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Effect of lipopolysaccharide on developmental competence of oocytes

Fumie Magata, Takashi Shimizu*

Graduate School of Animal and Food Hygiene, Obihiro University of Agriculture and Veterinary Medicine, 0808555 Obihiro, Japan

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ABSTRACT

In postpartum dairy cows, lipopolysaccharide (LPS) derived from gram-negative bacteria such as *Escherichia coli* causes uterine inflammation resulting in low fertility. The aim of this study was to determine the effect of LPS on the developmental competence of bovine oocytes in vitro. LPS perturbed the nuclear maturation of bovine oocytes by inhibiting meiotic progression. Although LPS did not affect the copy number of mitochondrial DNA, it decreased mitochondrial membrane potential in matured oocytes. LPS inhibited mitochondrial redistribution throughout the cytoplasm. Oocytes matured under LPS treatment showed decreased development to the blastocyst stage. Moreover, the trophoblast cell number of blastocysts was significantly lower when the oocytes were matured in the presence of LPS. Our findings suggest that LPS might impair the nuclear and cytoplasmic maturation of oocytes and obstruct subsequent embryonic development in dairy cows.

1. Introduction

Uterine bacterial infections commonly occur in postpartum dairy cows and cause infertility by disrupting uterine and ovarian functions [1]. Cows with metritis display slower growth of the first postpartum dominant follicle and lower peripheral plasma estradiol (E2) levels, and in ovulating animals, the peripheral plasma progesterone (P4) concentrations are lower [1].

Escherichia coli is one of the main bacteria of endometritis, and much of the tissue pathology is associated with the bacterial endotoxin, lipopolysaccharide (LPS) [1]. LPS has been detected in the follicular fluid of cows with endometritis [2] and metritis [3], suggesting a relationship between uterine infection, LPS production and follicular function. We have previously reported that the concentration of E2 was reduced in follicles with high levels of LPS compared to those with a low level of LPS, with drastic changes in the transcription levels of steroidogenesis-related genes [4]. Moreover, E2 production by granulosa cells and P4 production by theca cells was suppressed when these follicular cells were cultured with LPS [5,6]. These results indicate that the accumulation of LPS in follicular fluid perturbs steroid production in follicles, which might be one of the mechanisms of infertility in cows with uterine inflammation. However, the effects of LPS on follicle-enclosed oocytes or cumulus cells are not completely understood.

For successful fertilization and further embryonic development, oocytes must undergo nuclear maturation, progressing from the germinal vesicle (GV) stage until pausing at metaphase-II (MII). It has been shown that LPS increased the rates of meiotic arrest and germinal vesicle breakdown (GVBD) failure of bovine oocytes in vitro [7]. Moreover, addition of LPS to oocyte maturation medium reduced the proportion of bovine oocytes that became blastocysts after in vitro fertilization [8]. However, the mechanism by which LPS perturbs oocyte maturation is still not clear. In addition to nuclear maturation, cytoplasmic maturation is also critical for the developmental competence of oocytes. Mitochondrial changes during oocyte maturation including a drastic increase in mitochondrial number and their redistribution throughout the cytoplasm are essential processes for oocyte activation through fertilization and further embryonic development [9–11]. Here, we hypothesized that LPS might perturb both the nucleic and cytoplasmic maturation of oocytes, resulting in deleterious effect on developmental competence. To evaluate our hypothesis, we examined the effect of LPS on meiotic progression and mitochondrial status, as well as on the developmental competence of bovine oocytes in vitro.

Abbreviations: LPS, lipopolysaccharide; E2, estradiol; P4, progesterone; GV, germinal vesicle; GVBD, germinal vesicle breakdown; TLR4, Toll-like receptor 4; TLR2, Toll-like receptor 2; CD14, cluster of differentiation 14; MD2, Myeloid differentiation factor-2; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; MtDNA, mitochondria DNA; TE, trophectoderm cells; ICM, inner cell mass; COCs, cumulus-oocyte complexes.

* Corresponding author at: Graduate School of Animal and Food Hygiene, Obihiro University of Agriculture and Veterinary Medicine Inada-machi, Obihiro, Hokkaido 080-8555, Japan. Tel.: +81 155495419; fax: +81 155 49 5419.
E-mail address: shimizut@obihiro.ac.jp (T. Shimizu).

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2. Materials and Methods

2.1. Materials

All materials, unless otherwise stated, were purchased from Sigma-Aldrich Japan Inc. (Tokyo, Japan). L (+) Glutamine was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Bovine follicle-stimulating hormone (bovine FSH, biopotency 604 ng/IU) was obtained from the National Institute of Diabetes and Digestive and Kidney Diseases (Bethesda, MD, USA). IVF 100 and IVD 101 media were obtained from the Research Institute for Functional Peptides (Higashine, Japan). TRIzol reagent was obtained from Life Technologies Inc. (Carlsbad, CA, USA). PrimeScript RT Reagent Kit with gDNA Eraser and TaKaRa Taq kit were obtained from Takara Bio Inc. (Shiga, Japan). QIAamp DNA Micro Kit and QuantiTect SYBR Green PCR were obtained from QIAGEN GmbH (Hilden, Germany). Mito Tracker Orange CM-H2 TMRos was obtained from Invitrogen, Life Technologies Inc. (DriveRockville, MD, USA). JC-1 dye was obtained from ImmunoChemistry Technologies, LLC (Bloomington, MN, USA).

2.2. In Vitro Production of Embryos

Ovaries of multiparous Holstein cows were obtained from a local slaughterhouse and transported to the laboratory in phosphate buffered saline (PBS) containing 0.03 mg/ml streptomycin and 0.75 μg/ml amphotericin B at 20 °C. Cumulus-oocyte complexes (COCs) were aspirated from 2–8 mm follicles using a syringe with an 18G needle. COCs with at least three layers of cumulus surrounding a homogeneous cytoplasm were transferred to Medium 199 (Earl salts with 25 mmol/l HEPES and sodium bicarbonate) with 100 μg/ml kanamycin, 100 μg/ml L (+) Glutamine, 1 μg/ml estradiol (β-estradiol), and 0.02 IU/ml FSH (10 COCs/100 μl). The COCs were cultured for 23 h in humidified air with 5% CO_2 at 38.5 °C. After 23 h of culture, the matured oocytes were transferred to IVF 100 medium and fertilized with frozen-thawed bovine spermatozoa from the same bull (JP3H54654). Thirty oocytes were incubated with 5.0×10^6/ml spermatozoa in 100 μl droplets of fertilization medium overlaid with mineral oil for 8 h in humidified air with 5% CO_2 at 38.5 °C. After 8 h of fertilization, any remaining cumulus cells were removed from the presumptive zygotes by vortexing for 3 min in IVF 100 medium and followed by washing thrice prior to culture. Groups of 30 presumptive zygotes were transferred to 300 μl droplets of IVD 101 medium overlaid with mineral oil and were cultured for 9 days in humidified air with 5% CO_2, 5% O_2, and 90% N_2 at 38.5 °C. Cleavage was evaluated at 2 days after fertilization, and the blastocyst rate was calculated as the proportion of cleaved embryos that developed to the blastocyst stage at day 8 post-fertilization.

2.3. RNA Extraction, Reverse Transcription (RT) and Polymerase Chain Reaction (PCR)

To examine the mRNA expression of LPS receptors in bovine oocytes and cumulus cells, fresh and immature oocytes were denuded of cumulus cells by vortexing in PBS. The cumulus cell suspensions were centrifuged at 100 × *g* for 10 min at 4 °C and the supernatant was discarded. Total RNA was extracted from denuded oocytes and cumulus cells using TRIzol reagent according to the manufacturer's instructions and was stored at −80 °C until used. Before RT, the samples were treated with DNase, and single-strand complementary DNA was then reverse transcribed from total RNA using a commercial kit (PrimeScript RT Reagent Kit with gDNA Eraser). The RT conditions were as follows: 15 min of complementary DNA synthesis at 37 °C and 5 s of inactivation at 85 °C. The mRNA expression of *TLR2*, *TLR4*, *CD14*, *MD2* and *GAPDH* was analyzed by PCR on a Master cycler (Eppendorf, Hamburg, Germany) using a TaKaRa Taq kit. The primers for PCR were designed from bovine sequences using Primer-3 software (Table 1). The amplification program included 5 min of activation at 95 °C followed by 50 cycles of PCR (95 °C for 1 min, annealing temperature for 1 min, and 72 °C for 1 min), followed by a final extension of 5 min at 72 °C. The PCR products were electrophoresed on 2% agarose gels containing ethidium bromide, and visualized under UV light.

2.4. Assessment of Oocyte Nuclear Maturation

To determine the nuclear maturation of oocytes, meiotic progression was evaluated based on the presence of the first polar body and nuclear morphology. After 23 h of in vitro maturation, the oocytes were denuded of cumulus cells and fixed for 24 h in ethanol: acetic acid (3:1 v/v), and then stained with 1% acetic orcein. The nuclear stages were distinguished by the morphology of the chromatin material as described previously [12]. Oocytes with a second metaphase plate and the first polar body were classified as matured at the metaphase of the second meiotic cell division (MII).

2.5. Assessment of Mitochondrial DNA (MtDNA) Copy Number in Single Oocytes

Matured oocytes were collected at the end of 23 h of maturation and were denuded of cumulus cells by vortexing in PBS. Each oocyte was then treated with 5 mg/ml protease (protease type XIV from Streptomyces griseus) to remove the zona pellucida. The nude oocyte was washed thrice in PBS and total DNA was extracted using the QIAamp DNA Micro Kit according to the manufacturer's instructions. The relative *MTDNA* copy number of single oocytes was quantified by real-time PCR on an iQcycler (Bio-Rad Laboratories, Inc., Tokyo, Japan) using a commercial kit (QuantiTect SYBR Green PCR). The primers for real-time PCR were designed from bovine sequences using Primer-3 software (Table 1). The amplification program included 15 min of activation at 95 °C followed by 50 cycles of PCR (95 °C for 15 s, 55 °C for 30 s and 72 °C for 30 s). The starting quantity of *MTDNA* from each oocyte was determined using standard curves, and the relative gene expression levels were compared between LPS treatment groups.

2.6. Mitochondrial Staining

To determine the mitochondrial distribution pattern, oocytes were stained with MitoTracker (Mito Tracker Orange CM-H2 TMRos). After 23 h of culture, the COCs were stained with 300 nmol/l MtTracker for 30 min in humidified air with 5% CO_2 at 38.5 °C. The COCs were then denuded of cumulus cells by vortexing in PBS with 0.1% (w/v) bovine serum albumin (PBS-BSA), and the oocytes were fixed with 4% (v/v) paraformaldehyde for 15 min in humidified air with 5% CO_2 at 38.5 °C. After fixation, the oocytes were washed thrice in PBS-BSA, mounted on slides under cover slips without oocyte compression, and were examined immediately at room temperature by laser scanning confocal microscopy (Leica TCS SP5; Leica Microsystems GmbH, Wetzlar, Germany) using a 40× plan-apochromat objective. The 543-nm excitation band and 536–621 nm detector was used for mitochondrial visualization. The oocytes were classified into two categories according to the mitochondrial distribution pattern (peripheral or dispersed, Fig. 3A).

Mitochondrial membrane potential was determined by a mitochondrial permeability transition detection kit (JC-1 dye) according to the manufacturer's instructions. After 23 h of culture, the COCs were stained with JC-1 solution for 15 min in humidified air with 5% CO_2 at 38.5 °C. The COCs were then denuded of cumulus cells by vortexing in 1× assay buffer containing 0.1% (w/v) bovine serum