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Disruption of female reproductive function by endotoxins

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Short title: Endotoxemia-induced reproductive dysfunction

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Abstract

Endotoxemia can be caused by obesity, environmental chemical exposure, abiotic stressors, and bacterial infection. Circumstances that deleteriously impact intestinal barrier integrity can induce endotoxemia and controlled experiments have identified negative impacts of lipopolysaccharide (LPS; an endotoxin mimetic) on folliculogenesis, puberty onset, estrus behavior, ovulation, meiotic competence, luteal function and ovarian steroidogenesis. In addition, neonatal LPS exposures have transgenerational female reproductive impacts, raising concern about early life contacts to this endogenous reproductive toxicant. Aims of this review are to identify physiological stressors causing endotoxemia, to highlight potential mechanism(s) by which LPS compromises female reproduction, and identify knowledge gaps regarding how acute and/or metabolic endotoxemia influence(s) female reproduction.

Introduction to endotoxemia

Gram-negative bacteria protect themselves using two phospholipid membranes. The outermost facing membrane contains glucosamine-based phospholipid known as lipopolysaccharide (LPS), which is a recognized endotoxin, meaning it has toxic effects to the host after being shed from lysed bacteria (Raetz 1990, Rietschel, et al. 1994). Endotoxin elicits a well-characterized robust immune response in animals, but there is recent appreciation for its marked alteration of host metabolism (independent of overt immune modulation) in multiple laboratory models and humans.

LPS consists of a core oligosaccharide, O-antigens, and a lipid A moiety (depicted in Figure 1). The lipid A moiety portion of LPS is responsible for inducing the cellular response (Loppnow, et al. 1989). Systemic endotoxemia (increased circulating LPS) reflects either bacterial infection or compromised epithelial (skin, lung, gastrointestinal track, uterine, and mammary) barrier function. Metabolic endotoxemia is described as the physiological state when circulating LPS is 10–50 times lower than that observed during septic shock; (Cani, et al. 2007).

Unsurprisingly, endotoxemia is a consequence of infection by LPS-producing bacteria. There are also a myriad of environmental exposures that can cause endotoxemia and these include non-steroidal anti-inflammatory drugs (Arakawa, et al. 2012, Van Wijck, et al. 2012), mycotoxins (Alizadeh, et al. 2015, Assuncao, et al. 2016, Marin, et al. 2015) and alcohol (Hartmann, et al. 2012, Hartmann, et al. 2015). Indeed “leaky gut”, and resultant metabolic endotoxemia, has been associated with many pathologies such as inflammatory

bowel syndrome (Michielan and D'Inca 2015), cirrhosis (Fukui 2015, Lutz, et al. 2015), and cancer (Saggioro 2014). In addition, evidence that gut barrier function becomes compromised during obesity, resulting in metabolic endotoxemia, is firmly established (Al-Attas, et al. 2009, Amar, et al. 2008, Hawkesworth, et al. 2013). Although the etiology is not clear, low-grade, chronic inflammation caused by obesity-induced endotoxemia is thought to play a key role in the development of obesity-related disorders (Cani, et al. 2007, Hawkesworth, et al. 2013) including female reproductive dysfunction.

Heat stress is an abiotic stress that also induces endotoxemia. In an attempt to maximize radiant heat dissipation, heat-stressed animals redistribute blood to the periphery, and in order to maintain blood pressure, blood flow to the splanchnic tissues, including the gastrointestinal tract, is markedly reduced. The intestinal epithelial cells are extremely sensitive to oxygen and nutrient restriction (Rollwagen, et al. 2006). Heat stress thus causes marked hypoxic-induced conformational changes which ultimately reduces intestinal barrier integrity. Depending upon the severity and magnitude, heat stress can cause intestinally-derived endotoxemia (Pearce, et al. 2013a, Pearce, et al. 2012, Pearce, et al. 2013b, Pearce, et al. 2013c, Sanz Fernandez, et al. 2014). The duration of leaky gut is variable and transitory, for example, intestinal integrity is reduced as early as two hours after the onset of heat stress in pigs (Pearce, et al. 2014) and with removal of heat stress, intestinal integrity returned within days. Additionally, leaky gut can be caused by reduced nutrient intake and this has been demonstrated in multiple models (Kvidera, et al. 2017, Rodriguez, et al. 1996). Further, psychological and emotional stress also increases gastrointestinal tract barrier permeability (Vanuytsel, et al. 2014). Thus, endotoxemia is

relatively common and arises due to a variety of frequent initiators, but the severity of it depends on the source (epithelial barrier endotoxin infiltration versus bacterial infection) and duration of the inducing agent(s).

The major purpose of this review is to collectively describe experiments that have either directly tested the female reproductive effects of endotoxemia through *in vitro* culture models or *in vivo* experiments in which animals are administered LPS. Additionally, we will highlight research that has identified associations between physiological scenarios that compromise intestinal integrity (and concomitantly increase circulating endotoxin) with detrimental impacts on female reproduction. Studies evaluating the impact of metabolic and acute endotoxemia are included. Typically, controlled experiments to evaluate endotoxemia's impact on female reproduction have utilized the acute approach (i.e. an I.V. or I.M. LPS bolus). Further, we will describe how specific cells recognize and respond to LPS, characterize the systemic response to endotoxemia and the reproductive outcomes of LPS exposure, which have been examined in both traditional rodent and large animal models.

The systemic response to endotoxemia

Lipopolysaccharide-binding protein

Hepatic acute phase proteins (APP), which are produced as a secondary (non-local) response to a toxic stimuli, have been widely utilized as indicators of systemic and metabolic inflammation, including metabolic endotoxemia (Ceciliani, et al. 2012). Lipopolysaccharide-binding protein (LBP) is an APP, primarily produced in hepatocytes

(Grube, et al. 1994, Kirschning, et al. 1997), that interacts directly with the lipid A moiety of LPS (Schumann 2011, Tobias, et al. 1986, 1989). Interaction between LBP and LPS results in an LBP conformational change promoting recognition and transfer of LPS to macrophages (Wright, et al. 1989). Interleukin (IL)-6 (Grube, et al. 1994, Kirschning, et al. 1997), IL-1β and dexamethasone (Schumann, et al. 1996) stimulate hepatic LBP production but LBP can also be produced in lung epithelial cells (Dentener, et al. 2000, Klein, et al. 1998), gastrointestinal tract cells (Vreugdenhil, et al. 1999), kidney (Wang, et al. 1998), and the epididymis (Malm, et al. 2005). LBP acts as a soluble receptor and transports LPS to the appropriate toll-like receptor (TLR) to initiate intracellular signal cascades to elicit an immunological response (Schumann 2011). In humans, circulating LBP and plasma C-reactive protein (another broad biomarker of inflammation) are positively correlated (Tremellen, et al. 2015), thus providing rationale for using LBP as an inflammatory biomarker (Opal, et al. 1999).

The cellular response to endotoxemia

The lipid A moiety of LPS is highly conserved among species and it stimulates an inflammatory response because it is recognized by membrane bound TLR4 (Raetz 2008, Schumann 2011, Tobias, et al. 1989). Utilizing TLR4-deficient mice, it has been shown that TLR4 is required for LPS recognition and the subsequent cellular response (Hoshino, et al. 1999). However, other TLRs can also mediate a cellular response to LPS, dependent on the bacterial strain of origin. As an example, the LPS produced by *Leptospirosis* can instigate an intracellular response via TLR2, TLR4 or TLR5 (Faisal, et al. 2016, Goris, et al. 2011). In addition, host species can also differ in their response to LPS with some having variable

sensitivity to a specific LPS which impacts both the physiological response and development of mitigation strategies such as vaccine production (Werling, et al. 2009).

Toll-like Receptor 4

TLR4 is a membrane spanning protein bearing similarity to the interleukin 1 (IL-1) receptor (Aderem and Ulevitch 2000, Greenfeder, et al. 1995, Medzhitov and Janeway 2000). LPS binds to cluster of differentiation 14 (CD14) and is then transferred to a complex between TLR4 and myeloid differentiation factor 2 (MD-2) to initiate a cellular response (da Silva Correia, et al. 2001, Triantafilou and Triantafilou 2002). The MD-2 protein is a crucial component of LPS recognition as an extracellular piece of the TLR4 complex (Shimazu, et al. 1999). Soluble CD14 (sCD14) is integral for serum- and cell-mediated responses to LPS (Pugin, et al. 1993, Wright, et al. 1989) while the membrane-bound from (mCD14) is a glycosylphosphatidyl-inosital anchored protein (Haziot, et al. 1988, Simmons, et al. 1989) and works with TLR4 to transmit the LPS signal across the lipid bilayer to initiate a cellular response (Poltorak, et al. 1998). LBP was originally thought to be necessary for CD14 to bind LPS (Wright, et al. 1992), however, other studies suggest LPS directly activates CD14 or the MD-2-TLR4 complex (da Silva Correia, et al. 2001, Dentener, et al. 2000, Triantafilou and Triantafilou 2002), and LBP increases the rate of LPS binding to CD14 (Hailman, et al. 1994).

Following LPS recognition, TLR4 recruits proteins including TIR domain-containing adaptor protein (TIRAP), myeloid differentiation primary response gene 88 (MyD88), TIR domain-containing adaptor inducing interferon beta (TRIF), and TRIF-related adaptor molecule (TRAM) via its Toll-interleukin-1 receptor (TIR) domain causing downstream

pathway activation. TIRAP and MyD88 mediate MyD88-dependent signaling whereas TRIF and TRAM mediate MyD88-independent signaling. Both pathways involve phosphorylation of the REL-associated protein (RELA) subunit of nuclear factor kappa B (NFκB) although the MyD88-dependent pathway activates pro-inflammatory cytokine genes while the MyD88-independent signaling activates Type I interferon genes (Kawai, et al. 1999, Shimazu, et al. 1999). Phosphorylated RELA increases concomitant with increased LPS exposure demonstrating the ability of LPS to drive TLR4-mediated NFKB activation (Chow, et al. 1999). Interestingly, single nucleotide polymorphisms (SNPs) in the *TLR4* gene affects immune function and reproductive ability in dairy cows (Shimizu, et al. 2017), though the importance of *Tlr4* SNPs in humans remains vague (Gowin, et al. 2017, Hajjar, et al. 2017) and is an area of future interest regarding the biological response(s) to endotoxemia.

Detoxification of LPS by Acyloxyacyl Hydrolase

Acyloxyacyl hydrolase (AOAH) is a lipase that deacylates and detoxifies LPS within cells and (Hall and Munford 1983). AOAH releases secondary acyl chains from LPS regardless of the acyl chain structure or location on the diglucosamine backbone of LPS (Erwin and Munford 1990). AOAH is primarily produced in macrophages, neutrophils, and dendritic cells (Ojogun, et al. 2009) and converts hexaacylated LPS to pentaacylated or tetraacylated LPS rendering it unable to stimulate a response through TLR4 complex formation (Teghanemt, et al. 2005). AOAH activity increased in murine serum and hepatocytes following a 25 μg bolus of LPS (Ojogun, et al. 2009). In these mice, AOAH activity peaked after three days and returned to normal levels by day nine post LPS-injection (Ojogun, et al. 2009). Deacylated LPS (dLPS) can compete with LPS for LBP or CD14 binding (Kitchens

and Munford 1995a, b), however, binding of dLPS does not stimulate a cellular response (Kitchens, et al. 1992). Interestingly, LBP alone or in coordination with CD14 increases the susceptibility of LPS to AOAH detoxification (Gioannini, et al. 2007). *Aoah*-deficient mice have increased pulmonary damage in response to intranasal LPS exposure corroborating AOAH's protective role against LPS (Zou, et al. 2017). Thus, the chemical modification of LPS by AOAH partly regulates the immune response by decreasing the capacity of LPS to stimulate an intracellular signal cascade (Lu, et al. 2005).

AOAH cannot act on LPS when the fatty acyl chains are orientated to the inside of LPS aggregates or when LPS is anchored on the outer membrane of bacteria (Gioannini, et al. 2007). AOAH can act on LPS-LBP complexes as well as monomeric LPS-sCD14 complexes, suggesting a model where LBP and sCD14 transfer of LPS exposes fatty acyl chains to AOAH (Gioannini, et al. 2007). However, when LPS is transferred and bound to MD-2, the fatty acyl chains are less accessible, decreasing AOAH's ability to deacylate LPS and reduce TLR4 activation (Gioannini, et al. 2007). Whether the female reproductive tract has the capacity to locally detoxify LPS remains unknown though recently, the importance of AOAH in the lung (Zou, et al. 2017), urinary tract (Yang, et al. 2017) and colonic dendritic (Janelsins, et al. 2014) cells has been demonstrated.

Effects of LPS on female reproduction and fertility

Understanding the effects of LPS exposure on ovarian function is of interest in humans and production livestock species, since increased circulating LPS is associated with heat stress (Pearce, et al. 2013a, Pearce, et al. 2012, Pearce, et al. 2013b, Pearce, et al. 2014, Sanz

Fernandez, et al. 2014), obesity (Cani, et al. 2007), and bacterial infection. Uterine infections have been associated with various negative impacts on bovine fertility, including cystic ovaries (Bosu and Peter 1987, Peter, et al. 1989a, Peter, et al. 1989b), abnormal or delayed folliculogenesis after parturition (Huszenicza and Kegl 1999), a longer postpartum anestrus period (Bosu and Peter 1987), and a lengthened luteal phase (Peter and Bosu 1988). Interestingly, follicular fluid that surrounds and nourishes the maturing oocyte contains LPS levels reflective of the systemic circulation (Herath, et al. 2007). An accumulation of IL-6 and IL-8 in media collected after bovine granulosa cell or ovarian cortical strip culture was observed following LPS incubation, similar to the responsiveness of human immune cells (Bromfield and Sheldon 2013, Dentener, et al. 1993). Plasma LBP and follicular fluid IL-6 concentrations were also positively correlated, suggesting that systemic endotoxemia is associated with ovarian inflammation (Tremellen, et al. 2015). Thus, LPS can locate the ovary and potentially interact directly with the oocyte, though remains to be determined.

Impacts of endotoxemia on folliculogenesis

Bovine ovarian cortical explants exposed to LPS had reduced number of primordial follicles due to hyperactivation (Bromfield and Sheldon 2013). Similarly, mice exposed to LPS *in vivo* had reduced primordial follicle number which was described as TLR4-mediated, since *Tlr4*$^{-/-}$ mice are refractory to LPS-mediated primordial follicle depletion (Bromfield and Sheldon 2013) suggesting TLR4 in part regulates the ovarian LPS response. Phosphatase and tension homolog (PTEN) and Forkhead box O3 (FOXO3), both proteins involved in regulating primordial follicle activation, were translocated out of the oocyte nucleus of

primordial and primary follicles in cultured bovine cortical strips after LPS exposure (Bromfield and Sheldon, 2013). The aforementioned indicate premature primordial follicle activation, potentially leading to depletion of the ovarian follicular reserve. In rodent studies, altered protein abundance due to LPS exposure in neonatal rodents has been observed (Sominsky, et al. 2013). Furthermore, a diminished follicular reserve and earlier onset of ovarian senescence occurs in female rats neonatally exposed to LPS, raising concern about reproductive outcomes of bacterial infections early in life (Sominsky, et al. 2012).

Effects on the follicular stage of the estrous cycle, including ovulation

Immune challenges can disrupt the follicular phase in multiple species (Battaglia, et al. 2000, Kalra, et al. 1990, Peter, et al. 1990). LPS suppresses the hypothalamic-pituitary gonadal axis by decreasing pulsatile gonadotrophin-releasing hormone (GnRH) secretion (Hoshino, et al. 1999). LPS also blunts the 17β-estradiol (E_2) increase during the preovulatory phase, thus delaying subsequent luteinizing hormone (LH) and follicle stimulating hormone (FSH) surges, culminating in delayed or inhibited ovulation (Battaglia, et al. 2000, Peter, et al. 1989a, Peter, et al. 1990, Suzuki, et al. 2001). Using gonadectomized animals, it has been demonstrated that LPS suppresses GnRH release, thus disrupting the LH surge amplitude, frequency, and concentration (Coleman, et al. 1993, Ebisui, et al. 1992, Feng, et al. 1991, Kujjo, et al. 1995). In agreement with reduced E_2 compromising ovulation, when LPS was infused into the uterine lumen, the pre-ovulatory LH surge was attenuated (Peter, et al. 1989a). Furthermore, LPS-treated females had delays in the time to the LH surge (Fergani, et al. 2012) and lower ovulation rates (Williams, et al. 2008). Recently,

ovine kisspeptin/neurokinin B/dynorphin (KNDy) neuron activation has been demonstrated to be disrupted by LPS exposure, thus altering the hypothalamic-pituitary-ovarian axis (Fergani, et al. 2017).

LPS alters anterior pituitary hormones in circulation, through direct or indirect mechanisms. LPS infusion decreased LH but stimulated systemic prolactin (PRL) and cortisol levels in anestrous ewes and reduced mRNA abundance of the LH (LHβ) and LH receptor (LHr) (Herman, et al. 2010). Further, mRNA encoding FSH and the FSH receptor (FSHr), PRL and the PRL receptor were increased by LPS infusion (Herman, et al. 2010). Granulosa cells exposed to high levels of LPS had reduced mRNA expression of *LHr*, *FSHr*, and cytochrome P450 (CYP) 19A1 (*CYP19A1*) (Magata, et al. 2014a). Theca cells isolated from follicles exposed to high levels of LPS also had decreased mRNA abundance of *LHr*, *CYP17*, and *CYP11A1* but no difference in steroidogenic acute regulatory proteins (*StAR)* or 3β-hydroxysteroid dehydrogenase (*3β-HSD*) levels compared to theca cells from follicles exposed to low levels of LPS (Magata, et al. 2014b). LPS exposure did not impact cell number or androstenedione production from cultured theca cells from either small, medium or large ovarian follicles, but it did reduce E_2 production from cultured granulosa cells isolated from all three follicular sizes (Williams, et al. 2008). In addition, bovine follicles with high levels of LPS (> 0.5 EU/ml) had lower E_2 but elevated progesterone (P_4) levels, relative to follicles with lower LPS concentrations (Magata, et al. 2014a). In an *in vitro* system where bovine granulosa cells were cultured with LPS and provided with FSH and androstenedione, E_2 and P_4 conversion were reduced potentially due to decreased expression of *Cyp19a* mRNA and protein (Herath, et al. 2007). During the *in vivo* LH surge,

a threshold of E_2 is needed to induce behavioral display of estrus, however the amount of E_2 actually required for the behavioral estrus is thought to be at lower level than that required to induce ovulation (Saifullizam, et al. 2010) and LPS negatively impacts female estrus behavior and frequency (Battaglia, et al. 2000).

Post-ovulation impacts of LPS have also been demonstrated. Bovine oocytes subjected to *in vitro* maturation with LPS were less likely to successfully complete meiosis with intact meiotic structures (Bromfield and Sheldon 2011). In addition, increased levels of reactive oxygen species and apoptotic genes, and altered methylation patterns were observed in bovine oocytes as a result of LPS (Zhao, et al. 2017). Further, LPS negatively affected bovine oocyte nuclear maturation by compromising meiotic progression, mitochondrial membrane potential and mitochondrial cytoplasmic redistribution (Magata and Shimizu 2017). LPS also reduced blastocyst development of LPS-exposed oocytes and the trophoblast cell number of blastocysts (Magata and Shimizu 2017). These studies support the potential for LPS to negatively impact oocyte developmental competence.

Impact of LPS on luteal phase of the estrous cycle

Endotoxemia can compromise P_4 production and lead to decreased luteal function. Corpus luteum (CL) formation and the expected increase in P_4 was delayed in heifers exposed to LPS (Suzuki, et al. 2001). During a normal estrous cycle, in the absence of fertilization and pregnancy, prostaglandin F2α (PGF2α) causes CL regression and LPS can cause CL regression by inducing PGF2α production (Hockett, et al. 2000, Moore, et al. 1991). Not only does LPS administration delay ovulation, it also lengthens the time to luteinization, CL formation and sufficient P_4 production (Lavon, et al. 2011, Suzuki, et al. 2001), thus LPS has

numerous targets within the luteal phase. Additionally, CL size is reduced by LPS perhaps due to activation of pro-apoptotic pathways (Herzog, et al. 2012). The cannabinoid receptor type 1 (eCS) has recently been discovered to be involved in LPS-induced CL regression in mice as wild-type mice had increased uterine prostaglandin-endoperoxide synthase (PTGS2) and PGF2α expression, which resulted in reduced ovarian P_4 receptor abundance and regression of the CL, and these observations were absent in eCS deficient mice (Schander, et al. 2016).

Administrating LPS to goats during their luteal phase did not affect steroid hormone concentrations but did increase PGF2α metabolites (Fredriksson G 1985), and repeated uterine LPS infusions in dairy cows every 6 h from 12 h prior to ovulation until 9 d post-ovulation resulted in CL regression much sooner than controls (Luttgenau, et al. 2016). Culturing bovine luteal tissue *in vitro* with TNFα increased PGF2α in a dose-dependent manner (Benyo and Pate 1992). Additionally, porcine luteal tissue, when cultured *in vitro* with PGF2α, exhibits a feedback mechanism in which more PGF2α is produced (Guthrie, et al. 1979). Normally, the porcine CL acquires capacity to undergo luteolysis around day 13 of the luteal phase (Guthrie, et al. 1979), but multiple administrations of PGF2α can induce luteolysis in the porcine CL at an earlier time (Diaz, et al. 2000) suggesting LPS may accelerate luteolysis via TNFα and PGF2α induction in pigs, though this remains to be confirmed.

A temporal pattern of LPS affecting circulating P_4 has been demonstrated, whereby P_4 is initially increased and then declines in LPS-treated, relative to control females (Herzog, et

al. 2012). LPS exposure initially decreased but then did not affect P_4 production in bovine granulosa cells in culture (Herath, et al. 2007). Further, P_4 concentrations were increased in large bovine follicles, and it has been proposed that less P_4 is being converted to E_2 (Magata, et al. 2014a, Magata, et al. 2014b). However, others demonstrated that LPS *in vitro* can inhibit steroid secretion, specifically P_4 and androstenedione in thecal-interstitial cells (Taylor and Terranova 1995) suggesting endotoxemia could alter P_4 production, representing an endocrine disrupting effect.

Endotoxemia and pregnancy maintenance

P_4 is essential for pregnancy maintenance, and LPS reduces the P_4 receptor in uteri of pregnant mice (Agrawal, et al. 2013). The effect of LPS on the ability of P_4 to sustain gestation could cause spontaneous abortion, a phenotypic event frequently associated with physiological conditions in which LPS is elevated. Infection from gram negative bacteria or their outer wall components (including LPS) triggers pre-term labor in many species (Koga and Mor 2010) and in fact, intraperitoneal LPS injection is an established experimental model for inducing pre-term labor (Agrawal, et al. 2013, Deb, et al. 2004, (Elovitz and Mrinalini 2004)). In addition, infertility can be the result of reproductive tract infections in humans and production animals (Price, et al. 2013, Williams, et al. 2008). As mentioned earlier, LPS increases PGF2α release (Roberts, et al. 1975) leading to CL regression, a decline in P_4, and spontaneous abortion in goats (Fredriksson G 1985). LPS and bacterial infection also increase PGF2α in the mare (Fredriksson, et al. 1986) and the cow (Fredriksson, et al. 1985). Uterine epithelial and stromal cells express TLR4 and both produced PGF2α and prostaglandin E2 (PGE) after LPS exposure, a response abrogated by

using a TLR4 antagonist in bovine endometrial explants (Herath, et al. 2006). Endometrial epithelial and stromal cells can respond to LPS exposure via the TLR4- and MYD88-dependent pathways (Cronin, et al. 2012) and cows experiencing endometritis had increased endometrial expression of TLR4 and pro-inflammatory mediators in the first week post-partum (Herath, et al. 2009). TLR4 also mediates the local immune response in human (Hirata, et al. 2005, Rashidi, et al. 2015), feline (Jursza, et al. 2015) and canine (Silva, et al. 2012) endometrial cells. Recent evidence supports that metabolic stress, such as negative energy balance in lactating dairy cows, may alter the endometrial response to LPS (Sheldon, et al. 2017), a concern for animals experiencing the transition from gestation to lactation or for animals (and humans) who have metabolic perturbations.

Bovine embryos exposed *in vitro* to both LPS and PGF2α had reduced survival indicating the potential for LPS to alter pregnancy success (Soto, et al. 2003). Human trophoblast cells cultured with LPS increase pro-inflammatory macrophage production (Li, et al. 2016) and as mentioned earlier, there are fewer trophoblast cells in blastocysts that develop from LPS exposed oocytes (Magata and Shimizu 2017). Additionally, human decidual cells exposed to LPS produced TNFα and PGF2α which negatively affected cell growth. Further, when human amniotic fluid from normal relative to pre-term labor pregnancies were compared, there were increased amounts of TNFα in the pre-term samples, and LPS was detectable in 50% of preterm labor amniotic fluids (Casey, et al. 1989). Furthermore, as evidence that LPS can alter the maternal capacity to support pregnancy, LPS-induced changes to human and bovine endometrial epithelial cell protein abundance (which could affect implantation

at the critical time of maternal recognition of pregnancy) has been demonstrated (Cronin, et al. 2012, Jensen and Collins 2012)(Piras, et al. 2017).

Additional considerations:

Measuring circulating LPS should be interpreted with caution, since the limulus amebocyte lysate assay measures endotoxin biological activity and not LPS that is bound to inflammatory mediators such as soluble CD14 or LBP (Guerville and Boudry 2016). Additionally, the bacterial source of LPS remains undefined in these assays and there are interactions that can alter the assay interpretation (Guerville and Boudry 2016). Thus the usefulness of measuring LPS directly has been questioned (Gnauck, et al. 2015, 2016, Stadlbauer, et al. 2007). Also, most assays do not distinguish between LBP bound to LPS or that which is unbound, thus LBP data must also be appropriately interpreted and within context. Taken together, a lack of an effective and convenient LPS assay is limiting the immune-reproduction field and a collective approach in defining the physiological endotoxemia response is required.

Of additional interest and concern is that LPS causes hyperinsulinemia; either directly as an insulin secretagogue or indirectly by increasing glucose stimulated insulin secretion (Baumgard et al, 2016). Reasons why a catabolic signal like LPS increases an acutely anabolic hormone like insulin are not clear but reports suggest that insulin has potent anti-inflammatory effects (Chalmeh et al., 2013) and that immune cells are insulin sensitive (Maratou et al., 2007). Whether the ovary responds to hyperinsulinemia is unclear (Akamine, et al. 2010, Brothers, et al. 2010, Nteeba, et al. 2013, Wu, et al. 2012), however,

elevated insulin levels have been reported in both serum and follicular fluids of obese females (Robker, et al. 2009, Valckx, et al. 2012). Primordial follicle hyperactivation (similar to that caused by LPS exposure) has been documented in neonatal rat ovaries due to insulin administration (Kezele, et al. 2002). The negative effects of hyperinsulinemia and insulin resistance on female reproduction have been well-documented, largely as pertaining to obesity and polycystic ovary syndrome (Goodarzi, et al. 2011, Ogden 2016) and while not described herein in the interest of brevity, hyperinsulinemia could be a secondary consequence of endotoxemia with the potential to negatively influence female reproduction, though studies to specifically investigate this have not yet been performed. Hyperinsulinemia is not the sole secondary metabolic alteration observed due to endotoxemia: reduced circulating high density lipoprotein (HDL)-cholesterol was observed in dairy cows subjected to an acute exposure to LPS {de Campos, 2017 #7016} and, as discussed herein, LPS-induces an inflammatory response and inflammatory mediators could also impact reproduction as an indirect secondary consequence of elevated LPS.

Conclusion

In summary, endotoxemia negatively affects female fertility and fecundity and has many points of action within the reproductive tract. Endotoxemia originates from a variety of stressors and also during times of bacterial infection. Several studies investigating reproductive impacts of endotoxemia have used acute, bolus exposures, as summarized in Table 1, which may not accurately represent the temporal pattern of bacterial infection, or "leaky gut", thus more continuous chronic low-level LPS experiments are warranted in order to identify mitigation strategies to protect and/or improve mammalian female

reproductive function. *In vitro* experiments also are largely reflective of acute exposures since these levels are likely to be much higher than those that occur *in vivo* or those LPS concentrations that reach the follicular fluid and/or the oocyte. Additionally, endotoxemia that results from compromised intestinal integrity is accompanied by systemic exposure to additional intestinal components, many of which have not been characterized and identified and which may also be dynamic in response to the initiating stressor. Thus greater understanding of resident microbial populations and shifts to these populations will ultimately improve our understanding of the gut-hypothalamic-pituitary-ovarian-uterine axis.

Numerous questions remain to be clarified in our understanding of the impacts of endotoxemia on female fertility include but are certainly not limited to: 1) the level and/or duration required to impact fertility; the initiating insult to the reproductive tract, 2) the immune response within the reproductive tract that responds to endotoxemia, 3) the potential for tolerance to elevated LPS to develop, 4) the actual impact of LPS on the quality of the germ line, 5) potential effects on offspring (trans- and multi-generational) exposed to endotoxemia *in utero*, 6) the contribution or lack thereof of LBP on data derived from *in vitro* experiments. In addition, it is difficult to surmise the duration of metabolic endotoxemia which is likely to vary dependent on the physiological situation, but which ultimately has potential to impact physiological outcomes. Each of these areas are worthy of investigation with relevance to many facets of public health and production animal agriculture.

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Figure 1. Structure of lipopolysaccharide (LPS).

LPS is found on the cell wall of gram-negative bacteria, such as *Escherichia coli*. The lipid A region, depicted in red, elicits the immune response.

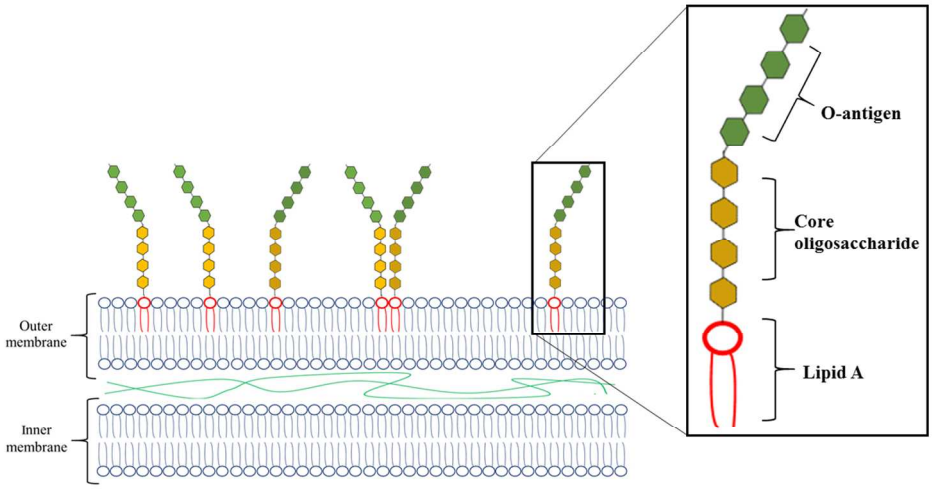

Figure 1. Structure of lipopolysaccharide (LPS).
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Table 1. Summary of LPS studies with effects on reproductive outcomes.

Species	Route	Duration	Dose	Citation	Findings
Ewes	intraamniotic	Single injection	0.1 - 10 mg	Newnham et al., 2005	Fetal death
			400 ng/kg	Battaglia et al., 1997	Ovariectomized. Increased P4, Decreased LH
	intravenous				
		26 h	300 ng/kg	Battaglia et al., 2000	Decreased E2 and LH
		2x (2 week interval)	40 ng/kg	Herman et al., 2010	Decreased LH, Increased prolactin, No effect on FSH
Rats	subcutaneous	Daily injections for 2 or 6 d	2 mg/kg or 20 µg/kg	Shakil et al., 1994	Decreased P4 and E2, Fewer large preovulatory follicles
Rhesus monkey	intravenous	2x daily for 5 d	150 µg	Xiao et al., 1999	Decreased P4
Trout	intraperitoneal	Single injection	3 mg/kg	MacKenzie et al., 2006	Induced apoptosis No effects on germinal vesicle break down
Gilts	permanent cannulas	Single injection	0.5, 1, 2, 3 µg/ kg	Cort, 1986	Abortions
			0.5, 1, 2, 3 µg/ kg	Cort et al., 1986	No change is cycle length. Decreased P4, increased $PGF_2\alpha$
			50, 250, or 1,250 µg	Tuo et al., 1999	No effect on P4 plasma, fetal survival or development. Increased fetal weight and amniotic fluid volume
	intrauterine		36 mg	Wrathall et al., 1978	Abortions
	intravenous				
	mixed into ration		40 mg	Cort et al., 1990	Increased $PGF_2\alpha$ No change in P4

Goats	intrauterine	injected 1 or 2x	0.1 - 5.2 µg/kg	Fredriksson et al., 1985	No hormonal changes
			0.1 - 5.2 µg/kg	Fredriksson et al., 1985	Increased $PGF_2\alpha$, decreased P4, abortions
	intravenous				
Heifers	intrauterine	every 6 h for 10 trts	5 µg/kg	Peter et al., 1990	Decreased E2 production, inhibited LH surge, no change in P4
			5 µg/kg	Peter et al., 1989	Inhibited LH surge and ovulation, caused ovarian cysts
		every 6 h for 9 d	3 µg/kg	Lüttgenau et al., 2016	Premature CL luteolysis, increased $PGF_2\alpha$ metabolites, decreased P4, reduced luteal size and blood flow
	intrauterine or intravenous	Single injection	5 µg/kg	Gilbert et al., 1990	Increased P4, PGF metabolites, cycle length was unchanged
	intravenous	Single injection	5 µg/kg	Suzuki et al., 2001	Decreased LH pulse frequency, E2, increased LH pulse amplitude, P4. Delayed LH surge
			0.01 µg/kg	Kujjo et al., 1995	Ovariectomized. Increased P4, decreased E2 and LH
Lactating Cows	intrauterine	2x @ 5 and 20 DIM	5 µg/kg	Peter et al., 1990a	Increased $PGF_2\alpha$ metabolites
	intravenous (iv) or intramuscular (im)	Single injection	0.5 ug/kg (iv) or 10 ug (im)	Lavon et al., 2008	No change in E2 yet delayed or inhibited ovulation
	intramuscular	Single injection	200 µg	Lüttgenau et al., 2016b	No change in P4, luteal size or luteal blood flow
			10 ug	Lavon et al., 2011	Decreased follicular E2, P4
Non-lactating Cows	intravenous	Single injection	0.5 µg/kg	Herzog et al., 2012	Decreased luteal size and luteal blood flow, Increased P4 and PGE
		6 h	1.0 or 2.5 µg/ kg	Giri et al., 1990	Abortions, increased $PGF_2\alpha$, decreased P4
Mice	intraperitoneal	Single injection	10 µg	Buhimschi et al., 2003	Preterm birth, stillborns

			50 µg/mouse	Fidel et al., 1994	Preterm birth
			0.5 µg/g BW	Ogando et al., 2003	Resorptions
			100 µg/mouse	Bromfield and Sheldon, 2013	Decreased primordial follicle pool, increased follicle atresia
			1.0 µg/g	Aisemberg et al., 2013	Resorptions, decreased P4
			0.4 - 2 mg/kg	Salminen et al., 2008	Preterm birth, stillborns
			2.4 mg/kg	Rounioja et al., 2005	Fetal defects
		Single injection or multiple at 1 - 6 h intervals d 12 - 17	0-100 mg	Kaga et al., 1996	Preterm birth
		2x	10 µg/kg then 120 µg/kg	Xu et al., 2007	Pre-treatment of LPS saved embryonic resorption
	intravenous	Single injection	10 µg	Harper and Skarnes, 1972	Abortions
			$7.5x10^6$ E.coli	Coid et al., 1978	Resorptions
			1.5 - 20 µg	Skarnes and Harper, 1972	Abortions
			2 - 5 ug	Rioux-Darrieulat et al., 1978	Abortions
			0.1 µg	Zhong et al., 2008	Abortions
	intraamniotic	Single injection	0.25 µg	Rounioja et al., 2003	Fetal defects
	intracervical	Single injection		Reznikov et al., 1999	Resorptions

	intrauterine	Single injection	250 µg	Elovitz et al., 2003	Preterm birth
	subcutaneous	Single injection	0.5 mg/kg	Chua et al., 2006	Resorptions, lower fetal weight
			0.25 mg or 0.147 mg	Coid, 1976	Resorptions, lower fetal weight

Abbreviations: iv = intravenous; im = intramuscular; E2 = 17β-estradiol; P4 = progesterone; LH = luteinizing hormone; FSH = follicle stimulating hormone; PGF2α = prostaglandin F2α; LPS = lipopolysaccharide; PGE = prostaglandin E