Investigating the role of inflammation on bovine oocyte health and development

by

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Abstract

In this study we investigated the effects of inflammation resulting from LPS exposure on oocyte health, maturation and subsequent developmental competence. Inflammation caused by infection in cattle is known to have negative impacts on reproduction and subsequent production efficiency. However, the mechanisms of action and the extent of damage remain to be well defined. Lipopolysaccharide (LPS) is a component of the bacterial cell wall of gram-negative bacteria such as *E. coli* and is known as the activator of the toll-like receptor 4 (TLR4) pathway and subsequent immune response. We hypothesize that granulosa cells are able to directly respond to an LPS challenge and that the response may perturb oocyte health and developmental competence.

Here we explore the ability of ovarian cells to respond to LPS via an inflammatory immune response in addition to the impacts of inflammation on the health and maturation of bovine cumulus-oocyte-complexes (COCs) treated with LPS and/or the TLR4 inhibitor, TAK-242, as a means of activating an innate immune response and to elucidate the potential role the TLR4 pathway plays in this process.

In conclusion, we found that ovarian cells are capable of mounting an inflammatory immune response in the presence of LPS via an increase in the expression of inflammatory cytokine markers. Additionally, LPS treated COCs demonstrated decreases in oocyte health marker expressions and poorer maturation outcomes.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations

ADCY3   adenylate cyclase 3

ANOVA   Analysis of variance

BSA   Bovine serum albumin

cAMP   cyclic adenosine monophosphate

CCL2   Chemokine ligand 2

CD14   Cluster of differentiation 14

cDNA   Complementary deoxyribonucleic acid

cGMP   cyclic guanosine monophosphate

COC   Cumulus oocyte complex

CTCF   Corrected total cell fluorescence

CYP19A1 Cytochrome P450 19A1

DCHF-DA   2'-7'-Dichlorodihydrofluorescein diacetate

DMEM/F-12   Dulbecco modified eagle medium: nutrient mixture F-12

DNA   Deoxyribonucleic acid

EFAF   Essentially fatty acid free

ERK1/2   Extracellular signal regulated protein kinases 1 and 2

GV   Germinal vesicle

GVBD   Germinal vesicle breakdown

ICM   Inner cell mass

IL	Interleukins
IL-6	Interleukin 6
IFNT	Interferon tau
IVF	*in vitro* fertilization
JNK	c-Jun terminal kinase
LH	Luteinizing hormone
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MD2	Myeloid differentiation 2
MYD88	Myeloid differentiation response gene 88
NADH	Nicotinamide adenine dinucleotide plus hydrogen
NFkb	Nuclear-factor-kappa-light-chain-enhancer of activated B cells
NPCC	natriuretic peptide precursor type C
p38	p38 mitogen-activated protein kinase
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
PD3A	phosphodiesterase 3A
PFA	Paraformaldehyde
qPCR	Real-time quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species

SD	Standard deviation
StAR	Steroidogenic acute regulatory protein
SOD	Superoxide dismutase
SOF	Synthetic oviductal fluid
Th1	T helper cell 1
TIR	Toll/interleukin receptor
TIRAP	Toll/interleukin receptor domain-containing adaptor protein
TLR	Toll like receptors
TRAM	Translocation associated membrane protein
TRIF	TIR-domain containing adaptor inducing interferon β
TNFα	Tumor Necrosis Alpha pg12
VEGF	Vascular endothelial family

I.   Literature Review

Inflammation and Reproduction in Cattle

The efficient production of cattle relies on maintaining optimal reproductive performance. Reproductive efficiency is one of the most important determinants of profitability in the cattle industry. Over the past few decades, the reproductive efficiency of cattle, particularly in the dairy industry, has declined across the world as producers select for cattle with increased milk production, a trait that is linked to increased infertility (Lucy 2001). It has been known for a long time that inflammation has negative correlations with reproductive outcomes. The exact nature and mechanisms of how these various infections impact reproduction are currently the focus of many research programs.

Endometritis is common in post-partum cows and is associated with impaired reproductive performance, including reduced first service conception and an increased risk of reproductive culling (Gilbert 1992). Endometritis is defined as an inflammation of the endometrium. It has been clearly demonstrated that endometritis results in reduced first-service conception rates and overall pregnancy rates (Gilbert et al., 1998; Hammon et al., 2001; Kasimanickam et al., 2004; Barlund et al., 2008; Cheong et al., 2011). Recently, and of particular concern, is that endometritis with both clinical and sub-clinical or asymptomatic (no cytological evidence) presentations have been shown to negatively affect reproductive outcomes (Dubuc et al., 2010a; Dubuc et al., 2010b).

Another common and economically significant inflammatory affliction in cattle is mastitis. Mastitis is defined as an inflammation of the mammary gland. The relationship between mastitis and reproduction has been well described. The literature supports mastitis negatively affecting reproduction in cattle through decreased pregnancy rates, alterations in the estrus cycle (Moore et al., 1991), early embryonic mortality (Risco et al., 1999), prolonged days open (Barker et al., 1998; Gunay and Gunay 2008), higher service numbers and decreased conception rates (Kelton et al., 2001; Hertl et al., 2010).

It is clear that inflammation negatively affects reproduction, most likely through a variety of mechanisms. One potential mechanism is through inflammation leading to a decrease in oocyte health and developmental potential. It has been shown that cows experiencing inflammation are less likely to ovulate the first dominant follicle (Sheldon et al., 2002). Bacterial infections or simulated bacterial infections have also been shown to decrease follicular growth (Peter and Bosu, 1988; Sheldon et al., 2002; Williams et al., 2008). One extremely interesting observation that was made while recovering embryos from cows that had previously been diagnosed with endometritis and those free of the condition was that the rate of unfertilized oocytes tended to be higher in the group that had previously had endometritis (Cerri et al., 2009). This finding is supportive of inflammation having a localized negative impact of oocyte health and developmental competence. The goal of this work is to produce new knowledge on the effect of inflammation caused by bacterial agents on oocyte health.

Oogenesis and Meiosis

Development of the oocyte is not limited to any one phase of reproductive development, but rather occurs throughout a female's reproductive lifetime. Oocyte development can be categorized into four phases, primordial germ cell mitotic division, nuclear arrest, cytoplasmic growth, and meiosis resumption (Jamnongjit and Hammes, 2005). The mitotic divisions of primordial germ cells occur prior to birth, establishing the lifetime reserve of oocytes for future reproduction by dividing into oogonia, which then divide further into primary oocytes (Jamnongjit and Hammes, 2005). As development continues, the primary oocytes enter into a nuclear arrest, often referred to as dictyate, at meiotic prophase I to prevent further maturation until puberty and subsequent ovulation onset (Jamnongjit and Hammes, 2005). This nuclear arrest is maintained by the presence of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP). cGMP is produced by cumulus cells that are stimulated by a secretion of natriuretic peptide precursor type C (NPCC) (Zhang et al., 2010) and helps maintain the necessary cAMP levels by passing through the existing gap junctions to block the hydrolysis of cAMP into phosphodiesterase 3A (PD3A) (Norris et al., 2009; Vaccari et al., 2009). High cAMP is generated within granulosa cells by the stimulation of adenylate cyclase from activated G-protein coupled receptors (Diluigi et al., 2008; Mehlmann et al., 2002). Mouse oocytes that were deficient for adenylate cyclase 3 (ADCY3) demonstrated a return to meiosis (Masciarelli et al., 2004), thus demonstrating its importance in maintaining meiotic arrest prior to maturation. During this phase of nuclear arrest, the

oocyte undergoes the process of cytoplasmic maturation. Throughout this phase of maturation, proteins, mRNA, and nutrients accumulate in the cytoplasm in an effort to create an environment that is conducive to embryonic development (Brevini-Gandolfi et al., 1999; Piccioni et al., 2005; Watson, 2006). After cytoplasmic maturation, the nuclear arrest is ended once the female experiences a surge in luteinizing hormone (LH) that coincides with the beginning of sexual maturity; i.e. puberty. The release of LH is thought to cause a dissociation between cellular gap junctions of the oocyte and its surrounding granulosa cells (Piontkewitz et al., 1993; Sela-Abramovich et al., 2006; Norris et al., 2008). Subsequently, this surge allows for the removal of granulosa inhibitors on the meiotic divisions of the oocyte, specifically cAMP and cGMP (Norris et al., 2009). During this time the nucleus will migrate to the peripheral portion of the oocyte. At this point the primary oocyte can develop into a secondary oocyte surrounded with proliferating granulosa cells and the zona pellucida (Jamnongjit and Hammes, 2005). This secondary development is also denoted by the presence of a polar body that forms after the separation of the chromatids, of which one half are stored inside the polar body, while the other half remain in the oocyte (Jamnongjit and Hammes, 2005). At this point, the oocyte has achieved maturity and is ready for fertilization. Throughout these processes the follicular environment plays an important role. Whereas it is known that an inflammatory environment perturbs these processes, the specific effects on oocyte health are not well described.

Embryogenesis in cattle

Following fertilization of the oocyte in the uterine tube, the resulting zygote is transported toward the uterus and undergoes the first mitotic cleavage to become a developing embryo. The embryo divides sequentially from the 2-cell, 4-cell, and 8-cell stages while moving through the uterine tube towards the uterus (Lonergan et al., 2016). By day 3-4 post-fertilization, the morula stage is reached and the embryo has arrived at the uterus (Lonergan et al., 2016). At this point, the embryo consists of a mass of at least 16 cells that have not yet differentiated. Around days 5-7 post-fertilization, the blastocyst stage is reached (Lonergan et al., 2016; Gilbert, 2000). During its formation, the cells are compacted to one side of the embryo in order to form the inner cell mass (ICM) as a result of the formation of a fluid filled cavity (blastocoel), along with the development of a trophoblast cell population (outer cell mass) (Gilbert, 2000). The ICM will eventually form the tissues of the fetus and the trophoblast cells will form the placental tissues (Marikawa and Alarcón, 2009). As the blastocoel continues to expand, the blastocyst will hatch and break through the zona pellucida around day 9-10 (Gilbert, 2000). Once the blastocyst has hatched, the conceptus will begin to elongate around day 15-18 of embryo development (Degrelle et al., 2005), increasing in size more than 1000-fold and covering the uterine horn (~ 60 mm) (Hansen et al., 2017; Betteridge et al., 1980; Grealy et al., 1996). Corresponding with this time the elongated embryo will produce an endocrine signal in order to establish maternal recognition of pregnancy and attachment to the uterine wall. It is important to note that the maternal immune system is suppressed locally during the pre-

attachment phase of pregnancy in order to promote embryo survival and successful attachment (Walker et al., 2010; Croy et al., 1988; Fisher et al., 1985). Secretions originating from the developed embryo include prostaglandin E2 (PGE2) and pregnancy recognition signal, interferon tau (IFNT) (Walker, et al., 2010; Croy et al., 1988; Fisher et al., 1985). Such compounds are known to inhibit the proliferation of lymphocytes (Walker, et al., 2010; Croy et al., 1988; Fisher et al., 1985). However, even with this localized suppression, there is an upregulation of bacterial permeability increasing protein (BPI) and lipopolysaccharide binding protein (LBP); proteins that are involved in the maternal immune response to bacterial infection (Walker, et al., 2010; Croy et al., 1988; Fisher et al., 1985).

Up to the blastocyst stage the embryo is largely autonomous with no contact and few direct environmental inputs required, which was confirmed by the ability to produce functional, apparently healthy blastocysts using completely *in vitro* methods. However, there is evidence that the maternal environment contributes to embryo health at this time by influencing embryo metabolism, gene expression, and survival (Rizos et al., 2002). Conversely, following blastocyst hatching and elongation the maternal environment is critical to continued development, as evidenced by a failure of embryo elongation, *in vitro* (Alexopoulos et al., 2005; Brandao et al., 2004). Many of the required factors appear to be reliant on the uterine gland production of critical factors, as experimentally ablated uterine glands prevents embryo elongation (Gray et al., 2002; Spencer, 2006).

Inflammation and Reproduction

Inflammation is intertwined intimately throughout the various processes of reproduction from ovulation to attachment and pregnancy. Inflammation is considered to be an immune response to pathogens, which involves the release of pro-inflammatory cytokines in addition to swelling caused by the dilation of blood vessels (Medzhitov, 2008). During ovulation, inflammation is a necessity, as it is the result of an increase in the production and release of pro-inflammatory cytokines from the tumor necrosis factor and interleukin families (Richards et al., 2015; Machelon et al., 1997). Specifically, interleukin 6 (IL-6) is an inflammatory factor known to play a role in cumulus cell expansion during ovulation. However, this particular cytokine is not solely responsible for this portion of ovulation, and therefore is not an exclusive indication of fertility (Richards et al., 2015; Machelon et al., 1997). Pro-inflammatory cytokines also play a role in follicular rupture by causing changes in blood flow and increasing the pressure within the follicular wall (Brannstrom et al., 1998) in addition to assisting with the breakdown of the extracellular matrix via the release of IL-6 from mast cells (Kathpalia et al., 1990). The inflammatory-like actions that occur during ovulation are mediated by secretions from the granulosa cells and macrophages including chemokines and cytokines via their pro-inflammatory actions, as well as members of the vascular endothelial cell family (VEGF) (Guimerà et al., 2009; Chowdhury et al., 2010; Gutman et al., 2008). Specifically, VEGFC and VEGFD work to promote the formation of new capillaries within the ovary in order to increase blood flow resulting in increased pressure and follicle swelling (Kim et al., 2017; Marconcini et al.,

1999; Witzenbichler et al., 1998). These inflammatory markers are also expressed during the process of implantation when the conceptus implants into the uterine wall (Mor et al., 2008). This is a well-known inflammatory process that has been theorized as an attempt to 'sterilize' the implantation site by activating immune cells to prevent the growth of bacteria and reduce the potential for infection (Mor et al., 2011) as well as assisting with the tissue remodeling that occurs within the uterus (Mor et al., 2011). Interestingly, during a sterile tissue response, non-infectious signals can act through the same Toll-like receptor pathway that a pathogen can in order to elicit an immune response (Mbitikon-Kobo, 2009). The inflammation that occurs in the endometrium during implantation is classified as a T helper cell 1 (Th1) response, which is mediated by pro-inflammatory cytokines in the interleukin family (including IL-6) and tumor necrosis factor alpha (TNFα) (Mor et al., 2002). Therefore, while inflammation can sometimes be perceived as a negative or destructive process, it can also be beneficial and has proven critical to the overall success of reproduction.

It is important to keep in mind that while inflammation can have positive effects on reproduction as previously mentioned, there is a fine balance in the body. If it shifts to a state of acute or chronic, ongoing inflammation, the outcome can be much different. Chronic inflammation can be particularly problematic when it is not accompanied by any easily detectable symptoms, as it will not be recognized and treated accordingly to prevent further damage;/l. A metabolomic study has shown that infertile heifers, with no obvious phenotype, had white blood cells with increased transcripts of the inflammatory cytokines

TNFa and *IL-6* compared with fertile heifers (Phillips et al., 2018). This immune response is indicative of a potential infection that could be negatively impacting fertility.

Furthermore, in humans, maternal inflammation has also been associated with pre-term labor and in some cases can cause infant mortality, however the specific causes and mechanisms remain to be understood (Li et al., 2010). It is important to note that the timing of an inflammatory infection can be crucial with respect to reproductive outcome as well. When an infection was introduced to cattle prior to breeding, it caused reductions in the capacity of the oocyte to become fertilized, successful development of embryos to the morula stage, as well as the embryo's ability to successfully elongate and secrete IFNT (Ribeiro et al., 2016). This observation is particularly interesting, as it strongly supports an effect of inflammation on oocyte health. Additionally, infection was noted to cause inflammatory-like transcription changes among embryonic cells, as well as decreases in pregnancy rates and number of calves produced per breeding (Ribeiro et al., 2016). The negative ovarian effects of the inflammatory immune response have been documented across mammalian species; however, the question still remains, how is a reproductive tract infection reaching and affecting the ovary? Whereas there has been no formal research to definitively determine how pathogens can reach the ovary, there have been some speculations. Many believe that these pathogens are able to reach such distant sites via general circulation or potentially via the countercurrent mechanism that is also used to transport endometrial prostaglandin to the ipsilateral ovary (Mapletoft et al., 1975). As with all physiological processes, balance is key to maintaining a healthy and functioning system.

Ovarian Dysfunction

The exposure to a bacterial pathogen via uterine infections can have significant effects on the cumulus oocyte complex (COC) and its subsequent cell populations. In addition to embryonic effects and ovarian cyclicity. Postpartum uterine infection can have negative impacts on ovarian function (Sheldon et al., 2002; Williams et al., 2007). For example, exposure to infection resulted in a reduction in the number of dominant follicles formed in addition to reduced follicle growth rates (Sheldon et al., 2002; Williams et al., 2007). This exposure to infection also decreased estradiol plasma concentrations and led to a reduction in size of the first postpartum corpus luteum with a subsequent decrease in progesterone (Williams et al., 2007). These ovarian effects could be the result of a significant reduction in luteinizing hormone (LH) once the pathogen enters the blood stream and reaches the brain in ewes (Williams et al., 2007), as LH is responsible for activating granulosa and theca cells to differentiate into luteal cells (Abedel-Majed et al 2019).

Lipopolysaccharide

Lipopolysaccharide (LPS) is a component of the bacterial cell wall of gram-negative bacteria, such as *Escheria coli* and *Salmonella*. The range of LPS concentrations found in the follicular fluid of cows with endometritis is found to be quite variable. The mean concentration for cows with subclinical endometritis was 4.8 ng/mL, and those with severe endometritis was 176 ng/mL (Herath et al., 2007). LPS has been known for a long

time to induce biological responses in cells (Hartwell et al., 1943). More recently it has been shown that this component acts through a family of receptors known as toll-like receptors (TLRs) (Miller et al., 2005; Bromfield et al., 2011; Magata et al., 2017; Miyake, 2007). These receptors were originally found in *Drosophila* in association with their innate immunity (Anderson et al., 1985; Lemaitre et al., 1996). TLRs are located on the surface of immune cells such as neutrophils and macrophages and consist of 11 members (Akira et al., 2006). When LPS-associated pathogen molecules (PAMPs) come into contact with a TLR, it initiates a pathway that triggers an inflammatory immune response within the body. This response involves the release of inflammatory cytokines such as *TNFa* and *IL-6* (Adams and Czuprynski, 1990). Of particular interest within the TLR family are TLRs 2 and 4 (Lu et al., 2008; Magata et al., 2017; Bromfield et al., 2011). These receptors were found to be active in response to the presence of LPS (Bromfield et al., 2011), allowing for the speculation that these are the specific pathways through which LPS initiates an immune response. It should be noted that LPS does not bind directly with TLR4; this pathogenic molecule initially comes into contact with CD14 (Lu et al., 2008). In order for the activation of TLR4 to occur, LPS must first bind with LPS binding protein (LBP) that is found in serum and follicular fluid (Schumann et al., 1990; Triantafilou et al., 2002; Ferrazza et al., 2017). Once bound, this binding protein will interact with CD14, a glycosylphosphatidylinositol-anchored protein bound to the outer membrane of the cell (Wright et al., 1990; Triantafilou et al., 2002). This protein assists with the interaction between the TLR4 and myeloid differentiation 2 (MD-2) complex, which allows for the

recognition of LPS (Shimazu et al., 1999; Nagai et al., 2002). The MD-2 protein is vital to the activation of the innate immune system in response to LPS. A study utilizing MD-2 knockout mice determined that upon exposure to lethal levels of LPS, the mice were found to be unaffected compared with the wild-type, of whom died within 10 hours of exposure with elevated levels of inflammatory cytokines such as TNF-Alpha and IL-6 (Nagai et al., 2002). This indicates that MD-2 plays a critical role in LPS recognition. Interestingly, granulosa cells express TLR4, CD14, and MD-2 suggesting they independently of migrating immune cells, can respond to LPS (Herath et al., 2007). Upon interaction between LPS and TLR4, a signal is perpetuated downstream to adaptor proteins, specifically TIR domain containing adaptor protein (TIRAP) and TRIF related adaptor molecule (TRAM) (Horng et al., 2001; Yamamoto et al., 2002). TIRAP activates myeloid differentiation primary response gene 88 (MyD88), thus initiating the MyD88-dependent pathway (Kawai et al., 2001; Fitzgerald et al., 2001). In a general sense, this pathway involves the activation of NFkb as well as MAPK (Lu et al., 2008), eventually leading to the activation and release of proinflammatory cytokines.
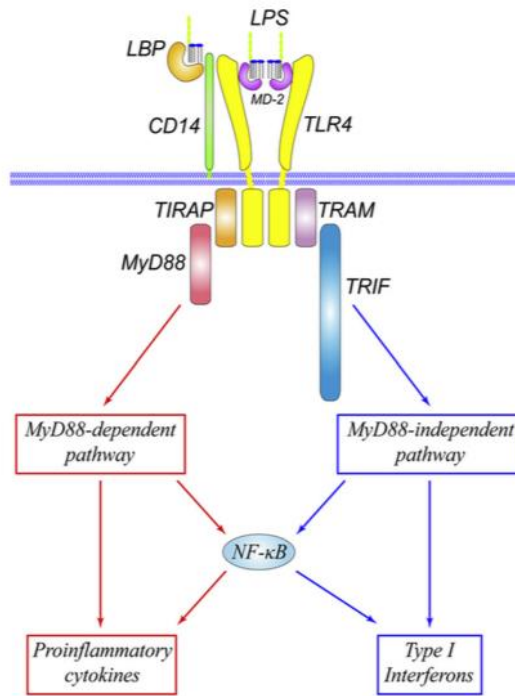
Figure 1: A simple depiction of the activation of the TLR4 pathway when activated by the binding of LPS. (Lu et al., 2008). Used with publisher's permission.

The Effects of LPS Exposure

Whereas temporary and tightly controlled forms of inflammation can be beneficial to reproduction, chronic ongoing inflammation can be detrimental to fertility. Specifically, effects can be seen within the ovaries with respect to ovarian functionality, oocyte developmental competence, and granulosa cell functionality to be discussed in greater detail in the following sections.

Oocyte Developmental Competence

When exposed to LPS, oocytes have some negative impacts concerning their overall health and development. Upon exposure to LPS, bovine oocytes reduced numbers of fertilized COCs that successfully reach the blastocyst stage of development, indicating a decrease in competence (Magata and Shimizu, 2017). Such effects could be attributable to the effects noted on LPS-treated oocytes that failed to fully mature and reach meiosis II, which is speculated to inhibit fertilization and subsequent cleavage (Magata and Shimizu, 2017; Bromfield and Sheldon, 2011). However, these effects are not limited to the nuclear level of maturation; there are also effects on the cytoplasmic maturation. LPS caused significant reductions in mitochondrial membrane potential as well as changes in the overall distribution of mitochondria throughout the cell, meaning the mitochondria was accumulated around the peripheral portion of the cell instead of dispersed throughout the cell as it would be in a healthy, mature oocyte (Magata et al., 2017). It is important to note that mitochondrial impairment has been associated with the premature arrest of embryos

(Thouas et al., 2004). Nonetheless, the copy number of mitochondrial DNA was unaffected by this treatment (Magata et al., 2017), which could indicate that LPS does not have an impact on the biosynthesis or deterioration of mitochondria, as well as on the capacity of the oocyte to be fertilized (Magata et al., 2017).

Granulosa Cell Endocrine Dysfunction

LPS has been shown to have significant impacts on granulosa cells and their functionality, specifically with respect to their steroidogenic abilities. Granulosa cells are one of the populations of cells surrounding the oocyte (Baumgarten and Stocco, 2018). These cells are responsible for the production of estrogen via the aromatization of androgens, and eventually progesterone, once luteinization of the cells occurs (Herath et al., 2007). Whereas granulosa cells are separated from vascularization, the porous basement membrane allows for the passage of LPS, subsequently exposing the granulosa cells to an infection present within the body (Herath et al., 2007). Exposure of granulosa cells to LPS inhibits the production and secretion of estrogen and progesterone from recruited and dominant follicles (Shimizu et al., 2012, Herath et al., 2007). These effects can be attributed to the decrease in the expression of aromatase (CYP19A1) (Herath et al., 2007), which is an enzyme that works to convert androgens to estrogens located within granulosa cells. Additionally, the presence of LPS can have negative impacts on the steroidogenesis of theca cells with regard to the reduced secretion of androstenedione and progesterone in an environment that is dominated by luteinizing hormone (Magata et al.,

2014). This reduction is a result of the downregulation of the steroidogenic acute regulatory protein (*StAR*) and cytochrome P450 17A1 (*CYP17*) transcripts which are involved in the process of steroidogenesis (Magata et al., 2014). Intriguingly, when the TLR4 pathway is blocked by an inhibitor such as ethyl (6R)-6-[N-(2-Chloro-4-fluorophenyl) sulfamayol]-cyclohex-1-ene-1-carboxylate (TAK-242), the effect of LPS on androgen production in theca cells reduces the negative effects of LPS on its decline (Samir et al., 2017), suggesting that TAK-242 is an effective inhibitor of the TLR4 pathway.

Granulosa Cell Immune Response

Interestingly, granulosa cells have been shown to demonstrate an immune response to the presence of inflammatory infection, even though this cell population is free of immune cells (Bromfield and Sheldon, 2011). Granulosa cells express pro-inflammatory immune markers such as interleukins (IL) 1, 6, and 8 in addition to TNFα (Bromfield and Sheldon, 2011; Herath et al., 2007). Interleukins are a family of pro-inflammatory cytokines functioning to differentiate and activate immune cells (Vaillant and Qurie, 2019). In order for this immune response to be elicited in the first place, the appropriate immune pathway must be activated. As previously mentioned, LPS binds to TLR4 in order to initiate an inflammatory immune response within the body. It is important to note that the expression of TLR4 has been confirmed in bovine granulosa cells (Magata et al., 2017; Bromfield and Sheldon, 2011). TLR2 has also been speculated to be involved in the mediation of LPS signaling (Magata et al., 2017; Yang et al., 1998), while others believe

that this receptor responds to signals from gram-positive bacteria (Bromfield and Sheldon, 2011). However, TLR4 remains to be the receptor of central focus in the LPS response. In order to definitively establish that LPS triggers an immune response via the TLR4 pathway, the receptor must be blocked and the subsequent immune response, if any, must be determined. TAK-242 is a small molecule inhibitor that is capable of selectively inhibiting TLR4 by blocking the phosphorylation of Erk1/2, p38 and the JNK portions of this specific TLR4 pathway and subsequently inhibiting its activation (Ii et al., 2006). As a result of this inhibition, researchers also noted a decrease in TNFα and IL-6 release from macrophages in both human and mice cells, indicating that the actions of this blocker appear to be conserved across mammalian species (Ii et al., 2006). Given this information, it is apparent that granulosa cells are capable of a defensive immune response to LPS via the TLR4 pathway.

Reactive Oxygen Species

Reactive oxygen species are formed by the reduction of an oxygen molecule and include molecules such as hydrogen peroxide, superoxide anion, and a hydroxyl radical (Ray et al., 2012). The superoxide anion is considered to be the precursor for most reactive oxygen species while hydrogen peroxide can be reduced into a hydroxyl radical, thus becoming a strong, yet unstable oxidant (Turrens, 2004). The primary source of reactive oxygen species is the mitochondria, specifically within the electron transport chain (Zhao et al., 2019). These molecules are formed when electrons leak out of the transport chain

and bind with oxygen to create hydrogen peroxide or a superoxide anion (Zhao et al., 2019). The majority of reactive oxygen species are produced from complexes I and III of the electron transport chain. Complex I is responsible for the transferring of electrons from nicotinamide adenine dinucleotide plus hydrogen (NADH) in the matrix to ubiquinone, creating a reduced product that is able to diffuse across the cell membrane while also creating a proton gradient with the transfer of protons (Zhao et al., 2019). In complex III, electrons are removed from ubiquinol and transported to cytochrome c in order to be delivered to complex IV (Zhao et al., 2019). In some cases, the delicate balance of ROSs within the body can change and the body can enter into a state of oxidative stress categorized by an overproduction of reactive oxygen species. This overproduction can be brought on by the overstimulation of NAD(P)H and the electron transport chain (Bhat et al., 2015). This increase in ROS can cause damage to the mitochondria itself, other cell structures and DNA (Bhat et al., 2015). Such mitochondrial dysfunction can even lead to cell apoptosis (Wang et al., 2013). These effects can be particularly problematic for the success of IVF as cell damage and apoptosis can inhibit embryonic development.

However, the cell does have lines of defense against the potentially harmful implications of ROS overproduction. The enzyme catalase works to break down the ROS, hydrogen peroxide, into water and oxygen, thus preventing cell apoptosis (Aebi, 1987). Without the help of this enzyme, hydrogen peroxide can cause apoptosis in important immune cells such as neutrophils (Kasahara et al., 1997). Whereas ROS are primarily known for inducing apoptosis, species that are generated via the TNF family might play a

role in preventing apoptosis (Wong et al., 1989). There is some theory that superoxide dismutase (SOD) is potentially expressed as a result of the activation of this pathway, in addition to catalase (Wong et al., 1989). SOD serves as a protective mechanism against the damaging effects of ROS and is considered to be a biomarker of oxidative stress (Jozwik M et al., 1999). This enzyme works to break down superoxide radicals into oxygen and hydrogen peroxide. Increased levels of SOD within follicular fluid were associated with oocytes that experienced failed fertilizations (Sabatini et al., 1999), and differences in levels of ROS within the peritoneal fluid of women may be a potential link to infertility (Wang et al., 1997). Additionally, infertile women have reduced concentrations of antioxidants compared with fertile women, again indicating that the overproduction of ROS could be impacting reproduction negatively (Polak et al., 2001).

Interestingly, ROS have a naturally occurring role throughout the various facets of reproduction including folliculogenesis and the maturation of follicles via its involvement in cell-signaling pathways involving cell differentiation, proliferation, and migration (Tamate et al., 1995; Plessis SS et al., 2008). However, upon its accumulation, ROS can cause granulosa cell apoptosis in the COC, thus creating a breakdown in the gap junction communication system that exists between granulosa cells and the oocyte (Chaube, et al 2014). This breakdown of communication can prevent the transport of important developmental signals in addition to the transport of nutrients to the oocyte (Chaube et al., 2014). The overproduction of ROS can not only be problematic for a natural reproduction, but it can also create complications with *in vitro* fertilization. Such an issue

can be combated by the use of antioxidants such as vitamin C and glutathione, in addition to reduced glucose concentrations to prevent such an overproduction (Wang et al., 2002; Iwata et al., 1998; Ali et al., 2003).

Project Rationale

Ensuring a sustainable supply of beef is one of the greatest challenges facing the beef industry. The cow-calf sector is faced with significant challenges, including land loss due to urban development and a growing population. One of the main factors improving the sustainability of beef production is the enhancement of reproductive efficiency. Heifer sub- and infertility lead to decreases in production efficiency, animal health, and the sustainability of the cow-calf sector. This decrease in fertility has been linked to inflammatory infections such as mastitis and endometriosis, which are especially prevalent in postpartum cattle. The bacteria commonly responsible for these types of infections is *E. coli*, as it has been detected in the follicular fluid of cattle experiencing endometriosis. LPS, derived from *E. coli*, is a component of the bacterial cell wall and is commonly used to elicit an inflammatory response in the laboratory setting.

In this study we sought to determine the impacts of inflammation on bovine oocyte health and development. Obtaining a better understanding of these impacts opens the opportunity for the development of preventative and therapeutic options. In order to better understand the local effects of infection on oocyte health, we exposed bovine COCs to LPS in order to re-create the signal of an infection. In doing this, we first analyzed

granulosa cells to determine their ability to respond directly to immune challenges. Upon successfully initiating an immune response from granulosa cells we focused on the effects of infection on well-established immune, health and ROS oocyte marker expression. We also looked at the effects of LPS treatment functionally on the viability and proliferation of granulosa cells in addition to the production of ROS in the oocyte which has been linked to cell damage, apoptosis and reductions in oocyte maturation.

Identifying the specific impacts of inflammatory infection on reproduction with respect to oocyte health and development can help solve the associated reproductive problems, thus allowing us to create solutions in an effort to improve the efficiency of cattle production.

Chapter 2: Immune Responsiveness of Ovarian Cells

Hypothesis

Ovarian cells *in vitro* can directly respond to LPS treatment by expressing inflammatory cytokines, creating the potential for negative effects on oocyte developmental competence.

Specific Objectives

1. Investigate the expression of *IL-6, TNFα* and *CCL2* in the cumulus-granulosa cells of COCs with high developmental competence and low developmental competence.

2. Investigate the ability of in vitro cultured granulosa to respond to LPS treatment by inducing the expression of *IL-6, TNFα* and *CCL2*

3. Investigate the effect of LPS challenge and the inhibition of TLR4 on the expression of *IL-6, TNFα* and *CCL2* in COCs during maturation.

4. Investigate the effect of LPS challenge and the inhibition of TLR4 on the production of TNFα by COCs during maturation.

Abstract

Inflammation is a costly problem within the cattle industry with negative repercussions on fertility. Such a reduction in reproductive function can have negative implications on production efficiency and could create difficulties for producers to keep up with the demand of an ever-growing population. LPS is a component of the bacterial cell wall of gram-negative bacteria like *E. coli*, and is used to replicate infectious conditions in cell culture. We hypothesized that LPS would elicit an inflammatory immune response in ovarian cells. Interestingly, cumulus cells biopsied from COCs that following IVF result in arrested embryos demonstrated increased expression of the inflammatory markers *TNFα, IL-6*, and *CCL2,* thus providing a potential link between inflammation induced by LPS and negative reproductive outcome. In order to further investigate the immune capabilities of ovarian cells, granulosa were exposed to various concentrations of LPS, with 1µg/mL proving to elicit the most consistent response, and therefore was used throughout all subsequent experiments. Under these conditions, bovine COCs demonstrated an immune response to LPS via increases in the expression of inflammatory markers *TNFα*, *IL-6*, and *CCL2*. Additionally, we detected increases in the release of TNFα following LPS treatment into oocyte maturation media further demonstrating the immune capability of granulosa cells. In conclusion, ovarian cells are capable of mounting an inflammatory immune response when exposed to LPS, thus, creating an opportunity for future damage to ovarian cells.

Introduction

Infections of the uterus or mammary glands by bacteria are common causes of disease in cattle and have been associated with negative reproductive outcomes (Sheldon et al., 2002; Herath et al., 2007; Williams et al., 2008; Lavon et al., 2011). Metritis and mastitis have been shown to alter follicular growth, resulting in reduced estradiol concentrations due to the decrease in granulosa cell proliferation, as well as extended luteal phases, and disrupting cyclicity (Sheldon et al., 2002; Herath et al., 2007; Williams et al., 2008; Lavon et al., 2011). One potential link between bacterial infections and ovarian alterations is the accumulation of LPS in the follicular fluid following an infection (Herath et al., 2007). A previous study has shown that bovine granulosa cells respond to LPS by producing IL-6 and IL-8 in a dose-dependent manner (Bromfield and Sheldon, 2011). They found that LPS was potentially acting through the Toll-like receptor 4 (TLR4) and perturbed meiotic progression (Bromfield and Sheldon, 2011).

The Toll-like receptors (TLRs) are a family of cellular receptors responsible for detecting and initiating the innate immune defense against bacterial, viral and fungal pathogens (Beutler, 2004; Takeuchi et al., 2010). Bovine granulosa cells have been shown to express the mRNA for TLR4 (Price et al., 2013; Bromfield and Sheldon, 2011). Recently, we have utilized a single cumulus-oocyte-complex in vitro fertilization (IVF) system to allow cumulus cell biopsies, removed from freshly isolated COCs, to be correlated to embryo developmental outcomes (Read et al., 2018). This allows the expression of transcripts in cumulus cells originating from high developmentally

competent oocytes to be compared with the cumulus from low developmentally competent oocytes (Read et al., 2018). Utilizing this system, we explored the expression of inflammatory cytokines in the two contrasting groups. We also explored the ability of bovine granulosa cells, cultured *in vitro* to respond to LPS by initiating the expression of inflammatory cytokines. Throughout the experiments we utilized the TLR4 inhibitor TAK242 to identify the potential role the TLR4 was playing in the LPS induced effects. Furthermore, we investigated the effect of LPS treatment during meiotic maturation on the expression of inflammatory cytokines in COCs. Finally, we determined the ability of COCs to produce TNFα in response to LPS challenge.

Materials and Methods

All reagents purchased from VWR (Radnor, USA) unless otherwise noted.

Single COC IVF System

Bovine ovaries were sourced from Brown Packing Co (Gaffney, SC) and transported back to Auburn University in 0.8% sterile saline solution. Upon arrival, COC samples were aspirated from follicles around 2-5mm with an 18-gauge, short-bevel needle and 10-mL syringe. The aspirated follicular fluid containing COCs and granulosa cells was collected into a 50-mL conical tube and remained at room temperature. Selected COCs had to meet the requirement of at least 3 surrounding layers of cumulus cells before being added to the wash media. This wash consisted of TCM-199, sodium bicarbonate (0.42M), hepes (0.42M), FBS (10%), gentamicine (50 µg/mL), and pyruvate (22 µg/mL). The selected

COCs were then washed additionally through 3 wash droplets (120 µL each) of maturation media held in place by mineral oil (Sigma Life Science, Darmstadt, Germany) in untreated cell culture plates. Prior to culture, small cumulus cell samples (~ 50-100 cells) were dissected under a microscope with an 18-gauge needle. The samples were snap frozen in liquid nitrogen. Each collected cumulus cell sample was correlated to the appropriate COC for future data analysis. Each experiment was repeated at least 3 times. After the cumulus cell biopsy was conducted, COCs were cultured in 20-µL drops of maturation media for 24 hours. Upon reaching maturation, the COCs were fertilized within individual droplets containing fertilization media with 10_6 spermatozoa/mL for 18-22 hours. In an attempt to eliminate variation resulting from the male constituent, semen from a single ejaculation from a bull with proven *in vitro* fertility was used. Fertilization media was composed of synthetic oviductal fluid (SOF, 1.17mM $CaCl_22H_2O$, 0.49mM $MgCl_26H_2O$, 1.19mM KH_2PO_4, 7.16mM KCl, 107.7mM NaCl, 25.07mM $NaHCO_3$, and 5.3mM Na-lactate) in addition to gentamicine (50 µg/mL), pyruvate (22 µg/mL), heparin (10 µg/mL), caffeine (194.2 µg/mL), and bovine serum albumin (BSA) fraction V essentially fatty acid free (EFAF) (6 mg/mL). After fertilization the presumptive zygotes were denuded via a glass pipette. Afterward, they were cultured in 20-µL droplets of culture media under mineral oil. The presumptive zygotes were not completely stripped of their cumulus cells, with a single layer of about 30 cells being deemed acceptable. Culture media was composed of SOF (5mL), BSA fraction V EFAF (6 mg/mL), glutamine (1.1 mM), myo-inositol (2.8 mM), sodium citrate (0.57 mM), pyruvate (22 µg/mL), gentamicin (50 µg/mL), essential

amino acieds (1X), and non-essential amino acids (1X). Cleavage and blastocyst rates were compared among the individually cultured presumptive zygotes in three separate IVFs, in both normal (5% CO_2, 95% air, n=79) conditions. Forty-eight hours after fertilization, cleavage rates were recorded based upon the beginning number of oocytes. Oocytes that failed to fertilize were included in the cleavage and blastocyst rates but excluded from all other analyses with those reaching the cleavage stage included. Eight days after fertilization, morula/blastocyst rates were recorded based on the starting number of oocytes. Zygotes that were able to reach the morula/blastocyst stage were determined to be of high developmental competence, while those stalled at the two-cell stage were deemed to be of low developmental competence.

COC Culture System

Bovine ovaries were sourced, aspirated, washed, and cultured as referenced in the Single COC IVF culture system section. Maturation media consisted of TCM-99, sodium bicarbonate (0.03M), FBS (10%), gentamicine (50 µg/mL), pyruvate (22 µg/mL), glutamax (0.5X), and EGF (10 ng/mL). After washing, the COCs were then placed in 90-µL drops of maturation media in groups of 20-25 for up to 22 hours (maturation) in their respective treatments. Multiple time points were chosen as a means to catch the acute response to LPS. At 1-hour maturation has just begun, by 6 hours the COC is the most transcriptionally active during maturation, and by 22 hours transcriptional activity is decreasing as the COC is reaching maturity. Treatment groups for this research consisted

of LPS (1 µg/mL), TAK-242 (1µM), and TAK-242 (1µM) plus LPS (1 µg/mL). COCs were cultured at 38.5º C and 5.0% CO_2 for their respective timepoints. Upon reaching the desired timepoint of culture, COCs were collected into 1.5-mL tubes with 0.5 mL of PBS and pelleted at $500 \times g$ for 5 minutes prior to removal of the supernatant and storage at -80ºC.

Granulosa Cell Culture System

Bovine ovaries were sourced from Brown Packing Co (Gaffney, SC) and transported back to Auburn University in 0.8% sterile saline solution. Upon arrival, COC samples were aspirated from follicles around 2-5 mm with an 18-gauge, short-bevel needle and 10-mL syringe. The aspirated follicular fluid containing COCs and granulosa cells was collected into a 50-mL conical tube and remained at room temperature. COCs were selected and isolated for other experiments, while the remaining follicular fluid was collected in 50-mL conical tubes and used to isolate granulosa cells. The follicular fluid was spun down at $500 \times g$ for 5 minutes to pellet the granulosa cells. Afterward the cells were re-suspended in 1 mL of wash media, washed in an additional 14 mL of media, and pelleted 3 times. This wash consisted of DMEM/F12 and anti-anti ($1\times$). A small portion of granulosa cells was diluted $10\times$ in PBS ($1\times$) and counted using a hemocytometer. The granulosa cells were plated at a density of 1.0×10^6 in maturation media with their respective treatment and cultured at 38.5º C and 5.0% CO_2. The following morning unattached cells were washed off the plate and the media was replaced with fresh media.

Following 48 hours of culture, the granulosa medium was replaced with media including varying concentrations of LPS, 0, 0.01, 0.1, 1, and 10 µg/mL. The granulosa media consisted of DMEM/F12, FBS (10%), and anti-anti (1×).

RNA isolation, cDNA synthesis and RT-PCR

The banked COC samples remained stored at -80º C until thawed for RNA isolation. RNA was isolated from these samples using the illustra™ RNAspin mini isolation kit (GE Healthcare Life Sciences) according to the manufacturer's protocol. Purity of isolated RNA samples was detected via a NanoDrop (ThermoFisher, Waltham, MA) with 260/280 values ranging from 2.21 to 2.25, and concentrations of isolated RNA were determined using a Qubit 4 fluorometer (ThermoFisher) according to the manufacturer's protocol. Afterward, the isolated RNA was reverse-transcribed into cDNA with qScript cNDA supermix (Quanta Biosciences Inc., Beverly, MA) according to the manufacturer's protocol.

qPCR

To account for the variations in RNA concentrations, C_q values from the PCR data of the samples were normalized to the C_qs of the reference gene *GAPDH* using the ΔΔCq method (Schmittgen and Livak, 2008). A Roche LightCycler 480 Real-time qPCR machine was utilized to compare the expression levels of the target transcripts using the delta-delta C_q method (Schmittgen and Livak, 2008). *GAPDH* was used as an internal loading control.

The qPCR reactions were ran using PerfeCTa SYBR Green Supermix (Quanta Biosciences Inc., Beverly, MA) according to the manufacturer's protocol. Primers for Gapdh, *TNFa*, and *IL-6* were tested for specificity and efficiency prior to use (Table 1).

TNFα Enzyme Linked Immunosorbent Analysis (ELISA)

An Invitrogen TNFα ELISA kit (Thermo Fisher, Catalog #: EBTNF) was utilized according to the manufacturer's protocol; to analyze the concentration of TNFα secreted from COCs treated for 22 hours in LPS (1 µg/mL), TAK (1 µM), or TAK (1 µM) and LPS (1 µg/mL) into cell culture media. Standard diluent B was diluted in order to generate the standard curve. Samples were diluted 2-fold before addition to the pre-coated well. After binding the antigen, biotin conjugate was added, followed by Streptavidin-HRP, a TMB substrate, and lastly the stop solution. Wells were washed accordingly between steps with 1 × wash buffer. Upon the addition of the stop solution, the plate was analyzed using an EMax® Plus microplate reader at a 450 nm absorbance.

Table 1: Primers used for detection of inflammatory cytokines in bovine COCs.

Name	Primer Sequence	NCBI Accession Number	Product size (bp)
GAPDH	F: CGTAACTTCTGTGCTGTGCC R: ATTGATGGCGACGATGTCCA	NM_001034034.2	136
TNFα	F: TCAAGCCTCAAGTAACAAGCC R: GTTGTCTTCCAGCTTCACACC	NM_173966.3	123
IL-6	F: TGAGTGTGAAAGCAGCAAGGA R: TCGCCTGATTGAACCCAGAT	NM_173923.2	100

Statistics

Statistical analysis was conducted via the use of GraphPad Prism (v8.4.3). One-way ANOVA with Tukey's multiple comparisons was utilized for the expression of inflammatory markers from the cumulus cells of arrested embryos (N=3) and the granulosa immune response to LPS (N=3). One-way ANOVA with post-hoc testing was used to analyze the inflammatory marker expression from granulosa cells and the TNFα secretion assay (N=3). Results were reported using the standard deviation (SD) from the mean and were deemed as statistically significant if $p < 0.05$.

Results

Cumulus cells from oocytes with low developmental competence have higher expression of inflammatory cytokines than those with high developmental competence.

Cumulus cells biopsied from bovine COCs were tested following categorization based on their developmental competence following IVF. Cumulus cells from oocytes that arrested at the 2-cell (low developmental potential) and morula/blastocyst (high developmental potential) stages were compared for expression levels of *TNFα, IL-6, and CCL2*. The cells were analyzed via qPCR. We found that the cumulus from oocytes that arrested at the 2-cell stage had higher expression of *IL-6* (6.68±2.20 fold, $p < 0.05$) than the cumulus from oocytes able to reach the morula or blastocyst stage (0.52±0.26 fold, $p < 0.05$). Similarly, cumulus from oocytes that arrested at the 2-cell stage had higher expression of *TNFα* (2.58±0.21 fold, $p < 0.05$) than the cumulus from oocytes able to reach the morula or blastocyst stage (0.84±0.11 fold). However, *CCL2* did not demonstrate any significant differences among developmental groups ($p > 0.05$) (Figure 2).
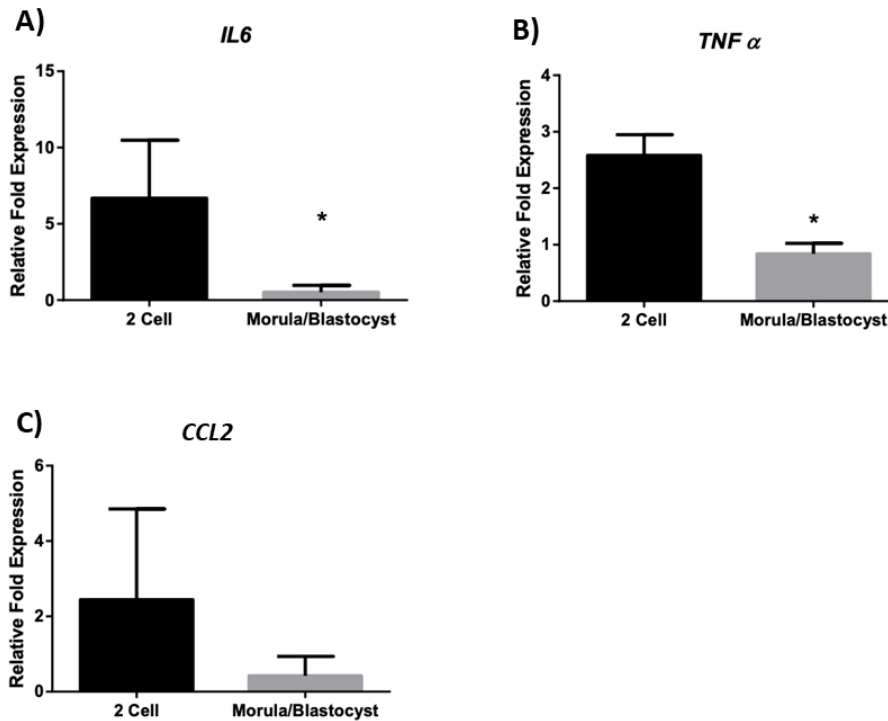
Figure 2: *Inflammatory marker expression in cumulus cell mRNA of arrested embryos.* Cumulus cells biopsied from embryos that arrested at either the 2-cell stage or morula/blastocyst stage, following IVF were analyzed via RT-PCR. Relative fold expression levels of cumulus cell mRNA that was normalized with a *GAPDH* internal control and analyzed for inflammatory markers *IL-6* (A), *TNFα* (B), and *CCL2* (C). With significant increases of IL-6 and *TNFα* in 2-cell stage arrested embryos and no significance for *CCL2*. (* denotes $p < 0.05$, N=3) Error bars are mean $\pm$ SD.

LPS elicits an immune response in culture granulosa cells

In order to determine the ability of cultured granulosa cells to directly elicit an immune response to the presence of LPS via an increase in the expression of pro-inflammatory cytokines, we cultured primary granulosa cells in the presence of various levels of LPS. Bovine granulosa cells were isolated and cultured for 48 hours prior to LPS treatment in order to reach ~80% confluence. They were then treated with 0, 0.01, 0.1, 1, and 10 µg/mL of LPS in a serum free medium for six hours. It was shown that the presence of LPS induced an immune response in the bovine granulosa cells. An induction of TNFα (8.65±0.76 fold, $p < 0.05$) was seen at 0.01 µg/mL of LPS and remained significant at 0.1 µg/mL (11.01±5.50 fold, $p < 0.05$), 1 µg/mL (16.62±7.08 fold, $p < 0.05$), and 10 µg/mL (18.23±8.23 fold, $p < 0.05$) in the granulosa cells (Figure 3).

Similarly, *IL-6* was significantly induced with the addition of 0.01 µg/mL of LPS (3.45±1.02 fold, $p < 0.05$) and remained significant at 0.1 µg/mL (3.45±0.64 fold, $p < 0.05$), 1 µg/mL (2.98±0.85 fold, $p < 0.05$), and 10 µg/mL (2.82±1.27 fold, $p < 0.05$) in the granulosa cells (Figure 4).

Finally, a significant increase in *CCL2* expression was seen in the granulosa cells with *CCL2* expression increasing to 3.22±1.46 fold ($p < 0.05$) (0.01 µg/mL), 10.36±8.13 fold ($p < 0.05$) (0.01 µg/mL), 11.08±7.84 fold ($p < 0.05$) (1 µg/mL). The response at 10 µg/mL was variable (7.82±6.00 fold) and found to not be significant ($p>0.05$) (Figure 5). Following these results 1 µg/mL of LPS was used for the remaining experiments. This concentration was selected due to its ability to elicit an inflammatory immune response, as

well as remaining relatively close to the existing concentration of LPS in cattle infected with endometritis.
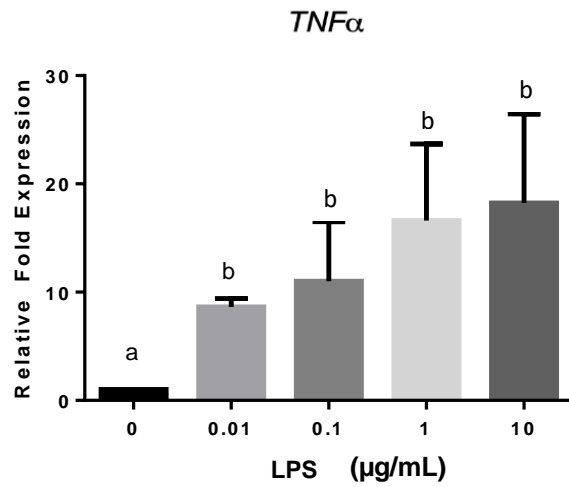
Figure 3: *Expression of TNFα mRNA in LPS treated granulosa cells.* Analysis of the ability of LPS to induce the expression of *TNFα* in granulosa cells via RT-PCR. Treatment for six hours with LPS resulted in the subsequent expression of *TNFα* in the granulosa cells (N=3). Significant differences (p<0.05) denoted by different letters as determined by ANOVA of the mRNA delta-CT values. Error bars are mean ± SD.
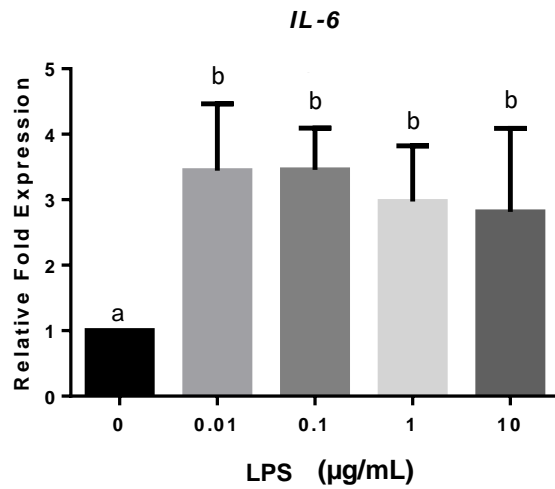
Figure 4: *Expression of IL-6 mRNA in LPS treated granulosa cells*. Analysis of the ability

of LPS to induce the expression of *IL-6* in granulosa cells via RT-PCR. Treatment for six

hours with LPS resulted in the subsequent expression of *IL-6* in the granulosa cells (N=4).

Significant differences (p<0.05) denoted by different letters as determined by ANOVA of

the mRNA delta-CT values. Error bars are mean ± SD.

Figure 5: *Expression of CCL2 mRNA in LPS treated granulosa cells.* Analysis of the ability of LPS to induce the expression of *CCL2* in granulosa cells via RT-PCR. Treatment for six hours with LPS resulted in the subsequent expression of *CCL2* increasing in the granulosa cells (N=4). Significant differences ($p<0.05$) denoted by different letters as determined by ANOVA of the mRNA delta-CT values. Error bars are mean ± SD.

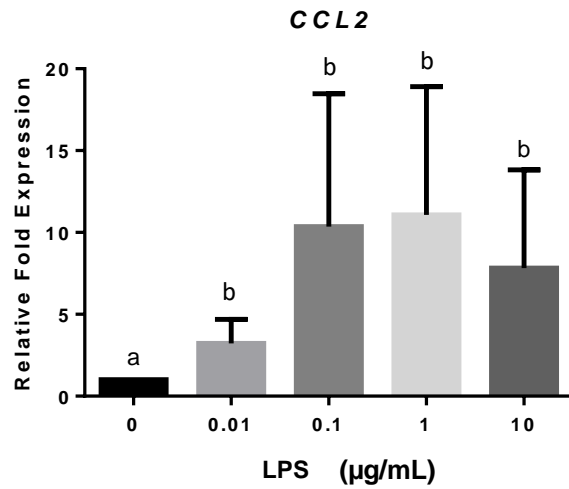**COCs respond to LPS challenge with increased inflammatory marker expression**

As a result of increased immune response in cultured granulosa cells treated with LPS, we decided to look for potential effects on COCs with a specific focus on the inflammatory markers *TNFα*, *IL-6*, and *CCL2*. The COCs were aspirated from abattoir-sourced *Bos taurus* cattle and treated with LPS, the TLR4 inhibitor TAK-242 (TAK) and TAK-242 plus LPS (TAK + LPS), and cultured for 1, 6, and 22 hours in maturation media. Each timepoint consisted of 20-25 COCs per replication. While there were no significant results with respect to each marker at 1 and 22 hours, the 6-hour mark demonstrated a significant increase in the expression of *TNFα* (2.08 ± 0.61 fold, $p < 0.05$), *IL-6* (4.03 ± 2.69 fold, $p < 0.05$), and *CCL2* (3.78 ± 2.98 fold, $p < 0.05$) in the LPS-treated group (Figures 4, 5, and 6). In all cases the presence of the TLR4 signaling inhibitor TAK242, either in the presence or absence of LPS, resulted in no significant increase in the inflammatory cytokines *TNFα*, *IL-6*, or *CCL2* (Figures 6, 7, and 8).

Figure 6: *Expression of TNFα mRNA in treated COCs.* Analysis of *TNFα* relative fold expression via RT-PCR in treated COCs at 1, 6, and 22 hours in maturation media, using *GAPDH* as an internal control. Treatment groups included no treatment (C), LPS, TAK-242 (TAK), or TAK plus LPS (TAK + LPS). Whereas, there was no significant differences at 1 and 22 hours, the 6-hour mark demonstrated a significant increase in *TNFα* with respect to the LPS treated group. N=3; Significant differences (p<0.05) denoted by different letters determined by ANOVA of delta-CT values. Error bars are mean ± SD.
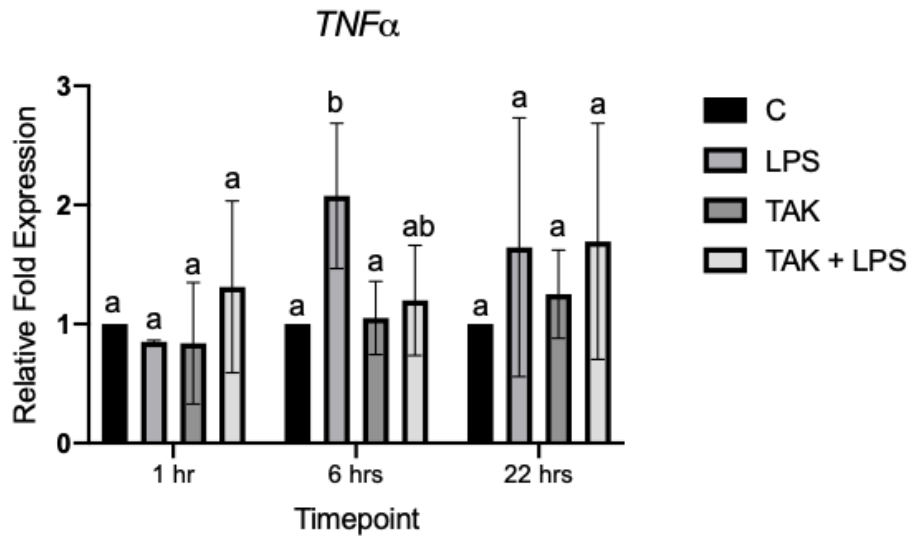
Figure 7: *Expression of IL-6 mRNA in treated COCs.* Analysis of *IL-6* relative fold expression via RT-PCR in treated COCs at 1, 6, and 22 hours in maturation media, using *GAPDH* as an internal control. Treatment groups included no treatment (C), LPS, TAK-242 (TAK), or TAK plus LPS (TAK + LPS). No significance demonstrated at 1 and 22 hours, however at 6-hours *IL-6* expression increased in the LPS treated group. N=3; Significant difference ($p < 0.05$) denoted by different letters, with respect to each individual timepoint, determined by ANOVA of delta-CT values. Error bars are mean $\pm$ SD.
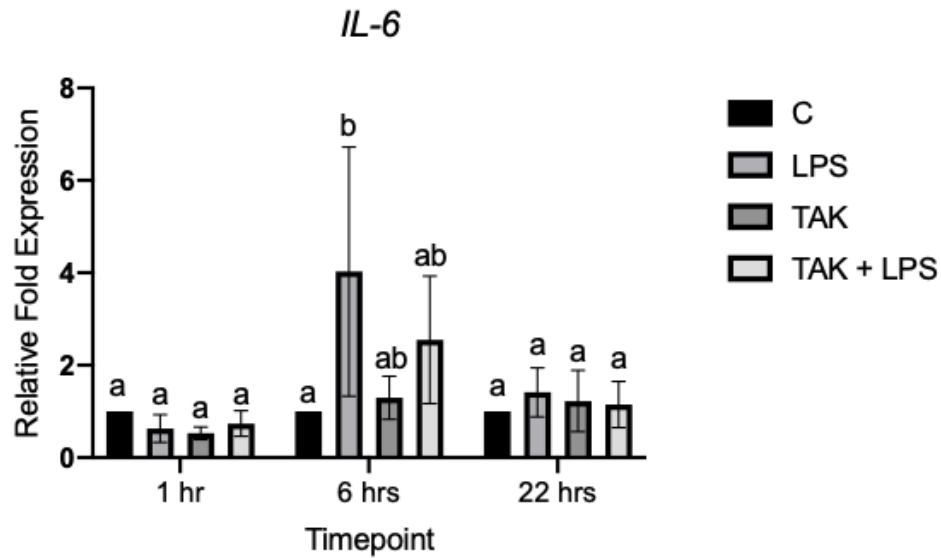
Figure 8: *Expression of CCL2 mRNA in treated COCs.* Analysis of *CCL2* relative fold

expression via RT-PCR in treated COCs at 1, 6, and 22 hours in maturation media, using

a *GAPDH* internal control. Treatment groups included no treatment (C), LPS, TAK-242

(TAK), or TAK plus LPS (TAK + LPS). No significance demonstrated at 1 and 22 hours,

however at 6-hours *CCL2* expression increased in the LPS treated group. N=3; Significant

difference (p<0.05) denoted by different letters, with respect to each individual time point,

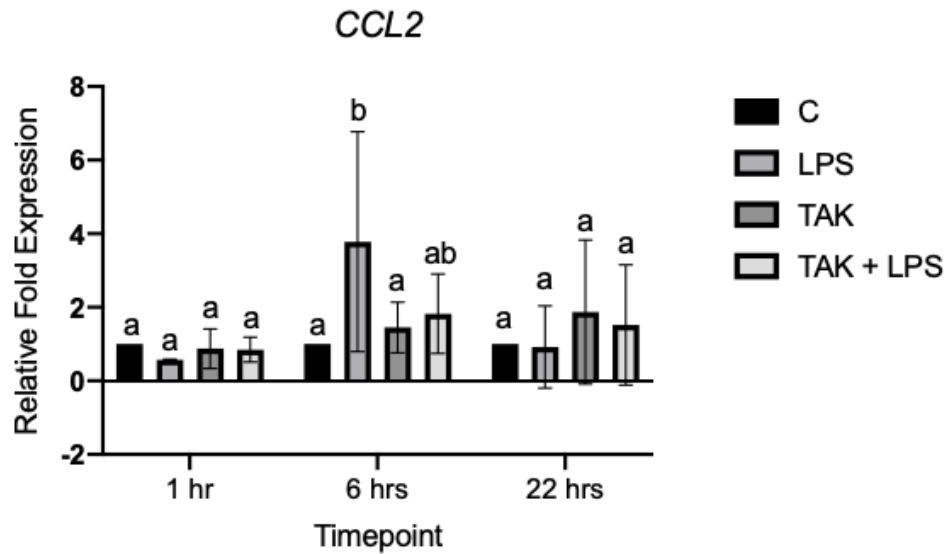determined by ANOVA of delta-CT values. Error bars are ± SD from mean.

COCs release TNFα following exposure to LPS

In order to verify the molecular results seen in granulosa cells and COCs concerning the inflammatory immune response, we conducted an ELISA to determine any potential differences in TNFα release in COCs. COCs were cultured in their respective treatment groups for 22 hours in groups of 10-15 COCs per droplet of maturation media. Then, the oocyte media was collected and used to measure TNFα. We found that the media from the LPS treated group had an increased secretion of TNFα into the culture media (0.29 ± 0.06 ng/mL, $p < 0.05$) compared with the control group (0.093 ± 0.10 ng/mL, Figure 9). Release of TNFα following treatment with TAK242 (0.22 ± 0.003 ng/mL, $p = 0.08$) or TAK242 with LPS (0.23 ± 0.02 ng/mL, $p = 0.07$) were trending towards significance with respect to the control.
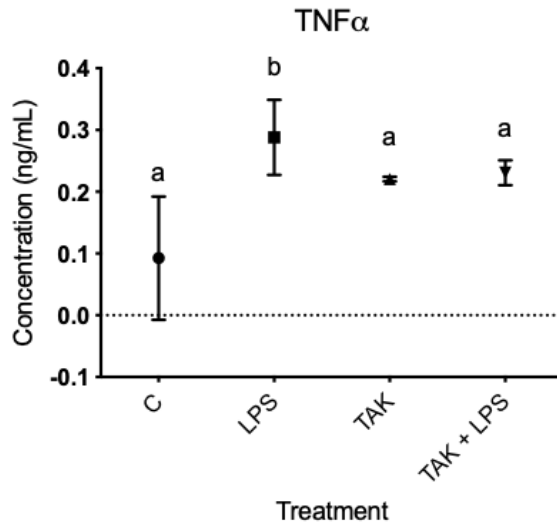
Figure 9: *Concentrations of TNFα protein secretion in cell media by COCs*. ELISA

measured concentrations of TNFα secretion by treated COCs into culture media (ng/mL).

Treatment groups included no treatment (C), LPS, TAK-242 (TAK), or TAK plus LPS

(TAK + LPS). The LPS treated group demonstrated a higher concentration of TNFα in

relation to other treatment groups. (Differing letters denote significance of $p < 0.05$) (C

and LPS, N=3) (TAK and TL, N=2). Error bars are mean ± SD.

Discussion

Bacterial infections leading to inflammation negatively impact follicular growth and endocrine function (Sheldon et al., 2002; Lavon et al., 2011). It is interesting that LPS has been found in follicular fluid from animals with uterine disease (Herath et al., 2007), raising the possibility that LPS may signal to the ovary leading to decreased oocyte health and developmental competence. In fact, several studies have demonstrated that granulosa cells inside follicles play direct roles in local innate immune responses (Price et al., 2013; Bromfeld and Sheldon, 2011). At present, the majority of the studies have focused on the ability of cultured granulosa cells to respond to LPS exposure by increasing inflammatory cytokine production. The present study was undertaken to confirm the ability of bovine cultured granulosa cells to respond to LPS exposure. We also sought to further clarify the ability of COCs during maturation to respond to LPS exposure by increasing their expression of inflammatory cytokines. This is an important step in linking the response to bacterial pathogens to oocyte health and developmental competence. Finally, we wanted to investigate the role that the TLR4 signaling pathway plays in responding to LPS challenge, in bovine COCs. Here we describe the immune responsiveness of cultured bovine granulosa cells induced via LPS. This response is signified by the increase in the expression of the inflammatory markers, *TNFα*, *IL-6*, and *CCL2* after 6 hours of treatment.

In addition to the increase in inflammatory marker expression observed, the presence of LPS increased the release of TNFα from the COCs into the culture media. Granulosa cells express TLR4, CD14, and MD-2 (Herath et al., 2007; Bromfield and

- 45 -

Sheldon, 2011), all of which are known to be required components of the TLR4-dependent response of immune cells to the presence of LPS (Wright et al., 1990; Nagai et al., 2002). In previous work, a novel small molecule inhibitor, TAK242, was found to selectively inhibit the induced expression of both *IL-6* and *TNFα* at both the mRNA and protein levels in human peripheral blood mononuclear cells, monocytes, and macrophages (Li et al., 2006). It was found to reduce the production of TLR4 ligands, but not the ligands for TLR2, -3, or -9 (Li et al., 2006). Due to TAK242 selectively inhibiting the production of inflammatory cytokines by blocking the phosphorylation of Erk1/2, p38 and JNK, we utilized it to investigate the role of TLR4 in mitigating the effects of LPS on bovine ovarian cells (Ii et al., 2006; Hussey et al., 2013). We found that LPS-treated COCs demonstrated significant increases in the expression of inflammatory markers. However, the expression of *TNFα*, *IL-6*, and *CCL2* in the TAK-LPS treated COCs remained near control values. Which supports observed effects of LPS on bovine COCs resulting from TLR4-dependent signaling. Previous work following a similar protocol has demonstrated that the LPS treatment of bovine granulosa cells results in increased *IL-6* (Bromfield and Sheldon, 2012). They also linked the resulting induction to TLR4 and demonstrated it by inhibition using siRNA (Bromfield and Sheldon, 2012). Additionally, researchers showed LPS negatively impacted the ability of bovine COCs to mature and increased the production of IL-6. Due in part to the negative effects of siRNA on COC developmental competence, we chose to utilize small-molecule inhibition of TLR4 to confirm its role in inducing LPS-triggered inflammatory cytokine production, and confirm it has negative consequences on

development. This finding supports that TLR4 plays an important role in bovine COCs in transducing the effect of LPS on cytokine production. In conclusion, ovarian cells are capable of mounting an immune response in the presence of LPS which acts via the TLR4 pathway and which can be inhibited via the use of TAK242. This *in vitro* platform can now be used to better understand the effect of inflammation on oocyte health and developmental competence. It is important to understand that these results could differ in an *in vivo* environment with varying degrees of inflammation and LPS concentrations, in addition to endocrine impacts throughout the body.

Chapter 3: Effect of LPS on Oocyte Health and Maturation

Hypothesis

Immune responsiveness of ovarian cells can have a negative impact on oocyte health and developmental competence.

Specific Objectives:

1. Investigate the effect of LPS challenge and the inhibition of TLR4 on the production of *BMP15*, *CYP19A1*, and *StAR* by COCs during maturation.

2. Investigate the effect of LPS challenge and the inhibition of TLR4 on the production of *SOD* by COCs during maturation.

3. Investigate the effect of LPS challenge and the inhibition of TLR4 on the maturation rate of COCs.

4. Investigate the effect of LPS challenge and the inhibition of TLR4 on granulosa proliferation and viability.

5. Investigate the effect of LPS challenge and the inhibition of TLR4 on the ROS in oocytes during maturation.

Abstract

Upon the determination that COCs have the ability to mount an immune response when exposed to LPS (Chapter 2), it was important to further investigate the effects of LPS on oocyte health and maturation. Notably, there were decreases in the expression of the health markers *BMP15*, *StAR*, and *CYP19A1* in the LPS treated COCs, indicating that this could be damaging to the oocyte. Additionally, there was an increase in the ROSs marker, *SOD,* which is indicative of an increase in ROS production within the COC. This indication was confirmed via LPS-treated, DCHF-DA stained oocytes showing an increase in ROS. Amongst all of the damage observed in the LPS treated COCs, granulosa cell viability and the capacity to proliferate was unchanged in the presence of LPS. However, LPS was associated with a decrease in the number of COCs that reached the MII phase of maturation suggesting that the activation of innate immune pathways in these cells affected the success of oocyte maturation.

Introduction

Infections leading to innate immune responses have been shown to negatively affect reproductive outcomes. Bovine granulosa cells express TLRs and are able to respond to LPS by initiating the production of inflammatory cytokines including IL-6 and IL-8 (Bromfield and Sheldon, 2012). During mammalian oocyte growth and maturation, the health of the oocyte is dependent on a highly ordered cascade of growth hormones, signaling molecules, and nutrients from the surrounding environment (Alvertini et al., 2001; Matzuk et al., 2002). The effect of LPS on the resulting function of the oocyte has been clearly shown to be detrimental to the maturation progression (Bromfield and Sheldon, 2012). We hypothesized that the inflammatory immune response resulting from LPS exposure would damage cell health and increase ROS, in addition to reductions in oocyte meiotic maturation.

Here we investigated the effect of LPS treatment on the timing of meiotic maturation progression *in vitro.* We also investigated effect of LPS during maturation on the health of the oocyte. We compared the proliferation and viability of granulosa treated with LPS to untreated controls. We also compared the expression of the oocyte-derived growth and differentiation factor bone morphogenic protein 15 (*BMP15*) and the granulosa steroidogenic markers steroid acute regulatory protein (*StAR*) and cytochrome P450 family 19 subfamily A member 1 (*CYP19A1*). Furthermore, we investigated the potential correlation between LPS-induced innate immune response and oocyte ROS levels as a

potential mechanism for the decrease in oocyte functioning caused by LPS. Finally, we compared the effect on the timing of meiotic maturation of the presence of LPS.

Material and Methods

COC Culture System

Bovine ovaries were sourced from Brown Packing Co (Gaffney, SC) and transported back to Auburn University in 0.8% sterile saline solution. Upon arrival, COC samples were aspirated from follicles around 2-5mm with an 18-gauge, short-bevel needle and 10-mL syringe. The aspirated follicular fluid containing COCs and granulosa cells was collected into a 50-mL conical tube and remained at room temperature. Selected COCs had to meet the requirement of at least 3 surrounding layers of cumulus cells before being added to the wash media. This wash consisted of TCM-199, sodium bicarbonate (0.42M), hepes (0.42M), FBS (10%), gentamicine (50 µg/mL), and pyruvate (22 µg/mL). The selected COCs were then washed additionally through 3 wash droplets (120 µL each) of maturation media held in place by mineral oil (Sigma Life Science, Darmstadt, Germany) in untreated cell culture plates. This maturation media consists of TCM-99, sodium bicarbonate (0.03M), FBS (10%), gentamicine (50 µg/mL), pyruvate (22 µg/mL), glutamax (0.5X), and EGF (10 ng/mL). After this wash, the COCs were then placed in 90-µL drops of maturation media in groups of 20-25 for up to 22 hours (maturation) in their respective treatments. Multiple time points were chosen as a means to catch the acute response to LPS. At 1-hour maturation has just begun, by 6 hours the COC is the most transcriptionally active during

maturation, and by 22 hours transcriptional activity is decreasing as the COC is reaching maturity. Treatment groups for this research consisted of LPS (1 µg/mL), TAK-242 (1µM), and TAK-242 (1µM) plus LPS (1 µg/mL). COCs were cultured at 38.5º C and 5.0% CO_2 for their respective timepoints. Upon reaching the desired timepoint of culture, COCs were collected into 1.5-mL tubes with 0.5 mL of PBS and pelleted at 500 × g for 5 minutes prior to removal of the supernatant and storage at -80ºC.

Granulosa Cell Culture System

Bovine ovaries were sourced from Brown Packing Co (Gaffney, SC) and transported back to Auburn University in 0.8% sterile saline solution. Upon arrival, COC samples were aspirated from follicles around 2-5 mm with an 18-gauge, short-bevel needle and 10-mL syringe. The aspirated follicular fluid containing COCs and granulosa cells was collected into a 50-mL conical tube and remained at room temperature. COCs were selected and isolated for other experiments, while the remaining follicular fluid was collected in 50-mL conical tubes and used to isolate granulosa cells. The follicular fluid was spun down at 500 × g for 5 minutes to pellet the granulosa cells. Afterward the cells were re-suspended in 1 mL of wash media, washed in an additional 14 mL of media, and pelleted 3 times. This wash consisted of DMEM/F12 and anti-anti (1×). A small portion of granulosa cells was diluted 10× in PBS (1×) and counted using a hemocytometer. The granulosa cells were plated at a density of 1.0×10^6 in maturation media with their respective treatment and cultured at 38.5º C and 5.0% CO_2. The maturation media consisted

of DMEM/F12, FBS (10%), and anti-anti (1×) in addition to an LPS treatment (1 µg/mL) derived from *E. coli*. Additionally, granulosa cells were treated with TAK242 (R&D Systems) and TAK242 plus LPS for viability and proliferation assays. The granulosa cells remained in culture until reaching confluence, about 72 hours.

Granulosa Cell Proliferation and Viability Staining

For each of these stains, the granulosa cells were treated for 6 hours, upon reaching confluence, and treated with LPS (1 µg/mL), TAK-242 (1 µM, and TAK-242 (1 µM) plus LPS (1 µg/mL).

Granulosa cells were stained for proliferation using the Click-iT™ EdU imaging kit with Alexa Fluor 488 dye (ThermoFisher) according to the manufacturer's instructions. Cultured granulosa cells were stained with an EdU (5-ethynyl-2'-deoxyuridine) working solution (10 µM) and incubated for 1 hour. Afterward, the cells were fixed with 3.7% paraformaldehyde (PFA), washed in PBS with 3% bovine serum albumin (BSA) and permeabilized with Triton-X 100 in PBS (1×). Lastly, the granulosa cells were treated with a Click-iT® reaction cocktail for 30 minutes, washed again with PBS and 3% BSA and stained with Hoechst 33342 for 30 minutes. After staining, the cells were imaged using an EVOS cell imaging system. This experiment was performed 3 times. Granulosa viability was determined via the use of Ready Probes™ cell viability imaging kit, blue/green (ThermoFisher) according to the manufacturer's instructions, by adding NucBlue® (living)

and NucGreen® (apoptotic) to cultured granulosa cells, incubated for 5 minutes and imaged with an EVOS cell imaging system 3 times.

RNA isolation, cDNA synthesis and RT-PCR

The banked COC samples remained stored at -80º C, until thawed for RNA isolation. RNA was isolated from these samples using the illustra™ RNAspin mini isolation kit (GE Healthcare Life Sciences) according to the manufacturer's protocol. Purity of isolated RNA samples was detected via a NanoDrop (ThermoFisher, Waltham, MA) with 260/280 values ranging from 2.21 to 2.25, and concentrations of isolated RNA were determined using a Qubit 4 fluorometer (ThermoFisher) according to the manufacturer's protocol. Afterward, the isolated RNA was reverse transcribed into cDNA with qScript cNDA supermix (Quanta Biosciences Inc., Beverly, MA) according to the manufacturer's protocol. Primers for *GAPDH*, *BMP15*, *StAR*, *CYP19A1*, and *SOD* were designed and tested for specificity and efficiency before use (Table 2).

Table 2: Primers used for detection of health and ROS markers in bovine COCs

Name	Primer Sequence	NCBI Accession Number	Product size (bp)
GAPDH	F: CGTAACTTCTGTGCTGTGCC R: ATTGATGGCGACGATGTCCA	NM_001034034.2	136
BMP15	F: GACACCCTAGGGAAAACCGC R: GGTATGCTACCCGGTTTGGT	NM_001031752.1	141
StAR	F: GACGTTTAAGCTGTGTGCCG R: TTGGTCGCTGTAGAGAGGGT	NM_174189.3	202
CYP19A1	F: TACCCGGTGAAAAAGGGGAC R: GGAAGCGTCTCAGAAGGGTG	NM_174305.1	220
SOD	F: CCACGTCCATCAGTTTGGAGA R: TATCCACGATGGCAACACCG	NM_174615.2	161

2'-7'-Dichlorodihydrofluorescein diacetate (DCHF-DA) Staining of Oocytes for ROS levels

To determine if intracellular levels of ROS were different following LPS challenge, treated oocytes were matured for 22 hours and were mechanically denuded using a glass pipette. The denuded oocytes were then collected and cultured for 30 min in PBS supplemented with 3% BSA, 10 µg/mL Hoechst 33342, and 10 µM 2'-7'-dichlorodihydrofluorescein diacetate (DCHF-DA). The stained oocytes were then washed briefly three times in PBS and imaged for fluorescence under an Evos FL Cell Imaging System (ThermoFisher Scientific, Waltham, MA) using the DAPI and GFP fluorescent filters. Staining intensity was quantified using ImageJ analysis software by calculating the corrected total cell fluorescence (CTCF, CTCF = Integrated Density–(Area of selected cell * Mean fluorescence of background readings)) of five areas of an individual oocyte and averaging them. The individual oocyte averaged CTCFs between the various treatments were then compared. Three biological repetitions were performed with averages and imaging done by the same researcher.

Statistics

Statistical analysis was conducted via the use of GraphPad Prism software (v8.4.3). One-way ANOVA analysis with multiple comparisons using post hoc testing for all of the oocyte health and ROS genetic markers. One-way ANOVA was also utilized with Tukey's multiple comparisons for granulosa viability and proliferation, maturation, and ROS

detection. Results were reported using standard deviation (SD) from the mean and were deemed statistically significant at $p < 0.05$.

Results

LPS exposure downregulates the expression of COC health markers

Whereas it has been clearly shown that granulosa can elicit an immune response little is known regarding the effects of this response on oocyte health. *BMP-15*, *StAR,* and *CYP19A1* are well known markers that are associated with oocyte health. Groups of 20-25 COCs were cultured for 1, 6, and 22 hours in maturation media with various treatments. Again, the 6-hour mark proved to be the most eventful with respect to the fold changes in oocyte health markers. Both *BMP15* (0.53 ± 0.22 fold, $p<0.05$) and *StAR* (0.55 ± 0.22 fold, $p<0.05$) demonstrated significant decreases in expression within LPS treated groups, while *CYP19A1* (0.68 ± 0.07, $p<0.1$) trended toward a significant decrease in the LPS group with respect to the control and TL groups. Meanwhile, the treatment groups involving TAK242 with and without LPS were not different from the control group with respect to each health marker (Figures 10, 11, and 12).
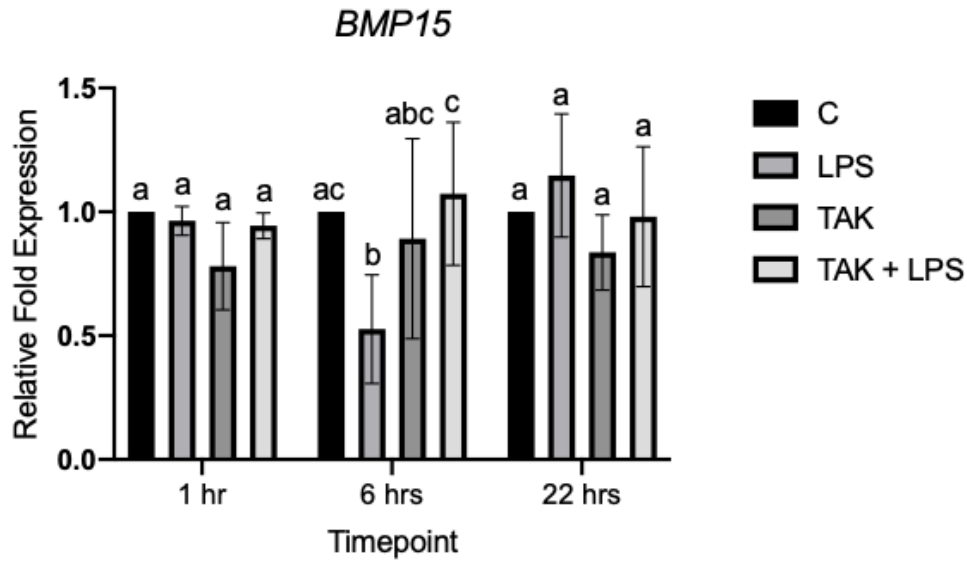
Figure 10: *Expression of BMP15 mRNA in treated COCs.* RT-PCR analysis of *BMP15* relative fold expression in treated COCs at 1, 6, and 22 hours in maturation media using *GAPDH* as an internal control. Cells were untreated (C), cultured in the presence of LPS (LPS), cultured in the presence of TAK-242 (TAK), or cultured with both LPS and TAK (TAK + LPS). No significant changes observed at 1 and 22 hours, however at 6-hours expression of *BMP15* was reduced in the LPS treated group. N=3; Significant difference ($p < 0.05$) denoted by different letters, with respect to each individual timepoint, determined by ANOVA of delta-CT values. Error bars are $\pm$ SD of mean.
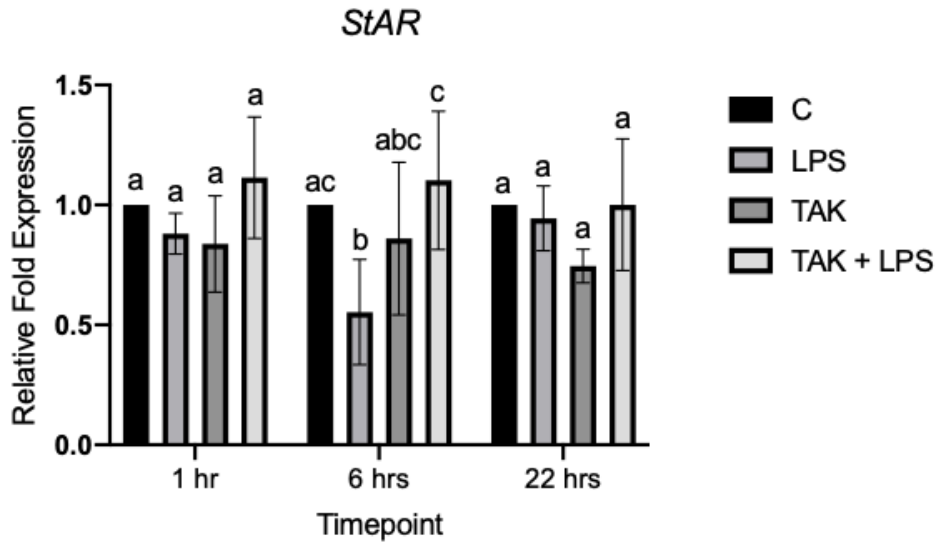
Figure 11: *Expression of StAR mRNA in treated COCs.* RT-PCR analysis of *StAR* relative fold expression in treated COCs at 1, 6, and 22 hours in maturation media using *GAPDH* as an internal control. Cells were untreated (C), cultured in the presence of LPS (LPS), cultured in the presence of TAK-242 (TAK), or cultured with both LPS and TAK (TAK + LPS). No significant changes observed at 1 and 22 hours, however at 6-hours *StAR* expression decreased in the LPS treated group. N=3; Significant difference ($p < 0.05$) denoted by different letters, with respect to each individual timepoint, determined by ANOVA of delta-CT values. Error bars are ± SD of mean.
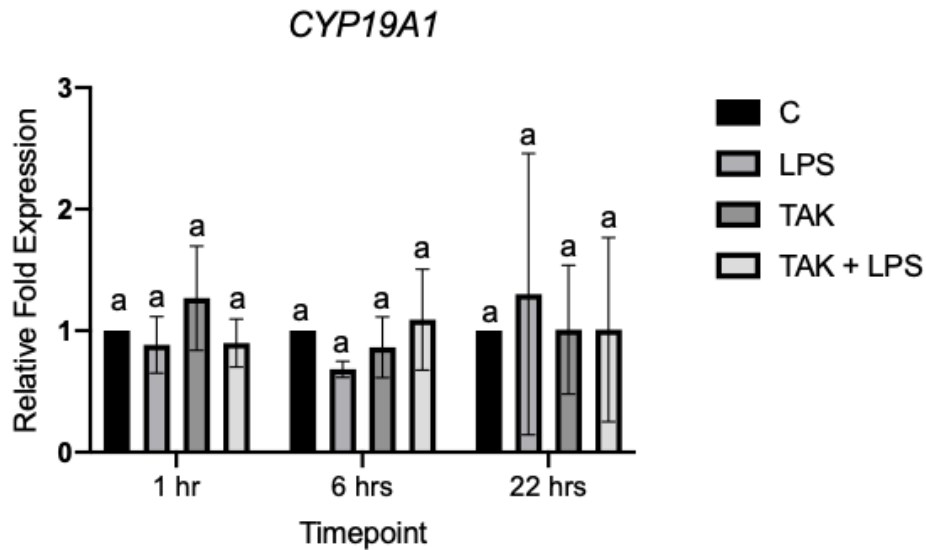
Figure 12: *Expression of CYP19A1 mRNA in treated COCs.* RT-PCR analysis of *CYP19A1* relative fold expression in treated COCs at 1, 6, and 22 hours in maturation media with *GAPDH* as an internal control. Cells were untreated (C), cultured in the presence of LPS (LPS), cultured in the presence of TAK-242 (TAK), or cultured with both LPS and TAK (TAK + LPS). No significant changes observed at any timepoint, however *CYP19A1* expression was trending toward a significant decrease in the LPS treated group. N=3; Significant difference ($p<0.05$) denoted by different letters, with respect to each individual timepoint, determined by ANOVA of delta-CT values. Error bars are mean $\pm$ SD.

Exposure to LPS increases the expression of *SOD*

As a means of further investigating the potentially damaging effects of inflammatory infection on the oocyte, we investigated the production of reactive oxygen species in treated COCs at 1, 6, and 22 hour timepoints. For this purpose, we selected the *SOD* marker to use as an indication of reactive oxygen species production. COCs were exposed to no treatment (C), LPS (1 µg/mL), TAK-242 (TAK, 1 µM), or TAK-242 plus LPS (TAK + LPS). There were no significant changes in *SOD* expression among the treatment groups at 1 and 22 hours. Interestingly, the LPS-treated COCs showed a significant increase in *SOD* expression (1.96 ± 0.77 fold, $p < 0.05$) at 6 hours compared with the control and TAK (0.91 ± 0.32 fold, $p < 0.05$)-treated groups and was not found to be significantly different from the TL (1.05 ± 0.25 fold, $p < 0.05$) treated group (Figure 13).
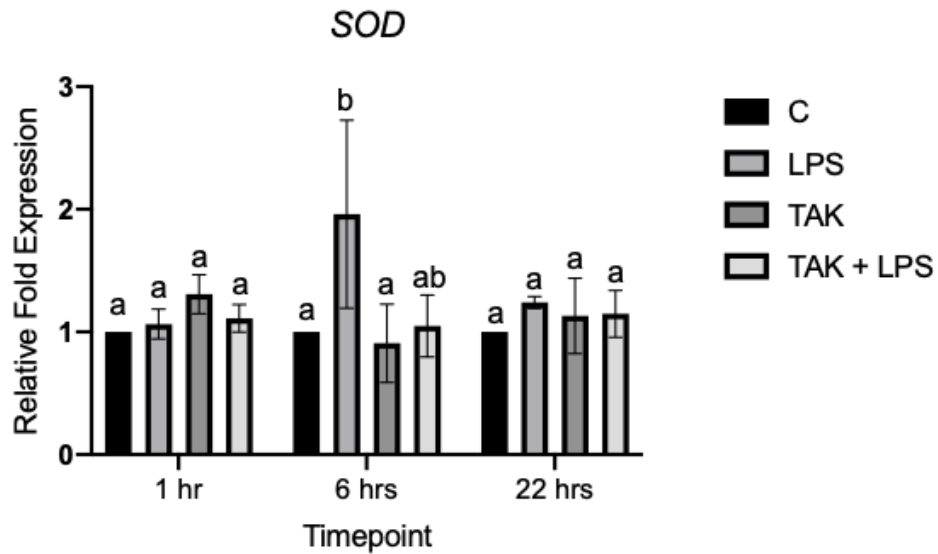
Figure 13: *Expression of SOD mRNA in treated COCs.* RT-PCR analysis of *SOD* relative fold expression in treated COCs at 1, 6, and 22 hours in maturation media with *GAPDH* as an internal control. Cells were untreated (C), cultured in the presence of LPS (LPS), cultured in the presence of TAK-242 (TAK), or cultured with both LPS and TAK (TAK + LPS). No significant changes observed at 1 and 22 hours, however at 6-hours *SOD* expression increased in the LPS treated group. N=3; Significant difference ($p<0.05$) denoted by different letters, with respect to each individual timepoint, determined by ANOVA of delta-CT values. Error bars are mean ± SD.

Exposure to LPS negatively impacts the rate of meiotic maturation in COCs

Now that we have seen the molecular effects of LPS on the oocyte, it is important to look at the functional aspects as well. We began by looking for potential effects of LPS on the meiotic maturation of COCs that were cultured in maturation medium in the presence of LPS (1 µg/mL), TAK242 (1 µM), TAK242 (1 µM) plus LPS (1 µg/mL) or in vehicle only control for 22 hours. The percentage of COCs that were at the germinal vesicle breakdown (GVBD) stage were increased in the LPS-treated group ($45.66\pm5.58\%$, $p < 0.05$) compared with the control ($19.14\pm1.63\%$) (Figure 14). However, the percentage of oocytes that reached the MII phase of meiotic maturation were significantly decreased in the LPS-treated group ($48.54\pm5.81\%$, $p < 0.05$) compared with the control ($75.15\pm2.08\%$). Interestingly, there was no difference from the untreated control in the stage of maturation between groups exposed to TAK242, regardless of the presence of LPS (Figure 14).
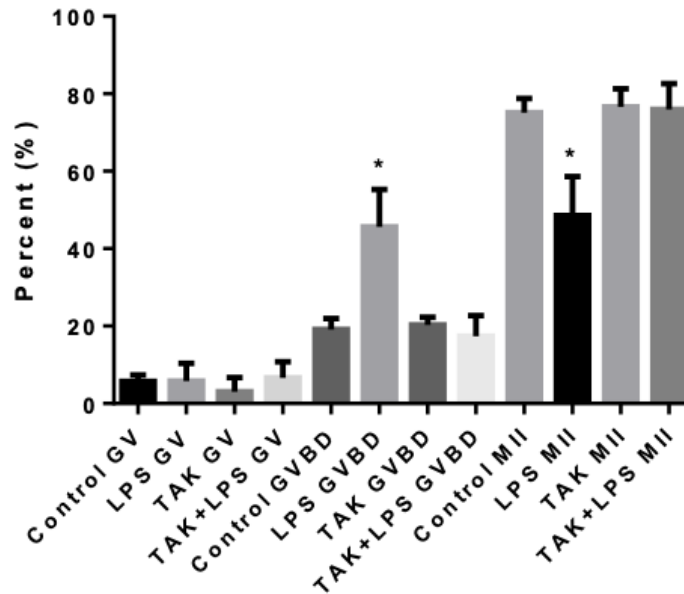
Figure 14: *Meiotic maturation of treated COCs.* Analysis of COCs stained with Hoechst 33342. The percentage of treated COCs throughout the phases of meiotic maturation, including germinal vesicle (GV), germinal vesicle breakdown (GVBD), and meiosis II (MII). No significant changes observed in GV groups, however there was an increase in the percentage of COCs at the GVBD phase of maturation in the LPS treated group. As well as, a decrease in the percentage of cells reaching the MII phase of maturation in the LPS treated group (* denotes $p < 0.05$, N=3).

LPS does not affect granulosa viability or proliferation during maturation

Because granulosa cells were shown to exhibit an immune response to LPS treatment, we were interested to see if this exposure had any effect on the ability of the granulosa cells to proliferate in addition to their overall viability. Granulosa cells were isolated and cultured until reaching >80% confluence (72 hours) and were treated with LPS (1µg/mL), TAK-242 (1 µM), or TAK-242 and LPS (1µM and 1 µg/mL). The cells were then stained using either a viability kit or proliferation assay.

There were no significant effects found among the various treatment groups pertaining to viability both among and between the biological repetitions (Figure 15), including C (1.77 ± 1.31% apoptotic, $p>0.05$), LPS (1.47 ± 0.68% apoptotic, $p>0.05$), TAK (1.40 ± 0.57% apoptotic, $p>0.05$), and TL (1.70 ± 1.25% apoptotic, $p>0.05$).

There were no significant effects found among the various treatment groups pertaining to proliferation both among and between the biological repetitions (Figure 16), including C (6.64 ± 1.55% proliferated, $p>0.05$), LPS (7.68 ± 2.03% proliferated, $p>0.05$), TAK (6.97 ± 1.59% proliferated, $p>0.05$), and TL (7.83 ± 1.71% proliferated, $p>0.05$).
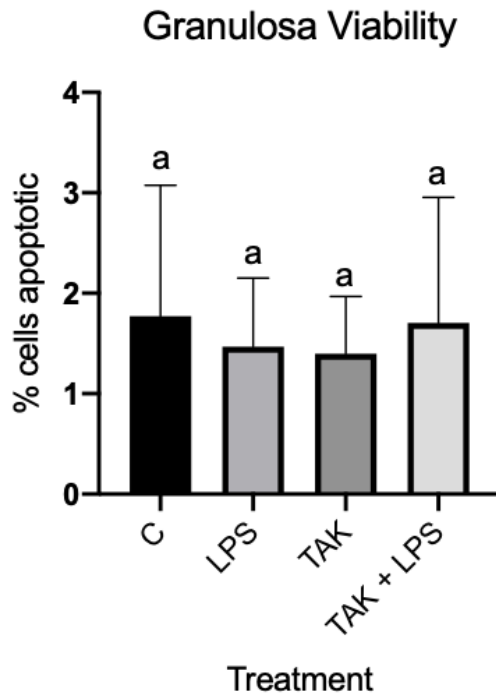
Figure 15: *Viability in treated granulosa cells.* Representation of the percentage of granulosa cells stained with a cell viability kit that were found to be apoptotic after a 6-hour treatment. Treatment groups included, no treatment (C), LPS, TAK-242 (TAK), or TAK plus LPS (TAK + LPS). No significant changes observed among treatment groups. N=3; Significance defined as $p < 0.05$. Error bars are mean $\pm$ SD.

Figure 16: *Examining proliferation in treated granulosa cells.* Representation of the percentage of total cells that proliferated after a 6-hour treatment and stained using an EdU and Alexa fluor 488 imaging kit. Treatment groups included, no treatment (C), LPS, TAK-242 (TAK), or TAK plus LPS (TAK + LPS). No significant changes observed among treatment groups. N=3; Significance defined as $p<0.05$. Error bars are mean $\pm$ SD.
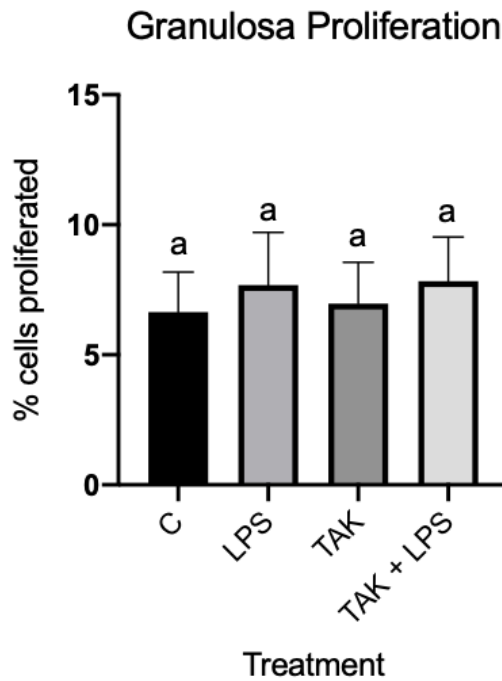
LPS exposure increase ROS in oocytes

In addition to the RT-PCR data accumulated on the production of ROS, we investigated the functional aspect of ROS production within the COC as well. Bovine COCs were treated in LPS, TAK-242, and TAK-242 plus LPS for 22 hours (maturation). Each group was stained with DCFH-DA in order to detect the presence of ROS via measures of corrected total cell fluorescence (CTCF). LPS-treated COCs had an increased fluorescence (50745 ± 5317 CTCF $p < 0.05$) compared with the control (34153 ± 4261 CTCF) indicating an increase in the presence of reactive oxygen species within the LPS-treated oocyte. There were no other significant differences among other treatment groups. (Figure 17).

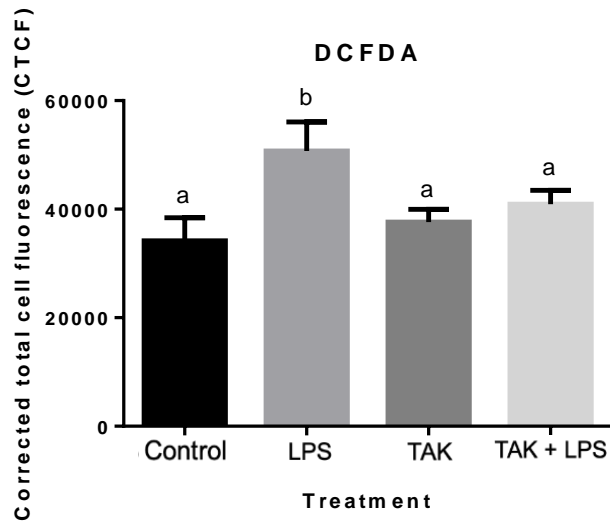**Figure 17:** *Presence of ROS in treated oocytes.* Measurements of corrected total cell fluorescence (CTCF) representing the presence of reactive oxygen species in oocytes stained with DCHF-DA. An increase in the CTCF value was observed in the LPS treated group. Significant difference (p<0.05) denoted by different letters, with respect to each individual timepoint, determined by ANOVA of delta-CT values. Error bars are ± SD from mean.

Discussion

      In the present study we investigated the effects of LPS on oocyte health and maturation. It is well understood that inflammatory infection has adverse effects on reproductive outcomes; however, the mechanisms through which this damage occurs are not well understood. In an attempt to better understand these processes, we investigated the effects of LPS on oocyte health and developmental competence. Beginning with oocyte health, we focused on the effects of LPS exposure on the expression levels of well-known ovarian health markers *BMP15*, *StAR*, and *CYP19A1*. BMP15 is a member of the TGFβ superfamily that is expressed in oocytes (Dube et al., 1998). BMP15 is expressed in primordial oocytes and increases with developmental competence. It has been shown to play important roles in ovarian function including granulosa proliferation, steroidogenesis, maturation, apoptosis, and cumulus expansion (Chang et al., 2016; Persani et al., 2014; Shimasaki et al., 2004; Hashimoto et al., 2005). StAR is known for its role in steroidogenesis, specifically being responsible for the transport of cholesterol into the inner mitochondria matrix, the rate-limiting step in steroidogenesis (Falck, 1960). *CYP19A1* is a cytochrome P450 enzyme that functions to convert androgens to estrogens (Shimodaira et al., 2012; Kamat, 1998). In our study we found both *BMP15* and *StAR* were significantly decreased in the LPS-treated group, in addition to *CYP19A1* trending toward a decrease.

      The decreases in expression of these markers indicate that there are problematic effects of LPS exposure on the health of oocytes and their ability to properly produce important hormones. Upon seeing the impacts of oocyte health, we decided to take a closer

look at the effects of LPS on oocyte maturation. We found that following 22 hours of maturation, the LPS-treated COCs had a higher percentage of COCs remaining at the germinal vesicle breakdown stage; however, there were fewer COCs overall that were able to successfully reach the MII phase of development. This finding indicates that the presence of LPS could be preventing the ability, or altering the timing, of oocytes to successfully mature in order to be effectively fertilized.

As we saw that oocyte development was altered, we investigated if there were any effects of LPS on the viability of granulosa cells or their ability to proliferate during culture. However, we did not find any significant differences among treatment groups, thus suggesting that LPS did not appear to have an effect on granulosa apoptosis or proliferation. Another potential source of damage to infected cells is the production of ROSs. In order to investigate this, the *SOD* marker was selected. *SOD* is typically produced in response to an overproduction of ROS in order to prevent cell damage and subsequent apoptosis (Yonus, 2018; Yasui and Baba, 2006). We found that the expression of *SOD* greatly increased in LPS-treated COCs, thus demonstrating that LPS could be causing an increase in ROS production. In support of these findings, we stained live COCs with DCHF-DA for the presence of ROS following 22 hours of maturation with and without exposure to LPS. We found that the LPS-treated group had an increase in ROS compared with all other treatment groups, thus indicating that LPS could potentially cause cell damage or apoptosis by increasing ROS production.

Final Conclusions and Future Directions

Inflammatory infection in cattle has long been a known culprit for reproductive dysfunction in cattle, it is also a costly issue that also negatively impacts production efficiency. In this study it was determined that the granulosa cells, surrounding the oocyte, are capable of mounting an immune response in the presence of inflammatory infection induced by LPS. This immune response is characterized by the increase in the expression of inflammatory markers TNFa, *IL-6*, and *CCL2*. Such an increase can be linked to the cell damage that was shown in COCs via a decrease in markers associated with cell health, including *BMP15*, *StAR*, and *CYP19A1*. This cell damage can also be attributed to the increased production of ROS shown via increases in *SOD* expression and the presence of ROS in live treated COCs. Subsequently, this impairment could lead to decreased developmental competence of COCs, preventing these cells from reaching the MII phase of development. Previous studies have also investigated the specific effects of LPS on bovine reproduction; however, the specific mechanisms through which this damage occurs is not well understood. Determining the specific aspects of the TLR4 pathway that LPS is affecting is critical to our understanding of exactly how this bacterial cell wall component is able to function through this pathway in addition to eliciting an opportunity to develop a solution to completely block the destructive effects of LPS in order to protect the reproductive function of cattle. Additionally, examining the effects of LPS on developing conceptuses both before and after fertilization could further illuminate the effects of inflammation due to infection on development.

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