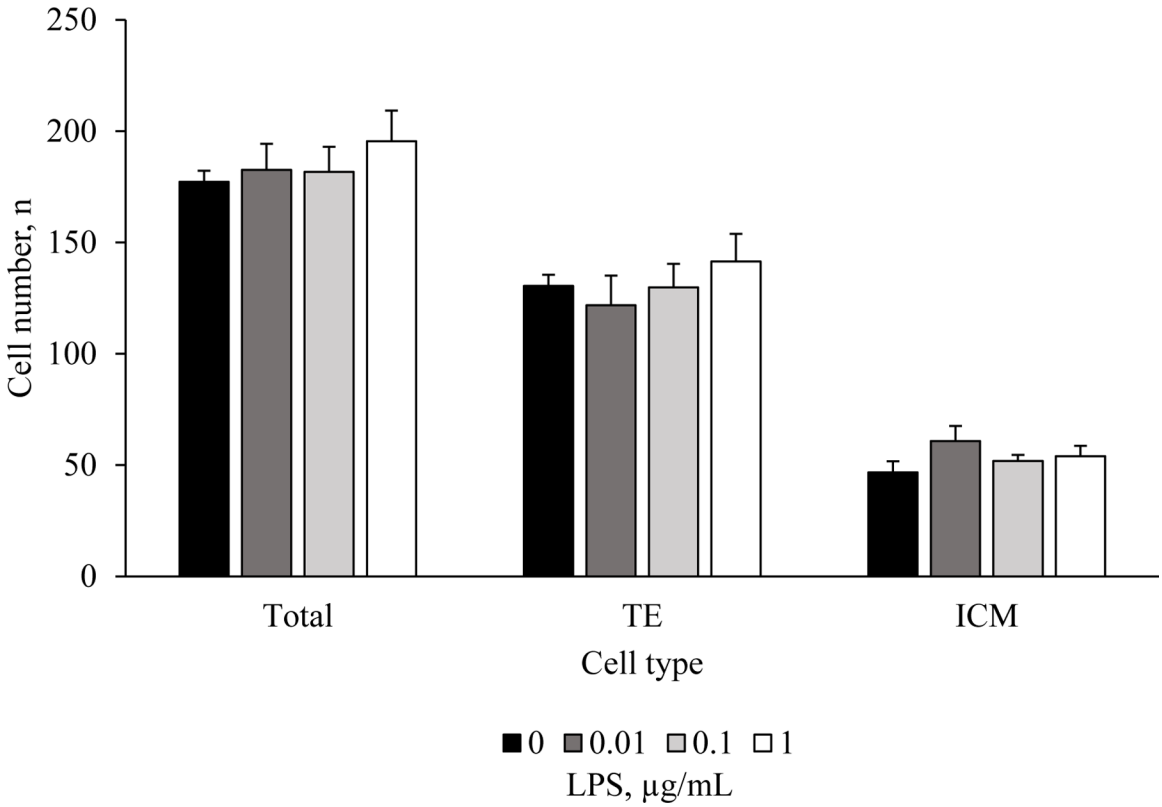

Figure 4.

Embryos derived from lipopolysaccharide (LPS)-matured oocytes did not differ in cell numbers at the blastocyst stage. Blastocyst cell counts evaluated included total cell number, trophectoderm (TE) cell number, and inner cell mass (ICM) cell number (n = 163; 5 independent replicates). Inner cell mass cell numbers were determined by subtracting trophectoderm cell counts from total cell numbers. Cell numbers for blastocysts derived from oocytes matured in 0, 0.01, 0.1, or 1 µg/mL LPS were evaluated using the cell count function of Fiji software. No significant treatment differences were observed in total (P = 0.86), TE (P = 0.83), or ICM (P = 0.21) among 0, 0.01, 0.1, or 1 µg/mL LPS treatment groups. Statistical analysis was performed using the GLM procedure of SAS. All values are shown as means ± SEM.

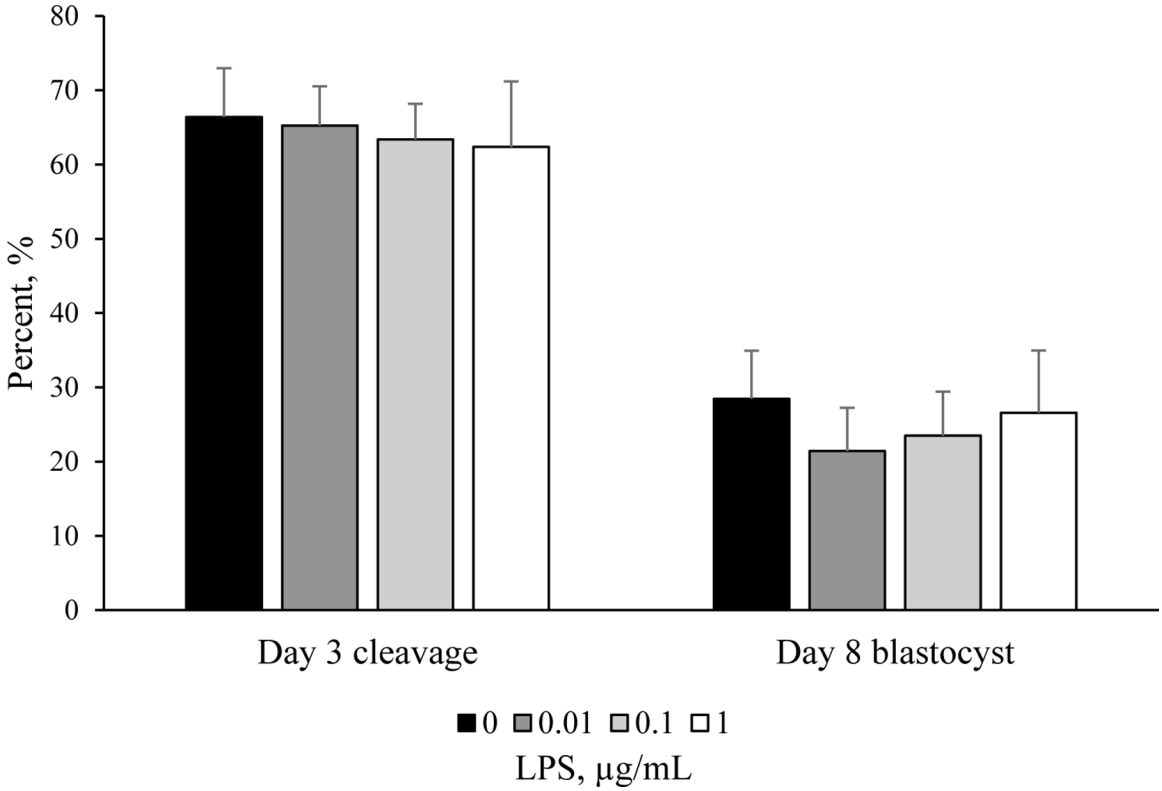

Figure 5.

Cleavage and blastocyst rates of bovine embryos from oocytes matured in 0, 0.01, 0.1, or 1 µg/mL lipopolysaccharide (LPS). Cleavage rates were measured on day 3 of in vitro culture and blastocyst rates were measured on day 8 of culture using traditional routine microscopy. Treatment differences were not detected for cleavage ($P = 0.97$) or blastocyst ($P = 0.88$) rates among 0, 0.01, 0.1, or 1 µg/mL LPS treatment groups (n = 300 embryos; 5 independent replicates). Statistical analysis was performed using the GLM procedure of SAS. All values are shown as means ± SEM.

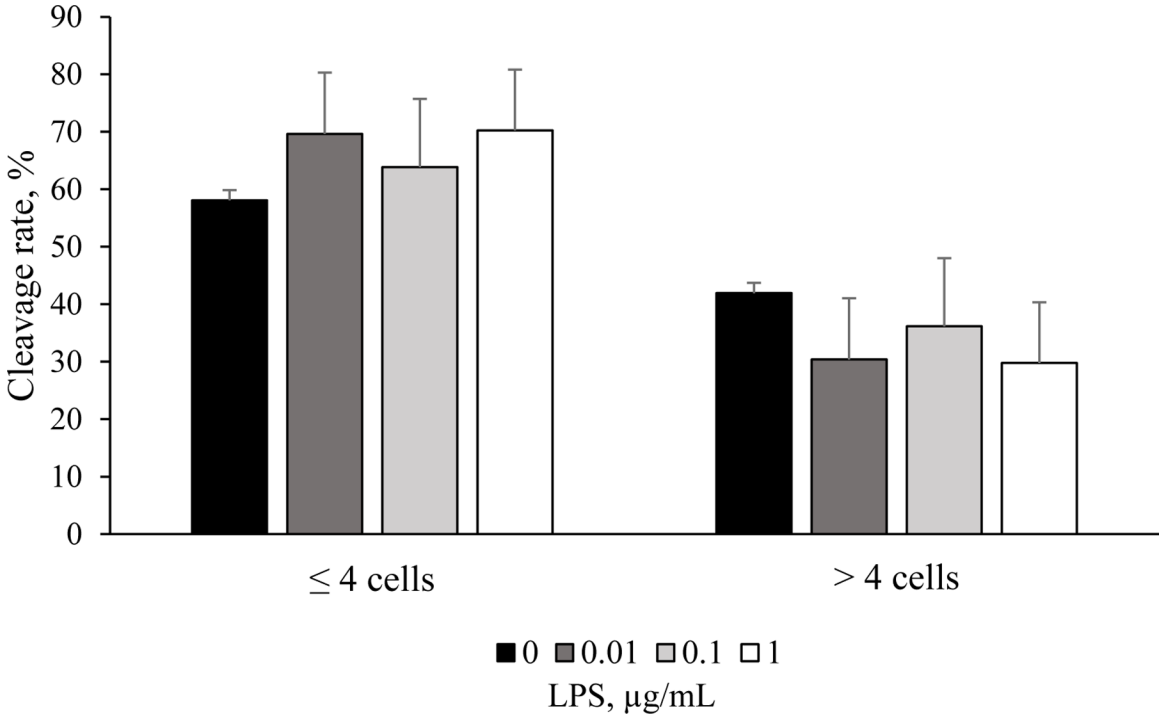

Figure 6.

Retrospective time-lapse evaluation of cleavage rate of bovine embryos at 72 h of culture was used as an estimator of embryonic success. Time-lapse embryos were retrospectively evaluated at 72 h of culture for cell number as a predictive measure for embryo viability (n = 153; 3 independent replicates). Embryos with greater than 4 cells at 72 h are more likely to continue development to the blastocyst stage, while those embryos containing 4 or fewer cells at 72 h of culture are less likely to develop into blastocysts. No detectable treatment differences were observed in embryos with 4 or fewer cells at 72 h among 0, 0.01, 0.1, or 1 µg/mL lipopolysaccharide (LPS) treatment groups ($P = 0.69$). Similarly, treatment differences were not observed in embryos with greater than 4 cells at 72 h among 0, 0.01, 0.1, or 1 µg/mL LPS treatment groups ($P = 0.69$). Statistical analysis was performed using the GLM procedure of SAS. All values are shown as means ± SEM.

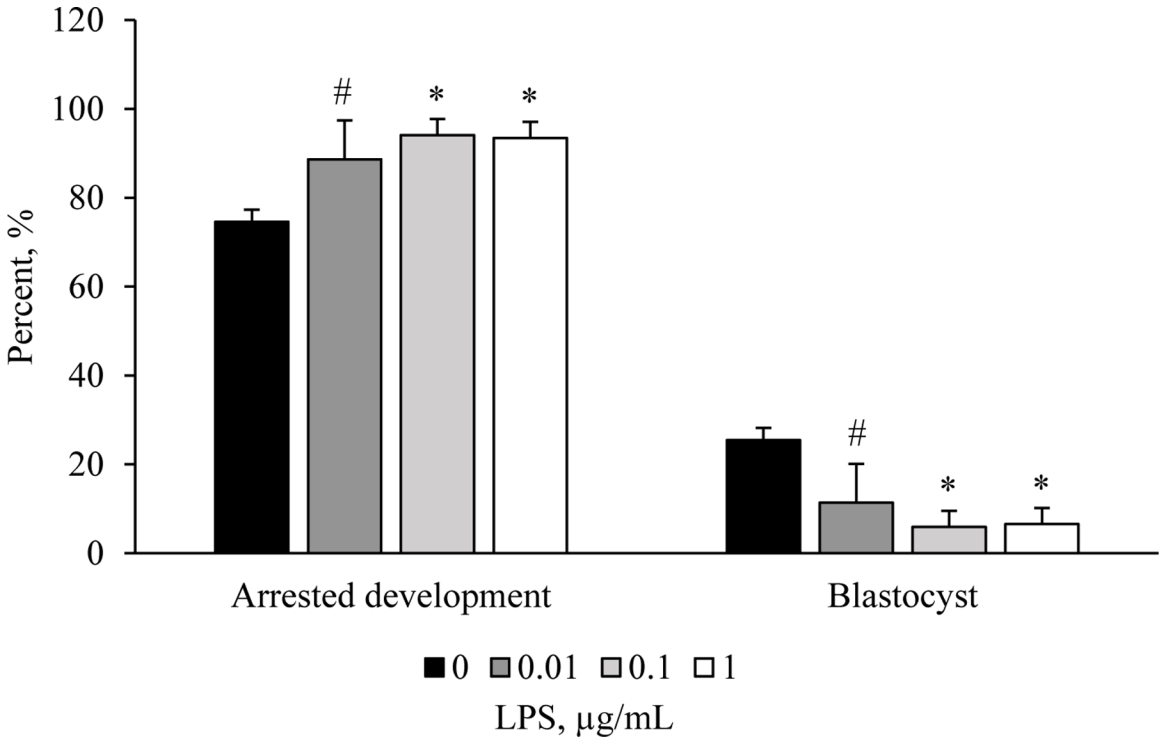

Figure 7.

Time-lapse evaluation of bovine embryo development from oocytes matured in 0, 0.01, 0.1, or 1 µg/mL lipopolysaccharide (LPS). Embryonic development was evaluated in relation to developmental arrest or successful development to blastocyst stage following 8 d culture (n = 153; 3 independent replicates). Embryos derived from LPS-challenged oocytes with 0.01 µg/mL LPS tended to experience increased arrest prior to the blastocyst stage compared to vehicle-treated controls ($^{\#}P = 0.06$). Embryos derived from 0.1 ($^{*}P = 0.03$) and 1 ($^{*}P = 0.03$) µg/mL LPS-challenged oocytes experienced increased arrest in development compared with vehicle-treated controls. Similarly, blastocyst development tended to decrease in embryos from oocytes matured in 0.01 µg/mL LPS ($^{\#}P = 0.09$) and significantly decreased in those from 0.1 ($^{*}P = 0.03$) and 1 ($^{*}P = 0.03$) µg/mL LPS compared with vehicle-treated controls. Statistical analysis was performed using the GLM procedure of SAS. All values are shown as means ± SEM.

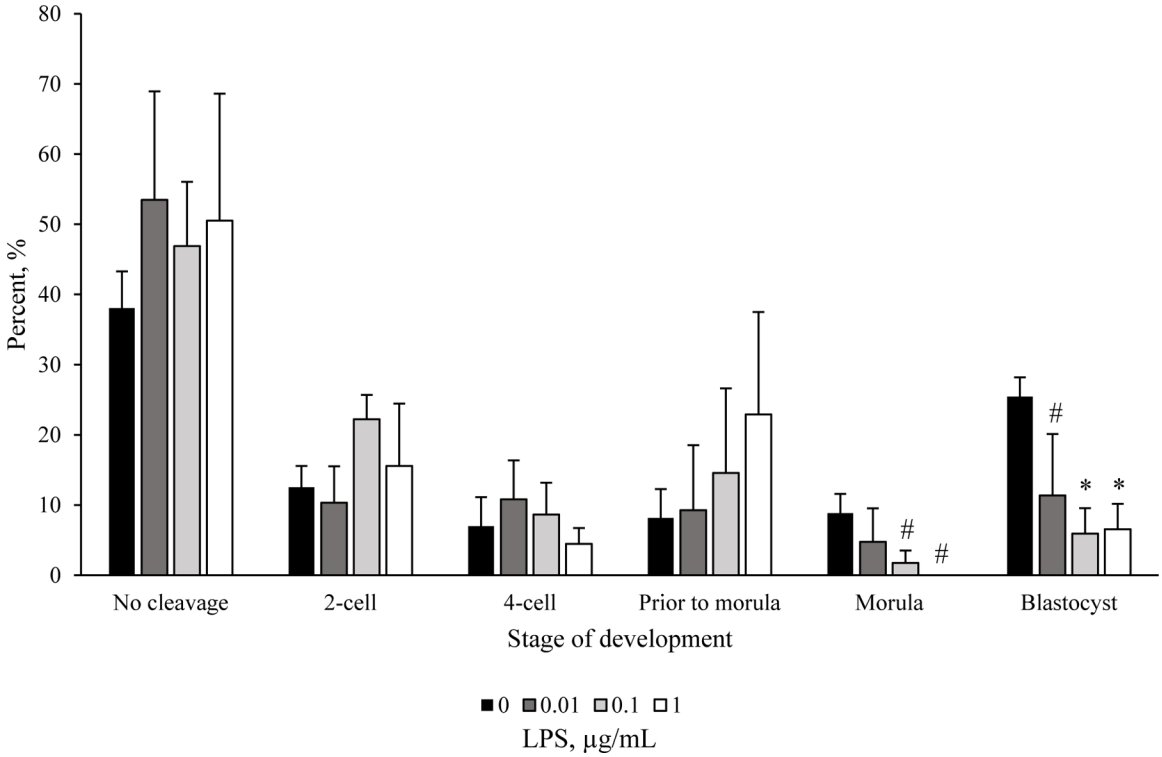

Figure 8.

Developmental stages of bovine embryos following 8-d culture evaluated with time-lapse microscopy. Percentage of bovine embryos that did not cleave (no cleavage), cleaved but arrested at 2-cell, 4-cell, prior to morula, or at morula stage, and percentage of embryos that successfully developed into blastocysts from oocytes matured in 0, 0.01, 0.1, and 1 µg/mL lipopolysaccharide (LPS). Embryos derived from oocytes challenged with 0.1 µg/mL LPS tended to have reduced development to the morula stage compared with vehicle-treated controls ($^{\#}P = 0.06$) with 0% of embryos from oocytes matured in 1 µg/mL LPS arresting at morula ($^{\#}P = 0.06$). A tendency for decreased blastocyst percentage was observed in embryos from 0.01 µg/mL LPS treatment group compared with vehicle-treated controls ($^{\#}P = 0.09$). Additionally, blastocyst percentage from embryos derived from oocytes matured in 0.1 and 1 µg/mL LPS significantly decreased compared with vehicle-treated controls ($^{*}P \leq 0.05$, respectively) (n = 153; 3 independent replicates). Statistical analysis was performed using the GLM procedure of SAS. All values are shown as means ± SEM.