# IDENTIFYING THE CONTRIBUTIONS OF INTERFERON RESPONSES TO DISEASE AND PREGNANCY IN CATTLE

By

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IDENTIFYING THE CONTRIBUTIONS OF

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Abstract: Two significant challenges the cattle industry is facing are respiratory disease and infertility. The type I interferon (IFN) pathway plays a pivotal role in both disease and pregnancy. The type I IFN pathway is interrupted by viral infection which reduces production of interferon stimulated genes (ISG). Interferon-tau (IFNT) is the pregnancy recognition signal in ruminants, which acts through the type I IFN pathway to activate ISG. Thus, understanding IFN and immune function could improve production efficiency. Studies described herein evaluate type I IFN and immune responses to bovine respiratory disease and pregnancy recognition in cattle.

The first experiment evaluated activation of the type I IFN pathway after infecting calves with bovine viral diarrhea virus type 1b (BVDV1b) and *Mannheimia haemolytica* (MH). Steady-state mRNA levels of *MX1*, *ISG15*, and *RTP4* were determined in peripheral blood leukocytes prior to BVDV1b exposure (d -4), prior to MH challenge (0 h), 12 h and 24 h after MH challenge. A significant time effect ($P < 0.05$) for all ISG was detected. At 0 h, *ISG15* levels increased 44-fold and remained elevated over 60-fold for 12 h and 24 h ($P < 0.01$). Likewise, *RTP4* and *MX1* increased at 12 h ($P < 0.05$) after BVDV challenge. Data suggests that the type I IFN pathway remains active after challenge with BVDV1b and MH.

The second experiment evaluated pregnancy rates after intrauterine, autologous transfer of IFNT-primed immune cells. Peripheral blood mononuclear cells were cultured overnight with 500 U/mL of IFNT, followed by autologous intrauterine transfer (IMMUNE; n = 97) on d 4 after estrus; controls received intrauterine infusion of saline (CONT; n = 82). On d 7, serum samples were collected for hormone analysis and embryos were transferred to all animals. Progesterone concentrations were similar for IMMUNE (4.1 ± 0.33 ng/mL) and CONT (3.7 ± 0.33 ng/mL) and were not different between pregnant and open cows ($P > 0.20$). Pregnancy rate for IMMUNE was 77% (75/97) compared with 57% (47/82) for CONT ($P < 0.01$). Results indicate that progesterone concentrations did not differ between groups and transfer of autologous IFNT-primed PBMC improved pregnancy rates after embryo transfer.

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# CHAPTER I

## INTRODUCTION

The population of the world is predicted to dramatically increase in the next 35 years, increasing the global demand for meat and animal products by 70% by the year 2050 (Alexandratos and Bruinsma, 2012). Due to limitations in land space, the increase in food production will need to be driven by new technologies. Currently, two of the largest issues that decrease production efficiency in livestock operations are respiratory disease and infertility. Thus, these issues are ideal targets for investigation to increase production efficiency. The interferon (IFN) pathway is a key modulator in respiratory disease and infertility; aberrations in IFN signaling could contribute to disease susceptibility and reduced reproductive rates. Interferons are immune-derived cytokines which regulate immune responses to pathogens and to pregnancy in domestic ruminants. Advances in our understanding of the IFN pathway and immune function could improve upon challenges associated with infertility and disease in domestic livestock production.

Bovine respiratory disease complex (BRDC) is typically composed of viral and bacterial pathogens affecting the respiratory tracts of calves. The two most common pathogens in Oklahoma are bovine viral diarrhea virus (BVDV) and *Mannheimia*

*haemolytica* (Fulton et al., 2002). Bovine viral diarrhea virus is a single stranded RNA virus, member of the *Pestivirus* genus, family *Flaviviridae* (Meyers and Thiel, 1996). Bovine viral diarrhea virus is separated into BVDV1a, BVDV1b and BVDV2 (Ridpath et al., 1994; Ridpath and Bolin, 1998). Calves can become infected with BVDV while still in utero; producing either transiently infected (TI) or persistently infected (PI) calves. If the dam is infected between 30 and 120 d of gestation with BVDV, calves are considered PI, and are unable to mount an immune response to BVDV (Bognar, 1972; Kahrs, 1973; McClurkin et al., 1984; Stokstad and Løken, 2002). If calves are infected after 120 d of gestation they are considered TI and are able to produce an immune response in utero (Bognar, 1972; Kahrs, 1973; McClurkin et al., 1984; Stokstad and Løken, 2002). There are two biotypes that produce BVDV, noncytopathic (ncp) and cytopathic (cp), depending on the virus' ability to cause cytopathogenicity, or pathological changes in the cell. Persistently infected calves can only be produced by ncp-BVDV, which is a lifelong disease causing calves to constantly shed the virus through nasal discharge and coughing (Dubovi, 1994). This is how BVDV is maintained and spread in the cattle population. In 2011, an estimated 16.2% of cattle in the United States were affected by respiratory disease, with 87.5% of the 16.2% being treated for the disease (NAHMS, 2011). Bovine respiratory disease is responsible for an estimated $800 to 900 million dollar loss each year, costing producers $23.60 per case, due to decreased feed efficiency, medicine costs, and death (Chirase and Green, 2000, NAHMS, 2011).

Another major problem the cattle industry is facing is infertility and subfertility. Cattle exhibit high fertilization rates, estimated at over 90%, but the rate of pregnancies carried to term can be as low as 40% (McMillan, 1998). A major cost and contributing

factor to failure in reproductive efficiency in both natural service and either artificial insemination or embryo transfer is the low rates of embryonic survival. Embryonic mortality is the losses that occur during the early embryonic period, extending from conception to differentiation, which is approximately 45 d (Committee on Bovine Reproductive Nomenclature, 1971), and most embryonic mortality can be attributed to inadequate endometrial receptivity (Hansen and Block, 2004). Early embryonic loss results in fewer calves being born, slower genetic progress, and a significant economic loss to the cattle industry (Dunne et al., 2000). Improving endometrial receptivity, decreasing embryonic loss, and increasing pregnancy rates, all work to improve the genetic selection, decrease production costs, increase reproductive efficiency, and ultimately increase production efficiency to feed the increasing population.

Reproductive technologies have evolved from natural mating to AI to embryo transfer (ET). Embryo transfer has become a large international business, as well as a tool to improve genetics and specific characteristics in cattle (Wu, 2012). Currently, AI has improved genetic selection but it is limited to single generation contributions at a time. Artificial insemination can be useful in breeding one superior bull to several superior females, but only one progeny is produced per superior female. Embryo transfer provides the opportunity to produce several progeny from one superior female and one or more superior males. There are an estimated 14,000 to 250,000 eggs produced in female cattle (Ireland et al., 2008; Machado et al., 2006; Suthar and Shah, 2009). Using ET technology, as many as 1,000 oocytes have been collected and 100 offspring have been produced by one cow in a year (Ireland et al., 2008; Machado et al., 2006; Suthar and Shah, 2009). This greatly increases the rate of genetic change in the cattle industry.

The use of reproductive technologies in the United States is still relatively low in the beef industry. There are several limiting factors stopping producers from implementing reproductive technologies, including labor, time, cost, lack of facilities, or degree of difficulty (NAHMS, 2007). In the United States, 37.7% of producers cited labor and time as the number one reason they do not use AI (NAHMS, 2007). In beef operations with greater than 200 cows, only 19.3% use estrus synchronization, 19.8% use AI, and 5.0% use ET (NAHMS, 2007). Use of these technologies is even less for all herd sizes in the United States at 7.9% use estrus synchronization, 7.6% use AI, and 1.6% use ET (NAHMS, 2007). It is conceivable that if reproductive rates were increased when utilizing reproductive technologies, then broader adoption of reproductive technology would be observed because there would be more return on time, labor, and facility investment for livestock producers. The increase and development of reproductive technologies will greatly increase the genetic improvement of cattle herds, as well as bridge the gap between the rise in global population and the need to increase food production.

Improving reproductive technology would increase genetic change, improve economically important traits of production animals, and increase production efficiency. There is a major opportunity for improving reproduction technology in order to meet future food demands. Type I interferon (IFN) activation, production, and signaling impacts both BRDC and fertility rates in cattle. The type I IFN pathway is active in both disease and pregnancy, though the functions and effects are different. Continued research into type I IFN signaling and effects could provide new opportunities for new technology to address BRDC rates and infertility.

# CHAPTER II

# LITERATURE REVIEW

## ESTROUS CYCLE

Bovine are a polyestrous animal entering estrus every 21 d with two distinct phases, the follicular phase and the luteal phase. The start of the estrous cycle is identified by estrus, or standing heat. Estrus lasts on average 15 h in cattle and marks the period of sexual receptivity by the female. Estrus is preceded by the follicular phase where follicles undergo two or three waves of growth and atresia to produce one dominant follicle for ovulation. Follicle stimulating hormone (FSH) and luteinizing hormone (LH) provide the necessary mechanisms by which follicular recruitment, selection, and dominance occurs via a negative feedback loop on the hypothalamic-pituitary-gonadal axis. The release of these hormones from the anterior pituitary is governed by the decapeptide, gonadotropin releasing hormone (GnRH) produced in the hypothalamus. The arcuate nuclei, also known as the tonic center, are responsible for the pulsatile release of GnRH (Clarke and Cummins, 1982). The preoptic area, or surge center, is responsible for the preovulatory surge of LH (Swanson and Hafs, 1971). Under the influence of FSH, follicles grow until one reaches dominance. The granulosa and

theca cells of the dominant follicle secrete estradiol and inhibin, which in turn work to decrease FSH production and increase LH secretion for luteolysis. Granulosa and theca cells in the follicle work together to produce the steroid hormone estrogen ($E_2$). Theca cells produce the androgens, androstenedione and testosterone from cholesterol, but lack the necessary enzymes to produce $E_2$. Androgens diffuse across the basement membrane to the granulosa cells and are converted to estrogens by the enzyme aromatase (Fortune and Quirk, 1988). Follicle stimulating hormone stimulates the production of inhibin from granulosa cells, which feeds back to the anterior pituitary to decrease the amount of FSH produced. Rising concentrations of $E_2$ cause a preovulatory surge of LH, inducing the follicle to rupture and release the oocyte. This rupture causes a subsequent cascade of events called luteinization to ultimately produce the corpus luteum (CL). The CL is a highly vascular structure that produces the hormone progesterone ($P_4$) and marks the beginning of the luteal phase of estrus (reviewed by McCracken et al., 1999).

During luteinization, theca and granulosa cells restructure to form luteal cells and the steroidogenic pathway is reprogrammed to primarily produce $P_4$. In ruminants' granulosa cells are differentiated into large luteal cells (LLC) and theca cells become small luteal cells (SLC) under the influence of LH. The SLC and LLC compose about 30% of the CL, with endothelial cells comprising about 50% (O'Shea et al., 1989; Lei et al., 1991). Progesterone is produced primarily from the LLC in the CL to act on the hypothalamus and anterior pituitary to block the pulsatile release of GnRH. This restricts the secretion of LH, $E_2$, and FSH to low frequency high amplitude amounts. Progesterone remains elevated for the duration of the luteal phase, and decreases about 48 to 60 h before ovulation (Baird, 1978). Progesterone, binding to progesterone receptors (PR)

blocks expression of both the estrogen receptors (ER) and oxytocin receptors (OTR) in the endometrium, thus preventing the increase of these hormones (McCracken et al., 1984). Progesterone down regulates its own receptor in the endometrial luminal epithelial (LE) and superficial ductal glandular epithelium (sGE) due to continual exposure to $P_4$ (Spencer et al., 1995a, b). The down regulation of $P_4$ effectively releases the block on the ER and OTR expression, allowing a return to estrus and follicular growth. Exogenous $E_2$ will cause luteolysis in ewes when administered between d 9 to 12 (Hawk and Bolt, 1970). When $E_2$ is administered between d 1 and 6, it is ineffective in causing a return to estrus by luteolysis (Hawk and Bolt, 1970). In sheep, this correlates with $P_4$ down regulating its own receptor at 8 to 10 d after ovulation and the up regulation of both the ER and OTR (Spencer et al., 1995a, b).

During the luteal phase, the CL undergoes a natural progression reaching full maturity and maximal $P_4$ production, to regression, or luteolysis and a marked decrease in $P_4$ production. Regression of the CL is caused by pulsatile secretions of the luteolytic hormone prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). To cause regression of the CL, ruminants must be exposed to 5 to 8 pulses of $PGF_{2\alpha}$ within a 24 h period, thus causing luteolysis and a return to estrus (McCracken et al., 1970; Nancarrow et al., 1973; Peterson et al., 1975). Endometrial-derived $PGF_{2\alpha}$ binds to receptors on luteal cells and initiates a signaling cascade that terminates $P_4$ production causing cellular death in the CL (reviewed by Silvia et al., 1991). Prostaglandin $F_{2\alpha}$ production is driven by luteal derived oxytocin (OT). Oxytocin binds OTR in luminal epithelium cells and stimulates the release of $PGF_{2\alpha}$ by activating the phosphatidyl inositol-diacylglycerol protein kinase C second

messenger system (Silvia and Homanics, 1988; Silvia and Raw 1993; Tysseling et al., 1998). Low pulses of $PGF_{2\alpha}$ stimulate the release of OT from luteal cells in the CL, which feeds back to increase the release of $PGF_{2\alpha}$ (McCracken et al., 1984). Release of $PGF_{2\alpha}$ is dependent on the presence of OTR in the endometrium. Increasing $PGF_{2\alpha}$ production eventually causes regression of the CL. Estrogen concentrations from the dominant follicle increase simulating the transcription and translation of OTR in the luminal epithelium (McCracken et al., 1984). Oxytocin binds OTR to stimulate the release of $PGF_{2\alpha}$ which drives both structural and functional regression of the CL (McCracken et al., 1984).

## PREGNANCY RECOGNITION AND MAINTENANCE

### *Progesterone*

Progesterone is essential for the continuation of pregnancy, and is necessary for implantation, placentation, and embryonic development. Progesterone acts on the endometrium to encourage functions necessary for conceptus growth and development (reviewed by Brook et al., 2014). In bovine, administration of $PGF_{2\alpha}$ lowered serum concentrations of $P_4$ and significantly decreased conceptus length on d 14 of gestation (Forde et al., 2011). The lack of conceptus growth in a low $P_4$ environment shows the importance of adequate $P_4$ concentration in pregnancy for functional growth and development of the conceptus. Cows ovariectomized between d 48 and 268 of pregnancy resulted in abortion of all fetuses (Estergreen et al., 1967), indicating that placental production of $P_4$ is not sufficient to sustain pregnancy. Ewes treated with the PR

antagonist RU486, significantly lowered blastocysts survival rate (Satterfield et al., 2006). It is clear that $P_4$ has an effect on the conceptus in early pregnancy and is required for sustaining pregnancy.

*Interferon-tau*

For pregnancy to be established and maintained the attenuation of the regression of the CL is vital for continued secretion of $P_4$. In cows, timing of conceptus entrance in the uterus in relation to luteal maintenance has been established to be between d 15 and 17 of pregnancy (Humbolt and Porta, 1984; Northey and French, 1980; Thatcher et al., 1984). This critical period calls for communication between the embryo and the recipient or dam. In essence, a signal must be sent to the dam to signal pregnancy to abrogate rejection of the conceptus and stimulate changes for growth and maintenance. Though the exact substance was unknown at the time, it was found that administering embryonic tissue or homogenates extended the lifespan of the CL. Cows with embryos removed at d 17, 18, or 19, exhibited longer interestrus intervals, while those with embryos removed at d 13 or 15 had on average 21 d interestrus periods (Northey and French, 1980), indicating, that embryos affect the return to cyclicity. When conceptuses were removed on d 9 or 14 after conception, the length of the estrous cycle is not affected (Humbolt and Porta, 1984). When embryos were removed at 16 d post conception estrus was prolonged by 7 d (Humbolt and Porta, 1984). An increase in $P_4$ and delayed regression of the CL occurred when homogenates of the embryos collected at d 17 or 18 were transferred to the uterine horn ipsilateral of the CL of non-pregnant cows (Northey and French, 1980). These data indicate that embryos secrete a substance that affects the CL and the

continuation of the release of $P_4$, suggesting that d 16 or older conceptuses exhibit antiluteolytic properties.

In sheep, trophoblastin, was identified as an antiluteolytic compound by intrauterine injection of homogenates from the embryo. Similar to studies in cattle, injections of extracts from trophoblasts extended the life of the CL in ovine, suggesting a complementary effect between the conceptus and the endometrium (Martal et al., 1979). A major secretory protein, identified as ovine trophoblast protein-1(oTP-1) in ovine, and bovine trophoblast protein-1(bTP-1) in bovine, is released by the unattached sheep conceptus between d 13 and 21 (Godkin et al., 1982; Imakawa et al., 1987). Ovine trophoblast protein-1 and bTP-1 have a molecular weight of about 17,000 (Godkin et al., 1982; Imakawa et al., 1987). These proteins are structurally related to type I IFN, such as IFNα, IFNβ, IFNε, and were later renamed interferon-tau (IFNT; Imakawa et al., 1987; Roberts et al., 1992b). Interferon-tau is secreted by the conceptus between d 10 and 25, with maximum secretion on d 14 to 16 in sheep and 16 to 19 in bovine (Helmer et al., 1987). Once IFNT had been identified as the pregnancy recognition signal in ruminants, subsequent work focused on mechanisms by which IFNT rescues the CL and maintains pregnancy.

Release of $P_4$ did not change in luteal tissue from non-pregnant cows incubated with recombinant bovine IFNα, indicating that IFNT acts elsewhere in the endocrine system and not on the CL directly to promote $P_4$ production (Barros et al., 1992). To determine where conceptus proteins exhibit their effect, endometrial tissues from cyclic cows were incubated with conceptus secretory proteins, or bTP-1 (Helmer et al., 1989). Endometrial produced $PGF_{2\alpha}$ is reduced by both conceptus secreted proteins and bTP-

1(Helmer et al., 1989). Danet-Desnoyers and others (1994) tested the effects of IFNT and OT on epithelial and stromal cells from the endometrium of cyclic cows 15 d post estrus. When epithelial cells were incubated with IFNT, with or without OT, $PGF_{2\alpha}$ production was decreased (Danet-Desnoyers et al., 1994). When OT was added $PGF_{2\alpha}$ production was increased, but was still significantly lower than controls (Danet-Desnoyers et al., 1994). Stromal cell production of $PGF_{2\alpha}$ was not affected by IFNT or by OT treatments, indicating that the major source of $PGF_{2\alpha}$ comes from the luminal epithelial cells (Danet-Desnoyers et al., 1994). Oxytocin receptors appear around d 14 of non-pregnant ewes, and decrease as $P_4$ increased during early luteal phase (Wathes and Hamon, 1993; Stevenson et al., 1994). The reduction of OTR is through a paracrine fashion involving several hormones and mechanisms. Interferon arrests the development of luteolytic mechanisms by acting on the endometrial epithelia to suppress transcription of the estrogen receptor and the oxytocin receptor, which are responsive to estrogen, in turn inhibiting the secretion of oxytocin (Beard and Lamming, 1994; Burgess et al., 1990; Spencer et al., 1995a, b). All of these mechanisms work synergistically to maintain and continue pregnancy and highlighted IFNT's role in successful pregnancy.

## TYPE I INTERFERON PATHWAY

Interferon-tau belongs to the type I IFN family that includes IFNα, IFNβ, and IFNΩ (Interferon Nomenclature, 1980). Interferons are signaling proteins belonging to the cytokine class that has antiviral and growth inhibitory effects (Isaacs and Lindenmann, 1957). The type I IFN pathway is activated when a virus invades the host animal. This invasion activates a signal transduction pathway that triggers transcription

of genes. These genes, known as interferon stimulated genes (ISG), assist the host animal to produce an antiviral response (Randall and Goodbourn, 2008). Type I IFN bind a common type I IFN receptor with two subunits, IFNAR1 and IFNAR2 located on the cell surface (Pestka et al., 1987). Upon binding to the type I IFN receptor, activation of the classic Janus activated kinas (JAK) signal transducer and activator of transcription (STAT), is initiated to signal a downstream cascade of events including induction of ISG (Binelli et al, 2001). The subunit IFNAR1is associated with the tyrosine kinase 2 (TYK2) and IFNAR2 is associated with JAK (Platanias, 2005). Both are ligand dependent, causing a rearrangement and dimerization of IFNAR1 and IFNAR2 followed by autophosphorylation (Platanias, 2005). Autophosphorylation of TYK2 and JAK activates the many STATs associated with type I IFN pathway including STAT1, STAT2, STAT3, and STAT5 (Darnell, 1997; Stark et al., 1998). Depending on the IFN, two STAT molecules are phosphorylated and dimerize with interferon regulatory factor-9 (IFR9) to form the complex IFN-stimulated gene factor-3 (ISGF3; Darnell, 1997; Stark et al., 1998). Interferon stimulated gene factor-3 binds to specific elements known as IFN-stimulated response elements (ISRE) that are present in certain ISG thereby initiating transcription of the genes (Platanians, 2005). Interestingly, type I IFN are generally induced by pathogens entering the host, causing an immune reaction, but IFNT represented a new class of IFN that is not pathogen induced (Martal et al., 1998). Instead IFNT is temporally secreted by the conceptus to signal pregnancy making it a cytokine as well as a reproductive paracrine hormone.

## INTERFERON SIGNALING IN THE UTERUS

There are several genes in the endometrium, CL, and blood that are differentially expressed between pregnant and non-pregnant ruminants; many of these have been identified to be related to IFNT. Several ISG have been identified, though their roles in pregnancy have not yet been elucidated. The ISG that are upregulated in response to IFNT are hypothesized to regulate endometrium receptivity, differentiation, and conceptus elongation and implantation (Bazer et al., 2009; Hansen et al., 1999; Hansen et al., 2010). Genes such as, *2'5'-oligoadenylate synthetase (OAS-1), MX dynamin-like GTPase 1 (MX1),* and *MX dynamin-like GTPase 2 (MX2)* have an increased expression due to stimulation by IFNT (Ott et al., 1998; Schmitt et al., 1993). This is in agreement with other studies that found a 15-kDa ISG in the uterus in relation to pregnancy (Johnson et al., 1999). In sheep, *ISG15 ubiquitin-like modifier (ISG15)* is expressed in the LE on d 10 or 11, as well as in the stratum compactum stroma and glandular epithelium (GE) on d 13 and 14 of pregnancy (Johnson et al., 1999). Interferon stimulated genes are also expressed in the CL during pregnancy. *Receptor (chemosensory) transporter protein-4* (*RTP4), ISG15, MX1*, and *OAS* have all been found in response to IFNT injections or in early pregnancy in the CL (Gifford et al., 2008; Oliveira et al., 2008; Spencer et al., 1999). These ISG are activated through the type I IFN pathway, much like a virus activates the pathway, but with some key differences. The type I IFN pathway is activated by IFNT, produced by the conceptus. The interferon regulatory factors are temporally and spatially regulated by IFNT during early pregnancy in the uterus. The interferon regulatory factors-1 and -2 (IRF1, IRF2) can only be found in the luminal epithelial (LE) and the sGE (Spencer et al., 1998). The expression of STAT1 and STAT2 as well as IRF9 were not detected in the LE and sGE during early pregnancy, but the

IRF2 protein expression is increased in the LE and sGE in response to IFNT (Choi et al., 2001). This suggests that IFNT is utilizing an unknown mechanism to up regulate expression of ISG, other than the STAT1, STAT2, and IRF9 pathway.

Recent work has brought to light the interactions of IFNT, prostaglandins (PG) and cortisol in the uterus during early pregnancy. The conceptus produces more PG than the endometrium when comparing cyclic to pregnant cattle (Lewis, 1989). The continual release of PG appears to help regulate growth and development of the conceptus. Receptors for PG are found in all cell types of the endometrium, indicating paracrine, autocrine, and potential intracrine effects on the endometrium to improve conceptus growth and elongation (Dorniak et al., 2011). For PG production to occur, the rate limiting enzyme cyclooxygenase 2 (COX-2) otherwise known as prostaglandin-endoperoxide synthase 2 (PTGS2) must be produced by the LE, sGE, and the conceptus (Kim et al., 2003; Ulbrich et al., 2009). In ovine, COX-2 is increased in pregnant ewes, with maximal production on d 12, but continued to be high through d 16 (Kim et al., 2003). The conceptus secretes COX-2 from d 8 to 17, with a maximum increase between d 14 and 16, declining after to become undetectable after d 25 (Charpigny et al., 1997). Prostaglandins are essential for conceptus growth, elongation, and implantation. Heifers that were injected with meloxicam (MEL), an inhibitor of COX-2, on d 15 after insemination, had reduced pregnancy rates compared to those heifers that were untreated (Erdem and Guzeloglu, 2010) indicating that PG has positive effects on the establishment of pregnancy.

Progesterone induces transcription of many genes that are involved with elongation and implantation of the conceptus. Genes include: *hydroxysteroid (11-beta)*

*dehydrogenase 1 (HSD11B1), lectin, galactoside-binding, soluble, 15 (LGALS15), and solute carrier family 5 (sodium/glucose cotransporter) member 1 (SLC5A1*; Satterfield et al., 2009). Relative to conceptus growth, HSD11B1 is involved with biologically activating cortisol in the endometrium and is implicated in conceptus elongation (Dorniak et al., 2012). Inhibiting COX-2 with MEL decreases the amount of cortisol and HSD11B1 found in the uterine lumen and endometrium (Dorniak et al., 2012). Infusing IFNT into the endometrium increases the amount of HSD11B1 and cortisol, but this effect is lowered when a combination of IFNT and MEL is infused (Dorniak et al., 2012). Inhibiting PG and the corresponding decrease of cortisol and HSD11B1 in the lumen and endometrium is indicative of uterine and conceptus interactions to release cortisol. It also indicates that IFNT can affect the release of cortisol, but inhibiting PG diminishes this effect. Infusing ewes with cortisol increased HSD11B1 and COX-2 activity, while inhibiting HSD11B1 by infusing PG915275, decreased cortisol (Dorniak et al., 2013). When cortisol and PG915275 were co-infused, COX-2 was diminished, IFNT increased HSD11B1 and cortisol, but PG915275 had no affect this increase (Dorniak et al., 2013). Furthermore, when ewes were infused with PG915275 conceptus length was severely inhibited and caused a reduction in several $P_4$ induced genes such as *HSD11B1* and *LGALS15* (Dorniak et al., 2013). Infusion of IFNT and PG915275 rescued this inhibition on conceptus growth (Dorniak et al., 2013). These studies exhibit the regulatory effects of IFNT on conceptus growth and elongation as well as the effects that cortisol, IFNT, and PG have on the uterine endometrium and conceptus.

There is more $PGF_{2\alpha}$ secreted after pregnancy than during the estrous cycle; yet this does not cause luteolysis because the CL is sensitive to pulses of $PGF_{2\alpha}$ rather than

basal concentrations. Pregnant sheep CL are actually exposed to a higher concentration of $PGF_{2\alpha}$ than cyclic sheep on d 13 of pregnancy than d 13 of the estrous cycle (Silvia et al., 1991). Pregnant sheep required a higher dose of $PGF_{2\alpha}$ than non-pregnant sheep, at 6 and 10 mg/58 kg of body weight and 4 mg/58 kg of body weight, respectively, to cause luteolysis (Silvia and Niswender, 1984) indicating a resistance to $PGF_{2\alpha}$ by the CL. This was further confirmed by examining the pattern of $P_4$ over time in response to $PGF_{2\alpha}$. Results indicated that the CL develops resistance to $PGF_{2\alpha}$ between d 10 and 12, and this continues to d 16 in sheep (Silvia and Niswender, 1986). The decrease in sensitivity could be explained by the escaping of IFNT from the uterus. Using a bioassay for type I IFN, Olivier and others discovered that type I IFN is released from the uterus of pregnant sheep through the uterine vein (Oliveira, et al., 2008). In addition, Gifford et al. (2008) observed that ISG were increased in the CL during early pregnancy. Building on this premise, it was speculated that infusion of IFNT would extend the life of the CL in ruminants. Non pregnant ewes were fitted with osmotic pumps to secrete recombinant oIFNT into the uterine vein for 72 h and then challenged with an injection of $PGF_{2\alpha}$ 24 h after pump installation (Antoniazzi et al., 2013). Progesterone levels were sustained in ewes receiving IFNT and challenged with $PGF_{2\alpha}$, indicating that IFNT decreases the CL's sensitivity to $PGF_{2\alpha}$.

## IMMUNE MODULATION DURING EARLY PREGNANCY

The conceptus is a foreign body that should be attacked by the maternal immune system, but this does not happen. Medawar (1953) first described the immunological significance of the pregnant mother being able to tolerate an allogeneic conceptus. The

conceptus relies upon the uterus to secrete products that will help implantation, and to create a suitable environment for growth and development. It is thought that the uterus and conceptus secrete cytokines during implantation, which are involved in the maternal cross talk between the fetus and the dam, and help to form collaborative relationships between the trophoblast, uterus and immune cells (Wegmann et al., 1993; Bai et al., 2012). T helper cells, active in immunoregulation, in one way can be classified as Th1 or Th2 based on the cytokine profile they induce. The Th1 response is associated with the inflammatory cytokines interleukin (IL) 2 and IFN-γ while Th2 response is associated with production of IL-4, IL-5, IL-6, IL-10 and IL-13 (reviewed by Saito et al., 2010). The Th1 profile is associated with a cell-mediated immunity, and Th2 profile is more of a humoral, anti-body response. To prevent immunological attack on the embryo, there is a shift from a Th1 immune response to a Th2 response. Early work suggested that IL-2, TNF-α, and IFN-γ, which are all Th1 associated cytokines, are deleterious to pregnancies in mice, causing abortion (Chaouat et al., 1990). Natural killer cells (NK) were found to cause fetal reabsorption in mice, and injection of anti-NK antibodies reduced the number of fetal adsorptions (Gendron and Baines, 1987). During pregnancy there is a shift towards Th2 cytokines release from the maternal-fetal interface; IL-3, IL-4, IL-5 and IL-10 cytokines are all found during each trimester of pregnancy (Lin et al., 1993). In late secretory phase of the menstrual cycle and early pregnancy, lymphocytes increase in number but do not exhibit classic T cell or natural killer cell markers (Bulmer et al., 1984, 1985). In early pregnancy leukocyte populations increased from 8.2% to 31.7% with over 75% of the leukocytes being CD56+ cells (Bulmer et al., 1991). This shift from

Th1 to Th2 cytokine profiles is considered essential for pregnancy maintenance and preventing rejection of the conceptus (Wegmann et al., 1993).

## SYSTEMIC ACTIVATION

While there are uterine specific changes in the immune system during pregnancy, there is also a systemic response to pregnancy. CD4+ and CD25+ are regulatory T-cells involved in preventing autoimmunity, and are implicated in suppressing immunological rejection of the conceptus (reviewed by Shevach, 2002). Cows in the stages of early pregnancy had greater populations of lymphocytes that were CD4+ and CD25+ in the peripheral blood when compared to non-pregnant cows (Oliveria and Hansen, 2008). In pregnant women the concentration of CD4+ and CD25+ cells more than doubled when compared to non-pregnant women (Somerset et al., 2004). Pregnant women exhibited greater serum concentrations of IL-4 and IL-10, both Th2 type responses, and decreased concentrations of IL-2 and IFN- $\gamma$, Th1 type responses, when compared to non-pregnant women (Marzi et al., 1996). These studies also indicate that there is a shift in the immune cell population and a shift from Th1 to Th2, indicating that immune cells assist in pregnancy maintenance. The antiviral protein, *MX1*, is up-regulated in pregnant ewes in peripheral blood mononuclear cells (PBMC) within 24 to 48 h of the IFN signal (Yankey et al., 2001). Peripheral blood leukocytes (PBL) from pregnant cattle showed *MX2*, *ISG15*, and *MX1* to by increased as early as d 16, 18, and 20, respectively (Gifford et al,. 2007). These studies indicate that IFN is activating genes throughout the body, and is not limited to the reproductive organs. Though the functions of these genes are not yet

known, it can be speculated that they are important to pregnancy function and maintenance.

Systemic activation of immune the system during pregnancy appears to be important for maternal physiological adaptations to pregnancy. Fujiwara and others (1993) postulated that PBMCs play a role in CL $P_4$ production by carrying information through blood circulation from the embryo to the CL. To test this theory they cultured luteal cells from pregnant and non-pregnant women with PBMCs isolated from the follicular phase, luteal phase, and early pregnancy of pregnant and non-pregnant women. Progesterone production was enhanced in luteal cells derived from both pregnant and non-pregnant women when cultured with PBMCs (Hashii et al., 1998). This effect was more significant in the cultures where PBMCs were derived from women in the early stages of pregnancy or those in the luteal phase (Hashii et al., 1998). There was a slight increase in $P_4$, but not significant, production by PBMCs derived from non-pregnant women (Hashii et al., 1998). This suggests that factors during pregnancy alter immune cells to aid in CL function and maintenance. The increase in $P_4$ caused by PBMCs suggests that they are involved in transmitting the presence of the embryo to the CL to facilitate CL transformation and the continual secretion of $P_4$.

## IMMUNE SYSTEM REGULATES ENDOMETRIAL RECEPTIVITY

The immune system also plays an active role in preparing receptivity of the uterine endometrium. Immune cells assist in $P_4$ production, which is vital to pregnancy, but without a receptive environment in which to attach pregnancy is impossible. Priming of the endometrial immune system is important for pregnancy recognition and conceptus

growth. One of the first priming events associated with reproduction is exposure of the endometrium to seminal plasma which is speculated to facilitate endometrial receptivity. Removal of accessory glands from male mice influences the percentage of pregnancies. When male mice with surgically removed seminal vesicles were mated with female mice, the number of pregnancies was reduced drastically, and the mean size of the pups was smaller than control groups (Pang et al., 1979; Peitz and Olds-Clarke, 1986). The lack of seminal vesicle fluid decreases the number of pregnancies in mice, potentially demonstrating the need for seminal fluid and uterine interaction in pregnancy. Seminal fluid's main function was traditionally thought to be the transport of sperm and fluids for sperm protection, but recent evidence shows that seminal fluid plays a role in the uterine immune response. There is an inflammatory response in human cervical and vaginal epithelial cell lines to seminal fluid, with cytokine-cytokine receptor interaction being the most prevalent of interactions. An increase in IL-6, IL-1α, IL-1β and interferon ε 1 genes was found when ectocervical epithelial were exposed to seminal fluid (Sharkey et al., 2007). There is a shift in the Th2 response in the para-aortic lymph nodes (LN) and mensenteric LN when mated with intact males, vasectomized males, or seminal vesicle-excised (SVX) males. The population of CD4+ and CD25+ cells increased 44% in the para-aortic LN compared to female mice in estrous, and there was no increase in the cell populations when mice were mated with vasectomized males or SVX males (Robertson et al., 2009). This suggests that the seminal fluid may be involved in shifting the uterine immune system to Th2 and away from Th1 response and assist in modulating the uterine environment to protect against the rejection of the allogenic conceptus.

Using splenocytes from pregnant and pseudopregnant mice, Takabatake and others (1997) were able to alter the implantation window and improve the implantation rates in this altered window in mice. Splenocytes were recovered from pregnant mice on d 4 and d 8 and administered by injection through the caudal vein on d 2 of pseudopregnancy to recipient mice that received embryos from donor mice (Takabatake et al., 1997). In pseudopregnant mice with no injection of splenocytes the receptive phase or implantation of the embryos was not seen until d 3 (Takabatake et al., 1997). In mice injected with splenocytes from either d 4 or d 8 pregnant donor mice significant implantation was seen on d 2 (Takabatake et al., 1997). Interestingly, splenocytes from the d 4 pregnant donor mice were more effective than those taken from d 8 pregnant mice in changing the implantation period to d 2 (Takabatake et al., 1997). This suggests that the presence of the embryo before implantation signals changes in the immune cells and endometrium differentiation. The change in implantation rate and the d of implantation suggests that immune cells facilitate endometrium differentiation and receptivity.

Systemic activation of maternal PBMC during early pregnancy is important for regulating uterine receptivity. The attachment of BeWo-cell spheroids, a human choriocarcinoma cell line, to endometrial epithelial cells (ECC) derived from human uteri was increased after co-culturing the ECC with PBMCs (Kosaka et al., 2003). Based on previous findings Yoshioka and others (2006) developed a novel approach to treat women with multiple in vitro fertilization (IVF) failures. Based on the observations that the immune system supports endometrial differentiation and embryo implantation, they developed an approach to utilize autologous PBMCs in repeated IVF failure patients (Yoskioka et al., 2006). Patients who had four or more IVF failures were separated into

control, receiving no immune cells, and those that received immune cells. Peripheral blood mononuclear cells were collected on d of oocyte retrieval and cultured with human chronic gonadotropin (hCG) for two d. The d before ET, fresh PBMCs were isolated and combined with the PBMCs from culture; the total cells were administered to the uterus of patients. All patients received no more than three fresh embryos of good quality. There was a significant difference in pregnancy between the women who received PBMCs and those who did not with pregnancy rates of 41.2% and 11.1%, respectively (Yoshioka et al., 2006). Live births and implantation rates were higher for the treated group compared to the non-treated group with 35.3% and 23.4% compared to 5.5% and 4.1%, respectfully (Yoshioka et al., 2006). This study showed that PBMCs had a positive effect on pregnancy for women with four or more IVF failures. Furthermore, Okitsu and others (2011) again demonstrated the beneficial effects of PBMCs on pregnancy when using embryos that were frozen and then thawed for ET. Using frozen thawed embryos, and PBMCs that were freshly isolated and not cultured with hCG, Okitsu and others, (2011) evaluated pregnancy rates in patients with a minimum of one IVF failure. Pregnancy rates did not differ significantly between treated and non-treated women, though, when pregnancy rates were broken down by the number of failed IVF attempts a difference was revealed (Okitsu et al., 2011). Women with three or more failed IVF attempts differed in pregnancy and implantation rates between the PBMC treated group and the non-treated group with rates of 42.1% and 25%, respectively, for the treated group and 16.7% and 9.38%, respectively, for the non-treated group (Okitsu et al., 2001). It is interesting to speculate that the lack of difference in pregnancy rates overall may be due to the lack of

culturing with hCG, or due to embryos being frozen and thawed, but more studies will be needed to test this theory.

Embryo transfer has been used by the cattle industry to capitalize on superior genetics, using eggs and sperm from high quality, valuable animals, to produce offspring that are genetically superior, higher quality, and of high value. Superovulation and IVF programs in cattle have made it possible to increase the number of viable high value embryos available for transfer. It is extremely important to producers who utilize embryo transfer to achieve high pregnancy rates in cattle to ultimately produce as many genetically superior calves as possible. Currently, pregnancy rates to embryo transfer in cattle have only reached a high of 70% (Hasler, 2007). In relation to the cattle industry and embryo transfer, only one study thus far has evaluated the effects of administering PBMCs into the uterine cavity before embryo transfer and the subsequent effects on pregnancy. Considering that humans and ruminants have similar mechanisms to establish pregnancy it is not unreasonable to consider that PBMCs would improve pregnancy rates in embryo transfer cattle.

Modeled after the experiment done by Yoshioka and others (2006), bovine PBMCs were isolated from heifers at d 3 of estrous cycle, cultured overnight, and administered to the uterine horn ipsilateral to the CL on d 4 of estrus, 3 d before ET (Ideta et al., 2010). Pregnancy rates for the PBMC treated group significantly differed from the non-treated group with 79.5% pregnant and 62.5% pregnant, respectfully (Ideta et al., 2010). These results indicate that PBMCs aid in pregnancy and implantation, possibly by changing endometrial receptivity for embryo invasion. Peripheral blood mononuclear cells could be eliciting these changes by priming the endometrial immune

system and recruiting cytokines to aid in endometrial differentiation, receptivity and embryo development (Ideta et al., 2010). This study is in agreement with those that were conducted in humans, though a key difference is the lack of priming cells with the maternal recognition signal, IFNT.

## CONCLUSION

To establish pregnancy, IFNT must be secreted to stop luteolysis and allow the continued secretion of $P_4$. The uterine environment must become modulated as to not reject the allogenic conceptus. Interferon-tau activates ISG in PBMC and the shift from a Th1 to a Th2 immune response aids in changing of the uterine environment. Previous work shows that the benefit of adding PBMC to increase pregnancy rates in cattle, but there is a lack of priming the cells. We speculate that priming the PBMC with IFNT will mimic uterine immune modulation and increase pregnancy rates in embryo transfer cattle.

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# CHAPTER III

## TYPE I INTERFERON RESPONSE IN COPPER DEFICIENT CALVES EXPERIMENTALLY INFECTED WITH BOVINE VIRAL DIARRHEA VIRUS TYPE 1B AND MANNHEIMIA HAEMOLYTICA


## ABSTRACT

Bovine respiratory disease complex (BRDC) is a major health issue facing the feedlot industry and is the leading cause of morbidity and mortality in feedlot cattle. The genome of bovine viral diarrhea virus (BVDV), a common agent in BRDC, contains an amino terminus protease (Npro) that inhibits the type I interferon (IFN) response *in vitro*, but *in vivo* work indicates that the type I IFN response is activated during BVDV infection. Dietary mineral status has been implicated in BRDC susceptibility in calves during shipping, but little is known regarding mineral supplementation and the IFN response. To determine if mineral deficiency or natural exposure to BVDV1b inhibits IFN signaling *in vivo*, Cu deficient (n = 6) or control (n = 6) calves were infected with BVDV1b and *Mannheimia haemolytica* (MH). Steady-state mRNA levels of *MX1, ISG15*, and *RTP4* were determined in peripheral blood leukocytes prior to BVDV1b exposure (d -4), prior to MH challenge (0 h), and 12 h and 24 h after MH challenge. Fold change relative to the average of d -4 values was calculated. No mineral effects were detected ($P > 0.10$) so mineral deficient and supplemented groups were pooled. A significant time effect ($P <$

0.05) for all interferon stimulated genes was detected. At 0 h, *ISG15* levels increased 44-fold and remained elevated over 60-fold for 12 h and 24 h ($P < 0.01$). Likewise, *RTP4* and *MX1* increased at 12 h ($P < 0.05$) after BRD challenge. Data suggests that regardless of mineral status, the type I interferon pathway remains active after being challenged with BVDV1b and MH *in vivo*.

## Introduction

Bovine respiratory disease complex (BRDC) is a major health and economic issue for feedlots in the United States. Bovine respiratory disease is responsible for roughly $800 to 900 million dollars in lost revenue each year due to decreased feed efficiency, medicinal costs, and death (Chirase and Green, 2000). In 2011, an estimated 16.2% of all feedlot cattle in the United States were affected by respiratory disease, and 87.5% of those animals required treatment for the disease (NAHMS, 2011). Treatment costs for BRDC have increased to approximately $23.60 per case which is double the cost observed in 1999 (NAHMS, 2011). Bovine respiratory disease typically results from a co-infection of both viral and bacterial pathogens. Common viral agents include bovine viral diarrhea virus type 1 and 2 , parainfluenza type 3, and infectious bovine rhinotracheitis, bacterial agents include, *Mannheimia haemolytica, Pasturella multocida, and Haemophilus* (Ellis, 2001) In Oklahoma, bovine viral diarrhea virus (BVDV) and

*Mannheimia haemolytica* are the two most common pathogens in BRDC cases (Fulton et al., 2002).

Viral infections activate the type I IFN pathway which in turn stimulates transcription of interferon stimulated genes (ISG; Randall and Goodbourn, 2008). Host pattern-recognition receptors (PRR) identify pathogen-associated molecular patterns (PAMPS) and stimulate innate immune activation and IFN production. Multiple PRR exist and are found in various cellular domains leading to a variety of pathways that can stimulate IFN production, but these pathways appear to converge at interferon regulatory factors- (IRF) 3 and -7 (reviewed by McNab et al., 2015). Work utilizing *in vitro* models demonstrates that BVDV prevents binding of IRF-3 to DNA, thus inhibiting type I IFN production (Baigent et al., 2002). Hilton et al. (2006) demonstrated that the NPro protein of BVDV blocks induction of IFN-β by degrading IRF-3. Collectively, these results suggest that BVDV can disrupt the type I IFN pathway, thereby reducing the production of IFN, but these works were conducted strictly *in vitro* only. Conversely, studies which expose cattle *in vivo* to laboratory cultured BVDV appear to maintain type I IFN production (Henningson et al., 2009; Palomares et al., 2013).

Bovine respiratory disease complex is most often observed in cattle being shipped, and studies have suggested that mineral supplementation can alleviate rates of BRDC in shipped cattle. For example, calves fed organic trace mineral supplements were found to have higher concentrations of eosinophils, suggesting that they would be better able to cope with an inflammatory response (Stanton et al., 2001). Furthermore, addition

of organic trace minerals to diets in feedlot cattle decreased the percentage of sick animals that needed second treatment of antibiotics for bovine respiratory disease (Kegley et al., 2012). This may indicate that while mineral status does not affect rate of morbidity, mineral deficiency potentially impairs the innate immune response. However, the effects of dietary minerals status on the type I IFN response have not been evaluated.

The objectives of the current study were to evaluate the type I IFN response in calves exposed to BVDV via exposure to a persistently infected (PI) calf and determine the effects of copper deficiency on type I IFN activation during viral exposure.

## Materials and Methods

### *Animals*

All procedures for this experiment were approved by the Oklahoma State University Institutional Animal Care and Use Committee (Animal Care and Use Protocol AG-12-5).

Twelve bull calves were selected from an Angus based commercial cow herd. Calves were individually tagged, surgically castrated, and vaccinated (Covexin 8; Merck Animal Health, Summit, NJ) for clostridial pathogens at the ranch of origin 80 d prior to the start of the experiment. Blood samples from all calves were seronegative to BVDV at 80 d and 24 d prior to the start of the experiment. All calves were tested for persistent infection of BVDV by immunohistochemical analysis (Oklahoma Animal Disease and

Diagnostic Laboratory, Stillwater, OK). Eleven d prior to the start of the experiment, calves were vaccinated for clostridial pathogens, infectious bovine keratoconjunctivitis (Autogenous Bacterin; Newport Laboratories, Worthington, MN) and viral pathogens, excluding BVDV, (Inforce; Zoetis, Florham Park, NJ). Calves were also treated for internal and external parasites (Ivermax Plus; Norbrook Laboratories, Lenexa, KS). Tilmicosin phosphate (300 mg per mL) was administered at the rate of 1.5 mL per 45.4 kg of BW (Micotil; Elanco Animal Health, Indianapolis, IN) and every calf was given a fly tag (Corathon; Bayer, Shawnee Mission, KS). Calves were then transported to the Animal Science Equine Center at Oklahoma State University for a 6-d weaning period.

After weaning, calves were transported to the Nutrition and Physiology Research Center (NPRC) at Oklahoma State University 5 d prior to the initiation of the experiment. Upon arrival calves were weighed, and, using BW and initial antibody titers to BVDV and MH, calves were allocated to experimental treatments of control (CONT) or copper deficient (CuDef). For 5 d calves were placed in individual metabolic stanchions with automatic waters and individual feed troughs to allow for adaptation. Calves were then randomly assigned to individual 3.05 x 3.66 m slatted floor pens for 42 d (d -46 of experiment; d 0 = MH challenge) with access to automatic water bowls and individual feed bunks. During the 42-d period prior to BRDC challenge, calves were individually fed diets that were not mineral supplemented or mineral supplemented (described below).

The BVDV and MH challenge was conducted as described by Burciaga-Robles et al. (2010) with minor modifications. Briefly, pre-BVDV peripheral blood leukocyte

(PBL) samples were collected on d -4 (d 0 = MH challenge), calves were comingled in a common pasture for 4 d with a persistently infected (PI) animal (Burciaga-Robles et al., 2010). On d 0 calves were gathered and placed in metabolic stanchions and pre-MH challenge PBL samples were taken. All calves received 10 mL of a solution containing 6 x $10^9$ CFU of MH serotype 1 that was reconstituted and grown prior to the challenge as described by Mosier et al. (1998). *Mannheimia haemolytica* was delivered via intratracheal bronchoalveolar by a licensed veterinarian (Dowling et al., 2002).

*Diet*

Prior to weaning, calves received no mineral supplementation. Upon arriving at the Horse Unit at OSU, calves were given *ad libitum* access to water, Bermuda grass hay, and a common receiving ration. After the calves were transported to the NPRC and placed in stanchions, Bermuda grass hay was removed, but the calves were still allowed *ad libitum* access to water and the common receiving ration. On d -46, a common dry supplement was formulated to meet or exceed NRC (2000) nutrient requirements except for Cu. Calves received this ration *ad libitum*, for the duration of the experiment, along with *ad libitum* access to water. Calves were either mineral supplemented or non mineral supplemented. The CONT calves received a ground corn top dress daily containing a common mineral supplement. The CuDef calves received a top dress of only ground corn daily, with no mineral supplementation. During the 4 d of the BVDV challenge, calves

were gathered at 0700 h each morning and sorted into their respective treatments and individually fed. Each calf received 11.3 kg of the common ration and their individual top dress. Non mineral supplementation resulted in CuDef calves becoming deficient in Cu only.

*Clinical Scores and Rectal Temperatures*

Rectal temperatures and clinical scores were documented for each calf at h 0 (at MH challenge), 2, 4, 6, 12, 18, and 24. Rectal temperatures were taken using a digital thermometer (GLA M-500; Agricultural electronics, San Luis Obispo, CA). All calves were monitored by trained personnel for clinical signs of BRDC and were based on criteria from the DART™ system (Pharmacia Upjohn Animal Health, Kalamazoo, MI) with some modifications as described by Step and others, (2008). Scores were assigned for each calf from 0 to 4 based where 1 = mild clinical signs; 2 = moderate clinical signs; 3 = severe clinical signs; 4 = moribund animals.

*Total RNA Isolation and cDNA synthesis*

Blood samples were collected at d -4, d 0 (equivalent to 0 h), 12 h and 24 h, and PBL were isolated and frozen at -80 $^{\circ}$C in 1 mL of TRIzol reagent (Life Technologies, Carlsbad, CA) according to procedures described by Gifford et al. (2007). Total mRNA

was isolated according to manufacturer's recommendations, and the integrity of RNA was assessed by gel visualization of 18S and 28S ribosomal RNA. The purity and quantity of RNA was determined using a NanoDrop, ND 1000 Spectophometer (Thermo Fisher Scientific, Wilmington, DE, USA). After isolation, 2 μg of mRNA was treated with DNase I Amplification Grade (Life Technologies) according to manufacturer's recommendation. Then cDNA was reversed transcribed from 2 μg of RNA using 1 μL of Superscript II Reverse Transcriptase (Life Technologies).

*Quantitative real time PCR*

Quantitative real time PCR (qRT-PCR) was used for analysis of three known interferon stimulated genes (ISG), *MX dynamin-like GTPase 1 (MX1), ISG15 ubiquitin-like modifier, (ISG15)* and *receptor (chemosensory) transporter protein-4 (RTP4).* Each cDNA sample was analyzed by qPCR utilizing primers and according to procedures reported by Gifford et al., (2007). *Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), peptidylprolyl isomerase A (Ppia),* and *beta-actin* were tested for stability using geNorm (Biogazell qbasePLUS2, Zwijnaarde, Belgium) and PPIA was selected.

*Statistical Analysis*

Fold-change of ISG mRNA abundance in PBL was calculated using the ΔΔCT method (Kubista et al., 2006). Fold-change for each gene was the dependent variable and was tested against treatment, time, and treatment x time using the MIXED procedure of SAS (Ver 9.2; SAS Institute). Significance level for all studies was set at $P < 0.10$. Steers served as the experimental units and was tested against time for clinical scores and temperature using the GLIMMIX procedure of SAS.

**Results**

Clinical severity (CS) scores were documented for all animals and a significant time effect was noted ($P < 0.01$) with scores peaking at about 1.1, 18 h after MH challenge (Fig. 1). By 48 h after MH challenge, most calves returned to a CS of 0, and by d 7 all CS were 0. Body temperatures rose after MH challenge to peak at just over 40 °C 12 h after MH challenge and all calves returned to below 39.5 °C by 24 h after challenge ($P < 0.01$; Fig. 2).

There was no effect of Cu deficiency on the production of ISG, nor was there a mineral by time effect, so data was pooled for all animals ($P > 0.10$). There was a significant effect of time ($P < 0.05$) for all ISG evaluated. At 0 h, *ISG15* levels increased 44-fold compared to d -4 and remained elevated over 60-fold for both 12 h and 24 h ($P < 0.01$; Fig. 3). *Receptor (chemosensory) transporter protein-4* increased ($P < 0.05$) after BRD challenge and was 6-fold greater than d -4 samples at 12 h (Fig. 4). Likewise, *MX1*

also increased ($P < 0.05$) after BRD challenge with a 12-fold greater change than d -4 at 12 h (Fig. 5).

## Discussion

Animals entering a feedlot are subjected to a range of environmental stressors including movement, comingling with sick animals, dehydration, short-term loss of appetite, and diet changes. Dietary minerals can impact immune function in cattle and mineral deficiency could potentially predispose animals to illness. Ten d after challenge with infectious bovine rhinotracheitis virus, Serum antibody titers for infectious bovine rhinotracheitis virus were greater on d 10, and remained higher on d 14 and 17 in calves that were Cu deficient when compared to Cu adequate calves (Stabel et al., 1993). Immunoglobulin M levels tended to be higher in Cu adequate calves than in Cu deficient calves and the Cu dependent enzyme, superoxide dismutase, activity was reduced in Cu deficient calves (Stabel et al., 1993). Serum immunoglobulin production, as well as Brucella abortus antibody titers, was reduced in Cu deficient calves challenged with Brucella abortus (Cerone et al., 1995). These studies suggest that Cu deficiency impedes the immune function of calves by delaying the antibody response, decreasing immunoglobulin levels, and decreasing Cu dependent enzymes for anti-inflammatory response. Additionally, the number of B lymphocytes was markedly reduced and monocytes were increased in Cu deficient calves, indicating that Cu status can impact

immune cell populations (Cerone et al., 1998). In calves that were Cu deficient a marked decrease in activity and phagocytosis of neutrophils was observed (Cerone et al., 1998). These studies indicate an impaired immune function in Cu deficient calves. However, in the current experiment Cu status had no effect on the IFN response in BVDV challenged calves.

Bovine viral diarrhea virus is a single stranded RNA virus, member of the *Pestivirus* genus, family *Flaviviridae* (Meyers and Thiel, 1996). Using a portion of the 5' untranslated region of BVDV isolates, by phylogenic analysis and PCR , BVDV was separated into BVDV1 with two sub genotypes, BVDV1a, BVDV1b and BVDV2 (Ridpath et al., 1994; Ridpath and Bolin, 1998). Calves can become infected with BDVD while still in utero. If the dam is infected between 30 and 120 d of gestation with BVDV, calves are considered persistently infected (PI) with BVDV (McClurkin et al., 1984; Stokstad and Løken, 2002). Persistently infected animals have lifelong infection and constantly shed the virus thereby maintaining and spreading the virus in the cattle population (McClurkin et al., 1984; Stokstad and Løken, 2002). If calves are infected after 120 d of gestation they are considered transiently infected (TI) and are able to produce an immune response (Bognar, 1972; Kahrs, 1973; McClurkin et al., 1984; Stokstad and Løken, 2002).

Interferons are among the first cytokines released in response to viral pathogens and function to combat viral infections by viral growth-inhibitory properties (Platanias, 2005). It is thought that BVDV evades the host immune response by interrupting the type

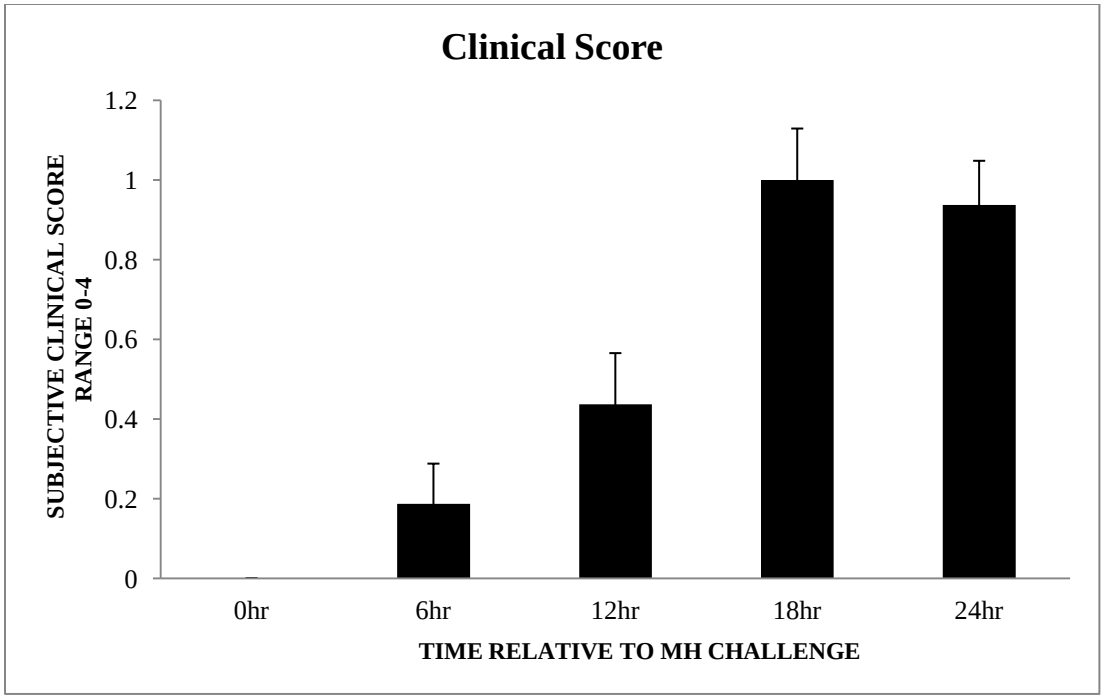
I interferon pathway. Non cytopathic-BVDV targets IRF-3, preventing it from binding to DNA, which in turn blocks the induction of IFN-β (Baigent et al., 2002). The amino terminus protease (NPro) of the BVDV genome targets IRF-3 for polyubiquitination and proteasomal degradation (Hilton et al., 2006) thus preventing release of IFNα and IFNβ. However, *in vivo* work suggested that the type I IFN production is maintained after BVDV infection. Work by Henningson and others (2009) evaluated the type I IFN pathway in calves challenged with either an NPro intact BVDV or an NPro deleted BVDV. Interestingly, it was observed that the NPro deleted BVDV caused an earlier up regulation of interferon concentrations than the NPro intact BVDV, with increased IFN concentrations d 3 to 4 and d 5 after virus challenge, respectively (Henningson et al., 2009). In the current study, ISG increase as early as 4 d after co-mingling with a PI calf which also indicates that the type I IFN pathway is recruited early after BVDV exposure. Calves that are transiently or persistently infected with BVDV2, also exhibit an increase in expression of IFNα/β after viral exposure (Charleston et al., 2002; Brackenbury et al., 2005). Interferon stimulated genes, *ISG15, 2'5' oligoadenylate synthetase-1 (OAS-1), ds RNA dependent protein kinase (PKR),* and *MX dynamin-like GTPase 2 (MX2)* levels increased in PI and TI cattle fetuses, as well as a PI steers infected with BVDV2 (Shoemaker et al., 2009). Due to the variability of the genetics and virulence of BVDV strains, it has been suggested that the differences in strains could cause a difference in immunosuppression, and subsequent effects on the type I IFN pathway (Palomares et al., 2013). Calves that were inoculated by intranasal aerosolization with a low virulence type

1a non-cytopathicBVDV or a high virulence type 2 non-cytopathic BVDV showed increased *MX1, ISG15, OAS-1,* and *PKR* in the spleen and trachea-bronchial lymph nodes indicating that the strain of BVDV does not change the effect on the type I IFN pathway (Palomares et al., 2013). In the U.S. feedlot industry, initial exposure to BVDV primarily comes from contact with PI animals. The current study attempted to mimic conditions of natural exposure found in shipment of cattle to the feedlot. Similar to other *in vivo* studies, a pronounced type I IFN response was observed with increased levels of ISG within 4 d after BVDV exposure. Of particular interest, is the up regulation of ISG at or before d 0, MH challenge, suggests that BVDV alone causes a type I IFN response.

**Conclusions**

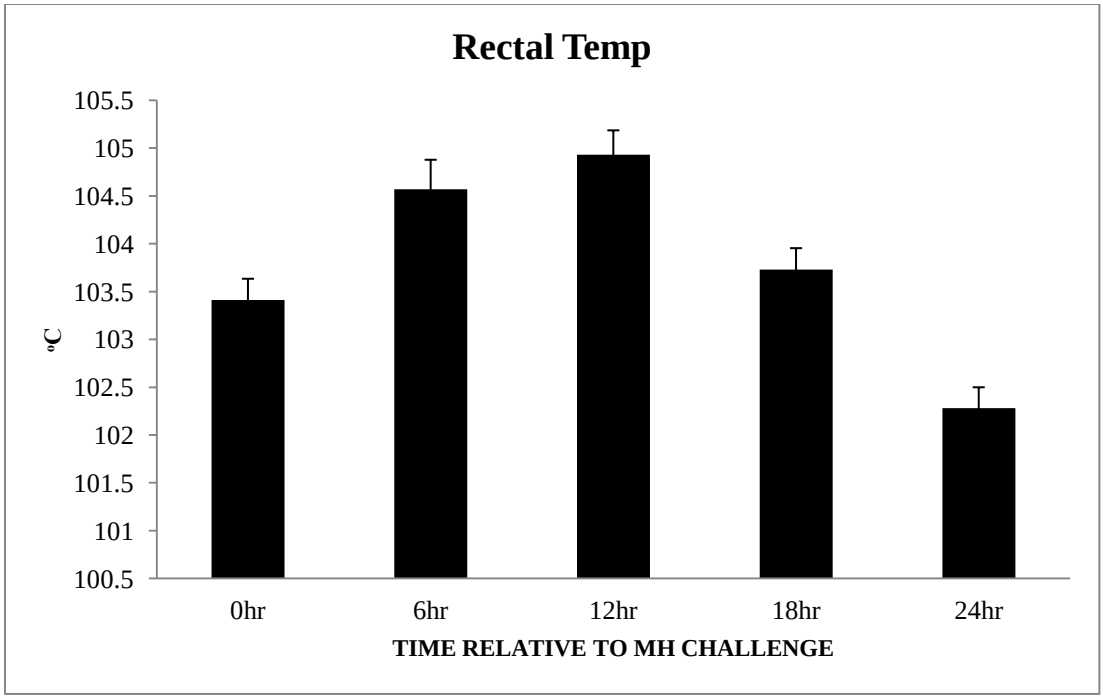
Though several studies have implicated mineral supplementation with immune function, mineral status did not appear to affect type I IFN signaling. Consistent with other *in vivo* work, calves exposed to a calf that was persistently infected with BVDV exhibited a rapid and robust type I IFN response. The observation of increased type I IFN activity is in contradiction to *in vitro* work which suggests BVDV impairs the type I IFN response. However, animals in the current experiment had relatively low clinical scores and it is possible that more severe cases of BRDC which involve BVDV could impair the type I IFN response.

**FIGURE 3.1** Clinical scores for the first 24 h after calves exposed to a Bovine viral diarrhea virus type 1b PI calf on d -4 and subsequently challenged with *Mannheimia haemolytica* (MH) at 0 h. Steers were monitored and recorded by trained personnel using the subjective criteria as follows: depression, abnormal appetite, and respiratory signs. Calves were given a score between 0 and 4 depending on the clinical signs and the severity of the signs. Time is in reference to the MH challenge at 0 h. Scores increased after 0 h to peak at 18 h before decreasing to a score of 0 by d 7. Scores were relatively low, but significant time effect was noted ($P < 0.01$). Mineral status was not significant ($P = 0.87$).


Clinical Score
SUBJECTIVE CLINICAL SCORE RANGE 0-4
1.2
1
0.8
0.6
0.4
0.2
0
0hr
6hr
12hr
18hr
24hr
TIME RELATIVE TO MH CHALLENGE

## FIGURE 3.1

**FIGURE 3.2** Rectal temperatures of the calves for the first 24 h, following exposure to a calf persistently infected with bovine viral diarrhea virus type 1b and subsequent *Mannheimia haemolytica* (MH) challenge. Temperatures were taken by digital thermometer. Time is in reference to the MH challenge at 0 h. There is an increase in temperature after the challenge, peaking at 12 h before decreasing to an average temp of 39.5 $^{o}$C at 24 h. There was a significant time interaction ($P < 0.01$) but mineral status was not significant ($P = 0.66$).


Rectal Temp
105.5
105
104.5
104
103.5
103
102.5
102
101.5
101
100.5
℃
0hr
6hr
12hr
18hr
24hr
TIME RELATIVE TO MH CHALLENGE

## FIGURE 3.2

**FIGURE 3.3** Interferon stimulated gene *ISG15 ubiquitin-like modifier (ISG15),* in response to calves exposed to a Bovine viral diarrhea virus type 1b PI calf and subsequent *Mannheimia haemolytica* (MH) infection. There was no effect of treatment so mineral supplement and mineral deficient were pooled. *ISG15* fold changes are relative to the average of d -4, and were was calculated using the ΔΔCt method. Time is relative to the MH challenge at 0 h. There was a marked increase in fold changes with maximum induction at 12 h. Fold changes stayed above 60 fold difference for 12 h and 24 h. Time was a significant factor ($P < 0.05$).

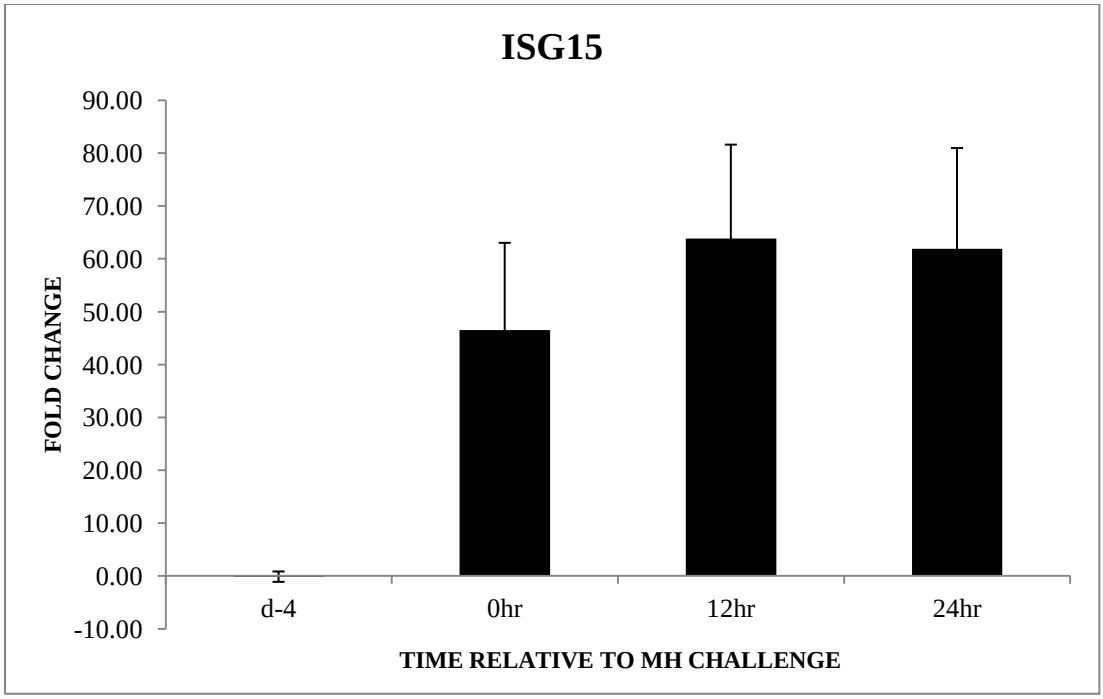

ISG15
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
-10.00
FOLD CHANGE
d-4
0hr
12hr
24hr
TIME RELATIVE TO MH CHALLENGE

**FIGURE 3.3**

**FIGURE 3.4** Interferon stimulated gene *Receptor (chemosensory) transporter protein 4 (RTP4),* in response to calves exposed to a Bovine viral diarrhea virus type 1b PI calf and subsequent *Mannheimia haemolytica* (MH) infection. There was no effect of treatment so mineral supplement and mineral deficient were pooled. *RTP4* fold changes are relative to the average of d -4, and were was calculated using the ΔΔCt method. Time is relative to the MH challenge at 0h. The greatest fold increase was at 12 h, with a 6 fold greater change compared to d -4. Time was a significant factor at ($P < 0.05$).

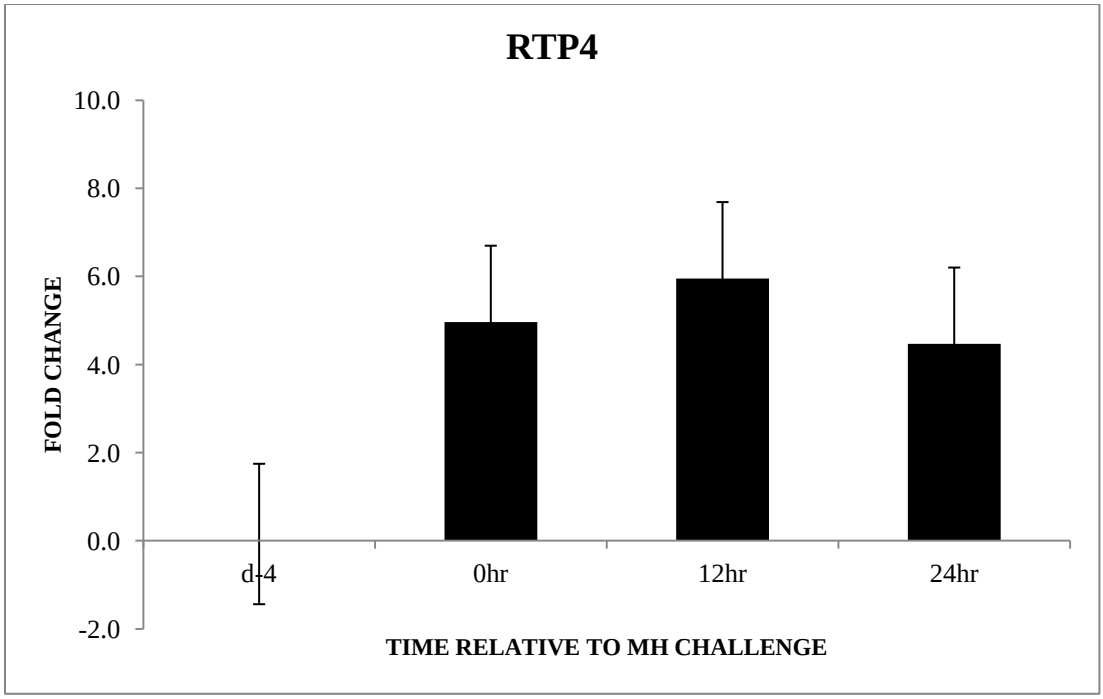

RTP4
10.0
8.0
6.0
4.0
2.0
0.0
-2.0
FOLD CHANGE
d-4
0hr
12hr
24hr
TIME RELATIVE TO MH CHALLENGE

**FIGURE 3.4**

**FIGURE 3.5** Interferon stimulated gene *MX dynamin-like GTPase 1 (MX),* in response to calves exposed to a Bovine viral diarrhea virus type 1b PI calf and subsequent *Mannheimia haemolytica* (MH) infection. There was no effect of treatment so mineral supplement and mineral deficient were pooled. *MX1* fold changes are relative to the average of d -4, and were was calculated using the ΔΔCt method. Time is relative to the MH challenge at 0 h. *MX1* had the greatest change at 12 h with a fold difference of 12 causing a significant time effect to be observed ($P < 0.05$).

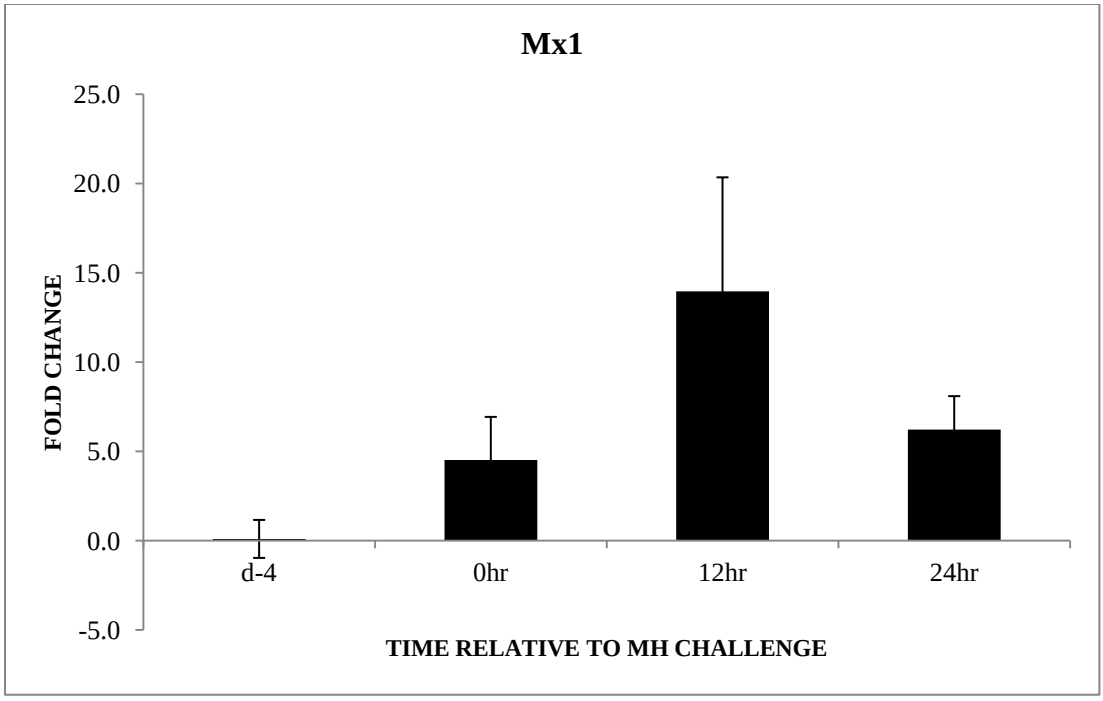

Mx1
25.0
20.0
15.0
10.0
5.0
0.0
-5.0
FOLD CHANGE
d-4
0hr
12hr
24hr
TIME RELATIVE TO MH CHALLENGE

**FIGURE 3.5**

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# CHAPTER IV

## INTRAUTERINE TRANSFER OF AUTOLOGOUS INTERFERON TAU-PRIMED PERIPHERAL BLOOD MONONUCLEAR CELLS INCREASES PREGNANCY RATES AFTER EMBRYO TRANSFER IN CATTLE


**ABSTRACT**

Early embryo loss costs livestock producers billions of dollars annually. As food production expands to meet future demand, reproductive efficiency of food animals will become even more important. Reproductive technologies are a valuable tool to rapidly expand the use of genetics from superior animals, but fertilization failure and embryonic loss limits successful implementation of progressive reproductive strategies. Interferon-tau (IFNT) is the pregnancy recognition signal in ruminants and up regulates interferon-stimulated genes (ISG) in the endometrium, corpus luteum, and peripheral immune cells during early pregnancy. To understand mechanisms of pregnancy loss, much work has focused on conceptus-endometrial interactions. However, there is an increasing body of evidence demonstrating that IFNT affects maternal peripheral blood immune cells and that these immune cells play an active role in establishing and maintaining pregnancy. In women and cattle, transfer of autologous immune cells to the uterus increases pregnancy rates. The current experiment tested the hypothesis that, intrauterine transfer of autologous, IFNT-primed, peripheral blood mononuclear cells (PBMC) will improve

pregnancy rates in cattle. Blood samples were collected at d 3 and PBMC were isolated utilizing Histopaque 1077 according to manufacturer's recommendation. Twenty to 40 million cells were cultured overnight in the presence of 500 U/mL of IFNT followed by autologous intrauterine transfer (IMMUNE; n = 97) on d 4; controls received intrauterine infusion of saline (CONT; n = 82). On d 7, serum samples were collected for hormone analysis and embryos were transferred to all animals. Pregnancy was determined on d 30 by transrectal ultrasonography, and progesterone quantified by RIA. Progesterone concentrations were similar for IMMUNE (4.1 ± 0.33 ng/mL) and CONT (3.7 ± 0.33 ng/mL) and were not different between pregnant and open cows ($P > 0.2$). Pregnancy rate for IMMUNE was 77% (75/97) compared with 57% (47/82) for CONT ($P < 0.001$). Results indicate that progesterone concentrations at d 7 did not differ between treatment groups and transfer of autologous IFNT-primed PBMC improved pregnancy rates after embryo transfer. These results illustrate that priming the maternal immune system resulted in enhanced pregnancy rates supporting the concept that immune function at the fetal-maternal interface affects pregnancy outcome.

## INTRODUCTION

Global meat demand is expected to increase dramatically in the next 35 years, increasing the demand for food by 70% by the year 2050 (Alexandratos and Bruinsma, 2012). This increase will need to be driven by new technologies in agricultural production systems. Advances in reproductive technologies can assist in capitalizing on superior animal genetics in the cattle industry to improve genetic improvement speed. Reproductive technologies have advanced from natural service to artificial insemination (AI), embryo transfer (ET), sexed semen, *in vitro* fertilization techniques, and cloning (Wu, 2012). Currently, the use of these techniques is limited in the United States with time and labor being the number one reason for not implementing these technologies (NAHMS, 2007). Improving the use of these technologies in the United States can improve the rate of genetic improvement to meet future food demands. A major issue facing the cattle industry, infertility and subfertility is slowing reproductive efficiency and increasing costs. With future increasing pressure on livestock industries to become even more efficient, subfertility and infertility could become even more costly. Livestock exhibit high fertilization rates, estimated at over 90%, but early embryo loss remains a challenge for efficient livestock production (McMillan, 1998). This loss could be attributed to inadequate endometrial receptivity (Hansen and Block, 2004). Because early pregnancy loss significantly contributes to subfertility, reducing early embryonic mortality will increase profitability and sustainability of livestock enterprises.

Pregnancy requires continued secretion of progesterone ($P_4$) by the corpus luteum (CL). To continue $P_4$ secretion the regression of the CL must be prevented. For this to occur a signal from the conceptus must be sent to the dam to communicate pregnancy. In ruminants, interferon-

tau (IFNT) is the pregnancy recognition signal which blocks luteolytic pulses of prostaglandin-$F_2\alpha$ ($PGF_2\alpha$; reviewed by Spencer and Bazer, 2004). To prepare the uterine environment for pregnancy, IFNT initiates complex signaling events that induce changes in the endometrium for adequate growth and maintenance of the conceptus (Bazer et al., 2010). Interferon-tau acts both locally in the uterus and systemically. A connection between the maternal immune system and IFNT has been demonstrated. Pregnancy increases interferon stimulated genes (ISG) in circulating immune cells in sheep (Yankey et al., 2001) and cattle (Han et al., 2006; Gifford et al., 2007). Interferon-tau was detected in uterine venous blood (Oliveira et al., 2008) suggesting that it is responsible for increased ISG in circulating immune cells (Bott et al., 2010). The physiological significance of systemic responses to early pregnancy is unclear.

The conceptus is an allogenic body that should be rejected by the maternal immune system, but the maternal immune system is modulated to accept the conceptus. Immune adaption to pregnancy is not limited to suppressing immune response to the allogenic conceptus, but immune cells may play an active role in establishing pregnancy (Kosaka et al., 2003). Fujiwara et al, (1993) postulated that peripheral blood mononuclear cells (PBMC) play a role in establishing and maintaining pregnancy. Women undergoing embryo transfer (ET) techniques exhibited increased pregnancy rates when human chorionic gonadotropin, the human pregnancy recognition signal, primed PBMC were transferred to the uterus prior to ET (Yoshioka et al., 2006). Similar results were found in pregnancy rates in cattle receiving ET, but the cells were not primed with IFNT (Ideta et al., 2010a). The objective of this study was to evaluate pregnancy rates in cattle that receive autologous, IFNT-primed intrauterine immune cells prior to ET. We hypothesize that intrauterine administration of immune cells will increase pregnancy rates in cattle undergoing ET.

## MATERIAL AND METHODS

*Animals*

All procedures were approved by Oklahoma State University Animal Care and Use Committee (protocol AG-14-28). Experiments were conducted at the Kiamichi Link Ranch, Finley, OK. One hundred twenty one Angus cows were used as embryo recipients. All recipient cattle were heat synchronized using a CIDR protocol. With respect to estrus (d 0), the CIDR (Zoetis, 1.38 g) was inserted at d -9 and removed at d -2; prostaglandin (Zoetis, 6 mL, 5 mg/mL) injection was administered i.m. Presence of healthy CL was confirmed by palpation, and freshly collected (n = 105) or *in vitro* fertilization derived (n = 16) embryos were randomly assigned to cows that received intrauterine administration of saline (CONT; n = 50) or intrauterine transfer of autologous IFNT-primed PBMC (IMMUNE; n = 71). Details of PBMC isolation and administration are described below. Embryos were transferred by a professional embryologist to the uterine horn ipsilateral to the CL on d 7, and blood samples were collected and serum stored frozen for $P_4$ analysis. Pregnancy was confirmed by transrectal ultrasonography at d 30.

For donor cows, a CIDR (Zoetis, 1.38 g) was inserted at d -12 with respect to estrus. Decreasing injections of FSH (Bioniche Animal Health, 400 mg/mL) were administered between d -8 and d -2. Either Estrumate or Lutalyse (Merck Animal Health, 2.3 mL, 250 mg/mL; Zoetis, 6 mL, 5 mg/mL) was given on d -3 and d -4; CIDR was removed d -4. Cows were inseminated, 12 to 16 h after the onset of estrus with 2 units of semen. A second service was conducted at 20 to 24 h with 1 unit of semen and an option of a third service at 30 to 36 h if cows displayed standing heat at the second service.

*Peripheral Blood Mononuclear Cell Isolation and Culture*

Peripheral blood mononuclear cells were isolated and treated with 500 U/ml IFNT, which approximates concentrations measured in the uterine vein of early pregnant cows (Oliveira et al., 2008). For isolation, 20 mL of blood was collected into EDTA tubes for PBMC isolation. Blood was mixed with 20 mL of RPMI 1640 (Sigma Aldrich, St. Louis, MO) medium and gently layered over 10 mL of Histopaque 1077 (Sigma Aldrich, St. Louis, MO). Tubes were centrifuged at 500 x g for 45 min at room temperature and PBMC collected. Cells were then subjected to red blood cell lysis (150 m*M* NH4Cl, 10 m*M* NaHCO3, 1m*M* EDTA, pH 7) for 2 to 5 min at 25$^{o}$ C depending on red blood cell contamination. After lysis, cells were washed with 20 mL of RPMI 1640, pelleted by centrifugation at 300 x g for 7 min. The resulting cell pellet was resuspended in 12 mL of RPMI 1640 containing 5% penicillin/streptomyocin (Gibco, Grand Island, NY) and 10% fetal bovine serum (Gibco, Grand Island, NY), with 500 U/mL IFNT (gift from Dr. Fuller Bazer, Texas A&M University, College Station, TX), and cultured in T75 flasks (Thermo Fisher Scientific, Waltham, MA) overnight at 35$^{o}$ C. To ensure culture conditions stimulated ISG, PBMC were collected and cultured as described above. Ten million PBMC were collected into 1 mL of Trizol (Life Technologies, Carlsbad, CA) before and after culture for 24 h with or without 500 U/mL of IFNT. Total RNA was extracted according to manufacturer's recommendation. Steady-state mRNA abundance of interferon stimulated gene-15 (*ISG15*) was analyzed utilizing qPCR as previously described and validated (Gifford et al., 2007).

Three d after estrus, PBMC were isolated and cultured in the presence of IFNT as described above. After approximately 24 h of culture, 20 to 40 million PBMC were centrifuged

at 300 x g for 7 min at 4$^{o}$C, washed with 9% saline solution, and resuspended in 450 uL of saline and drawn into ¼ cc artificial insemination (AI) straws and kept at room temperature for intrauterine transfer. Cells were then administered by AI equipment to the uterine body. On d 7, palpitation of the ovaries was performed to determine presence of CL. A non-surgical embryo transfer catheter was inserted transcervical and the embryo deposited in the ipsilateral horn to the CL.

*Data Analysis*

Fold change in *ISG15* mRNA abundance in immune cells after culture with IFNT for 24 h was calculated using the ΔΔCT method (Kubista et al., 2006). Effects of IFNT treatment on ISG relative fold change were analyzed using the MIXED procedure in SAS (Ver 9.2; SAS Institute). Immune cell transfer experiments were conducted four separate times over two years, with each time considered a replicate and cow considered experimental unit. Pregnancy data was analyzed using generalized linear mixed models methods where replicate was random effect and treatment was fixed effect. Progesterone concentrations were determined by RIA and were analyzed using the GLM procedures in SAS. Significance level for all studies was set at $P < 0.10$.

**RESULTS AND DISCUSSION**

As the global population increases, the demand for meat and other animal products will also increase. With the increase in global population driving the need for greater food production

(Alexandratos and Bruinsma, 2012), the production increase will need to be largely driven by new technologies that increase production efficiency. Reproductive technologies are tools that can rapidly expand genetics from superior animals, but successful implementation of reproductive technologies can be limited by early embryo loss (Looney et al., 2006). The negative economic impacts of early embryo loss are not limited to production practices that utilize reproductive technologies. Early embryonic loss also causes significant economic and productivity losses in current animal production operations that do not utilize reproductive technologies (Disken and Morris, 2008). Thus, reducing embryo loss would increase profitability and sustainability of current livestock operations as well as increasing the use of reproductive technologies to help meet the future global demand for animal products by expanding the use of superior genetics.

Progesterone is absolutely required for pregnancy; thus, pregnancy recognition requires rescue of the CL by the conceptus. Trophoblast cells of the conceptus secrete IFNT with maximal secretion on d 14 to 16 in sheep and 16 to 19 in cattle (Bartol et al., 1985; Bazer et al., 1997). In the uterus, IFNT upregulates ISG in the endometrium, and numerous ISG were shown to be spatially and temporally regulated in the endometrium during early pregnancy (Ott et al., 1998; Johnson et al., 1999; Hansen et al., 2003). Interferon stimulated gene-15 is expressed in the luminal epithelium of sheep on d 10 or 11, as well as in the stratum compactum stroma and glandular epithelium on d 13 and 14 of pregnancy (Johnson et al., 1999). Myxovirus resistance 1 (*MX1*; Ott et al., 1998) and 2’5’-oligoadenylate synthetase (*OAS-1*; Schmitt et al., 1993), are also increased in response to pregnancy and IFNT. Regulation of ISG is hypothesized to be important for endometrial receptivity, conceptus elongation, and implantation (Bazer et al., 2009; Hansen et al., 2010; Ott and Gifford, 2010). Although it is clear that conceptus signaling blocks

luteolytic pulses of $PGF_{2\alpha}$ to sustain $P_4$ production by the CL, work by Oliveira and others, (2008) also demonstrated that IFNT escapes the uterus and exogenous IFNT directly protects the CL from $PGF_{2\alpha}$ (Antoniazzi et al., 2013). Interestingly, work by Atkins and others, (2013) demonstrated that $P_4$ concentrations at d 7, which is before the embryo secretes IFNT, impact subsequent fertility. In the current experiment, intrauterine transfer of autologous IFNT-primed immune cells did not affect $P_4$ concentrations. For IMMUNE cows, $P_4$ concentrations averaged 4.1 ± 0.33 ng/mL compared with 3.7 ± 0.26 ng/mL for CONT ($P > 0.3$; Fig. 4.1). Moreover, when treatments were pooled, there was no difference in $P_4$ concentrations between pregnant (4.0 ng/mL) and open cows (3.6 ng/mL) at d 7 ($P > 0.2$, Fig. 4.2) indicating that $P_4$ concentration at d 7 is not an indicator of subsequent fertility after ET.

Medawar (1953) first described the immunological significance of the pregnant mother being able to tolerate an allogeneic conceptus without rejection. It is thought that the uterus and conceptus secrete cytokines during implantation, which are involved in the maternal cross talk between the fetus and the dam, and help to form collaborative relationships between the trophoblast, uterus and immune cells (Wegmann et al., 1993; Bai et al., 2012). The uterus undergoes changes, or a priming, to help prevent attack of the fetus and ensure a receptive environment for growth and development. One of the first priming events associated with reproduction is exposure of the endometrium to seminal plasma which is speculated to facilitate endometrial receptivity. Of 317 genes measured in human cervical and vaginal epithelial cell lines, an increase in IL-6, IL-1α, IL-1β and interferon ε 1 genes was found when cells were exposed to seminal fluid (Sharkey et al., 2007). The population of CD4+ and CD25+ cells increased 44% in the para-aortic lymph nodes of female mice mated to intact males, compared to female mice in estrous, and no increase in the cell populations when female mice were mated

with vasectomized males or SVX males (Robertson et al., 2009). This suggests that the seminal fluid may be involved in shifting the uterine immune system to one of Th2 from one of Th1 response. This response assists in modulating the uterine environment to protect against the rejection of the allogenic conceptus.

To prevent immunological attack on the embryo there is a shift from a Th1 immune response to a Th2 response in the uterus. Early work suggested that IL-2, TNF-α, and IFN-γ, which are all Th1 associated cytokines, are deleterious to pregnancies in mice, causing abortion (Chaouat et al., 1990). During pregnancy there is a shift towards Th2 cytokines release from the maternal-fetal interface, IL-3, IL-4, IL-5 and IL-10 cytokines are all found during each trimester of pregnancy (Lin et al., 1993). In early pregnancy leukocyte populations increased from 8.2% to 31.7% with over 75% of the leukocytes being CD56+ cells (Bulmer et al., 1991). This shift from Th1 to Th2 cytokine profiles is essential for pregnancy maintenance and stopping rejection of the conceptus (Wegmann et al., 1993).

While there are specific changes in the immune system within the endometrium during pregnancy, there is also a systemic response to pregnancy. CD4+ and CD25+ are regulatory T-cells involved in preventing autoimmunity, and are implicated in suppression of rejection of the conceptus (reviewed by Shevach, 2002). Cows in early stages of pregnancy had higher populations of lymphocytes that were CD4+ and CD25+ in the peripheral blood when compared to non-pregnant cows (Oliveria and Hansen, 2008). In pregnant women the concentration of CD4+ and CD25+ cells more than doubled when compared to non-pregnant women (Somerset et al., 2004). Pregnant women exhibited higher concentrations of IL-4 and IL-10, and decreased concentrations of IL-2 and IFN-γ, when compared to non-pregnant women (Marzi et al., 1996). This indicates the systemic change of immune profiles from one of Th1 to a Th2 profile. The

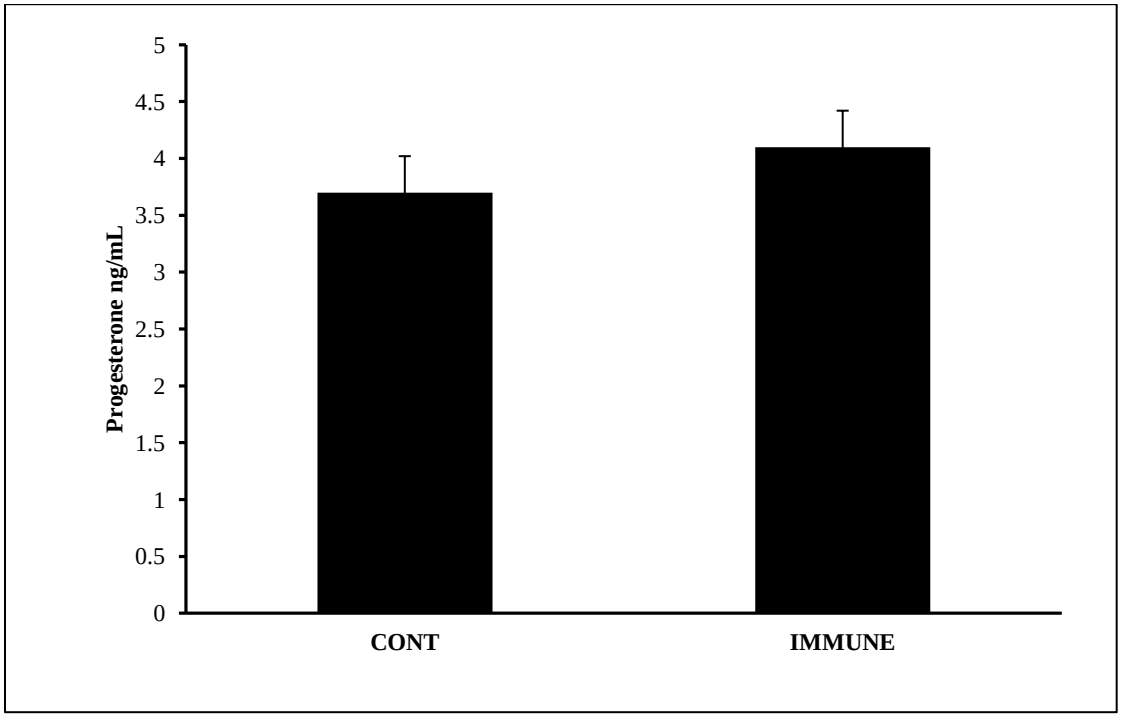
IFNT that escapes the uterus likely modulates the maternal systemic immune function. Yankey and others, (2001) first showed that the ISG, *MX1*, was up-regulated in peripheral blood leukocytes of pregnant ewes within 24 to 48 h of the onset of IFNT signaling. Expression of *MX2* was also increased as early as d 16 and, *ISG15*, and *MX1* were increased at d 18 and d 20 in peripheral blood leukocytes of pregnant cattle (Gifford et al., 2007). Although the functional significance of systemic immune activation is unclear, it is reasonable to speculate that conceptus regulation of the maternal immune system, both in the uterus and systemically, is an important adaptation for the mother to tolerate the allogenic conceptus.

There is a growing body of evidence to suggest that the maternal immune system plays an active role in establishing pregnancy. Attachment of BeWo-cell spheroids to endometrial epithelial cells (ECC) derived from human uteri was increased after co-culturing the ECC with PBMC (Kosaka et al., 2003), suggesting that PBMC aid in regulating endometrial receptivity. Pregnancy rates were increased in women when human chorionic gonadotropin-primed PBMC were combined with fresh PBMC and administered to the uterus 1 d prior to fresh ET (Yoshioka et al., 2006). In ET using frozen/thawed embryos and patients with 3 or more failed IVF sessions, fresh PBMC that were administered to the uterus 2 d before ET resulted in an increase in pregnancy rates (Okitsu et al., 2011). Similar to the experiment done by Yoshioka et al., (2006), when bovine PBMC were isolated from Holstein heifers at d 3 of the estrous cycle, cultured overnight, and administered to the uterine horn ipsilateral to the CL on d 4 of estrus, pregnancy rates by 17% for the PBMC treated group (Ideta et al., 2010a). However, Ideta et al., (2010a), did not culture the PBMC with IFNT. The current experiment expanded on previous results by treating PBMC with IFNT at concentrations observed in maternal circulation during early pregnancy. Treating PBMC with 500 U/mL increased ($P < 0.01$; Fig. 4.3) *ISG15* mRNA

abundance 250-fold above non-treated PBMC indicating that IFNT activates the type I IFN pathway in cultured PBMC. Intrauterine transfer of autologous IFNT-primed immune cells increased pregnancy rates in beef cattle undergoing ET procedures (Fig. 4.4). For IMMUNE cows, pregnancy rate was 77% (75/97) compared with 57% (47/82) for CONT ($P < 0.001$). Though the study was replicated four times there was zero variance between replicates. Data demonstrate that priming the maternal immune system resulted in enhanced pregnancy rates supporting the concept that immune function at the fetal-maternal interface affects pregnancy outcome. Interestingly, PBMC have been implicated in improving endometrial environment to promote conceptus development. Ideta and others (2010b) demonstrated that intrauterine administration of PBMC improves conceptus develop at d 15 of pregnancy in comparison to the control group. It is conceivable that improving the endometrial environment and early conceptus development could potentially improve development throughout pregnancy and translate into higher birth weights. This study evaluated the difference in birth weights of calves between IMMUNE and CONT cows and found no difference ($P > 0.6$; Fig. 4.5), suggesting that though there may be an early increase in development this does not continue.

Reproductive technologies have great potential for improving genetics, economics, and production efficiency. Both conventional and intensive reproductive management practices are affected by high rates of embryo loss. Results from the current study indicate that transfer of autologous interferon-tau-primed peripheral blood mononuclear cells increased success rates for embryo transfer indicating that maternal immune system plays a pivotal role in establishing pregnancy in cattle. Additionally, priming the maternal immune system during early pregnancy might be a method to decrease embryonic loss in domestic livestock.

**FIGURE 4.1** Serum progesterone at d 7 in cattle receiving autologous intrauterine transfer of Interferon-tau-primed immune cells (IMMUNE) or saline (CONT) on d 4 (d 0 = estrus). Intrauterine immune cell transfer did not influence progesterone concentrations ($P > 0.3$).


5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Progesterone ng/mL
CONT
IMMUNE

## FIGURE 4.1

**FIGURE 4.2** Serum progesterone at d 7 (d 0 = estrus) in cattle receiving an embryo and were subsequently diagnosed as open (OPEN) or pregnant (PREG) by transrectal ultrasonography on d 30. Day 7 progesterone concentrations were not indicative of subsequent fertility and were similar ($P > 0.2$) in open and pregnant cows.

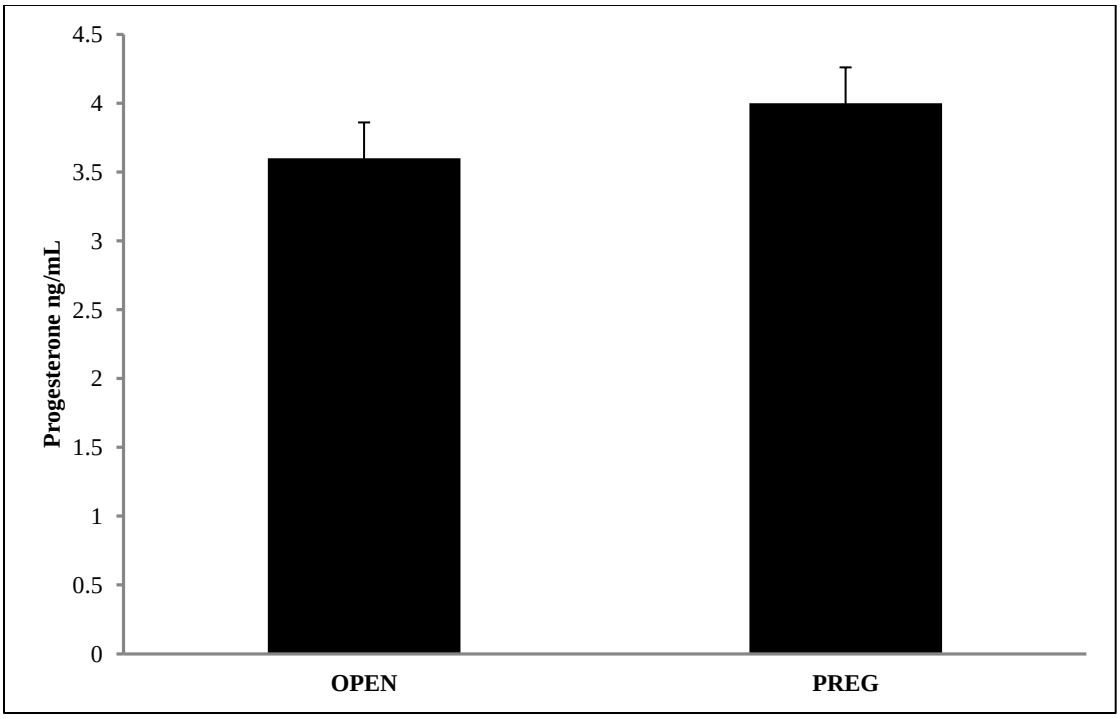



**FIGURE 4.2**

**FIGURE 4.3** Interferon stimulated gene-15 (*ISG15*), after treatment of peripheral blood mononuclear cells with Interferon-tau. Peripheral blood mononuclear cells from 6 feedlot steers were cultured with 500 U/mL interferon-tau overnight to evaluate the type I interferon pathway activation in response to interferon treatment. A sample was taken before culture (BC) and again after culture (AC) with interferon-tau. Steady-state mRNA levels of *ISG15*, a known target of the type I interferon pathway, were evaluated using qRT-PCR and fold change was calculated by the ΔΔCT method. Culture with interferon-tau increased ($P < 0.05$) *ISG15* levels over 250-fold.

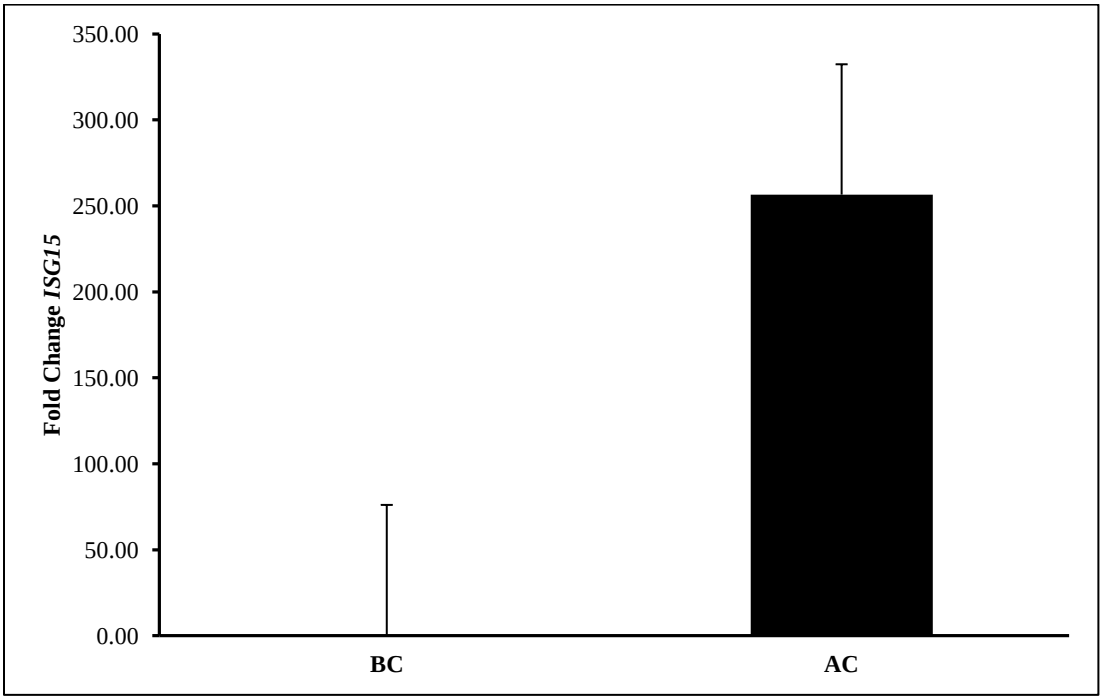

350.00
300.00
250.00
200.00
150.00
100.00
50.00
0.00
Fold Change ISG15
BC
AC

**FIGURE 4.3**

**FIGURE 4.4** Pregnancy rates of IMMUNE (n = 97) and CONT (n = 82) cows. IMMUNE cows received an intrauterine transfer of autologous peripheral blood mononuclear cells d 4 of estrus. CONT cows received an intrauterine transfer of saline solution. There was a significant difference in pregnancy rates between IMMUN and CONT ($P < 0.001$).

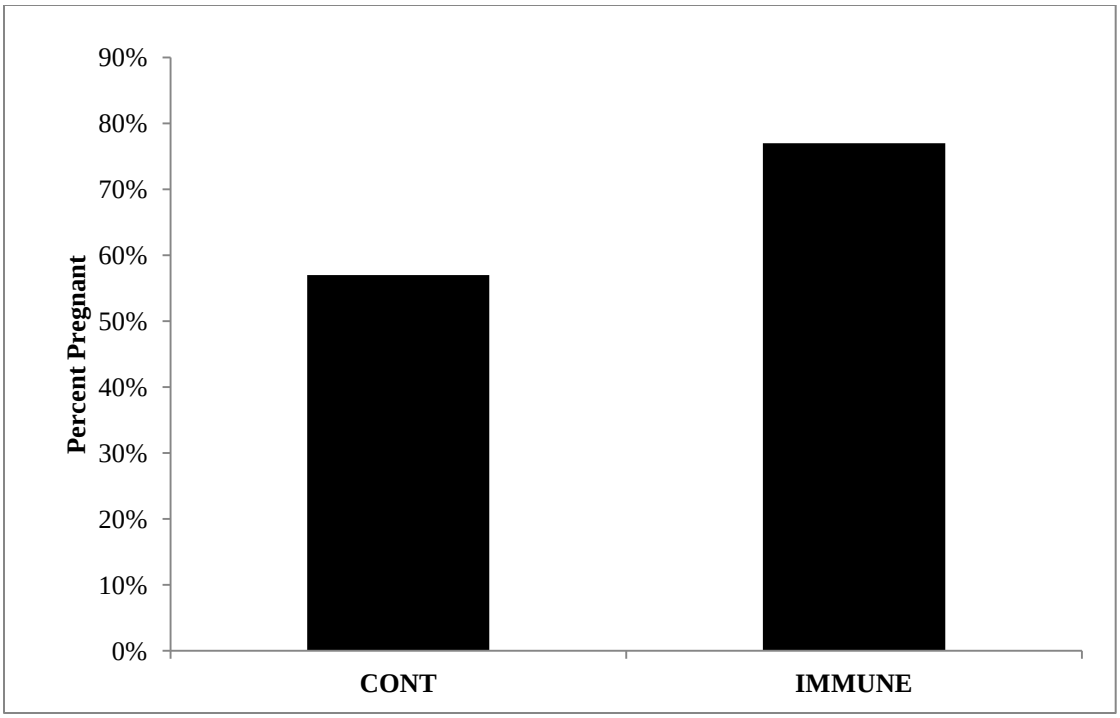



**FIGURE 4.4**

**FIGURE 4.5** Birth weights of calves from CONT cows (n = 34) and IMMUNE cows (n = 40). IMMUNE cows received an intrauterine transfer of autologous peripheral blood mononuclear cells d 4 of estrus. CONT cows received an intrauterine transfer of saline solution. No difference in birth weights was found between CONT and IMMUNE $P > 0.6$.

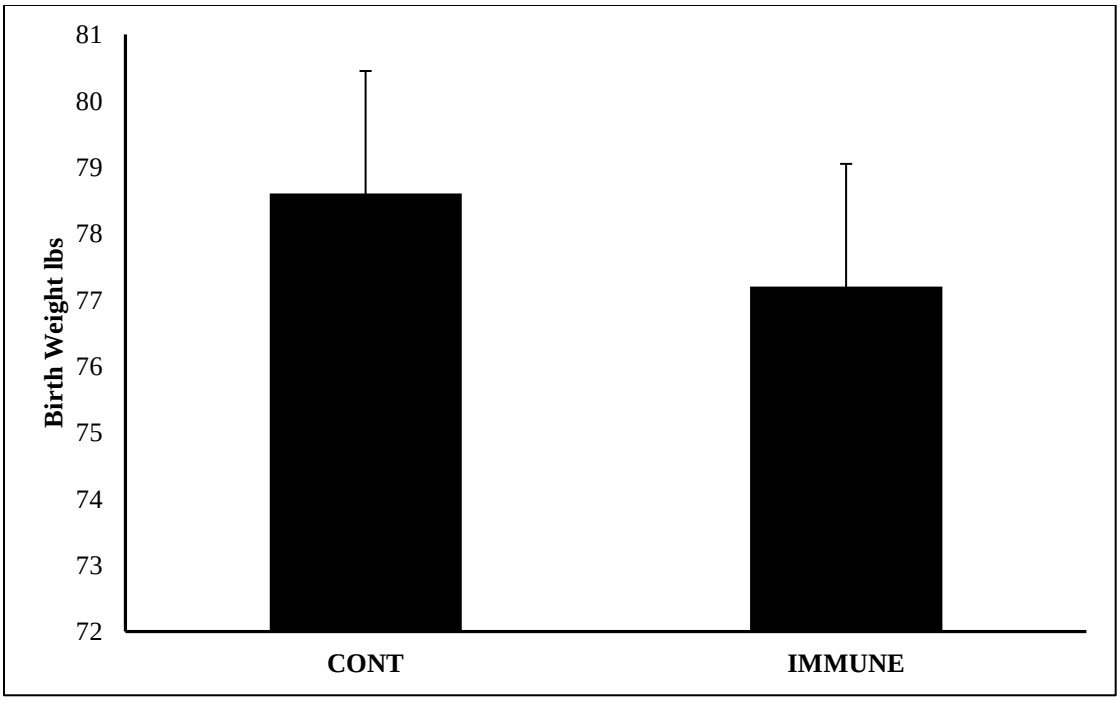



**FIGURE 4.5**

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# CHAPTER V

## SUMMARY AND CONCLUSION

The global population is expected to increase dramatically in the next 35 years, increasing the demand for food production. Meeting the increased demand for food will need to be driven by new technologies in agricultural production systems. Infertility and respiratory disease are two of the most economically important issues that limit production efficiency in today's livestock operations. The type I interferon (IFN) pathway is a key modulator in disease and infertility. Disruptions in the type I IFN signaling pathway could contribute to increased disease susceptibility and reduced reproductive rates. Investigation of the type I IFN pathway and its function in pregnancy and disease could reduce the losses associated with infertility and disease in the cattle industry.

Respiratory disease is one of the largest issues that feedlots are facing in the United States. Bovine viral diarrhea virus (BVDV) and *Mannheimia haemolytica* (MH) are two common pathogens of respiratory disease. The type I IFN pathway is activated when a viral pathogen enters the host body, and activation of the type I IFN pathway induces transcription of interferon stimulated genes (ISG). Bovine viral diarrhea virus is

thought to disrupt the type I IFN pathway, thus reducing transcription of ISG thereby circumventing the animal's immune system. Work *in vitro* has demonstrated a reduction in the production of IFN and ISG upon challenge with BVDV or proteins of the BVDV genome, but, work *in vivo* suggests that ISG are not reduced. Cattle that are shipped are often subject to short-term fasting, stress, and exposure to sick animals. Mineral supplementation or mineral deficiencies are thought to impact an animal's susceptibility to disease; however, little is known regarding mineral status and the IFN response. The purpose of study 1 was to evaluate the effects of BVDV and MH challenge in control and Cu deficient calves. Three known ISG were evaluated in response to BVDV and MH challenge. Times points ranged from BVDV exposure to 24 h after MH challenge. Results from study 1 demonstrated an increase in ISG shortly after BVDV exposure, regardless of Cu status. Results indicate that the type I interferon pathway is activated in response to BVDV.

Infertility and subfertility are a major problem for the cattle industry. Advances in reproductive technologies could increase production and the rate of genetic improvement. Most reproductive losses can be attributed to early embryonic loss from inadequate endometrial receptivity. Reducing embryonic loss can increase production and profitability in the cattle industry. Interferon-tau, a type I IFN, is the pregnancy recognition signal in ruminants. A connection between the immune system and IFNT has been identified, both within the uterus as well as systemically. In cattle and sheep, IFNT escapes the uterus and increases ISG in peripheral blood mononuclear cells (PBMC). Administering human chorionic gonadotropin, the pregnancy recognition signal in humans, primed PBMC intrauterine before embryo transfer has been found to increase

pregnancy rates in humans. Similar experiments in cattle demonstrated increased pregnancy rates in response to intrauterine transfer of PBMC, but these studies did not prime the cells with IFNT. Study 2 was preformed to evaluate the effects on pregnancy rates when IFNT-primed PBMC were transferred intrauterine before embryo transfer. Results indicate that PBMC have a positive effect on pregnancy rates, with an increase in pregnancies when compared to control cows. Progesterone ($P_4$) is essential for pregnancy establishment and maintenance, and work in the literature suggests that $P_4$ concentrations 7 d after estrus influence subsequent pregnancy rates. The current study also evaluated the effects of pregnancy and $P_4$ concentrations 7 d after estrus. There was no difference between pregnant and non-pregnant in control or experimental cows, indicating that $P_4$ concentrations at d 7 do not influence pregnancy status. It has been demonstrated that intrauterine transfer of PBMC increases the development and size of the conceptus, but studies have not evaluated if the development continues throughout pregnancy to birth. This study evaluated the effects of intrauterine transfer of IFNT-primed PBMC on calf weights at birth. There was also no difference in birth weights between the control and experimental calves, indicating that, though there might be early enhancement of conceptus growth, this does not continue to birth.

Greater understanding of the type I IFN pathway and immune responses to pregnancy and disease can provide new insight into physiological responses to the most economically important challenges facing livestock industries. Understanding disease pathways can lead to new treatments or early diagnosis of infections. Increasing pregnancy rates will increase reproductive efficiency, profits, and improve genetic improvement speed. Results from the current experiment demonstrated that the type I

IFN pathway is activated during bovine respiratory disease which indicates IFN is an important innate immune response to combat respiratory disease. Interferon therapies or identification of animals with deficient IFN responses could provide mechanisms to reduce bovine respiratory disease in US cattle herds. Furthermore, in experiment 2, IFN-treated peripheral immune cells primed the maternal endometrium making a more receptive uterine environment and led to increased pregnancy rates. Intrauterine transfer of peripheral immune cells could provide a novel method to increase pregnancy rates following the use of reproductive technology in the livestock industry. If pregnancy rates could be increased when utilizing reproductive technology, producers might be more willing to adopt these technologies. Broader use of reproductive technology would allow the U.S. cattle industry to be more competitive on the global market by accelerating genetic progress of traits important for both economic viability and production sustainability.

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