THE EFFECT OF LENGTH OF THE PREOVULATORY PERIOD ON MECHANISMS REGULATING EMBRYONIC SURVIVAL IN BEEF CATTLE

Thesis

Presented in Partial Fulfillment of the Requirements for the Degree Master of Science in the Graduate School of The Ohio State University

By

Lucas de Araujo Souto, B.S

Animal Science Graduate Program

*****

The Ohio State University

2010

Dissertation Committee:

Michael L. Day, Advisor
Carlos R. F. Pinto
Joy L. Pate
Steve C. Loerch
ABSTRACT

The effect of length of proestrus on estradiol and progesterone concentrations, embryonic protein and gene expression on d 17.5 of pregnancy and expression of interferon stimulated genes (ISG-15 and Mx2) in peripheral blood mononuclear cells (PBMC) from d 15 to 30 of pregnancy in cattle were investigated in two experiments. Beef cows were induced to have either normal (high estradiol; Hi-E) or deficient (low estradiol; Lo-E) concentrations of estradiol during the preovulatory period using a unique animal model that resulted in induced ovulation of follicles with similar age and diameter using GnRH.

In the first experiment, characteristics of the embryo on d 17.5 of pregnancy were compared in nonlactating multiparous beef cows. Concentrations of estradiol before and progesterone concentrations after, ovulation were quantified. Cows were slaughtered on d 17.5 of pregnancy and embryos and media used to flush embryos from the uterus were collected and analyzed for gene and protein expression of interferon tau (IFNτ) and chorionic somatomammotropin hormone – 1 (CSH-1). Expression of ISG-15 and Mx2 in PBMC on d 15, 16, 17 and 17.5 was determined. Estradiol concentrations before, and progesterone concentration after ovulation, amount of IFNτ in the uterine flush media and mRNA for ISG-15 in PBMC were greater in the Hi-E than Lo-E treatment. Gene expression for IFNτ and CSH-1 in the conceptus and Mx2 in PBMC did not differ between treatments. CSH-1 protein was undetectable in the media and conceptus.
In the second experiment, lactating primiparous beef cows were used to investigate the effect of the preovulatory estradiol concentrations on ISG-15 and Mx2 mRNA expression in PBMC from d 15 to 30 after ovulation in pregnant and cyclic cows. A deficiency in estradiol concentrations during the preovulatory period was not successfully created in the Lo-E treatment, therefore, treatments were combined and comparisons of ISG-15 and Mx2 gene expression between pregnant and cyclic cows were emphasized. Relative amounts of ISG-15 and Mx2 mRNA were greater in pregnant than cyclic cows after d 18 until d 30 of pregnancy. Maximum amounts of ISG-15 and Mx2 gene expression in pregnant cows were detected by day 20 followed by a decrease, and a subsequent biphasic pattern to day 30 of gestation.

In conclusion, peripheral progesterone concentrations, IFNτ activity in the uterine lumen and expression of ISG-15 mRNA in PBMC were greater in the Hi-E than Lo-E treatment, indicating that concentrations of estradiol achieved during the preovulatory period influenced function of the corpus luteum and embryonic function at 17.5 d of gestation. It is unknown at present whether these effects are through alterations of uterine function, a result of differences in progesterone concentrations and/or through alternative mechanisms. Further research is necessary to determine if these alterations in embryonic function will lead to its demise by d 30 of gestation. In the second experiment, it was demonstrated that ISG-15 and Mx2 mRNA is greater in pregnant than nonpregnant cows after d 18 of gestation/estrous cycle and thereafter appear to be a biphasic secretion pattern between d 18 and 30 in pregnant cows.
To Lica
ACKNOWLEDGMENTS

My Masters program would not be possible if it were not for the many people who were part of my life while living in US, sharing good and bad moments, and always providing essential support along the way here at The Ohio State University.

To my friend and advisor, Mike Day, thank you for your support and guidance in all aspects of my education, not only in terms of scientific learning, which for me was the best I could ever expect, but also personal and professional aspects. Thanks for always, even very busy, have time to talk, to listen and encouraging to express my ideas. I am also very thankful to the Day family, Toni, Travis and Leslie, for your friendship, support, and welcoming us into your home on several occasions, and more than that, to make me feel at home.

This program could not have been completed without the assistance and friendship of my officemates, Leandro Cruppe and Martin Maquivar, who were intensively present during my program time, and besides “making my life miserable” you were real friends. I could not forget of my other friends and grad mates, Brad Midendorf, Esbal and Yadira Jimenes, Fernanda Abreu, Matt Utt, Santiago and Valeria Bas, Courtney Kremmer, Mike Cressman, Alex Gress, Isain and Ileana Zapata, JJ Greene, Jim, Rusty Burgett. My thanks are extended to Dr. Dave Grum for all the help in the laboratory and the unique humor you provided. Special thanks go out to members of Dr. Joy Pate’s, Dr. Ott’s, Dr. Lee’s laboratory and Meat Lab. Without the guidance and
assistance of Dan Poole, Koji Toyokawa, Sadhat Walusimbi, Melanie Boretsky, Courtney Smith, Edyta Brzezicka, Shannon Boone and Julie Serr I would probably still be running real-time PCR and Western Blots, thus I can not thank you enough for the time you dedicated to my education. Without people such as Dr. Marty Mussard and Greg Fogle it would have been almost impossible had the entire farm job done with precision and quality. I extend to thank the others at the beef units here at Columbus, Jackson, and Belle Valley. To all my other colleagues here at The Ohio State University, I appreciate your support and friendship.

To Carlos and Aclecia Pinto, Marco and Tatiana, Alex and Ivanete Pires I would like to express my most sincere thanks for your friendship and to make me feel less far from Brazil, definitely the time spent in your companies were amazingly enjoyable and fun.

I thank the members of my advisory committee, Dr. Joy Pate, Dr. Steve Loerch and Dr. Carlos Pinto. Each of you contributed significantly to my development as a scientist and to the accomplishment I made. Special thanks go to Dr. Joy Pate and Dr. Ott for allowing me to become a “parasite” for couple weeks on their laboratory and providing the guidance and instruction needed to complete these projects.

I thank my family in Brazil for their understanding, support and love. Most importantly I thank my mom, Lica. I may not have got this far without your support, love and belief in me. I would have never been able to accomplish this if it were not for you all.
VITA

October 2, 1982. . . . . . . . . . . . . Born – Mogi Mirim, Sao Paulo

2003 - 2007 . . . . . . . . . . . . . . B.S. Veterinary Medicine, UNESP - Botucatu

2008 - 2010 . . . . . . . . . . . . . . Graduate Research Associate, The Ohio State University

PUBLICATIONS

Abstracts


Non-refereed Publications


FIELDS OF STUDY

Major Field: Animal Sciences
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>ii</td>
</tr>
<tr>
<td>Dedication</td>
<td>iv</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>v</td>
</tr>
<tr>
<td>Vita</td>
<td>vi</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xi</td>
</tr>
<tr>
<td>List of Figures</td>
<td>xii</td>
</tr>
<tr>
<td>Chapters:</td>
<td></td>
</tr>
<tr>
<td>1. Introduction</td>
<td>1</td>
</tr>
<tr>
<td>2. Literature Review</td>
<td>5</td>
</tr>
<tr>
<td>2.1 Introduction</td>
<td>5</td>
</tr>
<tr>
<td>2.2 Fertilization Rates and Embryonic Losses in Beef and Dairy Cattle</td>
<td>5</td>
</tr>
<tr>
<td>2.2.1 Fertilization Rates in Beef and Dairy Cattle</td>
<td>6</td>
</tr>
<tr>
<td>2.2.2 Early Embryonic Loss in Cattle</td>
<td>8</td>
</tr>
<tr>
<td>2.3 Follicles and Hormonal Associations Related to Fertility in Cattle</td>
<td>12</td>
</tr>
<tr>
<td>2.3.1 Follicular Development and Estrous Cycle</td>
<td>13</td>
</tr>
<tr>
<td>2.3.2 Artificial Insemination and Estrous Synchronization Programs</td>
<td>14</td>
</tr>
<tr>
<td>2.3.3 Proestrus and Relationships Associated with Fertility in Cattle</td>
<td>17</td>
</tr>
<tr>
<td>2.4 Hormonal Programming of the Uterus</td>
<td>23</td>
</tr>
<tr>
<td>2.5 Maternal Recognition of Pregnancy</td>
<td>26</td>
</tr>
</tbody>
</table>
2.6 Mechanisms of Uterus/Embryo Hormonal Signaling

2.6.1 Interferon tau Signaling of Interferon Stimulated Genes

2.6.2 Embryo Synthesized Proteins

2.7 Statement of the Problem

3. The effect of duration of the preovulatory period on the expression of IFN\(\tau\) and CSH-1 by embryos collected on day 17.5 of pregnancy and ISG-15 and Mx2 expression from days 15 to 17.5 in PBMC in beef cattle

4. The effect of the preovulatory estradiol concentrations on peripheral expression of ISG-15 and Mx2 mRNA, and progesterone concentration in lactating beef cows

5. General Discussion

References
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Primer sequences, annealing temperature, and number of PCR cycles for the genes RPL-19, IFNτ, CSH-1, Mx2 and ISG-15</td>
</tr>
<tr>
<td>3.2</td>
<td>Steady-state mRNA amounts (relative values) for RPL-19, interferon tau (IFNτ) and chorionic somatomammotropin hormone - 1 (CSH-1) in embryos collected on day 17.5 of pregnancy from cows with either normal (Hi-E) or deficient (Lo-E) preovulatory concentrations of estradiol</td>
</tr>
<tr>
<td>3.3</td>
<td>Antiviral activity for IFNτ expressed (U/mL) and embryo tissue score by individual animals in the Hi-E and Lo-E treatments</td>
</tr>
<tr>
<td>4.1</td>
<td>Primer sequences, annealing temperature, and number of PCR cycles for the genes RPL-19, Mx2 and ISG-15</td>
</tr>
</tbody>
</table>
### LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>3.3</td>
<td></td>
</tr>
<tr>
<td>3.4</td>
<td></td>
</tr>
<tr>
<td>3.5</td>
<td></td>
</tr>
<tr>
<td>3.6</td>
<td></td>
</tr>
</tbody>
</table>

3.1 Representative plate for quantification of antiviral activity for IFNτ, measured in duplicates and determined as the reciprocal of the dilution that prevented 50% destruction of the MDBK cells. Wells in black represent intact cells with no virus activity. Column 1 represents a positive control lane (no virus), while the following columns represent increasing dilutions of the uterine flush media for each cow. Pregnant cows are represented by letters B and D (in duplicates) and cow B has higher antiviral activity; consequently higher IFNτ concentration. Cows A and C are nonpregnant cows with complete destruction of the MDBK cells at all dilutions of uterine flush media. 

3.2 Circulating concentrations of estradiol during the preovulatory period in cows manipulated to have either normal (Hi-E; ♦) or deficient (Lo-E; ■) preovulatory estradiol concentrations. Cows were administered PGF on either h-72 (Hi-E) or h-48 (Lo-E), all cows received GnRH at h 0 to induce ovulation.

3.3 Circulating concentrations of progesterone following GnRH-induced ovulation in the Hi-E (♦) and Lo-E (■) treatments.

3.4 ISG-15 mRNA expression in PBMC ($2^{-\Delta CT} \times 100$) in the Hi-E (♦) and Lo-E (■) treatments on d15, 16, 17 and 17.5 of pregnancy.

3.5 Mx2 mRNA expression in PBMC expressed as $2^{-\Delta CT} (x100)$ following GnRH-induced ovulation in Hi-E (♦) and Lo-E (■) cows on d 15, 16, 17 and 17.5 of pregnancy.

3.6 Western blot analysis determining the absence of the chorionic somatomammotropin hormone - 1 (CSH-1) in concentrated uterine flush media of a pregnant cow on lane 4 (←). The positive controls are represented by recombinant CSH-1 on lane 1, with molecular weight of approximately 23 kDa (→). Lanes 2 and 3 represent extracted CSH-1 from a 60 d pregnant cow placentome with molecular weight of approximately of 32-33 kDa diluted 1:10 and 1:100 respectively.
3.7 A) Western blot containing on lane 1 a sample extracted from a 60 d pregnant cow placentome used as a positive control followed by samples extracted from Lo-E (Lanes 2 to 5) and Hi-E (Lanes 6 to 9) cows. Multiple bands can be observed on lane 1, however the more stained band represents to the molecular weight around 33 kDa for the CSH-1 (→). The samples of cows from both treatments did not show band matching to similar size of the positive control but presented a few additional bands around 28kDa size. B) The second figure represents a western blot containing the same samples used on figure 7a but using β-Actin as housekeeping protein. Bands with common molecular weight of approximately 42 kDa were observed in all lanes indicating right molecular weight for β-Actin and integrity of the protein samples.

3.8 Picture was taken from embryos collected on day 17.5 of pregnancy using the same scale of magnification. The embryo on the left (Hi-E treatment) was scored as 5 (1-5 scale where 1 is lesser tissue mass and 5 is the greatest tissue mass) and embryo in the right (Lo-E treatment) was scored as 1. Embryos had an AVA for IFNτ of 2,187,000 and 729,000 U/mL for the Hi-E and Lo-E embryos, respectively.

4.1 Circulating concentrations of estradiol during the preovulatory period in cows manipulated to have either normal (Hi-E; ▲) or deficient (Lo-E; ■) preovulatory estradiol concentrations. Cows were administered PGF on either h 72 (Hi-E) or h 48 (Lo-E), all cows received GnRH on h 0 to induce ovulation.

4.2 Circulating concentrations of progesterone following GnRH-induced ovulation in pregnant (▲) and cyclic (■) cows. No differences in progesterone concentrations between pregnant and cyclic cows were observed in the first 17 days; however progesterone concentration decreased after day 17 in cyclic cows.

4.3 Mx2 mRNA expression in PBMC expressed as $2^{-\Delta CT}$ (x100) following GnRH-induced ovulation in pregnant (▲) and cyclic (■) cows from days 15 to 30 of pregnancy.

4.4 ISG-15 mRNA expression in PBMC expressed as $2^{-\Delta CT}$ (x100) following GnRH-induced ovulation in pregnant (▲) and cyclic (■) cows on days 16, 18, 20, 22, 24, 26, 28 and 30 of pregnancy.
CHAPTER 1

INTRODUCTION

The United States has the largest fed-cattle industry in the world, and represents the world’s largest high-quality, grain-fed producer of beef. The beef sector is considered the largest single agricultural enterprise in the US, accounting in 2008 for 76 billion dollars of retail equivalent value. This amount corresponds to more than 20% of all agricultural receipts. In 2008, US cattle produced 26.6 billion pounds of carcass weight and 189.3 million of pounds of milk (USDA, 2010). Productivity in dairy and beef cattle is directly dependent upon reproductive efficiency because it affects milk production and number of calves born (Lamb et al., 2008; Wiltbank 2009). Fertilization rate and pregnancy losses are two important predictors of reproductive efficiency. Although data for fertilization rates in cattle has varied across different studies, it has been reported that failure in fertilization represents a minimal proportion of pregnancy failure, varying from 76 to 100% depending upon breed, day of embryo collection or parity (Ayalon, 1978; Maurer and Chenault, 1983). However, by d 30 of gestation viable embryos are present in only 50-60% of beef and 30-40% of dairy cows, representing early embryonic losses between 20 and 60%. Early embryonic loss, which is characterized by losses that occur until and around maternal recognition of pregnancy (MRP) which is about d 15 to 17 of pregnancy, is due to many factors such as genetic defects, reproductive diseases, and heat
stress (Sprott and Field 1998, Sartori et al., 2002; VanRaden and Miller 2006). However, early embryonic loss has been observed to be enhanced following timed-AI in both beef and dairy cattle and in high-producing lactating dairy cows. In both situations, deficiency of preovulatory estradiol and decreased concentrations of progesterone have been reported (Vasconcelos et al., 2001; Sartori et al., 2002a; Peters and Pursley, 2003; Sartori et al., 2004; Wolfenson et al., 2004; Perry et al., 2005). In high-producing lactating dairy cows, increased steroid metabolism has been associated with higher feed intake and hormonal clearance by the liver, resulting in decreased circulating concentrations of estradiol and progesterone (Sangsritavong et al., 2002; Vasconcelos et al., 2003). In cattle synchronized for timed-AI, failure to precisely control follicular dynamics in some of those animals leads to decreased steroid concentrations (Geary et al., 2000) and the induced ovulation of small follicles (Perry et al., 2005). The decrease in fertility caused by these aberrant steroid hormone characteristics could be due to altered oocyte quality or uterine deficiencies to support conceptus survival. Although quality of oocytes has been shown to play an important role in fertilization and pregnancy rates (Wiltbank et. al., 2002; Sartori et. al., 2002), reduced pregnancy rates are still observed in animals presenting high fertilization rates or animals implanted with an embryo by d 7 after ovulation (Diskin and Morris, 2008; Sartori et al., 2006). Therefore, these studies infer that a deficient uterine environment may be responsible for increased embryonic mortality.

Although many different factors such as heat stress or nutritional status could be related to deficiencies in uterine environment, deficiencies in steroids, particularly estradiol and progesterone appear to play a major role in determining fertility. In ewes,
conceptus survival was compromised when estradiol and progesterone concentration were limited or if the sequence of steroid exposure was altered (Miller and Moore, 1976a; 1976b; 1983). Estradiol has been shown to affect the uterus by either altering the morphology of the luminal and glandular epithelial cells in the endometrium or through actions that change the uterine responsiveness to other endocrine signals. Increased concentrations of estradiol prior to ovulation can be achieved by increasing the period of proestrus, which is the period of the estrous cycle characterized by low concentration of progesterone (initiation of luteolysis) and the preovulatory follicular phase (increase in estradiol resulting in LH surge and ovulation). Increasing the length of proestrus may be important for increased pregnancy rates (Mussard et al., 2002, 2003 and 2007). Based on these findings, the standard 7-Day CO-Synch + CIDR program was modified by decreasing the interval of use of the CIDR between GnRH and PGF from 7 to 5 days and increasing time from PGF and CIDR removal to timed-AI from 60 to 72 hours. Pregnancy rates were 9.1% and 13.3% greater in 2 years in the 5 d interval as compared with the 7 d interval (Bridges et al., 2008). In a further experiment, estradiol concentrations in the period between CIDR removal and GnRH induced ovulation were greater in the 5-Day compared to the 7-Day CO-Synch + CIDR program suggesting that increases in pregnancy rates may be due to different estradiol concentrations prior ovulation (Bridges et al., 2009).

The target of the work described in this thesis is to understand how increased preovulatory estradiol concentrations and/or length of proestrus enhances fertility in
cattle, with particular focus on aspects of conceptus function in the pre-
implantation period. Literature pertinent to this topic is reviewed below.
CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

This review addresses the principal physiological and applied aspects of cattle reproduction from the preovulatory period through early pregnancy. Initially, the indication of high fertilization rates in cattle followed by significant losses of embryos by d 30 of pregnancy is described. Subsequently, a summary of different findings that relate fertility to the concentration of estradiol prior ovulation is presented. Mechanisms of MRP and early gestation are discussed in sequence. A large portion of this review is focused on the steroids and proteins that play important role in the female reproductive tract and how these steroids coordinate uterine function to ensure conceptus development and survival. The review concludes with a problem statement and the rationale for conducting the research contained within this thesis.

2.2 Fertilization rates and embryonic losses in beef and dairy cattle

Failure in pregnancy rates can be attributed to either failure in fertilization of the oocyte or subsequent losses during pregnancy. If pregnancy losses occur before the end of the stage of differentiation, by d 42 of gestation in cattle, they are classified as early or late embryonic losses, while if it occurs after the differentiation stage, it is classified as
fetal loss. Early embryonic losses are considered losses occurring until and around MRP on days 15 to 17 in the bovine with late embryonic losses occurring after this period up to day 42 (Committee on Bovine Reproduction Nomeclature, 1972).

2.2.1 Fertilization Rates in Beef and Dairy Cattle

Fertilization rates in beef and dairy cattle have varied widely across reports; however, if insemination occurs at the appropriate time, it seems that fertilization failure represents only a minor proportion of pregnancy failure. When high-fertility semen is used, fertilization rates were shown to be greater than 90% in heifers, beef cattle and low-producing dairy cows (Diskin and Sreenan, 1980; Diskin and Morris, 2008). In high-producing dairy cows, fertilization rates appear to be slightly reduced. Results combined from different experiments showed fertilization rates in high-producing dairy cows to be nearly to 83% (610/732; combined results from Wiebold, 1988; Ryan et al., 1993; Tanabe et al., 1994; Sartori et al., 2002b; Cerri et al. 2009a, 2009b, 2009c).

Data from the last 50 years shows that the substantial increase in milk production has negatively affected reproduction of dairy cows (Lucy 2001). The major part of this effect is believed to be due to pregnancy losses; however fertilization failure may also play a role on decreased pregnancy rates in animals experiencing heat stress as observed by Sartori et al., (2002), where fertilization rates were decreased to 55.3%. Considering available reports in the literature, fertilization rates appear be slightly higher in lactating cows bred based upon detection of estrus (87.7%; Wiebold, 1988; Ryan et al., 1993; Tanabe et al., 1994; Sartori et al., 2002b; Cerri et al., 2009b) than when timed-AI is used (82.4%; Cerri et al., 2009a, 2009b, 2009c); however, additional studies with direct valid
comparisons are still needed. A possible reason for this slight decrease in fertilization rates observed in animals inseminated at timed-AI compared to estrus is aberrant follicular dynamics during estrous synchronization protocols (Geary et al., 2000) and the induced ovulation of small follicles in some animals (Perry et al., 2005) that could potentially influence oocyte quality. Additionally, the variation in the interval from artificial insemination (AI) to ovulation can impact conception rate by decreasing the likelihood of fertilization and/or reducing the embryo quality if AI is performed near to the time of ovulation (Dalton et al., 2000; Saacke et al., 2008).

Oocyte quality can be also affected in animals under altered nutritional or hormonal status (Fouladi-Nashta et al., 2007). In lactating dairy cows, insufficient energy supply resulted in reduced oocyte recovery and embryos reaching the blastocyst stage in vitro, suggesting poor reproductive performance due to reduced oocyte quality (Sinjder et al., 2000). It has been suggested that endocrine and metabolic signals that are responsible for follicular growth regulation can also affect oocyte development through changes in hormone/growth factor concentrations in follicular fluid or via granulosa-oocyte interactions (Webb et al., 1999; Walter et al., 2002). In cows induced to ovulate follicles that had prolonged dominance and consequently prolonged exposure to progesterone, oocyte quality was also compromised. One possible explanation was that the increased frequency of LH pulses in presence of low serum progesterone concentrations (Roberson et al., 1989) causes premature maturation of the oocyte (Revah and Butler, 1996; Cerri et al., 2009)

Deficiencies in fertilization rates can also be observed after insemination using semen with inferior fertility (Kini et al., 2010; Dalton et al., 2010). Although most of the
commercial semen available for artificial insemination attends the minimum criteria for characteristics such as progressive sperm motility, morphological abnormalities and number of sperm cells per straw, differences in fertility of commercial semen exists, while the mechanisms for this variation are poorly understood.

In summary, although fertilization rates vary in extreme situations of altered nutritional and hormonal states and appear to be slightly reduced in high producing dairy cows as compared to heifers or beef cows, a majority of data suggest that if high-quality semen is used, this is not a major contributor to pregnancy failure in cattle.

2.2.2 Early Embryonic Loss in Cattle

During early embryonic development, the period between d 15 to 17 of gestation is considered the most critical for establishment of pregnancy as this is the time of MRP. Embryos in this period have to undergo several mitotic divisions, be physiologically recognized by the cow and overcome maternal physiologic mechanisms of defense (Roberts et al., 1996). Embryo mortality has been reported to be the major cause of economic loss in all systems of cattle production reflected directly as a reduction in conception rates to a first service (Diskin and Morris, 2008). In two studies conducted by Diskin et al., (2006) using lactating dairy cows, early embryonic losses occurring by d 7 of pregnancy were significantly higher (28% and 40%) compared to late embryonic and fetal losses that occurred between days 28 to 84 of pregnancy (6.1% and 7.2%).

After syngamy, which is the fusion of male and female pronuclei, the zygote becomes an embryo and undergoes several mitotic divisions (referred to as cleavage), and other events prior to implantation that are necessary for its recognition by the maternal
system. The first cleavage division generates a two-celled (2-cell) embryo. Each cell, initially called blastomere, undergoes to subsequent divisions without changes in the total cytoplasmic mass. When individual blastomeres can no longer be counted accurately the early embryo is called a morula. Cells start to organize forming a fluid filled cavity called blastocoele and at this point, the embryo is referred to as a blastocyst (Senger, 2003). The rate of cleavage and the cell mass organization inside the embryo is often used as a predictor of embryo quality (Lundin et al., 2001). There is some indication that the pattern of embryo mortality in high-producing lactating dairy cows may differ compared to other categories of cattle such as heifers, nonlactating dairy cows or beef cows. Sartori et al., (2002) demonstrated that lower quality embryos were obtained from high-producing lactating dairy cows when compared directly to heifers (33% vs. 72%, respectively) or dry cows (53% vs. 83%, respectively). Low embryo quality was also observed by Wiebold, (1988) where 52% of the embryos from high-yielding dairy cows on days 6 to 7 after estrus were abnormal, based on morphological criteria. Ryan et al., (1993), studied the effect of high and low environmental temperatures on fertilization failure and embryos viability in lactating dairy cows. No differences in fertilization failure (17.6% and 20.5%) or proportion of viable embryos collected on d 6 or 7 (58.5% and 51.6%) were observed in between high and low temperatures respectively. However, in the same report the percentage of viable embryos collected on d 13 or 14 in cows exposed to high temperatures was lesser compared to cows exposed to low temperatures (27.1% and 59.5% respectively). In contrast, Sartori et al., (2002) reported a very high percentage of non-viable embryos on day 5 in high-producing lactating dairy cows in the summer (heat stressed; 62%) or winter (not heat stressed; 42%), as compared to heifers in
the summer (30%) and nonlactating dairy cows in the winter (20%). Although embryonic death prior to d 8 of gestation may be a greater concern in lactating dairy cows, these data suggest that embryonic loss by this stage of gestation is also occurring in heifers and nonlactating dairy cows. Similarly, data from beef cattle have shown that some embryonic loss, albeit a lesser proportion, do occur. Maurer and Chenault (1983) reported that 67% and 89% of the fertilized embryos collected between d 2 and 5 post-insemination were viable in nulliparous and multiparous cows, respectively, and this proportion was reduced to 55% and 76% when a different group of animals were collected by d 6 and 8 post-insemination in the same experiment. In a study conducted by Diskin and Sreenan (1980) using beef heifers, viable embryos collected on d 4 and 8 post-insemination were 100% and 92% respectively, showing more conservative results compared to Maurer and Chenault (1983).

The potential of using embryo transfer (ET) to reduce embryonic loss and enhance pregnancy rate has been investigated. Based upon evidence presented in the previous paragraph, it has been hypothesized that use of ET would increase the proportion of cows that are pregnant by avoiding much of the embryonic mortality that occurs between fertilization and d 7 of gestation. Although results vary between reports in the literature, in most cases ET has been shown to either improve or have no influence on pregnancy rate as compared to AI. For example, in a study performed by Sartori et al., (2006), when ET and AI were compared in lactating dairy cows throughout the year, no differences were observed in overall conception rates (35.6% and 40.3% respectively). In the same study, cows with multiple ovulations or corpus luteum (CL), regardless AI or ET treatment, had greater progesterone concentrations on d 7 of gestation and tended to
have greater conception rates compared to cows presenting one ovulation/CL. These findings suggested that higher progesterone concentrations on d 7 may positively affect embryo quality and consequently minimize embryonic losses after d 7 of pregnancy.

Similarly, no difference in pregnancy rates between ET and AI were observed by Drost et al., (1999) in lactating dairy cows when pregnancy was diagnosed by using progesterone concentrations on d 22 of pregnancy (cows with progesterone concentration > 1ng/ml on d 22 were considered pregnant). However, by d 42, animals subjected to ET had greater pregnancy rates when compared to cows that were artificially inseminated (35.4% and 21.4%, respectively). Interestingly, in cows considered pregnant at d 22 but diagnosed not pregnant at d 42, the inter-estrus interval (number of days between the first estrus on day 0 and the next detectable estrus) was between 28 and 35 days, suggesting that maternal recognition had occurred but embryos died shortly after this stage. On the other hand, it has been reported in a number of studies that ET increased pregnancy rate as compared to AI in lactating dairy cows (Putney et al., 1989; Ambrose et al., 1999; Al-Katanani et al., 2002; Vasconcelos et al., 2006; Demetrio et al., 2007). In heifers and beef cows, pregnancy rate from ET was either similar or enhanced slightly compared to timed-AI (Hinshaw 1999; Baruselli et al., 2000; Zanenga et al., 2000; Lamb, 2010).

While results above suggest that ET may reduce embryonic mortality in some situations, it is obvious that implantation of healthy embryos on d 7 will not completely restore the losses that occur between d 0 (high fertilization rates) and day 30 of gestation in cattle. The magnitude of embryonic losses that occur between d 8 and 42 of pregnancy have not been as extensively investigated as those during the first 8 days of gestation. In two studies conducted by Diskin and Sreenan (1980), in which embryos were collected
on a variety of days after fertilization and up to d 42 of gestation, embryo survival rates were excellent on d 8 (93%), but significantly reduced on d 12 (56%), d 16 (66%) and d 42 (58%) was observed. In crossbred Brahman heifers, it was demonstrated that 78% of the embryos were viable 16 d after insemination (Smith et al., 1982). Extended return to estrus following AI was observed in nonpregnant cows treated in a manner anticipated to increase embryonic mortality as compared to cows expected to have normal fertility (25.0 ± 0.8 vs. 21.2 ± 0.6) suggesting that embryos had survived through the initial stages of MRP but died shortly thereafter (Mussard et al., 2003a; 2003b).

In summary, embryonic losses have been reported to contribute to the major proportion of pregnancy failure. Whether major embryonic losses are occurring in the very first days of pregnancy or not it is still not clear, and may vary according to studies and animals. Although reports in the literature describing embryonic losses from d 7 to 30 of pregnancy are limiting, data from ET indicates that embryonic losses during this period are still occurring and suggests the uterus may be playing a major role in fertility.

2.3 Follicles and hormonal associations related to fertility in cattle

As shown previously, early embryonic losses represent an important proportion of the pregnancy failure that occurs in cattle. The factors associated with those losses are numerous and may be associated with decreased oocyte quality, age or size of ovulatory follicle, length of the period of proestrus, peripheral concentrations of steroids at key times during gestation or uterine function. It is very likely that more than one of those factors could be involved on the mechanisms of embryonic losses, and more importantly, that those factors may be acting simultaneously and/or associated among each other.
2.3.1 Follicular Development and Estrous Cycle

Follicle wave dynamics during the estrous cycle have been well described in cattle and have been recently reviewed (Evans, 2003; Adams et al., 2008). In general, length of estrous cycle in beef and dairy cows range from 18 to 24 d (Senger, 2003), which is largely influenced by length of luteal phase and number of follicle waves. The follicular wave pattern in the majority of the cattle (>95%) is of 2 or 3 waves, and factors that contribute to the number of follicular waves is related to factors such as breed or age (Ginther et al., 1989; Noseir et al., 2003). Thus, in most cows, follicle waves pass through a periods of emergence, selection and deviation, and dominance either two or three times during a given estrous cycle, with the dominant follicle of the final wave of the cycle progressing through ovulation. Each stage of follicle development is regulated by specific gonadotrophic and pituitary hormonal actions. The estrous cycle has been described (Peter et al., 2009) to have a short follicular phase (comprising approximately 20% of the estrous cycle), which consists of the periods of proestrus (approximately 2 d) and estrus (approximately 1 d). This phase is followed by a relatively long luteal phase, which represents approximately 80% of the estrous cycle, which consists of the periods of metestrus (approximately the following 5 day after ovulation) and diestrus (comprising approximately 13 d). Proestrus, the initial portion of the follicular phase, begins with the functional and structural regression of the corpus luteum and ends at the onset of the estrus where is characterized by the greatest circulating concentrations of estradiol. This phase is characterized by low peripheral concentrations of progesterone and increased concentrations of estradiol produced by the dominant/preovulatory follicle. Estrus is the
latter portion of the follicular phase which ends with the ovulation of the dominant follicle. The immediate time after estrus or initial portion of the luteal phase is known as metestrus. This period involves the luteinization of follicle cells of the previous dominant follicle into a corpus luteum. Finally, diestrus is the period after metestrus characterized by predominance of the corpus luteum and elevated concentrations of progesterone (Peter et al., 2009).

2.3.2 Artificial Insemination and Estrous Synchronization Programs

Artificial insemination is one of the most important and effective techniques for the genetic improvement of farm animals. In addition to enhancement of the genetic gain by making possible the maximum use of superior sires, other advantages such as prevention of infectious genital diseases, early detection of infertile bulls, use of semen from bulls that are unable to mate naturally due to age of physical defect and elimination of danger from handling bulls are often considered when adopting the technique. However, the necessity of estrus observation makes AI less efficient and more challenging for producers. In order to increase the use of AI in the cattle industry estrous synchronization and timed ovulation programs that allows AI at a predetermined time (timed-AI) rather than as determined by detection of spontaneous estrus have been created.

The development of protocols of timed-AI started from the basic discoveries in reproductive physiology dating from the early 1900s and from investigation in estrous synchronization for the last 60 years (Day et al., 2010). Initial attempts to synchronize estrus and ovulation started with the observation that progesterone prevents the
occurrence of estrus (Christian and Casida, 1948). Numerous studies investigating the effects of progestin on estrous response were conducted, however, a high variation in the subsequent fertility and estrus response was reported to exist (Zimbelman et al., 1970; Wishart, 1977). In addition to progestins, the characterization of prostaglandin F$_{2\alpha}$ (PGF) and its use as a luteolytic agent (McCacken et al., 1972; Lauderdale, 1975) in progestin short-programs, provided an alternative method to control and synchronize estrus in cattle, however time of estrus after those programs continued to vary due to the stage of estrous cycle that PGF administration was performed and efficacy of most of the programs was still not predicted (Wishart, 1974; Thimonier et al., 1975; King et al., 1982).

The better understanding of the follicular development in cattle through the characterization of the wave-like pattern (Savio et al., 1988; Sirois and Fortune, 1988; Ginther et al., 1989) and the association with the characterization of hormones capable to induce ovulation such as gonadotropin release hormone (GnRH) and estradiol-based hormones were essential for the rapid progress in the field of estrous synchronization and timed-AI programs. One of the major limitations to improve fertility in estrus synchronization protocols is related to the inefficiency in precisely control follicular development in some females. First attempts to synchronize emergence of follicular waves were performed after acute administration of progesterone and induction of atresia of persistence follicles that were induced after long period of progestin exposure (Stock and Fortune, 1993; Savio et al., 1993), however, this program was limited by the long duration of treatment of progestin to ensure presence of persistent follicle. An alternative method of induction of follicles atresia in a shorter period of time was achieved by using
an estradiol compound (17β-estradiol or estradiol benzoate for example) in presence of progesterone which results in emergence of a new follicular wave approximately four days later (Bo et al., 1995; Day and Burke, 2002). In the USA, GnRH is the major hormone used to induce synchrony of follicular waves due to lack of FDA-approved estradiol formulations to enhance or control reproduction in cattle. The administration of GnRH, with the intention of resetting follicular development, has been reported to induce ovulation in only some beef heifers (Atkins et al., 2008; 42% ovulated), dairy heifers (Moreira et al., 2000; 58.3% ovulated) and dairy cows (Vasconcelos et al., 1999; 64% ovulated). Failure of the GnRH to reset follicular development leads to aberrant follicular dynamics during the synchronization program (Geary et al., 2000) and greater occurrence of ovulation of small follicles within timed-AI protocols (Perry et al., 2005). Along with the GnRH used to synchronize emergence of follicular waves, PGF is used to regress the CL, when present, and a second administration of GnRH is used to induce ovulation of the dominant follicle. The ability of the first GnRH to reset follicular development and the stage of the estrous cycle at treatment has been linked to the size of the dominant follicle at the time of the second GnRH (Atkins et al., 2008, 2010). Programs of timed-AI using GnRH, PGF and another injection of GnRH were developed for dairy (Ovsynch; Pursley et al., 1997) and beef cows (CO-Synch; Geary et al., 1998). Although conception rates following estrus synchronization protocols have improved significantly due to modifications that enhance their efficacy (Larson et al, 2006; Bridges et al., 2008), research in this area is still needed in order to further improve fertility of cattle with these protocols.
2.3.3 Proestrus and Relationships Associated with Fertility in Cattle

The size of the dominant follicle from which ovulation occurs has been reported to influence pregnancy rate after AI in beef heifers (Perry et al., 2007) and suckled beef cows (Perry et al., 2005; Meneghetti et al., 2009), but this relationship is not always consistent. For example, the effect of the ovulatory follicle size on conception rate has been compared between a GnRH-induced ovulation and at spontaneous ovulation after estrus in postpartum cows and heifers by Perry et al., (2005, 2007). The diameter of the ovulatory follicle did not influence conception rate after detection of estrus but was positively associated with conception rate when ovulation was induced with GnRH in animals not exhibiting estrus. The effect of the diameter of the ovulatory follicle and whether or not estrus occurred on pregnancy rates in cows that were artificially inseminated by timed-AI was also investigated in Bos Indicus beef cows (Sa Filho et al., 2010). The diameter of the dominant follicle at AI was measured and cows were divided into four categories depending upon number of standard deviations (SD) they differed from the mean diameter of the ovulatory follicle across all cows (two SD below the mean, mean minus one SD, mean plus one SD, two SD above the mean). Pregnancy rates increased proportionally as the diameter of the dominant follicle increased (27.5%, 46.6%, 57.9% and 63.3%, respectively). Additionally, animals observed in estrus preceding timed-AI had significantly greater pregnancy rates compared to animals not observed in estrus (67.7% vs. 36.2%, respectively).

The influence of follicle size and age on fertility of cattle was investigated in a series of three experiments conducted by Mussard et al., (2003a, 2003b, 2007). It was hypothesized that the diameter of the ovulatory follicle would be the most appropriate
indicator of fertility and that animals ovulating a large follicle would have greater fertility compared to animals ovulating small follicles. In the first experiment cows that spontaneously ovulated were compared to cows that were induced to ovulate with GnRH when follicles had attained a diameter of 10 mm (“small” preovulatory follicles). Artificial insemination was performed 12 h after the onset of estrus in cows ovulating spontaneously and 12 h after the GnRH injection in the GnRH treatment. In the second experiment, the comparison was between cows induced to ovulate with GnRH with a follicle diameter of approximately 13 mm vs. 10 mm, and the third experiment, animals received the same treatment as in the second experiment but instead of AI 12 h after GnRH administration cows were implanted with an embryo 7 d later. The approach taken in the third experiment tested for effects of the uterus and CL on fertility, since there were no effects of oocyte on this experiment. In all experiments, lesser fertility was observed in cows induced to ovulate small as compared to large follicles. Although the hypothesis was confirmed within each individual experiment, greater fertility was observed with larger follicles, consideration of data across these experiments led to some interesting observations. The increase in fertility was not assort with an increase in follicular diameter, however, a stronger relationship between the interval from PGF to either a spontaneous or GnRH-induced LH surge (referred as “proestrus”) was observed, suggesting that the period of proestrus is a better predictor of fertility then follicle size. A fourth experiment was performed to investigate the effect of period of proestrus on fertility in cattle (Bridges et al., 2010) in cows that were induced to ovulate follicles of similar size and age, but that experienced either a short (1.25 d) or long (2.25 d) of
proestrus. Conception rates of cows in the long proestrus treatment were greater compared to short proestrus treatment (71% vs. 10%, respectively).

These findings were extended to development of a timed AI program to take advantage of increased fertility associated with a longer proestrus. In timed-AI programs, the period of “proestrus” actually refers to the interval from PGF (and progesterone device withdrawal when applicable) to GnRH administration. In the standard program, Bridges et al., (2008) proposed that decreasing the interval of CIDR between GnRH and PGF in the 7-Day CO-Synch + CIDR from 7 to 5 d and increasing the period from CIDR removal to timed-AI from 60 to 72 h would enhance pregnancy rates in postpartum beef cows. This modified protocol, referred to as the 5-Day CO-Synch + CIDR, resulted in a substantial increase in pregnancy rates of 13.3% and 9.1% in two different experiments when compared to the traditional 7-Day CO-Synch + CIDR, however two injections of PGF 12 h apart at the time of CIDR removal were used. The value of two, relative to one dose of PGF in a 5-Day CO-Synch + CIDR was investigated by numerous authors and pregnancy rates showed to be consistently greater (around 10 to 15%) in animals treated with one compared to two doses of PGF 8 h apart (Kasimanickam et al., 2009), 12 h apart (Souto et al., 2009; Cruppe et al., 2010a) or administered simultaneously (Cruppe et al., 2010b). Similar increase in pregnancy rates in a 5 vs. 7-Day CO-Synch + CIDR have been also observed in other experiments involving dairy cows (Santos et al., 2010) and beef heifers (Wilson et al., 2007). Additionally, high pregnancy rates have been reported with the 5-Day CO-Synch + CIDR in dairy heifers (Rabaglino et al., 2010).

The period of proestrus initiates with the removal of progesterone sources, such as from the CL and ends at the onset of the spontaneous LH surge (Peter et al., 2009). In
estrus synchronization programs, the period of proestrus would be equivalent to the period of progesterone removal from a CIDR and/or CL regression and ending with either a spontaneous or GnRH-induced LH surge. This reduction of progesterone is responsible for a series of hormonal changes that has important effects on regulation of the reproductive system. The frequency of LH pulses increases almost immediately in response to the decline in circulating concentrations of progesterone (Bergfeld et al., 1995). Release of LH from the anterior pituitary is primarily regulated by progesterone and estradiol and the negative association of progesterone and LH secretion has been well established (Kinder et al., 1996). Proestrus is characterized by LH pulses at an increasing frequency as proestrus progresses and the LH surge approaches (Imakawa et al., 1986). Pulsatile LH secretion is the primary factor that drives the final development of preovulatory follicles, leading to the growth of follicles and production of estradiol by granulosa cells (Walters and Schallenberger 1984). The changes in peripheral concentrations of estradiol during short (1.25 d) and long (2.25 d) proestrus was investigated by Bridges et al. (2010). The size of the ovulatory follicle size and age was similar and the magnitude of the GnRH-induced LH surge did not differ between treatments. However, concentrations of estradiol circulating were greater in the long than short proestrus treatment during the period preceding GnRH administration. In a subsequent experiment Bridges et al. (2009) compared follicular dynamics and estradiol concentrations between the 7 and 5-Day CO-Synch + CIDR program. Between these synchronization programs, the diameter of the ovulatory follicle at time of GnRH did not differ, but maximum estradiol concentrations during proestrus tended to be greater with the 5 d program.
Previous studies have focused on lengthening proestrus to achieve increased follicular stimulation by LH which results in increased preovulatory estradiol concentrations and fertility. However, other approaches that have the potential to increase follicular stimulation by gonadotropins and lead to the same end result have been investigated. These approaches include partial reduction in the circulating concentrations of progesterone, reducing negative feedback control of LH secretion in anestrous cows and providing exogenous gonadotropins such as equine chorionic gonadotropin (eCG). The relationship between concentration of progesterone and pulses of LH is dose-dependent. Kinder et al., (1996) investigated the effect of administration of different amounts of progesterone on LH secretion and estradiol. In this experiment, LH secretion and estradiol concentrations were less in cows with high (~8 ng/ml) as compared to low (~2 ng/ml) progesterone concentrations.

In estrus synchronization programs of Bos Indicus, studies have focused on programs that induce a partial reduce in progesterone concentration during the protocol, in this way follicular dynamics can still be controlled but negative feedback of progesterone on LH pulse would be reduced. In South America, EB-CIDR-ECP is a common program of estrus synchronization for Bos Indicus cattle and consists in administration of estradiol benzoate (EB) and CIDR insertion on d 0, PGF and estradiol cypionate (ECP) administration simultaneously with CIDR removal on d 9 and timed-AI on d 11. Two commonly used approaches are to either administer PGF before the end of the CIDR treatment and/or use a previously used CIDR that contains reduced progesterone concentration (Martinez et al., 2003; Peres et al., 2009; Meneghetti et al., 2009). In the first approach, the contribution of the progesterone coming from the CL is
removed, and the CIDR provides the only source of progesterone for the duration of time it remains in the vagina. Peres et al., (2009) and Meneguetti et al., (2009) investigated the effect of a new or a previously used CIDR for 18 days on pregnancy rates, diameter of ovulatory follicles and progesterone concentration at time of CIDR removal in post pubertal Nelore heifers and postpartum beef cows synchronized with EB-CIDR-ECP program. Both heifers and cows treated with a new CIDR presented lower pregnancy rates, higher concentration of progesterone at time of CIDR removal and a smaller follicle at timed-AI. In a subsequent experiment, the administration of PGF two days before the removal of the CIDR (on d 7) was compared to the normal time of PGF administration (d 9) in nonlactating beef cows. Cows receiving PGF on d 7 had lesser serum progesterone concentrations on d 9, a larger follicle at time of AI, and improved rates of ovulation, conception, and pregnancy (Peres et al., 2009).

In addition to reducing progesterone concentrations during estrus synchronization programs, administration of exogenous gonadotropins, such as eCG, at the appropriate interval before timed-AI or temporary calf removal are strategies that have been used in order to increase follicle stimulation or LH pulsatility (Williams et al., 1996; Baruselli et al., 2004). The influence of eCG given on d 9 of the EB-CIDR-ECP program has been recently tested in Nellore heifers and cows (Peres et al., 2009). In comparison to heifers and cows that received identical synchronization treatments but no eCG, injection of 300 IU of eCG increased diameter of the ovulatory follicle, ovulation rate and pregnancy rate to timed-AI in both heifers and cows. In postpartum beef cows, nutritional status and suckling may be one of the most important factors that inhibit ovulation. Rasby et al. (1992) have shown that nutrition restriction has a negative influence in LH release in
postpartum beef cows. The frequency of LH pulses is also inhibited by the suckling stimulus from the calf (Williams et al., 1996). Increased frequency of LH pulses can be achieved by restricting suckling or temporary weaning in cows presenting less than 30 postpartum (Yavas and Walton, 2000). Pregnancy rate was increased by approximately 22% after calf removal between the time of removal of a progesterone-releasing device and timed-AI (Barreiros et al., 2003).

In summary, fertility in cattle seems to be very dependent on the degree of gonadotrophic stimulation that animals receive before ovulation. Lengthening proestrus increases the length of stimulation of the ovulatory follicle, preovulatory estradiol concentrations and fertility. Modification of the GnRH-based synchronization programs to increase the length of proestrus substantially improves timed AI pregnancy rate. Modification of EB-CIDR-ECP programs to either reduce progesterone concentrations during the synchronization program or to increase gonadotrophic stimulation during proestrus with eCG or temporary weaning also enhances pregnancy rate to timed-AI.

2.4 **Hormonal programming of the uterus**

In domestic animals, progesterone and estrogens play key roles in regulation of a variety of physiological processes of normal growth, development and reproduction (Stormshak and Bishop, 2008). Coordination of uterine function during the estrous cycle is regulated by oscillating production of estradiol and progesterone by the ovary (Sorensen, 1979) and fertility has been reported to be drastically reduced if the sequential exposure of progesterone before estrus, increased estradiol concentration at estrus followed by progesterone concentration in the subsequent estrous cycle is altered in ewes.
(Moore, 1985; Wilmut et al., 1986) or cattle (Inskeep, 2004). Furthermore, conceptus survival was compromised when progesterone was limited during the luteal phase before estrus, estradiol was limited around estrus and progesterone was limited during the luteal phase after estrus (Miller and Moore, 1976; Moore et al., 1983). In the uterus, the action of estradiol has been associated with alteration of the endometrial morphology and consequent changes in responsiveness to other endocrine signals. Murray et al., (1992) studied the effect of estradiol on epithelial cells and uterine glands on sheep.

Ovariectomized sheep treated with estradiol for 6 days presented elongated mitochondria, Golgi complex and rough endoplasmatic reticulum more abundantly, and more lysosomal granules-like structures in the cytoplasm than sheep not treated with estradiol, suggesting significant changes in epithelial cell morphology and development of organelles responsible for protein synthesis. In a similar study using ovariectomized sheep, administration of estradiol was reported to increase the RNA:DNA ratio and proliferation of uterine epithelial cells (Johnson et al., 1997).

Reproductive processes in cattle are mainly regulated by steroids that act though binding to nuclear receptors that regulate gene expression (Clark et al., 1992). The predominant sex steroid receptors expressed in the uterus, estrogen receptor α (ER) and progesterone receptor (PGR), were reported to be up-regulated in response to the preovulatory estradiol surge in many different species (Lessey et al., 1988; Bazer et al., 1995). Additional studies describing the expression of ER and PGR in the uterus (endometrium) of bovine and ovine females during various periods of the estrous cycle showed that expression of both, ER and PGR, were found to increase during proestrus, reaching the maximal amounts at estrus and metestrus, and then decreasing during the
midluteal phase of the estrous cycle (Senior, 1975; Zelinski et al., 1982; Spencer and Bazer, 1995; Kimmins and MacLaren, 2001; Robinson et al., 2001; Ing and Zhang, 2004). Bridges et al., (2006) compared conceptus and uterine characteristics between beef heifers with normal and deficient preovulatory estradiol concentrations on d 15.5 of gestation. No differences in conceptus size and intrauterine concentrations of interferon tau (IFN\(\tau\)) between animals with different preovulatory estradiol concentrations were observed, but characteristics of uterine gene expression and protein localization for steroid receptors were different between heifers with high and low preovulatory estradiol concentrations. The intensity of staining for nuclear PGR in the deep glandular epithelium and amounts of mRNA for ER in the uterine endometrium was less in heifers that were deficient in preovulatory estradiol concentrations. Ing and Zhang (2004) suggested that the up-regulation of the progesterone receptors by the preovulatory estradiol was the main physiological process responsible for endometrial gland secretions to support embryo survival in response to increased levels of mid-luteal progesterone concentrations. On the other hand, the subsequent absence of PGR in the endometrial glandular epithelium is required for onset of differentiated function of the glands during pregnancy (Spencer et al., 2004). The role of preovulatory estradiol concentrations in determining lifespan of the CL are critical since lesser concentration of estradiol during the period of proestrus may alter the normal cyclic expression of the progesterone and estradiol receptor, resulting in up-regulation of oxytocin receptor expression and premature release of PGF (Garcia-Winder et al., 1986; Mann and Lamming, 2000; Robinson et al, 2001; Bridges et al., 2010). The effect of estradiol in inducing oxytocin receptor expression in different times of the estrous cycle was also shown in sheep
The administration of a large dose of estradiol during the middle of the estrous cycle increased the expression of oxytocin receptor 12 h later resulting in luteolysis.

2.5 Maternal recognition of pregnancy

In bovine females, ovaries are in constant follicular activity beginning during the last third of the gestation (Senger, 2003; Aerts and Bols, 2010). After animals reach puberty, dominant follicles that were entering to constant atresia are now able to ovulate (Day and Anderson, 1998). Ovulation is essential for pregnancy due to two main factors; it produces the oocyte that will be fertilized by a sperm cell and become the embryo, and results in formation of the CL. As discussed earlier in this chapter, oocyte quality is essential to success of pregnancy, and it can be affected negatively by numerous factors such as aberrant steroid function or heat stress. The CL is formed at the site of the previous dominant follicle after ovulation, through the structural and functional transformation of theca and granulosa cells into luteal cells (Peter et al., 2009). The rearrangement of granulosa cells into luteal cell results in production of progesterone, which is essential for maintenance of gestation in many species. Prostaglandin F$_{2\alpha}$ is the substance produced by the nonpregnant endometrium tissue that causes regression of the CL and the decline in progesterone concentration when embryos are not present around the period of MRP. Oxytocin induces release of PGF through activation of the oxytocin receptor (a 7-transmembrane, G-protein associated receptor) present in the endometrium by increasing inositol triphosphate turnover and the cytosolic calcium concentration, activation of protein kinase C and cyclooxygenase-2 (Flint et al., 1995; Asselin et al.,
The decline in progesterone concentration signals the hypothalamus to release GnRH and stimulates the pituitary to secrete gonadotropins that will induce ovulation and initiation of a new estrous cycle.

However, if the oocyte is fertilized by the sperm and the fusion of male and female pronuclei occurs, the zygote becomes an embryo and undergoes several mitotic divisions until it reaches the stage of blastocyst (already mentioned earlier in this chapter), and other events prior to implantation that are necessary for its recognition by the maternal system. After the blastocyst hatches, the embryo becomes portioned in two distinct cellular populations known as the inner cell mass and the trophoblast. The trophoblastic cells have a crucial role on MRP and on the structure of the embryo (Senger, 2003). Maternal recognition of pregnancy is a series of events where the presence of the embryo is responsible for altering the physiologic mechanisms of luteolysis and consequently allows the maintenance of pregnancy (Roberts et al., 1996). This process occurs around d 15-17 of pregnancy in the bovine and is associated with the initiation of production of INFτ by the trophoblastic cells starting as early as d 15 of pregnancy in bovine (Beterridge et al., 1978; Northey and French, 1980; Bartol et al., 1985). Significant increases in interestrus interval were observed after intrauterine infusion of INFτ in cyclic cows between d 15.5 and 21 of the estrus cycle (Plante et al., 1988). Interferon tau acts by suppressing the oxytocin-induced release of PGF in vitro (Danet-Desnoyers et al., 1994) and in vivo (Bazer and Johnson, 1991; Thatcher et al., 1992) through mechanisms involving down regulation of ER in the endometrial epithelium, which prevents the increase in oxytocin receptors (Bazer et al., 1994; Flint et al., 1994; Spencer et al., 1995). The molecular mechanisms of suppression of ER
transcription have not been fully defined, but it seems to involve the type I IFN receptor signal transduction system and IFN regulatory factors (IRF), such as IRF1 and IRF2 (Darnell et al., 1994; Spencer et al. 1996, 1997)

In addition to the paracrine actions of the IFNτ on the endometrium that alters release of PGF and prevents luteolysis, Bott et al., (2010) recently suggested that INFτ may have endocrine action on the CL. Infusion of either recombinant oINFτ or bovine serum albumin (BSA) into the uterine vein of nonpregnant ewes from d 10 to 11 post estrus resulted in greater expression of ISG-15 mRNA and protein in the CL of ewes receiving 24 h infusion of oINFτ compared to ewes receiving BSA. Prostaglandin F₂α was infused 12 h after beginning of the treatment in half of the animals of each treatment and progesterone concentrations were measured. In animals infused with BSA the decline in progesterone concentration were observed starting 6 h after infusion through 12 h, however, in ewes infused with recombinant oINFτ, a similar decline in progesterone concentration was observed at 6 h but concentrations returned to control values at 12 h. In a second experiment animals were infused with either BSA or recombinant oINFτ for 7 days beginning on d 10 of the estrus cycle. All ewes infused with BSA in the uterine vein returned in estrus by d 19 while 80% of the recombinant oINFτ -infused ewes maintained luteal phase of progesterone concentration through d 32.

2.6 Mechanisms of uterus/embryo hormonal signaling

2.6.1 Interferon Tau Signaling of Interferon Stimulated Genes

In addition to its role on MRP, IFNτ has been reported to stimulate numerous genes responsible for up-regulation of the endometrium in response to pregnancy that are
critical to the fetal-maternal communication and the maintenance of gestation (Charleston and Stewart, 1993; Austin et al., 1996; Spencer et al., 2004). Also known as interferon stimulated genes (ISGs), these genes are believed to be important for uterine function, receptivity to implantation, and conceptus development (Spencer et al., 2006; Bauersachs et al., 2006; Klein et al., 2006). The expression of ISGs occurs in a temporal and cell-specific manner. In addition to IFNτ, progesterone is believed to have a role in activation of ISGs. For most of the interferon actions on the uterus, progesterone works permissively to stimulation of ISGs (Bazer et al., 2008). If on one hand progesterone seems to be important to allow stimulation of ISGs by IFNτ, on the other hand implantation is preceded by loss of expression of PGR and estrogen ER receptors by uterine epithelia in different species of animals including cattle (Slayden and Keater 2007; Spencer et al., 2007, 2008). The progesterone-induced down-regulation of PGR in uterine luminal, superficial glandular and glandular epithelia of ewes is a prerequisite for expression of ISGs in the endometrium (Bazer et al., 2008). Ott et al., (1999) observed that uterine infusion of IFNτ in the presence of different steroids can stimulate differently the expression of ISGs in the uterus. The combination of IFNτ with progesterone has shown to have the greater stimulation compared to combinations with estradiol or only IFNτ infusion, respectively. Most of the interferon stimulation gene expression is limited to the glandular epithelia and endometrial stroma of pregnant ruminants. The absence showing to be absent in the luminal epithelia and superficial glandular epithelia is though to be due to the expression of the interferon regulatory factor 2 in this type of tissue (Choi et al., 2001).
Interferon stimulated gene 15 (ISG-15) plays an important role in adhesion and endometrial remodeling during the period of implantation of the conceptus into the uterine wall (Austin et al., 1996; Johnson et al., 1999c; Austin et al., 2004). The exact mechanisms of action and function of ISG-15 in the uterine endometrium is still unclear, however it has been demonstrated that ISG-15 conjugates to cytosolic endometrial proteins and is expressed at the uterine-placental interface throughout pregnancy in sheep (Joyce et al., 2005) and cows (Austin et al., 2004), which may facilitate important biological events during early gestation such as IFNτ signaling and regulation and/or modification of target proteins. Similar to ISG-15, the expression of another ISG known as Myxovirus resistance (Mx) has been described to be regulated by IFNτ in the uterine epithelium, stroma, myometrium, and in peripheral blood mononuclear cell (PBMC) of sheep and cattle (Charleston and Stewart, 1993; Ott et al., 1998; Hicks et al., 2003; Gifford et al., 2006). It has been demonstrated that Mx1 and Mx2 are both antiviral proteins, however the second is more strongly regulated in PBMC of pregnant cows than Mx1 (Gifford et al., 2006).

The mechanisms of action of ISGs are more fully reported in the uterus than in other tissues. Research using a receptor transporter protein that has shown to be up-regulated in presence of IFNτ has demonstrated that a similar gene response can be expressed by different tissues (Gifford et al., 2008). Receptor transporter protein 4 (RTP4) mRNA, due to its characteristics to be up-regulated in PBMC during early pregnancy, was observed to have higher gene expression in pregnant compared to nonpregnant ewes in the endometrium, CL and PBMC. Yang et al., (2010) reported the expression of ISG-15 was up-regulated in the bovine luteal cell in vitro during early
gestation in response of treatment with recombinant bovine IFNτ. The expression of ISG-15 and Mx-2 mRNA has been reported to higher in PBMC in response to IFNτ. The expression of Mx2 gene has shown to be increased 4 to 5 fold within 24–48 h of initial IFNτ signaling in pregnant sheep (Yankey et al., 2001). In heifers, a greater fold increase for peripheral Mx2 mRNA was observed from d 0 to 18 of pregnancy when compared to nonpregnant animals. Heifers that experienced pregnancy losses tended to have smaller fold increases in Mx2 mRNA expression than those that maintained pregnancy (Stevenson et al., 2007). Similarly, expression of ISG-15 was described to be detected in PBMC in response to IFNτ. In dairy cows, ISG-15 mRNA expression in peripheral cells was evident by day 15 with maximum amounts present from d 17 to 21 with subsequent decline until d 32 (Han et al., 2006). Gifford et al. (2007) also demonstrated that ISG-15, Mx2, and other ISGs were found to be less expressed in peripheral blood from d 18 nonpregnant dairy cows when compared with pregnant dairy cows at same period in pregnancy. In a similar experiment expression of ISG-15 and Mx2 were greater in pregnant than nonpregnant dairy cows on d 18 and 20 when compared to d 14 and 16 after AI. In the same study expression of ISGs among heifers, primiparous and multiparous dairy cows suggested that multiparous cows had a lesser ISG response than primiparous cows and heifers (Green et al., 2010).

2.6.2 Embryo synthesized proteins

In addition to IFNτ, other products that are produced by trophoblastic cells, such as placental lactogens, seem to also be important to the maintenance of the CL and regulation of pregnancy (Roberts et al., 1995). Ruminant placental lactogen, also known
as chorionic somatomammotropin hormone - 1 (CSH-1) due to its lactogenic and somatogenic proprieties, is a member of the growth hormones/prolactin gene family which is produced by binucleate cells of the conceptus trophoderm and secreted into both the maternal and fetal circulation (Gootwine, 2004; Alvarez-Oxiley et al., 2008). In ruminants, bovine CSH-1 is the only CSH-1 secreted in a glycosylated form. Although the specific role for glycosylation is not fully understood yet, it has been described that glycosylation can have many functions such as a role on the structure of the molecule, it is necessary to achieve the correct folding of glycoproteins, it can regulate the half-life of the molecules or it can mediate specific recognition by the receptors (Varki, 1993). The molecular weight of bovine CSH-1 was estimated to be 31-33 kDa in both placental explants or purified extract (Beckers et al., 1980; Bayatt, 1986) and it has been reported to have 200 residues with a 36 amino acid peptide sequence (Schuler et al., 1988). The molecular weight of the recombinant form of CSH-1 isolated and purified from E. coli is estimated to be around 24 kDa (Nakano et al., 2001), which is the theoretical mass of the protein core of the CSH-1 with no glycosylation.

Bovine CSH-1 mRNA is initially transcribed by binucleate cells and is secreted into both the maternal and fetal circulation by the time of implantation and throughout gestation. Binucleate cells are present in early fetal trophoderm of ruminants and secrete many placenta-specific proteins including placental lactogen and pregnancy-associated proteins (Zoli, 1992). Binucleate cells originate from the epithelia at the trophoderm basal lamina and their main characteristics are the presence of two large nuclei in an enlarged cell present in the trophoderm mononucleate epithelial sheet. Binucleate cells are unique by the process of migration from the placentome to fuse with uterine epithelial cells
forming the trinucleate cells, which are responsible for direct discharge of their placental-specific proteins into maternal circulation (Wooding and Beckers, 1987). Although Yamada et al., (2002) reported that CSH-1 was detected in trophoblastic tissue of the bovine beginning on d 36 of pregnancy, Kessler et al., (1991) suggested that CSH-1 can be expressed as early as d 17 of pregnancy. In ewes, detection is considered to happen around d 16 of pregnancy (Gootwine, 2004). Concentration of CSH-1 expression in the maternal plasma circulation is significantly greater in the ovine as compared to the bovine (peaks around 1-2 μg/ml and 2-3 ng/ml, respectively), and in cattle it can be detected only after d 60 of gestation using radioimmunoassay analysis (Beckers et al., 1982). The ratio between maternal and fetal concentration show that in all the species, with exception in cattle, level of maternal concentration of CSH-1 are higher than in the fetus circulation (Alvarez-Oxiley et al., 2008). The higher concentration of CSH-1 observed in the fetal rather than the maternal plasma in cattle, suggests that CSH-1 targets primarily fetal tissue in this species, but the reason is still unknown. Recombinant CSH-1 was administered subcutaneously in nonpregnant lactating dairy cows and the half-life was reported to be approximately 7.5 min (Byatt et al., 1992). Although not established in vivo yet, the glycosylated form of CSH-1 is also believed to have relatively short half-life due to acute decrease in CSH-1 levels in maternal circulation observed in cows 24 h after calving (Beckers et al., 1982).

In humans, CSH-1 may play an important role during pregnancy in the regulation of maternal carbohydrate, lipid, and protein metabolism (Handwerger and Freemark, 2000). In the fetus, CSH-1 has been suggested to act in the regulation of the fetal growth indirectly through alteration of the maternal metabolic environment, through transfer of
the maternal placental nutrients to the fetus or may mediate insulin-like growth factor 1 (IGF-1) stimulation (Oliver et al., 1992; Schoknecht et al., 1996). In the ovine, studies altering the levels of CSH-1 by different ways such as feeding restriction (Butler et al., 1987; Bauer et al. 1995), infusions of either of CSH-1 or antibodies against CSH-1 into maternal or fetal circulation and carunclectomy or fectomy (Oliver et al., 1996; Schoknecht et al., 1996; Waters et al., 1985), have suggested that the regulation of fetal growth occurs by stimulating maternal sharing of nutrients to the fetus (Anthony et al., 1995). A high rate of fetus growth has been associated to concentration of CSH-1 in ewes, especially in the last third of pregnancy. Therefore, in addition to its possible function on assistance of embryo growth and maintenance of pregnancy, CSH-1 has been extensively studied due to its potential for increasing cattle productivity by increasing of milk and meat production.

In ruminants, CSH-1 has somatogenic activity by activating heterologous systems. It has been suggested in sheep that CSH-1 acts through binding to both prolactin (PRL) and growth hormone (GH) receptors (Anthony et al., 1995) by mimicking the action of ovine PRL (Sakal et al., 1997) but not ovine GH. For GH receptors, it is felt that CSH-1 has antagonistic actions due to its capacity to occupy the homologous GH receptor site without inducing their homodimerization (Alvarez-Oxiley et al., 2008; Herman et al., 1999; Warren et al., 1999; Gertler and Djiane, 2002). In addition, a distinct receptor specific for CSH-1 has been reported to be expressed in ovine fetal liver (Freemark and Comer, 1989) and bovine endometrium (Galosy et al., 1991).

Throughout pregnancy, the placenta has an important role in transfer of nutrients, oxygen and waste between mother and her fetus, and also works as a barrier preventing
migration of cells between these two compartments. Bovine placentation is a complex mechanism involving a cascade of regulated gene expression where the attachment between maternal and fetal tissue begins between on d 20 to 30 of gestation and placentome is formed around d 50 of gestation (Greenstein et al., 1958; Hashizume, 2007). Additionally, the placenta is a very important autocrine, paracrine and endocrine organ which produce a large range of steroids and peptides hormones that, in addition to CSH-1, are responsible for regulating the development of fetomaternal unit and altering maternal physiology to support this process from the pre-implantation and implantation periods thru the end of gestation in the bovine (Gootwine, 2004).

In addition to CSH-1, GH and PRL also play a role in embryo growth regulation. In most mammals, GH is a single gene product, which is normally secreted in a pulse pattern by the pituitary gland (Baumann, 1991; Veldhuis, 2001) and targets many different cells. Growth and proliferation is affected both directly and indirectly by stimulation by IGF system. During pregnancy, the extensive proliferation of cells that is occurring in the placenta coincides with expression of receptor for GH in the placenta of cattle (Scott et al., 1992, Kolle et al., 1997) and sheep (Pratt and Anthony, 1995; Lacroix et al., 1999). In sheep placenta, expression of mRNA for GH receptors was observed between d 20 and 120 of pregnancy, with significant increase between d 23 and 43 (Lacroix et al., 1999). Until the fetal pituitary is mature enough to start to produce its own GH, which happens by d 50 to 60 of pregnancy in sheep, growth is mainly dependent upon GH produced by the mother (Gluckman et al., 1979). After this period, the main source of GH present in the placenta is produced by the fetus. In addition to GH produced by the maternal and fetal pituitary, in some species such as sheep, goats and
humans, GH can be also produced by the placenta. Although there is evidence of GH production by the trophoderm and syncytial cells of the placenta in sheep between d 27 and 75 of pregnancy (Lacroix et al., 1996), there is no evidence to date for expression in cattle when analyzed by microarray (Ishiwata et al., 2003, Band et al., 2002). Another important hormone produced by the pituitary is PRL. Prolactin is involved in the regulation of a variety of biological functions including growth and development, reproduction, metabolism, behavior and others (Bole-Feysot et al., 1998) but unlike GH, no receptor expression or production in the ruminant placenta has been reported.

In addition to CSH-1 and GH, prolactin-related protein (PRP) has been reported to be expressed by the placenta (Anthony et al., 1995). Because PRP and CSH-1 are structurally related to pituitary hormones such as GH and PRL, they are also known as members of the prolactin/growth hormone family. Prolactin-related protein gene was described to be expressed by the binucleate cells of the placenta of both cow and sheep (Anthony et al., 1995). More than six PRPs have been described to be produced by the placenta (Schuler and Kessler, 1992), however, the only member of the family translated in cattle is the PRP-1 mRNA (Kessler and Schuler, 1997). Furthermore, PRP-1 expression was detected on day 20 of gestation and before CSH-1 detection (Yamada et al., 2002).

2.7 Statement of the Problem

Early embryonic mortality has been reported to be a major cause of reduced pregnancy rates. Large economic losses in beef and dairy cows have been attributed to pregnancy failure in beef and dairy cattle. Decreased preovulatory estradiol
concentrations have been described in animals with reduced fertility such as beef and dairy cows synchronized to timed-AI and in high producing, lactating dairy cows. In timed-AI programs, decreased steroid concentrations can be attributed to the induced ovulation of immature follicles in some animals. In high producing lactating dairy cows, the increase in feed intake has been demonstrated to lead to an increase in hormonal clearance by the liver and consequent decreased peripheral concentrations of hormones such as progesterone and estradiol. In both cases, the decreased circulating concentration of estradiol seems to be related to decreased fertility and increased embryonic mortality. The uterine environment seems to be affected in animals with decreased fertility and embryo/uterus communication at and after MRP is very important to maintenance of pregnancy.

Recent studies have shown that a longer proestrus period is responsible for greater preovulatory estradiol concentrations and suggest that when estradiol is less, that decreased fertility may be the result of altered capacity of the uterus to support the developing embryo. The characteristics of the uterus were altered on day 5 and d 15.5 of gestation but differences in embryo function was not observed in animals presenting high compared to low preovulatory estradiol concentration (Bridges et al., 2006).

The central hypothesis of the research performed in this thesis is that reducing length of the preovulatory period leads to decreased preovulatory estradiol concentrations and results in decreased embryonic expression of IFN\textsubscript{\tau} and CSH-1, and these reduced protein and/or gene expression may be responsible for the previously observed reduction in fertility. Moreover, decreased progesterone concentration in the period after ovulation would be observed in animals with lower preovulatory estradiol concentrations.
Additionally, daily sampling of animals for ISG mRNA expression in PBMC from days 15 to 30 of gestation in experiment 2, would provide a rough estimation of time of pregnancy when embryonic losses occur and pregnant cows with higher preovulatory estradiol concentration would have up-regulation of ISG expression and lesser embryonic mortality. Determining the time that embryonic mortality occurr and a greater understanding of the mechanisms responsible for embryonic death before day 30 of gestation will contribute significantly to providing potential solutions for infertility in steroid deficient animals.

The overall objective of this work is to investigate the time when embryonic losses are occurring and the mechanisms responsible for embryonic losses in cattle that were exposed to decreased concentrations of estradiol prior to ovulation. Moreover, this study targets how reduced concentrations of estradiol prior to ovulation affects progesterone concentrations after ovulation, embryonic protein and/or gene expression of IFNτ and CSH-1 by d 17.5 of pregnancy, and Mx2 and ISG-15 gene expression from days 15 to 17.5 of gestation.
CHAPTER 3

EFFECT OF LENGTH OF THE PREOVULATORY PERIOD ON PERIPHERAL CONCENTRATIONS OF ESTRADIOL AND PROGESTERONE, EMBRYONIC EXPRESSION OF IFNτ AND CSH-1 AND EXPRESSION OF ISG-15 AND MX2 IN PBMC IN BEEF COWS

Abstract

The objective of the present experiment was to determine the influence of manipulation of preovulatory estradiol concentrations through alteration of the duration of the preovulatory period on gene expression and protein synthesis of interferon tau (IFNτ) and chorionic somatomammotropin hormone - 1 (CSH-1) by the conceptus on d 17.5 of pregnancy, and expression of interferon stimulated gene 15 (ISG-15) and Myxovirus resistance 2 (Mx2) in peripheral blood mononuclear cells (PBMC) from d 15 to 17.5 of pregnancy in cows. Nonlactating beef cows were presynchronized to a common day of estrus and ultrasonographic-guided follicle aspiration was performed 6.5 ± 0.3 d after estrus (day of aspiration = d 7 of experiment). The experiment was performed in two replications. Cows were assigned to receive 25 mg of prostaglandin F₂α (PGF, Lutalyse®) on either d 3 (high estradiol treatment; Hi-E), or on d 2 (low estradiol
treatment; Lo-E). All cows received 100 μg of GnRH (Cystorelin®) on day 0 (hr 0), resulting in a preovulatory period of either 3 (Hi-E) or 2 (Lo-E) days. Ovarian ultrasonography was performed on days -7, -3, 0, 2 and 6 to monitor emergence and diameter at ovulation of the preovulatory follicle, and formation of a new corpus luteum after GnRH. Blood samples collected at 12 h intervals from h -72 to h -12 and at h -6 and 0 were used to determine preovulatory concentrations of estradiol. Blood samples for progesterone analysis were collected on d 3, every other day from d -2 to 6 and d 9 to 13, daily thereafter and at slaughter on d 17.5. A second blood sample collected from days 15 to 17.5 of pregnancy was processed for analysis of ISG-15 and Mx2 gene expression in PBMC. Embryos collected from donor cows on d 7 of gestation following standard MOET procedures were implanted into experimental cows immediately after collection on d 7 of the experiment (7 days after GnRH). Cows were slaughtered on d 17.5, uteri were flushed and intact filamentous embryos collected from 4 cows in the Hi-E treatment and 8 cows in the Lo-E treatment. Antiviral activity (AVA) for IFNτ and immunoblotting analysis for CSH-1 was performed on flush media to estimate concentration of the respective protein. Gene expression was determined using RT-PCR for IFNτ, CSH-1, ISG-15, Mx2 and RPL19. Statistical analyses were conducted using mixed procedures of SAS with a model that included treatment and replication for gene expression. For analyses of progesterone and estradiol concentrations over time, the appropriate repeated measure of hour or day was included in the statistical model. Diameter of the ovulatory follicle on d 0 for the Hi-E (15.0 ± 1.0 mm) and the Lo-E (14.6 ± 1.1 mm) treatment did not differ. Concentrations of estradiol during the preovulatory period tended (P = 0.06) to be greater in the Hi-E than Lo-E treatment. A treatment x day interaction for
progesterone concentrations was detected. Concentrations of progesterone did not differ between the Hi-E and Lo-E treatments on d 0, 2, and 15, tended (P < 0.1) to be greater on d 11, 13 and 17 and were greater (P < 0.05) on days 4, 6, 9, 14 and 16 in the Hi-E than Lo-E treatment. Relative amount of mRNA did not differ between the Hi-E and Lo-E treatments for IFNτ (109 ± 23.5 and 181 ± 25.1 units, respectively) or CSH-1 (3.6 ± 1.7 and 15.7 ± 6.7 units), respectively. The Hi-E treatment tended (P = 0.07) to have greater AVA for IFNτ than in the Lo-E treatment (1,215,000 ± 561,184 vs. 405,000 ± 83,842; respectively). Expression of CSH-1 protein was not found in either the flush media or the conceptus. Expression of Mx2 mRNA in PBMC was similar between treatments but ISG-15 mRNA expression was greater (P < 0.01) in Hi-E than Lo-E. In conclusion, increasing the length of the preovulatory period resulted in increased preovulatory concentration of estradiol and concentrations of progesterone during the ensuing luteal phase. The Hi-E treatment tended to have greater AVA activity, indicative of enhanced concentrations of IFNτ in the uterine lumen, and tended to have greater expression of mRNA for ISG-15 in PBMC. Expression of IFNτ and CSH-1 mRNA by bovine embryos on d 17.5 of gestation did not differ between treatments. Therefore, concentration of estradiol during the preovulatory period influences the capacity of the embryo to produced IFNτ but other indicators of functional competence of the embryo were not altered. It is suggested that embryonic mortality, as a result of deficient preovulatory estradiol concentrations occurs after d 17.5 of gestation.
Introduction

Early embryonic mortality has been reported to be the major cause of pregnancy failure in ruminants. Large economic losses have been attributed to pregnancy failure in beef and dairy cattle (Diskin and Morris, 2008). A number of experiments have demonstrated that size of the ovulatory follicle is related to fertility in cattle (Mussard et al., 2002, 2003a, 2003b; Perry et al., 2005; Mussard et al., 2007; Perry et al., 2007; Meneghetti et al., 2009). However, collective consideration of many of these experiments (Mussard, 2010) and an additional study (Bridges et al., 2010) have suggested that the degree of gonadotrophic stimulation that follicles receive before ovulation has a strong relationship with fertility and this affect appears to be independent of follicle size.

Manipulation of length of the preovulatory period, at a constant follicle diameter, resulted in greater preovulatory estradiol concentrations and altered characteristics of the uterus on d 5 and 15.5 of gestation, but differences in embryonic function were not observed in animals presenting high compared to low preovulatory estradiol concentration (Bridges et al., 2005, 2006; Bridges, 2007).

The period of maternal recognition of pregnancy (MRP), around d 15-17 of gestation in cattle, is associated with initiation of production of IFNτ by the trophoblastic cells of the conceptus (Betteridge et al., 1978; Northey and French, 1980; Bartol et al., 1985) and also with suppression of PGF release through mechanisms involving down regulation of estrogen receptors in the endometrial epithelium, which prevents increased oxytocin receptors (Bazer et al., 1994; Flint et al., 1994; Spencer et al., 1995). A positive relationship of progesterone concentration, embryo size and concentration of IFNτ has
been described (Kerbler et al., 1997; Mann et al., 2006), however, determination of the minimal level of progesterone concentration necessary to ensure pregnancy in cattle has not been established. Interferon tau has also been reported to stimulate numerous genes of the endometrium in response to pregnancy which are critical to the fetal-maternal communication and the maintenance of gestation (Charleston and Stewart, 1993; Austin et al., 1996; Hansen et al., 1999; Spencer et al., 2004). Genes such as interferon stimulated gene 15 (ISG-15) and Myxovirus resistance gene 2 (Mx2) have been reported to be also expressed in PBMC (Han et al., 2006; Stevenson et al., 2007; Green et al., 2010). In dairy cows, ISG-15 mRNA expression was described to be evident by d 15 with maximum amounts present from d 17 to 21 and a subsequent decline until d 32 (Han et al., 2006). Stevenson et al. (2007) observed that in heifers, a greater increase in peripheral Mx2 mRNA was observed from d 0 to 18 of pregnancy when compared to nonpregnant females. Heifers that experienced pregnancy losses tended to have smaller increases in Mx2 mRNA expression than those that maintained pregnancy.

In addition to IFNτ, other products that are produced by the differentiated trophoblastic cells, such as placental lactogens, are also important to the maintenance of the CL and regulation of embryonic growth during pregnancy (Roberts and Anthony, 1994). Ruminant placental lactogen, which is also known as chorionic somatomammotropin hormone-1 (CSH-1) due to its lactogenic and somatogenic properties, is a member of the growth hormone/prolactin gene family and is produced by binucleate cells of the conceptus trophoderm and secreted into both the maternal and fetal circulation (Gootwine, 2004; Alvarez-Oxiley et al., 2008). Although CSH-1 was reported to be detectable in trophoblastic tissue of cattle beginning on d 36 of pregnancy (Yamada
et al., 2002), Kessler et al., (1991) suggested that CSH-1 can be detected as early as d 17 of pregnancy. In ewes, detection is reported to happen around d 16 of pregnancy (Gootwine, 2004). In humans, CSH-1 may play an important role during pregnancy in the regulation of maternal carbohydrate, lipid, and protein metabolism (Handwerger and Freemark, 2000). In sheep, it has been suggested to act in the regulation of fetal growth indirectly through alteration of the maternal metabolic environment, transfer of the maternal placental nutrients to the fetus, or CSH-1 may act by regulating insulin-like growth factor stimulation (Oliver et al., 1992; Schoknecht et al., 1996).

Therefore, the aim of this work is to address the mechanisms underlying embryonic loss in cattle by testing the hypothesis that a shortened preovulatory period would decrease preovulatory estradiol concentrations and lead to inappropriate gene expression and protein synthesis by the embryos in response to uterine changes, which ultimately results in the inability of the cows to maintain pregnancy.

**Material and Methods**

*Animals and treatments*

Thirty-six nonlactating crossbred (Angus x Simmental) beef cows from the OSU Beef Center (Dublin, OH) were used in this experiment which was performed in two replicates. All animals were handled in accordance with procedures approved by The Ohio State University Agricultural Animal Care and Use Committee. Cows had access, *ad libitum*, to grass pasture or hay. Calves were weaned at least fifteen days prior to the beginning of the experiment. Cows were synchronized to a common day of estrus and ultrasonographic-guided follicle aspiration was performed 6.5 ± 0.3 d after estrus (day of
aspiration = d -7 of experiment) to remove the dominant follicle of the first wave, and any other follicles with a diameter greater than 5 mm. Ovarian ultrasonography was performed on d -7, -3 and 0 to monitor emergence of a new follicular wave and presence of corpus luteum. Differences in preovulatory concentrations of estradiol were achieved by varying the time of luteal regression relative to the induction of ovulation with GnRH. Fifteen cows assigned to the treatment that was expected to result in normal preovulatory estradiol concentrations (Hi-E) received PGF on d -3 and twenty one cows receiving PGF on d -2 (Lo-E treatment) were expected to have lesser preovulatory estradiol concentrations. All cows received GnRH (100 μg) on d 0, resulting in either 3 or 2 d of proestrus preceding the GnRH-induced LH surge in the Hi-E and Lo-E treatments, respectively. Blood samples collected at 12 h intervals from h -72 to h -12 and at h -6 and 0 were used to determine the preovulatory concentrations of estradiol. Ovaries were examined by ultrasonography on d 4 of the ensuing estrous cycle to confirm ovulation and development of CL. Seven days following GnRH 8 cows from the Hi-E and 11 cows from the Lo-E treatment were implanted with fresh embryos. The rest of the cows that did not receive embryos (17 cows) were used in a different study as cyclic control animals and are not included on this experiment. Twenty seven embryos were collected from 3 multiparous crossbred (Angus x Simmental) donor cows using standard multiple ovulation, embryo transfer approaches. Estrous cycles of donors were synchronized and ultrasonographic-guided follicle ablation performed on d -6 of the experiment. Animals were superovulated with FSH (folltropin-V®, Bionich Animal Health, Belleville, Ontario, Canada) treated twice a day for four days starting on d -5 with 2.4 mg and decreasing 0.5 mg/day until d -1.5. A CIDR was inserted on d - 4.5 and removed on d -1.0, and PGF was
administered on d -2 and -1.5. Artificial insemination based on estrus was performed twice on h 12 and 24 (~ d 0 of experiment) after onset of estrus to provide embryos for transfer to experimental animals on d 7. Embryo quality was graded on a standard 1 to 5 scale (1 = excellent, 2 = good, 3 = fair, 4 = poor, and 5 = degenerate; Ahmad et al., 1995). Only embryos graded as 1 and 2 were implanted. Based on previous experience and report from Bridges et al. (2010), where a greater occurrence of shortened luteal phases occurred in heifers experiencing a short (1.25 d) vs. long (2.25 d) proestrus (50% vs. 12.5%, respectively), more cows were assigned to the Lo-E treatment. Two cows in the Lo-E treatment experienced a shortened luteal phase and were removed from the experiment. One cow in the Hi-E treatment presented double ovulation and was also removed from the experiment. On day 17.5 after GnRH the remaining 16 cows, 7 in the Hi-E and 9 in the Lo-E treatments, were slaughtered and the reproductive tract was removed. Thirteen embryos were recovered, 5 for the Hi-E and 8 for the Lo-E treatments. Embryos and tissue samples were collected as described below.

Blood samples for progesterone analysis were collected on d -3, every other day from d -2 to 6 and d 9 to 13, daily thereafter and at slaughter on d 17.5. Blood samples were stored on ice immediately after collection and then centrifuged at 1500×g for 20 min within 3 h after collection. Plasma was decanted and stored at -20°C until further analysis. Blood samples to measure ISG-15 and Mx2 mRNA in PBMC were collected on days 15, 16, 17 and 17.5 of pregnancy and immediately mixed into TRI-Reagent BD (Sigma-Aldrich®, St. Louis, MO, USA) and Acetic Acid 5M as described by the manufacturer’s procedures. Samples were immediately stored at -80°C for further analysis of mRNA expression.
Sample Collection

On d 17.5 of the experiment, cows were slaughtered in the OSU Meat Laboratory in the Department of Animal Sciences at The Ohio State University. Within 12.2 ± 3.5 minutes of when cows were stunned, the reproductive tracts were removed, placed on ice, and taken to the laboratory for processing. The ovaries and excess fat were removed from the reproductive tract and the uterine horns were separated. The uterine tract was placed into a clamp support and a small incision was made at the tip of the uterine horn ipsilateral to the CL and 40 mL of PBS was injected into the uterotuberal junction contralateral to the CL. The uterus was gently massaged, pushing the flush media through both uterine horns. The uterine luminal flush (ULF) media and conceptus were recovered through the incision. If a conceptus was not recovered, a second flush with 40 mL PBS was performed. The amount of ULF media that was recovered was quantified and snap frozen in liquid nitrogen for further analysis. If an embryo was present, it was photographed and immediately snap frozen in liquid nitrogen and stored at -80°C. The average time from stunning to snap freezing the last sample was 25.5 ± 3.7 minutes. Relative embryo mass was estimated from the pictures taken at collection using a scale of 1 to 5 (1 = least conceptus tissue to 5 = greatest conceptus tissue) by 10 individuals within our laboratory.

Hormone Quantification

Plasma concentrations of estradiol were determined by using a double-antibody, I\textsuperscript{125}-based assay that was previously described by Burke et al., (2003) but modified
recently in our lab. Standards were prepared by solubilizing purified 17β-estradiol (Sigma-Aldrich®) into phosphate buffered saline (PBS) containing 0.1% gelatin (Sigma®; 0.1% PBS-gel; pH = 7.4) then dilution according to the concentration of standards desired was performed. For standard curve concentrations, 200 μl of standard and 400 μl of charcoal stripped steer plasma were used per tube. The tubes receiving the pools and the samples to be analyzed received 400μl of the unknown sample or pools, and 200μl of 0.1% PBS gel. All tubes were extracted by adding 4ml of diethyl ether anhydrous (Sigma), re-suspended into 200 μl of 0.1% PBS gel and incubated overnight at 4°C. Estradiol labeled with I\textsuperscript{125} (E2D2), estradiol antiserum (E2D1) and precipitating solution (N6) were acquired from the commercially available RIA kit (Coat-a-Count® Double Antibody Estradiol, Siemens, Los Angeles, CA) and used in volumes of 100 μl, 60μl and 1ml, respectively, for each 400 μl of sample extracted. The estradiol radioimmunoassay was validated comparing the curves of samples containing a predetermined known to that of samples of unknown concentration using similar dilutions in 0.1% PBS gel. Equation of the curves and correlation (R\textsuperscript{2}) were (y = -1.0002x + 3.0772; 0.981) and (y = -1.079x + 3.2663; 0.987) respectively for known and unknown samples. Average intra-assay CV was 3.2%, and inter-assay CV’s (8 assays) for pooled plasma samples containing 2.42, 5.75, and 12.9 pg/mL of estradiol were 4.1%, 4.9% and 5.2%, respectively. The average sensitivity of the assays was 0.98 pg/mL. Analysis for estradiol using the peak and the area under the curve (AUC) were performed. The peak of estradiol concentration was calculated as the maximum concentration of estradiol observed during the preovulatory period. The AUC for estradiol concentration was calculated by using the trapezoidal method, where the trapezoidal area in between two intervals was calculated for each
interval of time from d -3 to GnRH administration. Plasma concentrations of progesterone were determined in two assays using a commercially available RIA kit (Coat-a-Count® Progesterone, Siemens) as described previously for our laboratory (Burke et al., 2003). Average intra-assay CV was 2.19%, inter-assay CV’s for pooled plasma samples containing 0.12, 1.06, and 3.09 ng/mL of progesterone were 3.8%, 4.2% and 4.4%, respectively, and the average sensitivity of the assays was 0.075 ng/mL.

*Antiviral Assay for IFNτ*

Amount of IFNτ in the collected ULF media was analyzed in the laboratory of Dr. Troy Ott at The Pennsylvania State University using an antiviral assay with Madin Darby bovine kidney cells (MDBK) as described in Ott et al., (1991). In brief, ULF media samples diluted 1:30 were serial diluted into a 96 well plate and incubated at 37°C with MDBK cells for 26 h. After the period of incubation the inhibition of viral replication was determined by using a cytopathic effect assay (antiviral assay). Cells were challenged with vesicular stomatitis virus in a dilution of 1:100 to achieve the cytopathogenic effect. Cells were stained with crystal violet stain solution #7 for 10 minutes and then rinsed in running water. Antiviral activity of the sample (in units/mL) was determined as the reciprocal of the dilution that prevented 50% destruction of the MDBK cells (Figure 3.1).

*Total RNA Extraction and RT-PCR*

In the embryonic tissue, gene expression for IFNτ and CSH-1 were analyzed using RT-qPCR expressing the relative amount of mRNA normalized by using the
housekeeping gene RPL-19 (Ndiaye et al., 2008). Gene expression for ISG-15 and Mx2 expressed in PBMC from days 15 to 17.5 were analyzed by semi-quantitative RT-PCR where samples were normalized by the housekeeping gene RPL-19 by using the $\Delta$Ct (target Ct – RPL-19 Ct) values (Livak et al., 2001; Schmittgen et al., 2008). Data from ISG-15 and Mx2 were analyzed and graphed using $2^{-\Delta \text{Ct}}$.

**Embryonic Samples**

Steady-state concentrations of mRNA for IFN$\tau$, CSH-1 and RPL-19 were determined in total RNA extracted from embryonic tissue using a 7500 Fast Real-Time PCR System (Applied Biosystem®, Foster City, CA) as described below. Oligonucleotide primers specific for the IFN$\tau$, CSH-1 and ribosomal protein L 19 (RPL-19) genes were designed to amplify the respective cDNA fragment (Table 3.1). Total mRNA was isolated from the whole embryonic tissue by using TRIZol reagent (Sigma- Aldrich®) following manufacturer’s procedures except the volume used was 5 or 7 ml of TRIZol, depending on the size of the embryo. Homogenization was performed manually by shaking the tubes vigorously for approximately 30 seconds after addition of TRIZol reagent. The clear phase containing total RNA was further processed following manufacture’s procedure and the final RNA precipitate was resuspended in RNAse free water. The pink phase containing protein and DNA was stored at -80°C for isolation of protein for CSH-1 western-blot analyses. The concentration of total RNA was determined by measurement of optical density at 260 nm in the UV/Vis spectrophotometer (NanoDrop® 2000, Thermo Fisher Scientific®, Waltham, MA). Two μg of total RNA was diluted in RNAse-free water (16 μl final volume) and treated with 2 μl of buffer solution and 2 μl of RQ1 RNAse-free DNase I (Promega®, Fitchburg, WI) in order to eliminate genomic
DNA contamination at 37°C for 30 min. Two μl of RQ1 DNAse Stop Solution (Promega®) was added to terminate the reaction and samples were incubated at 65 for 10 minutes at 95°C. Reverse transcriptase reaction were performed by using the Dynamo cDNA Synthesis Kit (New England Biolabs®, Ipswich, MA). Ten μl of the previous DNAse treated solution was mixed with 2 μl of reverse transcriptase enzyme, 1 μl of random hexamer and 10 μl of 5X buffer. Reverse transcription was performed using a GeneAmp PCR System 9700 under the following conditions: 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes and 4°C for 90 minutes. The reverse transcribed cDNA was then diluted 1:5 in RNAse free water. Quantitative PCR was performed using 5 μl of the diluted cDNA sample, 10 μl of SensiMix Plus Sybr Kit (Quantace®, Watford, UK), 3 μl of RNAse free water, 1 μl of forward primer (10 uM) and 1 μl of reverse primer (10 uM) for a total of 40 cycles under the following conditions: denaturing at 95°C for 30 sec; annealing at 60°C for 45 sec; and extension at 72°C for 45 sec, followed by an extra elongation of 5 min at 72°C. To verify the amplification of a single product, a melting curve analysis was performed after the end of the last PCR cycle and the PCR product was analyzed by gel electrophoresis. Standard curves prepared from purified IFNτ and CSH-1 cDNA PCR product were used to calculate the steady-state concentration of target gene mRNA in triplicate wells for each sample. For each message analyzed, RPL-19 was measured in the same q-PCR assay. The efficiency of amplification of standard curves, generated from cDNA of each gene of interest were compared to that of RPL-19 and found to be equivalent.
PBMC Samples

Total RNA was isolated from 200μl of whole blood by using TRI-Reagent BD (Sigma®) as described by the manufacturer’s procedures in order to analyze expression of ISG-15 and Mx2 mRNA. Relative expression of ISG-15 and Mx2 mRNA was determined in total RNA extracted from embryonic tissue by using a 7500 Fast Real-Time PCR System. Oligonucleotide primers specific for Mx2, ISG-15 and RPL-19 transcript were designed to amplify the respective cDNA fragment (Table 3.1). These primers were previously designed and reported by Gifford et al., (2007). The concentration of total RNA was determined by measurement of optical density at 260 nm in the UV/Vis spectrophotometer. Total RNA (0.3 μg) adjusted with DNAse free water was reverse transcribed by using the Dynamo cDNA Synthesis Kit. The reaction was performed by mixing 10 μl of the total RNA with 2 μl of reverse transcriptase enzyme, 1 μl of random hexamer and 10 μl of 5X buffer. Reverse transcription was performed in the GeneAmp PCR System 9700 machine under the following conditions: 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes and 4°C for 90 minutes. The reverse transcribed cDNA was then diluted 1:5 in RNAse free water. Real-time PCR was then performed using 5 μl of the diluted cDNA sample, 10 μl of SensiMix Plus Sybr Kit, 3 μl of RNAse free water, 1 μl of forward primer (10 uM) and 1 μl of reverse primer (10 uM) for a total of 35 cycles under the following conditions: denaturing at 95°C for 30 sec; annealing at 60°C (Mx2) or 58°C (ISG-15) for 45 sec; and extension at 72°C for 45 sec, followed by an extra elongation of 5 min at 72°C. To verify the amplification of a single product, a melting curve analysis was performed after the end of the last PCR cycle and the PCR product was analyzed by gel electrophoresis. A standard curve to determine the
efficiency of both the normalizer and the target gene was performed by using the purified Mx2 and RPL-19 cDNA PCR products. The efficiency of amplification of standard curves, generated from cDNA of each gene of interest, were compared to that of RPL-19 and found to be equivalent.

Protein Concentration and Western Blot Analyses

Western Blot analyses for detection of CSH-1 protein expression in ULF media and in the embryonic tissues were performed as described previously by Serr et al., (2009). In order to optimize detection of CSH-1 in the ULF, samples were concentrated by a precipitation method using trichloroacetic acid 100% (TCA). A volume of TCA (0.5 ml) was used for 4 volumes of ULF. Samples were incubated for 10 minutes at 4°C and then centrifuged at 14000 rpm for 5 minutes. Supernatant was removed and then the pellet was washed twice with cold acetone, dried for 5 min at 95°C and then resuspended in 2X Laemmli buffer (62.5 mM Tris, 1% SDS, 5% 2-mercaptoethanol, 12.5% glycerol, 0.05% bromophenol blue; Bio-Rad Laboratories®, Hercules, CA). Samples from embryonic tissue were extracted using TRIzol® and processed following the manufacturer’s procedure.

Proteins were separated by 12% SDS-PAGE and then wet-transferred to a polyvinylidene fluoride membrane (GE Biosciences®, Piscataway, NJ). The membranes were blocked in 5% nonfat dry milk in tris-buffered saline-Tween (TBST; 20 mM Tris, 150 mM NaCl, pH 7.4, plus 0.1% Tween-20) for at least 30 min at room temperature. The membranes were then incubated with the primary bovine CSH-1 antibody (1:3,000 dilution; product generously donated by Dr. Charles Wallace, The University of Maine,
previously validated for radioimmunoassay as described by Wallace, 1989) at temperature of 4ºC overnight. Specificity of the CSH-1 antibody was tested with recombinant CSH-1 purified protein (lot number 920514-10; Monsanto Co., St. Louis, MO, donated from Dr. Charles Wallace) and with bovine placentomal tissue collected on approximately d 60 of pregnancy. Protein from placentomal tissue was isolated using the procedure described previously for extraction from embryonic tissue. The membranes were washed in TBST 7 to 10 times and then were incubated in horseradish peroxidase-conjugated secondary antibodies (1:3,000 dilutions; Santa Cruz Biotechnology Inc.) for 1 h at room temperature. The membrane was again washed in TBST 7 to 10 times. The proteins were detected using ECL Plus (GE Biosciences®) and were then exposed to BioMax x-ray film (GE Biosciences®).

Data and Statistical Analysis

Statistical analyses were conducted by using the mixed procedures of SAS (2004) with a model that included the dependent variables ISG-15, Mx2, IFNτ antiviral activity, diameter of ovulatory follicle, and the independent variables treatment and replication. For analyses of progesterone and estradiol concentrations over time, the appropriate repeated measure of hour or day was included in the statistical model. Schwarz’s Bayesian Information Criteria (BIC; ‘smaller is better’) was used to determine the best variance-covariance structure for the models. The statistical model used was:

$$Y_{ijk} = \tau_i + \delta_{j:i} + \gamma_r + D_k + (\tau D)_{ik} + \epsilon_{ijk}$$

Where:

$Y_{ijk}$ = Observation of the $j$th cow in the $i$th treatment at the $k$th time point
\( \tau_i = \) Effect of the \( i \)th treatment

\( \delta_{ji} = \) Random effect of the \( j \)th cow within the \( i \)th treatment

\( \gamma_r = \) Effect of the \( r \)th replication.

\( D_k = \) Effect of the \( k \)th time point

\( (\tau D)_{ik} = \) The interaction effect of the \( i \)th treatment x the \( k \)th time point

\( \varepsilon_{ijk} = \) Residual term

Gene expression of IFN\( \tau \) and CSH-1 were analyzed by using RPL-19 as covariate. The statistical model for the analysis of covariate was:

\[
Y_{ij} = \tau_i + \gamma_r + \beta(X_{ij} - \bar{x}) + \varepsilon_{ij}
\]

Where:

\( Y_{ij} = \) Observation of the \( j \)th cow in the \( i \)th treatment

\( \tau_i = \) Effect of the \( i \)th treatment

\( \gamma_r = \) Effect of the \( r \)th replication

\( \beta(X_{ij} - \bar{x}) = \) variable regression coefficient

\( \varepsilon_{ij} = \) Residual term

In addition, Pearson’s correlation was performed by using the PROC CORR procedure in SAS between the ratio of gene expression (target gene and housekeeping gene), concentration of estradiol at 48 hours, peak and AUC, progesterone concentration on days 4, 6 and 9, and antiviral activity for IFN\( \tau \). Analysis for determination of outliers samples were performed based on the two standards deviation rule, where samples with values greater or smaller than two standard deviations were considered outliers and removed from the experiment.
Results

The proportion of implanted cows that had a viable embryo on d 17.5 in the Hi-E and Lo-E treatment were 71.4% (5/7) and 88.8% (8/9), respectively, and did not differ (P > 0.1). Only pregnant cows were included in the subsequent analyses. One cow from Hi-E treatment was considered an outlier and excluded from all analyses of expression of embryonic mRNA for RPL-19, CSH-1, IFNτ and progesterone concentrations.

Diameter of the ovulatory follicle on day 0 for the Hi-E (15.0 ± 1.0 mm) and the Lo-E (14.6 ± 1.1 mm) treatment did not differ (P > 0.1). During the preovulatory period, circulating concentrations of estradiol tended to be greater (P < 0.06), in the Hi-E treatment than the Lo-E treatment (Figure 3.2). Estradiol concentration also tended to be greater in the Hi-E than Lo-E treatment for both peak (12.5 ± 3.0 and 8.2 ± 0.8 pg/mL, respectively; P = 0.09) and AUC (424.02 ± 70.72 and 278.31 ± 29.33 units respectively; P = 0.06).Progesterone concentrations during the subsequent luteal phase were greater in the Hi-E than Lo-E treatment (treatment x d, P < 0.01). Concentrations of progesterone were greater in the Hi-E than Lo-E treatment on d 4, 6, 9, 14 and 16 (P < 0.05) and tended to be greater on d 11, 13 and 17 (P < 0.1; Figure 3.3). A positive correlation (P < 0.05) was observed between estradiol concentrations (at h -48 prior to GnRH and AUC) and progesterone concentrations on d 4, 6 and 9 after ovulation.

Relative amount of mRNA in the embryonic tissue did not differ between the Hi-E and Lo-E treatment for IFNτ (109 ± 23.5 and 181 ± 25.1 units, respectively) or CSH-1 (3.6 ± 1.7 and 15.7 ± 6.7 units, respectively; Table 3.2). Animals from the Hi-E treatment tended (P = 0.07) to have greater AVA units for IFNτ than the Lo-E treatment (1,215,000
± 83,842 vs. 405,000 ± 83,842; respectively), but no correlation was found between concentration of IFNτ in the ULF and mRNA expression for IFNτ in the conceptus. Although no correlation among embryo mass score and antiviral activity for IFNτ was observed, the two embryos with greatest AVA for IFNτ had also the greatest mass score of 4.6 and were from the Hi-E treatment (Table 3.3). Expression of mRNA for ISG-15 in PBMC from d 15 to 17.5 was greater (P = 0.01) in the Hi-E than Lo-E treatment (Figure 3.4) but no differences were observed in expression of Mx2 between treatments (Figures 3.5). No correlations were observed among estradiol or progesterone concentrations and mRNA expression for IFNτ, CSH-1, Mx2 and ISG-15, and AVA for IFNτ in the flush media.

In Western blots analysis, CSH-1 protein was not detectable in either concentrated ULF media or the conceptus. Bands representing the positive control samples were observed around 24 kDa for recombinant purified protein, and around 31-33 kDa for the glycosylated protein isolated from placentome tissue from a 60 day pregnant cow, but no band for concentrated ULF was detected in the same western blot (Figure 3.6). In a second western-blot, multiple bands were observed in the positive control lane, with a predominant band represented by the molecular weight around 33 kDa (Figure 3.7a). An additional non-specific band with size approximately of 28 kDa was observed in all samples isolated from the embryonic tissues either from Lo-E as from Hi-E treatments. The housekeeping protein β-Actin was expressed in all samples presenting similar molecular weight of 42 kDa among samples, indicating integrity of the proteins (Figure 3.7b).
Discussion

As expected, the diameter of the ovulatory follicle was similar between treatments and preovulatory estradiol concentrations tended to be increased in the Hi-E relative to the Lo-E treatment. Peripheral progesterone concentrations in the subsequent estrous cycle were greater in the Hi-E cows and a positive correlation with preovulatory estradiol concentrations was observed. No differences in embryonic gene expression for IFNτ and CSH-1 were observed but a tendency for higher concentration of IFNτ in the ULF was detected in the Hi-E treatment. Additionally, cows in the Hi-E treatment had a greater gene expression for ISG-15 in the PBMC as compared to the Lo-E animals, however no statistical difference in gene expression was observed for Mx2.

As the diameter of the ovulatory follicle was similar between treatments, but concentrations of progesterone differed, an association of progesterone concentration and size of the ovulatory follicle was not detected. However, one more day of “proestrus” promoted greater progesterone concentrations in the subsequent luteal phase in the present study. Differences in progesterone concentrations between treatments have been variable in previous studies using a similar approach (Bridges 2007). We have previously reported greater progesterone concentrations in cows with a longer proestrus (Mussard, 2010) but an important distinction of these experiments from the present study is that the follicle diameter was also greater in the cows with a longer proestrus. Therefore, effects of follicle size and length of proestrus are confounded in those experiments. In ewes that were induced to ovulate following a short proestrus and had reduced preovulatory estradiol concentrations prior to ovulation of follicles with similar diameter compared to
long proestrus (Murdock and Van Kirk, 1998), the number of granulosa cells within the follicle was reduced and the resultant CL were smaller, contained fewer large luteal cells, and produced lesser amounts of progesterone both in vivo and in vitro. One plausible explanation for the differences observed in the present study is that greater granulosa cell proliferation in the dominant follicles due to a longer proestrus resulted in a CL with a greater capacity to produce progesterone. Low progesterone has been associated with early pregnancy failure (Mann et al., 1999) and poor embryo development (Walton et al., 1990) while supplementing cows with progesterone has been shown to enhance conceptus development (Garrett et al., 1988). The association of higher progesterone concentrations in the beginning of the luteal phase in pregnant cows was related to higher production of INFτ and greater embryo size (Mann et al., 2006). In the present study, cows with greater progesterone concentrations had embryos that tended to produce more INFτ, but other measures of embryo function that were monitored were not influenced.

With the model used in the present study, Bridges (2007) observed that characteristics of the uterus differed between the Hi-E and Lo-E treatments on d 5 and d 15.5 of gestation but capacity of the conceptus to secrete INFτ into the uterine lumen did not differ between treatments in the early stages of MRP on d 15.5 of gestation. In the present study, a similar animal model was used to induce differing preovulatory estradiol concentrations and cows were slaughtered in the latter stages of MRP on d 17.5 of pregnancy. Unlike in the report by Bridges (2007), cows with greater preovulatory estradiol concentrations in the present study had greater luteal phase concentrations of progesterone, and greater INFτ activity was detected in the uterine lumen of the Hi-E than the Lo-E treatment. The two cows with the greatest INF in the uterine flush media
were in the Hi-E treatment and these cows also had the greatest estimated conceptus mass (Table 3 and Figure 8). Embryonic expression of mRNA for IFNτ did not differ between treatments. The other protein that was assessed as a measure of putative differences in embryonic development was CSH-1. Expression of mRNA was detected in the conceptus, but did not differ between treatments; however, the protein was not detected in either concentrated uterine flush media or in the conceptus. The precise time when embryos start to express CSH-1 mRNA and protein is unknown. Yamada et al. (2002) reported that expression of CSH-1 mRNA and protein was not detected in bovine trophoblastic cells from d 17 to 25 but was detectable after d 26 of gestation supporting similar findings from other authors (Gootwine, 2004; Alvarez-Oxiley et al., 2008). Differently, Kessler et al., (1991) reported that gene expression of CSH-1 was detected by d 17 and protein by d 18 of pregnancy in bovine conceptus. Similarly, expression of CSH-1 mRNA was present on d 17.5 in the present study, however, detection of CSH-1 protein in the uterine flush media may have been limited by the high dilution rate, and concentration of the protein did not aid in detection. Furthermore, we were unable to detect the protein extracted from the conceptus itself.

The IFNτ produced by the conceptus induces the expression of a variety of proteins and their mRNA (ISGs) in the uterus that are believed to be important for uterine function, receptivity to implantation, and conceptus development (Bauersachs et al., 2006; Klein et al., 2006). In PBMC, genes that have been studied extensively in ruminants are ISG-15 (Austin et al., 1996; Johnson et al., 1999; Hansen et al., 1999; Austin et al., 2004) and Mx2 (Charleston and Stewart, 1993; Ott et al., 1998; Hicks et al., 2003). In the present experiment, the cows with greater preovulatory estradiol and IFNτ
had greater expression of mRNA for ISG-15 in the PBMC (Figure 3). Expression of mRNA for Mx2 did not differ between treatments; however, numerically the Hi-E and Lo-E treatments had separated by d 17.5 of gestation (Figure 4). The greater expression of ISG-15 mRNA in Hi-E cows may be the result of the greater amount of IFNτ detected in the uterine flush media, however, correlations of IFNτ activity and expression of mRNA of ISG-15 or Mx2 in PBMC were not detected. Furthermore, we have observed (Souto and Day, unpublished) a similar trend for ISG-15 in cyclic cows in the Hi-E and Lo-E treatments on d 17.5 of the estrous cycle, suggesting that differences in preovulatory estradiol and/or luteal progesterone may contribute to differences in expression of ISG-15 mRNA in PBMC. It is believed that the peripheral stimulation of ISGs would reflect the stimulation of ISGs that should be occurring in the uterus in response to IFNτ. The action of steroids such as estradiol and progesterone is better reported in the uterus than their action in different tissues. However, research has demonstrated that a similar gene response can be expressed by different tissues, as showed by Gifford et al., (2008) when administration of IFNτ stimulated receptor transporter protein 4 mRNA in the endometrium, CL and PBMC in ewes. Ott et al., (1999) observed that uterine infusion of IFNτ in presence of different steroids can result in differential stimulation of Mx1 mRNA expression in the uterus. The combination of IFNτ with progesterone has shown to have greater Mx1 gene expression than when combined with estradiol or in the absence of both progesterone and estradiol. Perhaps, the greater concentrations of progesterone in the Hi-E treatment in the present experiment contributed to the increased expression of mRNA for interferon stimulated genes in the PBMC in the present study. The mechanism for actions of either greater preovulatory
estradiol or luteal phase progesterone concentrations to enhance expression of mRNA for ISG-15 and Mx2 in PBMC is unknown and requires further investigation.

In summary, the conceptuses of cows with deficient preovulatory estradiol concentrations tended to produce less IFNτ and these cows had lesser concentrations of mRNA for ISG-15 in their PBMC from day 15 to 17.5 of gestation. The amount of mRNA detected for IFNτ or CSH-1 on day 17.5 of pregnancy, and expression of mRNA for Mx2 in PBMC was not influenced by treatment. Significant correlations of IFNτ activity with gene expression for IFNτ or CSH-1 in embryos, ISG-15 or Mx2 in PBMC or peripheral progesterone concentration were not detected. However, peripheral progesterone concentrations in early gestation were greater in the Hi-E treatment and positively correlated with estradiol concentrations. It is concluded that while the conceptuses of cows with deficient preovulatory estradiol concentrations tend to produce less IFNτ and appear to have less embryonic mass, their function are not severely compromised on d 17.5 of gestation. The influence of the Hi-E and Lo-E treatment on functional characteristics of the uterus is the focus of an ongoing experiment. We suggest, based upon the findings of the present and previous research in our laboratory, that embryonic mortality in cows with deficient preovulatory estradiol concentrations occurs at a point after d 17.5 but before d 30 of gestation; probably due to defects in capacity of the uterus to sustain the embryo during the pre-implantation period.
<table>
<thead>
<tr>
<th>Message</th>
<th>Primer Sequence</th>
<th>Anneal (°C)</th>
<th>Cycles (#)</th>
</tr>
</thead>
</table>
| RPL-19  | Fwd- tccatgagatgtccagcagt  
         | Rev- tgttggagccagtgcaagcaga | 62.0 | 35 |
| IFNτ    | Fwd- atcgatgcacatgtatca  
         | Rev- gcgtgtcctctttggtcttag | 62.0 | 35 |
| CSH-1   | Fwd- tgttgcagggctcttctctctgtcctgctag  
         | Rev- acattcccttttggtcagcctatag | 62.0 | 35 |
| Mx2     | Fwd- ctgcagagacgcctcagtcg  
         | Rev- tgaagcagccaggaatagtg | 62.0 | 30 |
| ISG-15  | Fwd- ggtatccgagctgaagcagtt  
         | Rev- acctccctgtctcacttcaggt | 58.0 | 30 |

Table 3.1. Primer sequences, annealing temperature, and number of PCR cycles for the genes RPL-19, IFNτ, CSH-1, Mx2 and ISG-15.
### Treatment<sup>a</sup>

<table>
<thead>
<tr>
<th>Message&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Hi-E</th>
<th>Lo-E</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>RPL-19</em></td>
<td>92.12 ± 24.10</td>
<td>125.47 ± 8.20</td>
<td>0.13</td>
</tr>
<tr>
<td><em>IFN-τ</em></td>
<td>109.12 ± 23.54</td>
<td>181.66 ± 25.13</td>
<td>0.31</td>
</tr>
<tr>
<td><em>CSH-1</em></td>
<td>3.64 ± 1.72</td>
<td>15.71 ± 6.70</td>
<td>0.67</td>
</tr>
</tbody>
</table>

<sup>a</sup>Cows were induced with GnRH to ovulate dominant follicles of similar age and diameter either two, or three days after induction of luteal regression with PGF2α, which resulted in cows with either normal (Hi-E) or deficient (Lo-E) concentrations of estradiol during the preovulatory period, respectively.

<sup>b</sup>mRNA concentrations are indicated as relative values ± SEM

Table 3.2. Steady-state mRNA amounts (relative values) for *RPL-19*, interferon tau (IFNτ) and chorionic somatomammotropin hormone - 1 (CSH-1) in embryos collected on day 17.5 of pregnancy from cows with either normal (Hi-E) or deficient (Lo-E) preovulatory concentrations of estradiol.
<table>
<thead>
<tr>
<th>Cow</th>
<th>Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Antiviral Activity (U/mL)</th>
<th>Embryo&lt;sup&gt;b&lt;/sup&gt; Score (1-5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>203</td>
<td>Hi-E</td>
<td>243,00</td>
<td>2.8 ± 0.2</td>
</tr>
<tr>
<td>222</td>
<td>Hi-E</td>
<td>243,00</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>271</td>
<td>Hi-E</td>
<td>2,187,000</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>5429</td>
<td>Hi-E</td>
<td>2,187,000</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td><strong>1,250,000 ± 83,842&lt;sup&gt;c&lt;/sup&gt;</strong></td>
<td><strong>3.65 ± 0.6&lt;sup&gt;d&lt;/sup&gt;</strong></td>
</tr>
<tr>
<td>55</td>
<td>Lo-E</td>
<td>486,000</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>212</td>
<td>Lo-E</td>
<td>729,000</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td>308</td>
<td>Lo-E</td>
<td>486,000</td>
<td>4.4 ± 0.2</td>
</tr>
<tr>
<td>334</td>
<td>Lo-E</td>
<td>729,000</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>2606</td>
<td>Lo-E</td>
<td>243,000</td>
<td>2.0 ± 0.3</td>
</tr>
<tr>
<td>4118</td>
<td>Lo-E</td>
<td>162,000</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>5339</td>
<td>Lo-E</td>
<td>162,000</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>6512</td>
<td>Lo-E</td>
<td>162,000</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td></td>
<td><strong>405,00 ± 83,842&lt;sup&gt;c&lt;/sup&gt;</strong></td>
<td><strong>3.0 ± 0.5&lt;sup&gt;d&lt;/sup&gt;</strong></td>
</tr>
</tbody>
</table>

<sup>a</sup>Cows were induced with GnRH to ovulate dominant follicles of similar age and diameter either two, or three days after induction of luteal regression with PGF2α which resulted in cows with either normal (Hi-E) or deficient (Lo-E) concentrations of estradiol during the preovulatory period, respectively.

<sup>b</sup>Embryo mass score per group using a scale of 1 to 5 (1 = least conceptus tissue to 5 = greatest conceptus tissue) expressed as average ± SEM.

<sup>c</sup>Concentrations are indicated as the group average ± SEM. Cows in the Hi-E treatment tended (P = 0.07) to have higher antiviral activity for INFτ compared to the Lo-E treatment.

<sup>d</sup>Embryonic mass score did not differ between treatments.

Table 3.3 Antiviral activity for IFNτ expressed (U/mL) and embryo tissue score by individual animals in the Hi-E and Lo-E treatments.
Figure 3.1. Representative plate for quantification of antiviral activity for IFNτ, measured in duplicates and determined as the reciprocal of the dilution that prevented 50% destruction of the MDBK cells. Wells in black represent intact cells with no virus activity. Column 1 represents a positive control lane (no virus), while the following columns representing increasing dilutions of the uterine flush media for each cow. Pregnant cows are represented by letters B and D (in duplicates) and cow B has higher antiviral activity; consequently higher IFNτ concentration. Cows A and C are nonpregnant cows with complete destruction of the MDBK cells at all dilutions of uterine flush media.
Figure 3.2. Circulating concentrations of estradiol during the preovulatory period in cows manipulated to have either normal (Hi-E; ♦) or deficient (Lo-E; ■) preovulatory estradiol concentrations. Cows were administered PGF on either h -72 (Hi-E) or h -48 (Lo-E), all cows received GnRH at h 0 to induce ovulation [Treatment, $P < 0.05$].
Figure 3.3. Circulating concentrations of progesterone following GnRH-induced ovulation in the Hi-E (♦) and Lo-E (■) treatments. (treatment x d of experiment, \( P < 0.05 \); * denotes days that progesterone concentrations differed \( (P < 0.05) \) and **denotes that progesterone concentrations tended to differ, \( P < 0.1 \), on that day).
Figure 3.4. ISG-15 mRNA expression in PBMC $2^{-\Delta\text{CT}} \times 100$ in the Hi-E (♦) and Lo-E (■) treatments on d15, 16, 17 and 17.5 of pregnancy (Treatments differed, $P < 0.05$).
Figure 3.5. Mx2 mRNA expression in PBMC expressed as $2^{-\Delta\text{CT}}$ (x100) following GnRH-induced ovulation in Hi-E (♦) and Lo-E (■) cows on d 15, 16, 17 and 17.5 of pregnancy [Mx2 mRNA did not differ between Hi-E and Lo-E treatments].
Figure 3.6: Western blot analysis determining the absence of the chorionic somatomammotropin hormone - 1 (CSH-1) in concentrated uterine flush media of a pregnant cow on lane 4 (←). The positive controls are represented by recombinant CSH-1 on lane 1, with molecular weight of approximately 23 kDa (→). Lanes 2 and 3 represent extracted CSH-1 from a 60 d pregnant cow placentome with molecular weight of approximately of 32-33 kDa diluted 1:10 and 1:100 respectively.
Figure 3.7: A) Western blot containing on lane 1 a sample extracted from a 60 d pregnant cow placentome used as a positive control followed by samples extracted from Lo-E (Lanes 2 to 5) and Hi-E (Lanes 6 to 9) cows. Multiple bands can be observed on lane 1, however the more stained band represents to the molecular weight around 33 kDa for the CSH-1 (*→*). The samples of cows from both treatments did not show band matching to similar size of the positive control but presented a few additional bands around 28kDa size. B) The second figure represents a western blot containing the same samples used on figure 7a but using β-Actin as housekeeping protein. Bands with common molecular weight of approximately 42 kDa were observed in all lanes indicating right molecular weight for β-Actin and integrity of the protein samples.
Figure 3.8: Picture was taken from embryos collected on day 17.5 of pregnancy using the same scale of magnification. The embryo on the left (Hi-E treatment) was scored as 5 (1-5 scale where 1 is lesser tissue mass and 5 is the greatest tissue mass) and embryo in the right (Lo-E treatment) was scored as 1. Embryos had an AVA for IFNτ of 2,187,000 and 729,000 U/mL for the Hi-E and Lo-E embryos, respectively.
CHAPTER 4

THE EFFECT OF THE PREOVULATORY PERIOD ON ESTRADIOL CONCENTRATIONS AND PERSIPHERAL EXPRESSION OF ISG-15 AND MX2 mRNA, AND PROGESTERONE CONCENTRATION IN LACTATING BEEF COWS

Abstract

The objective of the present experiment was to determine the influence of manipulation of preovulatory estradiol concentrations through alteration of the duration of the preovulatory period on amounts of mRNA for interferon stimulated gene - 15 (ISG-15) and Myxovirus resistance - 2 (Mx2), and profile of progesterone secretion from d 15 to 30 of gestation in cows. Thirty six postpartum beef cows were presynchronized to a common day of estrus and ultrasonographic-guided follicle aspiration was performed 5.5 ± 0.1 days after estrus (day of aspiration = d -7 of experiment). Cows were assigned to receive 25 mg of prostaglandin F$_2$α (Lutalyse®) on either d -3 (high estradiol treatment; Hi-E), or on d -2 (low estradiol treatment; Lo-E). All cows received 100 µg of GnRH (Cystorelin®) on d 0 (h 0), resulting in a preovulatory period of either 3 (Hi-E) or 2 (Lo-E) days. Ovarian ultrasonography was performed on d -7, -3, 0, 2 and 6 to monitor emergence and diameter at ovulation of the preovulatory follicle, and formation of a new
corpus luteum after GnRH. Blood samples collected at 12 h intervals from h -72 to h -12 and at h -6 and 0 were used to determine the preovulatory concentrations of estradiol. Blood samples for progesterone analysis were collected on d -3, every other day from d -2 to 6 and d 9 to 13, and daily until d 30 of gestation. Embryos were collected from donor cows on d 7 of gestation following standard MOET procedures and implanted into 10 cows in the Hi-E (Hi-E-ET) and 12 cows in the Lo-E (Lo-E-ET) treatment immediately after collection on d 7 of the experiment (7 d after GnRH). Five cows in the Hi-E (Hi-E-0) and 9 in the Lo-E (Lo-E-0) treatment did not receive embryos and were used as cyclic control animals. Ultrasonography was performed on d 30 to confirm pregnancy status. Statistical analyses were conducted by using mixed procedures of SAS with a model that included the dependent variables of gene expression, size of ovulatory follicle and days postpartum, and the independent variables treatment and pregnancy. For analyses of progesterone and estradiol concentrations over time, the appropriate repeated measure of hour or day was included in the statistical model. Gene expression was determined by using RT-PCR for ISG-15, Mx2 and RPL-19. Eleven cows, 4 from Hi-E and 7 from Lo-E treatment, exhibited abnormal patterns of progesterone concentrations after GnRH, with basal concentrations below 1ng/mL until approximately day 11 and were removed from the experiment. One cow from Hi-E did not respond to the PGF administration before GnRH and one cow from the Lo-E presented short-cycle and both cows were removed from the experiment. This resulted in 4 cows for both the Hi-E-0 and Lo-E-0 treatments, and 6 and 9 cows for the Hi-E-ET and Lo-E-ET treatments respectively. Within the cows remaining in the experiment pregnant rates were 100% for both treatments at d 30 of pregnancy. Diameter of the ovulatory follicle on day 0 for the
Hi-E (13.4 ± 0.9 mm) and the Lo-E (13.4 ± 1.3 mm) treatment did not differ.

Concentrations of estradiol during the preovulatory period were greater (treatment x h; P = 0.01) in the Hi-E than Lo-E treatment at h -60 and -48, but did not differ in subsequent hours until ovulation. In thirteen cows (2 Hi-E-0, 3 Lo-E-0, 3 Hi-E-ET and 5 Lo-E-ET) the peak of estradiol concentration was reached at least 6 hours before administration of GnRH and was followed by a numerical decrease in estradiol concentration within that female. Progesterone concentrations did not differ between the Hi-E-ET and Lo-E-ET or Hi-E-0 and Lo-E-0 treatments. Relative amount of mRNA did not differ between the Hi-E and Lo-E treatments but were greater in pregnant compared to cyclic cows for both ISG-15 and Mx2 after d 18 until d 30 of pregnancy. A biphasic pattern of gene expression was observed for both ISG-15 and Mx2, where maximum amounts of gene expression in pregnant cows was detected by d 20 followed by a decrease, increase and finally a second decrease to d 30 of gestation. In conclusion, increasing the length of the preovulatory period in primiparous lactating beef cows successfully increased estradiol concentrations at h -48 and -60 before treatment with GnRH. However, it appeared that in a majority of animals, including animals in the Lo-E treatment, preovulatory estradiol concentrations were not limited as the majority of the cows seemed to have reached the peak of preovulatory estradiol with a subsequent decline in concentration before GnRH was given. Furthermore, no differences in progesterone concentrations between the Hi-E and Lo-E treatments were detected; inconsistent with findings presented in Chapter 3. Therefore, we conclude that we were unable to induce a deficiency in preovulatory estradiol concentrations in the Lo-E treatment probably due to the age and lactating status of the cows. As such, differences between pregnant and cyclic cows have been
emphasized. We conclude that amount of mRNA for ISG-15 and Mx2 do not differ between pregnant and cyclic cows from day 15 to 18, and are greater after d 18 to 30 in pregnant cows, reaching maximum expression on d 20 and subsequently declining to day 30 of pregnancy.

**Introduction**

Early embryonic mortality has been reported to be the major cause of failure of pregnancy in ruminants. Large economic have been attributed to pregnancy failure in beef and dairy cattle (Diskin and Morris, 2008). Although size and age of ovulatory follicles are reported to be related to fertility in cattle (Perry et al., 2005; Perry et al., 2007; Meneghetti et al., 2009), recent studies have suggested that the degree of gonadotrophic stimulation that animals receive before ovulation appear to have a stronger relationship to fertility (Mussard et al., 2002, 2003a, 2003b, 2007). Additional studies have shown that a longer proestrus period is responsible for greater preovulatory estradiol concentrations (Bridges et al., 2009, 2010). Furthermore, these findings suggest that decreased fertility may be the result of altered capacity of the uterus to support the developing embryo.

Interferon tau (IFNτ) is produced by the trophoblastic cells of embryo as early as d 15 of pregnancy in cattle and has been described to assist in maternal recognition of pregnancy (MRP) by suppressing prostaglandin F\textsubscript{2α} (PGF) released by the endometrium (Northey and French, 1980; Bartol et al., 1985; Bazer et al., 1994). In addition to its role in MRP, IFNτ has been reported to stimulate numerous genes responsible for up-
regulation of the endometrium that are critical to the fetal-maternal communication and the maintenance of gestation (Charleston and Stewart, 1993; Austin et al., 1996; Hansen et al., 1999; Spencer et al., 2004). These genes, known as interferon stimulated genes (ISGs), are expressed in a temporal and cell-specific manner and for most of their actions on the uterus, progesterone has a permissive role and IFN\(\tau\) stimulates the expression of ISGs (Bazer et al., 2008). Progesterone seems to be important to allow stimulation of ISGs while on the other hand implantation is preceded by loss of expression of progesterone and estrogen receptors by uterine epithelia in several mammal species (Slayden and Keater, 2007; Spencer et al., 2007, 2008). The progesterone-induced down-regulation of progesterone receptors in the uterine luminal, superficial glandular and glandular epithelia of ewes is a prerequisite for expression of ISGs in the endometrium (Bazer et al., 2008). Ott et al., (1999) observed that uterine infusion of IFN\(\tau\) in presence of different steroids can differently stimulate expression of ISGs in the uterus. The combination of IFN\(\tau\) with progesterone resulted in the greatest stimulation as compared to combination with estradiol or IFN\(\tau\) alone.

Although not fully understood, the mechanisms of action of ISGs are better reported in the uterus than its action in different tissues. Research using a receptor transporter protein that has shown to be up-regulated in presence of IFN\(\tau\) has demonstrated that a similar gene response can be expressed by different tissues, as showed by Gifford et al., (2008). Receptor transporter protein 4 (RTP4) mRNA, due to its characteristics to be up-regulated in PBMC during early pregnancy, was observed to have higher gene expression in pregnant compared to nonpregnant ewes in the endometrium, CL and PBMC. Yang et al., (2010) reported the expression of ISG-15 was up-regulated
in the bovine luteal cell \textit{in vitro} during early gestation in response of treatment with recombinant bovine IFN\(\tau\). The expression of genes such as interferon stimulated gene 15 (ISG-15) and Myxovirus resistance 2 (Mx2) mRNA have been reported to also be expressed in PBMC in response to IFN\(\tau\) (Yankey et al., 2001; Han et al., 2006; Stevenson et al., 2007; Green et al., 2010). The expression of the gene for Mx2 is increased 4 to 5 fold within 24 - 48 h of initial IFN\(\tau\) signaling in pregnant sheep (Yankey et al., 2001). In heifers, a greater fold increase for peripheral Mx2 mRNA was observed from d 0 to 18 of pregnancy when compared to nonpregnant animals. Heifers that experienced pregnancy losses tended to have smaller fold increases in Mx2 mRNA expression than those that maintained pregnancy (Stevenson et al., 2007). Similarly, expression of ISG-15 was described to be detected in PBMC in response to IFN\(\tau\) (Han et al., 2006; Green et al., 2010). In dairy cows, ISG-15 mRNA expression in peripheral cells was evident by d 15 with maximum amounts present from d 17 to 21 and a subsequent decline until d 32 (Han et al., 2006). Gifford et al. (2007) also demonstrated that ISG-15, Mx2, and other ISGs were found to be less expressed in peripheral blood cells from d 18 nonpregnant dairy cows when compared with pregnant dairy cows. In a similar experiment conducted by Green et al., (2010), expression of ISG-15 and Mx2 were greater in pregnant than nonpregnant dairy cows on d 18 and 20 when compared to d 14 and 16 after AI. In the same study, expression of ISGs among heifers, primiparous and multiparous dairy cows suggested that multiparous cows had a lesser ISG response than primiparous and heifers.

The hypothesis of the present study is that pregnant animals with reduced length of the preovulatory period would be deficient in preovulatory estradiol concentrations and have decreased ISG-15 and Mx2 mRNA expression from d 15 to 30 of pregnancy.
and that likelihood and timing of embryonic mortality could be estimated from these concentrations. An additional objective was to compare the profile of gene expression between pregnant and cyclic animals from d 15 to 30 of pregnancy.

Material and Methods

Animals and treatments

Thirty six primiparous crossbred (Angus X Simmental) postpartum beef cows from the OSU Beef Center (Dublin, OH) were used in this experiment. All animals were handled in accordance with procedures approved by The Ohio State University Agricultural Animal Care and Use Committee. Cows were 82.4 ± 16.6 d postpartum and had access, ad libitum, to grass pasture or hay. Cows were synchronized to a common day of estrus and ultrasonographic-guided follicle aspiration was performed 5.5 ± 0.1 d after estrus (day of aspiration = d -7 of experiment) to remove the dominant follicle of the first wave, and any other follicles with a diameter of greater than 5 mm. Ovarian ultrasonography was performed on d -7, -3 and 0 to monitor emergence of a new follicular wave and presence of corpus luteum. Differences in preovulatory concentrations of estradiol were achieved by varying the time of luteal regression relative to the induction of ovulation with GnRH. Fifteen cows assigned to the treatment expected to result in normal preovulatory estradiol concentrations (Hi-E) received PGF on d -3 and twenty one cows receiving PGF on d -2 (Lo-E treatment) were expected to have lesser preovulatory estradiol concentrations. All cows received GnRH (100 μg) on d 0, resulting in either 3 or 2 d of proestrus preceding the GnRH-induced LH surge in the Hi-E and Lo-
E treatments, respectively. Blood samples collected at 12 h intervals from h -72 to h -12 and at h -6 and 0 were used to determine the preovulatory estradiol concentrations. Ovaries were examined by ultrasonography on d 4 of the ensuing estrous cycle to confirm ovulation and development of CL. Seven days following GnRH, 10 cows from the Hi-E and 12 cows from the Lo-E treatment were implanted with fresh embryos (Hi-E-ET and Lo-E-ET). The cows that did not receive embryos, 5 from the Hi-E (Hi-E-0) and 9 from the Lo-E (Lo-E-0) treatments were used as nonpregnant control animals. Twenty three embryos were collected from 3 multiparous crossbred (Angus x Simmental) donor cows using standard multiple ovulation, embryo transfer approaches. Estrous cycles of donors were synchronized and ultrasonographic-guided follicle ablation performed on d -6 of the experiment. Animals were superovulated with FSH (folltropin-V®, Bionich Animal Health, Belleville, Ontario, Canada) treated twice a day for four days starting on d -5 with 2.4 mg and decreasing 0.5 mg/day until d -1.5. A CIDR was inserted on d -4.5 and removed on d -1.5, and PGF was administered on d -2 and -1.0. Artificial insemination based on estrus was performed twice on h 12 and 24 (~ d 0 of experiment) after onset of estrus to provide embryos for transfer to experimental animals on d 7. Embryo quality was graded on a standard 1 to 5 scale (1 = excellent, 2 = good, 3 = fair, 4 = poor, and 5 = degenerate; Ahmad et al., 1995). Only embryos graded as 1 and 2 were implanted. Based on previous experience and report from Bridges et al., (2010), where a greater occurrence of shortened luteal phases occurred in heifers experiencing a short (1.25 d) vs. long (2.25 d) proestrus (50% vs. 12.5%, respectively), more cows were assigned to the Lo-E treatment. Cows that experienced a shortened luteal phase were removed of the experiment. Differently from chapter 3, cows were not slaughtered on d 17.5 but instead
had blood samples collected daily from d 15 to 30 of pregnancy. Blood samples for progesterone were collected on days -3, -2, 0, every other day after GnRH until d 6 and from d 9 until d 14, and daily until d 30. Blood were stored on ice immediately after collection and then centrifuged at 1500×g for 20 min within 3 h after collection. Plasma was decanted and stored at -20ºC until further analysis. Blood samples to measure mRNA in PBMC were collected daily from day 15 until day 30 of pregnancy, but daily measurement was performed only for Mx2 while ISG-15 mRNA expression was measured every other day.

**Hormone quantification**

Plasma concentrations of estradiol were determined by using a modified double-antibody, \(^{125}\)I-based assay as described in more detail in chapter 3. Average intra-assay CV was 3.17%, and inter-assay CV (8 assays) for pooled plasma samples containing 2.78, 5.66 and 11.29 pg/mL were 5.26, 4.70 and 4.70 % respectively. Average sensitivity of the assays was 1.42 pg/mL. Plasma concentrations of progesterone were determined using a commercially available RIA kit (Coat-a-Count®, Siemens, Los Angeles, CA) as described previously for our laboratory (Burke et al., 2003). Average intra-assay CV was 2.21%, inter-assay CV’s (3 assays) for pooled plasma samples containing 0.12, 1.10 and 3.32 ng/mL were 1.8, 1.8 and 2.8% respectively. The average sensitivity of the assays was 0.043 ng/mL.
RNA extraction, cDNA synthesis and RT-qPCR

Total RNA was isolated from 200μl of whole blood by using TRI-Reagent BD (Sigma®, St. Louis, MO) as described by the manufacturer’s procedures in order to analyze expression of ISG-15 every other day from d 16 to 30, and Mx2 and ribosomal protein L 19 (RPL-19) mRNA daily from d 15 to d 30. Relative expression of ISG-15, Mx2 and RPL-19 mRNA was determined in total RNA extracted using a 7500 Fast Real-Time PCR System machine (Applied Biosystem®). Oligonucleotide primers specific for the Mx2, ISG-15 and RPL-19 genes were designed to amplify the respective cDNA fragment (Table 3.1). These primers were previously designed and reported by Gifford et al., (2007). The concentration of total RNA was determined by measurement of optical density at 260 nm in the UV/Vis spectrophotometer (NanoDrop® 2000, Thermo Fisher Scientific®, Waltham, MA). Total RNA (0.3 μg) adjusted for DNAse free water was reverse transcribed by using the Dynamo cDNA Synthesis Kit (New England Biolabs®, Ipswich, MA). The reaction was performed by mixing 10 μl of the total RNA with 2 μl of reverse transcriptase enzyme, 1 μl of random hexamer and 10 μl of 5X buffer. Reverse transcription was performed using a GeneAmp PCR System 9700 under the following conditions: 25°C for 5 minutes, 42°C for 30 minutes, 85°C for 5 minutes and 4°C for 90 minutes. The reverse transcribed cDNA was then diluted 1:5 in RNAse free water. Real-time PCR was performed using 5 μl of the diluted cDNA sample, 10 μl of SensiMix Plus Sybr Kit (Quantace®, Watford, UK), 3 μl of RNAse free water, 1 μl of forward primer (10 uM) and 1 μl of reverse primer (10 uM) for a total of 35 cycles under the following conditions: denaturing at 95°C for 30 sec; annealing at 60°C (Mx2) or 58°C (ISG-15) for 45 sec; and extension at 72°C for 45 sec, followed by an extra elongation of 5 min at
72°C. To verify the amplification of a single product, a melting curve analysis was performed after the end of the last PCR cycle and the PCR product was analyzed by gel electrophoresis. A standard curve to determine the efficiency of both the normalizer and the target gene was performed by using the purified Mx2 and RPL-19 cDNA PCR products. The efficiency of amplification of standard curves generated from cDNA of each gene of interest were compared to that of RPL-19 and found to be equivalent.

Data and statistical analysis

Statistical analyses were conducted using the mixed procedures of SAS (2004) with a model that included the dependent variables size of ovulatory follicle and days postpartum, and the independent variables of treatment and pregnancy. For analyses of progesterone and estradiol concentrations, and gene expression over time, the appropriate repeated measure of hour or day was included in the statistical model. Schwarz’s Bayesian Information Criteria (BIC; ‘smaller is better’) was used to determine the best variance-covariance structure for the models.

Gene expression for ISG-15 and Mx2 expressed in PBMC from d 15 to 30 were analyzed by the semi-quantitative RT-PCR method where samples were normalized by the housekeeping gene RPL-19 by using the ΔCt (target Ct – RPL-19 Ct) values (Livak et al., 2001, Schmittgen et al., 2008). Data from ISG-15 and Mx2 were analyzed and graphed by using 2^{-\text{deltaCt}}. The statistical model used was:

\[ Y_{ijk} = \mu + \tau_i + \delta_{ji} + D_k + (\tau D)_{ik} + \varepsilon_{ijk} \]

Where:

\( Y_{ijk} \) = Observation of the \( j \)th cow in the \( i \)th treatment at the \( k \)th time point
\( \mu = \) Grand mean

\( \tau_i = \) Effect of the \( i \)th treatment

\( \delta_{ji} = \) Random effect of the \( j \)th cow in within the \( i \)th treatment

\( D_k = \) Effect of the \( k \)th time point

\( (\tau D)_{ik} = \) The interaction effect of the \( i \)th treatment x the \( k \)th time point

\( \varepsilon_{ijk} = \) Residual term

Analysis for determination of outliers samples were performed based on the two standards deviation rule, where samples with values greater or smaller than two standard deviation were considered outlier and removed from the experiment.

**Results**

Eleven cows, 4 from Hi-E and 7 from Lo-E treatment, exhibited abnormal patterns of progesterone concentrations after GnRH with basal concentrations below 1ng/mL until approximately d 11 and were removed from the experiment. Statistical comparisons of diameter of the ovulatory follicle, days post-partum or progesterone concentration prior to GnRH administration between cows with normal and abnormal luteal phases did not offer possible explanations for failure to develop a CL in these 11 animals. Additionally, one cow from the Hi-E did not respond to the PGF treatment before GnRH induction and one cow from the Lo-E presented short-cycle, both cows were also removed from further analyses. This resulted in 4 cows for both the Hi-E-0 and Lo-E-0 treatments, and 6 and 9 cows for the Hi-E-ET and Lo-E-ET treatments respectively. All the cows that received an embryo were pregnant at d 30 of pregnancy.
Diameter of the ovulatory follicle on d 0 for the Hi-E (13.4 ± 0.9 mm) and the Lo-E (13.4 ± 1.3 mm) treatments did not differ. No correlation among day post-partum, peak of estradiol concentration or progesterone concentration at d -3, -2 and 0 was found. Concentrations of estradiol during the preovulatory period were greater (treatment x h; P = 0.01) in the Hi-E than Lo-E treatment at h -60 and -48, but did not differ in subsequent hours until ovulation (Figure 3.1). In thirteen cows (2 Hi-E-0, 3 Lo-E-0, 3 Hi-E-ET and 5 Lo-E-ET) the peak of estradiol concentration was reached at least 6 hours before administration of GnRH at h 0 (d 0) and was followed by a numerical decrease in estradiol concentration within that female. Progesterone concentrations did not differ between the Hi-E-ET and Lo-E-ET or the Hi-E-0 and Lo-E-0, and were thus combined. The pattern of progesterone for pregnant and cyclic cows, across treatment is illustrated in Figure 3.2.

Relative amount of mRNA did not differ between the Hi-E-ET and Lo-E-ET or between the Hi-E-0 and Lo-E-0 treatments, but were greater (Treatment x d; P < 0.001) in pregnant than cyclic cows for both ISG-15 and Mx2 from d 18 until 30 of pregnancy. A biphasic pattern of gene expression was observed for both ISG-15 and Mx2 in pregnant cows, where maximum amounts of gene expression in pregnant cows were detected for both genes by d 20 followed by a decrease, increase and finally decrease again by d 30 of gestation. The expression of Mx2 mRNA presented an acute increase from d 18 to 19 followed by a decrease from d 20 to 21 and a gradually recuperation of its levels until d 29 of gestation (Figure 3.3). Similarly, ISG-15 expression showed a significant increase from d 18 to 20 of pregnancy, a decrease on d 24 with a slight recuperation of its levels by d 26 and decrease again by d 30 (Figure 3.4). The fold difference used to compare
increasing in gene expression of pregnant vs. cyclic cows was determined by dividing the
greatest (from pregnant) by the smallest value (from cyclic cows). The peak of gene
eexpression, reached on day 20 for both Mx2 and ISG-15, was respectively 3.9 and 3.5
fold greater for pregnant than cyclic cows. Comparison between genes showed that Mx2
expression for pregnant cows was about 10 fold greater than for ISG-15.

Discussion

Gene expression of Mx2 and ISG-15 in PBMC for pregnant and cyclic cows from
d 15 to 30 of the experiment was characterized in the present study. Gene expression for
both Mx2 and ISG-15 did not differ until d 18 of pregnancy, was greater in pregnant than
cyclic cows from days 20 to 30 of pregnancy and fluctuated substantially during this time
period in a biphasic pattern for both genes. While we were successful in inducing some
difference in estradiol concentrations between the Hi-E and Lo-E groups, no differences
existed during the last 48 h before GnRH. Of greater concern was that estradiol
concentrations peaked at least h -6 in more than half of the animals and across both
treatments, suggesting that a deficiency in estradiol concentrations during the
preovulatory period was not achieved in the Lo-E treatment in the present study. In
addition, only one cow from the Lo-E treatment presented short-cycle in this experiment
comparing to 5 cows in chapter 3 and 50% of the cows with shortened period of proestrus
reported by Bridges et al., (2010). Neither progesterone concentrations nor gene
expression for Mx2 and ISG-15 differed between Hi-E and Lo-E treatments; within the
cyclic or pregnant cows. A viable explanation for the abnormal patterns of progesterone
that were detected in approximately 1/3 of the cows assigned initially to the experiment
was not evident and not noted in previous research with similar model (Bridges, 2007; Mussard, 2010).

Differences between treatments in progesterone concentration after ovulation or gene expression for Mx2 and ISG-15 in PBMC were not detected. High preovulatory estradiol concentration were reported to promote substantial changes in the uterine endometrium of beef heifers (Bridges et al., 2010) and changes in peripheral progesterone concentration of multiparous cows (Chapter 3), but the optimum manner of estradiol action, whether it acts through the peak or during a specific time prior to ovulation, has not been established yet. Furthermore, the length of the preovulatory period necessary to achieve maximum concentration of estradiol seems to be shorter for heifers (Bridges et al., 2010) compared to nonlactating multiparous cows (Chapter 3). For primiparous cows, an intermediary length in the preovulatory period between heifers and multiparous cows appears to be necessary, although lactating status could also have an important effect.

Preliminary results showing no differences between treatments for any of investigated variables on this research suggests the differences in estradiol concentration found on h - 60 and -48 were not sufficient to promote differences in peripheral progesterone concentration and gene expression.

In addition to its role on MRP, IFNτ has been reported to act on stimulation of numerous genes responsible for up-regulation of the endometrium in response to pregnancy that are critical to the fetal-maternal communication and the maintenance of gestation (Charleston and Stewart, 1993; Austin et al., 1996; Hansen et al., 1999; Spencer et al., 2004). Interferon stimulated genes such as ISG-15 and Mx have been described to be regulated by IFNτ in the uterine epithelium, stroma, myometrium, and in PBMC of
sheep and cattle (Charleston and Stewart, 1993; Hansen et al., 1999; Austin et al., 2004; Spencer et al., 2004; Gifford et al., 2006; Oliveira et al., 2008). Although no differences in ISG-15 and Mx2 expression between Hi-E and Lo-E treatment were observed on the present study, profile of gene expression between pregnant and cyclic cows from d 15 to 30 of gestation was characterized. Production of IFNτ mRNA and protein across pregnancy increases substantially from days 14 to 21 of gestation in cows and decreases rapidly coincident with placental attachment to the endometrium around d 25 (Farin et al., 1990; Ealy et al., 2001). Expression of Mx2 and ISG-15 in pregnant cows was not different from cyclic cows until after day 18, when levels increased and peaked on day 20 and decreased thereafter remaining higher in pregnant compared to cyclic cows until day 30 of pregnancy. In dairy cows, ISG-15 mRNA expression in peripheral white blood cells was detected by d 15 but maximum amounts were present from d 17 to 21 with a subsequent decline until d 32 (Han et al., 2006). Gifford et al. (2007) demonstrated that ISG-15, Mx2, and other ISGs were lesser in peripheral blood from d 18 nonpregnant dairy cows when compared with pregnant dairy cows at same period in pregnancy. In heifers, a greater fold increase for peripheral Mx2 mRNA was observed from d 0 to 18 of pregnancy when compared to nonpregnant animals. Heifers that experienced pregnancy losses tended to have smaller fold increases in Mx2 mRNA expression than those that maintained pregnancy (Stevenson et al., 2007).

Gene expression of Mx2, ISG-15 and 2’-5’ oligoadenylate synthetase 1 (Oas1) between pregnant and cyclic primiparous and multiparous dairy cows, and heifers were investigated by Green et al., (2010) in 3 different experiments. In experiment 1 gene expression between pregnant and cyclic cows did not differ on d 14 and 16 but were
greater in pregnant cows on d 18 and 20 of pregnancy. The shortest interval for pregnancy detection was studied in the next experiment where cows were bled on d 17 and 18, and differences between cows on days 17 and 18 were observed only in primiparous but not multiparous cows. In the third experiment a larger increase in ISG expression was observed in heifers but not in cows. The collective set of data suggests that ISGs responses are lesser in older animals on or before d 18 of pregnancy. Similarly, in the present study differences in ISG gene expression between pregnant and cyclic primiparous lactating beef cows occurred only after day 18 of gestation and no differences were observed between pregnant and cyclic multiparous nonlactating beef cows bled from d 15 to 17.5 of pregnancy (data unpublished from chapter 3).

In conclusion primiparous lactating beef cows seemed to be less likely to respond to the animal model used to induce different preovulatory estradiol concentrations. Differences in preovulatory estradiol concentrations at h -60 and -48 appeared to be insufficient to promote substantial differences in peripheral gene expression of ISGs, and progesterone concentration between Hi-E and Lo-E treatments from d 15 to 30 of gestation. However, comparison between pregnant and cyclic cows showed that differences in gene expression of Mx2 and ISG-15 started to occur after day 18 of gestation with pregnant cows peaking on d 20 with subsequent decrease in gene expression until day 30 of gestation.
<table>
<thead>
<tr>
<th>Figures Message</th>
<th>Primer Sequence (5’ – 3’)</th>
<th>Anneal (°C)</th>
<th>Cycles (#)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RPL-19</strong></td>
<td>Fwd- tccatgagatgctccagcagt Rev- tgttggagccactcgaga</td>
<td>62.0</td>
<td>35</td>
</tr>
<tr>
<td><strong>Mx2</strong></td>
<td>Fwd- cttcagagacgcctcaagtcg Rev- tgaagcagcccaffaatgtg</td>
<td>62.0</td>
<td>30</td>
</tr>
<tr>
<td><strong>ISG-15</strong></td>
<td>Fwd- ggtatccagctgaagcagtt Rev- acctccctgctgtcaggt</td>
<td>58.0</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 4.1. Primer sequences, annealing temperature, and number of PCR cycles for the genes RPL-19, Mx2 and ISG-15.
Figure 4.1. Circulating concentrations of estradiol during the preovulatory period in cows manipulated to have either normal (Hi-E; ▲) or deficient (Lo-E; ■) preovulatory estradiol concentrations. Cows were administered PGF on either h -72 (Hi-E) or h -48 (Lo-E), all cows received GnRH on h 0 to induce ovulation [Treatment, $P < 0.05$].
Figure 4.2. Circulating concentrations of progesterone following GnRH-induced ovulation in pregnant (▲) and cyclic (■) cows. No differences in progesterone concentrations between pregnant and cyclic cows were observed in the first 17 days; however progesterone concentration decreased after day 17 in cyclic cows.
Figure 4.3. Mx2 mRNA expression in PBMC expressed as $2^{-\Delta \text{CT}} \times 100$ following GnRH-induced ovulation in pregnant (▲) and cyclic (■) cows from days 15 to 30 of pregnancy [*Mx2 mRNA differed ($P < 0.05$) between pregnant and cyclic treatments].

Figure 4.3. Mx2 mRNA expression in PBMC expressed as $2^{-\Delta \text{CT}} \times 100$ following GnRH-induced ovulation in pregnant (▲) and cyclic (■) cows from days 15 to 30 of pregnancy [*Mx2 mRNA differed ($P < 0.05$) between pregnant and cyclic treatments].
Figure 4.4. ISG-15 mRNA expression in PBMC expressed as $2^{-\Delta \text{CT}} \times 100$ following GnRH-induced ovulation in pregnant (▲) and cyclic (■) cows on days 16, 18, 20, 22, 24, 26, 28 and 30 of pregnancy [*ISG-15 mRNA differed ($P < 0.05$) between pregnant and cyclic treatments].
CHAPTER 5

GENERAL DISCUSSION

The two experiments described in this thesis aimed to investigate the mechanisms affecting fertility in cows with differing preovulatory estradiol concentration. The research described in Chapter 3 focused on the embryonic changes caused by different preovulatory estradiol concentrations on d 17.5 of gestation in beef cows and chapter 4 focused on the peripheral interferon stimulated gene response to the IFNτ produced by the embryos from day 15 to 30 of gestation. Additionally, peripheral progesterone concentrations in the luteal phase after ovulation were also compared between treatments. An approach similar to that taken in chapter 3 was conducted previously by Bridges et al., (2006) where heifers received treatments that resulted in either high and low preovulatory estradiol concentrations, however, in the earlier study heifers were slaughtered on d 15.5 instead of d 17.5 of pregnancy. The authors did not find significant differences in the embryos, however the uterus of cows exposed to a low preovulatory concentration seemed to be compromised. The uterine changes observed by d 15.5 of gestation in the previous investigation led to the present study. It was proposed that differences in functional aspects of embryonic gene and protein expression would be expressed by d 17.5 of gestation in response to the uterine deficiencies previously noted by Bridges et al. (2006). Indeed, the conceptus of cows with deficient preovulatory
estradiol concentrations tended to produce less IFNτ and appeared to have less embryonic mass, but the lack of defects in other characteristics that were studied suggested that their function were not severely compromised on day 17.5 of gestation. The major focus of the research described in the present thesis was embryonic protein and gene expression that, if inadequately expressed, may be responsible for the decreased fertility after deficient preovulatory estradiol concentration, while the uterine environment that plays essential role on maintenance of the conceptus was less emphasized. Given that most of the factors secreted by the uterus and embryos that have been suggested to be required for conceptus survival in ruminants are not elicited until after maternal recognition of pregnancy during the peri-implantation period (~d 19 to 24), it is important that changes in both uterine and embryo function caused by reduced estradiol concentrations during this stage are better understood.

The higher progesterone levels observed in the Hi-E treatment cows in the subsequent luteal phase after ovulation suggested that preovulatory estradiol concentrations play an important role on the CL function that could be indirectly related to the higher levels of IFNτ in the uterine flush media and ISG-15 gene expression in PBMC. A positive relationship of progesterone concentration, embryo size and concentration of IFNτ has been described (Kerbler et al., 1997; Mann et al., 2006) and could be related to the findings observed on this experiment. Whether embryo mortality in cows with low preovulatory estradiol concentration occur due to reduced progesterone concentration, changes in the uterus morphology and/or embryonic tissue, it cannot be easily ascertained as these events often occur simultaneously and are intimately related.
In chapter 4 the effect of the different preovulatory estradiol concentrations on ISGs mRNA expression in PBMC and progesterone concentrations in the luteal phase after ovulation was investigated until day 30 of gestation. Differently from chapter 3 that used nonlactating multiparous beef cows, the animals used in chapter 4 were primiparous lactating beef cows and those animals seemed to respond differently to the experimental animal model used to induce different levels of preovulatory estradiol. A great portion of the cows (approximately 1/3) presented abnormal patterns of progesterone in the luteal phase after ovulation (the reason is unknown) and within the cows with normal luteal phase preovulatory, estradiol concentration seemed to be similar between treatments. This set of information led us to believe that the animal category and/or lactating status could be closely related to cows’ responses to the experimental model used. Since differences between treatments in preovulatory estradiol concentrations were subtle, at best, gene expression for ISG-15 and Mx2 in PBMC did not differ between treatments and this experiment became more fundamental to describe the patterns of ISG-15 and Mx2 mRNA expression in pregnant vs. cyclic cows.

Future investigations related to the coordination of uterine function by ovarian steroids and embryo derived signals after day 17.5 of pregnancy should provide additional insight for explaining infertility in cattle of varying endocrine status. In addition, which aspect of estradiol deficiency (peak vs. duration) has a greater impact on fertility and what is the best experimental model to induce animals with different preovulatory estradiol for each animal category may be addressed.
REFERENCES


on luteolysis and pregnancy rate to timed AI with the 5-d CO-Synch + CIDR program. J. Anim. Sci. 88 (E-Suppl. 2):767.


104


Lundin K., C. Bergh and T. Hardarson. Early embryo cleavage is a strong indicator of embryo quality in human IVF. Hum. Reprod. 16:2652-2657.


Wilson, K. N., M. L. Day, W. D. Whittier, R. Kasimanickam and J. B. Hall. 2007 Comparison of 5-day or 7-day CIDRbased estrous synchronization systems for fixed-time AI in beef heifers. J Anim Sci 85 (Supplement 2) 43 (abstract).


