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Lipopolysaccharide in ovarian follicular fluid influences the steroid production in large follicles of dairy cows

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ABSTRACT

In postpartum dairy cows, various inflammatory diseases depress reproductive performance. Lipopolysaccharide (LPS) derived from infections of the uterus or mammary gland with Gram-negative bacteria was shown to suppress steroid production in the granulosa cells of follicles *in vitro*. The aim of the study was to investigate the relationship between LPS in ovarian follicular fluid and steroidogenesis by the theca and granulosa cells of the large follicles *in vivo*. Bovine ovaries were collected from a slaughterhouse, and the largest (F1) and the second largest (F2) follicles were used (>8 mm in diameter, $n = 38$). LPS concentration in the follicular fluid was measured using quantitative kinetic assay. Follicular steroidogenesis was evaluated by measuring the estradiol (E2) and progesterone (P4) concentration in follicular fluid and by analysing transcription levels of steroidogenesis-related genes in theca and granulosa cells. LPS concentration detected in follicular fluid ranged from 0.2 to 2.0 EU/mL. In follicles with a high level of LPS (>0.5 EU/mL, $n = 15$), the concentration of E2 was lower and that of P4 was higher when compared to those in follicles with a low level of LPS (<0.5 EU/mL, $n = 23$), which was observed both in F1 and F2 follicles. Furthermore, in follicles with a high level of LPS, transcripts of steroidogenic enzymes such as CYP17 and P450arom were lower. In those follicles, the expression of caspase-3 was high, suggesting an association with follicular atresia. These findings indicate that LPS present in follicular fluid may cause ovarian dysfunction by inhibiting follicular activity.

1. Introduction

In postpartum dairy cows, various inflammatory diseases depress reproductive performance and cause impaired fertility. Metritis and mastitis are considered to be the most common infectious diseases during postpartum period; within 3 weeks after calving, 40% of cows develop metritis, whereas mastitis is present approximately 20–50% of all dairy cows. Infectious diseases often perturb the normal ovarian cyclic activity, including abnormal folliculogenesis as well as development of ovarian cysts, prolonged anestrus, and prolonged luteal phase (Opsomer et al., 2000; Mateus et al., 2002). It has been shown that cows with metritis had slower growth of the first postpartum dominant follicle and lower peripheral plasma estradiol (E2), and in ovulating animals, peripheral plasma progesterone (P4) concentrations were lower (Williams et al., 2007).

Escherichia coli (*E. coli*) is one of the main types of bacteria causing metritis and mastitis, and much of the

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tissue pathology is associated with the bacterial endotoxin, lipopolysaccharide (LPS). LPS was detected in the plasma, uterine fluid (Mateus et al., 2003), and follicular fluid (Herath et al., 2007) of cows with metritis and in the plasma and milk of cows with *E. coli* mastitis (Hakogi et al., 1989). It is reported that LPS acts at the hypothalamus or pituitary to suppress gonadotropin release and perturb follicle growth and function in the ovary of sheep (Battaglia et al., 1999) and cattle (Suzuki et al., 2001). Moreover, LPS is assumed to have a direct effect on the ovary, including follicular components such as the theca and granulosa cells or oocyte. In bovine follicles, granulosa cells (Herath et al., 2007; Shimizu et al., 2012) and theca cells (unpublished data in our study) express Toll-like receptor 4 (TLR4) which recognizes LPS, indicating that follicular cells are capable of responding to LPS. *In vitro* studies have shown that LPS suppressed E2 production with downregulation of transcripts for P450 aromatase in granulosa cells from large and small follicles (Herath et al., 2007; Shimizu et al., 2012). However, it remains to be unknown whether LPS present in follicular fluid affects follicular function in an animal body. Therefore, the objective of the present study was to investigate the relationship between LPS in follicular fluid and steroidogenesis by theca and granulosa cells *in vivo*.

2. Materials and methods

2.1. Sample collection

Ovaries ($n = 26$) of multiparous cows were collected from a local slaughterhouse immediately after slaughter. Regardless of the number of follicles coexisting within a set of ovary (right and left) or the presence of corpus luteum, the largest (F1, 14.1 ± 0.6 mm in diameter) and the second largest (F2, 11.4 ± 0.7 in diameter) follicles were used for the experiment (all follicles were >8 mm in diameter, $n = 38$). Follicular fluid was aspirated by using a syringe with a 22-gauge needle and centrifuged at $1500 \times g$ for 1 min at 4 °C. The supernatant (follicular fluid) was stored in endotoxin-free glass tubes at −20 °C until hormone or LPS measurement. Follicles were opened by making a small incision on the surface, and theca cells were then obtained by manually peeling the basal lamina. Granulosa cells were removed from peeled theca cells by gentle scraping with a medicine spatula and added to the cell-rich precipitate obtained by follicular fluid centrifugation. Then, granulosa cells were centrifuged at $100 \times g$ for 10 min at 4 °C and the supernatant was removed. Theca and granulosa cells were stored at −80 °C until total RNA extraction.

2.2. LPS measurement in follicular fluid

Concentrations of LPS in the follicular fluid were measured using the QCL-1000 Chromogenic Limulus Amebocyte Lysate (LAL) Endpoint Assay Kit following the manufacturer's instructions (Lonza Walkersville, Inc., Walkersville, MD, USA). Samples were thawed, diluted in endotoxin-free distilled water, and tested for non-specific LAL inhibition by comparing samples spiked with a known concentration of LPS with unspiked samples. In samples with evidence of LAL inhibition, proteinase K digestion was carried out as described by Petsch et al. (1998). Briefly, proteinase K was added to samples to get a final concentration of 0.4 mg/mL, and these mixtures were incubated for 18 h at 37 °C. Samples were then mixed with the LAL substrate reagent and assayed in endotoxin-free glass tubes. A standard curve for the endotoxin assay was prepared using endotoxin-free water with concentrations of 0.1, 0.25, 0.5, and 1 endotoxin units (EU)/mL (1 EU = 0.1-ng LPS). Internal recovery as determined using positively spiked samples was >80% and the limit of detection was 0.1 EU/mL.

2.3. Hormone assays in follicular fluid

The concentrations of E2 and P4 in follicular fluid were measured by enzyme immunoassay (EIA). Standard curve ranges were 2–2000 pg/mL for E2 and 0.05–50 ng/mL for P4. Culture medium was diluted with assay buffer when E2 and P4 concentrations were too high for the respective standard curve ranges. The diluted culture medium was then used for hormone assay. Concentrations of E2 and P4 were obtained from the appropriate standard curve, and the final concentrations were multiplied by the dilution factor. The respective intra- and inter-assay coefficients of variation averaged 7.7% and 4.9% for E2, and 6.5% and 8.7% for P4.

2.4. RNA extraction, reverse transcription (RT), and quantitative polymerase chain reaction (PCR)

Total RNA was extracted from theca and granulosa cells using TRIZOL reagent (Life Technologies, Inc., Drive-Rockville, MD, USA) according to the manufacturer's instructions and frozen at −80 °C. Samples which render sufficient amount and quality of total RNA were used for subsequent RT reaction. Before RT reaction, samples were treated with DNase and single-strand cDNA was then reverse transcribed from total RNA using a commercial kit (PrimeScript™ RT Reagent Kit with gDNA Eraser; TAKARA BIO INC., Shiga, Japan). The RT conditions were as follows: 15 min of cDNA synthesis at 37 °C and 5 s of inactivation at 85 °C. The mRNA levels of luteinizing hormone (LH) receptor (LHr), follicle stimulating hormone (FSH) receptor (FSHr), steroidogenic acute regulatory protein (StAR), cytochrome P450 side-chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-hydroxylase/17,20-lyase (CYP17), P450 aromatase (P450arom), caspase-3, and GAPDH were quantified by real-time PCR using an iQcycler (Bio-Rad Laboratories, Inc., Tokyo, Japan) and a commercial kit (QuantiTect™ SYBR® Green PCR; QIAGEN GmbH, Hilden, Germany). Primers for real-time PCR were designed from bovine sequences by using the software Primer-3 (Table 1). The amplification program included 15 min of activation at 95 °C followed by 50 cycles of PCR (95 °C for 15 s, 58 °C for 30 s, and 72 °C for 30 s). Values were normalized using GAPDH as the internal standard.

2.5. Statistical analysis

All data are presented as mean ± SEM. Statistical analysis was performed using Stat View 5.0 (SAS Institute Inc.,