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Lipopolysaccharide and tumor necrosis factor-alpha alter gene expression of oocytes and cumulus cells during bovine in vitro maturation

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Abstract

Communication between the oocyte and cumulus facilitates oocyte growth, cell cycle regulation, and metabolism. This communication is mediated by direct contact between oocytes and cumulus cells, and soluble secreted molecules. Secreted molecules involved in this process are known inflammatory mediators. Lipopolysaccharide (LPS) is detected in follicular fluid and is associated with reduced fertility, whereas accumulation of inflammatory mediators in follicular fluid, including tumor necrosis factor- α (TNF- α), is associated with female infertility. Maturation of oocytes in the presence of LPS or TNF- α reduces meiotic maturation and the capacity to develop to the blastocyst. Here we evaluated the abundance of 92 candidate genes involved immune function, epigenetic modifications, embryo development, oocyte secreted factors, apoptosis, cell cycle, and cell signaling in bovine cumulus cells or zona-free oocytes after exposure to LPS or TNF- α during in vitro maturation. We hypothesize that LPS or TNF- α will alter the abundance of transcripts in oocytes and cumulus cell in a cell type dependent manner. Exposure to LPS altered abundance of 31 transcripts in oocytes (including *ACVR1V*, *BMP15*, *DNMT3A*) and 12 transcripts in cumulus cells (including *AREG*, *FGF4*, *PIK3IP1*). Exposure to TNF- α altered 1 transcript in oocytes (*IGF2*) and 4 transcripts in cumulus cells (*GJA1*, *PLD2*, *PTGER4*, *STAT1*). Cumulus expansion was reduced after exposure to LPS or TNF- α . Exposing COCs to LPS had a marked effect on expression of targeted transcripts in oocytes. We propose that altered oocyte transcript abundance is associated with reduced meiotic maturation and embryo development observed in oocytes cultured in LPS or TNF- α .

Keywords

gene expression; inflammation; oocyte competence

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

1 | INTRODUCTION

Although the sperm cell and the oocyte contribute equally to the genetic material of the newly formed embryo, the necessary molecular and organelle machinery for growth and regulation of early embryonic development are established during oocyte maturation (Coticchio et al., 2015). Oocyte growth involves the increase of cell mass, proliferation of organelles, accumulation of maternal transcripts, and translational products that are required after fertilization, before activation of the embryonic genome (Li, Zheng, & Dean, 2010; Moussa, Shu, Zhang, & Zeng, 2015; Sirard et al., 1989; Sirard, Richard, Blondin, & Robert, 2006). Additionally, oocyte maturation requires changes to oocyte chromatin, repositioning of organelles, and facilitating meiotic resumption in response to the LH surge (Coticchio et al., 2015). Although infections cause infertility, little is known about how exposure of oocytes and cumulus cells to pathogen molecules alters their molecular signature, including exposure to lipopolysaccharide (LPS) or proinflammatory mediators such as tumor necrosis factor α (TNF- α).

The follicular environment is critical to the developmental competence of the growing oocyte. Oocyte growth and maturation depend on close communication between the oocyte and the surrounding cumulus granulosa cells. Transzonal projections (TZPs) play an important role in facilitating oocyte and cumulus cell communication by direct cell to cell contact before retraction at the time of the LH surge (Albertini, Combelles, Benecchi, & Carabatsos, 2001), whereas oocyte secreted factors act to modify cumulus cell function (Albertini et al., 2001; Matzuk, Burns, Viveiros, & Eppig, 2002; McGinnis, Limback, & Albertini, 2013). The interaction between the oocyte and surrounding cumulus cells is bidirectional; the cumulus cells respond to oocyte signals and the follicular environment to supply the oocyte with essential molecules for proper metabolism and regulation of meiotic maturation (Eppig, Freter, Ward-Bailey, & Schultz, 1983; Gilchrist, Ritter, & Armstrong, 2004; Su et al., 2008).

A large part of the communication between oocytes and cumulus cells is conveyed by molecules that are also known for their role as immune mediators. Oocyte secreted factors act on the granulosa cells to control proliferation, development and expansion (Matzuk et al., 2002). Subsequently, cumulus cells mediate oocyte development by regulating availability of metabolites and signaling factors (Eppig, 1991; Sugiura & Eppig, 2005). In parallel, Toll-like receptors (TLRs) are involved in the immune response to pathogens, and when activated by bacterial LPS increase expression of cytokines such as interleukin (IL)-1 β , IL-6, IL-8, and TNF- α . Granulosa cells of hens, pigs, cows, mice, and humans express TLRs and respond to bacterial LPS to increase expression of proinflammatory cytokines (Alvarez et al., 2006; Bromfield & Sheldon, 2011; Ibrahim, Kramer, Williams, & Bromfield, 2016; Price, Bromfield, & Sheldon, 2013). Uterine infections are common in cows, and subsequently LPS accumulates in follicular fluid, altering the microenvironment of oocyte development (Herath et al., 2007; Piersanti et al., 2019). In vitro maturation (IVM) of bovine cumulus-oocyte complexes (COCs) in the presence of LPS increases failure of the meiotic cell cycle (Bromfield & Sheldon, 2011; Zhao et al., 2017), and subsequently decreases blastocyst development (Soto, Natzke, & Hansen, 2003b). Similarly in human in vitro production (IVP) of embryos, the presence of LPS culture medium reduces clinical

pregnancy (Fishel, Jackson, Webster, & Faratian, 1988; Nagata & Shirakawa, 1996; Snyman & Van der Merwe, 1986). Interestingly, cumulus cells utilize endogenous ligands to TLR-4 to aid in final maturation of the oocyte and cumulus expansion (Shimada et al., 2008). Hyaluronic acid is a major component of the expanded COC that activates TLR-4 signaling to induce expression of IL-6 which acts as an autocrine regulator of cumulus cell function (Liu et al., 2009; Liu, Shimada, & Richards, 2008). The proinflammatory mediator, TNF- α , is upregulated in response to pathogenic components via TLR-4 signaling (Fock, Vinolo, de Moura Sa Rocha, de Sa Rocha, & Borelli, 2007). Infusion of TNF- α into the ovarian bursa in rats inhibits ovulation (Yamamoto et al., 2015), and presence of TNF- α during IVM increases meiotic failure in swine (Ma et al., 2010) and decreases blastocyst development in the cow (Soto, Natzke, & Hansen, 2003a). Elevated follicular fluid TNF- α is associated with decreased oocyte quality in women undergoing IVP (Lee et al., 2000), and patients with polycystic ovarian syndrome (PCOS) have elevated follicular fluid TNF- α concentrations compared with women not affected by PCOS (Amato et al., 2003).

Here we asked if LPS or TNF- α exposure during IVM of bovine COCs affected specific mRNA abundance in oocytes and cumulus cells. We targeted the expression of genes involved in immune function, epigenetic modifications, embryo development, oocyte secreted factors, apoptosis, cell cycle, and cell signaling. We hypothesized that exposure of COCs to LPS or TNF- α during IVM alters the abundance of transcripts in oocytes and cumulus cells in a cell type-dependent manner. These findings may aid in our understanding of how LPS or TNF- α reduces the competence of oocytes to develop to the blastocyst.

2 | RESULTS

The abundance of 92 cellular transcripts was independently evaluated in zona-free oocytes and cumulus cells following IVM for 24 hr in the presence of LPS or TNF- α . Heatmaps for the changes in gene expression compared with control medium are presented for oocytes (Figure 1a,b) and cumulus cells (Figure 1c,d) treated with LPS (Figure 1a,c) or TNF- α (Figure 1b,d). The heatmaps show that LPS had a greater effect on transcript expression than TNF- α , and that the effect of LPS and TNF- α was greater for the oocytes than the cumulus cells.

2.1 | Effect of LPS exposure during IVM on oocyte and cumulus cell gene expression

Collectively, a total of 31 genes in oocytes (Figure 2,3), and 12 genes in cumulus cells (Figure 4) were differentially abundant ($p < .05$) at a given concentration or using contrast analysis compared with those cultured in medium alone. Of the differentially abundant transcripts in oocytes, 24 genes increased and 7 decreased following exposure to LPS. The majority of differentially expressed genes in oocytes required contrast analysis to detect a significant difference from controls, with only 14 genes significantly altered at specific LPS concentrations. Abundance of *ACVR1B*, *ACVR2B*, *ADMA10*, *AY192564*, *BMP15*, *CDC42EP4*, *CDK1*, *CREM*, *DNMT1*, *DNMT3A*, *ESPRP1*, *H2AFX*, *HAS2*, *HDAC1*, *ILF3*, *ITPR2*, *MADD*, *NEDD4*, *NF2*, *POLR2D*, *PTEN*, *S100A1*, *SOX2*, and *XIAP* increased ($p < .05$) in oocytes following LPS exposure compared with control (Figure 2,3); whilst the abundance of *H2AFZ*, *HPSE*, *INHBA*, *MAB21L2*, *PLD2*, *POU5F1*, and *PRKAR2B*

decreased ($p < .05$) in LPS compared with control. A complete list of oocyte transcript abundance can be found in Table S3.

Of the differentially abundant transcripts in cumulus cells, the expressions of six genes increased and those of another six genes decreased following exposure to LPS (Figure 4). Half of the differentially abundant genes in cumulus cells required contrast analysis to detect a significant difference from control, with only six genes significantly altered at specific LPS concentrations. The abundance of *ACKR4*, *ACVR2B*, *ADAM17*, *NLRP5*, *POU5F1*, and *PTGER4* increased ($p < .05$) in cumulus cells following LPS exposure compared with control (Figure 4); whilst the abundance of *ACTA2*, *AREG*, *FGF4*, *HDAC8*, *IL6*, and *PIK3IP1* decreased ($p < .05$) following LPS exposure compared with control. A complete list of cumulus cell transcript abundance can be found in Supplemental Table 4.

2.2 | Effect of TNF- α exposure during IVM on oocyte and cumulus cell gene expression

Abundance of 92 cellular transcripts was independently evaluated in zona-free oocytes and cumulus cells following IVM for 24 hr in the presence of 1, 10, or 100 ng/ml TNF- α and compared with control. A total of only 4 genes in cumulus cells (Figure 5a–d), and 1 gene in oocytes (Figure 5e–h) were differentially abundant ($p < .05$) at a given concentration or using contrast analysis compared with control. Abundance of *GJA1* and *PLD2* transcripts were decreased ($p < .05$) in cumulus cells following TNF- α exposure, whereas *PTGER4* and *STAT1* transcripts were increased (Figure 5a–d). Abundance of *IGF2* transcript was increased ($p < .05$) in oocytes following exposure to 100 ng/ml of TNF- α (Figure 5g). A complete list of oocyte and cumulus cell transcript abundance can be found in Tables S5 and S6.

2.3 | Effect of LPS or TNF- α exposure during IVM on cumulus expansion

Exposure of COCs to LPS during IVM had little effect on complete cumulus expansion, except for an 8.3% reduction ($p < .05$) compared with medium alone following exposure to 1 ng/ml LPS (Figure 6a). Exposure of COCs to TNF- α reduced ($p < .05$) complete expansion by 22.1%, 21.0%, and 9.2% compared with medium alone after treatment with 1, 10, or 100 ng/ml, respectively (Figure 6b).

3 | DISCUSSION

Previous work has demonstrated that maturation of oocytes in the presence of LPS or TNF- α reduces meiotic competence and development to the blastocyst (Bromfield & Sheldon, 2011; Ma et al., 2010; Soto et al., 2003a, 2003b). The present experiment evaluated mRNA expression to assess how oocyte and cumulus cell transcript abundance was affected by exposure to LPS or TNF- α . Here, we hypothesized that exposure of COCs to LPS or TNF- α during IVM would alter the abundance of transcripts in oocytes and cumulus cell in a cell type-dependent manner which may be responsible for reducing oocyte developmental competence. We observed an effect of LPS exposure on oocyte and cumulus cell transcript abundance, with minimal effects of TNF- α exposure on the abundance of targeted genes. However, we did not evaluate the impact of LPS or TNF- α exposure during IVM on embryo

development, and as such direct mechanistic links between oocyte transcription and embryo development cannot be drawn from these current studies.

3.1 | Effect of LPS treatment during IVM on oocyte gene expression

Oocyte transcript availability is critical to bovine embryonic development before activation of the embryonic genome at the 8-cell stage and subsequent transcription of new mRNA (Graf et al., 2014). Exposure of COCs to LPS altered oocyte transcript abundance, with most transcripts being increased. Although the oocyte is considered to be transcriptional inactive during maturation, displaying a steady decline in transcript abundance over time, some transcripts do indeed increase in abundance between the germinal vesicle and MII stage of development (Reyes, Chitwood, & Ross, 2015), including those involved in oocyte maturation such as *CCNB1*, *WEE2*, *FBXO43*, and *MELK*. The changes in oocyte transcript abundance observed here may be associated with either increased oocyte transcription, decreased transcript degradation based on transcript polyadenylation (Su et al., 2007), or transcript transport from cumulus cells via transzonal projections (Macaulay et al., 2016). Exposure of COCs to LPS increased the abundance of oocyte genes involved in oocyte maturation and embryonic development including *BMP15* and *SOX2* (Gilchrist, Lane, & Thompson, 2008; Masui et al., 2007). A member of the TGF β superfamily, BMP-15 is a paracrine factor secreted by the oocyte that regulates cumulus granulosa cell function, including proliferation. Along with GDF-9 (Dong et al., 1996; Spicer, Aad, Allen, Mazerbourg, & Hsueh, 2006), another oocyte secreted factor, BMP-15 regulates oocyte maturation, cumulus cell metabolism, apoptosis, and expansion (Coticchio et al., 2015; Gilchrist et al., 2008; Matzuk et al., 2002). The function of SOX2 remains to be fully elucidated in the bovine, but it is known to be involved in pluripotency of mouse embryonic stem cells (Masui et al., 2007) and is essential for embryo development to the blastocyst stage in the rodent (Pan & Schultz, 2011). Similarly, in cattle, zygotes injected with *SOX2* siRNA have a decline in development to the blastocyst stage (Goissis & Cibelli, 2014). The present experiment did not carry mature oocytes forward through fertilization and embryo development which would be critical to recapitulate the work of others that have demonstrated that exposure of bovine COCs to LPS reduces the proportion of oocytes to reach the blastocyst stage of development at Day 8 from approximately 28–12% (Soto et al., 2003b).

The abundance of transcripts for genes involved in transcription and epigenetic regulation was affected in oocytes after exposure to LPS. Transcripts for *DNMT1*, *DNMT3A*, *ILF3*, *H2AFX*, *HDAC1*, and *POLR2D* increased in oocytes after LPS exposure, whereas *H2AFZ* transcript was depleted compared with control oocytes. Epigenetic reprogramming of the oocyte and the subsequent embryo is essential for embryonic development and heritable changes to the epigenome (Bromfield, Messamore, & Albertini, 2008). Epigenetic programming of the newly formed embryo genome and subsequent regulation of transcription include CpG DNA methylation and posttranslational acetylation, phosphorylation, and methylation of histones (Beaujean, 2014). The process of embryonic genome activation involves the erasure and settlement of epigenetic marks, as well as nonerasure and preservation of methylation in some areas within the genome (Chong & Whitelaw, 2004). This process may lead to epigenetic inheritance and potentially change the

fate of the embryo and subsequent phenotype of the offspring. The differential abundance of oocyte transcripts involved in epigenetic programming identified here could potentially disrupt this process and change how the epigenome is established in the developing embryo. The DNA methyltransferase DNMT1 maintains CpG methylation, whereas DNMT3A facilitates de novo CpG methylation. The process of DNA demethylation can either occur actively by 10–11 translocation methylcytosine dioxygenase (TET)1 and TET2 which were not evaluated here, or passively by decreasing the expression of *DNMT1* and *DNMT3A* (Hackett et al., 2013). Interestingly, both *DNMT1* and *DNMT3A* transcript expression increased in oocytes following LPS exposure, suggesting a possible dysregulation of CpG demethylation during oocyte maturation. The extent of either oocyte or embryo CpG methylation needs to be quantified in response to LPS exposure to determine if changes in transcript abundance have any direct effect on the epigenetic status of the embryo. Among other genes involved in transcription regulation, LPS exposure increased the abundance of oocyte *POLR2D* and *ILF3* transcript. The gene *POLR2D* encodes for a subunit of RNA polymerase II responsible for mRNA synthesis, suggesting LPS exposure could increase the oocytes ability for gene transcription after LPS exposure. In addition, *ILF3* is a double-stranded RNA binding protein that regulates gene expression and stabilizes mRNAs, further suggesting that LPS-mediated increased oocyte transcription. The abundance of oocyte *H2AFX* transcript was also increased after LPS exposure, which is an H2A histone family member involved in the regulation of gene expression and the cellular response to DNA damage and stress (Cloutier et al., 2015). Interestingly, the abundance of oocyte *H2AFZ* was decreased following exposure to LPS. H2AFZ is also a member of the H2A histone family, and depletion in mouse embryos results in a failure to develop, supporting the theory that appropriate chromatin structure is crucial for embryonic development (Faast et al., 2001). In addition, we observed an LPS-mediated increase in transcript abundance of the histone deacetylase *HDAC1*, suggesting possible dysregulation of histone posttranslational modifications. Interestingly, it has been reported that oocytes matured in the presence of LPS have reduced DNA methylation (5-mC) and histone H3 lysine 9 dimethylation (H3K9me2), whereas histone H3 lysine 4 dimethylation (H3K4me2) is increased (Zhao et al., 2017). It is interesting to postulate if the altered transcripts involved in epigenetic programming observed here may affect enduring epigenetic marks that could affect the phenotype of subsequent offspring.

Exposure to LPS increased the abundance of transcripts for genes associated with apoptosis (*MADD*, *NEDD4*, and *XIAP*) in oocytes. Neural precursor cell expressed developmentally downregulated protein 4 (NEDD4) is a ubiquitin ligase that regulates membrane channels and receptors by controlling their density and availability on the cell surface (Cao et al., 2008). *NEDD4* null mice present delayed embryonic development and lethality due to the role of NEDD4 in regulating insulin and insulin growth factor 1 (IGF-1) signaling (Cao et al., 2008). *MADD* is involved in the propagation of TNF- α proapoptotic signals (Schievella, Chen, Graham, & Lin, 1997) and is present in human GV stage oocytes (P. Zhang et al., 2007). While increased expression of *MADD* is critical to cancer cell survival, this is due to protein phosphorylation, and it is unclear what status MADD protein exists in the embryo (Jayarama et al., 2014). X-linked inhibitor of apoptosis protein (XIAP) is a protein that inhibits apoptosis and prevents cell death, even in response to TNF- α signaling (Duckett et

al., 1998). Collectively, it is unclear if the variable abundance of these apoptosis-related transcripts in oocytes ultimately affects cell survival in the embryo. Analysis of blastomere apoptosis in response to oocyte LPS exposure is warranted to further describe any downstream effects of altered transcript abundance.

Exposure to LPS increased oocyte transcript abundance of genes encoding the activin receptors, *ACVR1B* and *ACVR2B*. Oocyte development and follicle integrity increased in the bovine in response to activin supplementation in vitro (McLaughlin & Telfer, 2010; McLaughlin, Bromfield, Albertini, & Telfer, 2010). However, these data are in conflict with our proposed hypothesis that LPS exposure would decrease oocyte competence if we assume increased transcript abundance of activin receptor would mediate increased activin signaling.

3.2 | Effect of LPS treatment during IVM on cumulus cell gene expression

Of the 12 cumulus cell transcripts altered by LPS exposure only half increased, in contrast to oocyte transcript abundance in which 24 genes showed increased expression with exposure to LPS. Exposure to LPS decreased the abundance of cumulus cell transcript for the actin cytoskeleton gene, *ACTA2*, in a similar manner in which retinoic acid decreased expression in cumulus cells of the camel whereas simultaneously promoting maturation of oocytes (Saadeldin et al., 2019). Fibroblast growth factor-4 is involved in cell proliferation and developmental processes, and here transcript abundance of *FGF4* decreased in cumulus cells following LPS exposure. It is unclear the exact role cumulus FGF signaling has on oocyte development, but inhibition of FGF receptor signaling decreases subsequent embryo development (K. Zhang & Ealy, 2012). Cumulus cell expression of *Pik3ip1*, a negative regulator of PI3K, is lower in cumulus cells of small antral follicles compared to large antral follicles of the mouse (Wigglesworth, Lee, Emori, Sugiura, & Eppig, 2015). Interestingly, we observed a decreased *PIK3IP1* transcript abundance in cumulus cells following exposure to LPS, suggesting a developmental perturbation in these cells. The abundance of cumulus *POU5F1* transcript increased following exposure to LPS, which encodes the OCT-4 transcription factor that regulates the pluripotency of the embryo (Masui et al., 2007; Niwa, Miyazaki, & Smith, 2000). The transcript for the prostaglandin E2 receptor, *PTGER4*, has been previously reported to be expressed in bovine cumulus cells (Nuttinck et al., 2011), and its abundance increased in response to LPS exposure. Indeed, prostaglandin E2 signaling is vital to meiotic progression, cumulus expansion, and embryonic development (Nuttinck et al., 2011), and inhibition of this signaling system results in failure of these various cellular progressions. Finally, we hypothesized that the abundance of cumulus cell *IL6* transcript would be increased in response to LPS; however, the data suggest that only a mild expression of *IL6* transcript was present in cumulus cells and that statistically this expression was decreased in response to LPS. We view this particular result with some skepticism as the data appears skewed due to a single high abundance outlier in the control samples. We have previously demonstrated a robust IL-6 response of bovine mural granulosa cells to LPS (Bromfield & Sheldon, 2011), and others have shown increased *Il6* expression in murine COC in response to LPS and hyaluronan (Shimada et al., 2008).

3.3 | Effect of TNF- α treatment during IVM on gene expression of oocytes and cumulus cells

TNF- α is a potent cytokine with contradictory roles, it is involved in the proinflammatory response and can further activate inflammation, but it can also exhibit a role in controlling the extent of the immune response and inflammation (Akdis et al., 2016). Exposure of COCs to TNF- α increased the abundance of only one gene in oocytes, *IGF2*. The imprinted *IGF2* gene encodes for a growth factor important during embryo development in the bovine (Gebert et al., 2006). Tribulo, Siqueira, Oliveira, Scheffler, and Hansen (2018) reported that *IGF2* expression in the bovine endometrium is maximal on the day of estrus, suggesting potential effects on the oocyte, sperm, or fertilization. In parallel, IVM of bovine oocytes in fatty-acid free medium expressed increased *IGF2* transcript abundance in both oocytes and subsequent embryos compared to oocytes cultured in fatty-acid enriched maturation medium (Warzych, Wrenzycki, Peippo, & Lechniak, 2007). Interestingly, microglia increased *IGF2* expression to prevent TNF- α -mediated apoptosis (Nicholas, Stevens, Wing, & Compston, 2002), which has also been reported to occur in bovine embryos (Loureiro, Brad, & Hansen, 2007). It is possible that increased expression of *IGF2*, which is known to enhance cell proliferation and survival by signaling through the IGF1 and IGF2 receptors, in COCs exposed to TNF- α occurred as a protective mechanism to prevent cell death. Exposure to TNF- α decreased the cumulus cell expression of *GJA1* and *PLD2*. Connexin 43 (*GJA1*) is a component of cellular gap junctions found in abundance in granulosa-granulosa complexes (Johnson, Redmer, Reynolds, & Grazul-Bilska, 1999; Nuttinck et al., 2000). These granulosa gap junction complexes are fundamental for the developing oocyte in the avascular follicular environment, and mediate signal and metabolite transmission from the follicular fluid and granulosa to the growing oocyte (Albertini et al., 2001). Conversely, oocytes exposed to TNF- α increased *PTGER4* expression in cumulus cells in a similar fashion to exposure to LPS, as observed in this experiment. Interestingly, *STAT1* expression increased in cumulus cells after exposure to TNF- α which is involved in TNF- α induced apoptosis signal transduction (Jiang et al., 2017), and maybe involved in mediating cumulus cell apoptosis here, but this remains to be evaluated.

3.4 | Speculations for LPS or TNF- α effects on postnatal development

Previous data suggest that exposure of COCs to either LPS or TNF- α reduces progression to the blastocyst stage of development by 16 or 8 percentage points, respectively (Soto et al., 2003a, 2003b). Our work here and by others have demonstrated alterations in the machinery and status of the epigenome of the oocyte following exposure to LPS (Zhao et al., 2017). We hope that future experimentation will be able to evaluate the epigenetics and phenotype of cattle conceived following oocyte exposures. Indeed, moderate maternal protein restriction in mice for 3.5 days before conception significantly altered the phenotype of offspring to 1 year of age (Watkins, Lucas, Wilkins, Cagampang, & Fleming, 2011). Previous reports suggest that the presence of serum in maturation medium could reduce embryo development rates (Del Collado et al., 2015; Sagirkaya et al., 2007), as such it will be important to consider serum alternatives in future work as transcript abundance may be influenced by the specific maturation medium utilized. As such future experiments should consider using in vivo derived oocytes from animals with naturally or induced uterine infection, or evaluations using serum-free IVM medium. Phenotypic data from offspring conceived by dams with

uterine infections may aid in elucidating long-term effects of oocyte exposure to LPS or TNF- α . Alternatively, negative effects of LPS or TNF- α on the oocyte may be resolved during embryo growth due to the plasticity of the early developing embryo and consequently have little to no impact on the resultant offspring.

4 | MATERIALS AND METHODS

4.1 | Cumulus oocyte complex isolation and in vitro maturation

Ovaries were collected at a local abattoir and transported to the laboratory at 23°C in a 0.9% saline solution containing 100 IU/ml penicillin and 100 μ g/ml streptomycin (Caisson Labs, Smithfield, UT). Ovaries were processed within 4 hr of collection, and COCs were collected by bisecting 3–8 mm follicles using a sterile scalpel blade and vigorously washing the ovary in oocyte collection medium (BoviPRO, MOFA Global, Verona, WI). The medium containing COCs and cells was then passed through a 100 μ m filter (Corning Falcon, Tewksbury, MA) to collect the COCs, which were retrieved using a wiretrol pipette (Drummond Scientific Company, Broomall, PA) under a dissecting stereo microscope. An average of 10–15 COCs, having at least three layers of cumulus cells and an oocyte containing a homogeneous cytoplasm, were collected from each ovary and COCs from all ovaries were pooled as a single replicate. The COCs were washed three times in oocyte collection medium and groups of 10–15 COCs were matured in organ culture dishes (Corning Falcon) for 22–24 hr in 1 ml of oocyte maturation medium (Medium 199, 0.25 mM pyruvate, 10% fetal calf serum, 2 μ g/ml estradiol, 1% insulin/transferrin/sodium selenite solution, 50 IU/ml penicillin, 50 μ g/ml streptomycin, and 0.4 mM L-glutamine [all Thermo Fisher Scientific, Hampton NH] and 20 μ g/ml FSH [as Folltropin-V, Reproduction Resources, Walworth WI]) as previously described (Bromfield & Sheldon, 2011). Oocyte maturation was performed in humidified air containing 5% CO₂ at 38.5°C, in control oocyte maturation medium, or medium containing 1, 10, 100, 1000, or 10,000 ng/ml ultrapure LPS from *E. coli* 0111:B4 (InvivoGen, San Diego, CA), or 1, 10, or 100 ng/ml recombinant bovine TNF- α (R&D Systems, Minneapolis, MN). Every treatment replicates of COCs with either LPS or TNF- α was always carried out with a corresponding vehicle control treatment.

Following maturation, the COCs were washed three times in fresh Dulbecco's phosphate-buffered saline (DPBS, Thermo Fisher Scientific) containing 0.1% polyvinylpyrrolidone (PVP; Kodak, Rochester, NY). The COCs were examined and considered fully expanded when all cumulus layers had progressed away from the oocyte. Cumulus cells were removed from oocytes using 1000 U/ml of hyaluronidase in HEPES-TALP. The zona pellucida of denuded oocytes were removed with 0.1% protease from *Streptococcus griseus* (Sigma-Aldrich, St. Louis, MO) in DPBS, and zona-free oocytes were washed three times in fresh DPBS-PVP. Cumulus cells were obtained by centrifugation of the hyaluronidase medium following the removal of denuded oocytes. Zona-free oocytes from a single replicate were pooled together for analysis, as were all cumulus cells from a single replicate. Pooled zona-free oocytes and pooled cumulus cells were separately suspended in 350 μ l RLT buffer and stored at –80°C.

Each treatment was performed in four to six replicates per treatment. A replicate was defined as a single IVM procedure containing one dish per treatment. A total of 542 COCs were

subjected to IVM in the presence of LPS, 378 in the presence of TNF- α and 194 in control medium.

4.2 | Multiplex fluidigm analysis of oocyte and cumulus cell gene expression

Zona-free oocyte and cumulus cell RNA extraction was performed independently using the RNeasy Micro kit with DNase treatment (Qiagen) according to the manufacturer's instructions.

The Fluidigm quantitative polymerase chain reaction (qPCR) microfluidic device Biomark HD system (Fluidigm Co., San Francisco, CA) was used for gene expression assays at the University of Miami Miller School Of Medicine, Center for AIDS Research (CFAR). Primers were designed by Fluidigm Delta Gene assays (Fluidigm). Primer validation was performed using complementary DNA obtained from bovine oocytes, endometrium, peripheral white blood cells, granulosa cells, and ovarian cortex. All primers (Table S1) were validated using the Fluidigm primer quality control criteria applied to serially diluted test cDNA: $r^2 = 0.97$, the efficiency of 80–130%.

A total of 96 primers for Fluidigm analysis (Table S1 and S2) were validated for 4 housekeeping genes, 6 oocyte-specific genes, 29 genes involved in cell growth, and proliferation, 10 genes involved in the regulation of cell cycle, 19 genes related to immune response, 9 genes involved in control of gene expression and DNA modifications, 7 genes associated to apoptosis and cell death, and 12 other genes of interest.

Target specific preamplification after reverse transcription (RT-STA) was performed on all samples using the Preamp and Reverse Transcription Master Mix (Fluidigm) for 20 cycles. The procedure for real-time RT-PCR using the BioMark HD system (Fluidigm) (Dominguez et al., 2013) was as follows; primer sets and samples were loaded on an integrated fluidic circuit (IFC) plate and placed into a controller that prepares the nanovolume reactions. Real-time RT-PCR was carried out on the BioMark HD system. A total of 40 PCR cycles were performed using EvaGreen (Bio-Rad, Hercules CA) chemistry on the 96.96 dynamic arrays IFC developed by the manufacturer. Cycle threshold (C_t) values were calculated by the Fluidigm real-time PCR analysis software. The cutoff for undetectable genes was set at $C_t > 29$. The geometric mean of the four housekeeping genes was calculated and fold change relative to the geometric mean of the housekeepers was calculated for the 92 genes of interest using the $2^{-\Delta C_t}$ method.

4.3 | Statistical analysis

SPSS ver. 20.0 (IBM, New York, NY) and SAS ver. 9.4 software package (SAS Institute Inc., Cary, NC) was used for statistical analyses. Data obtained from PCR were analyzed using the generalized linear model of SPSS using treatment or concentration as the fixed effect. When multiple concentrations of a single treatment were used, the analysis was followed by a Dunnett's posthoc test comparing individual treatments to the control. Contrasts were performed grouping multiple concentrations of TNF- α or LPS and compared to the medium alone control, specifically increasing concentrations were combined and then compared to the control. Heatmaps and hierarchical clustering were generated using online

ClustVis tools (Metsalu & Vilo, 2015). The generalized linear mixed model procedure of SAS (GLIMMIX) was used to evaluate the effects of treatment on the percent of COCs to expand. Each COC was considered as an individual observation and expansion was considered as a binary variable (0 = did not expand; 1 = expanded). Treatment was considered as a fixed effect and replicate was used as a random effect. A $p < .05$ was considered statistically significant.

5 | CONCLUSIONS

We examined changes in transcript abundance in oocytes and cumulus cells when COCs were matured in the presence of LPS or TNF- α . The results provide evidence that LPS had a greater effect on transcript abundance than TNF- α , and the largest effect was that of LPS on oocyte transcripts. We assume that alterations to the abundance of maternal transcripts present in the oocyte will have some impact on pregnancy success or subsequent offspring phenotype, however, these specific studies will be required to derive a direct link between oocyte gene expression and pregnancy success. Further experimentation is required to define alterations to embryonic epigenetic programming, fetal development and offspring phenotype. These data highlight a need to understand the impact of bacterial components and inflammatory mediators on reproductive success in species where infection could have negative implications on reproductive performance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Comparative effect of lipopolysaccharides (LPS) or tumor necrosis factor (TNF) exposure during in vitro maturation on gene expression. Cumulus oocyte complexes were cultured in the presence of ultrapure LPS (a, c; ng/ml), or recombinant bovine TNF- α (c, d; ng/ml) for 24 hr. Expression of 92 genes was evaluated in oocytes (a, b) and cumulus cells (c, d) independently. Heatmaps were generated using the log² fold change of each gene compared with control cultures

FIGURE 2.

Effect of lipopolysaccharides (LPS) during IVM on oocyte gene expression. Cumulus oocyte complexes were cultured in the presence of 1, 10, 100, 1000 or 10,000 ng/ml of ultrapure LPS or control medium alone for 24 hr. Zona free oocytes were subjected to gene expression analysis. Data are presented as relative expression to the arithmetic mean of housekeeping genes (*SDHA*, *GAPDH*, *ACTB*, and *YWHAZ*). Each replicate is represented by a single circle, the mean for each treatment is represented by the horizontal line. Data were analyzed by generalized linear model followed by a Dunnett's posthoc test comparing individual treatments with the control. Contrasts were performed grouping multiple concentrations of LPS and compared with the control. Significant ($p < .05$) contrasts are indicated by the horizontal lines above the combined concentrations. * indicates a significant effect for the specific dose of LPS ($p < .05$) compared with the control

FIGURE 3.

Effect of lipopolysaccharides (LPS) during IVF on oocyte gene expression. Cumulus oocyte complexes were cultured in the presence of 1, 10, 100, 1000 or 10,000 ng/ml of ultrapure LPS or control medium alone for 24 hr. Zona free oocytes were subjected to gene expression analysis. Data are presented as relative expression to the arithmetic mean of housekeeping genes (*SDHA*, *GAPDH*, *ACTB*, and *YWHAZ*). Each replicate is represented by a single circle, the mean for each treatment is represented by the horizontal line. Data were analyzed by generalized linear model followed by a Dunnett's posthoc test comparing individual treatments with the control. Contrasts were performed grouping multiple concentrations of LPS and compared with control. Significant ($p < .05$) contrasts are indicated by the horizontal lines above the combined concentrations. * indicates a significant effect for the specific dose of LPS ($p < .05$) compared with the control

FIGURE 4.

Effect of lipopolysaccharides (LPS) during IVM on cumulus cell gene expression. Cumulus oocyte complexes were cultured in the presence of 1, 10, 100, 1000, or 10,000 ng/ml of ultrapure LPS or control medium alone for 24 hr. Cumulus cells were subjected to gene expression analysis. Data are presented as relative expression to the arithmetic mean of housekeeping genes (*SDHA*, *GAPDH*, *ACTB*, and *YWHAZ*). Each replicate is represented by a single circle, the mean for each treatment is represented by the horizontal line. Data were analyzed by generalized linear model followed by a Dunnett's posthoc test comparing individual treatments with the control. Contrasts were performed grouping multiple concentrations of LPS and compared with the control. Significant ($p < .05$) contrasts are indicated by the horizontal lines above the combined concentrations. * indicates a significant effect for the specific dose of LPS ($p < .05$) compared with the control

FIGURE 5.

Effect of tumor necrosis factor α (TNF- α) during IVM on oocyte or cumulus cell gene expression. Cumulus oocyte complexes were cultured in the presence of 1, 10, or 100 ng/ml of recombinant bovine TNF- α or control medium alone for 24 hr. Cumulus cells (a–d) and zona-free oocytes (e–h) were subjected to gene expression analysis. Data are presented as relative expression to the arithmetic mean of housekeeping genes (*SDHA*, *GAPDH*, *ACTB*, and *YWHAZ*). Each replicate is represented by a single circle, the mean for each treatment is represented by the horizontal line. Data were analyzed by generalized linear model followed by a Dunnett's posthoc test comparing individual treatments with the control. Contrasts were performed grouping multiple concentrations of TNF- α and compared with the control. Significant ($p < .05$) contrasts are indicated by the horizontal lines above the combined concentrations. * indicates a significant effect for the specific dose of TNF- α ($p < .05$) compared with the control.

FIGURE 6.

Effect of lipopolysaccharides (LPS) or tumor necrosis factor α (TNF- α) exposure during in vitro maturation on cumulus oocyte complex (COC) expansion. Cumulus oocyte complex expansion was evaluated following culture in the presence of (a) ultrapure LPS (1, 10, 100, 1000, or 10,000 ng/ml), (b) recombinant bovine TNF- α (1, 10, or 100 ng/ml), or control medium alone for 24 hr. Each replicate is represented by a single circle, the mean for each treatment is represented by the horizontal line. The percentage of COCs to fully expand was analyzed using the generalized linear mixed models procedure. * indicates a significant effect for the specific dose ($p \leq .05$) compared with the control