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Lipopolysaccharide-induced mechanisms of ovarian dysfunction in cows with uterine inflammatory diseases

Fumie MAGATA[1]

[1]Department of Veterinary Medical Science, The University of Tokyo, Tokyo 113-8657, Japan

Abstract. Uterine inflammatory diseases commonly occur in postpartum dairy cows, resulting in reduced reproductive performance due to aberrant uterine and ovarian activity. Infection of the uterus with gram-negative bacteria results in the detection of lipopolysaccharide (LPS) in the plasma and follicular fluid of cows along with uterine inflammation. LPS acts on follicular components such as theca cells, granulosa cells, and follicle-enclosed oocytes, leading to impaired follicular activity. Follicles with a high LPS environment exhibit reduced follicular steroidogenesis due to the inhibition of steroidogenic enzyme transcription. Primary cell cultures of bovine granulosa and theca cells have shown that LPS acts on follicular cells to impair steroid production, which may disturb follicle growth and/or reduce their ability to ovulate. Even if ovulation occurs, cows with uterine inflammation are less likely to conceive because in addition to uterine damage, LPS also impairs the developmental competence of oocytes. LPS perturbs the nuclear and cytoplasmic maturation of bovine oocytes. Moreover, oocytes matured using LPS treatment are less likely to develop into the blastocyst stage. Such oocytes also have a reduced number of trophoblast cells in blastocysts. Therefore, the detrimental effects of LPS on ovarian activity may be partly responsible for infertility in cows with uterine inflammation. Novel treatment and prevention strategies for uterine inflammatory diseases can be developed by advancing our knowledge of the pathophysiology underlying ovarian dysfunction, and this can only be achieved by further research. The present review outlines the molecular pathogenesis of LPS-induced ovarian dysfunction.
Key words: Dairy cow, Follicles, Lipopolysaccharide, Oocytes, Uterine inflammatory diseases

Introduction

In postpartum dairy cows, the prompt uterine involution and the onset of normal ovarian cycle activity are crucial for achieving optimal reproductive efficiency. However, uterine inflammatory diseases, such as metritis and endometritis, are common in high producing dairy cows and they cause infertility by disrupting uterine and ovarian functions [1]. Uterine inflammatory diseases result in huge economic losses due to compromised reproductive performance. In fact, the conception rates are approximately 20% lower in cows with endometritis, the calving to conception interval is 30 days longer, and 3% more cows are culled as they fail to conceive in a timely manner compared to healthy cows [2, 3].

On average, 20–40% of cows develop metritis within one week postpartum and 18.5–21% of cows show signs of systemic illness (e.g. pyrexia) [4]. Subsequently, 20% of cows present with a persistent clinical disease beyond 3 weeks postpartum (i.e. clinical endometritis), and approximately 30% have chronic inflammation of the uterus without clinical signs of uterine disease (i.e. subclinical endometritis)

[2, 4–7]. Even after successful treatment, cows that have experienced clinical endometritis are less fertile than cows that have not suffered from a postpartum clinical uterine disease [2]; this is probably owing to persistent subclinical endometritis.

In cows with uterine inflammation, decreased fertility is not only associated with uterine damage but also with diminished ovarian cyclic activity [8, 9]. Disrupted uterine involution causes significant adverse effects on ovarian function. Metritis cows are 11 times more at risk of prolonged luteal cycles during the postpartum period [8]. One study reported that cows with metritis showed a higher prevalence of persistent corpus luteum (CL) during the first postpartum luteal phase compared to healthy cows [10]. Moreover, cows with clinical endometritis are more likely to show anovulatory anestrus after calving or prolonged luteal phases compared to normal animals [1, 8].

Information regarding the pathophysiology of ovarian dysfunction is essential for the development of novel treatments and prevention strategies for uterine inflammatory diseases. However, there is surprisingly little information on the interactions between the uterus and the ovaries under pathological conditions. Thus, the aim of the present review was to outline the advances in our knowledge of the pathogenesis and consequences of bovine uterine inflammatory diseases, with a focus on the molecular effects of lipopolysaccharide (LPS) on ovarian function, due to bacterial infection of the uterus.

Uterine Inflammatory Diseases and Bacterial Infection

Sheldon *et al*. (2006) have proposed definitions for several uterine inflammatory diseases. Metritis is defined as a uterine inflammation that occurs within 21 days postpartum [5] and is characterized by an enlarged uterus with a fetid red-brown watery uterine discharge. Cows with metritis also exhibit pyrexia [5, 11]. Clinical endometritis is characterized by a purulent uterine discharge from the vagina for 21 days or more postpartum, and is not accompanied by any systemic symptoms [5]. Subclinical endometritis also occurs for 21 days or more postpartum and is defined as inflammation of the uterine endometrium, usually diagnosed by cytology, in the absence of clinical endometritis symptoms [5].

Uterine bacterial infections usually occur in early postpartum dairy cows [3]. Approximately 90% of cows have bacterial infection of the uterus within the first 2 weeks following parturition [12–14]. Bacterial infection in the uterine lumen decreases substantially in healthy cows 3 to 4 weeks postpartum [3]. The elimination of uterine bacterial infections is dependent on the involution of the genital tract, the regeneration of the endometrium, and the immune microenvironment of the uterus [5]. Failure to eliminate uterine bacterial infection leads to uterine inflammation resulting in embryonic damage, disruption of follicular function, and impaired fertility [3, 15].

The most common pathogenic bacteria responsible for causing uterine inflammation are *Escherichia coli*, *Trueperella pyogenes*, and a range of anaerobic bacteria, including *Fusobacterium nucleatum, Fusobacterium necrophorum*, and *Prevotella* spp. [4]. *T. pyogenes, F. necrophorum*, and *Prevotella* spp. act synergistically to exacerbate uterine inflammation [13, 16–18]. Infection of the uterine endometrium by *E. coli* precedes infection by *T. pyogenes* and is associated with harmful effects on the ovaries, the hypothalamic-pituitary axis, and fertility [1, 9, 19, 20]. The first dominant follicles of postpartum cows with more severe microbial infections grow slower and exhibit lower peripheral estradiol (E2) concentrations [9, 14, 21, 22], specifically due to the infection of the uterus with *E. coli* [9]. Cows with a uterine *E. coli* infection are less likely to ovulate and if ovulation occurs, these animals produce a smaller CL with lower peripheral plasma progesterone (P4) concentrations [9]. These data suggest that *E. coli* plays a crucial role in the development and pathology of uterine inflammatory disease, with subsequent knock on effects on ovarian function.

Uterine Inflammatory Diseases and LPS

LPS in peripheral blood

The inflammatory effects of *E. coli* are likely mediated through the endotoxin, LPS [23]. LPS is the major component of the cellular membrane of gram-negative bacteria and it provokes a local or systemic inflammatory response by stimulating the innate immune system. Plasma LPS concentrations increase in cows with subacute rumen acidosis [24], mastitis [25, 26], and uterine inflammation. LPS has been detected in the plasma of cows with metritis [27, 28] and endometritis [29], suggesting that it is absorbed from the uterus and transferred to the bloodstream.

Plasma LPS concentrations are consistently higher in cows with metritis than in healthy cows 0–3 weeks postpartum [27], despite the resolution of symptoms of systemic illness within 1 week postpartum. Usually, circulating LPS is immediately detoxified by hepatocytes in the liver. Thus, the continuous increase in plasma LPS concentrations in cows with metritis may suggest the possible accumulation of LPS in the bloodstream. Cows with hepatic lipidosis are not able to clear intravenously injected LPS, while clinically healthy cows clear LPS within 30 min [30]. These data suggest that LPS detoxification is impaired due to liver abnormalities. Hepatic dysfunction frequently occurs in high yielding dairy cows after calving and is associated with high incidence of uterine inflammatory disease. Further research examining the relationship between the health of the liver and changes in the plasma LPS concentrations of cows with uterine inflammation is required to elucidate the effect of postpartum metabolic disease on the long-term LPS accumulation in animals.

LPS in the follicular fluid

Although bacteria are rarely found in the ovaries, LPS can be found in the ovarian follicular fluid of cows with uterine inflammation [28, 31], suggesting that uterine derived LPS translocates to the ovaries and affects follicular function. However, LPS translocation into the follicle remains controversial [19, 29, 31]. In a clinical case of bovine metritis, LPS concentrations in the plasma, follicular fluid, and uterine fluid were investigated [28]. The results of this study showed that LPS concentrations in the follicular fluid and in the plasma were the same, while these LPS concentrations were lower than in the uterine fluid. Interestingly, in the same study, one of the seven follicles tested had an extremely high concentrations of LPS (12.40 EU/ml) compared to the others (0.62–0.97 EU/ml). No distinct differences in the size, appearance, vascularization, or location of the seven follicles were observed. These results suggest that some follicles may accumulate LPS selectively, passively, or by chance. We currently have no clear explanation for this bizarre observation. Further study is required to reveal the mechanism by which LPS accumulates in the follicular fluid.

LPS and Follicular Functions

The effect of LPS on the hypothalamus-pituitary-ovarian axis

The reduced ovulation rate in cows with uterine inflammation could be associated with the effects of the uterine infection on hypothalamic, pituitary, or ovarian function. LPS acts on the hypothalamus or pituitary by downregulating the release of gonadotropin and disrupting follicular growth in the ovaries of sheep [32] and cattle [33]. LPS suppresses the hypothalamic release of gonadotropin-releasing hormone (GnRH), the secretion of the luteinizing hormone (LH) from the pituitary, and the sensitivity of the pituitary to GnRH in sheep [34, 35]. These changes may result in reduced ovulation rates in cows with uterine inflammation.

Furthermore, LPS can directly act on the ovaries, including follicular components such as the theca and granulosa cells or oocytes. LPS is recognized by a specific receptor complex; toll-like receptor (TLR) 4, cluster of differentiation 14 (CD14), and myeloid differentiation factor 2 (MD2) [36]. Binding of LPS to this receptor complex results in the nuclear translocation of nuclear factor kappa-B components, which leads to the production of pro-inflammatory

cytokines and chemokines [23, 37]. In bovine follicles, granulosa cells [31, 38] and theca cells [39] express TLR4, CD14, and MD2, suggesting that follicular cells can respond to LPS. Indeed, bovine ovarian granulosa cells can initiate an inflammatory response to LPS via the TLR4 pathway. Granulosa cells respond to LPS via the acute phosphorylation of the TLR signaling components, p38 and extracellular signal-regulated kinase, and increased mRNA expression of *interleukin* (*IL*) *6* and *IL8* [40].

Association of LPS and steroidogenesis in follicles

In follicles with high concentrations of LPS (high-LPS follicles), E2 concentrations are lower and P4 concentrations are higher compared to those in follicles with low concentrations of LPS (low-LPS follicles). This observation was made both in the largest and the second largest follicles (Fig. 1) [41], thereby suggesting that LPS suppresses follicular activity irrespective of the developmental stage of large follicles. Moreover, the transcriptional levels of steroidogenesis-

related genes change drastically depending on the concentration of LPS in the follicular fluid (Fig. 1) [41]. In high-LPS follicles, the mRNA expression of gonadotropin receptors, such as the LH receptor (*LHr*) in theca cells and the follicle stimulating hormone (FSH) receptor (*FSHr*) in granulosa cells, is lower compared to that in low-LPS follicles. LH stimulates the production of P4 and androstenedione (A4) in the theca cells and FSH stimulates the production of E2 in granulosa cells through the activation of the cyclic adenosine monophosphate signaling pathway, which upregulates the transcription of steroidogenic enzymes [42, 43]. Decreased *LHr* and *FSHr* expression may suggest that LPS reduces the responsiveness of follicles to gonadotropins secreted from the pituitary. During follicular steroidogenesis, 17β-hydroxylase/17,20-lyase (CYP17) converts P4 into A4 in the theca cells, which is then transferred to granulosa cells and metabolized to E2 by the P450 aromatase. Interestingly, mRNA expression of *CYP17* in the theca cells and *P450 aromatase* in granulosa cells is downregulated in high-LPS follicles.
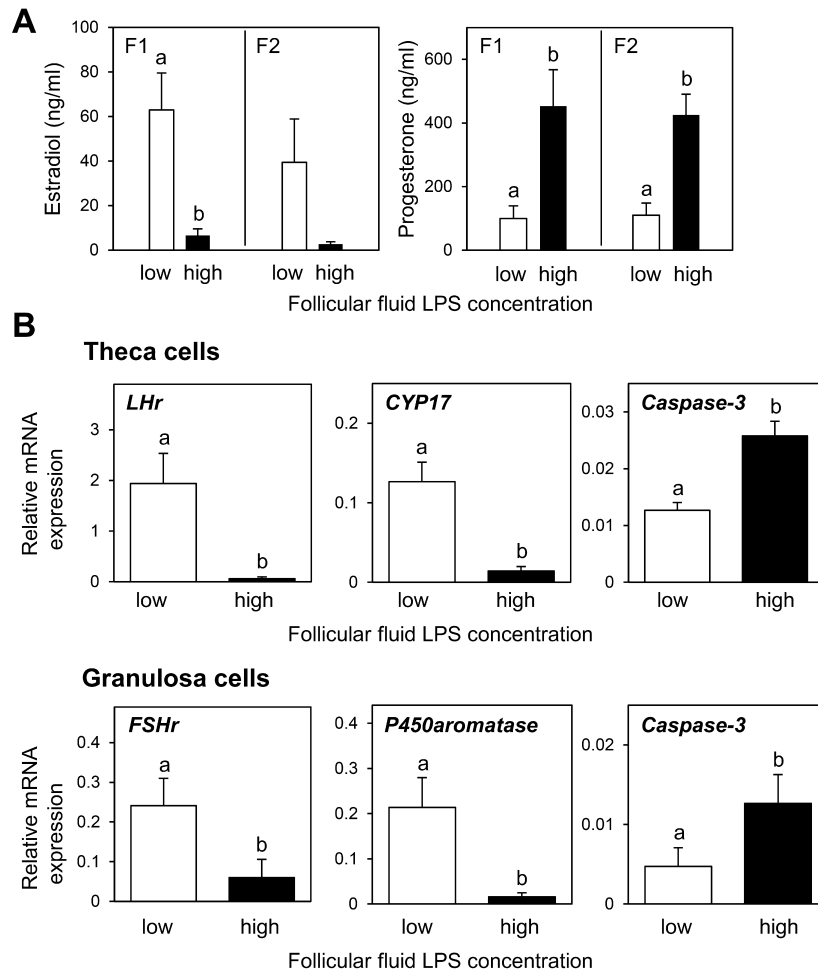

Fig. 1. Diminished activity in follicles with a high lipopolysaccharide (LPS) environment. (A) Estradiol and progesterone concentrations in the follicular fluid of the largest (F1) follicles or the second largest (F2) follicles in bovine ovaries. (B) Expression of steroidogenesis-related genes in the theca cells and granulosa cells of large follicles (> 8 mm diameter) in bovine ovaries. Cows with LPS concentration < 0.5 EU/ml in the follicular fluid were classified as 'low' and those with a concentration ≥ 0.5 EU/ml were classified as 'high'. Values with different letters (a, b) are significantly different between groups (P < 0.05). Modified from Magata *et al*. (2014) [41].

The downregulation of steroidogenic enzymes in high-LPS follicles is assumed to result in the reduction of E2 synthesis.

In granulosa cells, apoptotic cell death, a mechanism underlying cell loss during follicle atresia [44], is caspase-3-dependent in antral follicles [45]. The transcriptional level of *caspase-3* is higher in theca and granulosa cells of high-LPS follicles (Fig. 1). This suggests that detection of LPS in follicular fluid may be associated with follicular atresia. Bosu *et al*. (1996) reported that LPS triggered apoptosis in granulosa cells when early antral follicles, which had been isolated from bovine ovaries, were cultured with LPS [46].

Effect of LPS on the steroidogenesis of ovarian cells

In vitro studies have revealed that LPS acts directly on ovarian cells and impairs steroid production. Primary cell cultures of bovine granulosa and theca cells have been used to investigate the follicular mechanisms associated with uterine inflammation. In granulosa cells isolated from the small and large antral follicles, LPS was shown to suppress E2 production and cause the downregulation of the *P450 aromatase* gene [31, 38]. Moreover, treatment of theca cells with LPS impaired P4 and A4 production and reduced the mRNA expression of steroidogenesis-related genes (e.g. *StAR* and *CYP17*) [39].

The extended luteal phases noted in cows with uterine inflammatory disease could be associated with the effects on luteolysis or luteal cell function. In a study by Shimizu *et al*. (2016), luteinization of bovine granulosa and theca cells isolated from large follicles was induced *in vitro* and the effect of LPS on luteinizing follicular cells was investigated [47]. The results of this study showed that long-term exposure to LPS resulted in reduced P4 synthesis by the luteinizing theca cells, but not by granulosa cells, due to decreased expression of steroidogenic enzymes such as StAR and 3β-HSD. In a luteal cell culture isolated from buffalo corpora lutea, exposure to LPS inhibited P4 secretion and induced apoptosis via the mitochondrial pathway [48]. Another study reported that P4 secretion by cultured bovine luteal cells was stimulated by LPS [49].

The results of the aforementioned *in vitro* studies suggest that bovine theca and granulosa cells have LPS immune response cascades that support a mechanism against the direct action of LPS in the ovarian follicles, which impairs follicular development, ovulation, and the formation of the CL.

LPS and the Developmental Competence of Oocytes

Effect of LPS on the nuclear and cytoplasmic maturation of oocytes

The follicular environment is critical for the developmental competence of the growing oocytes. The accumulation of LPS in the follicular fluid may alter the microenvironment during oocyte development. Bovine oocytes and cumulus cells express the LPS receptor complex (i.e. TLR4, MD2, and TLR2), thus, they probably respond to LPS [50]. Oocytes must undergo nuclear and cytoplasmic maturation for successful fertilization and subsequent development into live offspring [51]. LPS challenge using the concentration detected in follicular fluid of cows with uterine inflammation, during *in vitro* maturation (IVM) of bovine cumulus-oocyte complexes (COCs) perturbs the nuclear maturation of bovine oocytes with inhibition of meiotic progression [50]. Failure of meiotic progression may be associated with fertilization obstruction and additional cleavage events of the zygotes.

During oocyte maturation, dynamic changes occur in the organelles of the oocyte cytoplasm, and these changes constitute the cytoplasmic maturation of oocytes. Cytoplasmic maturation is essential for further fertilization and embryonic development, as it is associated with oocyte activation and the pre-implantation development processes of embryos [51]. Mitochondria are the most abundant organelles in the oocyte cytoplasm and they play an essential role in adenosine triphosphate (ATP) production for oocyte activation. Mitochondrial maturation, redistribution, and ATP production during oogenesis are crucial processes for fertilization and successful development [52–56]. In bovine oocytes, mitochondrial redistribution from the periphery to the central cytoplasm occurs during IVM, which is essential for oocyte maturation [53]. LPS treatment during IVM affects mitochondrial redistribution and results in a reduced number of oocytes with dispersed mitochondrial distribution, which may cause poor cytoplasmic maturation [50] (Fig. 2A). Moreover, LPS exposure during
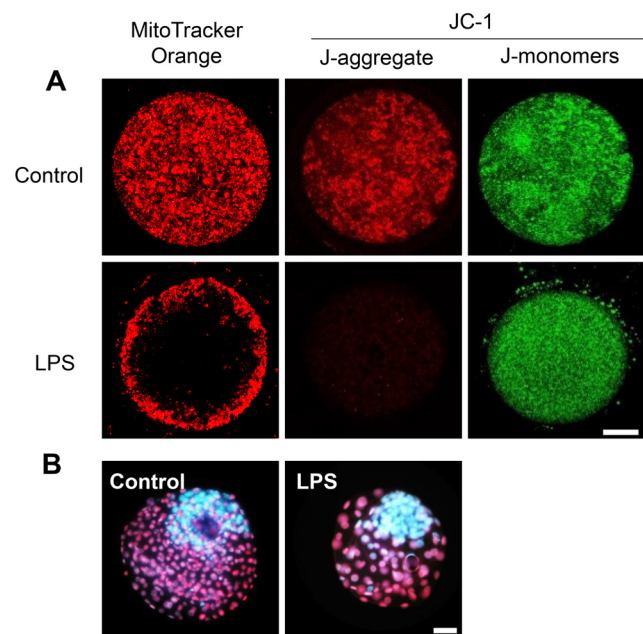

Fig. 2. (A) Representative images of oocytes after *in vitro* maturation. The mitochondrial distribution pattern was determined by MitoTracker staining. Lipopolysaccharide (LPS) decreased the number of oocytes with dispersed mitochondria and increased the number of oocytes with a peripheral distribution of mitochondria. The mitochondrial membrane potential was determined using a mitochondrial permeability transition detection kit (JC-1 dye). Rhodamine isothiocyanate (J-aggregate: high polarized mitochondria) and fluorescein isothiocyanate (J-monomer: low polarized mitochondria) staining were used. LPS decreased the mitochondrial membrane potential of oocytes. Scale bar = 25 μm. (B) Representative images of the differential staining of the inner cell mass (ICM) and trophectoderms (TEs) in day 7 blastocysts in which the ICM appears blue (Hoechst 33342 stain) and the TE pale red (propidium iodide). LPS reduced the cell number in TEs, but not in the ICM. Scale bar = 50 μm. Modified from Magata *et al*. (2017) [50].

IVM results in reduced mitochondrial membrane potential in mature oocytes [50] (Fig. 2A). Mitochondrial membrane polarity in mature oocytes has been associated with the developmental competence of oocytes. Thus, it has been suggested that the mitochondrial membrane potential is a key indicator of cellular viability [53, 57]. LPS-induced reduction in mitochondrial membrane potential may suggest that oocytes maturing in the presence of LPS produce less ATP, leading to developmental failure after fertilization. Fewer mitochondria or low mitochondrial DNA content, owing to inadequate mitochondrial biogenesis or cytoplasmic maturation, is known to adversely affect oocyte fertilizability [58]. LPS exposure during IVM does not affect mitochondrial DNA copy number suggesting that LPS may not affect mitochondrial biogenesis or degeneration [50].

Effect of LPS on embryonic development

During oocyte maturation, LPS acts to reduce oocyte cleavage and development following fertilization. Bovine oocytes matured in the presence of LPS show low potential to develop into blastocysts [50, 59]. In contrast, when LPS is added after fertilization has occurred, bovine oocytes are still able to develop into blastocysts [59], suggesting that the deleterious effects of LPS only occur during oocyte maturation and not during embryonic development.

Hill and Gilbert (2008) reported that the total cell number of blastocysts was reduced when bovine embryos were cultured in media conditioned by exposure to an inflamed endometrium [60]. The results of this study suggest that LPS involved in the uterine inflammation may negatively affect the quality of embryos. When bovine oocytes were induced to mature *in vitro* in the presence of LPS, the number of trophectoderm cells in day 8 blastocysts was reduced, whereas the number of cells in the inner cell mass was not affected by LPS treatment (Fig. 2B). These results suggest that LPS does not affect the viability of the developed embryo. However, a reduction in the number of trophoblasts would likely affect the placental size and function, which may cause implantation failure. Moreover, bovine trophoblasts produce the maternal recognition signal molecule, interferon tau (IFNT); thus, reduced trophoblast numbers may result in reduced IFNT production, and perhaps, reduced potential for embryonic survival and failure of pregnancy recognition.

Conclusion and Perspectives

Uterine inflammatory diseases commonly occur in high producing dairy cows resulting in reduced reproductive performance. Uterine bacterial infection-induced LPS acts directly on ovarian follicular cells and impairs steroidogenesis, which may disturb follicular growth or reduce their ovulatory ability. Even if ovulation occurs, cows with uterine inflammation are less likely to conceive, as LPS impairs the developmental competence of oocytes, in addition to causing uterine damage. The deleterious effects of LPS on ovarian activity may be partly responsible for infertility in cows with uterine inflammatory disease (Fig. 3). Cows experiencing uterine inflammation are less fertile even after a successful treatment; this is probably because of the long-term (i.e. carry-over) influence of LPS on reproductive functions, which lasts weeks after the resolution of the disease. Two hypotheses described below are postulated to explain the possible mechanisms of the long-term effect of LPS. First, LPS produced during the acute phase of uterine bacterial infection may get accumulated in the blood or follicular fluid for a long time, which would continuously affect follicular or oocyte function even after the inflammation has been resolved. To validate this, the LPS translocation from the uterus to
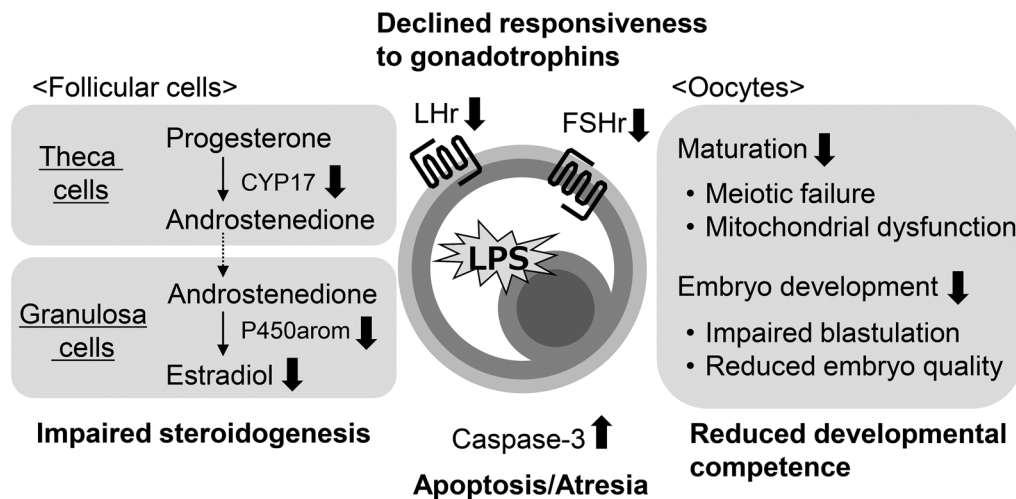

Fig. 3. Schematic illustration showing the possible mechanisms of ovarian dysfunction associated with uterine inflammation-derived lipopolysaccharide (LPS). In follicles with a high LPS environment (high-LPS follicles), the transcription of gonadotropin receptors, such as the luteinizing hormone receptor (*LHr*) in theca cells and the follicle stimulating hormone receptor (*FSHr*) in granulosa cells, was low, indicating the declining responsiveness to gonadotropins secreted from the pituitary. In high-LPS follicles, the estradiol concentration in the follicular fluid was low and mRNA expression of *caspase-3* was high, suggesting an association with follicular atresia. LPS acted directly on follicular theca and granulosa cells and impaired steroid production, which may disturb the follicular growth or reduce the ovulatory ability. Even if ovulation occurs, cows with uterine inflammation are less likely to conceive, as LPS negatively affects the developmental competence of oocytes. LPS perturbed the nuclear and cytoplasmic maturation of bovine oocytes and the subsequent embryonic development.

the ovarian follicles and the follicular fluid kinetics of LPS should be studied. Secondly, LPS may have a detrimental effect on follicles or enclosed oocytes during the early stage of follicular development. In cows, meiotic maturation from the germinal vesicle stage to metaphase II takes approximately 60 days [61]. The negative impact of LPS on early stage follicles would explain the compromised reproductive performance long after the resolution of bacterial infection. Further research that addresses the mechanism by which LPS acts on the reproductive axis and causes ovarian dysfunction is necessary for the development of novel treatment and prevention strategies for postpartum uterine inflammation and for the improvement of the reproductive performance of dairy cows.

Acknowledgments

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