EFFECTS OF HIGH PROTEIN DIET INTAKE, AMMONIA AND UREA CONCENTRATIONS ON MID-LUTEAL PHASE ENDOMETRIAL GENE EXPRESSION LEVELS IN POST-PARTUM DAIRY COWS

by

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B.V.Sc., The University of Peradeniya, Sri Lanka, 2007

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

MASTER OF SCIENCE

in

The Faculty of Graduate Studies

(Animal Science)

The University of British Columbia

(Vancouver)

April 2012

ABSTRACT

Fertility in high producing dairy cows has been decreasing over decades. Elevated ammonia and urea levels in circulation and reproductive fluids due to high protein intake is one of the major contributory factors for decreasing fertility observed in dairy cows. The objectives of this study were to examine the effects of a) ammonia and urea, and b) high protein diet intake on the mRNA expression levels of mid-luteal phase endometrial candidate fertility genes in lactating dairy cows.

In experiment I, the mRNA levels of endometrial candidate genes were measured using qRT-PCR after treating the endometrial tissues (100mg/well) with different concentrations of ammonium chloride (0, 75, 150, 300, 600 μM) or urea (0, 4, 8, 12, 16 mM), in-vitro. A high concentration of ammonium chloride (600 μM) or urea (16 mM) decreased ($P<0.05$) the expression levels of FGF2 and IGFBP1 genes when compared to the control. However, a mild concentration of ammonium chloride (150 μM) or urea (4 mM) increased ($P<0.05$) the expression levels of HSPA1A, IGFBP3, SERPINA14 and BCL2 genes. The expression levels of IGF1 and BAX genes were not affected ($P>0.05$) by any of the ammonium chloride or urea concentrations tested.

In experiment II, the mRNA levels of the candidate genes were measured using qRT-PCR in the mid-luteal phase endometrium of post partum dairy cows fed with a high (17.3% DM) or a low (14.8% DM) protein diet. The mRNA levels of all genes tested except IL1A were not different ($P>0.05$) between the two groups. The mean number of small and large follicles, mean size of large follicles, mean size of CL, and the number of days to first ovulation were negatively affected by high protein diet intake. Milk analysis

showed higher ($P<0.05$) MUN levels in the high protein group relative to that of low protein. Milk yield was not different ($P>0.05$) between the two groups.

I conclude from this study that the deleterious effect of excess dietary protein on dairy cow fertility may be due to alterations in follicular and CL dynamics and therefore embryo quality rather than any changes in the uterine environment.

PREFACE

A version of chapter 2 has been presented. Gunaretnam I, Pretheeban T, Cerri R, Veira D and Rajamahendran R. 2011: Effects of Ammonia and Urea on mRNA Expression Levels of Candidate Bovine Endometrial Genes In-Vitro. 44th Annual meeting of the Society for the Study of Reproduction, Portland, Oregon, 31 July - 4 August, 2011 (Abstract # 417). I designed and conducted the experiments and analyzed the results with the guidance of my supervisor Dr R. Rajamahendran. Dr T. Pretheeban assisted me in sample collection, conducting the experiments and reviewing the manuscript of chapter 2.

A version of chapter 3 has been submitted for presentation. Rajamahendran R, Gunaretnam I, Cerri R, Pretheeban T. 2012: The effects of high and low protein diet intake on the mRNA abundance of mid-luteal phase endometrial candidate genes and ovarian parameters in high producing lactating dairy cows. 17th International Congress on Animal Reproduction, Vancouver, British Columbia, 29 July – 2 August, 2012. I designed and conducted the experiments and analyzed the results with the guidance of my supervisor Dr R. Rajamahendran. Dr R. Cerri and Dr M.B. Gordon assisted me in conducting the experiments and sample collection for chapter 3.

Animal care committee, University of British Columbia approved the use of animals in the experiment for chapter 3. The experiment was conducted by following the guideline of Animal care committee, University of British Columbia and Canadian Council on Animal care. (Certificate #: A10-0332).

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LISTS OF ABBREVIATIONS

A	Adenine
A- site	Aminoacyl site
ADF	Acid detergent fiber
AI	Artificial insemination
ANOVA	Analysis of variance
ATP	Adenosine tri-phosphate
BAX	BCL2 associated x protein
BCL2	B-cell lymphoma 2
BCS	Body condition score
bIFNt	Bovine interferon tau
bp	Base pair
BUN	Blood urea nitrogen
C	Cytosine
CA	Canada
cDNA	Complementary DNA
CL	Corpus luteum
Cl^-	Chloride ion
CNCPS	Cornell net carbohydrate and protein system
CO2	Carbon dioxide
CP	Crude protein
Ct	Cycle threshold
D	Delta
DIM	Days in milk
dl	Decilitre
DM	Dry matter
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
ELISA	Enzyme linked immunosorbent assay
F	Forward
FGF2	Fibroblast growth factor 2
FSH	Follicular stimulating hormone
G	Guanine
GnRH	Gonadotropin releasing hormone
HP	High protein
HSPA1A	Heat shock protein 70
IGF1	Insulin-like Growth Factor 1
IGFBP1	Insulin-like growth factor binding protein 1
IGFBP3	Insulin-like growth factor binding protein 3
IgG	Immunoglobulin g
IL1A	Interleukin 1 alpha
IU	International unit
IVC	In-vitro culture
IVF	In-vitro fertilization
IVM	In-vitro maturation

K+	Potassium ion
Kg	Kilogram
LF	Large follicle
LH	Luteinizing hormone
LP	Low protein
MCal	Mega calorie
MF	Medium follicle
mg	Milligram
mg	Milligram
MHz	Mega hertz
ml	Milliliter
mM	Millimolar
mRNA	Messenger ribonucleic acid
MUN	Milk urea nitrogen
Na+	Sodium ion
Na+ /K+ ATPase	Sodium potassium adenosine triphosphatase
NE	Net energy
NEB	Negative energy balance
NEFAs	Non-esterified fatty acids
NH4Cl	Ammonium chloride
nm	Nano meter
NPN	Non-protein nitrogen
NRC	National research council
ºC	Degree celsius
ON	Ontario
P- site	Peptidyl site
pg	Picogram
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F2 alpha
PUN	Plasma urea nitrogen
qRT-PCR	Quantitative reverse transcription PCR
R	Reverse
RDP	Rumen degradable protein
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription polymerase chain reaction
RUP	Rumen undegradable protein
SEM	Standard error of the mean
SERPINA14	Serine protease inhibitor, clade A member 14
SF	Small follicle
Slc2a3	Solute carrier family 2, member 3
snRNA	small nuclear RNA
SUZ12	Suppressor of zeste 12 homolog
T°	Temperature
TCA	Tricarboxylic acid
TGF- ß	Transforming growth factor- beta

TMR	Total mixed ration
TNF	Tumor necrosis factor
tRNA	Transfer RNA
U	Uracil
UIP	Undegradable intake protein
VFAs	Volatile fatty acids
α	Alpha
µg	Microgram
µl	Microliter
µM	Micromolar

ACKNOWLEDGEMENTS

First and foremost, I would like to extend my deepest and sincerest gratitude to my supervisor Dr. R. Rajamahendran for his encouragement, guidance, and support throughout my graduate studies. Not only has Dr Rajamahendran been a great mentor, he has also extended his hand to me in friendship many times over. I am so very grateful for all that you have done. Secondly, I would like to thank my supervising committee members Dr Kimberly Cheng, Douglas Veira, and Ronaldo Cerri for reviewing my thesis and providing their constructive suggestions. My special thanks go to Dr. Ronaldo Cerri for his invaluable assistance with the endometrial biopsy procedures and ultrasonography throughout my experiments at the UBC Dairy Education and Research Center.

Besides my supervisor and committee members, I am extremely thankful to my friends Drs. T. Pretheeban and S. Pathmaniruban for making this dissertation possible. Your kindness towards my endeavours has meant a lot more to me than what I can say with mere words alone. It was Pretheeban who welcomed me to Canada and happily provided me with much needed support and encouragement in adapting to my new life in a new environment. I could not have asked for any better a friend or lab member from everything that I have gained from Pretheeban, I personally wish to thank you so much.

I gratefully acknowledge the funding sources of Dr. Rajamahendran's grants and the Guy Fowler Animal Science Grant for providing me with financial assistance during my study period.

I would like to thank Dr. Miriam Gordon for assisting me with designing and conducting my experiments at the UBC Dairy Education and Research Center. I also would like to extend my thanks to the manager, Mr. Nelson Dinn, and other staff members (Brad Duncan, Barry Thompson, Bill Kramer, Ted Toenders, Mike Duncan, Dan Peters, Andrew Bodnar, and Mary Ann la Fond) of the UBC Dairy Education and Research Center for supporting me in all the aspects of my experiments. My deepest appreciation also goes to Dr. Divakar Ambrose for providing me with the endometrial biopsy forceps for my experiments. Thank you to all for helping me out with the cleaning of the feed bins, ultrasonography, and all the other work that aided me in running my trial smoothly at the UBC Dairy Education and Research Center.

I also would like to take this opportunity to thank Ms. Sylvia Leung and Mr. Martin Hilmer for their assistance with ordering and receiving my laboratory supplies and the LFS graduate program coordinators Ms. Shelly Small and Ms. Lia Maria Dragan for handling my academic and financial matters. Many thanks go to all the LFS staff members who helped me in varying degrees of support during my studies at UBC. My special thanks go to Mr. Theepan Moorthy and Mr. Jaykumar Surendradas for proof reading my thesis and assisting with the statistical analysis of my data. Thank you to Dr. Geoffrey Hammond for providing me with the space to learn experimental techniques in his laboratory.

My wife, Erandika Prasadani, gave me encouragement, and provided faithful support during this study. This work is a success of her efforts as much as its mine and I thank her deeply for it. Most of all, my special thanks go to my parents for their love and encouragement, and my dear friends for their immense support towards the completion of my thesis. Thank you all.

DEDICATION

To My Parents

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Fertility is defined as the ability of an animal to conceive and give birth to viable offspring at regular intervals. Fertility in dairy cows can be assessed by monitoring the traditional fertility indices such as number of days open, calving interval, conception rate to first service, number of services per conception, estrus detection rate and calving rate (Royal et al. 2000). Decreasing fertility is one of the most challenging problems currently faced by farmers in the modern dairy industry (Roche, 2006). In lactating dairy cows, calving rate, first service conception rate, estrus detection rate and pregnancy rate have been reported to be declining (Butler, 1998; Norman et al. 2009; Royal et al. 2000; Washburn et al. 2002) over the past decades worldwide. However, milk production per cow has doubled over 40 years (Butler, 1998; Oltenacu and Broom, 2010) in many countries. Genetic selection of dairy cows for potential traits of high milk production coupled with improved nutritional management has drastically increased the milk yield per cow. This indicates that the improvements in genetic merits and nutritional management to increase milk production are negatively correlated with the fertility of dairy cows (Bonczeck et al. 1988). Follicular development and ovulation, corpus luteum (CL) and uterine function, fertilization and embryo development are tightly coordinated and interrelated reproductive events which should occur normally in order to ensure optimum fertility. Feed ingredients and their proportion in the diet have been shown to have a modulatory effect on one or more of the above reproductive processes leading to decreased fertility in dairy cows.

Post-partum dairy cows are fed with a diet containing high levels of protein (17 to 19 %) to support milk production (Butler, 2000). Feeding a diet formulated with high levels of protein has been shown to decrease fertility in dairy cows (Canfield et al. 1990; Elrod and Butler, 1993; Jordan and Swanson, 1979a). Feeding a high protein diet to lactating dairy cows results in excess urea and ammonia concentrations in circulation (Rajala-Schulz et al. 2001), follicular fluid (Sinclair et al. 2000), and the uterine environment (Hammon et al. 2005). These increased levels of urea and ammonia have been shown to affect proper functions of the reproductive organs such as the ovary and the uterus (Butler, 1998; Tamminga, 2006), thus resulting in decreased fertility in dairy cows. For example, ovarian follicular dynamics, CL function and uterine environment were adversely altered in dairy cows that were fed with a high protein diet (Carroll et al. 1988; Sonderman and Larson, 1989; Jordan et al. 1983). These alterations may result in the development of poor quality oocytes leading to compromised fertilization and embryo development (Bishonga et al. 1996; Blanchard et al. 1990).

Oviductal and uterine environments play crucial roles in the establishment of pregnancy especially during the early embryonic developmental stage. During this period, the embryo totally relies on the secretions of the oviduct and uterus for its survival and development. Uterine endometrial cells secrete a range of substances such as growth factors, cytokines, enzymes and transport proteins, collectively called as histotroph (Bazer, 1975; Igwebuike, 2009). Excess urea and ammonia in circulation due to a high protein diet intake in dairy cows have been shown to reduce the quality of uterine secretion, thereby creating an environment unsuitable for embryo development (Elrod et al. 1993; Jordan et al. 1983). The molecular mechanism by which the

endometrial secretions are modulated in the presence of high concentrations of urea and ammonia is not known. Therefore, in my first experiment, I investigated the effects of ammonia and urea on the mRNA levels of candidate fertility genes in the mid-luteal phase endometrium of lactating dairy cows. Recently, Pretheeban et al. (2011) have revealed that some growth factors, cytokines, immune and stress related genes were differentially expressed in the endometrium at mRNA and protein levels between lactating dairy cows and heifers. They attributed this difference to the decreased fertility observed in lactating dairy cows. Therefore, it is logical to believe that lowering the protein level in the diet would modulate the endometrial gene expression levels in dairy cows and improve their fertility. Therefore, my second experiment was carried out to investigate the effects of high and low protein diet intake on candidate endometrial gene expression at the mRNA levels in lactating dairy cows.

The rest of this chapter includes a review of literature on the bovine reproductive cycle, factors affecting fertility in high producing dairy cows, protein metabolism and the effect of protein intake on the reproductive performance of high producing dairy cows. In addition, it also includes a review of literature on gene expression, and the candidate fertility genes expressed by bovine endometrial cells.

1.2 Bovine reproductive cycle

1.2.1 Puberty and estrous cycle in cattle

In cattle, puberty is characterized as the time at which the first estrus behavior is manifested as a result of the ovaries becoming capable of releasing oocytes in response to increasing gonadotropic activity. Puberty occurs at around 8-10 months of age in

Holstein dairy cattle. However, the age of puberty is influenced by many factors including genotype, nutrition, environmental factors and growth rate. After reaching puberty, cattle exhibit regular cyclic changes in their reproductive organs which are manifested externally as estrus. The interval between two estrus periods is called an estrous cycle which is approximately 20 to 21 days in cattle. Estrous cycle is divided into 4 phases namely proestrus, estrus, metestrus and diestrus. Proestrus is the period of the last 3 to 4 days of a cycle during which regression of CL of the cycle and development of a preovulatory follicle from a dominant follicle occur. Therefore, this phase is endocrinologically characterized by declining levels of progesterone and rising levels of estradiol-17ß. Estrus is relatively a short period and on average lasts 18 to 19 hours in cows. During this period, the animal expresses the signs of estrus and becomes sexually receptive to the male. The most positive and reliable sign of estrus is the standing estrus, where a cow will stand for other cows to mount. In addition, other external signs of estrus include showing an interest in mounting other cows, restlessness, red and swollen vulval lips with clear glassy mucous discharge, frequent urination, frequent bellowing and drop in feed intake as well as milk production. During the estrus period, a rising level of estradiol-17ß secreted by the preovulatory follicle in association with the declining levels of progesterone triggers the leutinizing hormone (LH) surge resulting in ovulation. The next phase is the metestrus phase and lasts 3 to 5 days. During this period, ovulation occurs around 10 to 12 hours after the end of standing estrus. As CL is being formed from the ovulated follicle, the progesterone concentration in the circulation begins to rise during this period. A bloody vulval discharge known as metestrus bleeding can be observed mostly in heifers due to rupture of small blood vessels in the endometrium as a

result of a sudden drop in estradiol-17ß in the circulation. Diestrus is a relatively long period compared to the other phases and lasts for approximately 12 days with the presence of mature CL for 8 or 9 days. During early diestrus, blood progesterone concentration increases reaching the maximum before maintaining a constant level during the mid diestrus phase. Towards the end of this phase, PGF2α produced by the endometrium initiates a regression of the CL, the progesterone level then declines and the animal will enter into the next phase of proestrus to initiate the next cycle.

Based on the presence of CL or follicles in the ovary, the estrous cycle is more conveniently divided into two phases: the luteal phase and the follicular phase. The period of the presence of CL is called the luteal phase which is about 16 – 17 days in cows. Luteal phase begins with ovulation and lasts until the time of CL regression commences. Follicular phase is a relatively short period which is around 3 – 6 days in cows. The follicular phase begins with the onset of CL regression and lasts until ovulation.

Follicular growth, ovulation and CL formation are major events that occur during the estrous cycle. These events are initiated and properly regulated by reproductive hormones secreted by the hypothalamic-pituitary-ovarian axis. It has been well established, using ultrasound technology that follicular growth occurs in a wave-like pattern during the estrous cycle in cattle (Rajamahendran et al., 1994). During follicular growth, a cohort of follicles from the pool of primordial follicles is recruited around the time of estrus. Following a period of growth of this cohort, a single follicle emerges and continues to grow while it suppresses the growth of other follicles. On about 7-8 days of cycle, this dominant follicle reaches its maximum size and then begins to undergo atresia

between days 11 to 13. Following the regression of dominant follicle, the second wave of follicular growth occurs. Taylor and Rajamahendran, (1991a) reported that the length of the luteal phase is the determinant of number of waves of follicular growth during the estrous cycle. If circulating progesterone concentrations decrease while the second follicular wave is in its growth phase, then the second wave dominant follicle ovulates. On the other hand, this dominant follicle begins to regress and become atretic if circulating progesterone remains high even after it has grown up to its maximum size. If the second wave dominant follicle becomes atretic, the third follicular wave starts to grow.

Follicular stimulating hormone (FSH) and LH are gonadotropins secreted by the anterior pituitary. Biosynthesis and release of gonadotropins from the pituitary gland is primarily controlled mainly by the gonadotropin releasing hormone (GnRH) secreted by the hypothalamus (Senger, 2003). Neural and hormonal signals cause the hypothalamus to release GnRH into the hypothalamic-hypophyseal portal system which conveys GnRH to the anterior pituitary. GnRH is released in a pulsatile pattern from two different hypothalamic areas namely tonic and surge centers. Pulsatile release of GnRH from tonic and surge centers are responsible for the basal pulsatile and surge release of gonadotropins, respectively, from the anterior pituitary.

Basal pulsatile release of FSH stimulates the growth and development of ovarian follicles after they become gonadotropin dependent (Webb et al. 2004). In combination with LH, FSH enhances the maturation of the Graafian follicle (Hafez and Hafez, 2000). Further, FSH coupled with LH stimulates the production of estradiol-17ß and inhibin from growing follicles (Hafez and Hafez, 2000) which in turn suppress the basal release

of gonadotropins (Arai et al. 1996) by a negative feed back mechanism and hence inhibit the emergence of new follicular waves. At the end of the follicular phase, as progesterone concentrations decline with CL regression, a high concentration of estradiol-17ß triggers a surge release of LH resulting in ovulation of the dominant follicle (Hafez and Hafez, 2000). Furthermore, LH is also responsible for luteinization of the ovulated follicle and stimulation of progesterone secretion by the CL. As CL grows, progesterone increases and peaks during the mid cycle (Bearden and Fuquay, 1992). A high level of plasma progesterone inhibits a surge release of LH preventing ovulation during the luteal phase (Nanda et al. 1988; Taylor and Rajamahendran, 1994). At the end of the luteal phase, if a cow has not become pregnant, the estradiol-17ß secreted by developing follicles stimulates the expression of oxytocin receptors in the uterine endometrium resulting in the secretion of PGF2α by the endometrial cells. PGF2α, a substance responsible for the regression of CL, is transported to the ovary through a counter current mechanism between the utero-ovarian vein and the ovarian artery. Regression of CL allows a cow to repeat the estrous cycle, allowing for another opportunity to become pregnant.

1.2.2 Breeding, early pregnancy and post-partum period

After reaching puberty, dairy heifers are first bred at the age of 12- 14 months by natural service or artificial insemination. The Length of the gestation period of cattle ranges from 276 to 295 depending on the breed, sex of the calf, parity, twinning, and nutritional status (Hafez and Hafez, 2000). Breeding a dairy heifer by the age of 12-14 months allows farmers to have the first calf at the age of 24 months. After breeding, pregnancy is often diagnosed at around 25-35 days using trans-rectal ultrasonography (Taylor and Rajamahendran, 1991b). In addition, rectal palpations, non-return to estrus

and milk/plasma progesterone measurement are also used as methods for pregnancy detection.

A heifer after giving birth to her first calf is called a cow. The Early post-partum period is characterized by the recovery of the reproductive organs from the status of pregnancy as the hypothalamo-pituitary axis recovers from the negative feed back of progesterone. During this time, the uterus undergoes involution which may take about 30-40 days to be completed. Although the ovaries resume follicular growth as early as 1-2 weeks post-partum (Crowe, 2008; Rajamahendran and Taylor, 1990), regular ovarian cycles begin around days 50 after calving (Hafez and Hafez, 2000). However, this can be delayed by a number of factors such as suckling, milk production status of energy balance, body condition score and uterine disease. Generally, dairy cows are bred at their second or third estrous cycle at around 85-90 days post-partum so that inter calving intervals are maintained at around 365 days for optimum fertility and productivity.

1.3 Factors affecting fertility in high producing dairy cows

Decreasing fertility in dairy cows is a major challenge being faced by today's dairy industry worldwide (Royal et al. 2000; Lucy, 2001). The general goal of a dairy farmer is to get maximum milk yield from each cow with the minimum cost for feeding and management in order to increase profitability. Cows, like other mammals, give milk only after giving birth to a calf. Decreased milk production per cow and a lower number of calves born per year due to prolonged calving intervals and decreasing fertility are the main reasons for culling and profit loss in a dairy herd (Roche, 2006). Decreasing fertility is a problem associated with a number of factors and the mechanism(s) that lead to this

status in dairy cows is not clearly understood. Altered follicular dynamics and ovulation (Lucy et al. 1992 ; Salem et al. 2010) associated with poor estrus expression and/or detection (Lopez et al. 2004; Lucy, 2001), inadequate CL function (Howell et al. 1994; Leroy et al. 2008), poor oocyte competent and fertilization failure, altered uterine and oviductal environment (Butler, 1998; Pretheeban et al. 2011) and compromised embryo development and implantation (Rizos et al. 2010; Spencer at al. 2007) are implicated as possible reasons for decreasing fertility in lactating dairy cows (Lucy, 2007).

1.3.1 Altered follicular dynamics and ovulation

As described earlier, follicular growth occurs in a wave-like pattern during the estrous cycle in cattle. In general, post-partum dairy cows exhibit mostly two wave cycles (Townson et al. 2002; Taylor and Rajamahendran, 1991a). However, it is documented that cows with two waves of follicular growth ovulate an oocyte from an aged follicle (Ginther et al. 1989) which in turn results in reduced pregnancy rate (Townson et al. 2002). The number of waves per estrous cycle is found to be influenced by factors such as nutritional intake, parity, stage of lactation and plasma progesterone levels (Lucy et al. 1992; Murphy et al. 1991; Taylor and Rajamahendran, 1991a).

Early resumption of ovarian follicular dynamics and the first ovulation, in post-partum dairy cows, are of crucial importance for early conception. It is well known that even though ovarian follicular growth is resumed as early as 7-10 days post-partum, the first ovulation of the dominant follicle is delayed by many factors such as milk production, energy balance, dietary intake, body conditions, disease and parity (Crowe, 2008). During the first few weeks of the post-partum period, high yielding dairy cows experience an energy deficiency as the nutritional requirement is suddenly increased for

milk synthesis and the maintenance of normal bodily function. Therefore, high producing dairy cows enter into a metabolic status of negative energy balance (NEB) during the early post-partum period (Butler, 2000). Resumption of ovarian activity is found to be negatively associated with the status of energy balance in post-partum cows. Butler, (2000) reported that energy balance deprivation is correlated with low levels of blood metabolites and factors such as glucose, insulin, insulin like growth factor 1 (IGF1) and reduced LH pulse frequency, which in turn, results in low levels of estradiol-17ß production by the dominant follicle leading to delayed first ovulation in post-partum cows.

Extensive loss of body condition as measured by the Body Condition Scores (BCS) or over condition of dairy cows at or after calving is considered as another risk factor for delayed ovulation. The body condition of dairy cows should be between 2.75 to 3.0 at calving and a loss greater than 1 BCS unit within 60 days of post-calving is associated with the prolonged interval to first ovulation (Crowe, 2008). However, cows with body condition over the recommended unit at calving are also correlated with delayed ovulation due to severe NEB and extensive metabolism of fat (Rukkwamsuk et al. 1999).

Uterine diseases such as retained placenta, metritis and endometritis and other disease conditions such as acute mastitis and lameness in post-partum dairy cows are found to be associated with altered ovarian function leading to decreased fertility through various mechanisms (Sheldon et al. 2008). Acute stress caused by post-partum disease conditions are associated with a reduced secretion of GnRH and hence delayed ovulation due to inhibition of LH pulse frequency and low levels of estradiol-17ß secretion from

the dominant follicle (Crowe, 2008; Sheldon et al; 2008). In addition, uterine diseases are found to be directly associated with the inhibition of dominant follicular growth (Williams et al. 2007) and decrease the estradiol-17ß secretion from the dominant follicle (Herath et al. 2007; Sheldon et al. 2002; Williams et al. 2007) leading to delayed ovulation (Suzuki et al. 2001). The cystic ovarian follicle has been reported to be a major contributor to decreasing fertility by increasing the post-partum anestrous period. A cystic ovarian follicle is defined as any follicle measuring at least 25 mm in diameter and capable of maintaining its growth and steroid production without ovulation and present in the ovary for more than 10 days in the absence of a CL (Vanholder et al. 2006). Though physiology and etiology is not clearly understood, disturbance to the hypothalamus-pituitary- gonadal endocrine pathway is identified as the mechanism leading to the formation of ovarian cysts (Vanholder et al. 2006).

1.3.2 Poor estrus expression and detection

Expression of estrus behavior in dairy cows is mediated through a rising level of estradiol-17ß secreted by the pre-ovulatory follicle in association with declining levels of progesterone secreted by the regressing CL (Hafez and Hafez, 2000). Accurate detection of estrus is important to inseminate cows artificially during the exact time window to achieve high pregnancy rates. However, reduced intensity and duration of estrus due to low levels estradiol-17ß secreted by the dominant follicle in high producing dairy cows made detection of standing estrus difficult (Boer et al. 2010; Lopez et al. 2004). In addition, other external and internal stressors including confinement, floor surface, heat stress, high milk production and lameness are implicated with poor estrus expression in dairy cows (Diskin and Sreenan, 2000; Lopez et al. 2004, Lucy, 2001; Walker et al.

2008). The effect of high milk production on the duration and intensity of estrus expression is partly due to the reduced concentration of estradiol-17ß on the day of estrus (Lyimo et al. 2000), which in turn is probably due to the increased metabolism of steroid hormones in the liver (Sangsritavong et al. 2002).

1.3.3 Compromised CL function

Following ovulation, CL is formed from the ovulated follicle which consists mainly of two different cell types namely the theca and the granulosa. During CL formation, a mixture of theca and granulosa cells proliferate, reorganize and acquire a net work of blood supply to attain the ability to secrete progesterone. CL is a transient endocrine gland which produces progesterone, a key steroid hormone required for successful embryo development and the establishment of pregnancy in cattle (Mann et al. 2001). Reduced progesterone concentration in circulation has been shown to reduce the pregnancy rate in dairy cows (Folman et al 1983; Mann et al. 2001). Progesterone supports pregnancy through various mechanisms. Progesterone appears to promote the proliferation of uterine endometrial cells and enhance the secretion of a range of embryo supportive substances (Geisert et al. 1992) in order to prepare the endometrium for embryo implantation. In addition, the role of progesterone in maintaining the quiescence of the uterus through acting at the myometrium ensures the sustainability of pregnancy (Casey and MacDonald, 1997). Further, progesterone has also been found to have intracrine protective action on function and the survival of CL through inhibiting apoptosis induced by various agents including PGF2α (Goyeneche et al. 2003; Okuda et al. 2004). Given the action mechanisms of progesterone on pregnancy, it appears that the

success of pregnancy establishment during the early embryo development stage is greatly dependent on adequate concentrations of plasma and luteal progesterone.

Increased milk production, poor nutrition, extensive body condition loss and NEB are implicated as some of the risk factors for reduced circulating progesterone concentrations in dairy cows (Lucy, 2001). Increased milk production in association with changes in nutritional management for modern dairy cows accelerates the metabolic activity in the body (Lucy, 2001). As a result, circulating progesterone levels decreased in high producing modern dairy cows (Sartori et al. 2004; Vasconcelos et al. 2003) due to increased metabolism of progesterone in the liver (Lucy, 2001; Sangsritavong et al; 2002). Furthermore, severe NEB during the post-partum period appears to be negatively correlated with circulating progesterone concentration (Villa-Godoy et al. 1988). This may be partly due to a) the inhibitory effect of NEFAs on granulosa cell growth and metabolism, (Vanholder et al. 2005) and b) low levels of IGF1 (Butler, 2000) which is reported to have stimulatory effect on subsequent luteal progesterone secretion (Schams and Berisha, 2004). In addition, high protein intake during post-partum period is also found to be negatively associated with circulating progesterone concentration in dairy cows (Folman et al. 1983; Sonderman and Larson, 1989).

In addition to progesterone concentration, it is important that dairy cows maintain a proper length of luteal phase during the post-partum period. High yielding dairy cows are reported to have a longer luteal phase than that of moderate yielding cows (Opsomer et al. 1998) leading to the prolonged post-partum anestrous period. Conditions such as delayed uterine involution, uterine infection and other uterine abnormalities that reduce the PGF2α secretion from endometrium, NEB and heat stress are attributed as reasons for

a prolonged luteal phase in dairy cows (Lucy, 2001; Opsomer et al. 2000; Wiltbank et al. 2002). Another factor that leads to decreased fertility in high producing dairy cow is delayed post ovulatory rise of progesterone (Mann and Lamming, 2001). Green et al (2005) have reported that cows with high plasma progesterone concentration produced embryos with better developmental capacity as early as 5 days, post AI. Mann et al, (2006) reported that progesterone supplementation during 5 to 9 days post AI has significantly increased the embryo development and interferon-τ production than that of late (12 to 16 days post AI) progesterone supplementation. This indicates that delayed progesterone rise in association with low progesterone concentration is another major reason for early embryonic death in dairy cows.

1.3.4 Poor oocyte quality and fertilization failure

During folliculogenesis the follicle and its oocyte undergo changes which are characterized by follicular growth, nuclear and cytoplasmic maturation of oocyte, and stigma formation before ovulation (Hafez and Hafez, 2000). The follicles recruited from a pool of primodial follicles for further development consist of an oocyte which is arrested at the deplotene stage of prophase I of the first meiotic division. During nuclear maturation, the oocyte resumes division and progress to metaphase II of the second meiotic division where it is arrested again. The ovum is at metaphase II of second meiotic division (meiosis II) when it is ovulated. Completion of the meiosis II occurs at fertilization. During the course of cytoplasmic maturation, cytoplasm undergoes a number of characteristic changes which includes mRNA transcription, protein translation, and post transcriptional modification of proteins (Krisher, 2004). This is an essential process to support not only oocyte development (Brevini Gandolfi and Gandolfi, 2001)

but also embryo development (De Sousa et al. 1998). In addition, several ultra-structural changes in Golgi complexes, nuclear membrane, cortical granules and perivitelline space are found to occur at this stage (Merton et al. 2003). Therefore, any factors affecting the process of oocyte development may ultimately result in either fertilization failure or an embryo with poor developmental ability if fertilized.

Genetic merit for high milk production, nutrition, NEB, hormonal imbalance, parity, BCS, stage of lactation and heat stress are recognized as important factors affecting developmental capacity of an oocyte in high producing dairy cows (Lucy, 2001). Snijders et al. (2000) reported the reduced developmental capacity of embryos of fertilized oocytes from cows selected for high genetic merit for milk production. In a review article, Leroy et al (2008) stated that feeding energy rich diet, is both beneficial and detrimental to oocyte quality depending on the stages of lactation and the BCS of cows. A high energy diet would be beneficial to oocyte quality during the post-partum period in cows with poor body condition as it reduces the depth and duration of NEB and increases the availability of insulin and IGF (Adamiak et al. 2005; Butler, 2000; Gwazdauskas et al. 2000). On the other hand, a high energy diet would be detrimental during late post-partum period in cows with a moderately high body condition as it increases steroid metabolism (Adamiak et al. 2005; Sangsritavong et al. 2002). Furthermore, excessive ammonia and urea in follicular fluid as a result of high protein intake was also found to result in inferior oocyte quality (De Wit et al. 2001; Ocon and Hansen, 2003; Sinclair et al. 2000).

Follicular dynamics and hormonal imbalance are found to have a potential influence on oocyte quality. As mentioned earlier, the oocyte ovulated from a graafian

follicle of the second follicular wave would be aged when fertilized as it had to wait for a longer time before ovulation (Ginther et al. 1989). In addition, oocyte from a smaller follicle would be inferior in quality due to improper nuclear and cytoplasmic maturation (Marchal et al. 2002). Hormonal imbalances such as reduced LH pulse frequency, low levels of circulating insulin, and IGF1 are also reported to affect the oocyte developmental capacity.

According to Sartori et al. (2010), the current fertilization rate in high producing dairy cows remains to be closer to 83%. Thus, fertilization failure is not a major factor in accounting for the greater portion of decreased fertility rates observed in high producing dairy cows. Inaccurate timing of AI, however, is considered as a major reason for fertilization failure. The fertile lifespan of sperm is 30 to 48 hours in the female reproductive tract while that of the ovum is 20 to 24 hours. Ovulation occurs 10 to 12 hr after the end of standing estrus in cattle (Hafez and Hafez, 2000). Thus, inseminating cows at inappropriate times may contribute to the reduction of the sperm's or ovum's viability to participate in fertilization. In addition, fertilization rates have also been found to be reduced by poor oocyte quality which in turn could be affected by the many factors described previously. Heat stress, increased incidence of double ovulation, altered uterine and oviductal environment, poor quality sperm and improper handling of semen are some of the other factors contributing to fertilization failure (Sartori et al. 2002; Silva-del-Rio et al. 2009).

1.3.5 Compromised oviductal and uterine environment and embryo development

Early embryonic mortality is a major cause of profit loss in high producing dairy herds. Diskin and Moris, (2008) estimated that the total embryonic mortality in dairy cows including fertilization failure is up to 60%. Out of which, around 43% of the loss is due to early embryonic mortality. According to Dunne et al. (2000) most of the loss occurs between 8-16 days post insemination in dairy cows. However, the fertilization rate in high producing dairy cows is closer to 83% (Sartori et al. 2010), highlighting that early embryonic loss is the major cause of pregnancy failure in dairy cows. External factors such as heat stress, nutrition and semen quality and internal factors such as disease, oviductal and uterine environment, developmental competent of oocyte, milk production, BCS, parity and plasma progesterone concentration have been identified as contributors for embryonic mortality in dairy cows (Hansen, 2002; Lee and Kim, 2007).

Oviduct plays a pivotal role in early reproductive events as it provides a suitable environment for fertilization and embryonic development through various mechanisms (Aviles et al. 2010). The anatomical and physiological nature of the oviduct assists with the transportation of gametes and serves as site for sperm and oocyte maturation, sperm selection, and storage (Coy et al. 2008; Talevi and Gualtieri. 2010). After fertilization in the oviduct, the zygote begins to under go a series of mitotic cell divisions through the process called cleavage. As cell division continues, the embryo moves toward the uterus. During this time, oviduct and its secretion play a central role in embryo survival and development in order to make sure that the embryo is competent enough for further development in the uterus (Aviles et al. 2010; Ulbrich et al. 2010). Oviductal fluid is

composed of vast number of components including growth factors, cytokine and receptors, hormones and receptors, proteases and inhibitors, antioxidant protective agent, defense agent, chaperones and heat shock proteins and other proteins and enzymes which are necessary for optimal embryo development (Aviles et al. 2010). The presence of receptors in embryonic cells corresponding to molecules found in oviductal fluids suggests the importance of oviductal fluid in embryo development (Kane et al. 1997; Lee and Yeung, 2006). Further, Rizos et al. (2002) have shown a differential expression of developmentally important genes between embryos grown in in-vitro and in-vivo environments. They concluded that lack in some of the components of oviductal fluid in the in-vitro environment was the reason for aberrant gene expression in in-vitro produced embryos.

In cows, the fertilized egg reaches the uterus 4-5 days after ovulation when it is at the 8-16 cell stage. The morula, a solid mass of cells (blastomeres), is formed by 5 to 6 days after ovulation and at about day 7, the cells begin to differentiate to become a blastocyst. The blastocyst consist of two distinct cell types namely the trophoblast and the inner cell mass with a cavity, known as blastocoel. Trophoblast, the outer covering layer of blastocyst, is destined to become a placenta while the inner cell mass differentiate into a fetus. By the end of the first week, the blastocyst consists of about 120 cells and at about day 9 zona pellucida begins to fragment and blastocyst hatches out. This is then followed by blastocyst elongation and development of germ layers at about day 14. By day 16, the embryo signals its presence to the mother, a process called maternal recognition of pregnancy, by secreting an interferon called bovine interferon tau (bIFNτ)

to prevent luteolysis and maintain a continuous progesterone supply from the CL to establish successful pregnancy.

The first 2 weeks of pregnancy is a critical period of embryo development as the embryo completely relies on the oviduct and uterine environment for its survival. Thus, provision of optimal uterine environment is important to ensure normal growth and viability. Differential expression of endometrial genes during different stages of the estrous cycles and pregnancy indicates that the uterus undergoes dynamic changes during the cycle under the influences of ovarian steroids (Bauersachs et al. 2005; Klein et al. 2006; Mitko et al. 2008; Shimizu et al. 2010). The effect of steroid hormones and other factors such as cytokines and growth factors on pre-implantation endometrium are morphologically characterized by increased differentiation and proliferation of endometrial cells and glands (Gray et al. 2001; Groothuis et al. 2007). In order for the nourishment and development of the conceptus, the uterine endometrium secretes an array of substances such as growth factors, cytokines, lymphokines, enzymes, transport proteins, and other substances (Bazer, 1975; Igwebuike, 2009). A number of studies have clearly demonstrated the importance of uterine secretion as a primary regulator of conceptus development and pregnancy establishment (Bazer, 1975; Gray et al. 2001; Igwebuike, 2009; Spencer and Bazer, 2004).

Taken together, it suggests that any factors that alter the uterine and oviductal environments and their secretions could be detrimental to early conceptus survival and the embryo developmental process. Endometrial and oviductal secretions are found to be influenced by factors such as plasma estradiol-17ß and progesterone levels, and the presence of gametes and the embryo (Kodithuwakku et al. 2007; Geisert et al. 1992;

Georgiou et al.2007; Spencer et al. 2004). In addition, dietary nutrition, parity, BCS, and NEB are also reported to alter uterine endometrial function leading to decreased fertility in high producing dairy cows. High protein diet intake in ruminants has been shown to reduce the quality of uterine secretion consequently, creating an environment unsuitable for embryo development (Butler, 1998; Elrod et al. 1993; Jordan et al. 1983).

Previous research literature on the aspect of the relationship between nutrition and fertility indicates that dietary feed constituent and amount play a major role in reproduction in association with many other factors such as milk production, parity and NEB in high producing dairy cows. High protein intake is a major reason for reducing the fertility rate observed in high yielding dairy cows.

1.4 Protein metabolism and effect of high protein intake on reproductive performance of high producing dairy cows

1.4.1 Protein metabolism in ruminants

Protein is an essential nutritive element that provides amino acids for a wide range of important body functions including growth, reproduction, lactation and maintenance of normal bodily function in mammals. Unlike in non-ruminant animals, the protein metabolism in ruminants is a complex process which is typically characterized by microbial protein synthesis in the rumen by its inhabitants of different species of microbes: bacteria, protozoa and fungi. Protein content of the diet is generally referred as crude protein (CP) which includes both true protein and non-protein nitrogen (NPN) (NRC, 2001). The true protein ingested by ruminants can be classified into two categories based on their rumen degradability: a) ruminally degradable protein (RDP), the protein

which can be fermented by ruminal microbes, b) ruminally undegradable protein (RUP), the protein which can not be catabolized by microbes in the rumen. NPN, which is also classified under the category of RDP (Bach et al 2005), includes low molecular weight compounds such as amino acids, RNA, DNA, ammonia, urea, amines and amides (Bach et al. 2005; NRC, 2001).

The event of microbial protein synthesis involves degradation of RDP into peptides, amino acids and incorporation of amino acids into microbial protein (Bach et al. 2005). During RDP degradation, microbes attach with or ingest/absorb feed particles depending on solubility and size of feed particle and the type of microbes involved (Craig et al. 1987; Jouany and Ushida, 1999). Following this incorporation, the feed particles are catabolized into small peptides and free amino acids by proteolytic enzymes extra or intracellularly. Extracellular peptides and amino acids are absorbed by microbes where peptides undergo further degradation into free amino acids (NRC, 2001). A portion of free amino acids will be incorporated to form microbial proteins while the remaining will be deaminated to provide their carbon skeleton for the production of Volatile Fatty Acids (VFAs) (Bach et al. 2005). Deamination of amino acids and catabolism of NPN will result in the accumulation of ammonia in the rumen which may be used for microbial protein synthesis. Thus, at the end, the component of ruminal digestion is composed of ammonia, microbial protein and RUP. Microbial protein and RUP, in turn, will be available for passage as metabolizable protein to the small intestine (Bach et al. 2005; NRC, 2001). Large portion (50-80%) of the metabolizable protein available for absorption in small intestine is represented by microbial protein (Strom and Orskov, 1983). The metabolizable protein is then broken down by pancreatic enzymes into amino

acids and peptides and absorbed by the small intestine as in non-ruminant animals. Undigested protein and bacterial nitrogen is excreted through the feces.

High protein intake specifically consisting of excess RDP can apparently increases accumulation of ammonia within the rumen as a result of degradation of RDP. If microbes failed to assimilate the excessive amounts of ammonia for the production of microbial proteins, huge amounts of ammonia build up in the rumen will occur. Ammonia is absorbed through the ruminal and intestinal mucosa into the circulation and converted to urea in the liver for certain extend. This cascade process causes an increased amount of urea and ammonia to be in circulation which results in elevated blood/plasma and milk urea nitrogen (BUN/PUN/MUN) levels.

1.4.2 Effect of ammonia and urea on mammalian cell physiology

Ammonia and ammonium ions are toxic compounds produced as a by-product of protein metabolism during amino acids deamination. In order to remove the toxic ammonia from the body, the liver converts ammonia through urea cycle into urea which is a non-toxic and water soluble organic compound excreted through urine. Though urea is a non-toxic compound, high levels of circulating urea and ammonia are detrimental to several cell functions including cell growth, metabolism, and product expression (Ozturk, 2002). Hassell et al. (1991) have demonstrated that 2mM of ammonium chloride (NH_4Cl) suppressed the cell growth of different cell lines at variable degree. Presence of ammonia in culture media appeared to increase glycolysis and decrease the levels of pyruvate oxidation by the embryonic cells (Lane and Gardner, 2003). Similarly, Miller et al. (1988) reported decreased rates of oxygen uptake and increased rates of glycolysis and ATP production by hybridoma cells exposed to high concentrations of ammonia.

Expression levels of Slc2a3 (glucose transporter gene) and H19 (imprinted gene) in early embryos were perturbed by high a concentration of ammonia (Lane and Gardner, 2003; Zander et al. 2006).

Ammonia exerts its toxic effect on cell physiology through various mechanisms. It alters intra and extracellular pH, disturbs the membrane potential, and interacts with enzymes to alter metabolic pathways (Martinelle and Haggstrom, 1993; Ozturk, 2002). Presence of ammonia/ammonium in the extracellular fluid increases their concentrations in the cytoplasm and other cellular compartments. This, in turn, changes the intra and extra cellular pH (Martinelle and Haggstrom, 1993). Unlike ammonia, ammonium ion is unable to readily diffuse through the cell membrane, thus it is transported actively though Na^+ /K^+ ATPase and Na^+ K^+ $2Cl^-$ cotransporter transport proteins (Knepper et al. 1989; Post and Jolly, 1957). During transport, ammonium ions compete with K^+ for its binding site resulting in a disturbance to K^+ gradient over cytoplasmic membrane and hence membrane potential (Knepper et al. 1989; Ozturk, 2002). Ammonia is known to stimulate the conversion of α-ketoglutarate into glutamate in embryonic cells therefore reducing the amount of α-ketoglutarate entering into TCA cycle (Gardner and Lane, 1993). Taken together, this suggests that the major consequence of all the toxic pathways of ammonia is increased energy demand by cells for maintaining the ion gradients across the cell membrane (Martinelle and Haggstrom, 1993).

Urea is known to be a potent inhibitor of cell division. Buendia and Karsenti, (1992) and Stoppin-Mellet et al. (1999) reported that urea inhibits microtubule nucleation whereby preventing polymerization of tubulins into microtubules. This supports the result of De Wit et al. (2001) who found that bovine oocytes cultured in the presence of 6mM

of urea arrested oocyte division at metaphase I. High urea concentration is appeared to be stressful to cells and induces apoptosis in cell culture (Michea et al. 2000). Urea is readily permeable across the cell membrane and therefore it is not a hypertonic molecule (Burg et al. 2007). However, direct diffusion into the cell is reported to cause denaturation of protein and nucleic acids (Yancey et al. 1982).

All of the above data suggest that excess circulating ammonia and urea resulting from high protein intake could have adverse effects on reproductive processes starting from ovarian functions to embryo development in the uterus of dairy cows (Butler, 1998; Tamminga, 2006).

1.4.3 Effect of protein nutrition on reproductive functions of dairy cows

The diet composition of high producing dairy cows is changed at early lactation in order to meet nutrient requirements for high milk synthesis, maintenance, and to minimize negative energy balance during the post-partum period (Butler, 2000; Tamminga 2006). Thus, post-partum cows are fed with a diet containing high levels of protein (17 to 19 %) (Butler, 2000). On the other hand, it is also well documented that a diet formulated with high levels of protein is accompanied with decreased fertility in dairy cows (Canfield et al. 1990; Elrod et al. 1993; Jordan and Swanson, 1979a; Tamminga, 2006). Feeding a high protein diet to lactating dairy cows has been shown to result in excess urea and ammonia concentration in circulation (Larson et al. 1997; Rajala-Schulz et al. 2001; Roseler et al. 1993), follicular fluid (Moallem et al. 2011; Sinclair et al. 2000), and the uterine environment (McEvoy et al. 1997). These increased levels of urea and ammonia have been shown to affect reproductive functions such as follicular growth and ovulation, CL function, fertilization and embryo development,

maternal reorganization of pregnancy and implantation and uterine environment (Butler, 1998; Garverick et al. 1971; Lane and Garnder, 2003; Tamminga, 2006).

1.4.3.1 Effect of high protein intake on follicular dynamics and Ovulation

A protein diet consisting of excess CP and RDP has been shown to alter the follicular dynamics in dairy cows (Tamminga, 2006). Santos et al. (2001) have revealed that prepartum cows fed with a high protein diet had fewer numbers of small and large follicles in comparison with cows fed with a low protein diet. Carroll et al. (1988) have reported that first ovulation is delayed by 5 days when post-partum cows were fed with 20% of CP in comparison with that of cows fed with 13% of CP. This alteration in follicular dynamics could be the result of increased concentrations of urea (6.5 mM) and ammonia (267 μM) in the follicular fluid (Leroy et al. 2004; Sinclair et al. 2000). Rooke et al. (2004) reported an alteration in the growth and metabolism of granulosa cells when they were incubated in higher concentrations of ammonium chloride (200- 1600 μM) relative to control (0 μM). It has been suggested that energy balance deprivation due to high protein intake is correlated with low levels of blood metabolites and factors such as glucose, insulin and insulin like growth factor 1 (IGF1). High protein diet intake is also shown to reduce LH pulse frequency and, in turn, lower the levels of estrodiol-17ß production by the dominant follicle leading to delayed first ovulation in post-partum cows (Butler, 2000; Tamminga, 2006). However, other studies revealed that feeding a high protein diet is less likely to alter the dynamics of follicular development in dairy cows (Armstrong et al. 2001; Garcia Bojalil et al. 1994; Kaim et al 1983; Laven et al. 2004).

1.4.3.2 Effect of high protein intake on CL function

A functional corpus luteum is required for the maintenance of pregnancy. The Peripheral blood levels of progesterone and the size of the CL are good indicators of luteal function. The circulating concentration of progesterone decreases during the mid-luteal phase of the first estrous cycle in post-partum dairy cows fed with a high protein diet (Folman et al. 1983; Jordan and Swanson, 1979b; Sonderman and Larson, 1989). Moreover, Garverick et al. (1971) reported that CL obtained from Holstein-Friesian heifers fed with urea were lighter in weight and synthesized less amount of progesterone during in vitro incubation than that of animals fed with no urea. Altered growth and metabolism of granulosa cells (Rooke et al. 2004) due to elevated ammonia concentration in follicular fluid (Hammon et al. 2005) and elevated uterine PGF2α concentration (Butler, 1998) are possible causes which might explain the above observations. However, Blauwiekel et al. (1986) reported no deference in the mid-luteal blood progesterone concentrations of the two groups of non lactating cows fed with 25% and 15% of protein in the diet.

1.4.3.3 Effect of high protein intake on oocyte and embryo development

Decreased fertility observed in modern high producing dairy cows is partly attributed to increased PUN/BUN levels during the post-partum period (Canfield et al 1990; Melendez et al., 2000; Rajala-Schultz et al. 2001; Rhoads et al. 2006). Hammon et al. (2005) have reported that PUN levels greater and lesser than 20 mg/dl are associated with follicular fluid ammonia concentration 339 μM and 93.9 μM, respectively. Further, Sinclair et al. (2000) have reported that the PUN levels 19.6 mg/dl and 15.9 mg/dl are

associated with follicular fluid ammonia concentration 267 μM and 205 μM, respectively. Thus, low pregnancy rates observed in dairy cows fed high protein diet could be possibly due to the exposure of oocytes to the high concentration of ammonia and urea resulting in inferior quality oocytes and hence embryos with reduced developmental capacity. This hypothesis is confirmed by the observation that oocytes recovered from heifers and ewes fed with high protein or supplementary urea yielded poor quality embryos when used for in-vitro embryo production (Armstrong et al. 2001; Papadopolous et al.2001; Sinclair et al. 2000).

Rooke et al. (2004) reported that exposure of bovine granulosa cells to high concentrations of ammonium chloride (0 vs 200, 400, 800, 1600 μM) in-vitro adversely affected their growth and metabolism and resulted in the development of poor quality embryo. De Wit et al. (2001) and Ocon and Hansen, (2003) also demonstrated that increased urea concentrations (0 vs 5, 6, 7.5 mM) during in vitro maturation is associated with alterations in bovine oocyte nuclear maturation and subsequent embryo development. Such data suggest that high protein intake might affect the success of pregnancy by acting at the level of oocyte development.

Introduction of reproductive biotechnology such as multiple ovulation and embryo transfer, in-vitro maturation, fertilization and culture has made it possible to study the mechanisms by which various factors affect the normal physiology of embryo development. Various morphological characteristics such as color and shape of the embryo, cleavage rate, size and shape of the blastomeres, development rate to morula and blastocyst, blastocyst cell number, inner cell number and embryonic cell apoptosis are widely used to asses the embryo quality as a part of embryo transfer procedure (Merton,

2002). In addition, many researchers have attempted to use physiological and genetic characteristics such as embryo metabolism, intracellular pH, and embryo gene expression level as measurements of embryo quality in recent years (Zander et al. 2006; Pretheeban et al. 2009).

A number of studies have been carried out to investigate the direct effect of protein nutrition and its metabolites, ammonia, and urea on embryo development. Blanchard et al. (1990) have reported that a higher percentage of unfertilized and non-transferable embryos were recovered from superovulated cows fed with high levels of RDP when compared with that of low levels of RDP. Moreover, Dawuda et al. (2002) revealed that cows fed with supplementary urea from the day of insemination to embryo collection reduced both quality and quantity of recovered embryos. Bishonga et al. (1996) and McEvoy et al (1997) reported that elevated plasma urea (~ 3 vs ~7 mM and 2 vs 7.1 mM, respectively) and ammonia (50.9 vs 90.4 μM) levels in superovulated ewes fed with urea reduced embryo recovery rate and viability in-vivo and in-vitro. However, it is not clear if urea and ammonia act throughout the oocytes and embryo development or only at certain specific stages.

Examining the effect of ammonia and urea during various developmental stages of embryo in-vivo and in-vitro would allow determining the time points where embryo development is affected. Rhoads et al. (2006) have reported that reduced conception rates observed in lactating dairy cows with PUN level > 19 mg/dl is due to the toxic effects of urea on oocyte or embryos less than 7 days old. Moreover, Fahey et al. (2001) demonstrated that dietary urea exerts its deleterious effects through acting at oocyte or oviductal environment in ewes. This indicates that the development of oocytes and

embryos at oviduct in ewes and during the first week of insemination in cows is highly sensitive to increased PUN level.

When bovine oocytes and embryos were exposed to different concentrations of ammonia (concentrations (0, 29, 88, 132, 176, 356 μM) were selected to simulate normal and excessive physiological levels) during in-vitro maturation (IVM), fertilization (IVF) and culture (IVC), Hammon et al. (2000) found that high concentrations adversely affected the cleavage and blastocyst development rate. However, exposure of oocytes to moderate concentrations (29 and 88 μM) of ammonia during IVF increased the subsequent developmental capacity of embryos (Hammon et al. 2000). Gardner and Lane, (1993) have demonstrated that the presence of ammonia greater than 75 μM and 150 μM in mouse embryo culture media reduced the blastocyst cell number and development rate of zygotes to morula and blastocyst, respectively. Lane and Gardner, (2003) and Zander et al. (2006) also found that the presence of ammonia in mouse embryo culture media resulted in a significant reduction cell number of inner cell mass, and an increase in the number of apoptotic cells in the blastocyst. Further, it has also been revealed that the presence of ammonia or urea in the environment of embryos significantly decreased the intracellular pH, and perturbed the metabolism and candidate gene expression levels by ovine and mouse embryos (Lane and Gardner, 2003; McEvoy et al. 1997; Zander et al. 2006).

As described earlier, ammonia and urea are believed to have detrimental effects on embryonic cells through various mechanisms. However, the out come of all these pathways result in costing valuable energy to individual cells. The energy deprivation

during compaction and blastulation when energy demand is high may result in poor quality embryo production (Hammon et al. 2000).

1.4.3.4 Effect of high protein intake on uterine environment

Optimum uterine environment is crucial for embryo viability. High protein diet intake in lactating dairy cows have been shown to create uterine environment unsuitable for embryo development (Butler, 1998). In cows and ewes, excess CP or urea/ammonia intake/infusion is reported to be associated with elevated urea and ammonia levels in uterine and oviductal fluid (Butler, 1998; Kenny et al. 2002; McEvoy et al. 1997; Jordan et al. 1983). This elevation might subsequently be detrimental to embryo development in two ways: 1) ammonia and urea could directly exert their toxic effect on embryonic cells (De Wit et al. 2001; Lane and Gardner, 2003; Ocon and Hansen, 2003; Yuan and Krisher, 2010; Zander et al. 2006), 2) they could indirectly act on embryos by altering the functions of uterine endometrium and in turn, the uterine milieu (Blanchart et al. 1990; Elrod and Butler, 1993; Jordan et al. 1983; McEvoy et al. 1997; Rhoads et al. 2004).

Excess CP or RDP intake have been shown to decrease and tend to decrease uterine pH at day 7 and at day of estrus, respectively in dairy cows and heifers (Erold and Butler, 1993; Elrod et al. 1993). Similarly, Rhoads et al. (2004) demonstrated that jugular infusion of urea decreased uterine pH from 7.06 to 6.88 over 12 hours. Mineral composition of uterine fluid is found to be affected by high protein intake. Uterine fluid K, Mg and P concentrations were lower during luteal phase in cows fed with high protein diet (Jordan et al. 1983). This observation is supported by the results of Kenny et al (2002) who found that oviductal calcium concentration is reduced when heifers were infused with urea. However, it has also been shown that uterine fluid protein, PGF2α,

prostaglandin E_2 concentrations were not altered as PUN concentration (22.6 vs 16.6 mg/dl) increased (Rhoads et al. 2004) though PGF2α secretion is reported to be increased by endometrial cells treated with urea in-vitro.

Considering the findings of the above studies, it is clear that elevated systemic PUN associated with high CP intake could be detrimental to embryo development through altering uterine fluid pH and composition in addition to their direct effect on embryos. However, the mechanism where by ammonia and urea resulting from high protein intake alters the uterine secretion at gene expression level remains to be elucidated.

1.5 Gene expression and candidate fertility genes expressed by endometrial cells

1.5.1 Overview of gene expression in eukaryotic cells

Gene expression is a complex process that takes the information encoded in the gene to synthesize specific gene products such as protein and RNA. Steps involved in protein coding gene expression for protein synthesis includes transcription, RNA splicing, translation, and post translational modification of the protein. However, expression of non-protein coding gene results in production of functional RNAs such as rRNA and tRNA where gene transcribed into RNA will not be translated. At cellular level, gene expression is essential for a cell to control its structure and function. For an organism, gene expression is essential for an array of functions including cell differentiation, morphogenesis and versatility.

Transcription is a cascade of events whereby a complementary single strand mRNA is produced from a sequence of DNA by the action of RNA polymerase. Factors

involved in transcription include DNA, transcription factors, RNA polymerase and ATP. The portion of the DNA used to produce mRNA is called the transcription unit, which consist of protein coding region, exon and non-coding region, intron. During transcription, transcription factors bind with DNA to a region called TATA box which promotes binding of RNA polymerase to the DNA and the formation of transcription complex in association with other transcription factors. Using the energy provided by the reduction of ATP, RNA polymerase moves along the DNA strand breaking hydrogen bonds between complementary nucleotides. Further, while moving, RNA polymerase adds RNA nucleotides which are complementary to DNA bases to the RNA sugar-phosphate back bone and form pre-mRNA template. Once transcription is completed, the pre-mRNA will be released out of DNA strand for RNA splicing.

RNA splicing is the process of post transcriptional modification of the pre-mRNA. Like the gene from which it was transcribed, pre-mRNA contains regions of intron which interrupt the coding region of the exon. Intron must be removed from pre-mRNA before it is released into cytoplasm as mature mRNA for translation. mRNA splicing is mediated through splicesome which consists of several small nuclear ribonucleic acid (snRNA) and proteins complexes. At the beginning of the splicing, two snRNA protein complexes bind to pre-mRNA at or near intron- exon boundary leading to whipping of pre-mRNA from 5'- end of the intron towards 3'-end. Then, three other snRNA protein complexes bind to previously bound snRNA protein complexes to form splicesome which finally splices the pre-mRNA at intron-exon intersections and ligate two exons together to form mature mRNA. Eventually, a mature mRNA migrates from the nucleus to the cytoplasm for translation.

Translation is the process whereby protein is synthesized from a mature mRNA template. Molecules involved in this process include mRNA, small and large subunits of ribosome, tRNA and release factor. The process is divided into three major events: initiation, elongation and termination. Mature mRNA contains methylated cap at 5'-end, poly A tail at 3'-end and several codons for specific amino acids in between. During the initiation, the small subunit of the ribosome attaches to methylated cap and moves towards the first codon of RNA which is called translation initiation site. tRNA contains anti-codon which is complementary to the codon on mRNA that specifies a particular amino acid. First mRNA codon is typically AUG which corresponds to the amino acid methionine. First tRNA with anti-codon of UAC carrying methionine binds with first codon of mRNA. Large subunit of ribosome contains two binding sites, P and A site. P site of the ribosome bind with first tRNA while A site is occupied by 2^{nd} tRNA with an anti codon complementary to 2^{nd} codon on mRNA. Methionine is then transferred to the amino acid carried by 2nd tRNA. Then, as the first tRNA exits from the P site, 2^{nd} tRNA moves from A site to P site leaving A site available for 3^{rd} tRNA which carries another amino acid to the peptide chain. As ribosome moves along the mRNA, the process of polypeptide chain elongation continues until stop codon is reached. When the stop codon reaches the A site, the release factor enters A site and terminate the elongation process leading to the completion of translation. Then, ribosome disintegrates from mRNA and newly formed protein is released.

Post translational modification is a process whereby a protein after translation undergoes chemical modification before becoming functional. This modification includes

processes such as the addition of other functional molecules (i.e. carbohydrates and lipids), removal of amino acid from amino end, and formation of disulfide bonds.

Regulation of gene expression is essential for cells to control the quantity and timing of production of a protein. Gene expression could be regulated at several points in the pathway from DNA to protein: transcription, mRNA splicing, mRNA transport, mRNA degradation, translation and post translational modification of protein. Ability to control gene expression allows cells to control their structure and function based on the need. Measurement of gene expression is a powerful tool for assessing the activity of cells at molecular level. Gene expression is measured quantitatively and qualitatively at both mRNA and protein levels using a number of techniques such as RT-PCR, Northern and Western blotting and DNA microarrays.

1.5.2 Bovine endometrial candidate fertility genes and their function

1.5.2.1 Cell growth, proliferation and differentiation related genes: Insulin like Growth Factors (IGFs) and binding proteins (IGFBPs) and Fibroblast Growth Factor (FGF)

The Insulin-like Growth factor (IGF) system is reported to be expressed in many reproductive tissues (Geisert et al. 1991; Kirby et al. 1996) and believed to play a pivotal role in the regulation of bovine endometrium during the estrous cycle (Robinson et al. 2000). IGF1, IGFBP1 and IGFBP3 are some of the important members of the IGF system. The IGF1 is known as a factor having potential influence on tissue differentiation and proliferation and metabolism of various cell types including the bovine endometrial cells (Robinson et al. 2000; Stewart and Rotwein, 1996). Moreover, IGF-I is reported to enhance cell division and blastocyst formation in the preimplantation mouse embryo

(Harvey and Kaye, 1989). Expression of IGF I and II by conceptus (Watson et al. 1992) during the pre-implantation period and their ability to increase the production of IFNτ by ovine embryo (Ko et al. 1991) indicate their plausible importance during embryo development. IGFBP1 and IGFBP3 are two of six IGFBPs which control the bioavailability of IGFs to target cells. In addition to this function, they are also reported to act independent of IGF to stimulate cell migration in different cell types including the trophectoderm cells (Gleeson el at. 2001; Jones et al. 1993; Simmons et al. 2009).

The fibroblast growth factors are thought to be involved in a wide range of cellular activities including apoptosis, cell survival, chemotaxis, cell adhesion, angiogenesis, migration, differentiation and proliferation of various cells and tissues (Itoh, 2007; Power et al. 2000). Until recently, at least 23 members of the FGFs have been identified in mammals (Itoh, 2007). Expression of FGFs in the endometrium and their receptor in early embryos suggest the involvement of FGFs during embryo development and pregnancy (Cooke et al. 2009; Michael et al. 2006). FGF2 is an important member of FGFs which plays a key role during early embryogenesis (Gospodarowicz, 1991). In cows and ewes, expression of FGF2 is detected throughout the early embryo stages including blastocyst (Daniels et al. 2000; Gospodarowicz, 1991) and in the endometrium, and shown to increase IFNτ mRNA and protein abundance in the trophectoderm cells (Cooke et al. 2009; Michael et al. 2006; Ocon-Grove et al. 2008). Moreover, supplementation with FGF2 alone or together with TGF- ß increased the blastocyst development rate (Fields et al. 2011; Larson et al., 1992) while the addition of an inhibitor of FGF receptor kinase activity decreased blastocyst development rate in vitro (Fields et al. 2011).

1.5.2.2 Apoptosis and stress related genes

The apoptosis is a process of programmed cell death during which cells undergo characteristics morphological changes before cell death. Among numerous gene transcripts involved in apoptosis, members of the BCL2 gene family play a major role in regulating apoptosis (Adams and Cory, 1998). The BCL2 gene family is categorized into two groups: anti-apoptotic and pro-apoptotic (Adams and Cory, 1998). Within the BCL2 family, the BCL2 gene is characterized as a pro survival and apoptosis-suppressing factor whereas the BAX gene is identified as an apoptosis-promoting factor (Banerjee et al. 2002; Hockenbery et al. 1990). The ratio between these two gene transcripts determines the fate of cells and therefore considered as a good indicator of assessing the degree of apoptosis in cells and tissues. Studies have shown that apoptosis regulates remodeling of endometrium during estrous/menstrual cycle and implantation (Okano et al. 2007; Gompel et al. 1994). Endometrial cell proliferation and differentiation is an important event that prepares the uterine internal environment for implantation. Expression levels of anti and pro apoptotic genes between the receptive and the non receptive endometrium have been studied by Salilew-Wondim et al. (2010) and have reported that higher expression levels of anti and pro apoptotic genes in the receptive and the non receptive endometrium, respectively. Similarly, Pretheeban et al. (2011) have shown higher levels of BCL2 expression in the endometrium of dairy heifers than lactating cows in which the uterine environment is thought to be less receptive to embryos compared to heifers (Pursley et al. 1997).

The Heat shock proteins (HSPs) are molecular chaperones ubiquitously found in mammalian cells. In addition, they are highly expressive in response to the cells and

tissues being exposed to extreme conditions such as hyperthermia and a variety of toxic conditions (Santoro, 2000). They function through assisting the correct folding of native and stress accumulated misfolded non-native proteins to repair the damaged protein and allowing the cells to survive in extreme environmental conditions (Schmitt et al. 2007). HSPs can prevent stress induced inappropriate cell death through modulating apoptotic signaling pathway (Schmitt et al. 2007). HSP70, a distinct family of heat shock proteins is encoded by HSPA1A in mammalian cells. Tabibzadeh et al. (1996) described the expression of full complement of HSP70 in the endometrium during the menstrual cycle. Proteomic analysis has shown a significant increase in HSP 70 protein level in the endometrium of infertile women compared to that of fertile women (Nip et al. 1994). Similarly, Choe et al. (2010) have demonstrated an up-regulation of HSP 27 in the endometrium of cows with endometritis at both mRNA and protein levels. Possible explanation is that accumulating leukocytes during endometritis could produce high levels of reactive oxygen species and cytokines which can modulate the expression levels of HSP70 (Jacquier-Sarlin et al. 1994).

1.5.2.3 Cytokines

The IL1A and the TNF, members of the cytokine family, are identified as factors involved primarily with immune response and inflammatory process in several cells and tissues (Pasparakis et al. 1996; Takacs et al. 1988; Vassalli, 1992). In addition, they are also thought to play a pivotal role in reproductive processes such as ovulation (Okuda and Sakumoto, 2003; Rae et al. 2004), embryo implantation (Fazleabas et al. 2004; Yelavarthi et al. 1991) and most importantly in CL maintenance (Majewska et al. 2010; Okuda and Sakumoto, 2003; Pretheeban et al. 2010; Tanikawa et al. 2005). Endometrial expressed

IL1 and TNF reported to act locally as modulators of cross talk between embryo and maternal environment and prostaglandins secretions (Hunt, 1993; Murakami et al. 2001; Okuda and Sakumoto, 2006; Simon et al. 1994; Tanikawa et al. 2005). In addition, in combination with prostaglandins they also function as modulators of luteal functions (Niswender et al. 2000). Treatment with IL1A lengthened the lifespan of CL and increase the progesterone output by luteal cells through modulating endometrial secretion of prostaglandins (PGF2α, PGE2) (Majewska et al. 2010; Okuda and Sakumoto, 2003; Tanikawa et al. 2005). TNF is also found to modulate prostaglandins secretion from endometrium and therefore stimulate the CL progesterone production and lengthen the CL lifespan in cattle (Skarzynski et al. 2003).

1.5.2.4 Immune related gene

SERPINA14, a member of super family Serine Protease Inhibitor (serpins) is secreted by the uterine endometrial epithelial cells under the influence of progesterone and estradiol-17ß in many species, including the bovine (Padua and Hansen, 2010; Ulbrich et al., 2009). Secretions of higher of SERPINA14 by the endometrium during pregnancy indicate its plausible important role in the establishment and maintenance of pregnancy (Mitko et al. 2008). It has been shown that SERPINA14 performs a variety of biological functions during the process of pregnancy including suppression of local immune system resulting in protection of allogenically distinct conceptus from local immune reaction (Hansen and Tekin, 2005; Hansen, 2007). SERINA14 is involved in local Immuno suppression through variable mechanisms. Lymphocytes proliferations and functions were inhibited when exposed to SERINA14 (Hansen and Tekin, 2005; Skopets and Hansen, 1993). Activity of natural killer cells was reported to be reduced by

SERPINA14 in-vitro (Liu and Hansen, 1993). Moreover, SERPINA14 is reported to bind with ovine IgA and IgM in a time and concentration dependent manner (Hansen and Newton, 1988) indicating the suppressive effect of SERPINA14 on antibody mediated immune reaction against embryos (Ulbrich et al., 2009).

1.6 Hypothesis and objectives

Feeding a diet with high levels of protein is associated with decreased fertility in dairy cows. Excess systemic urea and ammonia levels resulting from high protein intake were shown to be detrimental to embryo development through altering uterine fluid pH and composition, in addition to their direct effects on embryos. Wathes et al. (2009) demonstrated that NEB in dairy cows causes alteration in the expression of some immunologically important genes, leading to delay in involution of the uterus and infertility. Pretheeban et al. (2011) have revealed the differential expression of growth factors, cytokines, and immune and stress related genes in the endometrium between lactating dairy cows and heifer. They attributed this difference to the decreased fertility observed in lactating dairy cows. Therefore, I believe that lowering the protein level in the diet would modulate the endometrial gene expression levels in a way of improving the uterine environmental quality in lactating dairy cows.

1.6.1 Research hypothesis

High protein diet intake and associated high levels of ammonia and urea in circulation could negatively alter the mid-luteal phase endometrial candidate fertility gene expression levels in post-partum dairy cows.

1.6.2 Research objectives

1) To examine the effects of ammonia and urea on mid-luteal phase candidate bovine endometrial gene expression in-vitro.
2) To examine the effect of high and low protein intake on mid-luteal phase candidate bovine endometrial gene expression in post-partum dairy cows.

CHAPTER 2: EFFECTS OF AMMONIA AND UREA ON mRNA LEVELS OF CANDIDATE BOVINE ENDOMETRIAL GENES IN-VITRO

2.1 Introduction

Fertility in high producing dairy cows has been decreasing over the past few decades. This reduction in fertility coincided with the considerable increases in milk production which was due to intensive genetic selection along with improved nutrition and management of dairy cows (Butler, 2000; Dobson et al. 2007; Lucy, 2001). Increased culling rate due to decreased fertility accounts for the major portion of profit loss in dairy herds (Roche, 2006). Decreased fertility is a multi-factorial problem and the mechanisms behind it are not clearly understood in dairy cows. Altered uterine and oviductal environment (Butler, 1998; Pretheeban et al. 2011) leading to compromised embryo development and implantation (Rizos et al. 2010; Spencer et al. 2007; Wiebold, 1988) are implicated as possible reasons for decreased fertility in ruminants (Lucy, 2007). Butler, (2000) and Roche, (2006) are of the view that improper nutritional management is the most important cause for the disturbance of proper function in reproductive organs, including the uterine environment, leading to decreased fertility in dairy cows.

Post-partum dairy cows are fed a diet containing high levels of crude protein (17 to 19 %) to support milk production (Butler, 2000). It is well documented that diet formulated with high levels of crude protein results in reduced fertility in dairy cows (Canfield et al. 1990; Elrod and Butler, 1993; Jordan and Swanson, 1979a). One possible hypothesis was that the reduced fertility observed in cows fed with high protein diet could be due to the effect of elevated ammonia (McEvoy et al. 1997) and/or urea (Butler, 1998; Rhoads et al. 2004) concentrations in circulation and in the uterine environment.

Excess ammonia and urea in the environment of embryo is toxic to embryonic cells (De Wit et al. 2001; Lane and Gardner, 2003; Ocon and Hansen, 2003; Yuan and Krisher, 2010; Zander et al. 2006). In addition, excess ammonia and urea indirectly act on embryo by altering the functions of the uterine endometrium, and in turn, the uterine milieu as well (Blanchart et al. 1990; Elrod and Butler, 1993; Jordan et al. 1983; McEvoy et al. 1997; Rhoads et al. 2004). In particular, excess urea was reported to increase the secretion of endometrial prostaglandin F2 alpha (PGF2α) (Butler, 1998) which is involved in corpus luteum regression.

Uterine endometrium synthesizes and secretes a variety of substances, including growth factors, cytokines, hormones, enzymes and transport proteins, collectively called as "histotroph" (Bazer, 1975; Igwebuike, 2009). Components of histotroph act as crucial regulators of conceptus growth and development (Igwebuike, 2009). Any factors affecting uterine secretion and therefore creating suboptimal uterine milieu might be detrimental to embryonic development. I believe that elevated urea and ammonia in circulation resulting from high protein intake could alter the histotroph composition by exerting their effects on endometrial gene expressions. Therefore, the objective of the present study is to examine the effects of ammonia and urea on the mRNA levels of candidate fertility genes in the mid-luteal phase endometrium of lactating dairy cows.

2.2 Materials and methods

2.2.1 Tissue collection and culture

Mid-luteal phase bovine uteri were collected from a local abattoir. The stage of estrous cycle was determined based on the ovarian morphology (Ireland et al. 1980,

Singh et al. 2008). Briefly, the phase of uteri were classified as mid-luteal phase of estrus cycle when there was a fully developed corpus luteum (CL) present in the ovary, with a bright orange or yellow color mass of tissue when dissected. Uteri were transported on ice to the laboratory within an hour of collection. Uteri were washed three times with normal saline containing penicillin (100 IU/ml) and streptomycin (100µg/ml) before and after longitudinal incision of the horn ipsilateral to the CL. Endometrial tissues were cut into small pieces and placed in a culture dish containing 10 ml of Dulbecco's modified eagle's medium (DMEM) containing 0.1% bovine serum albumin, penicillin (100 IU/ml) and streptomycin (100 µg/ml). One hundred milligrams of explants were weighed and incubated in one milliliter of the above media at 37°C and 5% CO_2 in two separate 12 well tissue culture plates for 20 hours. After incubation, the media was replaced and explants were treated with 0, 75, 150, 300, 600 µM of ammonium chloride (n=6) or 0, 4, 8, 12, 16 mM of urea (n=5) and incubated for a further 24 hours. Ammonia and urea concentrations were selected based on previous in-vitro studies (De Wit et al. 2001; Hammon et al. 2000; Ocon and Hansen, 2003; Zander et al. 2006). Explants were harvested, snap frozen in liquid nitrogen, and stored at -80°C until further processing. Culture media was collected and stored at -80°C until further processing.

2.2.2 RNA extraction

RNA extraction of frozen endometrial tissues was performed using total RNA isolation solution, Tri Reagent (Sigma Aldrich). RNA was extracted by following the single step RNA isolation method (Chomczynski and Sacchi, 1987) with the slight modifications (Pretheeban et al. 2011, Singh et al. 2008). Frozen explants were briefly thawed at room temperature and thoroughly homogenized in liquid nitrogen using a

mortar and pestle. Homogenates were transferred into corresponding RNase and DNAse free two milliliter eppendorf tubes. One milliliter of Tri reagent was added into each tube and the contents were thoroughly mixed through vortexing for 30 seconds before allowing them to incubate for 10 minutes at room temperature. Then, the samples were vigorously agitated after adding 200 µl of chloroform (Sigma Aldrich) and incubated further for 15 minutes at room temperature. Samples were centrifuged at 12000 rpm for 15 minutes at 4°C and the supernatant containing total RNA was transferred into another set of corresponding eppendorf tubes. Samples were then gently mixed after adding 750 µl of propanol by inverting the tubes three times and allowed to stand at room temperature for 30 minutes. Supernatant was removed taking care not to disturb the pellet at the bottom after centrifuging the mix at 12000 rpm for 15 minute at 4ºC. Resultant RNA pellets were washed twice with 1 ml of 70 % cold (-20°C) ethanol. At each washing, samples were centrifuged at 7500 rpm for 5 minutes at 4ºC. After draining off the last few drops of the ethanol, the pellets were allowed to air dry by leaving the tubes horizontally at room temperature for 15 minutes. Then the RNA pellet was dissolved in 100 µl of nuclease free DEPC treated water (Invitrogen), incubated at 37ºC for a few minutes and stored at -80ºC until further processing. RNA quality and quantity was assessed by using Nanodrop ND-1000 spectrophotometer at absorbances of 260 and 280 nm.

2.2.3 Reverse transcription

Commercially available first strand cDNA synthesis kit (Bio-Rad) was used for reverse transcription of RNA, following manufacture's protocol. Briefly, 20 µl of reverse transcription reaction mixture was prepared by combining 4 µl of 5x iScript Select

reaction mix, 2 µl of random primer, 1 µg of RNA sample, 1 µl of iScript reverse transcriptase and nuclease free water. Then, contents were incubated for 5 and 30 minutes at 25ºC and 42ºC, respectively. Finally, the reaction was stopped by incubating the samples at 85ºC for 5 minutes to inactivate the activity of transcriptase. The cDNA product was stored at -20ºC until further processing.

2.2.4 qRT-PCR

Relative quantification of mRNA transcripts was performed using qRT-PCR as previously described by Pretheeban et al. (2011). Primer sequence, size of amplified fragment, annealing temperature and gene accession number of all genes are shown in Table 2.1. Suppressor of zeste 12 homolog (Drosophila) (SUZ12) was used as the internal control to normalize the mRNA levels of each gene. PCR was performed by running 25 µl of PCR reaction mixture consisting of 12.5 µl of SYBR Green Super Mix (BioRad), 2 µl of gene specific forward and reverse primers (Pretheeban et al. 2011), 8 µl of PCR water (Sigma Aldrich) and 2.5 µl of cDNA sample in iCycler, through a CFX96 Real-Time PCR Detection System (Bio-Rad). The following reaction conditions were used for PCR: an initial step of 95°C for 5 minutes for denaturation, followed by 40 cycles of 95°C (15 s), 60°C (1min), and 72°C (20 s) for amplification. Cycle threshold (Ct) value of each transcript was determined when there was a steep increase in fluorescence level above the background during the log-linear phase of reaction. Contamination of amplicon of each gene was ruled out by confirming the presence of a single peak at melt curve analysis. Before running the PCR, the qRT-PCR efficiency of reference and each target gene was determined using the same reaction conditions (Pfaffl, 2001). Briefly, cDNA samples from all five treatments were pooled into two different

eppendorf tubes for ammonia and urea and the pooled sample was serially diluted with PCR water as follows: 1:4, 1:16, 1:64, and 1: 256. Standard curve (Ct value versus cDNA input concentration) for each gene was developed by running PCR using the above serially diluted samples and corresponding PCR efficiencies were calculated using the slope of the curve according to the equation: $E = 10^{[-1/slope]}$ (Pfaffl, 2001). Obtained Ct values were analyzed using the RT-PCR efficiency correction method (Pfaffl, 2001) based on the PCR efficiencies of each gene to calculate the relative gene expression.

2.2.5 Enzyme-linked immunosorbent assay (ELISA)

Prostaglandin F2α concentrations in culture media were measured using a commercially available PGF2α high sensitivity Enzyme Immuno Assay kit (Enzo Life Science). Briefly, standards (0, 1.95, 7.8, 31.25, 125, 500 and 2000 pg/ml) were prepared using known concentrations of PGF2α by serially diluting PGF2α solution in culture media. One hundred µl of standards or samples were added into appropriate wells of a microtiter plate coated with secondary antibody, donkey anti-sheep IgG. Twenty five µl of PGF2α conjugated with alkaline phosphatase was added into appropriate wells followed by 25 µL of primary antibody specific to PGF2α. Then, the plate was incubated at 4ºC overnight after incubating it at room temperature on a plate shaker for 5 minutes at 500 rpm. After emptying the contents, wells were washed 3 times by adding 400 µL of wash buffer. Then 200 µL of substrate (P-nitro phenyl phosphate) was added to each well and incubated at 37 °C for 3 hours. Then, 50 µl of stop solution containing trisodium phosphate was added to each well and PGF2α concentrations were calculated by measuring optical densities of samples at 405 nm wave length using an ELISA plate reader.

2.2.6 Statistical analysis

Gene expression and PGF2α concentration data were statistically analyzed using one-way Analysis of Variance (ANOVA) and the differences between treatment groups were analyzed using Tukey's post-hoc test. The results were expressed as mean ± SEM and considered to be statistically significant at $P<0.05$.

2.3 Results

2.3.1 Relative mRNA levels of cell growth, proliferation and differentiation related genes (IGFBP1, FGF2, IGF1 and IGFBP3)

Figure 2.1 shows the effects of ammonium chloride and urea on endometrial mRNA levels of IGFBP1, FGF2, IGF1 and IGFBP3. IGFBP1 mRNA expression level was lower ($P< 0.05$) in explants treated with 600 µM of ammonium chloride when compared with the control (Fig.2.1 1A). The expression level of IGFBP1 gene was lower ($P=0.0662$) in explants treated with 75 µM of ammonium chloride when compared to the control. None of the urea (Fig.2.1 1B) concentrations tested affect the mRNA levels of the IGFBP1 ($P>0.05$). The relative mRNA level of FGF2 was lower ($P<0.05$) in explants treated with high concentration (600 µM) of ammonium chloride (Fig.2.1 2A) when compared to the control and in explants treated with high concentration (16 mM) of urea (Fig.2.1 2B) when compared to lower concentrations (0 and 4 mM). Also, a trend towards decrease in the mRNA levels of FGF2 was observed in the explants treated with 75 µM ($P=0.0681$) and 300 µM ($P=0.089$) of ammonium chloride (Fig.2.1 2A) when compared to the control. The relative mRNA levels of IGF1 were not affected ($P>0.05$) by any of

the ammonium chloride (Fig.2.1 3A) or urea (Fig.2.1 3B) concentrations tested, even though 4 mM of urea tend to stimulate its expression (P=0.0976). IGFBP3 mRNA levels tend to be lower in explants treated with high concentration (600 μM) of ammonium chloride (Fig.2.1 4A) when compared to the control (P=0.0522) and low concentration (75 μM) (P=0.0633). However, the mRNA levels of IGFBP3 was higher (P< 0.05) in explant treated with 4 mM of urea (Fig.2.1 4B) when compared to the control or other concentrations.

2.3.2 Relative mRNA levels of apoptosis and stress related genes (BCL2, BAX, BAX: BCL2, HSPA1A)

Effects of different concentrations of ammonium chloride and urea on endometrial relative mRNA expression of apoptotic (BCL2, BAX) and heat shock protein (HSPA1A) genes are shown in figure 2.2. BCL2 mRNA levels were higher (P<0.05) in explants treated with 150 μM of ammonium chloride (Fig.2.2 1A) compared to explants treated with 0, 75 and 300 μM. The levels of BAX were not affected by any of these concentrations (Fig.2.2 2A). Relative mRNA expression levels of BCL2 and BAX were not affected (P>0.05) by any of the urea concentrations tested (Figs.2.2 1B and 2B). In addition, no effects of ammonium chloride (Fig.2.2 3A) and urea (Fig.2.2 3B) were observed on BAX: BCL2 ratio. mRNA levels of HSPA1A were higher (P<0.05) in explant exposed to mild concentrations of ammonium chloride (150 μM) (Fig.2.2 4A) and urea (4 mM) (Fig.2.2 4B).

2.3.3 Relative mRNA expression of immune related gene (SERPINA14)

Figure 2.3 shows the effect of ammonium chloride and urea on mRNA expression levels of SERPINA14. Levels of SERPINA14 were higher (P<0.05) in explants exposed

to mild concentrations of ammonium chloride (150 μM) (Fig.2.3 A) and urea (4 mM) (Fig.2.3 B). In addition, a slight increase in the levels of SERPINA14 was observed when treated with 8 mM (P=0.0527) and 16 mM (P=0.0698) of urea when compared to the control.

2.3.4 PGF2α levels in culture media

The effect of ammonium chloride and urea on PGF2α secretion by endometrial explants into the culture media is shown in figure 2.4. PGF2α secretion by endometrial explants was not affected (P>0.05) by any of the ammonium chloride (Fig.2.4 A) or urea (Fig.2.4 B) concentrations tested.

2.4 Discussion

In dairy cows, excess protein intake has been shown to increase the levels of ammonia and urea in circulation and in the reproductive fluids, including uterine fluid (Jordan et al. 1983; Sinclair et al. 2000). Elevation of ammonia and urea in the uterine fluid could result in compromised embryo development due to their extreme toxic effect on embryos (Hammon et al. 2000; Lane and Gardner, 2003; Zander et al. 2006) and altered uterine fluid composition which creates a sub optimal uterine environment (Butler, 1998; Erold and Butler, 1993; Jordan et al. 1983; McEvoy et al. 1997). In cows with elevated levels of plasma urea and ammonia, the quality of the uterine fluid could be negatively modulated in two ways: 1) Alteration of uterine pH due to increased ammonia 2) Direct toxic effect of ammonia and urea on endometrium. Our study examined the effect of ammonia and urea on the mRNA levels of endometrial fertility genes.

Expression of the Insulin-like Growth factor (IGF) system is reported in many reproductive tissues (Geisert et al. 1991; Kirby et al. 1996) and believed to play a pivotal role in the regulation of endometrium during the bovine estrous cycle (Robinson et al. 2000). IGF1, IGFBP1 and IGFBP3 are some of the important members of the IGF system. The data in the present study demonstrated that exposure of endometrial explants to high (600 µM) concentrations of ammonium chloride decreased the mRNA levels of IGFBP1 when compared to the control (0 µM). IGFBP1 is one of six IGFBPs which control the bioavailability of IGFs to target cells (Rutanen et al. 1988). Considering the above function, our data demonstrates an interesting result that 600 µM of ammonium chloride might have positive effects on endometrial functions and subsequent embryo development by increasing the availability of IGFs. However, IGFBP1 is also found to be engaged in stimulating cell migration independent to IGF in different cell types including trophoblast cells (Gleeson el at. 2001; Jones et al. 1993), indicating that high concentration of ammonia exposure to endometrial cells may impair trophoblast function. No urea concentrations tested had significant effect on the endometrial mRNA expression level of IGFBP1 in this study. IGFBP3 functions as the regulator of freely available IGFs to their receptors (Rutanen et al. 1988). In addition, IGFBP3 is reported to have pro-apoptotic (Butt et al. 2000; Rajah et al. 1997; Williams et al. 2007), and anti-proliferative (Alami et al. 2008; Oh et al. 1995; Silha et al. 2006) functions on various tissues. In our study, up regulation of IGFBP3 mRNA, was observed when treated with a mild concentration (4 mM) of urea. Therefore, 4 mM urea could be more toxic than other concentrations used by exerting negative effects on proliferation and cell survival of developing embryo. IGFs are identified as factors having potential influences on tissue

differentiation, proliferation and metabolism of various cell types (Stewart and Rotwein, 1996). No significant treatment effects were observed on mRNA levels of IGF1 among different concentrations of ammonium chloride or urea. This finding is in agreement with the observation made by Amsrtong et al. (2001) who showed high protein intake failed to affect the circulating IGF1 concentrations in cows. However, a slightly higher mRNA level was found in explants treated with 4mM of urea when compared to the control. This observation implies that mild urea concentrations may have positive effects on endometrial cells. Supporting this observation, Hammon et al. (2000) showed that mild to moderate concentrations of ammonium chloride in the culture media could have positive impact on embryo development.

Fibroblast growth factor (FGF) is thought to be involved in a wide range of cellular processes including apoptosis, cell survival, chemotaxis, cell adhesion, migration, differentiation and proliferation. FGF2 is a member of the FGF family and plays a key role during early embryogenesis (Gospodarowicz, 1991). In cows, expression of FGF2 is detected throughout the early embryo stages including blastocyst (Daniels et al. 2000; Gospodarowicz, 1991) in the endometrium, and is also shown to increase IFNτ mRNA and protein abundance in the trophectoderm cells (Michael et al. 2006). In our study, relative expression levels of FGF2 decreased with increasing concentrations of ammonium chloride and urea. Particularly, the expression level was significantly lower ($P<0.05$) in explants treated with a higher concentration (600 μM) of ammonium chloride and a higher concentration (16 mM) of urea compared to lower concentrations. This finding indicates that exposure of endometrium to higher levels of ammonium chloride and/or urea may have detrimental effects on endometrial epithelial cell migration,

differentiation and proliferation through decreased mRNA expression levels of FGF2, resulting in the alteration of uterine milieu during the mid-luteal phase. This finding is supported by the fact that increasing ammonia concentration affected cell growth, metabolism, and caused apoptosis in a variety of cell types (Ozturk et al. 1992; Rooke et al. 2004; Schneider et al. 1996).

Apoptosis is a process of programmed cell death during which cells undergo characteristic morphological changes before cell death. Among numerous gene transcripts involved in apoptosis, members of the BCL2 gene family play a major role in regulating the apoptosis mechanism (Adams and Cory, 1998). The BCL2 gene family is categorized into two groups: anti-apoptotic and pro-apoptotic (Adams and Cory, 1998). Within the BCL2 family, the BCL2 gene is characterized as a pro survival and apoptosis-suppressing factor whereas the BAX gene is identified as an apoptosis-promoting factor (Banerjee et al. 2002; Hockenbery et al. 1990). The ratio between these two gene transcripts determines the fate of the cells and thereby is considered as good indicator of assessing the degree of apoptosis in tissues. Hammon et al. (2000) have observed that exposure of ova to moderate concentrations of ammonium chloride (22 and 88 μM) during in-vitro fertilization increased blastocyst development rate while exposure during in-vitro maturation, fertilization and culture increased the morula development rate. This may be the consequence of reduced incidence of apoptosis in embryonic cells during the development when exposed to moderate concentrations of ammonia. In support of the above observation, we found BCL2 mRNA expression levels to be higher in explants treated with a moderate concentration (150 μM) of ammonium chloride compared to that of low or high concentrations (0, 75 and 300 μM). However, the ammonium chloride

concentrations that caused positive effects on both studies are different. This difference may be due to the degree of sensitivity of cells to ammonium chloride. In contrast to above observations, Lane and Gardner, (2003) have reported that levels as low as 18.8 μM of ammonia increased apoptosis in blastocyst cells. Furthermore, Zander et al. (2006) showed that exposure of embryos to 300 μM of ammonia increased the incidence of apoptosis. In the present study, expression of BAX was not affected by any amount of ammonia concentrations tested and BAX: BCL2 ratios were also not affected by any of the ammonia or urea concentrations tested; indicating that high concentration of ammonia or urea is less likely to affect the early embryo development by increasing the incidence of apoptosis in endometrial cells.

Heat shock proteins are molecular chaperones expressed in response to exposure of cells and tissues to extreme conditions such as hyperthermia and variety of toxic conditions (Santoro, 2000). They function through assisting the correct folding of native and stress accumulated misfolded non-native proteins to repair the damaged protein and allowing the cells to survive in extreme environmental conditions (Schmitt et al. 2007). In addition, HSPs can prevent stress induced inappropriate cell death through modulating apoptotic signaling pathway (Schmitt et al. 2007). HSP70, a distinct family of heat shock proteins is encoded by HSPA1A in mammalian cells. In our study, HSPA1A mRNA levels increased when ammonia (150 μM) and urea (4 mM) concentrations were moderately high, indicating that HSPA1A is inducible at the above concentrations of ammonia or urea exposure. Our finding is in agreement with the fact that HSP expression is stimulated in response to extreme environmental conditions (Santoro, 2000; John, 2010). However, after showing a higher expression at the above concentrations, the

mRNA levels of HSPA1A were found to decrease with increasing concentrations of ammonia (> 300 μM) and urea (> 8mM). This could be partly due to the detrimental effects of high concentrations of ammonia and urea on the transcription mechanism of HSPA1A (Ozturk et al. 1992). Therefore, high concentrations of ammonia or urea could lead to cell death by preventing anti-apoptotic actions of HSPA1A (Schmitt et al. 2007).

Secretion of SERPINA14 by endometrium (Padua and Hansen, 2010) is important for the establishment and maintenance of pregnancy in ruminants. SERPINA14 performs a variety of biological functions during pregnancy including suppression of local immune system resulting in the protection of allogenically distinct conceptus from local immune reaction (Hansen and Tekin, 2005; Hansen, 2007). Our study showed that the mRNA levels of SERPINA14 was increased when explants were exposed to mild concentrations of urea (4 mM) or ammonia (150 μM). Then as concentration increased, a decreasing pattern of expression was observed even though they were not different among them. The highest concentration of ammonia and urea used in this study is not enough to cause a significant down regulation of SERPINA14 mRNA levels. Our study suggests that exposure of explants to mild concentration of urea and ammonia is likely to have a supportive effect on embryo development through immunosuppressive action by increasing the mRNA levels of SERPINA14 (Hansen and Tekin, 2005).

Collectively, our study shows that mRNA levels of some candidate genes are: 1) up-regulated (IGF1, IGFBP3, BCL2, HSP1A1, and SERPINA14) when explants were treated with moderate concentrations of ammonia and urea compared to control suggesting that exposure to moderate concentrations may have a positive effect on the uterine environment, however, IGFBP3 appeared to show a negative effect, 2) down-

regulated (IGFBP1, IGFBP3, FGF2 and SERPINA14) when explants were treated with high concentrations of ammonia and urea compared to control, suggesting that exposure to high concentrations have a negative effect on the uterine environment.

Elevated levels of PGF2α in circulation or in the culture media were found to be correlated with poor embryo development and increased embryonic mortality (Scenna et al. 2004; Schrick et al. 1993; Seals et al. 1998). In addition, elevated urea concentration in the endometrial environment has shown to increase the secretion of PGF2α by endometrial cells (Butler, 1998). However, Rhoads et al. (2004) showed that increasing the BUN level to 22.6 mg/dl by infusing urea failed to affect the secretion of PGF2α by the endometrium in lactating dairy cows. Our study also revealed that neither the urea nor the ammonia concentrations tested affected the secretion levels of PGF2α by endometrial explants. Therefore, the mechanism by which elevated ammonia and urea in the circulation reduce the pregnancy rate may be primarily due to the negative effects on endometrial gene expression levels rather than increased PGF2α secretion.

2.5 Conclusion

Elevated ammonia and urea concentrations, associated with high protein intake, in the circulation and in the reproductive fluids including uterine “histotroph” appeared to be detrimental to embryo development and pregnancy. Based on my results, I conclude that exposure to high concentrations of ammonia and urea have negative effects on some candidate endometrial genes, while moderate concentrations have positive effects. The detrimental impact of high concentration of ammonia and urea on endometrial mRNA

levels may be one of the possible mechanisms leading to the reduced fertility observed in dairy cows fed with a high protein diet.

Table 2.1 Oligonuleotide sequence and amplicon size of gene specific primers used in qRT-PCR

Gene ontology classification	**Gene symbol**	**Primer sequence (5'-3')**	**Annealing T °**	**Fragment length**	**Gene accession number**
Cell growth, proliferation and differentiation related genes	IGF1	F- TCAGTTCGTGTGCGGAGACA R- ACTTCCTTCTGAGCCTTGGG	56	222bp	NM001077828
	IGFBP1	F-CTACAAAGTGCTGGACAGATTAGCC R- GTAGACACACCAACAGAGCCC	60	157bp	X54979
	IGFBP3	F- AAGAAAGGTCATGCCAAGGACAGC R- TTGTCGCAGTTGGGAATGTGGATG	60	199bp	AF305199
	FGF2	F- TACAACTTCAAGCAGAAGAG R- CAGCTCTTAGCAGACATTGG	56	214bp	NM174056
Apoptosis and stress related genes	BAX	F- TGCTTCAGGGTTTCATCCAG R- AACATTTCAGCCGCCACTC	58	223bp	U92569
	BCL2	F- TTCGCCGAGATGTCCAGTCAGC R- GTTGACGCTCTCCACACACA	62	156bp	U92434
	HSPA1A	F- CACTTCGTGGAGGAGTTCA R- GGTTGATGCTCTTGTTGAGG	58	376bp	AY149619
Immune related gene	SERPINA14	F- ATATCATCTTCTCCCCCATGG R GTGCACATCCAACAGTTTGG	60	126bp	L22095
House keeping gene	SUZ12	F- GAACACCTATCACACACATTCTTGT R- TAGAGGCGGTTGTGTCCACT	60	130bp	XM582605

F- Forward Primer; R- Reverse Primer; bp- Base pair; T ° - Temperature.

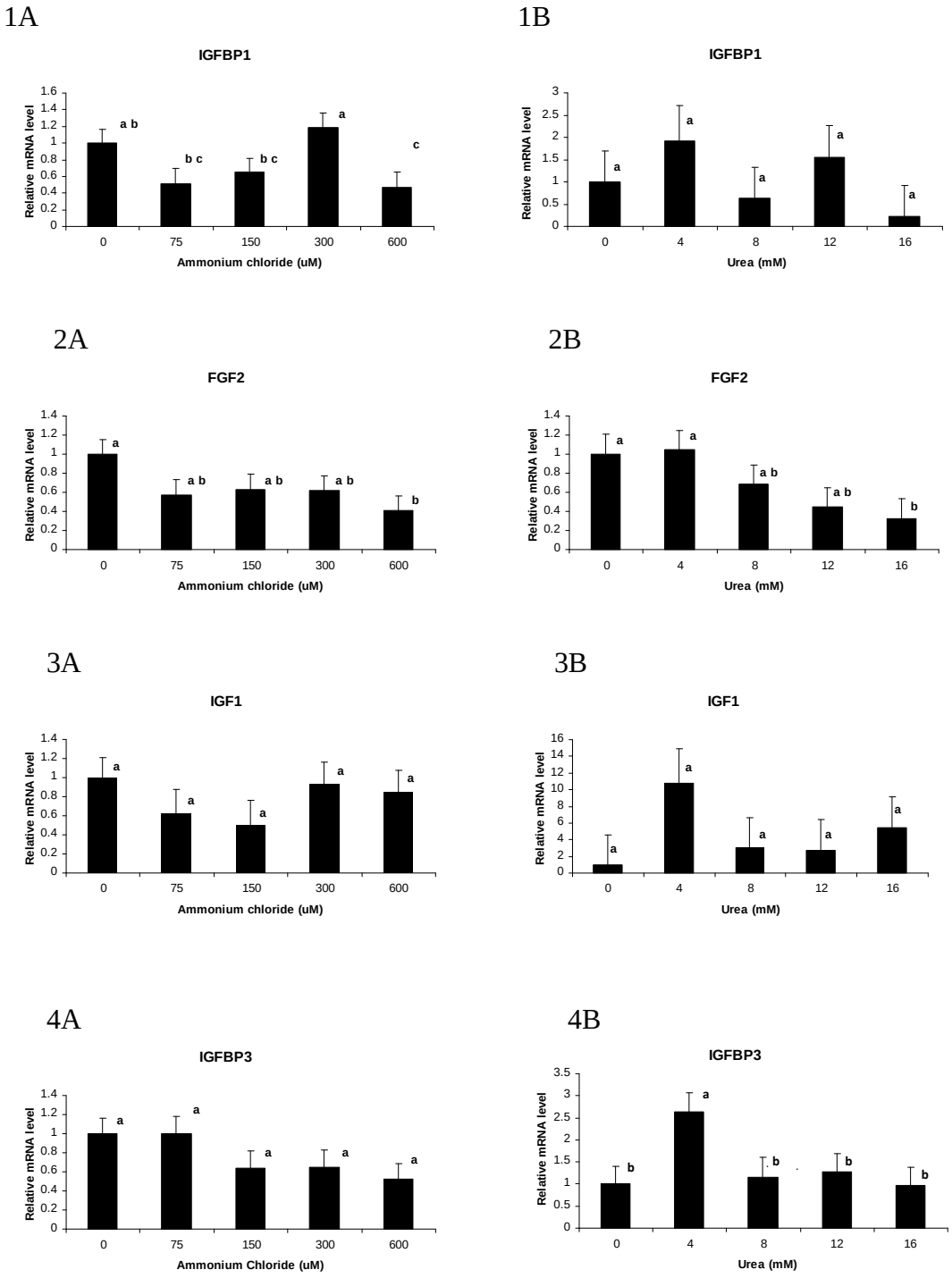
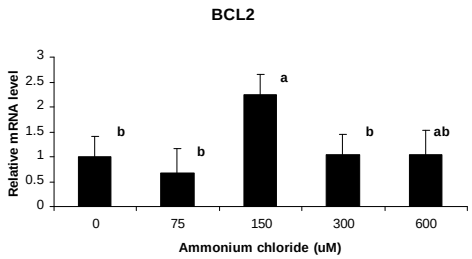
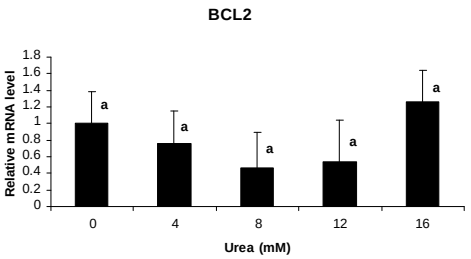


Figure 2.1 Effects of ammonium chloride (A) and urea (B) on mRNA abundance of cell growth, proliferation and differentiation related genes: IGFBP1 (1), FGF2 (2), IGF1 (3), IGFBP3 (4). Different letters indicate statistical significance ($P<0.05$).

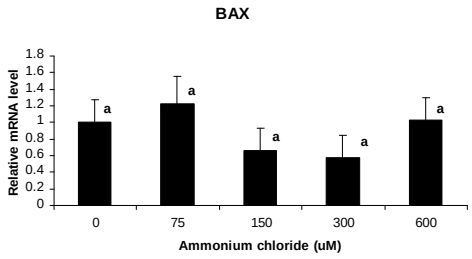
1A



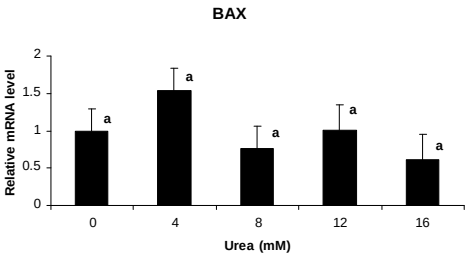
1B



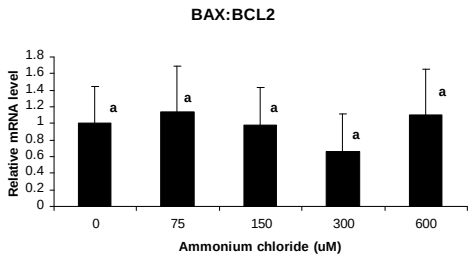
2A



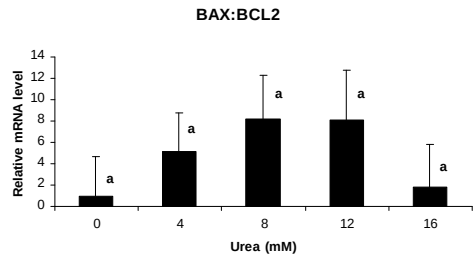
2B



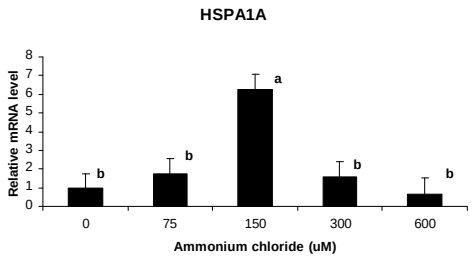
3A



3B



4A



4B

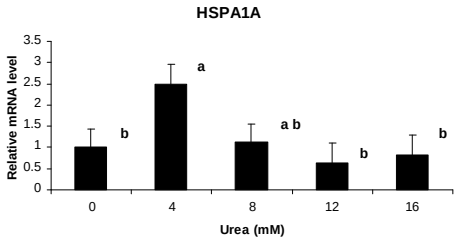


Figure 2.2 Effects of ammonium chloride (A) and urea (B) on mRNA abundance of apoptosis and stress related genes: BCL2 (1), BAX (2), BAX: BCL2 (3) and HSPA1A (4). Different letters indicate statistical significance ($P<0.05$).

A B

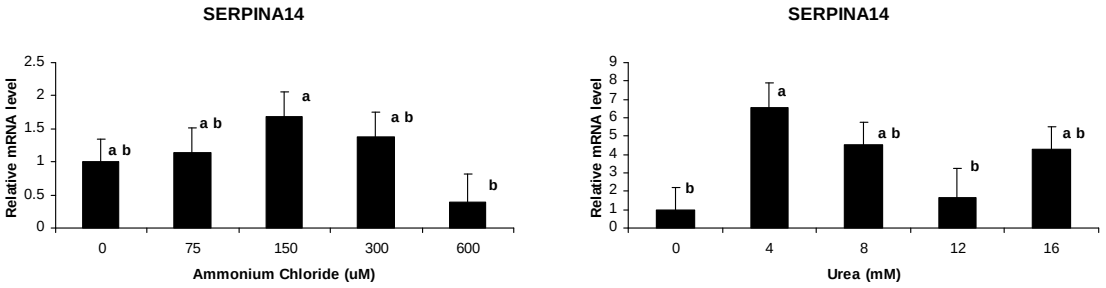


Figure 2.3 Effects of ammonium chloride (A) and urea (B) on mRNA abundance of immune related gene: SERPINA14. Different letters indicate statistical significance ($P<0.05$).

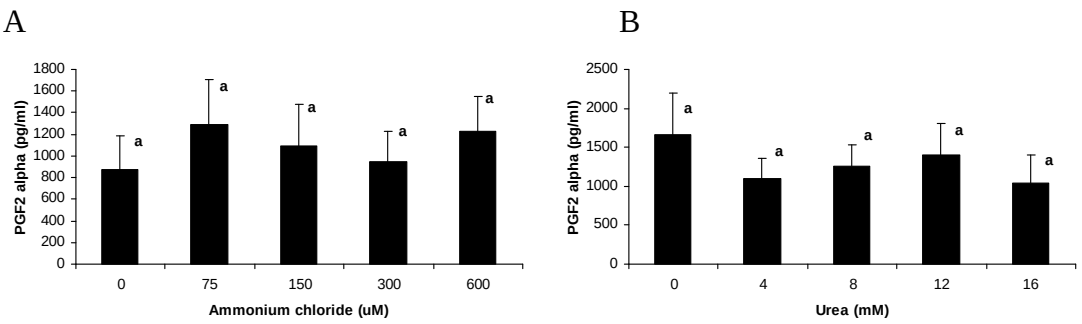


Figure 2.4 Effects of ammonium chloride (A) and urea (B) on PGF2α secretion by endometrial explants. Different letters indicate statistical significance ($P<0.05$).

CHAPTER 3: EFFECTS OF HIGH AND LOW PROTEIN INTAKE ON THE mRNA ABUNDANCE OF MID-LUTEAL PHASE ENDOMETRIAL CANDIDATE GENES, MILK UREA NITROGEN LEVELS, FOLLICULAR AND CORPUS LUTEAL DYNAMICS, AND MILK PRODUCTION IN HIGH PRODUCING LACTATING DAIRY COWS

3.1 Introduction

Fertility in high producing dairy cows has been decreasing over decades concomitant with considerable increase in milk production due to intensive genetic selection and improved nutritional management (Butler, 2000; Dobson et al., 2007; Lucy, 2001). Decreasing fertility is a multi-factorial issue and the factors/mechanism that lead to this state in dairy cows is not clearly understood. However, altered follicular dynamics and ovulation (Lucy et al. 1992 ; Salem et al. 2010; Wolfenson el at. 2004) associated with poor estrus expression and/or detection (Lopez et al. 2004; Lucy, 2001), inadequate corpus luteum (CL) function (Howell et al. 1994; Leroy et al. 2008), altered uterine and oviductal environment (Butler, 1998; Pretheeban et al. 2011) and compromised embryo development and implantation (Rizos et al. 2010; Spencer at al. 2007; Wiebold, 1988) are implicated as some of the possible reasons for decreasing fertility (Lucy, 2007). In addition, feeding a high protein diet is implicated as the most important contributing factor that leads to decreasing fertility through disturbing one or more of the above reproductive functions (Butler, 1998, 2000; Tamminga, 2006).

The diet fed to lactating dairy cows in early lactation is formulated to meet the nutritional requirements of high milk production, the maintenance of normal body functions and to minimize the negative energy balance during the postpartum period

(Butler, 2000; Tamminga, 2006). Thus, post-partum cows are fed with a diet containing high levels of protein (17 to 19 %) (Butler, 2000). On the other hand, it is also well documented that a diet formulated with high levels of protein is accompanied with decreased fertility in dairy cows (Canfield et al. 1990; Elrod et al. 1993; Jordan and Swanson, 1979a; Tamminga, 2006). Feeding high protein diet to lactating dairy cows has been shown to result in excess urea and ammonia concentration in circulation (Larson et al. 1997; Rajala-Schulz et al. 2001; Roseler et al. 1993), follicular fluid (Hammon et al. 2005; Moallem et al. 2011; Sinclair et al. 2000), and uterine fluid (Hammon et al. 2005; McEvoy et al. 1997). These increased levels of urea and ammonia have been shown to affect reproductive functions such as follicular growth and ovulation, CL function, fertilization and embryo development, maternal reorganization of pregnancy, and implantation (Garverick et al. 1971; Lane and Garnder, 2003; Tamminga, 2006). A high protein diet fed to dairy cows consists of two fractions: Rumen Degradable Protein (RDP) and Rumen Un-degradable Protein (RUP). High protein intake consisting of excess RDP has been shown to alter the follicular dynamics in dairy cows (Carroll et al. 1988; Santos et al. 2001, Tamminga, 2006). This alteration may result in the development of poor quality oocytes leading to compromised fertilization and embryo development (Bishonga et al. 1996; Blanchard et al. 1990; Dawuda et al. 2002; Rhoads et al. 2006). However, other studies revealed that feeding an excess protein diet is less likely to alter the dynamics of follicular development (Garcia Bojalil et al. 1994; Laven et al. 2004). Proper CL function is of crucial importance for the establishment of a successful pregnancy. High protein diet intake has been shown to reduce plasma progesterone concentrations in dairy cows (Folman et al. 1983; Sonderman and Larson,

1989). Although the impact of high protein intake on plasma progesterone concentration has been studied, only few studies have focused on the impact of high protein intake on CL dynamics. Garverick et al. (1971) reported that the weight of CLs obtained from Holstein Friesian heifers fed with urea was lighter than that of heifers fed with no urea. Further studies need to be carried out to determine the effect of high protein diets on follicular and CL dynamics.

The uterine endometrium synthesizes and secretes a variety of substances, including growth factors, cytokines, hormones, enzymes and transport proteins, collectively called as histotroph (Bazer, 1975; Igwebuike, 2009). Components of histotroph act as crucial regulators of conceptus growth and development (Igwebuike, 2009). Any factors affecting uterine secretion and therefore creating suboptimal uterine milieu might be detrimental to early embryonic development. High protein intake by lactating dairy cows has been shown to reduce the quality of uterine secretion and therefore creates an environment unsuitable for embryo development (Butler, 1998; Elrod et al. 1993; Jordan et al. 1983; Rhoads et al. 2004). I believe that high protein intake associated with excess ammonia and urea could alter histotroph composition through exerting its effects at the gene expression levels of the endometrial cells. Recently, Pretheeban et al. 2011 have revealed that the expression levels of candidate genes in the endometrium were different between lactating dairy cows and heifers. They attributed this differential expression of candidate genes as a possible reason for reduced fertility observed in lactating dairy cows. Therefore, I believe that lowering the protein level in a diet would modulate the endometrial gene expression levels in dairy cows and improve their fertility. Therefore, the objectives of the present study were to compare the effects

of high and low protein intake on a mid-luteal phase endometrial candidate genes involving in various cell functions: cell growth, proliferation and differentiation (Insulin like Growth Factor 1 (IGF1), Insulin like Growth Factor Binding Protein 1 and 3 (IGFBP1, IGFBP3), Fibroblast Growth Factor 2 (FGF2)), apoptosis and stress tolerance (B-Cell Lymphoma 2 (BCL2), BCL2 Associated X protein (BAX), Heat Shock Protein 70 (HSPA1A)), corpus luteum maintenance (Interleukin 1α (IL1A), Tumor Necrosis Factor (TNF)), and immune function (Serine Protease Inhibitor (SERPINA14)). In addition, another objective of this study was to compare the effects of high and low protein intake on follicular and CL dynamics, milk production, and milk urea nitrogen level in dairy cows.

3.2 Materials and methods

3.2.1 Experimental animals, housing and treatment

This study was performed at the Dairy Education and Research Center, University of British Columbia, Canada during the period between February, 2011 and June, 2011. Twenty-four multiparous prepartum Holstein cows were assigned into 12 blocks, having two cows in each block based on their parity, previous milk production and body condition score (BCS). Body condition was determined using a 5 points scoring system developed by Ferguson et al. (1994). Cows in each block were randomly assigned to receive one of two isocaloric diets, total mixed ration (TMR) containing either high (CP=17.3%, n=12) or low (CP=14.8%, n=12) levels of crude protein (Table 3.1) once they calved. All cows were housed in a single experimental pen having 24 free stalls fitted with rubber mattresses bedded with cleaned sand. The pen was equipped with an

automated electronic feed and water intake system (Insentec, Marknesse, Holland) which included twelve feed and two water bins. This system was connected to a central computer. All cows had free access to feed and water. To be identified by the electronic system, each cow was tagged with their own passive transponder in their ear tag. A panel fitted above each bin was able to read a cow's transponder and thereby allow it to reach feed/water if she was assigned to access a particular bin. The system was also able to capture the feed and water intake of individual cows. All cows were fed with experimental diets starting from the day of calving until the day of endometrial biopsy.

During the treatment, cows reaching 33-42 days in milk (DIM) were synchronized for ovulation using the "Double-Ovsynch" method (Souza et al. 2008). Each Ovsynch protocol consisted of two GnRH (100 μg; Fertiline, Vetoquinol Canada Inc., Lavaltrie, QC, Canada) injections 9 days apart with one $PGF_{2\alpha}$ (25 mg; Lutalyse, Pharmacia Animal Health, Orangville, ON, Canada) injection 48 hours before the second GnRH injection. Additionally, one $PGF_{2\alpha}$ injection was given 24 hours before the second GnRH injection in the second Ovsynch. Cows that failed to respond to this Ovsynch treatment, confirmed by ultrasonography for ovulation, were resynchronized until they ovulated. In all, uterine endometrial biopsies were obtained, 11 to 12 days after synchronized ovulation, from 12 cows in high protein group and 10 cows in low protein group.

3.2.2 Milk production and milk sampling

Milk production of experimental cows was recorded daily. Milk samples were collected weekly starting from the first week until the end of the study. Briefly, evening (pm) and morning (am) milk samples were collected into a plastic container containing a dissolvable milk fat preservative (BroTab10, Systems Plus Ltd., Ontario, CA) during the

normal milking time. Milk samples were sent to Ontario Dairy Herd Improvement Cooperative (Guelph, ON) to determine the milk urea nitrogen levels.

3.2.3 Ultrasonography

Transrectal real time ultrasonography, using a portable ultrasound scanner with 7.5-MHz linear-array transducer (Aloka-SD500, Aloka Co., Tokyo, Japan), was used to monitor ovarian and uterine structures weekly after calving. With the onset of Ovsynch treatment, ultrasonography was carried out on the day of GnRH and PGF2α injection and 2 days after the GnRH injection in order to determine ovulation response. During the procedure, the uterus was assessed for the status of involution and any other abnormalities and the ovaries were assessed for follicles and CL. Follicular number and diameter, and CL diameter on both ovaries were recorded. Follicles were classified into three groups based on diameter. Follicles with a diameter size < 5 mm, 5-10 mm and > 10 mm were categorized as small, medium and large follicles, respectively.

3.2.4 Feed sampling and analysis

Feed samples were collected twice a week and pooled together into weekly samples. Then samples were dried at 55 º C for 48 hours to determine the DM content, and ground thoroughly before pooling weekly samples to biweekly samples. Pooled samples were sent to A & L Canada Laboratories Inc., London, Ontario for diet ingredients analysis (Table 3.1).

3.2.5 Endometrial biopsy

Endometrial biopsy was performed at 11 or 12 days post-synchronized ovulation. On the day before biopsy, animals were scanned to confirm the presence of CL and its side. On the day of biopsy, following epidural anesthesia using 3-4 ml of 2 % Lidocaine

hydrochloride (Lidocaine, Bimeda-MTC), the external genitalia were thoroughly cleaned using paper towels and the endometrial biopsy forceps (Kevorkian-Younge, Fine Surgicals, and 3.5 X 8 mm bite and 50.8 cm shaft), covered with a plastic sheath, was introduced into the cervix through the vagina. While the tip of the forceps was in the cervix, the plastic sheath was broken and the gun was extended into the uterine horn ipsilateral to the CL. Around 100-200 mg of endometrium was biopsied by manipulating the uterine horn via the rectum. The biopsy gun was thoroughly cleaned in an order using mild soap solution, tap water, 70 % alcohol, distilled water and 0.9% Nacl before using it on another cow. The samples were placed in DNAse and RNase free cryo-vials, snap froze in liquid nitrogen and stored at -80°C until further processing.

3.2.6 RNA extraction

RNA extraction of frozen endometrial tissues was performed using total RNA isolation solution, Tri Reagent (Sigma Aldrich). RNA was extracted by following the single step RNA isolation method (Chomczynski and Sacchi, 1987) with the slight modifications (Pretheeban et al. 2011, Singh et al. 2008). Frozen explants were thoroughly homogenized in liquid nitrogen using a mortar and pestle. Homogenates were transferred into corresponding RNase and DNAse free two milliliter eppendorf tubes. One milliliter of Tri reagent was added into each tube and the contents were thoroughly mixed by vortexing for 30 seconds before allowing them to be incubated for 10 minutes at room temperature. Then, samples were vigorously agitated after adding 200 µl of chloroform (Sigma Aldrich) and incubated for further 15 minutes at room temperature. Samples were centrifuged at 12000 rpm for 15 minutes at 4°C and the supernatant containing the total RNA was transferred into another set of corresponding eppendorf

tubes. The contents were gently mixed after adding 750 µl of propanol (Sigma Aldrich) by inverting the tubes three times and allowing them to stand at room temperature for 30 minutes. Supernatant was removed taking care not to disturb the RNA pellet at the bottom of the tube after centrifuging the contents at 12000 rpm for 15 minutes at 4ºC. Resultant RNA pellets were washed twice with 1 ml of 70 % cold (-20°C) ethanol. At each washing, samples were centrifuged at 7500 rpm for 5 minutes at 4ºC. After draining off the remaining ethanol at the end of last washing, the pellet was left on a bench top to air dry at room temperature for 15 minutes. Then the RNA pellet was dissolved in 100 µl of nuclease free DEPC treated water (Invitrogen), incubated at 37ºC for a few minutes and stored at – 80ºC until further processing. RNA quality and quantity was assessed by using Nanodrop ND-1000 spectrophotometer at the absorbance of 260 and 280 nm.

3.2.7 Reverse transcription

Commercially available first strand cDNA synthesis kit (Bio-Rad) was used for reverse transcription of RNA, following manufacture's protocol. Briefly, 20 µl of reverse transcription reaction mixture was prepared by combining 4 µl of 5x iScript select reaction mix, 2 µl of random primer, 1 µg of RNA sample, 1 µl of iScript reverse transcriptase and nuclease free water. Then, the contents were incubated for consecutive 5 and 30-minute periods at 25ºC and 42ºC, respectively. Finally, the reaction was stopped by incubating the samples at 85ºC for 5 minutes to inactivate the activity of transcriptase. The resultant cDNA product was stored at -20ºC until further processing.

3.2.8 qRT-PCR

Relative quantification of mRNA transcripts was performed using qRT-PCR as previously described by Pretheeban at al. (2011). Suppressor of zeste 12 homolog

(Drosophila) (SUZ12) was used as the internal control to normalize the mRNA level of each gene. PCR was performed by running 25 µl of a PCR reaction mixture consisting of 12.5 µl of SYBR Green Super Mix (BioRad), 2 µl of a gene specific forward and reverse primers (Pretheeban et al. 2011), 8 µl PCR water (Sigma Aldrich), and 2.5 µl of cDNA sample in iCycler, through a CFX96 Real-Time PCR Detection System (Bio-Rad). The following reaction conditions were used for PCR: an initial step of 95°C for 5minutes for the denaturation program, followed by 40 cycles of 95°C (15 s), 60°C (1min), and 72°C (20 s) for the amplification program. The cycle threshold (Ct) value of each gene was determined when there was a steep increase in the fluorescence level above the background during the log-linear phase of reaction. Contamination of the amplicon of each gene was ruled out by confirming the presence of a single peak at melt curve analysis. Candidate genes were classified into four categories based on their role in embryo development. Primer sequence, size of amplified fragment, annealing temperature and gene accession number of all genes are shown in Table 3.2.

3.2.9 Analysis of data

3.2.9.1 qRT-PCR

The relative mRNA level of each gene was quantified using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Leroy et al. 2010; Pretheeban et al. 2010). Briefly, the ΔCt for each gene was calculated by subtracting the Ct value of the house keeping gene from that of the target gene. ΔΔCt was calculated by subtracting the highest sample ΔCt value taken as an arbitrary constant, from all the other ΔCt values. The formula $2^{-\Delta\Delta Ct}$ was used to determine the fold changes in mRNA levels of each target gene in relation to the internal control gene. For each target gene, the statistical differences in the mRNA abundance

levels between the high and low protein groups were calculated using the Welch's or pooled two sample t-test, depending on the equality of variance in the observations between the two groups using Microsoft Excel. The results were expressed as mean ± SEM and considered to be statistically significant at $P<0.05$.

3.2.9.2 Milk production and milk urea nitrogen (MUN)

The statistical significance of the difference between the two means of average daily milk production and weekly MUN levels were determined by two way repeated measures of ANOVA using Sigmaplot statistical package. The results were expressed as mean ± SEM and considered to be statistically significant at $P<0.05$.

3.2.9.3 Follicular and CL dynamics

The effects of high and low protein intake on the mean number of small, medium and large follicle(s), mean diameter of large follicle(s), days to first CL appearance, and mean diameter of CL appeared during the first six weeks of calving, were determined by the two sample pooled t-test using Microsoft Excel. The results were expressed as mean ± SEM and considered to be statistically significant at $P<0.05$.

3.3 Results

3.3.1 Relative mRNA expression levels

Figure 3.1 shows the effect of high and low protein diet intake on the relative endometrial mRNA abundant levels of cell growth, proliferation, and differentiation related genes (IGF1, IGFBP1, IGFBP3, FGF2). Figure 3.2 shows the effect of high and low protein diet intake on the relative mRNA abundant levels of apoptosis and stress related genes (BAX, BCL2, BAX: BCL2, HSPA1A). None of the above gene expression

levels were different between the high and low groups P>0.05). Figure 3.3 shows the effect of high and low protein intake on the mRNA levels of cytokines (IL1A, TNF) and immune related (SERPINA14) genes. The relative endometrial mRNA abundant level of IL1A was lower in cows fed with a high protein diet compared to that of low protein (P<0.05) fed cows. However, the relative mRNA abundance of TNF and SERPINA14 did not differ between the two groups (P>0.05).

3.3.2 Milk production

Figure 3.4 shows the relationship between the dietary protein levels and milk production during the first 16 weeks of lactation. Average daily milk production did not differ (P>0.05) between cows fed with high or low levels of protein (46.6±1.8 kg/day vs. 44.6±1.9 kg/day, respectively).

3.3.3 Milk urea nitrogen

The relationship between protein intake and MUN levels throughout the study period is shown in Figure 3.5. Weekly average MUN levels were higher (P< 0.05) in the high protein (9.49 ± 0.2 mg/dl) group when compared to the low protein (6.96 ± 0.2 mg/dl) group throughout the study period.

3.3.4 Follicular and CL dynamics

The relationship between the dietary protein levels and the number of small, medium and large follicles that appeared during the first 6 weeks of feeding is shown in figure 3.6. The mean number of small follicles was lower in cows fed with the high protein (1.2± 0.2) diet when compared with that of the low protein (2.5± 0.3) diet (P< 0.0005). The mean number of medium follicles was not affected (P>0.05), while that of large follicles tended to be lower in the high protein (1.1± 0.1) group compared to that of

the low protein (1.3± 0.1) group (P= 0.096). Moreover, most of the cows (7 out of 12) in the low protein diet group had at least one large follicle during the first 2 weeks of feeding where as in the case of the high protein group, only one cow had a large follicle during this period. Figure 3.7 shows the effect of high and low protein intake on the diameter of large follicles that developed during the first 6 weeks of feeding. Average follicular diameter was lower (P< 0.05) in cows fed with high protein (14.38 ± 0.43 mm) when compared with that of the low protein (16.07 ± 0.61) fed cows.

Figures 3.8 and 3.9 show the effect of high and low protein intake on the CL diameter that appeared during the first 6 weeks of feeding, and days in milk to the first CL appearance, respectively. The mean CL diameter and the mean days to the first CL appearance were lower (P<0.05) and tended to be longer (P = 0.089), respectively, in cows fed with a high protein diet.

3.4 Discussion

Vigorous genetic selection coupled with feeding high protein diets to increase milk production is associated with decreased fertility in dairy cows. Alteration in the biochemical properties of the uterine histotroph leading to a sub-optimal uterine environment during early embryo development has been suggested as one factor causing the reduction in fertility of dairy cows (Butler, 1998). Investigation of high protein intake induced changes in mRNA abundant levels of fertility related candidate genes in the mid-luteal phase endometrium may elucidate the underlying mechanism whereby the high protein intake associated with suboptimal uterine environment reduces fertility.

IGF1 and FGF2 are expressed in many reproductive tissues including the uterine endometrium (Geisert et al. 1991; Kirby et al. 1996; Michael et al. 2006) and play a key role in embryo development (Michael et al. 2006; Robinson et al. 2000). These two growth factors have been reported to potentially influence cell differentiation, proliferation, migration, adhesion and metabolism of various cell types (Gospodarowicz, 1991; Stewart and Rotwein, 1996). IGFBP1 and IGFBP3 are two of six IGFBPs which control the bioavailability of IGFs to target cells. In addition to this function, they are also reported to act on IGF independently to stimulate cell migration in different cell types including trophectoderm cells (Gleeson el at. 2001; Jones et al. 1993; Simmons et al. 2009). This study found no significant differences in the mRNA levels of any of the above genes between the two groups of cows. This observation is supported by the finding that high protein or urea intake failed to affect the circulating IGF1 concentration (Amsrtong et al. 2001, Laven et al. 2004).

Apoptosis is a process of programmed cell death during which cells undergo characteristic morphological changes before cell death. BCL 2 and BAX proteins are members of the BCL2 family and have been characterized as apoptosis-suppressing and apoptosis-promoting factors, respectively (Banerjee et al. 2002; Hockenbery et al. 1990). The ratio between these two members determines the fate of a cell (Korsmeyer, 1995). The results of this study revealed that mRNA expression levels of BAX, BCL2 and BAX: BCL2 ratio did not vary between the two groups of cows. This status may be possibly due to the fact that the MUN levels observed in cows fed with high and low protein were lower than levels which have been shown to reduce fertility in dairy cows (Canfield et al. 1990; Rajala-Schulz et al. 2001). It is also possible that apoptosis may also be elicited by

some other pathways where the BCL2 family is not involved (Groebner et al. 2010; Thorburn, 2004). Consistent with these observations are the results from our previous in vitro study (Chapter 2) showing that the BAX: BCL2 ratio did not change when endometrial explants were exposed to high concentrations of ammonia and urea. Heat shock proteins are molecular chaperones expressed in response to the exposure of cells and tissues to extreme conditions such as hyperthermia and a variety of toxic conditions (Santoro, 2000). No differences were observed in HSPA1A mRNA expression levels between the two groups in our study. This observation suggests that bovine endometrial cells can tolerate the levels of urea and ammonia resulting from the high and low protein levels used in our study.

IL1A and TNF, members of the cytokine family, are identified as a factor involved primarily with immune response and the inflammatory process in several cells and tissues (Pasparakis et al. 1996; Takacs et al. 1988; Vassalli, 1992). In addition, endometrial secreted IL1A and TNF were thought to play a pivotal role in reproductive processes such as ovulation (Okuda and Sakumoto, 2003; Rae et al. 2004) and most importantly in CL maintenance (Majewska et al. 2010; Okuda and Sakumoto, 2003; Pretheeban et al. 2010; Tanikawa et al. 2005). Treatment with IL1A appeared to lengthen the lifespan of CL and increase the progesterone output by luteal cells through modulating the endometrial secretion of prostaglandins (PGF2α, PGE2) (Majewska et al. 2010; Okuda and Sakumoto, 2003; Tanikawa et al. 2005). Low levels of IL1A mRNA abundance in the endometrium of the high protein fed cows in our study suggests that high protein intake may be a contributory factor to reduced fertility in dairy cows. In agreement with this observation, Pretheeban et al. (2011) have recently shown that low

levels of IL1A expression in the endometrium may be a factor leading to the reduced fertility observed in lactating dairy cows. TNF is also found to stimulate progesterone production and lengthen the CL lifespan when treated the endometrium with TNF during the luteal phase (Skarzynski et al. 2003). SERPINA14 is a member of the Serine Proteinase Inhibitor superfamily, reported to be expressed by the endometrium during pregnancy (Padua and Hansen, 2010). It has been identified that SERPINA14 is involved with local immune suppression in the uterus resulting in protection of allogenically distinct conceptus from local immune reaction (Hansen and Tekin, 2005; Hansen, 2007). No differences were observed at endometrial mRNA expression levels of TNF and SERPINA14 between the two groups of cows in this study.

Collectively, we found that none of the genes (IGF1, IGFBP1, IGFBP3, FGF2, BAX, BCL2, HSP1A1, TNF and SERPINA14) tested except IL1A were differentially expressed in the endometrium of cows fed either high or low protein diets. This may be due to the fact that the two protein levels (17.3% and 14.8%) used in this study were not different enough, and that the MUN levels observed in both groups remained below the levels which have been shown to reduce fertility. However, Pretheeban et al. (2011) have recently shown that mRNA abundance levels of IGF1, FGF2, BCL2, HSP1A1, TNF and SERPINA14 in the endometrium of heifers fed diets with protein levels of around 10% of dry matter were higher when compared with that of lactating dairy cows fed with around 18% (of dry matter) of protein suggesting that high protein intake may be a factor leading to the reduced fertility observed in lactating dairy cows.

Ovarian activity is a complex process which is not only influenced by hormones and growth factors produced by the follicles but also by environmental factors such as

nutrition, climate, and stress (Webb et al. 2004). High protein intake and the associated increase in ammonia and urea level in circulation are believed to have a negative influence on ovarian activity (Butler, 1998; Santos et al. 2001; Tamminga, 2006). Santos et al. 2001 have revealed that pre-partum cows fed a high protein diet had fewer numbers of small follicles (3-5mm) in comparison with cows fed with a low protein diet after calving. In agreement with this observation, our study revealed that the average number of small and large follicles that appeared during the first 6 weeks of calving were lower and tended to be lower, respectively, in cows fed a high protein diet relative to the cows fed the low protein diet; although no difference was observed in number of medium size follicles between the two groups. The precise mechanism by which the initiation of follicular growth and the number of primary follicles emerging from a pool of primordial follicles, to start the follicular wave is not understood. However, it is believed that there may be involvement of local factors such as growth factors, hormones, enzymes and other proteins secreted by the follicle(s) (Webb et al. 2004). The low number of small follicles observed in the high protein group may be due to the detrimental effect of high protein intake on secretion of these local factors. Finding that the high protein group had very few numbers of large follicles during the first two weeks of feeding may be possibly due to the consequence of a lesser number of small follicles observed in that group. However, other studies have shown that the number of follicles developing during the postpartum period is not related to the level of protein intake (Garcia Bojalil et al. 1994; Laven et al. 2004; Santos et al. 2001). Further, the current study also revealed that the average size of large follicles observed during the first 6 weeks of DIM was lower for cows fed a high protein diet. Large follicles produce more estradiol-17 ß which in turn

enhances the expression of estrus, uterine involution, and early resumption of ovarian activity (Cerri et al. 2004; Spicer and Zinn, 1987). The observed difference in the sizes of large follicles between the two treatment groups in this study may be a result of increased concentration of urea and ammonia in the follicular fluid (Hammon et al. 2005; Leroy et al. 2004; Sinclair et al. 2000), which might alter the growth and metabolism of granulosa cells (Rooke et al. 2004) and possibly also alter the interaction between the FSH and its receptor. Considering these results, the current study suggests that the altered follicular dynamics caused by high protein intake may be associated with the reduced fertility observed in lactating dairy cows.

The number of days to the first ovulation and the size of the CL are two important measures used to assess CL dynamics. Our study revealed that the high protein intake tends to delay the interval to first ovulation and reduces the average CL size during the first 6 weeks of lactation. Elevated ammonia concentrations in follicular fluid (Hammon et al. 2005) leading to altered growth and metabolism of granulosa cells (Rook et al. 2004), lower levels of endometrial IL1A mRNA abundance, and the smaller sizes of the large follicles observed in the current study may be possible causes to explain the above observations. Consistent with our study, Garverick et al (1971) observed that heifers that were fed urea showed lighter weight CL than that of heifers fed no urea. This may be a reason why circulating progesterone concentrations during the mid-luteal phase was shown to be lower in cows fed with high protein diets (Folman et al. 1983; Jordan and Swanson, 1979; Sonderman and Larson, 1989).

The concentration of protein in the diet of dairy cows is strongly correlated with milk production. Canfield et al. (1990) and Howard et al. (1987) observed an increase in

milk production when cows were fed a high protein diet (19-20% of dry matter) when compared to that of low protein diets (15-16% of dry matter). However, in the present study, no difference was observed in milk production between cows fed high and low protein diets throughout the study period. Using information available on feed composition, digestion and metabolism, ruminant diet formulation has been greatly improved in order to enhance the production efficiency (NRC, 2001). This improvement enabled nutritionists to predict the nutritional requirements needed for animal performance in a specific feeding situation (Tylutki et al. 2008). The Cornell Net Carbohydrate Protein System (CNCPS) is a very popular diet formulation system used to predict the metabolizable protein requirements of dairy cows in a specific feeding situation (Sniffen et al. 1992; Tylutki et al. 2008). Manipulation of diet during formulation using the above system in the present study probably resulted in lower protein intake having a minimal risk of reducing milk production.

Urea and ammonia, as byproducts of protein metabolism, are toxic to cells when their levels are high in the circulation. Urea level in the blood is measured as Blood Urea Nitrogen (BUN). As urea can freely be defused into milk, the MUN level is reported to be closely correlated to the corresponding BUN level (Baker et al. 1995; DePeters and Ferguson, 1992; Roseler et al. 1993). Testing for urea nitrogen levels in milk is relatively inexpensive and offers a more rapid method relative to BUN. Therefore, measuring urea nitrogen levels in milk to assess protein metabolism is more commonly used in the dairy industry. Several studies show that elevated BUN/MUN level is associated with reduced fertility in dairy cows (Larson et al. 1997; Rajala-Schulz et al. 2001; Roseler et al 1993). BUN and MUN concentrations greater than 20 mg/dl and 19 mg/dl, respectively have

been linked with reduced fertility in dairy cows (Canfield et al. 1990; Elrod and Butler 1993; Ferguson and Chalupa, 1989; Kenny et al. 2002). Further, Rajala-Schulz et al. 2001 observed that cows with MUN levels less than 10 mg/dl were two and a half times more likely to become pregnant relative to cows with a higher MUN level (15.4 mg/dl). Similarly, Ferguson and Chalupa, (1989) also reported that BUN levels greater than 20 mg/dl appeared to reduce the conception rate by three times when compared to lower BUN levels. In the current study, MUN levels were higher in cows fed with high protein when compared to that of low protein fed cows, throughout the study period. However, even the highest levels of MUN observed in the high protein group remained below the MUN levels that have been shown to reduce fertility in dairy cows. This may be again due to improved diet formulation, which could be efficiently used by animals to reduce the urea nitrogen production.

A number of studies have been published demonstrating that high levels of dietary protein act at different levels of the female reproductive axis; including the hypothalamus, pituitary, ovary and uterus resulting in reduced fertility. I studied the relationship between dietary protein levels and ovarian and uterine function and found that high levels of dietary protein are more likely to alter ovarian function over uterine environment. Urea nitrogen level in plasma is more strongly correlated to the amounts found in follicular fluid (r^2=0.86) than within uterine fluid (r^2=0.17) (Hammon et al. 2005). The impairment observed in ovarian function, but not in uterine environment, in our study may be attributed to the effect of urea in follicular fluid rather than in uterine fluid. In our present study, however, the MUN levels even within the high protein group remained within the range that has been shown to not reduce fertility in dairy cows. This

lower concentration might have been enough to cause alteration in ovarian activity but not in the uterine environment. Our observation is in agreement with the finding that the excess dietary urea associated with reduced pregnancy rates is mostly due to alteration in oocyte development and/or oviductal environment rather than the alteration in the uterine environment of sheep (Fahey et al. 2001). Further more, our previous study showed that < 4mM of urea did not have any detrimental effect on mRNA levels of most of the genes tested when endometrial explants were treated with urea. In agreement with that observation, the current study showed that the mean MUN 10 mg/dl which is closer to the 4 mM of Urea observed in the high protein group did not affect mRNA abundance of genes tested, excluding IL1A.

3.5 Conclusion

Many factors including nutrition are implicated in the reduced fertility observed in high producing dairy cows. A number of studies have shown relationship between excess dietary protein associated increased BUN/MUN and reduced PR in lactating dairy cows. This current study intended to unveil the underlying mechanism where by excess dietary protein reduces fertility. Taking the results of this study together as a whole, I suggest that the deleterious effect of excess dietary protein on fertility is mainly due to alterations in follicular and CL dynamics rather than changes in the uterine environment. However, further studies are required to better understand the mechanism by which ammonia and urea affect ovarian dynamics and function at the molecular level. In addition, a large scale study should be carried out to investigate if lowering the protein level to 14.8 % would increase PR in lactating diary cows.

Table 3.1 Diet ingredients and composition. The chemical composition of the diet is presented as the mean of all feed samples collected through out the study period.

Ingredient	**Unit**	**HP**		**LP**	
Corn silage	(% of DM)	25.38		25.4	
concentrate	(% of DM)	49.74		49.7	
Grass silage	(% of DM)	16.02		16.03	
Alfalfa hay	(% of DM)	8.86		8.86	
Chemical composition		**Mean**	**SEM**	**Mean**	**SEM**
Dry Matter	%	100.0	0.00	100.0	0.00
Crude Protein	%	17.3	0.22	14.8	0.17
Soluble Crude Protein	% of CP	56.2	0.54	55.0	0.59
ADF-CP	%	0.3	0.02	0.3	0.02
UIP (Bypass Protein)	Est % CP	23.8	0.31	24.7	0.32
Acid Detergent Fiber	%	22.5	0.30	25.4	0.23
Neutral Detergent Fiber	%	34.8	0.47	39.1	0.50
Total Digestible Nutrients	%	72.1	0.21	70.0	0.16
NE Lactation	MCal/Kg	1.7	0.00	1.6	0.00
NE Maintenance	MCal/Kg	1.8	0.01	1.7	0.01
NE Gain	MCal/Kg	1.1	0.01	1.0	0.01
Calcium	%	1.0	0.04	0.9	0.03
Copper	ug/g	22.4	1.75	20.9	1.49
Phosphorus	%	0.3	0.01	0.3	0.01
Potassium	%	1.6	0.03	1.7	0.04
Sulphur	%	0.2	0.00	0.2	0.00
Magnesium	%	0.3	0.01	0.3	0.01
Zinc	ug/g	122.5	5.43	121.6	7.49
Iron	ug/g	381.0	13.89	413.5	17.08
Manganese	ug/g	73.3	1.12	77.5	3.43
Sodium	%	0.4	0.01	0.4	0.01

Table 3.2 Oligonuleotide sequence and amplicon size of gene specific primers used in qRT-PCR

Gene ontology classification	Gene symbol	Primer sequence (5'-3')	Annealing T °	Fragment length	Gene accession number
Cell growth, proliferation and differentiation related genes	IGF1	F- TCAGTTCGTGTGCGGAGACA R- ACTTCCTTCTGAGCCTTGGG	56	222bp	NM001077828
	IGFBP1	F-CTACAAAGTGCTGGACAGATTAGCC R- GTAGACACACCAACAGAGCCC	60	157bp	X54979
	IGFBP3	F-AAGAAAGGTCATGCCAAGGACAGC R- TTGTCGCAGTTGGGAATGTGGATG	60	199bp	AF305199
	FGF2	F- TACAACTTCAAGCAGAAGAG R- CAGCTCTTAGCAGACATTGG	56	214bp	NM174056
Apoptosis and stress related genes	BAX	F- TGCTTCAGGGTTTCATCCAG R- AACATTTCAGCCGCCACTC	58	223bp	U92569
	BCL2	F- TTCGCCGAGATGTCCAGTCAGC R- GTTGACGCTCTCCACACACA	62	156bp	U92434
	HSPA1A	F- CACTTCGTGGAGGAGTTCA R- GGTTGATGCTCTTGTTGAGG	58	376bp	AY149619
Cytokines	ILIA	F- CTCTCTCAATCAGAAGTCCTTCTATG R- CATGTCAAATTTCACTGCCTCCTCC	58	424	NM174092
	TNF	F- GAAGCTGGAAGACAACCA R- TCCCAAAGTAGACCTGCC	60	338	NM173966
Immune related gene	SERPINA14	F- ATATCATCTTCTCCCCCATGG R- GTGCACATCCAACAGTTTGG	60	126bp	L22095
House keeping gene	SUZ12	F- GAACACCTATCACACACATTCTTGT R- TAGAGGCGGTTGTGTCCACT	60	130bp	XM582605

F- Forward Primer; R- Reverse Primer; bp- Base pair; T ° - Temperature.

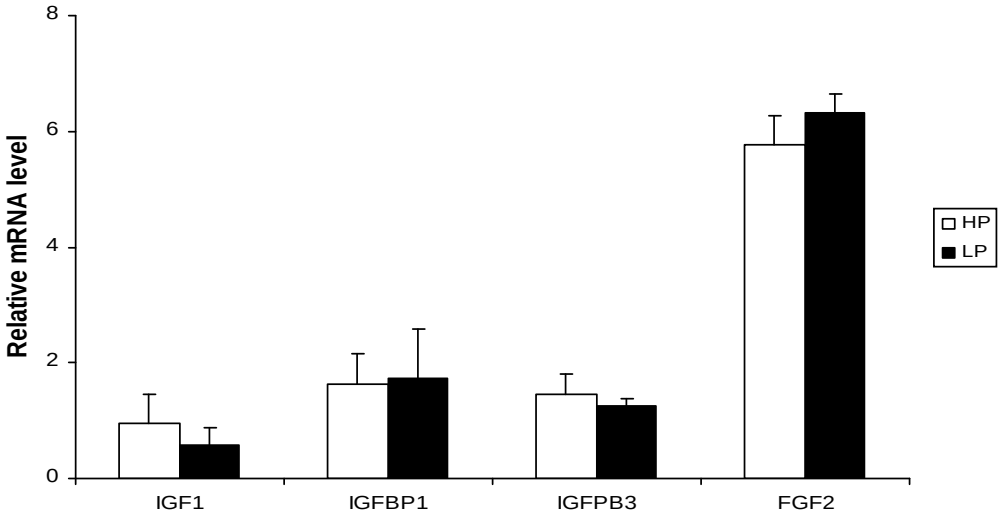


Figure 3.1 Effects of high (HP n=12) and low (LP n=10) protein intake on relative endometrial mRNA levels of IGF1, IGFBP1, IGFBP3 and FGF2 in lactating dairy cows. Data expressed as mean ± SEM.

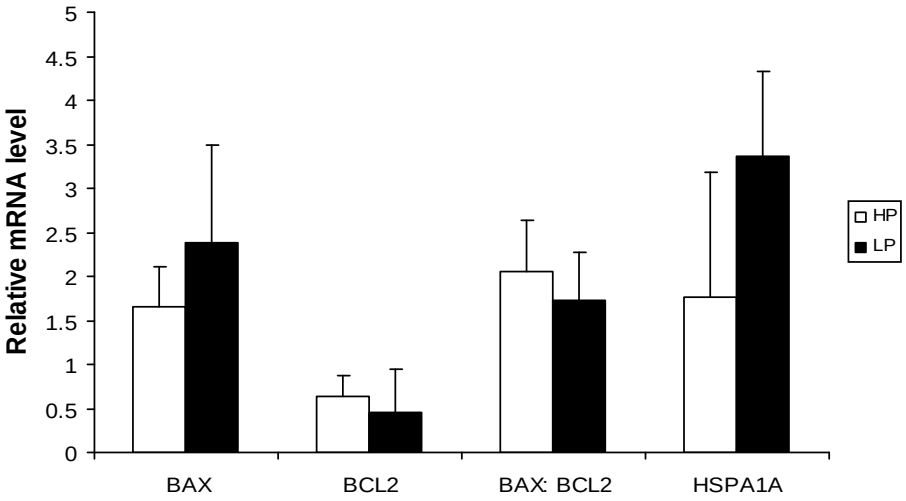


Figure 3.2 Effects of high (HP n=12) and low (LP n=10) protein intake on relative endometrial mRNA levels of BAX, BCL2, BAX: BCL2 and HSPA1A in lactating dairy cows. Data expressed as mean ± SEM.

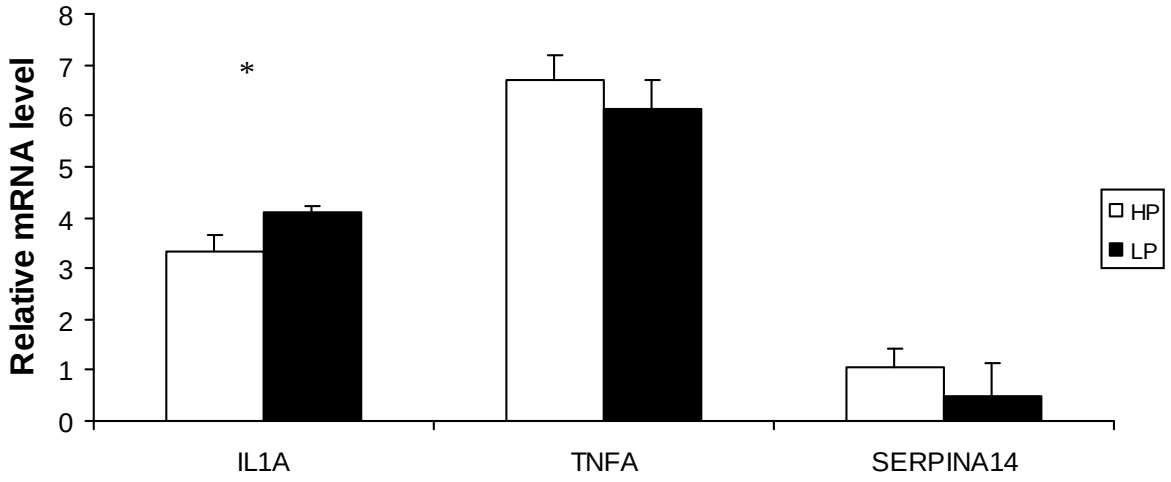


Figure 3.3 Effects of high (HP n=12) and low (LP n=10) protein intake on relative endometrial mRNA levels of IL1A and TNF and SERPINA14 in lactating dairy cows. Data expressed as mean ± SEM. * $P < 0.05$.

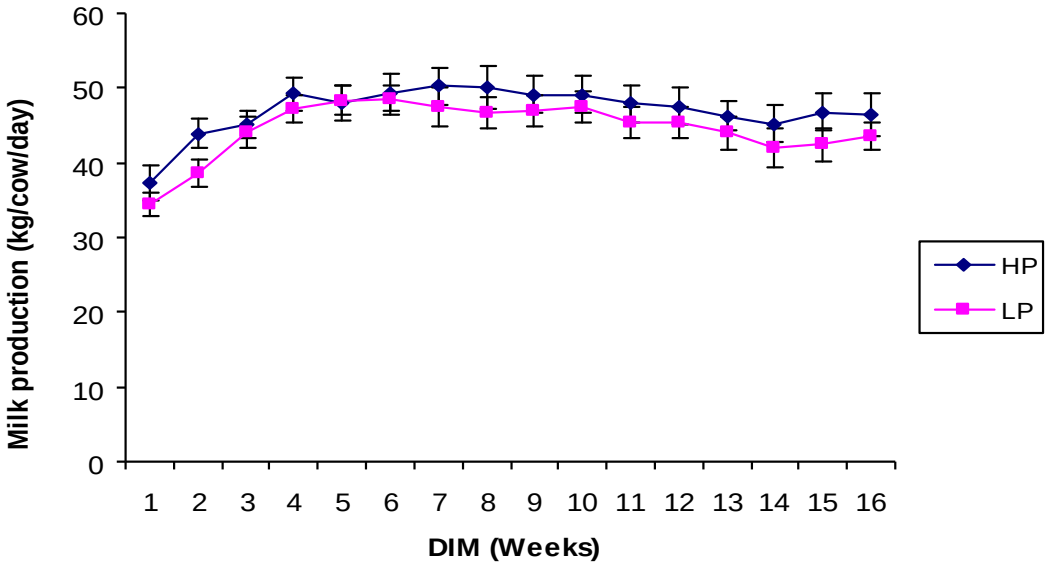


Figure 3.4 Effects of high (HP n=12) and low (LP n=10) protein intake on milk production in lactating dairy cows. Data expressed as mean ± SEM.

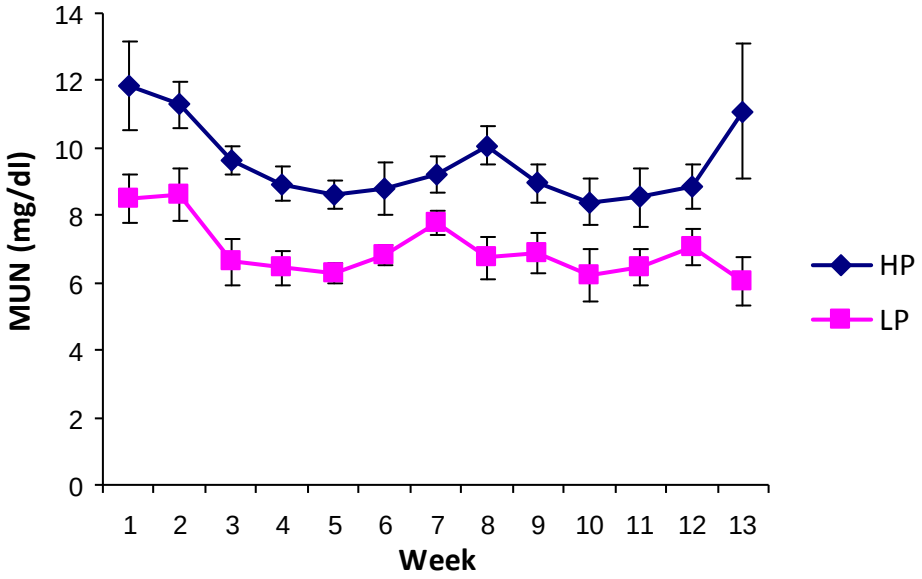


Figure 3.5 Effects of high (HP n=12) and low (LP n=10) protein intake on milk urea nitrogen (MUN) level. Data expressed as mean ± SEM.

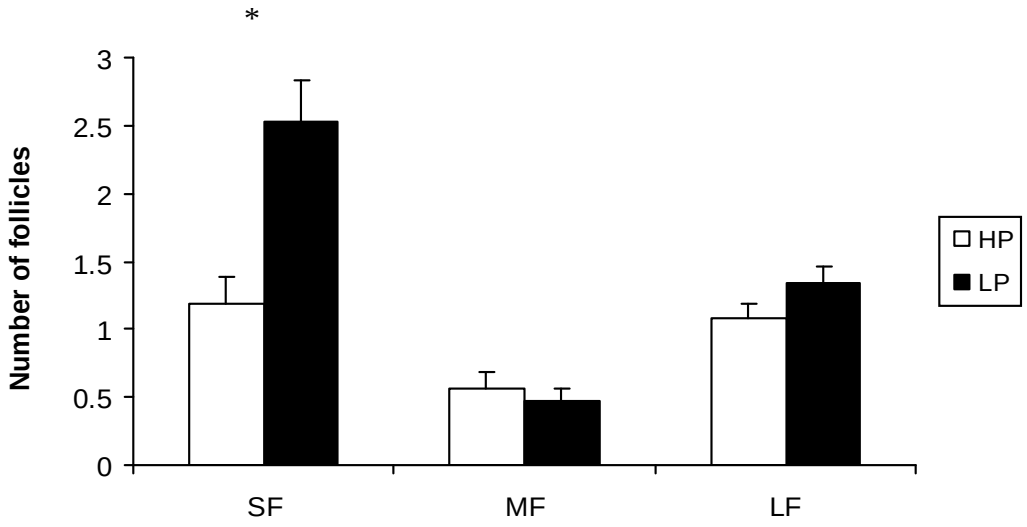


Figure 3.6 Effects of high (HP n=12) and low (LP n=10) protein intake on number of small (SF), medium (MF) and large (LF) follicles appeared during first 6 weeks of feeding in lactating dairy cows. Data expressed as mean ± SEM. *P<0.05.

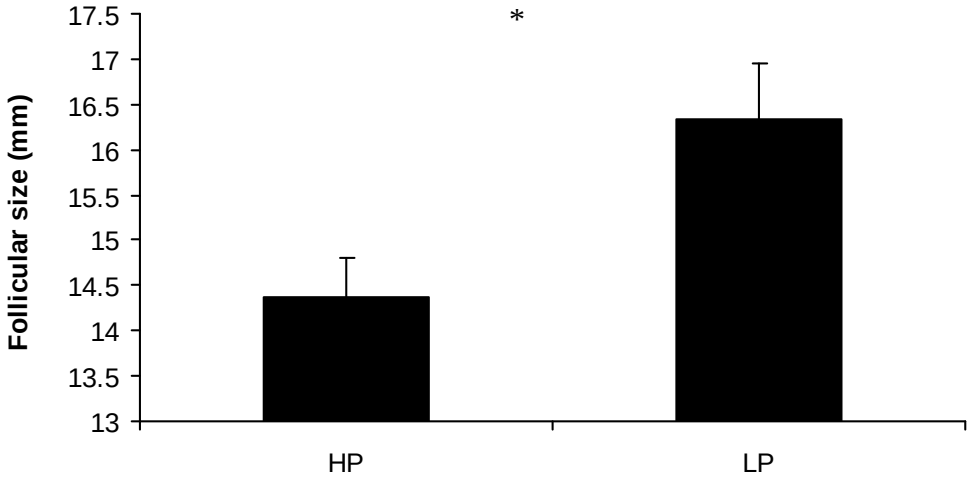


Figure 3.7 Effects of high (HP n=12) and low (LP n=10) protein intake on diameter of large follicles appeared during the first 6 week of feeding in lactating dairy cows. Data expressed as mean ± SEM. * P< 0.05.

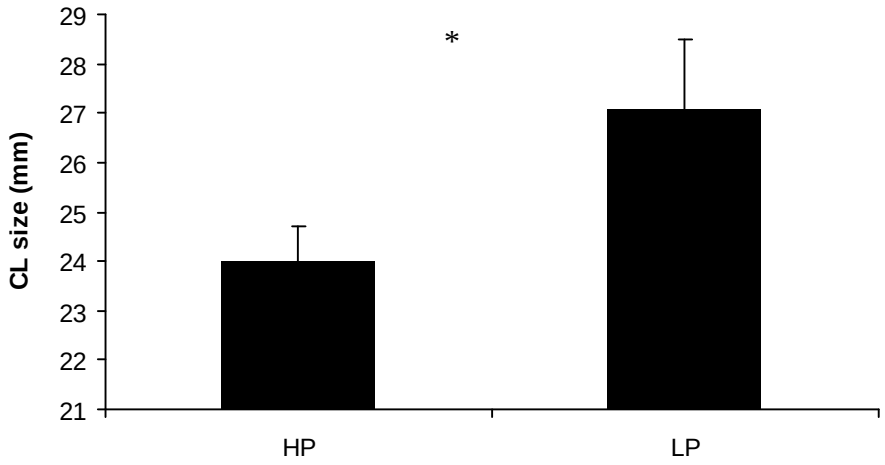


Figure 3.8 Effects of high (HP n=12) and low (LP n=10) protein intake on mean CL size in lactating dairy cows. Data expressed as mean ± SEM. * P< 0.05.

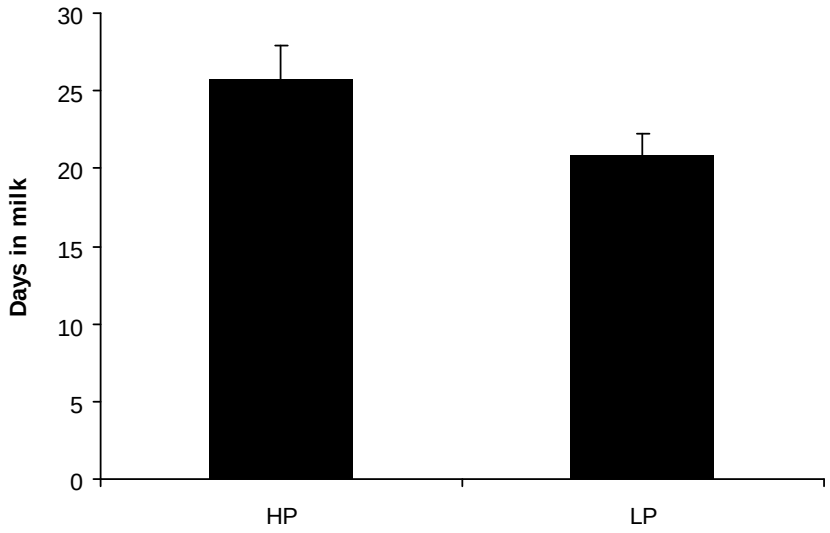


Figure 3.9 Effects of high (HP n=12) and low (LP n=10) protein intake on days to appearance of the first CL in lactating dairy cows. Data expressed as mean ± SEM.

CHAPTER 4: GENERAL DISCUSSION AND CONCLUSION

4.1 Summary

Decreasing fertility observed in lactating dairy cows is a significant challenge faced by dairy farmers. Over decades, dairy cattle breeding program has mainly focused on genetic selection of cows for improved milk production. Coupled with good nutritional management practice, genetic selection has increased milk production of dairy cows. As a part of management, post partum dairy cows are typically fed with a diet containing high levels of protein (17-19% of dry matter) to support high levels of milk production. However, high protein intake is also implicated as a contributory factor for the decreased fertility observed in lactating dairy cows. High protein intake and the associated excess ammonia and urea concentrations in circulation, follicular and uterine fluids are believed to affect normal functions of the reproductive system such as the ovary and the uterus. The quality of the uterine endometrial secretions is found to be sub-optimal in dairy cows fed with a high protein diet. In the past, a number of tests such as uterine fluid pH, mineral composition, and PGF2α concentrations have been used to assess the quality of endometrial secretions in-vivo, and in-vitro (Butler, 1998; Elrod et al. 1993; Jordan et al. 1983; Rhoads et al. 2004). Examining the effect of high protein intake and the associated ammonia and urea concentrations on uterine secretions at the gene expression level would be a novel approach which may unveil the molecular mechanism of high protein intake and associated decreased fertility in dairy cows. This study was carried out with the aim of investigating the effects of high protein intake, ammonia and urea concentrations on selected candidate gene expression levels in the mid-luteal phase endometrium of lactating dairy cows.

The results of Experiment I revealed that 8 out of 10 candidate genes were differentially expressed in vitro in response to exposure of the endometrial tissues to different concentrations of ammonium chloride or urea. However, the results of Experiment II showed that only 1 out of 12 candidate genes was differentially expressed in the endometrium of dairy cows fed high vs. low protein diets. Experiment II also revealed that follicular and CL dynamics were negatively affected by the high protein diet. In addition, MUN levels were higher in the high protein group. No difference in milk yield was observed between the two groups. Findings of these two studies and their importance with regard to decreased fertility in dairy cows are discussed below.

Considering the findings of ammonia and urea on cell growth, proliferation and differentiation related genes (IGF1, IGFBP1, IGFBP3, and FGF2), it was observed that exposure of endometrial cells to higher concentrations of ammonia and urea decreased the expression levels of IGFBP1, FGF2 when compared with control or lower concentrations. This suggests that the presence of excess ammonia and urea in circulation or uterine fluid may affect embryo development by altering the functions of uterine endometrial cells. However, when it comes to apoptotic genes (BAX, BCL2), expression levels were not affected at any of the concentrations of ammonia and urea tested, indicating that the presence of even higher concentrations of ammonia or urea in circulation or uterine fluid is less likely to cause apoptosis of endometrial cells. In contrary to this observation, Lane and Gardner, (2003) and Zander et al. (2006) reported that exposure of embryos to as low as 18.8 μM of ammonia increased the incidence of apoptosis. The contradictory nature of these observations could be due to the degree of sensitivity of the two types of cells to ammonium chloride. Considering the functions of

stress related gene HSPA1A and the IGFBP3 gene, expression of these genes increased when treated with lower concentrations of ammonium chloride or urea suggesting that lower concentrations are also toxic to the endometrial cells. However, as ammonia and urea concentrations increase, a declining pattern of the expression levels were observed though they were not different from lower concentrations. This may probably be due to the effect of ammonia and urea on transcription mechanisms of HSPA1A and IGFBP3. Interestingly, I also found that expression levels of immune related gene SERPINA14 along with IGF1 and BCL2 genes were up-regulated at lower concentrations of ammonia and urea. This finding suggests that ammonia (150 μM) and urea (4mM) also have beneficial effect on uterine environment when they exist in lower concentrations. Collectively, Experiment I provides evidence that exposure of endometrial cells to high concentrations of ammonia (600 μM) and urea (16 mM) have negative effects on some endometrial genes expression levels, while moderate concentrations have positive effects.

Results of Experiment II indicated that of the 12 genes examined only IL1A was differentially expressed in cows fed with high or low protein diet. Endometrial IL1A gene is thought to play a pivotal role in CL maintenance though modulating the prostaglandins (PGF2α, PGE2) secretion to lengthen the lifespan of the CL and increase the progesterone output (Majewska et al. 2010; Tanikawa et al. 2005). Finding low levels of IL1A mRNA abundance in the endometrium of cows fed with a high protein diet suggests that this diet may affect CL function and therefore fertility. However, the expression levels of other genes were not different between the high and low protein groups even though MUN levels were higher in high protein treated cows. This may be due to levels of MUN in the high (9.49 ± 0.2 mg/dl) and low protein (6.96 ± 0.2 mg/dl)

groups being lower than the levels (16.8 mg/dl) that have been shown to affect the uterine environment (Canfield et al. 1990; Jordan et al. 1983).

The follicular and CL dynamics were found to be negatively affected when cows were fed with a high protein diet in Experiment II. The average number of small and large follicles and mean size of large follicles appearing during the first 6 weeks post-partum were negatively affected by high protein intake. In addition, number of days to first appearance of CL and average size of CL were also negatively affected by high protein intake. This indicates that ovarian structures are more sensitive to MUN levels than the endometrial cells. Collectively, Experiment II indicates that high protein diet and the associated MUN levels more likely to reduce fertility through altering the ovarian function rather than the uterine function. This view is in agreement with Fahey et al. 2001 who concluded that excess dietary urea reduced PR in sheep mainly due to alteration in oocyte development and/or oviductal environment rather than alteration in the uterine environment. In addition, a recent study has also shown that PR was not different between dairy heifers fed with a diet containing ~10% CP and lactating dairy cows fed with a diet containing 18% CP, when they were implanted with good quality embryos (Gordon et al. 2009).

4.2 Significance of dissertation

This is the first study that investigated the effects of high protein intake and the associated ammonia and urea concentrations on selected candidate gene expression levels in the mid-luteal phase endometrium of lactating dairy cows. I have shown that a) high concentrations of ammonia and urea have negative effect on the mRNA expression of

some endometrial genes while moderate concentrations have positive effect in vitro, b) lowering the protein level from 17.3% to 14.8% in the diet did not alter the mRNA expression levels of endometrial candidate fertility genes except the IL1A gene, c) lowering the protein level in the diet to 14.8% increased the mean number of small and large follicles, mean size of large follicles, mean size of CL, and decreased the number of days to first ovulation in, and d) lowering the protein level in the diet to 14.8% did not affect milk production. In addition, this study helps to identify the appropriate ammonia concentrations which must be maintained in oocyte and embryo culture media during the process of in-vitro fertilization to achieve better fertilization rate and good quality embryos.

4.3 Limitation and future direction of dissertation

The protein levels used in Experiment II were perhaps not as different as would be needed to show effects on gene expression. This may be a reason why I did not observe differences in candidate endometrial gene expression levels between cows fed with a high or low protein diet. However, Pretheeban et al. (2011) have recently shown that some of the genes that we tested in our study were differentially expressed in the endometrium of dairy heifers fed with ~10% CP and lactating dairy cows fed with ~18% CP. They attributed this differential expression as a possible reason for reduced fertility observed in lactating dairy cows. Therefore, it would have been more appropriate if I had another treatment group consisting of heifers for comparison. Further, I did not measure the ammonia concentration in plasma or uterine fluid. Measuring plasma or uterine fluid ammonia concentration would have been helpful to compare the results with our in vitro

study. This study has shown that lowering the protein level in the diet has beneficial effect on ovarian function and did not affect milk production. Therefore, a large scale study should be carried out to investigate if lowering the protein level to 14.8% would increase PR in lactating diary cows. In addition, further investigations are warranted to study the effects of high protein diet intake and associated ammonia and urea on follicular cells, oocyte, embryo and CL at the gene expression level.

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