COMPARISON OF GENE EXPRESSION LEVELS IN EMBRYO, ENDOMETRIUM AND CORPUS LUTEUM OF DAIRY HEIFERS AND LACTATING DAIRY COWS AND MANIPULATION OF ENDOMETRIAL GENE EXPRESSION IN-VITRO

by

Pretheeban Thavaneetharajah
B.V.Sc., The University of Peradeniya, Sri Lanka, 2005

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate Studies
(Animal Science)

THE UNIVERSITY OF BRITISH COLUMBIA
(Vancouver)
November 2011

© Pretheeban Thavaneetharajah, 2011
ABSTRACT

Fertility in high producing dairy cows has declined over the past sixty years. Even though, fertility has dropped in lactating dairy cows it remained high in dairy heifers. There is increasing evidence to suspect that embryo quality, corpus luteum (CL) function and endometrial environment are major factors affecting fertility in lactating dairy cows.

The main objective of this study was to compare the quality of the embryo, endometrium and CL by examining selected genes at mRNA and/or protein levels between dairy heifers and lactating dairy cows. In embryos, interferon tau (IFNτ) and heat shock protein 70 (HSPA1A) were highly expressed in heifers compared to lactating dairy cows. Also, differences were observed in the expression levels of glucose transporter 5 (GLUT5) and sodium-potassium adenosine triphosphatase (Na/K-ATPase) between grade 1 and 2 embryos of lactating dairy cows. The CL studies revealed better maintenance, angiogenesis and steroidogenesis in terms of mRNA expression levels (interleukin 1 alpha: IL1A, insulin like growth factor 1: IGF1, fibroblast growth factor 2: FGF2, vascular endothelial growth factor: VEGF, b-cell lymphoma 2: BCL2 and 3-beta-hydroxy steroid dehydrogenase: 3βHSD) in the CL of heifers than lactating dairy cows. Endometrium of lactating dairy cows and heifers showed differences in the expression of candidate genes (BCL2, HSPA1A, IGF1, FGF2, IL1A, tumor necrosis factor alpha: TNF, and serpin peptidase inhibitor, clade A member 14: SERPINA14) on day 11 of the estrous cycle with more expression in heifers than lactating dairy cows. We further studied the effect of recombinant bovine IFNτ (rbIFNτ) on mid luteal phase endometrium in-vitro to manipulate the expression levels of above endometrial genes. Expression levels of genes (IGF binding protein 3: IGFBP3, TNF, SERPINA14, FGF2,
oxytocin receptor: OXTR) and prostaglandin F2 alpha: (PGF2α) levels in the media were inhibited at higher concentrations of rbIFNτ.

Collectively, differences observed in the candidate gene expression levels of embryo, CL and endometrium between dairy heifers and lactating dairy cows suggest that they may regulate reduced fertility observed in lactating dairy cows. Additionally, treatment of endometrium with IFNτ may potentially regulate the luteal survival mechanism and endometrial function during mid luteal phase in bovines.
PREFACE

A version of chapter 2 has been published. Pretheeban T, Gordon MB, Singh R, Perera R and Rajamahendran R. 2009. Differential mRNA expression in in-vivo produced pre-implantation embryos of dairy heifers and mature cows. Mol Reprod Dev 76:1165-1172. I designed and conducted the experiments in consultation with my supervisor Dr. R. Rajamahendran. In addition, I analyzed the results and wrote the manuscript. MB. Gordon and R. Perera assisted in the experiments. Dr. R. Singh helped reviewing the manuscript.

A version of chapter 3 has been published. Pretheeban T, Balendran A, Gordon MB and Rajamahendran R. 2010. mRNA of luteal genes associated with progesterone synthesis, maintenance, and apoptosis in dairy heifers and lactating dairy cows. Anim Reprod Sci 121:218-224. I designed and performed the experiments in consultation with Dr. R. Rajamahendran. In addition, I analyzed the results and wrote the manuscript. A. Balendran and MB. Gordon assisted in sample collection.

A version of chapter 4 has been published. Pretheeban T, Gordon MB, Singh R, Rajamahendran R. 2011. Comparison of expression levels of candidate genes in endometrium of dairy heifers and lactating dairy cows. Can J Anim Sci 91:255-264. I designed and performed the experiments in consultation with Dr. R. Rajamahendran. In addition, I analyzed the results and wrote the manuscript. MB. Gordon assisted in experiments. Dr. R. Singh helped reviewing the manuscript.

All animal experiments were conducted by adhering to the guidelines outlined by the University of British Columbia Animal Care Committee and Canadian Council on Animal Care. Animal care certificate number: A06-1551.
TABLE OF CONTENTS

ABSTRACT ................................................................................................................................. ii
PREFACE....................................................................................................................................... iv
TABLE OF CONTENTS .................................................................................................................. v
LIST OF TABLES ........................................................................................................................... ix
LIST OF FIGURES ........................................................................................................................ x
LISTS OF ABBREVIATIONS .......................................................................................................... xii
ACKNOWLEDGEMENTS ............................................................................................................... xiv

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW .................................................. 1
1.1 Introduction ............................................................................................................................. 1
1.2 Brief overview of bovine estrous cycle .................................................................................. 6
1.2.1 Hormonal control of estrous cycle .................................................................................... 8
1.3 Factors influencing fertility in dairy cows .............................................................................. 10
1.3.1 Anestrus ............................................................................................................................ 10
1.3.2 Oocyte developmental competence ................................................................................ 12
1.3.3 Corpus luteum function ..................................................................................................... 14
1.3.3.1 Formation of corpus luteum .......................................................................................... 14
1.3.3.2 Maintenance and function of corpus luteum ................................................................. 15
1.3.3.3 Role of progesterone on establishment of pregnancy .................................................. 17
1.3.3.4 P4 and embryonic loss .................................................................................................. 18
1.3.4 Fertilization failure ........................................................................................................... 18
1.3.5 Embryonic loss in cows ..................................................................................................... 19
1.3.5.1 Genetic factors involved in embryonic mortality .......................................................... 22
1.3.5.2 Embryo quality and pregnancy loss ............................................................................. 23
1.3.5.3 Current methods evaluating embryo quality ............................................................... 23
1.3.5.4 Events reflecting embryo quality during pre-implantation development .................... 24
1.3.5.5 Embryonic genome activation ....................................................................................... 25
1.3.5.6 Embryonic gene expression ......................................................................................... 26
1.3.5.7 Blastocyst formation and expansion .......................................................................... 26
1.3.5.8 Energy metabolism ....................................................................................................... 27
1.3.5.9 Expression of interferon tau ........................................................................................ 29
1.3.5.10 Stress related gene expression .................................................................................... 30
1.3.5.11 Apoptosis and embryo quality ................................................................................... 32
1.3.6 Oviductal environment ..................................................................................................... 34
1.3.7 Uterine environment ......................................................................................................... 35
1.3.7.1 Role of endometrial secretions during pre-implantation period .................................... 36
1.3.7.2 Insulin like growth factor system (IGFs) ..................................................................... 36
1.3.7.3 Fibroblast growth factor ............................................................................................... 37
1.3.7.4 Endometrial cytokines .................................................................................................. 38
1.3.7.5 Serpin peptidase inhibitor, clade A member 14 ............................................................ 39
1.3.7.6 Endometrial apoptosis and heat stress .......................................................................... 40
1.4 Manipulation of uterine environment ................................................................................... 41
1.4.1 Use of interferon tau to manipulate uterine functions ....................................................... 42
1.5 Rationale, hypothesis and objectives of the dissertation ...................................................... 43
1.5.1 Chapter 2: Differential mRNA expression in in-vivo produced pre-
implantation embryos of dairy heifers and cows .................................................. 44
1.5.2 Chapter 3: mRNA of luteal genes associated with progesterone synthesis,
maintenance, and apoptosis in dairy heifers and lactating dairy cows .................. 44
1.5.3 Chapter 4: Comparison of expression levels of candidate genes in
endometrium of dairy heifers and lactating dairy cows........................................ 45
1.5.4 Chapter 5: Recombinant bovine interferon tau alters candidate gene expression
in mid luteal phase endometrium of cows ........................................................... 45

CHAPTER 2: DIFFERENTIAL mRNA EXPRESSION IN IN-VIVO PRODUCED
PRE-IMPLANTATION EMBRYOS OF DAIRY HEIFERS AND LACTATING
COWS ................................................ .......................................................... 46
2.1 Introduction ........................................................................................................ 46
2.2 Materials and methods ..................................................................................... 48
  2.2.1 In-vivo embryo production ......................................................................... 48
  2.2.2 Embryonic gene expression ..................................................................... 49
  2.2.3 RNA extraction ......................................................................................... 49
  2.2.4 Reverse transcription ................................................................................. 50
  2.2.5 Semi quantitative PCR ............................................................................ 50
  2.2.6 Quantitative real-time PCR .................................................................... 51
  2.2.7 Statistical analysis ....................................................................................... 52
2.3 Results ............................................................................................................... 52
  2.3.1 Expression of genes in embryos of heifers vs. lactating cows .................. 52
  2.3.2 Expression of genes in grade 1 vs. grade 2 quality embryos ..................... 53
2.4 Discussion ......................................................................................................... 53

CHAPTER 3: mRNA OF LUTEAL GENES ASSOCIATED WITH
PROGESTERONE SYNTHESIS, MAINTENANCE, AND APOPTOSIS IN DAIRY
HEIFERS AND LACTATING DAIRY COWS ..................................................... 64
3.1 Introduction ........................................................................................................ 64
3.2 Materials and methods ..................................................................................... 66
  3.2.1 Animals and treatments ......................................................................... 66
  3.2.2 Corpus luteum enucleation and processing ............................................. 66
  3.2.3 RNA extraction ......................................................................................... 67
  3.2.4 Reverse transcription ................................................................................. 68
  3.2.5 Quantitative real-time PCR .................................................................... 68
  3.2.6 Progesterone RIA .................................................................................... 69
  3.2.7 Statistical analysis ....................................................................................... 70
3.3 Results ............................................................................................................... 70
  3.3.1 mRNA expression of apoptotic molecules and heat shock protein .......... 70
  3.3.2 mRNA expression of steroidogenic enzymes ......................................... 70
  3.3.3 mRNA expression of genes associated with angiogenesis ..................... 71
  3.3.4 mRNA expression of genes associated with luteal maintenance .......... 71
  3.3.5 Progesterone assay ................................................................................... 71
3.4 Discussion ......................................................................................................... 71
CHAPTER 4: COMPARISON OF EXPRESSION LEVELS OF CANDIDATE GENES IN ENDO METRIUM OF DAIRY HEIFERS AND LACTATING DAIRY COWS ................................................................. 78

4.1 Introduction ........................................................................................................ 78
4.2 Materials and methods ....................................................................................... 81
  4.2.1 Animal procedures and tissue collection ....................................................... 81
  4.2.2 RNA extraction ............................................................................................ 82
  4.2.3 Reverse transcription .................................................................................... 83
  4.2.4 Primer design ............................................................................................... 83
  4.2.5 Semi-quantitative PCR ................................................................................. 83
  4.2.6 Quantitative real-time PCR ......................................................................... 84
  4.2.7 Immunohistochemistry ................................................................................. 85
  4.2.8 Statistical analysis ....................................................................................... 86
4.3 Results ................................................................................................................ 87
  4.3.1 Relative mRNA abundance of apoptotic genes and HSPA1A ..................... 87
  4.3.2 Relative mRNA abundance of growth factor genes .................................... 87
  4.3.3 Relative mRNA abundance of cytokine genes ........................................... 87
  4.3.4 Immunohistochemistry ................................................................................. 88
4.5 Discussion .......................................................................................................... 89

CHAPTER 5: INTERFERON TAU ALTERS CANDIDATE GENE EXPRESSION AND PROSTAGLANDIN CONCENTRATIONS IN MID LUTEAL PHASE BOVINE ENDOMETRIUM IN-VITRO ........................................................................ 97

5.1 Introduction ........................................................................................................ 97
5.2 Materials and methods ....................................................................................... 99
  5.2.1 Animals and collection of reproductive tracts .............................................. 99
  5.2.2 Endometrial tissue culture ......................................................................... 99
  5.2.3 Treatment with recombinant bovine IFNτ .................................................. 100
  5.2.4 RNA extraction .......................................................................................... 100
  5.2.5 Reverse transcription ................................................................................. 101
  5.2.6 Quantitative real-time PCR ...................................................................... 101
  5.2.7 ELISA for PGF2α and PGE2 .................................................................... 102
  5.2.8 Statistical analysis ..................................................................................... 103
5.3 Results ................................................................................................................ 103
  5.3.1 Effect of rbIFNτ on the mRNA expression levels of endometrial genes ...... 103
    5.3.1.1 Apoptotic genes and heat shock protein .............................................. 103
    5.3.1.2 Growth factor genes .......................................................................... 103
    5.3.1.3 Cytokine and immune function related genes .................................. 104
    5.3.1.4 Oxytocin receptor .............................................................................. 104
  5.3.2 Effect of rbIFNτ on the secretion of prostaglandins .................................... 104
5.4 Discussion .......................................................................................................... 104

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS ....................... 114

6.1 Summary ......................................................................................................... 114
6.2 Comparison of embryonic, endometrial and CL gene expression between dairy heifers and lactating cows. 115
6.3 Effect of IFN\(\tau\) on endometrial gene expression ........................................ 122
6.4 Significance of dissertation and potential application ..................................... 123
6.5 Limitations of dissertation and future directions ............................................ 123
6.6 Final conclusions ......................................................................................... 124

BIBLIOGRAPHY .................................................................................................. 126

APPENDICES ....................................................................................................... 175
  Appendix A ......................................................................................................... 175
  Appendix B ......................................................................................................... 176
LIST OF TABLES

Table 2.1 Details of the gene specific primers used for PCR amplification of embryonic genes .................................................................................................................................................. 58

Table 3.1 Details of the gene specific primers used for PCR amplification of corpus luteum genes ................................................................................................................................................. 76

Table 4.1 Details of the primers used for PCR amplification of endometrial genes ........ 93

Table 5.1 Details of the gene specific primers used for PCR amplification of endometrial genes .................................................................................................................................................. 111
LIST OF FIGURES

Figure 1.1 Diagrammatic summary of the factors affecting fertility in dairy cows.............. 4

Figure 1.2 Schematic of major factors contributing to the early embryo development prior to implantation in bovines. CL, corpus luteum; MRP, maternal recognition of pregnancy. ........................................................................................................ 5

Figure 1.3 Schematic representation of the hormonal control of bovine estrous cycle.... 7

Figure 1.4 Schematic presentations of the components contributing to reduced fertility in dairy heifers and lactating cows between day 1 of gestation and parturition. ................. 21

Figure 2.1 a) The representative gel photograph shows the PCR products of gene transcripts. b) The mRNA expression levels of genes in embryos of heifers (HP) and lactating cows (CP). ........................................................................................................ 59

Figure 2.2 a) The representative gel photograph shows the PCR products of gene transcripts. b) The mRNA expression levels of genes in embryos of heifers (H1) and lactating cows (C1). ........................................................................................................ 60

Figure 2.3 a) The representative gel photograph shows the PCR products of gene transcripts. b) The mRNA expression levels of genes in embryos of heifers (H1 and H2). ........................................................................................................ 61

Figure 2.4 a) The representative gel photograph shows the PCR products of gene transcripts. b) The mRNA expression levels of genes in embryos of lactating cows (C1 and C2). ........................................................................................................ 62

Figure 2.5 a) The mRNA expression levels of BAX and BCL2 genes from real-time PCR in a) pooled embryos of heifers (HP) and lactating cows (CP), b) grade 1 quality embryos of heifers (H1) and lactating cows (C1) and c) grade 1 and 2 quality embryos of heifers (H1 and H2). Third column in each experiment represents the BAX: BCL2 ratio of corresponding groups. ........................................................................................................ 63

Figure 3.1 Amounts of mRNA of genes associated with a) apoptosis and heat shock, b) steroidogenesis, c) angiogenesis and d) luteal maintenance, in dairy heifers and lactating dairy cows. ........................................................................................................ 77

Figure 4.1 The mRNA levels of endometrial genes in heifers and lactating dairy cows. 94

Figure 4.2 Immunohistochemical localization of endometrial IL1A (A), TNF (B) and FGF2 (C) proteins on day 11 of estrous cycle in heifers (a, b, c) and lactating dairy cows (d, e, f). ........................................................................................................ 95
Figure 4.3 The distribution of immunostaining intensity (HSCORE) in different regions of the bovine endometrium for IL1A, TNF and FGF2 during the mid luteal phase........ 96

Figure 5.1 The mRNA levels of bovine endometrial genes after treatment with rbIFNτ (0, 1.25, 5 and 25 ng/ml)........................................................................................................................................ 112

Figure 5.2 Prostaglandin concentrations in endometrial culture media after 24 h treatment with rbIFNτ (0, 1.25, 5 and 25 ng/ml). a) PGF2α and b) PGE2......................... 113
# Lists of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>3βHSD</td>
<td>3-beta-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin converting enzyme</td>
</tr>
<tr>
<td>AI</td>
<td>Artificial insemination</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>BAK</td>
<td>BCL2 antagonist</td>
</tr>
<tr>
<td>BAX</td>
<td>BCL2 associated x protein</td>
</tr>
<tr>
<td>BCL2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BCS</td>
<td>Body condition score</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenetic protein</td>
</tr>
<tr>
<td>bp</td>
<td>Base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>bTP-1</td>
<td>Bovine trophoblast protein-1</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>Caspase</td>
<td>Cysteinyl aspartic acid-protease</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complimentary DNA</td>
</tr>
<tr>
<td>CL</td>
<td>Corpus luteum</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>Cx43</td>
<td>Connexin 43</td>
</tr>
<tr>
<td>CYP11A1</td>
<td>Cytochrome P450 family 11 subfamily A polypeptide 1</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethylpyrocarbonate</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified essential medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>E₂</td>
<td>Estradiol-17β</td>
</tr>
<tr>
<td>Fas L</td>
<td>Fas ligand</td>
</tr>
<tr>
<td>Fas</td>
<td>Fas antigen</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>FGFR</td>
<td>Fibroblast growth factor receptor</td>
</tr>
<tr>
<td>FLT1</td>
<td>Fms-related tyrosine kinase 1</td>
</tr>
<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>g</td>
<td>Gravity</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GH</td>
<td>Growth hormone</td>
</tr>
<tr>
<td>GLUT</td>
<td>Glucose transporter</td>
</tr>
<tr>
<td>GnRH</td>
<td>Gonadotropin releasing hormone</td>
</tr>
<tr>
<td>h</td>
<td>Hour(s)</td>
</tr>
<tr>
<td>HSPAA1A</td>
<td>Heat shock protein 70</td>
</tr>
<tr>
<td>IETS</td>
<td>International embryo transfer society</td>
</tr>
<tr>
<td>IFN-I</td>
<td>Type I interferons</td>
</tr>
<tr>
<td>IFNα</td>
<td>Interferon alpha</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IFNτ</td>
<td>Interferon tau</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin like growth factor</td>
</tr>
<tr>
<td>IGFBP</td>
<td>Insulin like growth factor binding protein</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
</tbody>
</table>
IL1A   Interleukin 1 alpha
IL1R   Interleukin 1 receptor
IM     Intramuscular
kb     Kilo bite
KDR    Kinase insert domain receptor
LH     Luteinizing hormone
LLC    Large luteal cell
mg     Milligram
mL     Milliliter
mM     Millimolar
mRNA   Messenger ribonucleic acid
MRP    Maternal recognition of pregnancy
Na/K-ATPase Sodium-Potassium Adenosine Triphosphatase
ng     Nanogram
NK     Natural killer
OT     Oxytocin
oTP-1  Ovine trophoblast protein-1
OXTR   Oxytocin receptor
P₄     Progesterone
PBS    Phosphate buffered saline
pg     Picogram
PG     Prostaglandin
PGE2   Prostaglandin E2
PGF₂α   Prostaglandin F2 alpha
PGFS   Prostaglandin F2 alpha synthase
PR     Pregnancy rate
Q-RTC    Quantitative real time PCR
rbIFNτ  Recombinant bovine interferon tau
RT-PCR  Reverse transcription polymerase chain reaction
SERPIN14 Serpin peptidase inhibitor, clade A member 14
SLC    Small luteal cell
SPARC  Secreted protein, acidic, cysteine-rich
STAR   Steroidogenic acute regulatory protein
SUZ12  Suppressor of zeste 12 homolog
TNF    Tumor necrosis factor alpha
VEGF   Vascular endothelial growth factor
µg     Microgram
µL     Microliter
µM     Micromol
ACKNOWLEDGEMENTS

First, and foremost, I am very grateful to my supervisor Dr. R. Rajamahendran for guiding and supporting me academically and personally for the past five years in his lab. He has been a great mentor to me and gave the opportunity and freedom to grow as a researcher. He also allowed me to teach and train students during my time in the lab. Secondly, sincere thanks to my supervisory committee members Dr. Kimberly Cheng, Dr. Geoffrey Hammond and Dr. Keith Choi for overseeing my research progress and providing valuable time and effort to make this dissertation a success.

I would like to express by deepest appreciation and gratitude for the financial assistance provided to me from Elizabeth R Howland Fellowship, University Graduate Fellowship, Viterra Graduate Fellowship and my supervisor’s grants and the facilities provided by the Faculty of Land and Food Systems at UBC.

Next I would like to thank past and present members of the Dr. Raja’s lab for giving me a warm environment and support during my time in the lab. Special thanks to Ms. Anusha Balendran and Ms. Ruwanie Perera for their kindness and support. Thanks to Dr. Ravinder Singh for helping me learn scientific methods and providing valuable critics to my research. Thanks to Ms. Miriam Gordon for her continuous support to my experiments in Agassiz and Mr. Gunaretnam Iyadurai for assisting in experiments and for the company during the years of stay at UBC.

I also take this opportunity to thank Dr. Divakar Ambrose and Dr. Marcos Colazo for their assistance in embryo recovery and endometrial biopsy procedures. My thanks also go to Mr. Nelson Dinn and the staff of the UBC Dairy Education and Research Center for providing me with research subjects, space and shelter and helping me during the experiments. Thanks to Dr. Eduardo Jovel, Ms. Sylvia Leung, Mr. Gilles Galzi and Mr. Martin Hilmer for their technical assistance. Thank you to Mr. Theepan Moorthy for proof reading some of the chapters of this dissertation. I also thank past and present LFS graduate program managers Ms. Kirsten Cameron, Ms. Allison Barnes and Ms. Shelly Small and Ms. Lia Maria Dragan for their help in my academic and financial matters at UBC. Also thanks to Dr. Nelly Ausperg and Dr. Peter Leung for giving me the opportunity to learn experimental techniques and to Ms. Roshni Nair for helping me follow courses through Reproductive and Developmental Sciences program.

I am extremely thankful to my parents, Thavam and Uma, in-laws, relatives and friends for their love and support given to me and for my accomplishments. It would not be possible to complete this dissertation without the relentless love, encouragement and understanding provided by my charming wife Manaja.
Dedicated To My Parents
CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The modern high producing lactating dairy cow is considered to be sub-fertile because the pregnancy rates (PR) after artificial insemination (AI) have declined significantly over the past 60 years worldwide (reviewed by Lucy, 2001; Pryce et al., 2004; Norman et al., 2009). Even though the PR of lactating dairy cows has dropped drastically from 66% to 40% it has remained unaltered or even slightly increased (70%) in dairy heifers (Pursley et al., 1997; Pryce et al., 2004). The PR is considered to be an efficient tool in evaluating the fertility of farm animals, including cows. In addition, other reproductive parameters such as number of breedings per conception, the days open after calving, and calving interval are also indicative of reduced fertility in high producing lactating cows (Washburn et al., 2002).

Reduced fertility of dairy cows in a farm results in increased reproductive culling and replacement costs, as well as an inconsistent number of cows in the milking herd throughout the year, and thus eventually lower milk production and economic loss to the farmer. It has been reported recently in the USA that an estimated value of a new pregnancy is $278 and an average loss of pregnancy costs $555 for high producing dairy cows (de Vries, 2006).

Over the past 60 years, the average milk production of a dairy cow has increased drastically worldwide. According to the latest report by the United States Department of Agriculture the mean annual milk production of a dairy cow was 5333 lbs in the year 1951 and 21148 lbs in year 2010 (National Agricultural Statistic Service, 2011). It is
suspected that intense genetic selection for milk production over several decades has contributed to the reduced fertility in dairy cows (Lucy et al., 2001). Even though, increase in milk production is associated with reduced fertility in dairy cows, the causes for the sub-fertility are multi-factorial (Grohn and Rajala-Schultz, 2000). Therefore, the reduced fertility in high producing lactating dairy cows is considered to be due to an array of causes such as physiological, environmental and management factors (Figure 1.1).

The general and reproductive health of the animal, genetics, ovarian and endocrine functions, estrus expression, oocyte, sperm and embryo quality, oviductal and uterine environments are all important physiological factors that could possibly affect the fertility of a post-partum dairy cow; while nutrition and environment are the other major contributing factors that could hinder the optimal fertility of a dairy cow (Royal et al., 2008).

Investigating the reduced fertility in lactating dairy cows would be a mammoth task requiring immense skills and effort, as well as thought process from various angles and proper funding. Several research groups are actively involved in the effort of identifying the causes of reduced fertility and seek to develop methods to improve fertility in dairy cows. Embryo quality, corpus luteum (CL) function and the interaction between the embryo and the uterine environment are some of the niche topics of current investigation as they regulate the establishment and maintenance of pregnancy (Figure 1.2). Among several tools and approaches available to investigate the quality and or functions of the above components, examining them at a molecular level using gene expression profiles is at the forefront of contemporary research. Vast numbers of genes
are identified and their temporal and spatial expressions in different reproductive tissues have been studied in mammals including bovines. Several of those genes are implicated as developmentally or functionally important for the fertility in embryos, CL and endometrium.

Analysing the gene expression levels of those important genes at specific stages reveals the developmental or functional capacities of those tissues, therefore, in this study, I have compared the quality of the pre-implantation embryos, CL and uterine environment between dairy heifers and lactating cows using candidate gene expression in order to understand whether poor quality embryos, compromised CL and endometrial functions are the reasons for the reduced fertility observed in lactating dairy cows. I also studied the in-vitro effect of recombinant bovine interferon tau (rbIFNτ) on the candidate endometrial gene expression in cattle in order to enhance endometrial function. The findings of this study will help understand the quality of above factors between dairy heifers and lactating dairy cows as well as means to improve fertility in bovines.

In this section, reproductive physiology of cattle is reviewed with specific emphasis on early embryo development. The factors influencing fertility in dairy cows such as estrous cycle, fertilization, embryo development and maternal environment are discussed. Subsequently, CL function, embryo quality, and endometrial function are reviewed with specific emphasis on gene expression. In addition, the role of IFNτ during early embryo development and its use to manipulate endometrial functions are reviewed. Finally, the rationale, hypothesis and the objectives of the dissertation are clearly briefed at the end of this section.
Figure 1.1 Diagrammatic summary of the factors affecting fertility in dairy cows. CL, corpus luteum.
Figure 1.2 Schematic of major factors contributing to the early embryo development prior to implantation in bovines. CL, corpus luteum; MRP, maternal recognition of pregnancy.
1.2 Brief overview of bovine estrous cycle

As in other domestic mammals, cattle exhibit regular cyclic changes in reproductive organs and sexual behaviour. The sexual cycle is called the estrous cycle and the duration of estrous cycle is approximately 21 days, with a range of 18 to 24 days in bovines (Hafez and Hafez, 2000). Cows exhibit multiple estruses throughout the year however, not cycle usually during pregnancy and disease conditions (Dhali et al., 2005). A typical estrous cycle of a cow consists of four phases: estrus, metestrus, diestrus and proestrus. Proestrus occurs on 19-21 days of the cycle and lasts for 3 days; it starts with regression of CL and reduction in progesterone (P₄) levels (Hafez and Hafez, 2000). Also proestrus is characterized by follicular growth and estradiol (E₂) production by the ovary (Shearer, 2003). Estrus is the sexually receptive phase and this period begins with the acceptance of the male and on an average lasts for 18 h varying within a range of 18 to 24 h (Hafez and Hafez, 2000). Characteristic signs of estrus for descriptive purposes could be divided into two groups: behavioural and physiological signs. Cows in estrus would stand for mounting by other cows or a bull. The supplementary signs of estrus are discharge of clear, glassy strings of mucus from the vulva, restlessness, swelling and reddening of the vulval mucosa, and slight reductions of feed intake and milk yield (Ball and Peters, 2004). Metestrus lasts for 5 or 7 days and occurs on 1-5 days of the cycle. Ovulation occurs within the metestrus phase that is 24 - 30 h after the onset of estrus. Diestrus is the lengthiest phase and occurs on 6-17 days of the cycle. During this time the CL will be functional and produces increasing levels of P₄. If pregnancy occurs, the CL will be maintained throughout the pregnancy period, if not the CL will be maintained until 17 or 18 d of the cycle and regresses thereafter (Shearer, 2003).
Figure 1.3 Schematic representation of the hormonal control of bovine estrous cycle. The synthesis and release of gonadotropin releasing hormone (GnRH) from hypothalamus are regulated by three feedback mechanisms. An ultra short loop involves regulation by GnRH itself within the hypothalamus. In the short loop, levels of pituitary hormones (follicle stimulating hormone; FSH and luteinizing hormone; LH) regulate the secretion of GnRH. In the long loop, estradiol (E₂) and progesterone (P₄) influences the secretion of GnRH. Both E₂ and P₄ also regulate the secretion of pituitary hormones. Inhibin influences the secretion of FSH. Oxytocin (OT) from both pituitary and CL induces the secretion of uterine prostaglandin F2α (PGF2α) which in turn acts on CL. Red and green lines are indicative of negative and positive feedback mechanisms, respectively.
1.2.1 Hormonal control of estrous cycle

The estrous cycle in cows, similar to other animals, is controlled by the reproductive hormones secreted by the hypothalamo-hypophyseal-ovarian axis. Even though the estrous cycle consists of four different phases it can also be divided into two major phases: namely, the follicular and luteal phases (Hafez and Hafez, 2000). The follicular phase is relatively short in duration (3 to 4 days) and commences with the onset of regression of the CL and ends with ovulation. The luteal phase commences with ovulation and lasts for about 15 to 16 days, at which time regression of the CL commences. Synthesis and secretion of hormones in hypothalamo-hypophyseal-ovarian axis are controlled by both positive and negative feedback mechanisms (Figure 1.3).

Gonadotropin releasing hormone (GnRH) is the main neuroendocrine reproductive hormone secreted by the hypothalamus (Ball and Peters, 2004). The synthesis and secretion of gonadotropins: luteinizing hormone (LH) and FSH from the anterior pituitary are controlled by GnRH and by the feedback mechanisms operated within the hypothalamo-hypophyseal-ovarian axis (Langley, 1977). GnRH is released in a pulsatile manner every 1 to 3 h from the hypothalamus. FSH and LH are responsible for various events within the ovary such as oogenesis, folliculogenesis, ovulation, CL function and production of E\textsubscript{2} and P\textsubscript{4} (Hafez and Hafez, 2000). Pulsatile GnRH secretion stimulates the pulsatile secretion of LH and FSH. The levels of GnRH fluctuate during various phases of an estrous cycle being at basal levels during diestrus and peaks at estrus (Sorensen, 1979).

During proestrus FSH and LH are released from the anterior pituitary at smaller concentrations upon GnRH stimulation. Recruitment of follicles takes place after they
become gonadotropin dependent at the stage of 4-6 mm in diameter (Driancourt, 2001). Ovarian follicles grow in a cohort and produce E_{2} in response to FSH. One follicle diverges from the cohort at the stage of 8-9 mm in diameter and increases in size to become the dominant follicle (Savio et al., 1988). The dominant follicle acquires more LH receptors and contains higher E_{2} and inhibin-A, and lowest insulin like growth factor binding proteins (IGFBPs) and follistatin concentrations than the subordinate follicles (Austin et al. 2001). The higher concentrations of E_{2} and inhibin-A secreted by the dominant follicle suppress the secretion of FSH from pituitary through negative feed back mechanism (Pawson and McNeilly, 2005). Deprivation from FSH induces atresia of subordinate follicles. Thereafter, the dominant follicle is mainly responsive to LH and continues to grow and secrete E_{2} unless inhibited by reduced levels of LH due to the production of P_{4} by a functional CL (reviewed by Forde et al., 2010). Increasing plasma E_{2} and decreasing plasma P_{4} concentrations at the end of diestrus are responsible for the manifestation of estrus behaviour in cows and causes a surge release of LH and FSH by directly acting on pituitary and indirectly by inducing the secretion of GnRH from hypothalamus (Hansel and Echternkamp, 1972). The surge release of LH results in ovulation of dominant follicle and subsequent formation of CL (Bearden and Fuquay, 1992).

The newly formed CL secretes P_{4} and characterizes the luteal phase of the estrous cycle. Increasing levels of plasma P_{4} suppresses the secretion of FSH and LH and prevents ovulation. Maximal levels of P_{4} are reached during the mid cycle and maintained at higher levels until luteolysis (Bearden and Fuquay, 1992). In the case of a non-pregnant cow, PGF2\(\alpha\) is secreted from the endometrium and causes the regression of CL
and cessation of P₄ production between d 16 and 19 of the estrous cycle. PGF₂α secretion in uterus is induced by OT at the end of luteal phase. Secreted endometrial PGF₂α is then transferred to the CL by the counter current mechanism and causes luteolysis (Forde et al., 2010). Luteolysis is associated with reducing levels of plasma P₄. Concurrently, E₂ concentrations start to increase. However, if conception is successful the CL will continue to produce P₄ throughout the pregnancy and suppresses estrus (Hafez and Hafez, 2000).

1.3 Factors influencing fertility in dairy cows

1.3.1 Anestrus

High yielding dairy cows especially during the post partum period experience anestrous and irregular estrous cycles due to the endocrine imbalances and pathologic conditions of the uterus and ovary that affect the regular cyclic functions (reviewed by Peter et al., 2009). Major causes for the delayed resumption of ovarian activity are postpartum reproductive diseases and nutritional status of the animals. Negative energy balances, inflammatory diseases of the uterus, ovarian cysts, persistent dominant follicle and/or CL are some of the contributing factors.

In bovines, follicles grow in a wave like pattern and culminate either in ovulation or atresia (Rajakoski., 1960; Ireland et al., 2000). Generally Bos taurus cows exhibits two to three follicular waves per estrous cycle. Each wave is accompanied by a synchronous growth of a cohort of follicles, selection of the dominant follicle and establishment of dominance. In a three wave cycle, the dominant follicle of each of the first two waves undergoes atresia while that from the third wave ovulates. However, the dominant follicle of the ultimate follicular wave in postpartum anestrous cows does not ovulate (Taylor and
Rajmahendran, 1994; reviewed by Lucy, 2007). The control of follicular growth and ovulation depends on numerous factors including hormones (FSH, LH, E₂, P₄, Inhibin, Insulin etc.) and growth factors (insulin like growth factor 1: IGF1, IGFBPs, bone morphogenetic proteins BMPs, etc.). Any defects in the above systems can affect the normal pattern of follicular growth and may lead to anovulation or give rise to a suboptimal quality oocyte and sub-functional CL. Bleash et al. (2004) have reported that increased duration of ovulatory follicle development from the time of dominance to estrus (fewer follicular waves) is associated with reduced PR following AI in dairy cows undergoing spontaneous oestrous cycles. Extending the period of dominance of the ovulatory follicle by even 1.5 days compromises the embryo quality in cows (Cerri et al., 2009). Nutrition and lactation are two important factors that indirectly affect the follicular dynamics in dairy cows (Lucy et al., 1992). Wolfenson and others have reported longer duration (four days) of dominance for large follicle in lactating cows compared to heifers (Wolfenson et al., 2004).

The estrus (heat) period is characterized as the time interval when a cow exhibits sexual desire and acceptance of a bull to mate and impregnate within natural settings. With the introduction of AI and its wide spread use in the cattle industry it is imperative to properly identify the window of estrus in cows in order to achieve greater PR. However, accuracy of identifying and inseminating a cow in estrus is still a huge challenge in large scale dairy farms and is found to be the most serious problem in achieving better PR. Breeding cows that are not in estrus around the time of insemination has little chance of achieving better PR.
Reduced duration and intensity of estrus is a major problem in the identification of cows for breeding, and this eventually negatively affects the reproductive efficiency. The modern Holstein dairy cow exhibits estrus for a shorter duration compared to their counterparts 30 years ago (7 h vs. 14.9 h; Esslemont and Bryant, 2004; Lopez et al., 2004). The hormone responsible for estrus behaviour is E\textsubscript{2}, and its concentrations in blood plasma are positively correlated with the behavioural signs of estrus in dairy cattle (Roelofs et al., 2004). Reduced E\textsubscript{2} production by the dominant follicle, reduced P\textsubscript{4} production that fails to remove the refractory state of the hypothalamus to E\textsubscript{2} and stress are major causes of reduced expression of estrus in postpartum dairy cows (reviewed by Boer et al., 2009).

1.3.2 Oocyte developmental competence

Developmental competence of an oocyte is characterized as the ability to resume meiosis, to undergo cleavage after fertilization, to develop into a blastocyst, and progress through pregnancy and appear as a healthy offspring. However, embryo quality overlaps with oocyte quality after the beginning of cleavage stage (Sirard et al., 2006). Therefore, oocyte quality can be considered to be one of the determinants of embryo quality, controlled mainly by the events that occur prior to ovulation (Lonergan et al., 2001). Quality, or developmental competence, is acquired during growth and maturation of oocyte (Kanka, 2003).

Reduced developmental capacity of oocytes has been reported in dairy cows. High genetic merit for milk production, lactation, negative energy balance, milk-stimulating diet are important factors found to influence the oocyte quality (reviewed by Leroy et al., 2008a & b). In addition, the ability of the oocyte to produce good quality
embryos is determined by various factors including age of dam, parity, body condition score (BCS) and season (Salamone et al., 2000, Snijders et al., 2000, Sinclair et al., 2000 and Al-Katanani et al., 2002). Alterations in the follicular dynamics and temporal changes in the endocrine hormones also regulate the competence of oocytes (Saivo et al., 1993; Revah and Butler, 1996; Austin et al., 1999; Mihim et al., 1999).

Quality of the oocyte is extensively controlled by the state of follicular growth and associated endocrine and paracrine factors. As mentioned previously, the period of dominance and contents of follicular fluid influences the maturation of oocytes in cows. Defective nuclear and cytoplasmic maturation are important causes of reduced oocyte quality. Oocytes that have not undergone nuclear maturation can not be fertilized, and cytoplasmic maturation is essential for acquiring developmental competence and for further development to the embryonic stage (Krisher, 2004). The mRNA and proteins accumulated during the growth phase of arrested oocytes are important to support and regulate embryo development after fertilization (De Sousa et al., 1998). The embryo is largely dependent on the oocyte transcription factors and proteins until it reaches late four-cell to eight-cell stage (Memili and First, 2000).

Several methods proposed to evaluate oocyte developmental competence include morphological, cellular and molecular predictors. Relative to classical methods using morphology (ooplasm appearance, cumulus layers and meiotic spindle morphology), cellular and molecular characteristics (gene expression profiling of follicular and cumulus cells and analysis of follicular fluid content) are found to be more reliable (Wang and Sun, 2007).
1.3.3 Corpus luteum function

1.3.3.1 Formation of corpus luteum

Formation and proper function of the CL is essential for the early embryonic development and establishment of pregnancy in many species including cows. The development of CL begins around pre-ovulatory period with changes occurring in the thecal and granulosa cells of the follicle following the surge release of LH. Proliferation, reorganization and neovascularisation are the main events that take place in an ovulated follicle as it is transformed into a corpus haemorrhagicum and eventually forms a CL. In order to evaluate the function and quality of CL in cows it is necessary to understand the molecular mechanisms behind its formation and maintenance. A CL consists of two types of steroidogenic cells named large luteal cell (LLC) and small luteal cell (SLC) (Donaldson and Hansel, 1965; Alila et al., 1984) that are derived from the granulosa and theca cells of the follicle respectively. Steroidogenic cells are closely associated with the extensive vascular network of the CL and these cells are intermixed within the luteal tissue (O’Shea et al., 1989). The nonsteroidogenic cell types are fibroblasts, endothelial cells, pericytes and blood cells (Priedkalns and Weber, 1968; Farin et al., 1986). The pre-ovulatory LH surge causes the luteinisation of theca and granulosa cells which start to synthesize and secrete P₄ (Wuttke et al., 1997). The transition from E₂ secretion to P₄ is due to the transformation that occurs in the steroidogenic pathway of luteal cells. The synthesis of P₄ by the CL and the, P₄ concentration in blood are found to be dependent on the amount of steroidogenic tissue in CL, blood flow to CL and the capacity of steroidogenic tissue to synthesize and secrete P₄ (reviewed by Niswender et al., 2000).
1.3.3.2 Maintenance and function of corpus luteum

The CL is maintained by various factors of luteoptrophic events within the CL, as well as factors that protect against luteolytic factors. Cellular communication is an essential part in the maintenance of CL function. Gap-junctional and humoral pathways of cellular contact are found to occur between luteal cells (Grazul-Bilska et al., 1997). Connexin 43 (Cx43) is the major connexin molecule found in the borders of luteal cells and forms gap junctional intracellular communication.

It has been established that LH is the principle luteotrophic hormone in farm animals supporting formation of CL, its maintenance, and P₄ synthesis and secretion (Hoffman et al., 1974; Rajamahendran and Sianangama, 1992; Kawate et al., 2000). Studies in ewes demonstrated the necessity of LH for the maintenance of normal expression of genes encoding steroidogenic acute regulatory protein (STAR), cytochrome P450 family 11 subfamily A polypeptide 1 (CYP11A1; formerly known as P450scc) and 3-beta-hydroxysteroid dehydrogenase (3βHSD) enzymes which are responsible for the transport of cholesterol and conversion of cholesterol to P₄ in luteal cells (Haworth, 1997; Bao and Garverick, 1998).

In bovine luteal cells, IGF1 activates an intrinsic tyrosine kinase (Chakravorty et al, 1993) which is thought to modify cytoskeleton and increase the secretion of P₄. IGF1 also maintains luteal weight by preventing cellular death (Davis et al., 1996). Together with growth hormone GH, IGF1 exhibits an anti-apoptotic effect in porcine luteal cells (Ptak et al., 2004).

Angiogenesis is another very important process in the formation and maintenance of rapidly growing CL (Fraser and Wulff, 2003). Neovascularisation and migration of
blood cells into the luteal tissue occur post-ovulation and continue until the regression of CL. Vascular endothelial growth factor (VEGF) is the principle angiogenic substance involved in the formation of luteal vasculature; acting as a mitogen and regulating the cell migration and tube formation of endothelial cells thereby influencing CL growth and maintenance, and P₄ secretion (Kamada et al., 2004). VEGF acts through two tyrosine kinase receptors; namely the fms-related tyrosine kinase 1 (FLT1) and the kinase insert domain receptor (KDR) (Ferrara et al., 2003). Other factors associated with angiogenesis are fibroblast growth factor 2 (FGF2) and IGF1 with similar functions as VEGF (Robinson et al., 2007); secreted protein, acidic, cysteine-rich (osteonectin) (SPARC) a matrix cellular protein identified in ovine CL found to stimulate endothelial cell proliferation and angiogenesis (Smith et al., 1996a; Jendraschak and Sage, 1996), and the angiotensin converting enzyme (ACE) that is found in the endothelial cells of bovine CL (Kobayashi et al., 2001b). Another category of luteotropic factors found in CL are the cytokines including interleukins (IL), and tumor necrosis factors and IFNs (Petroff et al, 1999; Nishimura et al, 2004). IL1 alpha (IL1A), IL1 beta (IL1B) and tumor necrosis factor alpha (TNF) differentially function as local modulators to regulate prostaglandin F2 alpha (PGF2α) and prostaglandin E2 (PGE2) production in bovine CL (Skarzynski et al., 2003).

Apoptosis of luteal cells is a well characterized feature in the process of luteal regression. Apoptosis is associated with the increased expression of apoptotic genes. Fas antigens (Fas) are cell surface receptors capable of inducing apoptosis when bound to Fas ligands (Fas L) (Nagata, 1997). The presence of Fas mRNA was recently demonstrated in the bovine CL, but Fas L alone does not induce apoptosis in bovine luteal cells.
(Tanigushi et al., 2002). The later report also demonstrated that the anti-apoptotic action of Fas L can be escalated by IFNγ and modulated by TNF in bovine luteal cells. In addition to Fas, apoptosis is regulated by the expression of other genes, such as B-cell lymphoma 2 (BCL2), BCL2 associated x protein (BAX) (Rueda et al., 1997) and cysteinyi aspartic acid-proteases (caspases) (Pru and Tilly, 2001) in luteal tissue. Rueda et al., 1999, demonstrated that the caspase-3 mRNA levels are increased in the CL after induction of luteolysis by PGF2α treatment. Cells are protected from apoptosis by BCL2 expression, but an elevated level of BAX expression increases cell death (Tilly, 1996).

**1.3.3.3 Role of progesterone on establishment of pregnancy**

Progesterone is indispensable in creating a suitable endometrial environment for early embryo development, implantation, and the maintenance of pregnancy. During pre-implantation embryo development, P4 increases embryo growth and induces the secretion of IFNτ by acting on the endometrium to stimulate the production of a variety of embryotrophic factors (Geisert et al., 1992). Furthermore, P4 influences the timing of luteolysis in cows and poses a risk to the developing embryo. Late post ovulatory P4 rise gives less time for the P4 exposure to the endometrium which leads to a poorly developed embryo that is unable to secrete sufficient amounts of IFNτ to inhibit luteolytic cascade (Mann and Lamming, 1999). In addition, P4 is necessary to maintain uterine quiescence that allows for proper implantation of the embryo within the endometrium and for fetal development (McDonald et al., 1952). Another role of P4 is to act locally to regulate luteal function. In cows it is believed that intra-luteal P4 is implicated in a survival pathway in the CL by stimulating prostaglandins (PGs), oxytocin (OT) and its own production in a stage specific style and inhibiting apoptosis (Skarzynski et al., 2008).
1.3.3.4 \( P_4 \) and embryonic loss

It has long been established that the outcome of AI is dependent on sufficient concentration of \( P_4 \) that is maintained during early pregnancy. Many studies have demonstrated that reduced concentrations of \( P_4 \) either in milk (Lamming et al., 1989; Mann et al., 1995) or plasma (Mann et al., 1995 & 1996; Buttler et al., 1996; Mann et al., 2001) are associated with reduced PR in cows. In a review, Inskeep, (2004) has pointed out four major time periods during embryo development in cows that are susceptible to lower concentrations of \( P_4 \), which results in embryonic mortality. They are: early postovulatory period (when lower \( P_4 \) exists during preceding luteal phase); days 4 through 9 after mating; the period of maternal recognition of pregnancy (days 14 through 17 with slightly elevated \( E_2 \) concentration), and the late embryonic period (days 28 to 42). In addition, it is well documented that a delay in the post ovulatory \( P_4 \) rise is most important for embryo development rather than the concentrations achieved at later stages in cows and ewes (Garrett et al., 1988; Nephew et al., 1991). Even though studies reported lower concentrations of \( P_4 \) in plasma or milk during luteal phase or pregnancy of lactating dairy cows, it is unclear whether this is due to reduced production by CL or increased metabolism of secreted \( P_4 \). Supplementation with \( P_4 \) during early pregnancy has given unequivocal results with most studies reporting increased PR in lactating cows while no or reduced effects in heifers (Van Cleeff et al., 1991 &1996; Macmillan and Peterson, 1993; Villarroel et al., 2004; Larson et al., 2007).

1.3.4 Fertilization failure

Oocytes and sperm can fail to fertilize due to a number of reasons. The success depends on oocyte and sperm quality as well as the uterine and oviductal environments
that process and transport the gametes. The importance of oocyte health and oviductal and uterine environments are described elsewhere in the introduction section. Timing of AI is an important factor that determines the viability and quality of gametes and successful fertilization. Thus in dairy herds, fertilization failure due to inaccurate timing of AI is associated with poor reproductive management and inadequate estrus expression in cows. Available evidence in cows suggests a fertile window of 6 to 24 h after ovulation for ovum and 12 to 24 h in the oviduct for sperm (Hafez and Hafez, 2000; Gordon, 2003).

Aged sperm often exhibits loss of acrosome integrity, alterations in the cytoplasmic content and structural defects in the chromosomes. In addition, structural barriers within the female reproductive tract that prevent the union of gametes can cause fertilization failure. However, animals with those anatomical abnormalities are usually culled from the herd after several attempts of breeding as nulliparous cows (Hafez and Hafez, 2000).

Even though the PR has declined, the fertilization rates observed in dairy cows are generally high (88 – 90%). The majority of studies and unpublished data from our lab reported similar fertilization rates for lactating dairy cows and heifers (Gordon, 2003); however, Sartori and others have shown that during summer fertilization rates are higher in heifers compared to lactating cows (Sartori et al., 2002b).

1.3.5 Embryonic loss in cows

An obvious reason for the reduced PR in high producing lactating cows over the years is embryonic mortality. Embryonic loss can be caused by intrinsic and/or extrinsic factors. Reduced developmental competence of the embryo and chromosomal defects are
the main intrinsic causes. Alterations in the maternal environment, asynchrony between embryo and mother, and failure of mother to respond to embryonic signals are the extrinsic factors which compromise the embryonic survival (Hansen, 2002). In bovines the early embryonic stages are more vulnerable than the later developmental stages. Considerable amount of embryonic mortality is observed in dairy cows between days 1 and 42 of pregnancy resulting in reduced calving rates of about 55% (Peters, 1996). Higher rates of embryonic loss in bovines are reported during the first two weeks of pregnancy and up to 40% of total embryonic losses occurs between days 8 and 17 of pregnancy (Dunne et al., 2000) indicating that early embryonic mortality is the major cause of pregnancy failure (Humblot, 2001). Therefore early embryo development is critical for the successful implantation and establishment of pregnancy. Fertilization failure and pregnancy losses of dairy cows are depicted in a chart (Fig 1.4). Proper embryo development and implantation depend on several factors such as embryos with qualities favourable of growing to the blastocyst stage and hatching, adequate endometrial receptivity, and successful interaction between the embryo and the endometrium (Chen et al., 1999).
Figure 1.4 Schematic presentations of the components contributing to reduced fertility in dairy heifers and lactating cows between day 1 of gestation and parturition. FR fertilization rate, PR1 pregnancy rate on day 21 of gestation, PR2 pregnancy rate on day 75 of gestation, CR calving rate (Modified from Walsh et al., 2011).
1.3.5.1 Genetic factors involved in embryonic mortality

It is considered that embryonic mortality serves as an efficient way of eliminating genetically aberrant embryos before birth thus reducing the incidence of chromosomally abnormal infants (Jacobs, 1982). From an agricultural point of view, embryonic mortality among domestic animals causes a reduction in PR and leads to economic loss. Embryos produced with chromosomal defects are unable to continue development and lost progressively during early developmental stages.

The major chromosomal abnormality reported in embryos is an abnormal chromosomal number (aneuploidy; King, 1991). Abnormal segregation during gametogenesis and cleavage of embryos and polyspermy are the main reasons for the occurrence of aneuploidy. This can be reflected as polyploidy or mixploidy or monosomy. A true polyploid embryo, i.e., one in which all the cells of an embryo consisting of polyploid chromosomes, would not survive beyond the eight-cell stage (Hyttel et al., 2001), while those with mixploid chromosomes with up to 25% polyploid trophoblast cells are compatible with pregnancy (Hare et al., 1980). In addition, Kawarsky and others have shown that development rates (assessed by cell numbers) were slowest in haploid and polyploidy embryos, intermediate in aneuploid embryos, and fastest in mixoploid and diploid embryos in cows (Kawarsky et al. 1996).

Furthermore, embryonic loss in cows has been attributed to homozygosity for certain blood groups and substances (β-globulin, J-antigen; uridine-5′-monophosphate synthase; Zavy, 1994; Hafez and Hafez, 2000). Inbreeding is believed to be a potential cause for chromosomal abnormalities that produced recessive lethal genes (Hansen, 2002).
1.3.5.2 Embryo quality and pregnancy loss

In cattle, embryo quality determines whether or not the growing embryo continues to develop and produce a live calf. Other than the inputs from oocyte and sperm, intrinsic factors of the developing embryo are crucial determinants of embryonic quality. During early and late embryonic development, embryonic genome activation, compaction, blastocyst formation, elongation of the trophoblast, secretion of IFNτ, differentiation of placental tissues, and development of the inner cell mass into a fetus take place. The quality of an embryo depends on the coordinated occurrence of these events which in turn determine the success of pregnancy.

Reduced embryo quality is one of the important causes of embryonic mortality that leads to pregnancy loss in mammals including bovines. Many studies have reported that good quality embryos produced better PR compared to poor quality ones in cows (Donaldson, 1986; Hasler, 2010). There are also studies reporting no differences in PR derived from different quality embryos graded according to their morphology (Spell et al., 2001).

1.3.5.3 Current methods evaluating embryo quality

External morphology of the embryo has been widely used to assess their quality prior to transfer in many species (Hasler, 1998), but, the predictive value of embryo morphology before the expression of the embryonic genome is limited in bovines (Rijnders and Jansen, 1996). Overall morphological assessment of embryos includes parameters such as size and shape of the blastomeres, presence of extruded cells or fragmentation, compaction, colour of the embryo, timing of development and stage at a certain time after fertilization (Merton, 2002). In addition, the thickness of the zona
pellucida and the size of the perivitelline space can be used to evaluate the quality of embryos.

Currently, measuring the metabolic activity of embryos using culture medium is considered to be a non-invasive and feasible method to assess embryo quality. Oxygen consumption, nutrient uptake, production of metabolites and secretion of various factors by the embryo can be measured and used to indirectly reflect the quality of embryos (Abe and Hoshi, 2003; Sakkas and Gardner, 2005). Vital staining using fluorescent probes to evaluate membrane integrity and cryoresistance are some of the partially invasive methods used to evaluate bovine embryos. Furthermore, invasive techniques such as cytogenetic analysis, differential staining, TUNEL staining, electron microscopy and mRNA expression are employed to evaluate the quality of embryos more precisely using specific functions of the developing embryo (Van Soom et al., 2001). Since morphology assessment is of lower predictive value in assessing the quality of embryos between studies, it is essential to develop higher quality assessment methods in the future.

1.3.5.4 Events reflecting embryo quality during pre-implantation development

After fertilization in the oviduct, the zygote moves towards the uterus while undergoing several cell divisions. At the 8-16 cell-stages, about 4 to 5 days after ovulation, the bovine embryo reaches the uterus. The morula stage is reached by day 5 to 6 post ovulation, and on day 7 the first differentiation and formation of the blastocyst occur. By the end of the first week of development, the embryo is at the blastocyst stage and consists of blastocoele and two distinct cell types: the trophectoderm, the outer layer
of rapidly dividing cells, and the inner cell mass, which comprises undifferentiated cuboidal cells (King, 1991).

Blastocyst formation is initiated at compaction and characterized by cellular polarization and differentiation (Pratt et al., 1982). In the bovine embryo, compaction occurs around the 16 to 32 cell stage (Betteridge and Flechon, 1988), and is dependent on the well-orchestrated expression of factors controlling cell adhesion and differentiation of trophoblast cells (Watson et al., 1999).

1.3.5.5 Embryonic genome activation

Embryonic genome activation is an important event during the development of the pre-implantation embryo. After fertilization, maternal mRNA from the ooplasm will be used until the fourth/fifth cell cycle of the growing embryo (Barnes and First, 1991; Plante et al., 1994; Memili and First, 1998). With the activation of the embryonic genome, maternal mRNAs are replaced by embryonically expressed ones (Kanka, 2003) and this switch from maternal to embryonic transcription is called the maternal-embryonic transition (Badr et al., 2007). Bovine embryonic genome activation takes place at two time points. First, the minor gene activation occurs between 1 to 4 cell stage embryos, which is evident by low level of transcriptional and translational activity (Memili et al., 1998), similar to that seen in mouse and rabbit embryos (Telford et al., 1990). Secondly, the major genome activation takes place, coinciding with the maternal-embryonic transition which takes place during the period of the fourth cleavage cycle (8-16 cell stage) in bovine embryos. This is characterized by increased transcriptional activity (King et al., 1988; Pavlok et al., 1993), functional organization of the nucleolus (Laurincik et al., 2000), and changes in protein synthesis (Frei et al., 1989). At the end of
embryonic genome activation the embryo is dependent on transcripts from its own genome for the rest of its development (De Sousa et al., 1998b).

### 1.3.5.6 Embryonic gene expression

Most of the genes in early embryos are expressed in a stage-specific manner exhibiting two major patterns: starting after the onset of genomic activity or expression throughout the period before and after embryonic genome activation (Niemann and Wrenzycki, 2000). Expression of developmentally important genes is related to the two cell lineages (inner cell mass and trophectoderm) of the early embryo (Wrenzycki et al., 2003). This well controlled spatial gene expression pattern has a role in the regulation of pre-implantation development. Currently, arrays of genes that are expressed in pre-implantation embryos are under investigation in many species to unravel their exact role in the early stages of embryonic development. In addition, analysis of gene expressions through measurements of the relative abundance of mRNAs in embryos is considered to be a tool to estimate embryo quality (Rizos et al., 2002a, b & 2003; Jones et al., 2008).

### 1.3.5.7 Blastocyst formation and expansion

Formation of the fluid-filled cavity (cavitation) in the early embryo occurs at the initiation of blastocyst development, and is mediated by fluid transfer across the blastomeres that are present on the peripheral area of the embryo (Watson, 1992). Cavitation occurs in embryos before the formation of the epithelial trophectoderm and the inner cell mass (Watson, 1992). The cavitation process requires a polarized epithelium in order to bring water in and develop the blastocoel (Kidder and Watson, 2005).
Na/K-ATPase actively transports ions across the trophectoderm and contributes to the formation of the fluid filled cavity (Watson, 1992). Na/K-ATPase is made up of two subunits, a catalytic, α-subunit and a β-subunit (Kidder and Watson, 2005). Both α and β subunits are important for the blastocyst formation and the activity of the Na/K-ATPase enzyme (Jorgensen, 1986; Madan et al., 2007). Betts and others demonstrated the expression of Na/K-ATPase subunit isoform (α & β) during pre-implantation embryo development in bovines (Betts et al., 1997). They also showed that ouabain, a strong inhibitor of Na/K-ATPase, affected blastocyst formation in early bovine embryos. The accumulation and composition of fluid in the blastocoele during early embryonic development is critically regulated by Na/K-ATPase activity and is essential for differentiation of inner cell mass and trophectoderm cell types (Watson, 1992; Betts et al., 1997 & 1998).

1.3.5.8 Energy metabolism

Two different mechanisms: (a) an active, sodium-dependent transport (SGLT) which couples glucose uptake with Na\(^+\) influx against a concentration gradient, and (b) passive energy independent transport via facilitative hexose transporters (GLUT) mediate transport of energy substrates across plasma membranes in mammalian cells. The family of facilitative hexose transporters, currently comprising several isoforms (GLUT1 - 14), have common structural features (Douard and Ferraris, 2008).

Embryonic genome activation in pre-implantation embryos coincides with a change in metabolism, which switches from the utilization of lactate and pyruvate to glucose and fructose as the main energy source (Rieger et al., 1992; Khurana and Niemann, 2000). An embryo which undergoes compaction, cavitation and first
differentiation, demonstrates marked changes in energy substrate utilization with increased hexose consumption that requires specific regulation of hexose transport by the early embryo. As with glucose, fructose is utilized by the bovine embryos in-vitro (Guyader-Joly et al., 1996) and is present in the uterine fluid of a cow (Suga and Masaki, 1973), human (Casslen and Nilsson, 1984) and sow (Bazer et al., 1991). Moreover, it has been reported that fructose supplementation in the culture medium improves pre-implantation embryo development and quality in bovines (Bhuiyan et al., 2007; Chaubal et al., 2008).

Hexose transport is up-regulated by the early embryo through increased expression of facilitative hexose transporters in bovine embryos (Augustin et al., 2001 and Leppens-Luisier et al., 2001). Thus far in bovines, transcripts for GLUT1 -5 and -8 have been reported in pre-implantation embryos (Lequarre et al., 1997, Navarrete Santos et al., 2000, Augustin et al., 2001 and Wrenzycki et al., 2001 & 2003). GLUT1, -3 and -8 are expressed throughout bovine pre-implantation embryonic stages while GLUT5 and -2 are demonstrated at early morula and late blastocyst stages, respectively. GLUT5 is the best-characterized fructose transporter and its mRNA expression starts at the 8 to 16 cell stage embryo in cattle. Beginning of GLUT5’s expression coincides with the time of embryonic genome activation (Augustin et al., 2001) and increases significantly from morula towards the blastocyst stage (Lonergan et al., 2003; Camero & et al., 2005). Furthermore, GLUT5 mRNA levels are described as a potential marker of developmental competence in pre-implantation bovine embryos (Gutierrez-Adan et al., 2004).
1.3.5.9 Expression of interferon tau

The term maternal recognition of pregnancy has been used in its broadest sense to define how the mother responds to the presence of a conceptus within her reproductive tract and, particularly, how such events allow the pregnancy to advance successfully (Short, 1969). The important outcome of the embryo-maternal interaction in most mammals is the extension of the functional lifespan of the CL and thereby the production of uterotrophic steroid hormones especially P₄ which provides a conducive environment for embryo development. Absence of embryo signalling within a critical period of time will result in loss of the pregnancy and seems to be higher in farm animal species (Wilmut et al., 1986; Roberts et al., 1990).

Godkin and others (Godkin et al., 1982) purified a low molecular weight protein released into culture medium by day 13-21 ovine conceptuses. This secretory protein was eventually designated as ovine trophoblast protein-1 (oTP-1) (Godkin et al., 1984). Shortly thereafter, it was demonstrated that bovine conceptuses produce a similar complex of proteins, bovine trophoblast protein-1 (bTP-1) (Bartol et al., 1985) that cross-react with oTP-1 antiserum (Helmer et al., 1987).

Ovine and bovine trophoblastic proteins were identified as type I Interferons (IFN-I) by molecular cloning of complimentary DNA (cDNA) (Imakawa et al., 1987 & 1989) and protein sequencing (Stewart et al., 1987; Chapigny et al., 1988). This revealed a novel role for IFN in the normal physiology of early embryonic development. The trophoblast IFNs (IFNτ) possess a number of unique features which not only suit them well for their role in the establishment of pregnancy but also distinguish them from other
well characterized type I IFNs (Stewart et al., 1989, Klemann et al., 1990 and Charlier et al., 1991).

The trophoblast IFNs are secreted by the conceptus in large amounts between days ~12 and 21 of gestation in sheep (Hansen et al., 1988; Farin et al., 1990) and days ~14 and 25 of gestation in cattle (Helmer et al., 1987; Farin et al., 1990). They are involved in triggering a series of maternal responses such as prolonging the lifespan of the CL (Godkin et al., 1984b; Knickerbocker et al., 1986a; Thatcher et al., 1989), changes in endometrial protein synthesis (Godkin et al., 1984a; Vallet et al., 1987; Salamonsen et al., 1988; Sharif et al., 1989), and inhibition of OT induced PGF2α production by the uterus (Fincher et al., 1986; Knickerbocker et al., 1986b; Salamonsen et al., 1988). These trophoblast proteins also possess potent antiviral and antiproliferative activities (Roberts et al., 1989).

It is established that IFNτ is the primary agent responsible for the maternal recognition of pregnancy in cattle (Roberts et al., 1992). Bovine embryos begin to express IFNτ immediately after the formation of blastocyst (Farin et al., 1990) and its production is primarily dependent on the presence of a functional trophectoderm (Hernandez-Ledezma et al., 1993). Therefore, the level of IFNτ secretion has been discussed as a parameter for the assessment of embryo quality. More information on the properties, actions and the use of IFNτ are described in later sections (1.4.1 and chapter 5).

1.3.5.10 Stress related gene expression

Heat shock responses were first discovered in the fruit fly *Drosophila melanogaster*, manifested as puffing in salivary gland chromosomes (Ritossa et al.,
Twelve years later the gene products responsible for the heat shock response were identified as heat shock proteins (HSPs) (Tissiere et al., 1974). Later the sequence, structure, location and mode of interaction of HSPs were identified (Westwood et al., 1991).

Four major families of HSPs are identified according to their molecular size: HSP90, HSP70 (HSPA1A), HSP60 and HSP27. Recently a high molecular size HSP (HSP105) was identified during mouse embryo development (Hatayama et al., 1997). HSPs serve two major functional roles. Firstly they: 1) act as intracellular house-keeping proteins (molecular chaperones) by regulating transport of proteins into cellular compartments, 2) cause the folding of proteins, 3) cause the degradation of unstable proteins, 4) act towards the prevention of protein aggregation, 5) take control of regulatory proteins and the refolding of miss-folded proteins (Bakau and Horwish, 1998).

Secondly HSPs are synthesized in response to a wide variety of cellular injuries that are induced by changes in temperature as well as other stress conditions, such as the presence of free oxygen radicals, infections, heavy metals, ethanol, and ischemia (Linquist, 1986 and Welch, 1992). Increased HSP70 expression is associated with increased ability of the stressed cells to protect them from cell death (Mosser et al., 1997; Nollen et al., 1999) by interacting with apoptotic mechanisms (Buzzard et al., 1998; Li et al., 2000; Saleh et al., 2000; Parcellier et al., 2003).

Heat stress is one of the environmental factors that can affect reproductive efficiency in animals. Several studies in mice, rats and humans have demonstrated the expression and importance of HSPs during spermatogenesis (Dix, 1997; Allen et al., 1988 a & b; Lee, 1990; Dix et al., 1996; Werner et al., 1997) and oogenesis (Cruci et al., 1962).
1987 & 1991; Lenz et al., 1983; Baumgartner and Chrisman, 1981). In mice, although the HSP70 family has received special attention, members of the 60kD and 90kD HSP families are also synthesized in pre-implantation embryos (Bensaude et al., 1983). Spontaneous HSP70 expression begins with the onset of mouse zygotic genome activity but inducible HSP70 expression is still absent (Morange et al., 1984). The predominant HSP expressed up to the blastocyst stage is HSP70 and it can be induced by heat shock from the blastocyst stage onward (Wittig et al., 1983).

Fertility of cows was compromised when they were exposed to increased environmental temperature. Embryo development found to be affected by elevated ambient temperatures (Ealy et al., 1993). It has also been demonstrated that, bovine 2-cell embryos were more severely affected by heat shock than other stages (Edwards and Hansen, 1997). Since embryos were very sensitive to environmental stresses during their development they produced factors associated with thermo protection such as heat shock proteins (HSP70) to protect themselves (Kawarsky et al., 1998; Wrenzycki et al., 1999). HSP70 was produced due to the transcription of the heat inducible gene (HSP70) of the HSP family and its expression pattern and function are demonstrated during early embryonic development (Kawarsky and King, 2001).

**1.3.5.11 Apoptosis and embryo quality**

Apoptosis, the process of programmed cell death occurs in multi-cellular organisms. Wide variety of stimuli either physiological or pathological in origin can induce apoptosis and control the growth and differentiation of cells (Elmore, 2007). Development, aging, immune reaction, infection, toxicity, heat shock etc. are some of the common causes of apoptosis (Antonsson, 2001).
During apoptosis molecular and biochemical changes take place and result in the morphological alteration of the cells (Saraste, 2000). Shrinkage and deformation of cells are common morphological observation in apoptosis. Chromatin aggregation occurs at the nuclear membrane; plasma membrane undergoes blebbing/budding and eventually the cells will be fragmented into pieces bound by membrane (Kerr et al., 1972; Schimke et al., 1994; Ko and Prives, 1996; Edwards, 1998).

Apoptotic process involves two main pathways the mitochondrial pathway and the death receptor pathway (Ingo et al., 2000). Several gene transcripts are found to be involved in the apoptotic process in which the BCL2 gene family is the most important one. The BCL2 family of proteins are involved in the regulation of the mitochondrial apoptotic pathway. These genes are classified into two groups, anti-apoptotic (BCL2, BCLW, BCL-XL, AL, MCL1) and pro-apoptotic (BAX, BAK, BOK, BIK, BLK, HRK, BNIP3, BIM, BAD, BID, BCLXs) in mammals (Cory and Adams, 1998). Among them BCL2 and BAX genes are found to regulate apoptosis and thereby the cell survival. BAX was the first pro-apoptotic homolog of BCL2: it is found in B cell lymphoma 2 and accelerates cell death by heterodimerizing with BCL2, and thus neutralizes the effects of BCL2 on cellular survival (Oltvai et al., 1993).

Evidences for the occurrence of apoptosis in embryos are found in many species including bovines (Hardy, 1999). Inadequate culture conditions and lack of survival factors are considered as major causes of apoptosis in early stage embryos in-vitro. However, in-vivo, maternal environment, hormones, growth factors, heat stress, metabolic changes etc. can contribute to the apoptotic state of the developing embryo. Bovine embryos undergo apoptotic cell death by exhibiting typical morphological
changes during their early stage of development (Yang and Rajamahendran, 1999). Unequal sized blastomeres and cellular fragmentations, which are typical signs of apoptosis, were observed in the developing embryos. These embryos are poor in quality and unable to continue development to establish a viable pregnancy.

The expression of apoptotic genes (BCL2 and BAX) have been demonstrated in pre-implantation embryos of many species including cows (Kolle et al., 2002; Yang and Rajamahendran, 2002; Jurisicova et al., 2003). Studies by Jurisicova et al., (1998) and Exley et al., (1999) revealed that changes in the expression of BCL2 and other members of the BCL2 family were associated with fragmentation of embryos at the pronucleate and blastocyst stages. It was demonstrated that the relative expression of BCL2 and BAX proteins in the bovine pre-implantation embryos were indicative of their quality (Yang and Rajamahendran, 2002). The later study revealed that healthy and fragmented embryos expressed BCL2 and BAX proteins in excess, respectively. A study by Lonergan et al, (2003) also found that the expression of the BAX gene in the blastocysts might represent the blastocyst quality, and therefore can be used as a marker to assess embryo quality.

1.3.6 Oviductal environment

Oviduct is the site of fertilization and the first site that comes into contact with the early embryo. It actively participates in the transport of early embryonic stages toward the uterus. Several important events such as sperm capacitation, fertilization and early embryo development take place within the oviduct; therefore, the oviduct has the potential to contribute necessary factors that affect fertility. In vivo and in vitro studies have demonstrated the profound importance of the oviductal environment for early
embryonic development in many species (Boland, 1984; Ectors et al., 1993; Polge et al., 1972; Enright et al., 2000; Galli and Lazzari, 1996). Various paracrine and autocrine systems involving growth factors, cytokines and PGs secreted by oviductal epithelial cells have been shown to mediate embryo growth by directly acting on the embryo and promoting action of the oviductal microenvironment (Martus et al., 1997; Pushpakumara et al., 2002; reviewed by Ulbrich et al., 2010).

1.3.7 Uterine environment

The uterus is an important organ in the reproductive tract of mammals and has a multifaceted role during the active reproductive phase. Regulation of the estrous cycle, transport and capacitation of spermatozoa, early embryo development, implantation and support of pregnancy throughout gestation are the known main functions of the uterus. Growth and development of the conceptus is controlled to some extent by the maternal uterine environment (Wilmut and Sales, 1981; Garett et al., 1988; Lawson et al., 1983). Many factors including steroid hormones, cytokines and growth factors have been shown to control endometrial differentiation and proliferation during the period of pre- and post-implantation (Chen et al., 1999). Uterine regulation of conceptus development could occur through the selective transport of serum growth factors into the uterine lumen and/or through local endometrial secretion of polypeptide growth factors under the influence of the ovarian secretion of P₄.

Holistic transcriptome and proteome studies of the uterus have revealed quantitative and qualitative changes in the genes at different stages of the estrous cycle and pregnancy (Katagiri and Takahashi, 2004, Bauersachs et al., 2005; Klein et al., 2006; Michael et al., 2006). These spatial and temporal changes were mainly due to the
expression pattern of sets of genes in the uterine environment, where the endometrium is especially important for the embryo-maternal interaction and successful establishment of pregnancy. Endometrial secretory components synthesized and released during the pre-implantation period were regulated by hormones such as P₄, E₂ and OT as well as by the secretions of the embryo itself (Spencer et al., 2004).

**1.3.7.1 Role of endometrial secretions during pre-implantation period**

In cows, the embryo enters the uterus as early as 4 - 5 days post ovulation, but implantation takes place only after 20 days of gestation. In contrast, in primates the embryo attaches immediately after it enters the uterus. Therefore, the histotroph secreted by the endometrial glands is important in cows to nourish the embryo until implantation. Many enzymes, growth factors, hormones, cytokines, amino acids, energy substrates, transport proteins and other substances have been identified in histotroph (Bazer and Roberts, 1983; Kim et al., 2011).

**1.3.7.2 Insulin like growth factor system (IGFs)**

IGF-I and –II are members of the insulin family and are called somatomedins (LeRoith and Roberts, 1993). IGFs have had multiple functions in the growth, proliferation and differentiation of cells and in protein and carbohydrate metabolisms in tissues (Zapf and Froesch, 1986; Simmen et al., 1989; LeRoith et al., 1991). IGF-I and -II were expressed temporally in the bovine endometrium (Robinson et al., 2000). Ovarian steroids have been known to regulate in part the levels of endometrial IGF mRNA levels and luminal IGF content during the estrous cycle and early pregnancy in sows (Simmen et al., 1990) and cows (Robinson et al., 2000). The bioavailability of the IGFs is regulated
by a family of six binding proteins designated as IGFBP-1 through to IGFBP-6 (Giudice et al., 1994; Hossner et al., 1997). IGF-I and II were found to increase the production of IFNτ by embryos in ovines in-vitro (Ko et al., 1991).

1.3.7.3 Fibroblast growth factor

The FGF family has many autocrine and paracrine factors that have a variety of biological functions in multicellular organisms (Ornitz and Itoh, 2001). In many species a group of the FGF family regulates the trophectoderm development during early pregnancy. In mice, FGF4 originated from inner cell mass inhibits the differentiation of trophectoderm (Arman et al., 1998 and Feldman et al., 1995). Porcine FGF7 derived from endometrium stimulated the proliferation of trophoblast cells during embryonic development (Ka et al., 2001). Basic FGF (FGF2) acted as a mitogen, morphogen, and angiogenic factor to regulate early embryogenesis (Gospodarowicz, 1991). Expression of FGF2 and its receptor, FGFR2, were found during the blastocyst stage embryo development in cows (Daniels et al., 2000 and Lazzari et al., 2002). Treatment with FGF2 increased the size of mouse trophectoderm outgrowths (Haimovici et al., 1991; Tanigushi et al., 1998), stimulated gastrulation in rabbit conceptuses (Harbed et al., 1995), and enhanced embryo growth to blastocysts when given together with another growth factor, TGFβ (Lin and Hansel, 1996; Larson et al., 1992). The endometrial-derived FGF2 has been shown to regulate neovascularization during placental attachment and syncytium formation in ovines (Reynolds and Redmer, 2001). Recently it was reported that FGF2 expressed by the bovine endometrium during the estrous cycle as well as early pregnancy, and mediated trophectoderm proliferation and regulated IFNτ production by trophectoderm cells of the blastocyst stage embryo (Michael et al., 2006).
1.3.7.4 Endometrial cytokines

Expression of pro-inflammatory cytokines such as TNF and IL1 in the endometrium of many animal species including bovines has been well documented (Tabibzadeh and Sun. 1992; Kover et al. 1995; reviewed by Okuda and Sakumoto. 2006). Even though, IL1A and TNF were established as major mediators of immune response (Tzianabos and Wetzler, 2004), they also regulated reproductive functions (Simon and Polan, 1994; Ingman and Jones, 2008). TNF and IL1 were expressed in the endometrium throughout the estrous/menstrual cycle and during pregnancy and were assumed to have multi-functional role in reproduction such as uterine and placental functions and embryonic development (Tartakovsky and Ben-Yair. 1991; reviewed by Hunt et al. 1993).

In cattle, TNF and IL1 are expressed in the endometrium in a temporal manner and influence uterine functions, as reviewed by Okuda and Sakumoto, (2006). In their review the authors discussed the functional role of TNF and IL1 in the secretion of PGs in the bovine endometrium. TNF and IL1 were considered luteoprotective agents during the mid-luteal phase of the estrous cycle by regulating the ratio between PGE2 and PGF2α secretion (Okuda and Sakumoto, 2006). In bovines, PGE2 increased luteal survival whereas PGF2α was a luteolytic agent (Thibodeaux et al. 1992; reviewed by Miyamoto and Shirasuna, 2009). The PGs were secreted by the endometrium and transferred to the CL through a counter current mechanism (from uterine vein to ovarian artery) and exert their effects (McCracken et al. 1999). Apart from the direct effect of TNF and IL1 on endometrial PG secretion, they were also transferred to CL and regulated luteal function during the estrous cycle.
1.3.7.5 **Serpin peptidase inhibitor, clade A member 14**

Serpin peptidase inhibitor, clade A member 14 (SERPINA14) also known as uterine milk protein is a member of the clade A serine proteinase inhibitor super family and is known to be expressed mainly in the endometrium of certain mammals. Even though other members of the serpin family were inhibitory of serine proteases, SERPINA14 exhibited no such activity in the uterus (Padua and Hansen, 2010).

SERPINA14 is expressed in the endometrium during the estrous cycle and pregnancy and was found to be regulated by E<sub>2</sub> and P<sub>4</sub> in pigs, ewes and cows (Leslie and Hansen, 1991; Bauersachs et al., 2005; Padua and Hansen, 2010). The endometrium of pregnant sheep secreted large quantities of SERPINA14 (Moffatt et al., 1987) and was found to modulate immune function by inhibiting lymphocyte proliferation (Skopets et al., 1992) and the cytotoxic activity of natural killer (NK) like cells (Liu and Hansen, 1993) and thereby prevented fetal rejection. In pigs, SERPINA14 is involved in iron transport to the fetus by binding to and stabilizing the iron-binding protein uteroferrin during pregnancy (Padua and Hansen, 2010).

In cows, SERPINA14 was expressed at higher concentrations during estrus, is reduced to a lower level and then gradually increases from the mid luteal phase and is present at higher concentrations throughout the pregnancy (Ulbrich et al., 2009a). In the later study, it was demonstrated that SERPINA14 secretion was mainly regulated by E<sub>2</sub> during estrus and then by P<sub>4</sub> during the luteal phase even though it was believed to be regulated only by P<sub>4</sub> previously (Leslie and Hansen, 1991).
**1.3.7.6 Endometrial apoptosis and heat stress**

Apoptosis has been widely studied in primate and rodent endometrium since their menstrual or estrous cycle undergoes prolific changes and implantation is more invasive compared to domestic animals. Apoptosis was negligible during the proliferatory phase, progressively increased during the secretory phase and peaked in the menstrual phase in human endometrium (Tabibzadeh et al., 1994).

As described for other tissues, apoptosis may influence the remodelling of endometrium in cows during the estrous cycle and pregnancy. In a recent study, Salilew-Wondim and others (Salilew-Wondim et al., 2010) assessed the nature of apoptosis using transcriptome fingerprinting. They showed in cows that anti-apoptotic genes associated with cell proliferation (e.g.: BCL2) were elevated in receptive endometriums and pro-apoptotic genes associated with cell death were higher in non-receptive endometriums in cows. In addition, previous findings also indicated the importance of the anti-apoptotic gene BCL2 for the survival of endometrial glandular cells, and those findings suggested that enrichment of anti-apoptotic genes and reduced levels of pro-apoptotic genes in receptive endometriums may indicate faster cellular proliferation and differentiation to prepare the endometrium for substantial remodelling (Gompel et al., 1994; Tabibzadeh et al., 1995; Daikoku et al., 1998).

Furthermore, P₄ in human, rat, ewe and cow endometrium was found to regulate the process of apoptosis during the luteal phase and early pregnancy. Reduced apoptosis at the morphological and molecular levels was demonstrated in the endometrium after treatment with external P₄ in humans (Lovely et al., 2005) and cows (Wang et al., 2003).
In all mammalian cells, heat shock 70 kDa protein 1 (HSPA1A) and other members of the HSP family has a role as a housekeeping gene by controlling apoptosis, protein folding and responding to internal as well as external stressors. In addition, HSPA1A were associated with cytoplasmic sex steroid receptors in the endometrium during the secretory phase of the menstrual cycle (Nanbu et al., 1996). HSPs are induced by many stressors such as hyperthermia, free oxygen radicals, heavy metals, ethanol, inflammation and infection (Neuer, 2000). Heat shock or stress is an important factor that induces the expression of HSPs. Elevated environmental temperature and heat produced due to high metabolism during lactation are predominant stress conditions in cows. Effect of heat stress has been demonstrated in the uterus of cows previously (Malayer and Hansen, 1990) where heat shock (elevated temperature) treatment of the endometrium induces the expression of HSPs.

1.4 Manipulation of uterine environment

Several approaches and methods have endeavoured to increase the PR in lactating dairy cows by improving the quality of the reproductive tract including the uterine environment. Pharmacological and nutritional manipulations of the uterine functions have been widely researched in lactating dairy cows and yield unequivocal results in terms of pregnancy success.

Many of the uterine manipulations were aimed either at preventing the luteolytic signal produced by the endometrium or extending the secretion of P₄ by CL in cows (Binelli et al., 2009). Since the anti-luteolytic signal originates from the developing embryo, the functional capacity of the embryo to produce sufficient amounts of that signal is critical at the time of maternal recognition of pregnancy. In addition, the
functional capacity of the endometrium and the optimal interaction between the embryo and the endometrial environment determine the efficient shutdown of the luteolytic cascade during early pregnancy.

**1.4.1 Use of interferon tau to manipulate uterine functions**

As mentioned before, pre-implantation embryo development depends heavily on the proper function of the endometrium. The developing embryo is nourished by various factors secreted by the endometrium and these factors were induced by the luteal P₄ and/or the embryo. IFNτ has been found to be an important agent that regulated the expression and secretion of various proteins in the endometrium (Bazer et al., 2008). Most of the studies have examined the effect of IFNτ on the uterine environment during the period of maternal recognition of pregnancy (from day 16 of pregnancy). Since IFNτ secretion begins at the blastocyst stage of embryos, it may be important in endometrial functions prior to maternal recognition of pregnancy.

Studies in cattle and sheep have used recombinant IFNτ or IFNα of either ovine and/or bovine origin to extend the lifespan of the CL. Interferons were infused into the uterine lumen or given as intravenous or intramuscular injections around the time of maternal recognition of pregnancy (Meyer et al., 1995; Bleach et al., 1998; Binelli et al., 2001; Bott et al., 2010). Unequivocal results were observed. The above treatments have increased the inter-estrus interval, reduced/not affected pulsality and concentration of PGF2α, or changed the blood P₄ concentrations in the subjects. Intramuscular injection of IFNα which can also block luteolysis decreased PR in heifers (Barros et al., 1992) because IFNα has several adverse actions such as causing hyperthermia (Newton et al., 1990). However, large scale studies have not yet been attempted to evaluate the effect of
IFN\(\tau\) on the fertility of cows. Also the effect of IFN\(\tau\) on the bovine endometrium during mid luteal phase (prior to maternal recognition of pregnancy) has not been studied. Therefore, it is worth investigating the role of IFN\(\tau\) on endometrial function during pre-implantation embryo development.

1.5 Rationale, hypothesis and objectives of the dissertation

Reduced fertility in high producing lactating dairy cows is a concern for farmers and the dairy industry. Although a great deal of research has investigated this problem with hopes of overcoming it, it unfortunately still remains a struggle to identify effectively the causes and their remedies. Even though fertility has gradually declined in lactating cows that of dairy heifers has remained at high levels for the past 60 years. Decline in fertility, and specifically the PR, is mainly due to the embryonic loss in cows and occurs mainly during the period of pre-implantation development (d 8 - 17). Embryo quality, uterine environment and luteal support are the known major determinants of embryo development during this period and affect pregnancy success in cows.

Embryo quality, uterine and CL functions are known to be dependent on an array of factors expressed and/or secreted by themselves in conjunction with the cascade of interactions that occur between theses factors within the reproductive system. Genes with specific functions in the above tissues are known to be expressed in a temporal and spatial manner and control the coordinated development of the conceptus during early pregnancy. Hence, an aberrant expression of important genes in the embryo, endometrium, or CL would compromise their quality and function and therefore PR. Therefore, it is rational to think that reduced PR observed in lactating dairy cows could
be due to altered expression of genes controlling key functions in the embryo, endometrial and CL compared to dairy heifers.

The primary goal of my research was to compare the quality of the embryo, the endometrium and the CL at molecular levels during the period of pre-implantation embryo development between lactating dairy cows and heifers. The secondary goal was to manipulate the endometrial gene expression using IFNτ as a candidate agent in order to enhance the endometrial function in bovines. The above questions were investigated in four separate studies, detailed in Chapters 2, 3, 4 and 5. The scope and aim of each study are briefly described here.

1.5.1 Chapter 2: Differential mRNA expression in in-vivo produced pre-implantation embryos of dairy heifers and cows

The hypothesis of this study was that the expression of candidate genes of embryos will be different between dairy heifers and lactating cows. The aim of this study was to compare the expression levels of developmentally important genes at mRNA levels in the embryos of dairy heifers and lactating cows. This study provides information in regards to the quality of the d7 blastocyst stage embryos from above groups of cows.

1.5.2 Chapter 3: mRNA of luteal genes associated with progesterone synthesis, maintenance, and apoptosis in dairy heifers and lactating dairy cows

The hypothesis of this study was that the expression of candidate genes of CL will be different between dairy heifers and lactating cows. The aim of this study was to compare the expression levels of candidate genes associated with P₄ synthesis and development of CL at mRNA levels in dairy heifers and lactating cows. Findings of this
1.5.3 Chapter 4: Comparison of expression levels of candidate genes in endometrium of dairy heifers and lactating dairy cows

The hypothesis of this study was that the expression of candidate genes of the endometrium will be different between dairy heifers and lactating cows. The aim of this study was to compare the expression levels of candidate genes of endometrium at mRNA and/or protein levels in dairy heifers and lactating cows. This study provides insight into the functional capacity of the endometrium at the molecular level in the above group of animals.

1.5.4 Chapter 5: Recombinant bovine interferon tau alters candidate gene expression in mid luteal phase endometrium of cows

The hypothesis of this experiment was that the treatment of bovine endometrium with recombinant bovine IFNτ will improve the expression levels of candidate genes in-vitro. The objective of this study was to investigate the effect of IFNτ on the expression levels of candidate genes in the mid luteal phase bovine endometrium in-vitro. The findings of this study provide information about the potential application of rbIFNτ to enhance uterine function in cows. In addition, findings will contribute to the knowledge about the IFNτ function in the endometrium.
CHAPTER 2: DIFFERENTIAL mRNA EXPRESSION IN IN-VIVO PRODUCED PRE-IMPLANTATION EMBRYOS OF DAIRY HEIFERS AND LACTATING COWS

2.1 Introduction

Modern high producing dairy cows are considered to be sub-fertile as their reproductive efficiency has declined considerably over the past sixty years, and the causes appear to be multifactorial. In contrast milk production has increased several folds during the same period, with higher milk yield being shown to have an inverse relationship with the pregnancy rates (PR) observed in dairy cows (Faust et al., 1988). It is important to note that even though the PR of lactating dairy cows has declined drastically from 66% to 40% over the past fifty years it has remained unaltered (70%) in heifers (Pursley et al., 1997). A recent study (Balandran et al., 2008), found that, after first and second inseminations, PR were greater in dairy heifers (84.3%), and declines with increasing parity (51.5%, 31.4%, and 19.5% for the 1st, 2nd, and 3rd/4th parity cows respectively).

Embryonic loss is a known major cause of reduced PR in cows. It may occur due to the reduced developmental competence of the embryo, chromosomal defects, alterations in the maternal environment, asynchrony between the embryo and dam, and failure of the dam to respond to embryonic signals (Hansen, 2002). Proper embryo

---

development is critical for successful implantation and establishment of pregnancy and requires good quality embryos, adequate endometrial receptivity, and successful interaction between the embryo and the endometrium (Chen et al., 1999). Further, embryonic quality is defined as the ability of the growing embryo to continue development, establish pregnancy, and to produce a calf. Morphological characteristics are the common method of assessing embryo quality (Rijnders and Jansen, 1998; Van Royen et al., 1999; Gardner et al., 2000), however novel approaches such as analysis of the gene expression pattern during early embryonic development, cryotolerance of embryos, and blastomere count etc. have also been widely used (Van Soom et al., 2003).

Embryonic genome activation, compaction and cavitation, elongation of the trophoblast, secretion of IFNτ, development of the inner cell mass into the fetus, and differentiation of placental tissues are important events during the embryonic development. For an embryo to be of good quality, the above processes must take place in a proper manner, and depends on the coordinated expression of various proteins by the embryo. Sodium/Potassium-ATPase (Na/K-ATPase; Betts et al., 1997), GLUT5; Wrenzycki et al., 2003), IFNτ (Imakawa et al., 1987 & 1989), HSPA1A (Neuer et al., 1999), and apoptotic molecules, BAX and BCL2 (Kolle et al., 2002; Yang and Rajamahendran, 2002) are some of the known important factors expressed in the blastocyst during pre-implantation that regulated growth and function of embryos.

Several studies using both in-vitro and in-vivo models have shown that the quality of embryos was related to their gene expression profiles, and that altered expression of certain genes may affect the quality and the developmental potential of pre-implantation embryos (Lonergan et al., 2000; Knijn et al., 2005; Corcoran et al., 2007). In order to
understand whether parity and poor quality of embryos is a reason for the reduced PR observed in lactating dairy cows, we have compared the gene expression pattern of pre-implantation embryos in dairy heifers and lactating cows.

2.2 Materials and methods

2.2.1 In-vivo embryo production

This study was conducted at the UBC Dairy Education and Research Centre in Agassiz, British Columbia, Canada. All handling and management of animals were in accordance with the guidelines of the Canadian Council on Animal Care (1993). Forty five lactating Holstein Friesian cows (n=20; 2\textsuperscript{nd}/3\textsuperscript{rd} parity) and heifers (n=25) with regular cyclic conditions were synchronized using Ovsynch (Pursley et al., 1995), and superovulated with intramuscular injection of FSH (Folltropin-V, Bioniche; 35mg), twice daily, starting on day 9 (Day 0=day of expected estrus) for five days at a decreasing dose followed by a dose of PGF2\textalpha{} (Lutalyse, Pharmacia; 35mg) on day 12 (Rajamahendran et al., 1987). Cows and heifers were inseminated twice on the day of estrus using semen from a proven bull. Embryos were flushed on day 7 using a non-surgical method (Elsden et al., 1976) and evaluated for fertilization and quality according to their morphology using the standard procedures set forth by the International Embryo Transfer Society (IETS). Transferable embryos (grade 1 and 2) were snap frozen in liquid nitrogen and stored at -80\degree{}C until processing for the analysis of transcript abundance.
2.2.2 Embryonic gene expression

Prior to the comparison of gene expression levels in in-vivo produced embryos, experimental conditions were standardized using embryos produced in-vitro in our lab using established procedures (Giritharan et al., 2007). Following culture, embryos were harvested and subjected to RNA extraction and RT-PCR procedures.

Four experiments were done at different time points to evaluate the gene expression profile of candidate genes (BAX, BCL2, IFNτ, HSPA1A, GLUT5 and Na/K-ATPase) in in-vivo produced embryos. In experiment I, grade 1 and 2 quality embryos were pooled together (n=6/pool, each from different animals) of heifers (HP) and lactating cows (CP). Experiment II, consisted of pools of grade 1 embryos (n=5, each from different animals) of heifers (H1) and lactating cows (C1). For experiments III and IV, pools of grade 1 or 2 quality embryos (n=7, each from different animals) of heifers (H1 and H2) and lactating cows (C1 and C2) were used respectively. For all experiments, three replicates of PCR per group were used.

2.2.3 RNA extraction

Total RNA was extracted from pools of embryos of heifers and cows using the PicoPure RNA Isolation Kit (PicoPure RNA Isolation Kit, Arcturus) according to the manufacturer instructions. Briefly, embryos were lysed using 10µL of lysis buffer at 42°C for 15 minutes. The extract was centrifuged (3000g for 2 min) and the supernatant was transferred into new microcentrifuge tube. An equal volume of ethanol was added and the mixture was pipetted into a preconditioned purified column. The column was centrifuged (100g for 2 min and then 16000g for 30 seconds), washed with wash buffers twice, and the RNA was extracted by centrifuging (1000g for 1 min) with elution buffer (11µL). The
quality and quantity of the RNA were assessed using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific). Following extraction, the RNA samples were DNase-treated (DNase, Cells-to-cDNA II Kit, Ambion). Extracted RNA samples were stored at -80°C until processing.

2.2.4 Reverse transcription

Reverse transcription (RT) was performed using first strand cDNA synthesis kit (Cells-to-cDNA II Kit, Ambion). The RT reactions were performed by following the kit manufacturer’s protocol with slight modification (Girittharan et al. 2007). RNA samples were DNAse-treated and first-strand cDNAs were synthesized by incubation of a 20 µL reaction mixture containing 9 µL of total RNA, 2.5 µM of random decamers, 0.25 mM of deoxyribonucleoside triphosphate mixture, 2 µL of X10 RT buffer (pH 7.4), 0.5 U/µL of RNase inhibitor, 0.5 U/µL of M-MLV reverse transcriptase and nuclease free water at 42°C for 1 h. The reverse transcriptase was inactivated by incubation of the reaction mixture at 94°C for 10 min and the product was stored at -20°C for future use in polymerase chain reaction (PCR) amplification.

2.2.5 Semi quantitative PCR

The PCR analyses were performed using Jumpstart REDTaq ReadyMix PCR reaction mix (Jumpstart, Sigma) and gene specific primers for Na/K-ATPase, GLUT5, BAX, BCL2, HSPA1A and IFNτ, as well as the house keeping gene glyceraldehydes-3-phosphate dehydrogenase (GAPDH). All the primer sequences were obtained from previously published data. The primer sequence, fragment size, annealing temperature, number of PCR cycles, and gene reference identification number for each gene are given
in the Table 2.1. The PCR reaction was as per the manufacturer’s protocol except for a slight modification. Briefly, gene specific primers (10 µM), MgCl₂ (0.5 mM), nuclease free water and cDNA template (2 µL) were added to 12.5 µL of Jumpstart REDTaq ReadyMix PCR reaction mix to make a 25 µL reaction mixture. The typical reaction cycles consisted of an initial denaturation step at 94 °C for 2 min, followed by 31 to 40 cycles of denaturation at 94 °C for 30 sec, annealing at 57 to 62 °C for 30 sec, and elongation at 72 °C for 45 sec, with a final elongation step at 72 °C for 5 min. The PCR products were analyzed by gel electrophoresis using ethidium bromide (0.2 µg/mL) stained 2% agarose gels. The gels were photographed under ultraviolet illumination and the optical densities of individual bands were analyzed using Scion Image Beta 4.02 software (http://www.scioncorp.com/). Relative optical density of each gene was normalized to the house keeping gene, GAPDH. The possibility of PCR cross contamination and genomic DNA amplification were ruled out as no PCR products were observed in the negative controls (without template and without reverse transcriptase).

2.2.6 Quantitative real-time PCR

Transcript abundance was compared for the apoptotic genes BAX and BCL2 using copy DNA from embryos of heifers and lactating dairy cows (HP, CP, H1, C1, H2) with three replicates per group using quantitative real time PCR (Q-RT PCR). PCR analyses were performed using the ABI PRISM 7300 real time cycler (Applied Biosystems), SYBR Green Master Mix (Power SYBR Green PCR Master Mix, Applied Biosystems) and gene specific primers (Table 2.1). For each sample the PCR reaction consisted of 2.5 µL of cDNA (fractions of 1/20 of original cDNA), 10 µM of each primer
in a final volume of 25 µL per reaction. Reaction conditions included an initial steps of 50°C (2 min) and 95°C (10 min), followed by 40 cycles of 95°C (15 sec) and 60°C (1 min). Q-RT-PCR data (Ct values) were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). First, expression values (Ct values) were adjusted according to corresponding GAPDH control/housekeeping gene expression values (Ct values). Later the relative abundances of the mRNAs were calculated by dividing each pool expression value by the baseline value.

2.2.7 Statistical analysis

Expression data were analyzed using the JMP 7.0.2 (SAS Institute Inc.) software package. Transcript abundances were compared between experimental groups using one-way analysis of variance (ANOVA) and the differences were analyzed by Student $t$-test. The results were reported as the mean values for each set of data ± SEM and the level of statistical significance was defined at probability level less than 0.05.

2.3 Results

2.3.1 Expression of genes in embryos of heifers vs. lactating cows

The mRNA expression levels of developmentally important genes (GLUT5, Na/K-ATPase and HSPA1A) were similar between the pooled embryos of heifers (HP) and lactating cows (CP) in experiment I (Fig. 2.1). In contrast, the relative abundance of IFNτ transcripts were greater (P<0.05) for heifer than lactating cow embryos. Transcript abundance of apoptotic genes BAX and BCL2 did not differ between pooled embryos both in semi-quantitative and real time PCR analysis including BAX: BCL2 ratio (Figs. 2.5).
Expression of mRNA levels of developmentally important genes except HSPA1A did not show any differences in grade 1 quality embryos of heifers and lactating cows. Whereas, HSPA1A, mRNA levels were higher (P<0.05) in heifer embryos (H1) than that of lactating cows (C1) in experiment 2 (Fig. 2.2).

**2.3.2 Expression of genes in grade 1 vs. grade 2 quality embryos**

The transcript abundance of the developmentally important genes showed a distinct pattern between different quality embryos of heifers when compared to that of lactating cows. In heifers the GLUT5, IFNτ and Na/K-ATPase genes did not show a difference in mRNA expression between grade 1 (H1) and grade 2 (H2) quality embryos. However, HSPA1A was more expressed in grade 1 quality embryos compared to that of grade 2 quality embryos (Fig. 2.3).

Expression of mRNA of developmentally important genes (GLUT5 and Na/K-ATPase in lactating cows was high (P<0.05) in grade 1 (C1) quality embryos compared to that of grade 2 (C2) quality. But HSPA1A and IFNτ expression levels did not differ between grades 1 and 2 quality embryos of cows (Fig. 2.4). Also apoptotic genes BAX and BCL2 expression and the BAX: BCL2 expression ratio did not show any differences (P>0.05) between grade 1 and 2 quality embryos both in heifers and lactating cows (Fig. 2.5).

**2.4 Discussion**

In the present study, we were able to show the differential mRNA expression of some developmentally important, and apoptotic genes of the pre-implantation embryos produced in-vivo, between dairy heifers and lactating cows. Quality of an embryo plays a
key role in its developmental potential, and the assessment of quality using molecular tools has been viewed as a novel approach, which has greatly enhanced the spectrum of knowledge and applications to improve fertility of mammalian species not only in the in-vitro culture conditions, but also in in-vivo models.

Pregnancy rates observed in the Holstein dairy cows in recent years, when compared to few decades back (Spalding et al., 1974; Pursley et al., 1997) exhibits a marked difference between heifers and lactating cows. Among many other fertility parameters, embryo quality is considered as an important characteristic for continuous embryonic development, maintenance of pregnancy, and production of a live calf in cattle. In this study, we used a set of marker genes which are known to play key physiological roles during the pre-implantation embryo development such as maternal recognition of pregnancy, compaction and cavitation, energy metabolism, protection from heat stress, and apoptosis. These genes have been used in the past to evaluate embryos produced in different culture conditions and nutritional management systems (Rizos et al., 2002 & 2003).

The rate of energy substrate utilization by the pre-implantation embryos is considered as both a quality and a viability parameter (Rieger, 1992; Wrenzycki et al., 2000), and in bovine embryos GLUT5 is expressed from day 5 and is critical for the uptake of fructose from the uterine fluid. The lowered expression of mRNA for GLUT5 in grade 2 compared to grade 1 embryos of lactating cows in this study, could be an indicator of the reduced ability of grade 2 embryos of lactating cows to transport fructose and to synthesize nucleotides which are essential during the onset of transcription (Augustin et al., 2001; Gutierrez-Adan et al., 2004).
The bovine embryo begins to express IFNτ immediately after the formation of the blastocyst (Farin et al., 1990) and its production is primarily dependent on the presence of a functional trophectoderm (Hernandez-Ledezma et al., 1993), therefore the level of IFNτ secretion has been discussed as a parameter for the assessment of embryo quality. In the present study IFNτ is more expressed in the pooled embryos of heifers than those of lactating cows. This could be due to the enhanced function of the trophectoderm in heifers compared to cows. In contrast an early and higher mRNA expression of IFNτ during the pre-implantation embryo development indicates poor quality of the bovine embryo (Kubisch et a., 1998; Wrenzycki et al., 2001). However, there was considerable variability observed in the expression of IFNτ between individual embryos (Hernandez-Ledezma et al., 1992). Additionally, Larson et al., (2001), demonstrated a sexual dimorphism in the secretion of IFNτ by early blastocysts in which female embryos produced more than that of male embryos. Therefore, the expression pattern observed in this study could be a result of these effects, and a more prudent approach should be taken to analyze this gene transcript.

When HSPA1A is expressed under various stress conditions, its synthesis enhances the ability of stressed cells to cope with increased concentrations of unfolded or denatured proteins. HSPA1A has also been shown to inhibit apoptosis thereby increasing the survival of cells exposed to a wide range of lethal stimuli (Mosser et al., 1997) by directly interacting with various components of programmed cell death (Saleh et al., 2000; Parcellier et al., 2003). In this experiment HSPA1A mRNA was more expressed in grade 1 heifer embryos as compared to lactating cows. Also, lowered expression was observed in grade 2 embryos of heifers than grade 1 embryos. This indicates a possible
correlation between good quality embryos and increased expression of HSPA1A in dairy cows and higher expression of HSPA1A observed in grade 1 heifer embryos is probably of benefit to the developing embryo.

Na/K-ATPase plays a key role in the formation of blastocysts in early embryonic development. The accumulation and composition of fluid in the blastocoele is critically regulated by Na/K-ATPase activity and is also essential for the differentiation of the inner cell mass and trophectoderm cell types (Watson, 1992; Betts et al., 1997 and 1998). In our study, mRNA for Na/K-ATPase was more expressed in grade 1 than grade 2 quality embryos of lactating cows. The lowered expression in the grade 2 quality embryos may possibly compromise the quality and the subsequent developmental potential.

Expression of apoptotic genes reflects the morphological changes that occur during the early embryo development, and messenger RNA expression of pro-apoptotic gene BAX and anti-apoptotic gene BCL2 is considered an indicator of blastocyst quality in bovine pre-implantation embryos (Lonergan et al., 2001; Yang and Rajamahendran, 2002). Additionally, the expression ratio of BAX to BCL2 in a tissue can determine whether cells will be protected from apoptosis or will succumb to it (Wall et al., 1999). In the present study, there were no differences observed in the mRNA expression between heifer and cow embryos or even between grade 1 and 2 quality embryos both in the semi-quantitative as well as quantitative PCR methods. This may be due to the origin of embryos in this study, where all of them were in-vivo produced and transferable embryos. Also, the demonstration of differential apoptotic gene or protein expression in above studies could be due to the effect of post-transcriptional control mechanisms.
controlling translation rates or degradation of BAX and BCL2 proteins (Meirelles et al., 2004). Furthermore, Vandaele et al., (2008) have shown that mRNA expression of BAX and BCL2 genes may not be reliable markers of apoptosis in bovine pre-implantation embryos; therefore measuring the mRNA expression levels of above genes in this study may not have reflected the apoptotic status of the embryos. Furthermore, our real-time PCR results of the BAX gene was comparable to the expression levels measured using the semi quantitative PCR method, and validates the measurements of mRNA expression levels by this method.

In the present study (unpublished data) as well as of others (Sartori et al., 2002b; Silva at al., 2002) the percentage of viable embryos produced in the lactating cows was lower than that of heifers. From our data, the percentage of grade 2 quality embryos produced in lactating cows was higher as compared to heifers and when the mRNA expression data was examined, the grade 2 quality embryos from lactating cows were found to be inferior to that of the grade 1 quality embryos from lactating cows. This could be possibly be correlated to the reduced PR observed in the lactating cows as compared to heifers.

In summary, the differential expression of mRNA abundances in pre-implantation embryos may explain the potential role of these genes in the reduced PR observed in lactating cows in the field. This study could be expanded to incorporate more developmentally important genes in order to understand the depth of molecular mechanisms involved as well as the functional aspects of genes examined in this study.
<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Primer sequence (5'-3')</th>
<th>Annealing Temperature (°C)</th>
<th>PCR cycle</th>
<th>Fragment length</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>5' TGTTCCAGTGATGATTCACC  3' AGGAGGCATTGCTGACAATC</td>
<td>58</td>
<td>34</td>
<td>318bp</td>
<td>U85042</td>
</tr>
<tr>
<td>BAX</td>
<td>5' TGCTTCAGGGTTTCATCCAG  3' AACATTTCCAGCCGCACTC</td>
<td>58</td>
<td>34</td>
<td>223bp</td>
<td>U92569</td>
</tr>
<tr>
<td>BCL2</td>
<td>5' TCGCCGAGATGTCAGTCAGC  3' GTTGACGCTCTCCACACACA</td>
<td>62</td>
<td>37</td>
<td>156bp</td>
<td>U92434</td>
</tr>
<tr>
<td>NA/K-ATPase</td>
<td>5' ACCTGTGGGGCATCCGAGAGAC  3' AGGGGAAGGCACAGAACCACCA</td>
<td>58</td>
<td>31</td>
<td>336bp</td>
<td>NML012504</td>
</tr>
<tr>
<td>GLUT5</td>
<td>5' CATCTCCATCATCGTCCTCA  3' GTAGATGGTGAGGAGGAGAC</td>
<td>57</td>
<td>40</td>
<td>531bp</td>
<td>AF308830</td>
</tr>
<tr>
<td>HSPA1A</td>
<td>5' CACTTCGAGGAGGTCA      3' GGTTGATGCTCTGTTGAGG</td>
<td>58</td>
<td>38</td>
<td>376bp</td>
<td>AY149619</td>
</tr>
<tr>
<td>IFNτ</td>
<td>5' GACTCTCCTCCTATCCCTGTCT  3' GGCTTCATCATCTCCACTCT</td>
<td>57</td>
<td>35</td>
<td>386bp</td>
<td>AF196325</td>
</tr>
</tbody>
</table>
Figure 2.1 a) The representative gel photograph shows the PCR products of gene transcripts. N= 2 pools/group, each pool (HPa, b & CPa, b) consists of six grade 1 and grade 2 quality embryos. b) The mRNA expression levels of genes in embryos of heifers (HP) and lactating cows (CP). The data are represented as mean ±SEM. *P<0.05.
Figure 2.2 a) The representative gel photograph shows the PCR products of gene transcripts. Each pool consists of five grade 1 (H1a, b & C1a, b) quality embryos. b) The mRNA expression levels of genes in embryos of heifers (H1) and lactating cows (C1). The data are represented as mean ±SEM. *P<0.05.
Figure 2.3  a) The representative gel photograph shows the PCR products of gene transcripts. N=2 pools/group, each pool consists of seven grade 1 (H1a, b) or grade 2 (H2a, b) quality embryos. b) The mRNA expression levels of genes in embryos of heifers (H1 and H2). The data are represented as mean ±SEM. *P<0.05.
Figure 2.4  

a) The representative gel photograph shows the PCR products of gene transcripts. N=2-4 pools/group, each pool consists of seven grade 1 (C1a-b) or grade 2 (C2a-d) quality embryos. 

b) The mRNA expression levels of genes in embryos of lactating cows (C1 and C2). The data are represented as mean ±SEM. *P<0.05.
Figure 2.5 a) The mRNA expression levels of BAX and BCL2 genes from real-time PCR in a) pooled embryos of heifers (HP) and lactating cows (CP), b) grade 1 quality embryos of heifers (H1) and lactating cows (C1) and c) grade 1 and 2 quality embryos of heifers (H1 and H2). Third column in each experiment represents the BAX: BCL2 ratio of corresponding groups. The data are represented as mean ±SEM and P<0.05.
CHAPTER 3: mRNA OF LUTEAL GENES ASSOCIATED WITH PROGESTERONE SYNTHESIS, MAINTENANCE, AND APOPTOSIS IN DAIRY HEIFERS AND LACTATING DAIRY COWS

3.1 Introduction

Reduced pregnancy rates (PR) in high yielding dairy cows is a world wide problem and a matter of great concern to dairy producers due to immense economic losses (Lucy, 2001). This has led to increased research efforts to further understand the underlying mechanisms of reduced PR in lactating dairy cows. Although PR in lactating dairy cows have declined gradually over the past 50 years, PR of dairy heifers have remained constant (Pursley et al., 1997). In a recent study, Balendran et al., (2008), showed that PR in heifers after first and second artificial inseminations (AI) was 83.4% and it declined in lactating dairy cows from 51.5% in the first parity to 19.5% in the third/fourth parity. The causes for the reduced PR in lactating dairy cows seem to be multifactorial and include disruptions in the ovarian follicular dynamics, CL function (Opsomer et al., 1998; Wolfenson et al., 2004), poor estrus behaviour and/or detection (Lopez et al., 2004), reduced oocyte/embryo quality (Lucy, 2007), and compromised oviductal and uterine environments (Gustafsson and Larsson, 1985; Binelli et al., 1999; Spencer et al., 2007). Formation of CL and secretion of P₄ are of paramount importance for the early embryonic development, implantation and maintenance of pregnancy. It has

been long established that P₄ concentrations during early pregnancy have a marked effect on PR. Many studies have demonstrated that lower concentration of P₄ in milk (Lamming et al., 1989; Mann et al., 1995) and plasma (Butler et al., 1996; Mann and Lamming, 2001) of cows is associated with higher rates of pregnancy failures.

The formation, function and maintenance of CL are regulated by various luteotropic factors before and after ovulation, as well as by the inhibition of several luteolytic factors during the period of active CL function (Stocco et al., 2007). These regulatory mechanisms are mediated by the cyclic expression of key genes during the lifespan of CL, and therefore, determine the functional capacities, as well as the lifespan of CL tissue. Major categories of genes that participate in the formation, maintenance, secretion and regression of CL are those involved in extracellular matrix and cytoskeleton formation, transcriptional regulation, angiogenesis, steroidogenesis, oxygen metabolism, apoptosis, and inflammation (Casey et al., 2005; Goravanahally et al., 2009). Therefore, it is expected that any alteration in the coordinated expression of luteal genes could compromise the optimal development and function of the CL.

In this study we compared the mRNA expression of candidate genes in the CL during mid luteal phase, between cycling dairy heifers and second/third parity lactating dairy cows. These genes are involved in steroidogenesis (STAR, CYP11A1; formerly known as P450scc, and 3βHSD), angiogenesis (VEGF, IGF1, and FGF2, luteal maintenance (IL1A and TNF) and apoptosis or heat stress (BAX, BCL2 and HSPA1A). We hypothesized that expression of aforementioned gene transcripts differ between CL of dairy heifers and lactating dairy cows.
3.2 Materials and methods

3.2.1 Animals and treatments

This study was conducted at UBC Dairy Education and Research Centre in Agassiz, British Columbia, Canada. Dairy heifers (14 months of age) and second or third parity lactating dairy cows (60 days postpartum, BCS-2.75) of Holstein Friesian breed with regular ovarian activity and no reproductive abnormalities were treated with a protocol to synchronize ovulation (Aali et al., 2004). Briefly, animals were treated with GnRH (Fertiline, Vetoquinol; 100 µg; im) followed by PGF2α (Lutalyse, Pharmacia; 25 mg; im) 7 days later, and a second dose of GnRH (100 µg; im) was administered 48 h after PGF2α treatment. All animals were examined once a day from the day of first GnRH treatment until enucleation for, ovulation (Day 0) and development of CL using ultrasonography and CL were enucleated on Day 10 from five animals in each group. Animals were housed in free-stall barns and fed a total mixed ration of corn and grass silage, hay and concentrates. Lactating dairy cows were fed twice daily and dairy heifers were fed once daily according to their dry matter requirements (NRC, 2001). All handling and management of animals used in this study were in accordance with the guidelines of the Canadian Council on Animal Care (1993) and approved by the Animal Care and Use Committee of the University of British Columbia.

3.2.2 Corpus luteum enucleation and processing

Epidural anesthesia was induced in lactating dairy cows using lidocaine hydrochloride (Lidocaine, Bimeda-MTC; 2%; 4 to 5mL) and an incision was made in the ventral wall of the vagina (colpotomy) and CL removed as previously described (Aali et
al., 2004). In heifers, CL was removed surgically through a flank incision under paralumbar block and using (Lidocaine, Bimeda-MTC; 2%; 4 to 5mL). In all animals (n=10) CL were located and enucleated by applying gentle pressure to the ovarian area surrounding the gland. All animals were moved and housed in individual free stalls for recovery and observed continually during the first 12 h after surgery, and were kept in individual pens for 3 to 5 days. Soon after the enucleation, CL was snap frozen in liquid nitrogen and stored at -80°C until processing. Blood samples were taken from the tail vein in all the animals on day 8 post ovulation and on the day of enucleation (Day 10) for the determination of P₄ concentrations. Plasma was separated by centrifuging the blood samples at 1000 × g at 4°C for 10 min and stored at -20°C until radioimmunoassay.

3.2.3 RNA extraction

Total RNA was extracted using a single step RNA isolation method (Chomczynski and Sacchi, 1987), using a total RNA isolation solution, Tri Reagent (Sigma). Briefly, luteal tissues (100 mg) were pulverized in liquid nitrogen using mortar and pestle, then immediately transferred into sterile nuclease-free microcentrifuge tubes containing 1000 µL of Tri Reagent. Contents were mixed thoroughly by vortexing and allowed to stand for 5 min at room temperature. Then 200 µL of chloroform was added to each tube, and agitated for 30 sec. The tubes were allowed to stand at room temperature for 15 min and were centrifuged at 12000 × g for 15 min at 4°C. The supernatant was carefully transferred into a new set of nuclease free centrifuge tubes and added 750 µL of isopropanol to each tube. Contents were mixed by slightly inverting the tube thrice, and again allowed to stand at room temperature for 30 min before centrifugation at 12000 × g for 10 min at 4°C. Supernatant was discarded and the pellet at the bottom of the tube was
washed twice with 1000 µL of ice-cold 75% ethanol by centrifugation at 7500 × g for 5 min, air dried for 10 to 15 min thereafter, and finally dissolved in 100 µL of sterile diethylpyrocarbonate (DEPC) treated water. The quantity and quality of RNA was assessed by measuring the optical densities using Nanodrop ND-1000 spectrophotometer and by observing clear bands for 28S, and 18S, ribosomal RNA species on ethidium bromide stained agarose gel (1%). Total RNA was stored at -80°C for cDNA synthesis.

3.2.4 Reverse transcription

Reverse transcription (RT) of RNA was performed using a first strand cDNA synthesis kit (Cells-to-cDNA II Kit, Ambion). The RT reactions were performed by following the kit manufacturer’s protocol with slight modification (Giritharan et al. 2007). RNA samples were treated with DNase and first-strand cDNA was synthesized by incubating a 20 µL reaction mixture containing 2.5 µM of random decamers, 1.25 µM of oligo dT primers, 0.25 mM of deoxyribonucleoside triphosphate mixture, 2 µL of 10 X RT buffer (pH 7.4), 0.5 U/µL of RNase inhibitor, 0.5 U/µL of M-MLV reverse transcriptase, 2 µg of total RNA and nuclease free water at 42°C for 1 h. Inactivation of reverse transcriptase was performed by incubating the reaction mixture at 94°C for 10 min and the product was stored at -20°C to use in the PCR amplification.

3.2.5 Quantitative real-time PCR

Expression of mRNA was compared for all the genes in this study using quantitative real time PCR (Q-RT PCR) and GAPDH as the housekeeping gene. PCR was performed using the iCycler, CFX96 Real-Time PCR Detection System (Bio-Rad), iQ SYBR Green Super Mix (Bio-Rad) and gene specific primers (Table 3.1). For each
sample PCR reaction consisted of 2.5 µL of cDNA (fractions of 1/20 of original cDNA), 10 µM of each primer in a final volume of 25 µL per reaction. Reaction conditions included an initial step of 95°C (5 min), followed by 40 cycles of 95°C (15 sec), 60°C (1 min) and 72°C (20 sec). Melt curve analysis was performed for each gene to confirm the presence of a single peak. Q-RT-PCR data (Ct values) were analyzed using the comparative Ct method (Livak and Schmittgen, 2001). Expression values (Ct values) of all genes for each sample were adjusted according to corresponding housekeeping/control gene (GAPDH) expression values (Ct values). Calculations of relative quantitation were performed using the sample with the lowest value in the group as a control group and using the formula $2^{-\Delta\Delta ct}$. The PCR products were confirmed by gel electrophoresis using ethidium bromide (0.4 µg/mL) stained 1% agarose gel. The gels were photographed under ultraviolet illumination. At the beginning of the experiment, all the primer sets were standardized using semi-quantitative RT-PCR and commercially available JumpStart RED Taq Ready Mix PCR reaction mix (Sigma) from slaughter house CL samples. Amplification of single bands was confirmed and the PCR products were sequenced to confirm their identity. The primer sequence, fragment size, annealing temperature, number of PCR cycles, and gene accession number are provided in Table 3.1.

3.2.6 Progesterone RIA

Radioimmunoassay was performed using a Coat-A-Count P₄ kit (Diagnostic Products). Reference standards or serum samples (100 µL) were added to P₄ antibody coated tubes. Buffered I¹²⁵-labelled P₄ (1.0 mL) was added to all tubes and vortexed. After 3 h of incubation, tubes were decanted and counted for radioactivity on a gamma
counter for 1 min and P₄ concentrations were detected. Coefficient of variation (CV) within (intra) and between (inter) assays were 7% and 9%, respectively. The sensitivity of the assay was 0.03 ng/mL.

### 3.2.7 Statistical analysis

Data analysis for mRNA expression and P₄ concentration were performed by one-way ANOVA followed by Student t-test. The results were reported as the mean values for each set of data ± SEM and statistical significance declared at probability level (P) less than 0.05.

### 3.3 Results

#### 3.3.1 mRNA expression of apoptotic molecules and heat shock protein

The quantitative RT-PCR results of luteal tissue mRNA levels for apoptotic molecules (BAX and BCL2) and HSPA1A are shown in Figure 3.1a. Analysis of variance revealed higher mRNA expression of BCL2 (P<0.05) in heifers, compared to that of lactating cows. However, the expression levels of BAX and HSPA1A did not differ between the two groups. The expression ratio of BAX: BCL2 was higher in lactating dairy cows, in comparison to that of dairy heifers.

#### 3.3.2 mRNA expression of steroidogenic enzymes

Expression of mRNA for the steroidogenic enzymes in CL were examined using quantitative real time PCR. No differences were observed for the expression of STAR and CYP11A1 mRNA between dairy heifers and lactating dairy cows. There was a higher
expression of mRNA for 3βHSD (P<0.05) in dairy heifers compared to that of lactating dairy cows (Fig 3.1b).

### 3.3.3 mRNA expression of genes associated with angiogenesis

The quantitative real time PCR results of luteal tissue mRNA levels for genes associated with luteal angiogenesis (VEGF, IGF1 and FGF2) were higher (P<0.05) in dairy heifers compared to that of lactating dairy cows (Fig. 3.1c).

### 3.3.4 mRNA expression of genes associated with luteal maintenance

When the mRNA expression of genes associated with luteal maintenance were analyzed, IL1A was highly expressed in the CL of dairy heifers, compared to that of lactating cows (P<0.05). TNF, mRNA expression was not different between these two groups (Fig 3.1d).

### 3.3.5 Progesterone assay

Plasma concentrations of P₄ on days 8 and 10 of the estrous cycle (mid luteal phase) did not show any significant differences between dairy heifers and lactating dairy cows (Appendix A). P₄ levels in heifers and cows on day 8 were 3.11 vs. 2.11 ng/mL and on day 10 were 5.07 vs. 2.83 ng/mL.

### 3.4 Discussion

Investigation of reduced PR in high producing dairy cows is a complex procedure and comparing the quality of CL using gene expression profiles will give an insight into some of the underlying causes at the molecular level. Proper formation, function and maintenance of CL are essential for adequate P₄ secretion which is critically important
for the early embryo development, implantation and maintenance of pregnancy in many animal species including cattle (Niswender et al., 2000; Spencer et al., 2007).

Apoptosis is an important process that occurs at all stages of the CL lifespan. The degree of apoptosis differs between different stages especially between CL development and regression. The apoptotic molecules of the BCL2 family (activator, BAX and inhibitor, BCL2) are a determinant of apoptotic status and the fate of cells in distinct tissues including CL (Korsmeyer, 1995; Tilly, 1996). Findings in the present study indicated greater BCL2 mRNA and a lower ratio of BAX: BCL2 mRNA in heifers in contrast to lactating dairy cows. Therefore apoptosis in CL may occur at a greater rate in cows than heifers. It is further supported by the studies in human CL that demonstrated the greatest amounts of BCL2 mRNA in the midluteal functional CL and the greatest amounts of BAX mRNA in the regressing CL (Rueda et al., 1997; Sugino et al., 2000).

Heat shock proteins are molecular chaperones that help other proteins to undergo folding, transport, and assembly into multi-protein complexes, or to refold after heat shock or other stresses (Didelot et al., 2007). It has been widely recognized that HSPA1A promotes cell survival in a variety of tissues by preventing apoptosis caused by different means such as heat shock, UV radiation, inadequate nutrition etc. (Beere, 2004, 2005). There is evidence for protective actions of HSPA1A on luteal cells during induced luteolysis in sheep (McPherson et al., 1993), as well as a mediator in luteal regression in rats (Narayansingh et al., 2004). When considering the stressors, especially heat shock, even though both heifers and cows are managed under similar conditions the effect of milk production causes an increase in the body temperature in lactating dairy cows (Sartori et al., 2002b). Therefore, it was expected that an increased expression of
HSPA1A mRNA in cows compared to heifers because of HSPA1A’s inducible nature by which it resists subsequent apoptotic stimuli (Dimmeler and Zeiher, 1997). However, in the present study, amount of HSPA1A mRNA was not different between heifers and lactating dairy cows. This might reflect the susceptibility of CL in lactating dairy cows for apoptosis, compared to dairy heifers and also evident from the expression of apoptotic genes in the present study. Furthermore, it has been shown that nitric oxide mediated inhibition of apoptosis in rat pre-ovulatory granulosa cells is associated with an induction of HSPA1A (Yoon et al., 2002).

Steroid biosynthesis in CL is controlled by the expression of various factors, mainly by STAR, CYP11A1 and 3βHSD which are recognized as luteal markers (Stocco et al., 2007). In the present study, there were greater amounts of 3βHSD mRNA in CL of heifers in contrast to lactating dairy cows, which perhaps indicated an enhanced capability of P₄ production by heifer CL. A gradual decline in amounts of 3βHSD mRNA has lead to a decrease in P₄ production which in turn could trigger the release of intraluteal PGF₂α (Stocco and Deis, 1998; Arosh et al., 2004). Casey et al., (2005) has demonstrated a sharp reduction in the amount of STAR, CYP11A1 and 3βHSD mRNA in regressed CL in contrast to non-regressed CL of same stage of the estrous cycle.

Angiogenesis plays an important role in the formation, maintenance and function of CL and several angiogenic factors are involved in this process including VEGF, FGF2 and IGF1 (Schams and Berisha, 2002). We compared amounts of mRNA of the aforementioned genes in CL of heifers and lactating dairy cows, because inadequate luteal function is associated with decreased luteal vascularity (Redmer and Reynolds, 1996). Lesser amounts of mRNA found in the present study for all three factors in
lactating dairy cows may indicate reduced vascularity in the CL of cows in contrast to heifers. Furthermore, FGF2 acts as mitogen and induces the secretion of P₄ in CL of cattle during the mid luteal phase (Miyamoto et al., 1992). The greater amounts of BCL2 mRNA in the present study could also be due to the anti-apoptotic effects of FGF2 and VEGF in endothelial cells (Pardo et al., 2002; Bufalo et al., 2003). It is speculated that stimulation of steroidogenesis and inhibition of apoptosis in the CL of lactating dairy cows would be compromised due to the lesser amounts of IGF1 mRNA compared to that of heifers (Neuvians et al., 2003; Townson, 2006).

The role of cytokines such as IL1A and TNF in CL survival and luteolysis is an active area of research. Treatment of endometrium with IL1A increases the production of PGE2, a luteotrophic factor and thereby the PGE2:PGF2α ratio in cows (Tanikawa et al., 2005; Okuda and Sakumoto, 2006; Skarzynski et al., 2008). Also, it increases the production of P₄ and the lifespan of the CL. Greater amounts of IL1A mRNA in heifers in the present study suggests a greater probability of survival and function of their CL compared to that of lactating dairy cows. Furthermore, endometrial production of PGE2 increases during early and mid luteal phases in cattle (Miyamoto et al., 2000; Murakami et al., 2001). This is further supported from recent findings, where the expression of the IL1A gene is greater in the endometrium of dairy heifers as indicated by amounts of mRNA and protein compared to lactating dairy cows (Pretheeban et al., 2009b). Luteal survival and P₄ secretion were also enhanced after treating endometrium with TNF in cattle (Miyamoto et al., 2000; Skarzynski et al., 2000, 2003) during the mid luteal phase of the estrous cycle. However, the present study did not reveal any difference in the expression of luteal TNF mRNA on Day 10 between heifers and lactating dairy cows.
Timing of post ovulatory P₄ rise, luteal survival and continuous P₄ production are more important for the establishment and maintenance of pregnancy than the final concentrations of P₄ during pre implantation development (Goff, 2004; Inskeep, 2004; Robinson et al., 2005). In the present study, the P₄ concentrations during the mid luteal phase did not differ between dairy heifers and lactating dairy cows. Optimal P₄ concentration for enhancing PR in cattle is not well established (Nogueira et al., 2004); not only sub optimal concentrations of P₄ can cause luteolysis but greater concentrations during early luteal phase can also trigger premature release of PGF2α and luteolysis (Nogueira et al., 2004). Furthermore, P₄ concentration during the previous estrous cycle can affect the embryo survival of the preceding cycle (Shrestha et al., 2004). Factors such as feed intake and milk yield can alter the metabolism of P₄ (McCann and Hansel, 1986; Wiltbank et al., 2000; Rabiee et al., 2001) and thereby the peripheral P₄ concentrations might differ considerably from the local concentrations needed for the establishment and maintenance of pregnancy.

From the present study, it is concluded that mRNA of genes in the mid luteal phase CL differs between dairy heifers and lactating dairy cows. Genes regulating angiogenic, steroidogenic and luteotropic factors are more expressed in heifers than in lactating dairy cows, whereas apoptosis seemed to be more evident in CL of lactating cows. Collectively these findings suggest that CL of second/third parity lactating dairy cows is compromised in luteotropic as well as steroidogenic capacities on Day 10 of the estrous cycle. However, the post transcription regulation of aforementioned factors should be further studied in order to determine whether they have a role in the reduction of PR observed in lactating dairy cows.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Sequences (5’-3’)</th>
<th>$T_a$°C</th>
<th>Gene Accession Number</th>
</tr>
</thead>
</table>
| GAPDH | F- TGTTCCAGTATGATTCCACCC  
R- AGGAGGCCATTGCTGACAATC | 58 | U85042 |
| VEGF | F- TGTAATGACGAAAGTCTGCAG  
R- TCACCGCTCCGGGTTTCACA | 60 | M32976 |
| IGF1 | F- TCAGTTTCGTTGCGGAGACA  
R- ACTTCCTTCTGAGCCTTGGG | 56 | NM001077828 |
| FGF2 | F- TACAACCTCAAGCAGAAGAG  
R- CAGCCTCTTACAGACCATTTG | 56 | NM174056 |
| StAR | F- CATGGTGCTCCGCCCCTTGCT  
R- CATTGCCACAGACCTCTTTGA | 58 | BC110213 |
| 3βHSD | F- TGTTGGTGAGGAGGACAGG  
R- GGCCTCTTGGGATGATCT | 58 | BC111203 |
| CYP11A1 | F- AACGTCCCTCCAGAATCTGTACC  
R- CTTGCTTATTGCTCCCTCCTGCC | 58 | BC133389 |
| IL1A | F- CTCTCTCAATCAGAAGTCTTCTATG  
R- CATGTAAATTTCAGCTGCTCCTCC | 58 | NM174092 |
| TNF | F- GAAGCTGGAAGACAACCA  
R- TCCCAAAATGACCTGCTC | 60 | NM173966 |
| BAX | F- TGCTTACGGGTCTCATCCAG  
R- AACATATTCCAGCCCGCACT | 58 | U92569 |
| BCL2 | F- TTCCGGAGATGTCCAGTCAGC  
R- GTTGACGCTCTCCACACACA | 62 | U92434 |
| HSPA1A | F- CACTTCGTTGAGGAGTTCA  
R- GGTTGATGCTCTTGGGAG | 58 | AY149619 |

F: Forward primer; R: Reverse primer; $T_a$: Annealing temperature
Figure 3.1 Amounts of mRNA of genes associated with a) apoptosis and heat shock, b) steroidogenesis, c) angiogenesis and d) luteal maintenance, in dairy heifers and lactating dairy cows. The data are represented as mean ±SEM. *P<0.05.
CHAPTER 4: COMPARISON OF EXPRESSION LEVELS OF CANDIDATE GENES IN ENDOMETRIUM OF DAIRY HEIFERS AND LACTATING DAIRY COWS

4.1 Introduction

The marked decline in the reproductive performance of high yielding, lactating dairy cows has been a world wide problem. The reasons for the decline in reproductive performance in dairy cows seem to be multi-factorial and not entirely associated with an increase in milk production (Grohn and Rajala-Schultz, 2000; Lucy, 2001). It is important to note that even though the pregnancy rates (PR) of lactating dairy cows have declined drastically from 66% to 35% over the past fifty years, they have remained unaltered (70%) in heifers (Spalding et al., 1975; Pursley et al., 1997). The decreased PR observed in dairy cows could be due to an array of causes, such as compromised health and immune status, poor estrus expression and/or detection, extended anovulatory periods, low fertilization rates, and increased embryonic mortality (Hansen and Arechiga, 1999; Grohn and Rajala-Schultz, 2000; Lucy 2001). In addition, parity was found to influence the quality of the oocytes, embryos and CL function (Sartori et al., 2002b; Rizos et al., 2005; Pretheeban et al., 2009 & 2010) as well as the pregnancy outcome (Balendran et al., 2008) in dairy cows.

In bovines and other species the early developmental stages of embryos are more vulnerable than the later developmental stages. Higher rates of embryonic losses occur during the first two weeks of pregnancy and among them up to 40% occur between days 8 and 17 post insemination (Maurer and Chenault, 1983; Dunne et al., 2000; Humblot, 2001). Maternal uterine environment plays a major role in the embryonic growth and development in mammals (Wilmut and Sales, 1981; Lawson et al., 1983; Garrett et al., 1988). Steroid hormones, cytokines and growth factors are essential for regulating the differentiation and proliferation in the endometrium during the period of early embryonic development, implantation and pregnancy. Optimal secretion of embryotrophic factors especially the uterine histotrophs at the right time is important for proper embryo development; therefore, a suboptimal uterine environment might be a likely cause of pregnancy loss in lactating dairy cows (Gustafsson and Larsson, 1985). Studies of mRNA and protein expression in the endometrium of many species revealed quantitative and qualitative changes in the expression of key regulatory factors at different stages of the estrous cycle and during pregnancy (Katagiri and Takahashi, 2004; Bauersachs et al., 2005; Klein et al., 2006; Michael et al., 2006).

Some of the candidate genes have been shown to influence the function of endometrium and therefore the fertility in cows and other mammals. These candidate genes are apoptotic molecules (BCL2 and BAX), HSPA1A, cytokines (IL1A and TNF, uterine serpins (SERPINA14, previously known as UTMP) and growth factors (insulin-like growth factor 1 (IGF1), insulin-like growth factor binding protein 1 (IGFBP1), insulin-like growth factor binding protein 3 (IGFBP3) and FGF2.
Apoptosis is an important process by which programmed cell death occurs in tissues including endometrium of human and animals (Tabibzadeh, 1995; Wang et al., 2003; Groebner et al., 2010). Lovely et al (2005) demonstrated that, P4 plays an important role in the regulation of apoptosis in human endometrium including the pro- and anti-apoptotic genes, BAX and BCL2, which are involved in the initiation of apoptotic process and thereby determining cell fate decisions (Elmore, 2007). Studies using different mammalian tissue types have shown that HSPA1A expression is inducible by heat, and the thermotolerance of the tissues increases with increased expression of HSPA1A (Neuer et al., 1999; Dokladny et al., 2006). The bovine endometrium in response to heat shock expresses heat shock proteins including HSPA1A, which protects early embryos from thermal stress and prevents endometrial protein denaturation (Malayer and Hansen, 1990; Tabibzadeh and Broome, 1999).

In bovines, IGF1 of either embryonic or endometrial origin was found to stimulate embryonic development and suppress apoptosis (Moreira et al., 2002; reviewed by Velazquez et al., 2009). Further, IGFBP1 and IGFBP3 proteins have been found to regulate the availability of IGF1 in endometrium as well as acting independently on the embryo-maternal communication (Rutanen et al., 1988; Simmons et al., 2009). Fibroblast growth factor 2 acts as a mitogen, morphogen, and angiogenic factor that regulates early embryogenesis (Gospodarowicz, 1991) and improves bovine blastocyst development (Larson et al., 1992; Lim and Hansel, 1996; Reynolds and Redmer, 2001). In bovines, TNF and IL1A play a significant role in the endometrial PGs production by regulating their secretion during the estrous cycle (reviewed by Okuda and Sakumoto, 2006). SERPINA14, a member of the serpin family of proteinase inhibitors, participates in the
modulation of the maternal immune system during the pregnancy. It inhibits natural killer cell (NK) like activity, protects the embryo from maternal cytotoxic lymphocytes, and is regulated during the pregnancy in the endometrium (Stewart et al., 2000; Tekin and Hansen, 2002).

The objective of our study is to analyze the endometrial mRNA levels of BCL2, BAX, HSPA1A, IL1A, TNF, SERPINA14, IGF1, IGFBP1, IGFBP3 and FGF2 genes and the protein expression of candidate genes in order to determine whether the endometrial environment of lactating dairy cows is different from the endometrial environment of dairy heifers.

4.2 Materials and methods

4.2.1 Animal procedures and tissue collection

This part of the study was conducted at the UBC Dairy Education and Research Centre in Agassiz, British Columbia, Canada. All handling and management of animals were in accordance with the guidelines of the Canadian Council on Animal Care (1993) and approved by the Animal Care and Use Committee of the University of British Columbia. Holstein-Friesian dairy heifers (Age, 14 months; cycling regularly) and second/third parity lactating dairy cows (>60 days post partum; BCS, 2.75) were enrolled in this experiment. Animals were housed in free-stall barns and fed a total mixed ration of corn and grass silage, hay and concentrates. Lactating dairy cows were fed twice daily and dairy heifers were fed once daily according to their dry matter requirements (National Academy of Science-National Research Council, 2001). All animals were synchronized for estrus by administering 25 mg PGF2α (Lutalyse, Pharmacia; 5mg/ml)
twice intramuscularly at 12 days intervals (Aali 2003). Ovulation and the presence of CL were monitored by ultrasound imaging and per rectal examination and ten animals (5 from each group) were selected for biopsy.

Endometrial biopsies were performed 11 days post estrus (during the mid-luteal phase) after inducing epidural anesthesia by administering 4 to 5 mL, 2% (wt/vol) lidocaine hydrochloride (Lidocaine, Bimeda-MTC) into the epidural space. Uterine biopsy forceps (3.5 X 8 mm cutting area; Kevorkian-Younge, Fine Surgicals) covered with a plastic sheath was introduced into the vagina and subsequently passed through the cervix after rupturing the sheath by manipulation per rectum. Upon entering the uterus and the chosen site of biopsy (approximately halfway between the uterine body and uterotubal junction in the uterine horn ipsilateral to the ovary containing the CL), two small samples (100 to 200 mg) of endometrial tissue were obtained by gently guiding the uterine inner wall to the biopsy instrument per rectum. The biopsy instrument was gently removed and the collected tissue samples were either snap frozen in liquid nitrogen and stored at -80°C for RNA extraction or placed in 10% formalin solution for immunohistochemistry.

4.2.2 RNA extraction

Endometrial tissues were processed for RNA extraction following a single RNA isolation method (Chomczynski and Sacchi, 1987) with modifications (Singh et al., 2008) using a commercially available total RNA isolation solution, Tri Reagent (Sigma). The quantity and quality of RNA was assessed by measuring the optical densities using Nanodrop ND-1000 spectrophotometer and by observing clear bands for 28s and 18s ribosomal RNA species on ethidium bromide stained agarose gel (1%, wt/vol).
4.2.3 Reverse transcription

First strand cDNA was generated from extracted RNA using the iScript Select cDNA synthesis kit (Bio-Rad). The reverse transcription reactions were performed by following the kit manufacturer’s protocol. Briefly, RNA samples were DNase-treated and first-strand cDNA was synthesized by incubating a 20 µL reaction mixture containing 2 µL of random primer, 4 µL of 5x iScript select reaction mix, 1 µL of reverse transcriptase, 1 µg of total RNA and nuclease free water at 25°C for 5 min then at 42°C for 30 min. Inactivation of reverse transcriptase was performed by incubating the reaction mixture at 85°C for 5 min and the product was stored at -20°C to use in PCR amplification.

4.2.4 Primer design

All the primers except for IGFBP1 and IGFBP3 were derived from the literature. For IGFBP1 and IGFBP3 forward and reverse primers were designed from bovine mRNA sequences (NCBI) using PrimerQuest PCR Design Tool (Integrated DNA Technologies, Inc.). The primer sequence, annealing temperature, fragment size and gene accession number are provided in Table 1.

4.2.5 Semi-quantitative PCR

At the beginning of the experiment, specificity of all primer sets was examined using PCR (JumpStart RED Taq Ready Mix PCR reaction mix; Jumpstart; Sigma) and agarose gel electrophoresis using slaughter house endometrial samples. Amplification of single band was confirmed and the PCR products were sequenced to confirm their identity. The possibility of PCR cross contamination and genomic DNA amplification
were ruled out as no PCR products were observed in the negative controls (without template and without reverse transcriptase).

4.2.6 Quantitative real-time PCR

Levels of mRNA for all genes were analyzed using quantitative real time PCR (Q-RT PCR) and suppressor of zeste 12 homolog (SUZ12; Walker et al. 2009) and GAPDH as the housekeeping genes. Polymerase chain reaction was performed using the iCycler, CFX96 Real-Time PCR Detection System (Bio-Rad), iQ SYBR Green Super Mix (Bio-Rad) and gene specific primers (Table 4.1). For each sample, reaction mixture consisted of 2.5 µL of cDNA (original cDNA was diluted 3 folds), 10 µM of each primer in a final volume of 25 µL per reaction. Reaction conditions included an initial step of 95°C (5 min), followed by 40 cycles of 95°C (15 sec), 60°C (1 min) and 72°C (20 sec). Melt curve analysis was performed for each gene to confirm the presence of a single peak. Q-RT-PCR data (Ct values) was analyzed using the comparative Ct method (Livak and Schmittgen 2001). Expression values (Ct values) of all genes for each sample were adjusted according to corresponding housekeeping gene’s (SUZ12 and GAPDH) expression values (Ct values). However, the values normalized only by SUZ12 are presented. Calculations of relative quantitation were performed using the sample with the highest adjusted value in the group as a control group and using the formula \(2^{-\Delta\Delta Ct}\). The PCR products were confirmed by Gel electrophoresis using GelRed Nucleic Acid Gel Stain (1 µL/mL; Biotium,) stained 2% (wt/vol) agarose gels. The gels were photographed under ultraviolet illumination.
4.2.7 Immunohistochemistry

From the preliminary studies performed using Q-RT PCR, IL1A, TNF and FGF2 genes showed highest differences for the relative mRNA levels among all genes analyzed between heifers and lactating cows. Therefore, we have selected the aforementioned genes to determine their protein expression in the different areas of the endometrium in heifers and lactating dairy cows.

Immunohistochemistry was performed using established methods (Singh et al. 2008) with modifications. After overnight fixation in 10% (v:v) formalin, endometrial biopsy samples were transferred to 70% (v:v) ethanol, paraffin embedded and sectioned to a thickness of 5µm. Bovine lung and testicular tissues collected from the slaughter house were also treated in a similar manner and used as positive controls for IL1A, TNF and FGF respectively. Glass mounted sections were then deparaffinized in xylene (15 min) hydrated quickly in a series of ethanol solutions (100, 95, 80 and 70% ethanol, v:v) and stained using standard immunohistochemistry procedures. Tissue sections were rinsed in PBS (pH 7.2 to 7.4) for 5 min and antigen retrieval was performed by placing the tissue sections in 10X citrate buffer (preheated to 98ºC, pH 6.0) at 98ºC for 30 min, cooled at room temperature for 20 min and rinsed with PBS for 15 min. Endogenous peroxidase activity was quenched upon incubation for 10 min with 0.3% H₂O₂ (v:v) in PBS and then rinsed with PBS for 15 min. Sections were blocked for 1 h at room temperature with animal-free blocker (Vector Laboratories, Burlingame, USA), washed and incubated in a 1:25 dilution of antibodies (Santa Cruz Biotechnology) specific for IL1A (sc-1253), TNF (sc-1351) and FGF2 (sc-1360) in 1% (wt/vol) bovine serum albumin (BSA) in PBS at 4ºC for 24 h. Negative controls were used with pre immune
IgG (sc-2028; Santa Cruz Biotechnology). Sections were washed in PBS, incubated with horseradish peroxidase labeled secondary antibody (PI-9500; Vector Laboratories), diluted 1:200 in 1% BSA (wt/vol) in PBS for 1 h at room temperature, and rinsed with PBS. Sections were visualized for peroxidase using Nova Red (Vector Laboratories), incubated for 10 min to complete the reaction, counterstained with haematoxylin, dehydrated in a graded series of ethanol (70, 80 and 95% ethanol, v:v), cleared with xylene and then covered with a coverslip placed over Vectamount mounting medium for evaluation by a light microscope (Leitz, Orthoplan).

The staining intensity of tissue sections was evaluated and graded (0: absence; 1: weak; 2: moderate; 3: strong) in a blind fashion by two independent examiners at X 100 magnification. The luminal epithelium, glands, stroma and blood vessels of the endometrium were scored individually by examining five fields per section. Semiquantitative histological score (HSCORE) method was used to assess the intensity and distribution of staining (Lessey et al. 2006). HSCORE was calculated for each region using the following equation for heifers and lactating dairy cows: HSCORE = Σ Pi (i+1), where i is the staining intensity and Pi the percentage of stained cells or area at each level of intensity.

4.2.8 Statistical analysis

Messenger RNA and protein expression data were analyzed using the JMP 7.0.2 (SAS Institute Inc.) software package. Abundance of gene transcripts was compared between experimental groups using a one-way ANOVA and the differences were analyzed by Student t-test. Least square analysis of covariance was performed to analyze the immunohistochemistry data, and parity, examiner and parity X examiner were used as
variables in the modeling procedure. The results were reported as the mean values for each set of data ± SEM and the level of statistical significance was defined at probability level less than 0.05.

4.3 Results

4.3.1 Relative mRNA abundance of apoptotic genes and HSPA1A

Transcript abundance of BCL2, BAX and HSPA1A were compared in the endometrial biopsy of heifers and lactating dairy cows. Endometrial expression of BCL2 and HSPA1A mRNAs were greater (P<0.05) in heifers than lactating dairy cows. There was no difference observed for BAX mRNA level (Fig. 4.1a). Also, there was no difference found in the BAX: BCL2 ratio between the two groups.

4.3.2 Relative mRNA abundance of growth factor genes

Transcript levels of growth factors (IGF1, IGFBP1, IGFBP3 and FGF2) were analyzed in the biopsied endometrium of heifers and lactating dairy cows. Both FGF2 and IGF1 mRNA levels were more (P<0.05) expressed in the heifer endometrium in comparison to the endometrium of lactating dairy cows (Fig. 4.1b). IGFBP1 and IGFBP3 mRNA levels did not show any differences between the two groups.

4.3.3 Relative mRNA Abundance of cytokine genes

Transcript abundance of cytokine genes (SERPINA14, IL1A and TNF) in the endometrium was compared between heifers and lactating dairy cows. Significant differences were found on the levels of mRNA transcripts of aforementioned cytokines between the two groups. Levels of mRNA for SERPINA14, IL1A and TNF were
significantly higher (P<0.05) in heifer endometrium in comparison to that of lactating dairy cows (Fig. 4.1c).

4.3.4 Immunohistochemistry

Protein expression of IL1A, TNF and FGF2 were analyzed in the different areas of the endometrium in heifers and lactating dairy cows. The immunohistochemical staining of aforementioned genes in the endometrium is shown in Fig. 4.2. Columns A, B, and C are of representative photographs of the staining of IL1A, TNF and FGF2 proteins. Immunostaining is indicated in the luminal epithelium (thin black arrows), glands (thick black arrows), stroma (black arrowheads) and blood vessels (blue arrows) of the endometrium, luminal epithelium, sub-mucosa and interstitial spaces of the lung and interstitium, and the cells of the seminiferous tubules of testis. Photographs of negative (g, h & i) and positive (j, k & l) controls are also shown in Fig. 4.2. Distribution of immunostaining intensity in different regions of the endometrium between heifers and lactating dairy cows are given as HSCORE in Fig. 4.3. The glands and stroma of the endometrium in heifers showed a higher immunostaining (P<0.05) of IL1A, TNF and FGF2 compared to lactating dairy cows. Luminal epithelium showed a higher expression (P<0.05) for TNF and FGF2, but not IL1A in the heifer endometrium when compared to that of lactating dairy cows. The blood vessels of the heifer endometrium showed more immunostaining (P<0.05) for FGF2, but not for either IL1A or TNF when compared that of lactating dairy cows.
4.5 Discussion

Declining PR in modern, high producing dairy cows has resulted in increased calving intervals, number of services per conception, premature culling, replacement costs and decrease in calf crop and total milk production. The reproductive parameter, PR, has gone down gradually with increasing parity and has remained unaltered in dairy heifers over the past sixty years. The ability of the uterine environment to nourish and maintain the pregnancy from the early embryo stage is an important function, and the quality of the uterine environment is dependent on the coordinated secretion of essential factors during the different stages of pregnancy. In this study, we have demonstrated the presence of differentially expressed endometrial genes at mRNA and protein levels in lactating dairy cows and dairy heifers.

It has been established that the proteins in the BCL2 family (activator, BAX and inhibitor, BCL2) play an important role in programmed cell death in tissues. The proportion of inhibitors to activators in a cell may determine the fate of the cell to undergo apoptosis (Korsmeyer, 1995). In our study, BCL2 was more expressed in the heifer endometrium compared to that of lactating dairy cows. However, the BAX: BCL2 mRNA expression ratio was found to be similar in heifers and lactating dairy cows. This might be indicative of an equivalent status of endometrial apoptotic process in both groups of animals, and it is of non-apoptotic in nature. This is supported by the findings of Wang and others (Wang et al., 2003), where they have shown that experimentally induced apoptosis is suppressed by P₄ treatment (mimicking the luteal phase) in bovine endometrium. Further, there could be involvement of other apoptotic processes such as fas and caspases (Nagata and Goldstein, 1995; Selam et al., 2001), however, a recent
study (Groebner et al., 2010) revealed that, neither an activation of a caspase cascade nor an increase of apoptotic cells occurs in luteal phase and peri-implantation endometrium of cows.

The increment in body temperature of lactating dairy cows in response to increase in environmental temperature is higher in comparison to that of heifers (Sartori et al., 2002b). Also the higher energy metabolism and heat production due to milk synthesis induce heat stress in lactating dairy cows and decrease the PR observed post AI (Chebel et al., 2004). Because of the inducible nature of HSPA1A to heat shock (Malayer and Hansen, 1990), we expected a higher HSPA1A mRNA level in lactating dairy cows to heifers in our study. However, HSPA1A mRNA levels were more in heifers than lactating dairy cows. Low levels of HSPA1A can also affect the assembly, transport, or binding activities of steroid receptors (Johnson et al., 1996; Sabbah et al., 1996) which can compromise the optimal endometrial function in cows.

IGF1 is shown to be expressed in the bovine uterus (Geisert et al., 1991; Kirby et al., 1996; Robinson et al., 2000) and to stimulate embryo survival (Block et al., 2007). In our study the expression level of the IGF1 mRNA was lower in lactating dairy cows than heifers. Studies using bovine embryos have demonstrated that, IGF1 can act as a survival factor by increasing the total cell number and reducing the percentage of apoptosis in pre-implantation embryos exposed to heat shock (Fabian et al., 2004; Jousan and Hansen, 2004). Recent study with cows and ewes reveal that IGFBP1 and IGFBP3 most likely regulate conceptus elongation by stimulating migration and attachment of the trophectoderm in cows and ewes (Simmons et al., 2009). Though, the mRNA levels of IGFBP1 and IGFBP3 were not different between heifers and cows in our study, reduced
level of IGF1 mRNA in the endometrium may be a factor contributing to pregnancy failures in lactating dairy cows.

The FGF family consists of many autocrine and paracrine factors that play variety of roles in multicellular organisms (Ornitz and Itoh, 2001). It has been established that FGF2 acts as a weak mediator of trophectoderm proliferation and a strong regulator of IFNτ production in the trophoblast cells of blastocyst stage embryos of cows (Michael et al., 2006). In our study, there was a higher level of mRNA and protein for FGF2 present in heifer endometrium when compared to lactating dairy cows, and this could reflect a better endometrial environment for embryo viability in heifers compared to lactating dairy cows. However, in our immunohistochemistry findings the FGF2 immunostaining was also different in the blood vessels of the endometrium. Therefore it is not possible to eliminate the contribution by the systemic circulation.

In the present study, TNF is more expressed in the heifer endometrium in comparison to that of lactating dairy cows both at mRNA and protein levels. It has been demonstrated in the past that TNF plays a dual and opposing roles in luteal maintenance a) at lower concentrations acts as a luteolytic agent by inducing the secretion of PGF2α and b) at higher concentrations acts as a luteotropic agent by stimulating the secretion of PGE2 in the endometrium of bovines (reviewed by Okuda and Sakamoto, 2006). The relative levels of PGE2 and PGF2α secretions have been shown to play a key role in CL survival (Murakami et al., 2001; Tanikawa et al., 2005; Okuda and Sakamoto, 2006). IL1A also plays a dual role in luteal function during the estrous cycle. CL is maintained by increased PGE2: PGF2α ratio during early and mid-luteal phases, however, decreasing the PGE2: PGF2α ratio during late luteal phase supports luteolysis, both induced by IL1A
(Tanikawa et al., 2005; Skarzynski et al., 2008). In our study, the IL1A mRNA and protein expression were higher in the heifer endometrium in comparison to lactating dairy cows. Therefore, the higher expression of TNF and IL1A in heifers indicates a better probability of CL survival and in turn, a more viable pregnancy compared to that in lactating dairy cows.

During the early period of pregnancy, P₄ inhibits the uterine immune function by inducing endometrial secretion of SERPINA14 and protects and maintains the pregnancy (Hansen, 1998). In our study, mRNA level of SERPINA14 was higher in the heifer endometrium than lactating dairy cows. Therefore, lower levels of SERPINA14 at midluteal phase could compromise the pregnancy status in lactating dairy cows compared to heifers.

From this study, we are reporting lower expression of key endometrial factors at mRNA and protein levels in lactating dairy cows compared to that of dairy heifers. These factors are important for the growth and survival of the pre-implantation embryos, proper function of the uterine environment, and the survival of the CL. In conclusion, our findings clearly indicate a differential expression pattern of candidate genes in lactating dairy cows and potentially cause a compromised uterine environment for embryo development. Further studies are warranted to determine whether these factors have a role in the lower PR observed in lactating dairy cows.
<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer sequences (5’-3’)</th>
<th>Annealing °C</th>
<th>Amplicon size (bp)</th>
<th>Gene Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BAX</strong></td>
<td>F- TGCTTCAGGGTTCATCCAG</td>
<td>58</td>
<td>223</td>
<td>U92569</td>
</tr>
<tr>
<td></td>
<td>R- AACATTTCCAGCCGCACTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BCL2</strong></td>
<td>F- TTCGCGAGATGTCCAGTCAGC</td>
<td>62</td>
<td>156</td>
<td>U92434</td>
</tr>
<tr>
<td></td>
<td>R- GGTTGATGCTCTTGTTGAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HSPA1A</strong></td>
<td>F- CACTTCTGGAGAGTTCA</td>
<td>58</td>
<td>376</td>
<td>AY149619</td>
</tr>
<tr>
<td></td>
<td>R- GGTTGATGCTCTTGTTGAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IGF1</strong></td>
<td>F- TCAGTTCGTGTGGAGAGACA</td>
<td>56</td>
<td>222</td>
<td>NM001077828</td>
</tr>
<tr>
<td></td>
<td>R- ACTTCCTTGAGCCTTGGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IGFBP1</strong></td>
<td>F- CTACAAAGTGCTGGACAGATTAGCC</td>
<td>60</td>
<td>157</td>
<td>X54979</td>
</tr>
<tr>
<td></td>
<td>R- GTAGACACACCCAGAGCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IGFBP3</strong></td>
<td>F- AAGAAAGGTTGATGCAAAGAACACAGC</td>
<td>60</td>
<td>199</td>
<td>AF305199</td>
</tr>
<tr>
<td></td>
<td>R- TTGTCCAGTTGGGAATGTGGATG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>FGF2</strong></td>
<td>F- TACAACCTTCAAGCAGAGAG</td>
<td>56</td>
<td>214</td>
<td>NM174056</td>
</tr>
<tr>
<td></td>
<td>R- CAGCTCTTAGCAGACATGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>IL1A</strong></td>
<td>F- CTCTCTCAATCAGAGTCTCTATG</td>
<td>58</td>
<td>424</td>
<td>NM174092</td>
</tr>
<tr>
<td></td>
<td>R- CATGTCAAATTTTCACTGCTCTCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TNF</strong></td>
<td>F- GAAGCTGGAAGAACAACCA</td>
<td>60</td>
<td>338</td>
<td>NM173966</td>
</tr>
<tr>
<td></td>
<td>R- TCCTAAATCTACAGCTGACC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SERPINA14</strong></td>
<td>F- ATATCATCTTCTCCCCCATGG</td>
<td>60</td>
<td>126</td>
<td>L22095</td>
</tr>
<tr>
<td></td>
<td>R- GTGCACATCCAACATTTTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SUZ12</strong></td>
<td>F- GAACACCTATCACAACACATTCTGT</td>
<td>60</td>
<td>130</td>
<td>XM582605</td>
</tr>
<tr>
<td></td>
<td>R- TAGAGGCCGTTGTGTCCACT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GAPDH</strong></td>
<td>F- TGTTCCAGTATGATTCCACACC</td>
<td>58</td>
<td>318</td>
<td>U85042</td>
</tr>
<tr>
<td></td>
<td>R- AGGAGCCATTGCTGACAATC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.1 The mRNA levels of endometrial genes in heifers and lactating dairy cows. The data are represented as mean ±SEM. *P<0.05.
Figure 4.2 Immunohistochemical localization of endometrial IL1A (A), TNF (B) and FGF2 (C) proteins on day 11 of estrous cycle in heifers (a, b, c) and lactating dairy cows (d, e, f). a, b, c, d, e and f at 100X magnification. g (100X), h (100X) and i (250X) are negative controls using pre immune IgG and j (lung; 100X), k (lung; 250X) and l (testis; 100X) are positive controls for IL1A, TNF and FGF2 respectively. Note immunostaining in luminal epithelium (black thin arrows), glands (black thick arrows), stroma (black arrowheads) and blood vessels (blue arrows) of endometrium; luminal epithelium, submucosa and interstitial spaces of lung and interstitium and cells of the seminiferous tubules of the testis.
**Figure 4.3** The distribution of immunostaining intensity (HSCORE) in different regions of the bovine endometrium for IL1A, TNF and FGF2 during the mid luteal phase. The data are represented as mean ±SEM. LE, Luminal epithelium; GL, Glands; ST, Stroma; BV, Blood vessels. *P<0.05 between heifers and lactating dairy cows.
CHAPTER 5: INTERFERON TAU ALTERS CANDIDATE GENE EXPRESSION AND PROSTAGLANDIN CONCENTRATIONS IN MID LUTEAL PHASE BOVINE ENDOMETRIUM IN-VITRO

5.1 Introduction

Major losses of pregnancy occur during the pre-implantation period in (d 8 – 17 of gestation) in cows (Walsh et al., 2011). This period is critical in terms of embryo development, establishment of embryo-maternal communication and the maternal recognition of pregnancy. Endometrium plays a major role in the establishment of pregnancy by providing the developing embryo with embryotrophic substances through the secretion of large amounts of “histotroph” (Roberts et al., 2008). In addition, the endometrium receives signals from the embryo and processes them through various pathways by which it recognizes the pregnancy and thereby prepares for the implantation.

Many studies have attempted to investigate the receptivity of the endometrial environment to the embryo at molecular levels during estrous cycle and early pregnancy. Transcriptomic and proteomic approaches are employed to better understand the role played by the endometrium in embryo development (Bauersachs et al., 2005 & 2006; Gray et al., 2006; Klein et al., 2006). These studies have looked at the effects of pregnancy, P₄ concentrations, location (caruncular vs. intercruuncular endometrium) etc. on the endometrial gene expression in cows. Salilew-Wondim and others have compared changes in the gene expression in pre-transfer endometrium that resulted in calf delivery with those that resulted in no pregnancy in heifers (Salilew-Wondim et al., 2010). In this regard, our lab recently compared the expression of candidate genes in the endometrium
of dairy heifers and lactating cows on day 11 of the estrous cycle (Pretheebean et al., 2011). Findings of our study revealed several candidate genes to be differently regulated between the above groups of animals. In the present study, therefore we used IFNτ to manipulate the spatial and temporal expression of candidate genes in bovine endometrium.

Interferon tau, secreted by the mononuclear trophoblast cells of the bovine and ovine embryos is considered to be the primary factor of maternal recognition of pregnancy (Roberts et al., 1992). It prevents the luteolytic mechanism from taking place and thereby extends the survival of the CL in ruminant species. In addition, IFNτ, acts on the endometrium and CL to induce changes that either protect the CL and/or prepare the endometrium for embryo survival and implantation (Ahn, 2008).

The actions of natural or recombinant IFNτ that prevent the luteolytic cascade from taking place have been widely investigated in sheep and cattle. The established theory for the anti-luteolytic mechanism in sheep is that IFNτ inhibits the pulsatile release of “luteolysin” PGF2α from the endometrium by preventing the up-regulation of estrogen receptor-alpha (ERα), and thereby reducing the oxytocin receptor (OXTR) number (Spencer et al., 2007). However, in cattle whether the down regulation of OXTR takes place through the inhibition of ERα is unclear (Roberts et al., 2008).

Apart from its effect on prostaglandin synthesis, IFNτ exerts a multitude of changes in the endometrium during early pregnancy (Chen et al., 2006; Bauersachs et al., 2006; Spencer et al., 2008). Biological functions of the endometrium, which are not limited to apoptosis, angiogenesis, cellular growth and inflammation, are regulated by IFNτ during the pre-implantation period in ruminants. Therefore, we have selected IFNτ
as the candidate agent to study its effect on endometrial genes, which we previously
demonstrated to be differentially expressed in dairy heifers and lactating cows (Chapter
4).

5.2 Materials and methods

5.2.1 Animals and collection of reproductive tracts

Reproductive tracts from Holstein Friesian cows were collected at a local
abattoir within 20 min of exsanguination. The stage of the estrous cycle was determined
by examining the utero-ovarian morphology. Briefly, mid-luteal phase (d 8 – 12, d 0 =
ovulation) uteri were identified and selected according to the colour, crown size, and
surface appearance of the CL and also by the colour of the uterine lining (Ireland et al.,
1980; Miyamoto et al., 2000; Singh et al., 2009 and 2011). Reproductive tracts were
transported to the laboratory on ice within 1 – 1.5 h after collection.

5.2.2 Endometrial tissue culture

Endometrial tissue culture was performed following previously established
procedures (Singh et al. 2010). Immediately upon arrival at the laboratory, uterine horns
ipsilateral to the CL were washed three times with sterile saline containing penicillin (100
IU mL$^{-1}$) and streptomycin (100 µg mL$^{-1}$); excised longitudinally; the intercurncular
endometrium was dissected from the myometrial layer. The tissues were then cut into
small pieces (1 – 2 mm$^2$) with a scalpel; washed again with sterile saline; placed in
Dulbecco’s modified eagle’s medium (DMEM; Sigma) containing 0.1% BSA (Sigma),
penicillin (100 IU mL$^{-1}$) and streptomycin (100 µg mL$^{-1}$). One hundred mg of
endometrial tissues per well were incubated at 37°C in 5% carbon dioxide (CO₂) in a 12 well tissue culture plate (Becton Dickinson) in 2 mL of the same media.

5.2.3 Treatment with recombinant bovine IFNτ

After incubation for 20 h, the culture medium was changed and the endometrial tissues were treated with different concentrations (0, 1.25, 5 and 25 ng/mL) of recombinant bovine IFNτ (rbIFNτ; CR2058, Cell Sciences) for a further 24 h. Concentrations of rbIFNτ and treatment length used in the present study were determined based on previous studies (Desnoyers et al., 1994; Meyer et al., 1996; Johnson et al., 1999; Takahashi et al., 2003). Each treatment was performed in duplicate. The antiviral activity of the rbIFNτ used was 4.8 X 10⁷ IU/mg. At the end of the 24-hour incubation, endometrial tissues and the culture media from each treatment group were collected and stored at -20°C until RNA extraction or PG analysis, respectively. The experiment was repeated five times using reproductive tracts from different animals over a period of four months.

5.2.4 RNA extraction

Total RNA was extracted from endometrial tissues using a commercially available total RNA isolation solution, Tri Reagent (Sigma Aldrich, USA) and following the method described by Chomczynski and Sacchi, (1987) with modifications (Sing et al., 2011). The quantity and quality of the RNA was assessed by measuring the optical densities using a Nanodrop ND-1000 spectrophotometer and by observing clear bands for 28S and 18S ribosomal RNA species on an ethidium bromide stained agarose gel (1%, wt/vol).
5.2.5 Reverse transcription

First strand cDNA was generated from extracted RNA using the iScript Select cDNA synthesis kit (Bio-Rad). The reverse transcription reactions were performed by following the kit manufacturer’s protocol. Briefly, RNA samples were DNase-treated and first-strand cDNA was synthesized by incubating a 20 µL reaction mixture containing 2 µL of random primer, 4 µL of 5x iScript Select reaction mix, 1 µL of reverse transcriptase, 1 µg of total RNA and nuclease free water at 25°C for 5 min then at 42°C for 30 min. Inactivation of reverse transcriptase was performed by incubating the reaction mixture at 85°C for 5 min and the product was then stored at -20°C until use in the PCR amplification.

5.2.6 Quantitative real-time PCR

Quantitative real time PCR (Q-RT PCR) was performed to analyze the mRNA levels of all genes using the iCycler, CFX96 Real-Time PCR Detection System (Bio-Rad), iQ SYBR Green Super Mix (Bio-Rad) and gene specific primers. A Suppressor of Zeste 12 homolog (Drosophila) (SUZ12) was used as the housekeeping gene. For each sample, the reaction mixture consisted of 2.5 µL of cDNA (original cDNA was diluted 3 folds), 10 µM of each primer in a final volume of 25 µL per reaction. Reaction conditions included an initial step of 95°C (5 min), followed by 40 cycles of 95°C (15 sec), 60°C (1 min) and 72°C (20 sec). Dissociation curves were analyzed for each gene to confirm the presence of a single peak. Q-RT-PCR data (Ct values) was analyzed using the comparative Ct method (Livak and Schmittgen 2001). Expression values (Ct values) of all genes for each sample were adjusted according to the corresponding housekeeping gene’s (SUZ12) expression values (Ct values). Calculations of relative quantitation were
performed using the $2^{\Delta \Delta \text{ct}}$ method. The PCR products were confirmed by gel electrophoresis using GelRed Nucleic Acid Gel Stain (1 μL/mL; Biotium) stained 2% (wt/vol) agarose gels. Gels were photographed under ultraviolet illumination. The list of genes and the details of the primers; annealing temperature, fragment size and gene accession number are provided in Table 5.1.

**5.2.7 ELISA for PGF2α and PGE2**

Concentrations of prostaglandins F2α (PGF2α) and E2 (PGE2) in culture media were measured using high sensitivity enzyme immunoassay kits (PGFα: 930-069; PGE2: 930-001, Assay Designs). Briefly, 100 μL of samples or standards or culture media were placed in the multi-well strips coated with secondary antibodies raised in donkey against sheep IgG (PGF2α) or goat against mouse IgG (PGE2). A volume of 50 μL of respective tracers and 50 μL of primary antibodies were added to each sample. After overnight incubation at 4°C all wells were washed with 400 μL of wash buffer three times. P-nitrophenyl phosphate (200 μL) was added to each well; sealed and incubated at 37°C for 3 h (PGF2α) or 1 h (PGE2). At the end of incubation a solution (50 μL) containing trisodium phosphate in water was added to stop the reaction and the plates were read immediately by the plate reader. Readings were taken at 405 nm wave length and a four-parameter logistic curve fitting program was used to calculate the PG concentrations. The intra and inter assay coefficient of variations were 6.5 and 11% for PGF2α and 3.1 and 8.1% for PGE2.
5.2.8 Statistical analysis

Data were expressed as mean ± SEM. Statistical evaluation of mean differences of PGs and real-time RT-PCR expression levels among different treatment groups were analyzed by ANOVA, followed by the multiple comparison tests using JMP 7.0.2 (SAS Institute Inc.) software. P value of <0.05 was considered statistically significant.

5.3 Results

5.3.1 Effect of rbIFNτ on the mRNA expression levels of endometrial genes

5.3.1.1 Apoptotic genes and heat shock protein

After a 24-hour treatment, there was no change in the expression of BAX, BCL2 and HSPAIA mRNA levels at any rbIFNτ doses (Fig 5.1). However, the BAX: BCL2 expression ratio was increased at 1.25 ng/ml and 25 ng/ml doses of rbIFNτ. At 5 ng/ml the ratio was higher compared to the control, however, not statistically significant (P = 0.14).

5.3.1.2 Growth factor genes

Treatment with rbIFNτ for 24 h down regulated the relative mRNA abundance of FGF2 and had no effect on IGF1 mRNA (Fig 5.1). The highest concentration (25 ng/ml) of rbIFNτ, decreased FGF2 mRNA expression compared to the control. At a 5 ng/ml concentration, the expression level was low however, not statistically significant (P=0.10). None of the rbIFNτ concentrations affected the mRNA expression of IGF1, but the binding protein for IGF1 (IGFBP3) was down regulated in all the treatment groups compared to the control. No treatment effect was observed in IGFBP1 mRNA levels.
5.3.1.3 Cytokine and immune function related genes

The expression levels of the cytokines (IL1A, TNF and SERPINA14) are given in figure 5.1. There was no change observed in the mRNA expression levels of IL1A at any doses of rbIFNτ compared to the control. The mRNA levels of TNF did not change at lower doses of rbIFNτ treatment; however, the highest dose (25 ng/ml) down regulated the relative abundance of TNF mRNA. The mRNA levels of SERPINA14 also decreased by rbIFNτ treatments at 5 ng/ml and 25 ng/ml doses compared to the control.

5.3.1.4 Oxytocin receptor

Endometrial OXTR mRNA levels were down regulated by the rbIFNτ (Fig 5.1). All the concentrations of rbIFNτ (1.25, 5 and 25 ng/ml) were effective in decreasing OXTR mRNA levels compared to the control after 24 h incubation.

5.3.2 Effect of rbIFNτ on the secretion of prostaglandins

Enzyme immunoassay results for prostaglandins revealed decreased levels of PGF2α in the culture media with higher doses of IFNτ (5 and 25 ng/ml) compared to the control (Fig 5.2a). PGE2 levels did not change with any of the concentrations studied (Fig 5.2b)

5.4 Discussion

The endometrial environment plays an important role in early embryo development and the establishment of pregnancy in mammals. Growth factors, cytokines and many other proteins secreted by the endometrium act on the embryo and the CL in a paracrine and/or endocrine manner. Histotroph secreted by the endometrium is induced by hormones, predominantly by P₄ and other embryonic factors. The IFNτ secreted by the
pre-implantation bovine embryo interferes with the endometrial luteolytic cascade and induces alteration to the protein synthesis and secretion by the endometrium. In our previous study (chapter 4), we found differences in the expression of the selected candidate genes in day 11 endometrium between dairy heifers and lactating cows. Hence, we wanted to examine whether treatment with pharmacological agent in-vitro affected the expression of the candidate genes (BAX, BCL2, IGF1, IGFBP1, IGFBP3, FGF2, TNF, IL1A, SERPINA14 and HSPA1A). Since we found differences in the expression of IFNτ, between the pooled embryos of heifers and lactating dairy cows in our first experiment (chapter 2) and it’s well known that embryonic derived IFNτ induces various changes in the endometrium, we selected IFNτ as an agent to manipulate bovine endometrial gene expression in-vitro.

Findings of the present study reveal minimal changes in the expression of the candidate genes in the endometrium with the treatment of rbIFNτ. They are a) an increase of the BAX: BCL2 ratio, b) down-regulation of mRNA levels of FGF2, IGFBP3, SERPINA14, TNF and OXTR at higher concentrations and c) reduction in the secretion of PGF2α at higher concentrations. These changes indicate that IFNτ might play a minimal role in the endometrial gene expression during the mid-luteal phase in cows.

Even though there were no changes observed in the mRNA levels of individual apoptotic genes, the in-vitro treatment with IFNτ increased the BAX: BCL2 ratio in the endometrium. Our finding is supported by a previous study (Wang et al., 2003), where apoptosis was induced in bovine endometrial cells by IFNτ, however, this effect was diminished when co-treated with P₄. In addition, recent studies showed an up-regulation of pro-apoptotic genes without apparent apoptosis in bovine endometrium during pre-
implantation embryo development (Groebner et al., 2011; Forde et al., 2011). The later authors also observed an increase in the expression of anti-apoptotic genes. Therefore, a mechanism must be in place in the endometrium to prevent conceptus induced apoptosis and this can be explained by the anti-apoptotic effects of P₄. In our study, we did not supplement the endometrial explants with exogenous P₄ which could have prevented the increase in the BAX: BCL2 expression ratio.

In the present study, IFNτ treatment did not affect the expression of IGF1 in the endometrium. A similar result was reported in ewes where neither pregnancy nor IFNτ infusion changed the expression of IGF1 in-vivo (Gray et al., 2006). In a recent study, Forde and others have demonstrated that IFNτ does not regulate the expression of IGF1 and IGF2 in endometrial stroma prior to day 16 of pregnancy in cows (Forde et al., 2011). The IGF binding proteins (IGFBP1 and 3) modulate the availability of uterine IGFs and regulate conceptus development during early pregnancy in ruminants (Bazer et al., 2008). In the present study, although IFNτ treatment did not affect the mRNA levels of IGFBP1 in the endometrium, regardless of dose it inhibited the mRNA levels of IGFBP3. Pregnancy and the day of the estrous cycle regulate the expression level of IGFBPs, predominantly IGFBP1 which is induced by P₄ and stimulated by the presence of conceptus in sheep and cattle. Nevertheless, the stimulation of IGFBPs by IFNτ alone is found to be minimal and requires combinatorial action (Simmons et al., 2009). Their study and others (Dorniak et al., 2011), suggest that conceptus stimulation of IGFBP1 in ruminant endometrium could be mediated through the conceptus and/or endometrium derived PGs rather than IFNτ. Furthermore, conceptus stimulation of IGFBP1 is apparent only after day 13 of pregnancy in cows (Simmons et al., 2009) and that would explain...
why IFNτ had no effect on IGFBP1 mRNA levels in our study. Since IGFBP3 is the main binding protein that controls the bioavailability of IGF1 in tissues, our results indicate a potential increase in the IGF1 levels due to decreased levels of IGFBP3 mRNA in mid-luteal phase endometrium. Also, IGFBP3 may regulate conceptus development, independent of the IGF pathway (Burger et al., 2005). In addition to the local factors, insulin also regulates the expression of IGFBPs in tissues (Royal et al., 2000).

Fibroblast growth factor 2, which is a potent stimulator of IFNτ in the embryo, is secreted by the bovine endometrium under the influence of ovarian steroids (Michael et al., 2006). In the present study, FGF2 mRNA levels were unchanged after being treated with low and mid doses of IFNτ, but decreased with higher doses. In ewes, it was reported that pregnancy status does not change the mRNA levels of endometrial FGF2 until placental attachment, and protein levels do not change until maternal recognition of pregnancy (Ocon-Grove et al., 2006). In contrast, FGF2 mRNA levels were induced by IFNτ around the time of maternal recognition of pregnancy in ewes infused with physiological concentrations of IFNτ into the uterus for five days (Dorniak et al., 2011).

During the luteal phase, the endometrium secretes various cytokines and glycoproteins which are responsible for many different functions during embryo development. In the present study, IL1A expression was not affected by any of the IFNτ doses tested and is supported by the findings of microarray data (Forde et al., 2011), which reported no change in the expression of IL1 neither due to pregnancy nor IFNτ treatment on days 5, 7 and 13 post estrus in heifers. Also, TNF expression was not altered in their study; however, its expression had direct links to other up-regulated genes on day 16 pregnant animals when interaction analysis was performed. We found the same in our
study where TNF expression was not altered at low and mid IFNτ doses, but down regulated at a higher dose. Similarly, Chen et al. (2007) failed to report any differential expression of IL1A or TNF in ovine endometrial epithelial cells after IFNτ treatment. In addition, our results are in agreement with others where treatment with IFNτ at higher dose decreased TNF expression in luteal phase bovine endometrium in-vitro (Okuda and Sakumoto, 2006). Also, we observed reduced levels of PGF2α in the media after treatment with higher doses of rbIFNτ. Hence, the action of IFNτ on PGF2α secretion may have been mediated through TNF expression.

SERPINA14 was down regulated at higher doses of IFNτ in the present study. In contrast, SERPINA14 mRNA was up-regulated on day 18 and 15 of pregnancy in cows (Klein et al. 2006) and ewes (Stewart et al. 2000) respectively. SERPINA14 expression is moderate during the mid-luteal phase, and not detected between days 15 and 21 of pregnancy. It then increases as pregnancy progresses in cows. Our observation could be due to the effect of the day of the estrous cycle, dose of IFNτ or other factors such as P₄, prolactin, placental lactogen and GH, which all play a major role in regulating SERPINA14 expression in endometrium (Moffatt et al., 1987; Leslie and Hansen, 1991; Spencer et al., 1999; Stewart et al., 2000). An abundant expression of SERPINA14 is found in uterine fluid only later in pregnancy in cows and ewes (Padua and Hansen, 2010). In a recent study, expression of bubaline endometrial SERPINA14 was similar during early pregnancy and the mid-luteal phase of the estrous cycle; this supports the notion that IFNτ might not be responsible in inducing SERPINA14 expression during early pregnancy (Kandasamy et al., 2010).
We used OXTR as a control for the IFN\(\tau\) action on endometrium, where OXTR mRNA was down-regulated with all treatment groups compared to the controls. During a normal estrous cycle OT binds to endometrial OXTR and initiates the pulsatile secretion of PGF2\(\alpha\) which results in luteal regression (Flint et al., 1994). In addition, we observed a reduction in PGF2\(\alpha\) secretion by the endometrium. These findings support the fact that during pregnancy conceptus derived IFN\(\tau\) suppresses the expression of OXTR and prevents the pulsatile secretion of PGF2\(\alpha\). This in turn maintains the CL and assures continuous P\(_4\) production (reviewed by Spencer et al., 2008). However, other studies emphasize that OXTR may play only a supporting role in the prevention of luteolysis; thus other mechanisms may be present to diminish PGF2\(\alpha\) secretion and to enhance the survival of the CL in ruminants (Okuda et al., 2002; Arosh et al., 2004a & b; Krishnaswamy et al., 2009). Although, PGE2 secretion was not affected, the PGE2/PGF2\(\alpha\) ratio had increased with IFN\(\tau\) treatment (5 ng/mL) in the present study. Zitta et al (2007), reported a similar observation in bovine stromal and epithelial cell co-culture, treated with 100 ng/ml rbIFN\(\tau\). It is generally accepted that a shift from PGF2\(\alpha\) towards PGE2 is beneficial for luteal survival (Arosh et al., 2004a), however, the debate is still on going in regards to the exact role of PGs and their regulation during early pregnancy (Ulbrich et al., 2009b).

In summary, we demonstrate a direct role for exogenous IFN\(\tau\) treatment on the expression of some candidate genes in mid-luteal phase bovine endometrium in-vitro. Some of the changes observed in gene expression could be due to supra physiological concentration of rbIFN\(\tau\) used in this study. In addition, treatment effects on the expression of TNF, OXTR and secretion of PGs demonstrate the importance of IFN\(\tau\)
action in modulating luteal survival during early embryo development. Further studies are warranted to confirm IFNτ action and to elucidate the underlying molecular mechanisms controlling endometrial gene expression.
Table 5.1 Details of the gene specific primers used for PCR amplification of endometrial genes

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer sequences (5'-3')</th>
<th>Annealing °C</th>
<th>Amplicon (bp)</th>
<th>Gene Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAX</td>
<td>F- TGCTTCAGGGTTTCCATCCAG</td>
<td>58</td>
<td>223</td>
<td>U92569</td>
</tr>
<tr>
<td></td>
<td>R- AACATTTACGCGCCACTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCL2</td>
<td>F- TCGCGCGATGTACCGTACGC</td>
<td>62</td>
<td>156</td>
<td>U92434</td>
</tr>
<tr>
<td></td>
<td>R- GGTGACGCTCTCCACACACACA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HSPA1A</td>
<td>F- CAGTTCGAGGAGGAGGTTCA</td>
<td>58</td>
<td>376</td>
<td>AY149619</td>
</tr>
<tr>
<td></td>
<td>R- GGTGATGCTCTGTGTGAGGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF1</td>
<td>F- TCAGGTCGTGCAGGAGACA</td>
<td>56</td>
<td>222</td>
<td>NM001077828</td>
</tr>
<tr>
<td></td>
<td>R- ACTTCTCTCAGCTCCTG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGFBP1</td>
<td>F- CTGCAAGGTGTGAGGAGATTGC</td>
<td>60</td>
<td>157</td>
<td>X54979</td>
</tr>
<tr>
<td></td>
<td>R- GAGACACAAAGGACAGCAGCCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGFBP3</td>
<td>F- AAGAAGGACATGCAAGGACAGC</td>
<td>60</td>
<td>199</td>
<td>AF305199</td>
</tr>
<tr>
<td></td>
<td>R- GGTCGCAATGGGAATGATGGATG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF2</td>
<td>F- TACACTTTCAAGCAGAAGAG</td>
<td>56</td>
<td>214</td>
<td>NM174056</td>
</tr>
<tr>
<td></td>
<td>R- CAGCTTACTCACAGAGCAGAGA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL1A</td>
<td>F- CTCTCTACAGAAGGCTTTATG</td>
<td>58</td>
<td>424</td>
<td>NM174092</td>
</tr>
<tr>
<td></td>
<td>R- CATGTCGAAATTCAGCTCCAT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF</td>
<td>F- GAAAGCTGGAAGACAACCA</td>
<td>60</td>
<td>338</td>
<td>NM173966</td>
</tr>
<tr>
<td></td>
<td>R- TCCGTTACTGACCTGCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SERPINA14</td>
<td>F- ATATCATCTTCCTCCCCCATGG</td>
<td>60</td>
<td>126</td>
<td>L22095</td>
</tr>
<tr>
<td></td>
<td>R- GTGCACTCACAGATTTGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SUZ12</td>
<td>F- GAACACCTACACACATCTTGT</td>
<td>60</td>
<td>130</td>
<td>XM582605</td>
</tr>
<tr>
<td></td>
<td>R- TAGAGGCAGTTGCTCCTACT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OXTR</td>
<td>F- AAGATGACCTTCATCGTGG</td>
<td>60</td>
<td>318</td>
<td>AF101724</td>
</tr>
<tr>
<td></td>
<td>R- CGTGAAGAGCAGATCATCCAG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

F: Forward primer; R: Reverse primer; bp: base pair
Figure 5.1 The mRNA levels of bovine endometrial genes after treatment with rbIFNτ (0, 1.25, 5 and 25 ng/ml). The data are represented as mean ±SEM. Difference between control and treatments are indicated by asterisk. P<0.05.
Figure 5.2 Prostaglandin concentrations in endometrial culture media after 24 h treatment with rbIFNτ (0, 1.25, 5 and 25 ng/ml). a) PGF2α and b) PGE2. Data are presented as mean ±SEM. Difference between control and treatments are indicated by asterisk. P<0.05.
CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

6.1 Summary

Over the past sixty years, fertility in lactating dairy cows has declined gradually to a point that is less profitable to the dairy industry in terms of milk production and management costs. This phenomenon has been observed in high producing lactating dairy cow world wide and has been attributed to increased genetic selection for high milk yield over the years, but the underlying causes are believed to be multifactorial in nature (Lucy et al., 2001; Bousquet et al., 2004). Interestingly, the reproductive performances of dairy heifers are far better when compared to lactating dairy cows. Therefore, physiological, nutrition and management factors must be contributing to the differences observed in the reproductive efficiency of the dairy cattle.

The majority of reproductive failures occur during the early stages of pregnancy, and there is a considerable uncertainty about the physiology of early embryo development in lactating dairy cows. Since, embryo quality, uterine environment and CL functions are considered to be major elements in the determination of proper embryo development; they have been under immense scrutiny in dairy cows. In recent times, a substantial amount of information regarding the genes and proteins involved in reproductive processes has become available in many animal species including farm animals particularly bovines. This has led to the development and application of novel strategies and techniques for comprehensively investigating physiological processes such as early embryo development, uterine and CL functions.

This research was done in order to understand the nature of embryo quality, uterine and CL functions at molecular levels in dairy heifers and lactating cows, and to
identify strategies to improve their quality in cows. First, we focussed on comparing the quality of the embryo (chapter 2), CL (chapter 3) and endometrium (chapter 4) by gene expression studies using selected candidate genes between dairy heifers and 2\textsuperscript{nd} and/or 3\textsuperscript{rd} parity lactating dairy cows. Our results are suggestive of better quality embryos, CL and endometrium in dairy heifers compared to lactating cows in terms of the expression of several candidate genes examined in this study. There is a wide agreement in the scientific circle in regards to the quality differences observed in the above tissues, and our results contribute knowledge to the molecular basis of this. Secondly, in the endometrial study we manipulated the expression of genes in-vitro in cows using recombinant bIFN\textsubscript{τ}, and this altered the expression of a few candidate genes which might augment the endometrial environment and make it more conducive to early embryo development. However, further research is needed to elucidate the exact role of exogenously administered rbIFN\textsubscript{τ} on endometrial function and early embryo development in cows. The following discusses further our findings related to the quality and function of the above tissues and supports the complexity of factors involved in early embryo development of lactating dairy cows.

6.2 Comparison of embryonic, endometrial and CL gene expression between dairy heifers and lactating cows.

The prevalence of pregnancy loss in lactating dairy cows is mostly observed during the period of pre-implantation embryo development as early embryo stages are more vulnerable than the later stages due to physiological, nutritional and environmental inadequacies and stresses (Diskin and Morris, 2008). Embryo quality and maternal environment are important considerations in determining the proper development of the
conceptus and pregnancy outcome. In addition, embryo-maternal interaction is an essential process that should take place prior to implantation and involves various factors secreted by the embryo, endometrium and CL. In our study, we found differences at mRNA and/or protein levels of candidate genes in the above tissues between dairy heifers and lactating cows. Some of these differences might have a role in the decreased PR observed in lactating dairy cows.

External examination of morphological characteristics is generally considered to be the standard procedure for determining embryo quality in many animal species including bovines. However, the subjective nature of the above technique and inconsistent results has led to the use of new methods such as gene expression profiling of embryos (Badr et al., 2007). In the recent past, many candidate genes reflecting the key functions and quality of embryos have been discovered from a vast amount of genes expressed in embryos at different time points. Gene arrays are widely used to filter those genes and the results are then confirmed by more sensitive techniques such as hybridization, PCR and blotting (Evans et al., 2008). Accordingly, we used the PCR method to compare the embryo quality through examining the expression levels of candidate genes in embryos of heifers and lactating dairy cows.

Our study revealed differences in the expression levels of IFNτ, HSPA1A, Na/K-ATPase and GLUT5 genes either between heifers and lactating cows or between different grades of embryos. Expression of IFNτ by blastocyst stage embryos reflects the functional capacity of the trophoblast, therefore day 7 blastocysts of heifers in our study could be considered superior in quality than lactating dairy cows (Hernandez-Ledezma; Rizos et al., 2003). In a recent study, Lee et al. (2011) demonstrated higher expression of
IFNτ in day 8 good quality blastocysts compared to lower quality ones. They also demonstrated a similar observation for GLUT5 mRNA, which in our study did not differ between heifer and lactating cow embryos, although it was highly expressed in grade 1 blastocysts than grade 2 of lactating cows. In addition, cow embryos showed differences in the expression of Na/K-ATPase which may explain the quality differences observed morphologically, because our finding is consistent with other studies demonstrating a good correlation between Na/K-ATPase and a good embryo quality (Wrenzycki et al., 2003; Hua et al., 2010). Even though, poor quality embryos expressed increased levels of BAX, no differences were found in any of the groups in our study. The above difference is mainly observed in in-vitro conditions and in our case may not be evident because we used only grade 1 and 2 quality in-vivo produced blastocysts.

Elevated temperatures can be detrimental to embryonic development, and the embryonic cells produce HSPs to neutralize the harmful effects. HSPA1A is one of the genes expressed at the beginning of embryo development and is considered to be essential to negate heat stress, apoptosis and to maintain cellular homeostasis (Neuer et al., 1999). Higher levels of HSPA1A mRNA in heifer grade 1 compared to heifer grade 2 or cow grade 1 blastocysts suggest that it could be a marker of bovine embryo quality (Pederson et al., 2005). However, there is disagreement in the literature regarding the level of expression of HSPA1A and embryo quality. Because of its inducible nature by heat, HSPA1A expression appears to be higher in heat stressed embryos compared to the controls (Oliveira et al., 2006; Mortensen et al., 2010). In contrast, a higher expression is regarded as beneficial since it prevents cells from undergoing apoptosis and eventually embryonic death (Lonergan et al., 2003; Oliveira et al., 2006). The core body temperature
of lactating cows is higher than that of heifers; however, HSPA1A expression in grade 1 embryos was not correlated with the above observation leaving cow embryos susceptible to heat stress compared to heifers. Furthermore, Rodriguez-Alvarez et al. (2010) demonstrated higher expression of HSPA1A and IFNτ genes as a function of embryonic cells in elongated bovine embryos. Moreover, our endometrial study revealed an increment in the expression of HSPA1A in heifers compared to lactating cows; however, no changes were observed in the CL. Therefore, HSPA1A expression in an embryo and the endometrium supports the notion that embryo and endometrial quality is better in heifers than cows. In addition to gene expression, other techniques examining the quality of the embryos also demonstrated better quality embryos produced in heifers compared to lactating cows (Leroy et al., 2005). They also showed a higher percentage of grade 2 quality embryos produced in lactating cows compared to that of heifers and this is in agreement with our unpublished data (Pretheeban et al.,) examining morphological quality.

The recipient’s ability to support embryo development is dependent mainly on the secretion of histotroph by the endometrium. Many studies have characterized endometrial gene expression at different time points during the estrous cycle and pregnancy in bovines; however, there were no data available comparing heifers and lactating cows. In our study, endometrial biopsies were taken on day 11 of the estrous cycle to represent the mid-luteal phase, which is critical for the embryo’s survival. Heifer endometrium showed up-regulation of several factors involved in vital functions compared to lactating cows, suggesting a better endometrial environment for pregnancy. Expression levels of IGF1,
FGF2, HSPA1A, SERPINA14, IL1A and TNF were higher in heifer endometrium than that of lactating cows.

Although, BCL2 showed a similar profile, the ratio between BAX and BCL2 did not differ among any of the embryo groups. Also, we have confirmed the expression of FGF2, IL1A and TNF at their protein levels. Comparing that with other data in the literature, we were able to confirm the functional importance of some of the above candidate genes, related to embryo development, $P_4$ profile and uterine receptivity in cows (Clemente et al., 2009; Neira et al., 2009; Salilew-Wondim et al., 2010). However, no studies are available for a direct comparison. In addition, gene expression data in the endometrium across studies may not be consistent because of variations due to timing and location of the endometrial samples taken (Bauersachs et al., 2005). The role of IL1A in the bovine endometrium is multifaceted because it is highly expressed after ovulation and at the time of luteolysis as an inflammatory response; but during the mid-luteal phase it acts as a luteotrophic factor for the survival of the CL. We found higher expression in heifer’s than lactating cows. Interestingly, levels of the IL1A transcript are also associated with clinical and subclinical metritis (Gabler et al., 2009). None of our animals showed clinical metritis, but we can not rule out the possibility of subclinical metritis. Furthermore, increments in endometrial IGF1 and FGF2 observed in our study could enhance embryo development in heifers than lactating cows as evident in in-vitro studies (Michael et al., 2006; Neira et al., 2009). We also found differences in the expression of SERPINA14 which would provide better immunosuppression for the survival of embryo in heifers than lactating cows, but its role in bovines needs to be further elucidated.
Studies comparing the uterine environment and PR in cows after embryo transfer tend to indicate that the heifer endometrium is more conducive for embryo survival than that of lactating cows (Hasler et al., 2001; Block et al., 2010). A recent study in our lab also reflects a similar outcome on PR after transfer of frozen and fresh heifer embryos to both heifers and lactating cows (Pretheeban et al., unpublished data). Therefore, differential expression of some endometrial genes might regulate directly or indirectly the pregnancy outcome in cows. The differences in the endometrial and embryonic gene expression observed in our study could be also attributed to several other factors such as timing of P₄ rise and its concentrations, metabolic effects of lactation, nutritional effects such as uterine pH and ammonia/urea concentrations. High protein diets fed to lactating dairy cows is considered to have a negative influence on reproductive efficiency and this effect could be mediated through the altered secretion of histotroph by elevated ammonia or urea concentrations (Kenny et al., 2002; Ball and Peters, 2004). A recent study (Gunaretnam et al., 2011) in our lab looked at the effect of ammonia and urea on endometrial gene expression, and found altered levels of FGF2 expression at higher concentrations in-vitro.

When considering that P₄ regulates the secretion of histotroph and indirectly controls embryo development and survival, it can be concluded that CL function is critical during early pregnancy. Hence, we examined the expression of candidate genes in mid-luteal phase CL. Our findings indicate a better CL survival and steroidogenesis in heifers than lactating cows according to the expression of several candidate genes. The mRNA levels of 3βHSD were higher in CL of heifers than lactating cows in our study indicating a better steroidogenesis in heifers. However, we did not find any differences in
the plasma P₄ concentrations on day 8 and 10 of the luteal phase. As described in other studies, the timing of P₄ rise is more critical than the actual P₄ concentrations for embryo survival (Wijayagunawardane et al., 1996; Hein et al., 1998). The rise in P₄ was observed to be critical between day 0 and 7 post-ovulation.

Furthermore, the local P₄ concentrations within the uterus might vary compared to the peripheral levels observed in cows due to an altered metabolic clearance (Parr et al., 1993a & b; Vasconcelos et al., 1998). Reduced secretion of P₄ by the CL could be due to luteal insufficiency caused by compromised structural and functional capacities (Shelton et al. 1990). Heat stress can be another cause of reduced P₄ concentration in cows as plasma P₄ concentrations differ between cool and warm seasons (Sartori et al., 2002a). In a recent study, Luttgenau and others have demonstrated that plasma P₄ concentrations in the mid-luteal phase are dependent on luteal size and not on the mRNA levels of steroidogenic and angiogenic factors or luteal blood flow (Luttgenau et al. 2011). However, there was evidence for an association between elevated P₄ concentration and increased mRNA levels of steroidogenic factors in luteal cells on day 6-10 of estrous cycle in-vitro (Rekawiecki et al. 2005). In addition, feed intake and milk yield are two important factors that could alter the plasma P₄ concentration in cows (reviewed by Inskeep, 2004). Therefore, it is very important to consider above factors when comparing the plasma P₄ concentrations in cows.

In our study, heifers exhibited better angiogenic and lower apoptotic properties in CL compared to lactating dairy cows. Higher IGF1, VEGF, and FGF2 and IL1A mRNAs levels are indicative of better angiogenesis and luteal maintenance in heifer CL than that of lactating cows. In addition, the BAX: BCL2 mRNA ratio was higher in the CL of
lactating cows than that of heifers. Therefore, heifer CL would survive longer and function better than that of lactating dairy cows, allowing P₄ to be secreted continuously resulting in a more effective pregnancy.

6.3 Effect of IFNτ on endometrial gene expression

The ruminant conceptus secretes the pregnancy recognition signal, IFNτ, which prevents luteolysis and prolongs the secretion of P₄. Hence, IFNτ expression and secretion are critical in terms of early embryo development and establishment of pregnancy. IFNτ not only inhibits the luteolytic cascade from taking place but also, acts on the endometrium and CL to induce/suppress the expression of genes that are involved in the regulation of early embryo development, implantation, CL maintenance and P₄ synthesis and secretion. Therefore, the deficiency of an embryo to secrete adequate amounts of IFNτ will hinder the above processes and in turn affect the pregnancy. Since, IFNτ has the potential to induce changes in the endometrium prior to implantation; we treated the endometrium with rbIFNτ to improve the expression of candidate endometrial genes, which we examined in our endometrial study (chapter 4).

Treatment with rbIFNτ induced minimal changes in the expression of the candidate genes in our study. A few recent studies have demonstrated that the effect of IFNτ on endometrial gene expression is mainly measureable around the time of maternal recognition of pregnancy in cows and ewes (Dorniak et al., 2011; Forde et al., 2011). However, some of the changes observed in our study are indicative of potential IFNτ action on luteolysis/luteal survival (expression of PGE2, PGF2α and OXTR) and apoptosis (BAX: BCL2 ratio). Inhibitory effects observed on the expression of FGF2 and SERPINA14 expression could be due to the use of supra physiological concentrations of
IFN\(\tau\). Furthermore, it is important to note that the changes we observed in this study are direct effects of IFN\(\tau\) on the endometrium in-vitro; however, other factors such as ovarian steroids, embryonic and systemically derived factors may influence its action in-vivo.

6.4 Significance of dissertation and potential application

Pregnancy success is a major component of reproduction and production efficiency in dairy herds. There is enough evidence is available to demonstrate the differences in PR and reproductive efficiency between lactating dairy cows and heifers. It has been shown that embryo viability is hugely dependent on an optimum uterine environment and on good quality embryos. The focus of this study was to identify selected early embryonic, CL and endometrial gene expression profiles in heifers and lactating cows and to find methods to improve their expression levels. Findings of this study will help to: (a) understand the endometrial and CL gene expression as a basis for differences in embryo survival rate; (b) understand embryonic gene expression as a basis for differences in embryo quality; (c) produce data that might help to identify key markers of embryonic development useful for screening purposes, and (d) develop methods to improve endometrial function.

6.5 Limitations of dissertation and future directions

Even though, we demonstrated quality differences in the embryo, endometrium and CL at molecular levels between dairy heifers and lactating cows, we did not directly translate our findings in-vivo. For the embryo study, embryos could be biopsied for gene expression analysis and then transferred to recipient animals to examine the embryo
survival rate. Likewise, embryo survival rate could be assessed on the subsequent cycle/s by transferring embryos to those animals used for endometrial biopsies. It is also important to consider the possible effect of superovulation on embryo quality; therefore, it will be important to examine the quality of a single embryo derived from spontaneous ovulations. Furthermore, we could have accommodated more genes that are involved in determining the quality of the embryo, endometrium and CL, and examined their expression at the protein level as well. Moreover, examining the gene expression profiles of the endometrium and CL and the ovarian hormones at several time points during the luteal phase and during early pregnancy would improve understanding about the regulation and control of the genes used in our study. In addition, the source of the animals used for the interferon study could have accounted for some variability of gene expression data. Also the effect of rbIFNτ on endometrial gene expression and subsequent PR could be examined in-vivo.

6.6 Final conclusions

Understanding the reasons for reduced fertility in dairy cows has been a challenging area of research. Differences in the quality of embryo, CL and endometrium between heifers and lactating cows at molecular levels were found.

The key findings of this dissertation are as follows:

1. Expression of the following candidate genes differed between heifers and lactating cows.

   a) Day 7 blastocyst - IFNτ and HSPA1A; grade 1 vs. grade 2 cow blastocyst - GLUT5 and Na/K-ATPase.
b) Mid-luteal phase CL - 3βHSD, IL1A, TNF, FGF2, IGF1, VEGF, BAX: BCL2 ratio.

c) Mid-luteal phase endometrium - IL1A, TNF, FGF2, IGF1, SERPINA14, and HSPA1A.

2. Treatment with rbIFNτ altered the expression of candidate genes (IGFBP3, TNF, FGF2, SERPINA14, and OXTR) and the secretion of PGF2α in mid-luteal phase endometrium.

The conclusions derived from this dissertation have increased our understanding of the molecular basis of early embryo development and the information is of potential use to guide new research to investigate and develop strategies to improve the PR of lactating dairy cows. In addition, our findings will be useful to other species, including humans in investigating the causes of reduced fertility.
BIBLIOGRAPHY


Ahn HW. 2008. Progesterone and interferon tau regulated genes in the endometrium of the ovine uterus and expression of interferon stimulated genes in the corpus luteum during early pregnancy in sheep. MSc Thesis, Texas A&M University, College Station, Texas.


Corcoran D, Fair T, Park S, Rizos D, Patel OV, Smith GW, Coussens PM, Ireland JJ, Boland MP, Evans ACO, Lonergan P. 2006. Suppressed expression of genes involved in


Gordon IR. 2003. Biotechnology in Agriculture Series, No 27. Laboratory production of cattle embryos. 2nd ed. CABI publishing, Cambridge, MA, USA.

Gordon IR. 2004. Reproductive technologies in farm animals. CABI publishing, Cambridge, MA, USA.


Perry G. 2000. The bovine estrous cycle. South Dakota State University-Cooperative Extension Service-USDA.


Rijnders PM, Jansen CAM. 1998. The predictive value of day 3 embryo morphology regarding blastocyst formation, pregnancy and implantation rate after day 5 transfer following in-vitro fertilization or intracytoplasmic sperm injection. Hum Reprod 13:2869-73.


Wall NR, Mohammad RM, Al-Katib AM. 1999. BAX: BCL2 ratio modulation by bryostatin 1 and novel antitubulin agents is important for susceptibility to drug induced


APPENDICES

Appendix A:
Representative photographs of agarose gel electrophoresis for the genes ( chapters 2, 3, 4 and 5) analyzed by real-time PCR. Bands show the specific product size of the primers used.
Appendix B:

Progesterone levels of heifers and lactating dairy cows on day 8 post-ovulation and on the day of corpus luteum enucleation, day 10 (chapter 3). Data is presented as mean±SE.

![Graph showing progesterone levels for heifers and lactating dairy cows on day 8 and day 10 with p-values.](image-url)