EFFECTS OF MILKING FREQUENCY ON MILK YIELD, COMPOSITION AND INDICES OF MAMMARY GLAND METABOLISM IN LACTATING DAIRY COWS

By

Abraham Puthenparampil Alex

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STATEMENT BY AUTHOR

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SIGNED …… Date: 9/28/09
Abraham Puthenparampil Alex

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This thesis has been approved on the date shown below:

……. Date: 09/28/09
Dr. Robert. J. Collier, PhD
Professor of Animal Sciences

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DEDICATION

I dedicate this to the almighty GOD, who blessed me to fructify my dream of pursuing graduate studies in the United States at the University of Arizona. I also dedicate this to my family and all my teachers in INDIA and to my dear India, the love of my heart.

Where the mind is without fear and the head is held high;
Where knowledge is free;
Where the world has not been broken up into fragments by narrow domestic walls;
Where words come out from the depth of truth;
Where tireless striving stretches its arms towards perfection;
Where the clear stream of reason has not lost its way
into the dreary desert sand of dead habit;
Where the mind is led forward by thee into ever-widening thought and action--
Into that heaven of freedom, my Father, let my country awake.

Rabindranath Tagore
(From Geetanjali, 1910)

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LIST OF ABREVIATIONS

ACTB	Beta-actin
AIF	Apoptosis inducing factor
APAF	Apoptosis-activating factor
AZ- DHI	Arizona dairy herd improvement
BM	Basement membrane
CAD	Caspase activated DNAse
cDNA	Complementary deoxyribonucleic acid
CIS	Cytokine-inducible SH2-containing protein gene
CP	Crude protein
CN	Casein
DISC	Death-inducing signaling complex
DMI	Dry matter intake
ECM	Extracellular matrix
FADD	Fas-associated death domain protein
FASL	Fas ligand
FDA	Fat-depleted adipocyte
FIL	Feedback inhibitor of lactation
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GJ	Gap junction
GLUT-1	Glucose transporter type-1
GAT	Galactosyl transferase
GOI	Gene of interest
HPRT	Hypoxanthine phosphoribosyl transferase
Hsp 70	Heat shock protein 70
HIAA	Hydroxy indole acetic acid
IAP	Inhibitor of apoptosis proteins
IGF-I	Insulin like growth factor -I
IL	Interlukin
IFN	Interferon
JAK	Janus kinase
ME	Myoepithelial cell
MEC	Mammary epithelial cell
MFG	Milk fat globule
NBT	Nitro blue tetrazolium solution
NRF	Nuclear respiratory factor
PC	Plasma Cell
PCD	Programmed cell death
PPP1R11	Protein phosphatase-1, regulatory (inhibitor) subunit 11
PTHrP	Parathyroid hormone-related peptide
PMN	Polymorphonuclear
RT-PCR	Reverse transcription polymerase chain reaction

RPS15A	Ribosomal protein subunit
RER	Rough Endoplasmic Reticulum
SGLT-1	Sodium glucose co-transporter type-1
SER	Smooth Endoplasmic Reticulum
STAT	Signal transducers and activators of transcription
SDH	Succinate dehydrogenase
SV	Secretory vesicle
SCC	Somatic Cell Count
SNF	Solids not fat
SOCS	Suppressors of cytokine signaling
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRADD	Tumor necrosis factor receptor-associated death domain
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TJ	Tight junction
USDA	United States Department of Agriculture
UXT	Ubiquitously-expressed transcript

ABSTRACT

Six primiparous Holstein cows were assigned to a half udder design (n=6) 40 days prior to parturition. Beginning at parturition, one udder half was milked once daily (24hr interval) and the other four times daily (6hr interval). Udder halves were biopsied at days 15, 60, 120, and 230 of lactation for mammary tissue to perform mitochondrial staining and apoptosis studies. Increasing the milking frequency from 1x to 4x elevated the 4x udder half milk yield at early (d1-45) ($P<0.0001$), mid (d46-150) ($P<0.0001$) and late (d151-230) ($P<0.0001$) lactation. Milk protein percent ($P= 0.013$), lactose percent ($P=0.004$) and SNF percent ($P=0.006$), were elevated in milk from 4x udder halves over milk from 1x udder halves. We did not detect an effect of increased milking frequency on milk fat percent ($P=0.25$); however, yield of all components was increased. Increased milking frequency also increased mitochondrial numbers in mammary cells from 4x udder half ($P=0.002$) compared to 1x. We did not detect an effect of increased milking frequency on mammary apoptosis percentage. We also did not detect a difference in the abundance of gene transcripts for SOCS1, SOCS2, SOCS3 and CIS in milk; but could find an increase in α-lactalbumin($P=0.04$) and β-casein ($P=0.001$) 4x udder half gene transcripts.

INTRODUCTION

Lactation persistence is accepted as one of the most important determinants of profitability in a dairy herd (Stelwagen, 2001). Lactation persistence has been defined as the degree to which peak milk yield is maintained throughout a single lactation (Grossman et al., 1999). Improving persistence will enhance profitability, improve sustainability of the dairy industry, enhance dairy cow welfare and ultimately reduce the environmental impact of dairy farming on our nation's ecosystems. The improvements in sustainability and animal welfare come from the fact that improved persistence produces healthier cows that live longer. If the herd is provided with adequate nutrition to support the increased milk production, then the benefits are maintained over time. Increasing the milking frequency of dairy cows results in increased milk production (Amos et al., 1985). The percentage of herds using increased milking frequency to boost milk yields has increased, according to USDA sire summaries and in countries where bovine somatotropin supplementation is prohibited, such as Canada, the use of increased milking frequency may be even more accepted than in the United States (Wood, 1967).

In dairy animals, administration of exogenous growth hormone (GH) or increased milking frequency enhances lactation persistence (Amos et al., 1985). Milk production in dairy cows declines after peak lactation by a phenomenon referred to as secretory diminution and is associated with dramatic changes in mitochondrial oxidative damage (Hadsell et al., 2006). Developing novel strategies to enhance persistence should permit increased calving intervals, reduce stress and enhance longevity in dairy cows. This increased longevity is expected to translate into reduced culling rates and a reduced need

for replacement animals (Van Amburg et al., 1997). Delayed rebreeding and somatotropin treatment dramatically enhance persistence in a manner that is not only cost-effective and profitable, but beneficial to animal health and welfare (Amos et al., 1985). Despite these benefits, dairy scientists still do not fully understand how somatotropin or IGF-I promote lactation and under what circumstances they are more or less effective. The objectives of the study were to investigate the effects of increased milking frequency on milk yield, composition, mammary epithelial cell apoptosis and mitochondrial numbers in lactating dairy cows.

CHAPTER 1

LITERATURE REVIEW

Milk is considered as a food source and humans included it in their diet some 9000 years ago with the domestication of the goat (Boutinaud et al., 2003). The early 20th century marked a high focus on increasing the profitability of the dairy industry (Annen et al., 2007). Methods often used to increase income are increasing the herd size or increasing production by increased concentrate feeding; the latter method usually increases feed costs (Wilde et al., 1987). Milk production costs have increased dramatically during the past decade forcing dairy producers to look for methods to increase net income (Hale et al., 2003). Milk yield in dairy cows can be increased by a variety of methods like improving genetic background, environment, feed, hormone treatment and other factors (Bar Peled et al., 1995). One of the most fundamental methods to increase milk yield is by increasing milking frequency (Collier & Romagnolo, 2002). Decreased milking frequency causes a drop in milk synthesis in species such as rats and humans (Neville et al., 1991). Tucker et al., (1966) suggested that the mechanism through which these effects are mediated was not clearly established, but involved a biophysical component (intra gland milk pressure) and one or more biochemical components (metabolic negative feedback). When milking frequency is increased during early lactation, the increase in milk yield is retained even after the treatment ended, thereby exhibiting a carry-over effect on milk yield during later lactation (Bar-Peled et al., 1995). Lactation studies in cattle show that maintenance of peak milk yield and increase in persistency depend heavily on the rates of cell proliferation and apoptosis (Capuco et al., 2001).

Lactation persistence: Definition

Lactation persistence is defined as the degree to which peak milk yield is

maintained throughout a single lactation (Grossman et al., 1999). Numerous definitions for persistency of lactation yield are used in the literature, “the rate at which milk yield falls off from the maximum”, (Sordillo et al., 1988), “the degree to which the milk yield in early lactation is maintained”(Norgaard et al., 2005), “the ability of the cow to continue to produce at a high level throughout her lactation” (Capuco et al., 2003), “the extent to which peak yield is maintained” (Wood, P.D.P. 1967), or “the ability to maintain a more or less constant yield during the lactation”(Hale et al., 2002). A typical lactation curve increases from initial yield at calving to maximum or peak yield, maintains peak yield, and decreases from peak yield to the end of lactation (Kamidi et al., 2005). A cow with a flatter lactation curve is more persistent than a cow with the same total yield but with a curve decreasing rapidly after the peak (Tucker et al., 1973). In dairy goats, mammary growth and differentiation during early lactation account for increasing milk yield during the ascending portion of the lactation curve, whereas after peak lactation, loss of mammary cells largely accounts for declining milk yield (Knight and Peaker, 1984). Milk yield and shape of the lactation curve are determined by the extremely high dynamic nature of the mammary secretory cell (Stelwagen et al., 2001). Persistency is a heritable trait, which is greater in primiparous than in multiparous animals and is improved by increasing the milking frequency and growth hormone injection and reduced by poor nutrition and concurrent pregnancy (Bar Peled et al., 1995).

Secretory diminution: Definition

In dairy cows, decreasing the daily frequency of milk removal accelerates secretory diminution, whereas increasing the frequency has the opposite effect (Knight et

al., 1994). Secretory diminution in dairy animals also involves the transformation of active secretory cells into resting cells, which in turn undergo apoptosis. Secretory diminution in dairy cows is directly proportional to the loss of mammary DNA and decreased secretory activity of existing cells present within the gland (Capuco et al., 2003). In dairy animals, this loss is controlled by the frequency of milk removal, exogenous growth hormone administration (Van Amburgh et al., 1997), photoperiod and reproductive status. Among these factors, milking frequency is cited as the most important regulator (Knight et al., 1994). Oxytocin and growth hormone, when given exogenously are found to slow down secretory diminution in dairy cows and the effect of these hormones is quite small and is only about 10 - 20 % (Bauman et al., 1999).

Cell types of mammary gland

The basic structural component of mature mammary gland is the alveoli lined with milk-secreting cuboidal cells which are surrounded by myoepithelial cells (Watson et al., 2008). The alveoli join together to form lobules with lactiferous ducts (Hens et al., 2005). The myoepithelial cells contract under the stimulation of oxytocin, expelling the milk secreted from alveolar units into the lobules leading to lactiferous ducts which finally drain into gland cistern and teat cistern (Fleet et al, 1995). The epithelial cell matrix, together with the adipocytes, fibroblast and inflammatory cells constitute the mammary stroma (Wiseman et al., 2002). Somatic cells in milk consist of neutrophils, macrophages, lymphocytes, eosinophils and various epithelial cell types of the mammary gland (Marcus and Shuster, 1994). In a healthy lactating mammary gland, macrophages are the predominant cell type in milk whereas neutrophils are the major cell population during early inflammation (Hill, 1981). Milk somatic cells play a protective role against

infectious disease in the bovine mammary gland (Akers et al., 1987). During inflammation, cytokines including interleukin-1 (IL-1), IL-6, IL-8, tumour necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) which are important in eliciting acute phase response are released, allowing accumulation of leukocytes at the site of infection (Gin et al., 1984)

Synthesis of milk components

The precursors of milk components leave the blood, enter the fluid and are taken up through the basolateral membrane of the epithelial cells (Neville and Peaker, 1981). Milk precursors enter milk by five routes in the alveolar lumen, including uptake of amino acids (I), uptake of milk fat precursors (II), uptake of sugars and salts (III), uptake of immunoglobulins (IV) and the paracellular pathway (V) (Figure 1.1), (Neville, 1995). Amino acids are covalently bind through peptide bonds to form proteins in the ribosomes and transferred to rough endoplasmic reticulum (RER), where milk proteins like casein, ß-lactoglobulin, and a-lactalbumin are synthesized (Doepel et al., 2004). Casein (CN), secreted as a micelle, is an aggregate of protein surfactant molecules dispersed in the colloidal solution of milk. Casein (αS1, αS2, β, κ forms), formed in golgi apparatus is the predominant phosphoprotein in milk (Turner et al., 1992). Glucose and UDP-galactose, precursors of milk sugar, enter golgi apparatus which results in the formation of lactose and drawing in of water to the cell (Neville et al., 1990). Acetate and ß-hydroxy butyrate, precursors of fatty acid synthesis in ruminants are absorbed through the basolateral membrane of the mammary cells (Clegg et al., 2000). Milk fat triglycerides are synthesized in the smooth endoplasmic reticulum (SER) which forms small droplets and fuse together to form milk fat globule (MFG), entering the lumen of the alveolus via

pathway II (Moore and Christie, 1979). Immunoglobulins, (especially IgA) are transported to the apical side of the cell through the endocytic vesicles which releases them into the lumen of the alveolus (Hayward, 1983). Because of tight junctions between epithelial cells, there is little flow of substances between the cells, except water and some ions (Neville, 1995). The paracellular pathway allows passage of substances between the epithelial cells, rather than through them (Lin et al., 1995; Morton, 1994). When oxytocin causes milk ejection, tight junctions become leaky allowing lactose and potassium to move from the lumen into the extracellular space, and sodium and chlorine to move into the lumen from the extracellular space (Allen, 1990).

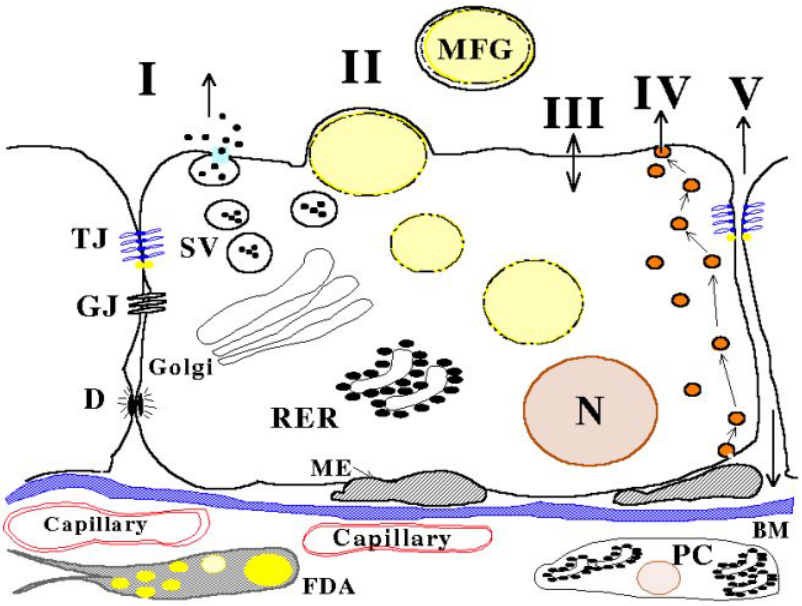

Figure 1.1, Alveolar Cell from lactating mammary gland. N, nucleus; TJ, tight junction; GJ, gap junction; D, desmosome; SV, secretory vesicle; FDA, fat-depleted adipocyte; PC, Plasma Cell; BM, basement membrane; ME, cross section through process of myoepithelial cell; RER, rough endoplasmic reticulum. Pathway I (exocytosis), II (lipid), III (apical transport), IV (transcytosis) and V (paracellular pathway) (Neville, 1995).

Somatic cell count (SCC) and its significance in dairy industry

When an infection is present in a mammary gland, milk- secreting cells are damaged and their capacity for producing milk is lowered (Harmon, 1994). The use of Somatic Cell Count (SCC) to monitor udder health over the last few decades has proven to be a valuable tool for the identification of cows with major contagious pathogenic infections and is being proposed useful for selection decisions for breeding programs of dairy cattle (Shook, 1985). The current legal limit for somatic cell count in US market is 750,000 cells/ ml of milk (Eberhart et al., 1982) which is higher than legal maximum for SCC in other dairy exporting countries like Canada (500,000 cells/ml) and European Community (400,000 cells/ml), (Smith et al., 1998). The factors affecting SCCs are infection status, lactation number, stage of lactation, estrus, exercise, heat stress, stray voltage and day-to-day variation in milk production (Harmon, 1994). An uninfected udder typically has SCC less than 100,000 cells/ml and when the SCC is 100,000 to 199,999 cells/ml, the presence of infection is ruled out by bacteriological testing (Smith et al., 2001). Since SCCs reflect infection, they are used to estimate production losses and Eberhart et al, (1982) suggest that SCC of 500,000 cells/ml and 1,000,000 cells/ml cause production losses of 6% and 18% respectively. Because of the difficulties of accurately detecting and recording clinical mastitis, genetic selection for cows with low SCC has been proposed as a method to reduce the incidence of mastitis (Shook, 1989).

Hormones and Growth factors affecting lactation

Prolactin

Prolactin is a significant player in ruminant lactation persistency (Knight, 1992). Prolactin signaling is essential for mammogenesis and differentiation of the mammary gland during pregnancy in laboratory species and without prolactin, the steroid hormones

like estrogen and progesterone, failed to stimulate mammogenesis (Topper et al., 1980). In cattle and goats, prolactin secretion does not limit the secretion of milk as it's concentration in blood of cows is only slightly correlated with milk yield (Tucker, 1973), Neville et al., (2002), suggests that supplemental prolactin has little effect on milk yield in rat and mice. Bromocriptine and cold temperatures also markedly reduce the secretion of prolactin without suppressing the milk yield in cows (Smith et al., 1967). In cattle, increasing the daily light from 8 to 16 h/d increases the concentrations of prolactin several folds and also increases milk yield by 6 to10% (Lynch et al., 1991). Recent research by Dahl et al., (2002) suggested that long-day photoperiods stimulate secretion of prolactin in cows.

Progesterone

Progesterone induces the lobule-alveolar growth during pregnancy in cattle (Neville et al., 2002). Progesterone also stimulates DNA synthesis at the end buds and also along the walls of the mammary ducts (Bresciani, 1968), where its receptors are located (Haslam et al., 1979). Estrogen, in combination with progesterone when administered near calving, induces lactation in about 70% of cows treated and the induced lactation is about 70% of normal milk production (Smith, 1996). Although progesterone inhibits the initiation of lactation, once lactation is established, administration of progesterone has no effect on milk yield (Herrenkohl, 1972). Progesterone plays no role in the maintenance of lactation because the progesterone receptor is not expressed in the mammary glands during the late lactation stage (Meyer et al., 2006). Capuco et al., (2003), have shown that progesterone has greater affinity for milk fat than for its own intracellular receptor, which minimizes its action at the mammary gland.

Placental Lactogen

Bovine placental lactogen, which is a part of the somatotropin and prolactin gene family (Dewhurst and Knight, 1993) stimulates mammogenesis by binding primarily to the prolactin receptor (Elliott, 1959). Administration of placental lactogen has little effect on metabolism in lactating cows (Bruckmaier, 2001), but it stimulates lactogenesis in mammary explants in midpregnancy (Neville et al., 2002) and is equipotent to bovine prolactin and human somatotropin (Stelwagen et al., 1996). Placental lactogen has both somatogenic and lactogenic properties in vivo, depending on the specificity of the somatotropin and prolactin receptors and specific binding sites for it are found in the uterine endometrium of pregnant cows (Kahl et al., 1995).

Glucocorticoids

Cortisol causes differentiation of the lobule-alveolar system in the bovine mammary gland (Tucker, 1981). Glucocorticoids in combination with prolactin is essential for inducing the synthesis of milk proteins (Thatcher & Tucker, 1968). In non lactating cows with well-developed lobule-alveolar systems, it induces the onset of lactation (Collier et al., 2002). Therapeutic doses of synthetic glucocorticoids suppress milk yields in cattle (Braun et al., 2002). The release of glucocorticoids is induced by milking stimuli in cows, a pattern that is maintained throughout lactation (Tucker et al., 1973). In cattle, mammary uptake and binding of glucocorticoids increases with the onset of lactation and are positively correlated with the uptake of glucose into mammary tissue (Gorewit & Tucker, 1977).

Milking frequency effects on production performance in dairy cows

Milking cows once daily reduces farm expenses, particularly labor costs and improves the health and welfare of cows, through increased body condition (Woolford et al., 1985; Davis et al., 1987). Cows milked once daily carry a greater volume of milk in their udder than twice daily milked cows (Lacy-Hulbert et al., 2005). The major disadvantage of once-daily milking is reduced milk production and losses reported in previous experiments ranged from 7 to 38% for periods of once-daily milking (Davis et al., 1987) and losses from full lactations ranged from 22 to 35% (Carruthers et al., 1989). In cattle, increasing milking frequency from 2x to 3x or 4x increases milk yields (Bar-Peled et al., 1995, Hale et al., 2003, Norgaard et al., 2005).

Table 1.1 Effect of milking frequency on milk yield in different species, using half-udder experiments (change expressed as a percentage of the 2x-milked control gland).

	Milking frequency per udder half			
Species	1x	3x	4x	Reference
Cow	–32			Ayadi, 2003
Cow (early lactation)	–38			Stelwagen and Knight, 1997
Cow (late lactation)	–28			Stelwagen and Knight, 1997
Cow			+14	Knight et al., 1992
Cow		+7		Morag, 1973)
Cow		+32		Cash and Yapp, 1950
Cow	–19.6			Patton, 2006
Cow	–13			Lacy-Hulbert, 1999
Goat	–18			Salama, 2003
Goat		+10		Knight, 1992
Goat		+14		Knight et al., 1990
Goat		+20		Wilde et al., 1987
Goat	–26			Wilde and Knight, 1990

Differences in response within a certain milking frequency may depend on differences in stage of lactation, production level, breed, duration of the milking regimen, and individual udder characteristics (Stelwagen, 2001, J. Dairy Sci. 84: 204-211).

Milking more than 2x results in increased milk yield and milk secretion rate in dairy cows (Hillerton et al., 1990) and goats (Knight et al., 1990). Milking cows three times daily (3x) increases production ranging from 3 to 26% (Andersen et al., 2003). Previous research by Elliott (1959), also indicate that 3x milking increases production by 39%. Pearson et al.,

(1979), reports that cows milked twice daily consume slightly more feed but produces less milk than cows milked 3x. But milking cows 6x did not improve milk yields over 3x (Van Baale et al., 2005), possibly because of time away from feed in the 6x milked group. Knight, (1992) reported that four times daily milking delayed the onset of estrus and ovulation compared to 2x milking.

Effect of milking frequency on mammary tight junction permeability

The most important member of the junctional complex between epithelial cells is tight junction (TJ), or zonula occludens which form semi permeable barriers between adjacent epithelial and endothelial cells (Stelwagen et al., 1994). In mammary epithelium, intact TJ prevent paracellular leakage of blood serum components into milk and prevent milk components from crossing into blood. TJ also maintains a small trans epithelial potential difference between blood and milk (Peaker, 1977). TJ also restricts the passage of ions and small molecules between adjacent cells (Schneeberger & Lynch, 1992) and are actively involved in the regulation of paracellular transport (Madara, 1988). Stelwagen et al., (2001), observed that TJ in cows became leaky during a 24-h milking interval and the moment they became leaky (approximately 17 to 18h after milking) coincided with the moment rate of milk secretion start to decline, suggesting that TJ play a role in milk loss associated with 1x milking. A proposed mechanism for TJ action with varied milking frequency is presented in Figure 1.3. Reduction in milking frequency increases cell stretching and decrease in prolactin secretion, leading to down regulation of a factor called parathyroid hormone-related peptide (PTHrP) (Daifotis et al., 1992), which blocks apical calcium channels needed for TJ integrity (Jovov et al., 1994).

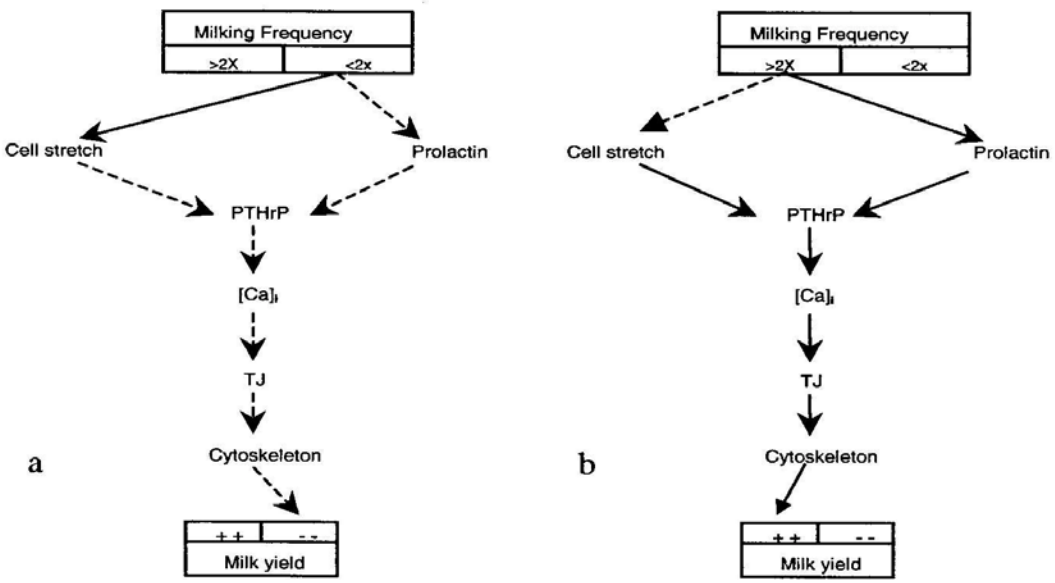

Figure 1.2 Proposed model of mammary tight junctions (TJ) role in the effects of 1x milking (a) or continous milking (b) on milk yield, mediated during the first 24 h. Stippled arrows indicated a decrease and solid arrows indicate an increase. Adapted from Stelwagen et al., 2001. (J. Dairy. Sci. 84: 204-211).

Milking frequency effects on milk composition

Carruthers et al., (1993) suggest that dairy cows milked 1x daily produce relatively more concentrated milk with increased fat, protein and less lactose. Milk of 1x ewes also has a greater percentage of protein and lower percentage of lactose than milk of 2x ewes, with little difference in fat percentage (Knight et al., 1990). Milk casein also is more concentrated in 1x dairy cow milk (Lacy-Hulbert et al., 1999). But, recent research suggests that there is no difference in milk casein between cows milked 1x and 2x during early (Remond et al., 1992), late and whole lactation (Sorensen, 2003). A decrease in milk lactose content is the most consistent change induced by 1x milking due to leaky tight junctions (Davis et al., 1999). Previous research suggests that once daily milking for

14 days (Stelwagen et al., 1994a), 26 days (Lacy-Hulbert et al., 1999), 3weeks (Remond et al., 1992), 10 weeks (Carruthers et al., 1993), and whole lactation (Davis et al., 1998) did not affect milk somatic cell count compared with 2x in dairy cows. Similarly, 1x milking throughout lactation in dairy cows did not affect milk somatic cell count (SCC) (Holmes et al., 1992). The increase in SCC during 1x is not associated with damage to epithelial cells, but may be due to the impairment of the tight junctions (TJ) barrier which facilitates a para-cellular influx of somatic cells into milk (Stelwagen et al., 1994b). Reduced milking frequency increases SCC in cows with high initial SCC in early lactation (Kamote et al., 1994).

Effect of pregnancy on milk production

Pregnancy causes a significant decline in milk yield of dairy cows and the mechanism by which pregnancy influences milk yield is not fully understood, but is believed to be caused by the estradiol and progesterone hormones that maintain the pregnancy (Stefanon et al., 2002). Peaker and Linzell (1974) found that administration of estrogen in late pregnancy caused a significant decline in milk yield in goats. Also, estradiol injection before drying off accelerated mammary tissue remodeling and involution in dairy cows by promoting plasminogen activation (Athie et al., 1996). Placental lactogen also mediates the effect of pregnancy on mammary development in declining lactation and thereby on lactation persistency (Stefanon et al., 2002). In addition to hormonal effect, milk yield losses can also be attributed to the increasing energy demand of the fetus, especially after d 190 of pregnancy in dairy cows (Bell et al., 1995). Few studies have examined the effect of pregnancy on milk yield and milk composition. In dairy cows, some authors (Coulon et al., 1995; Olori et al., 1997; Roche

2003) reported milk yield losses from the 5th to 6th month of pregnancy onwards, whereas other authors (Bormann et al., 2002) reported a decline in milk yield as early as 3rd month of pregnancy. The effect of pregnancy depends on the stage of lactation when pregnancy occurs (Brotherstone et al., 2004). Cows that conceive in the 1st, 2nd, or 3rd month of lactation suffered more milk losses during a 300-d lactation period that non-pregnant cows or cows becoming pregnant in mid or late lactation. Similarly, Tekerli et al., (2000) demonstrated that cows which conceived shortly after calving had lower lactation persistency and that the decline in the slope of the lactation curve after peak yield decreased with increased days open. Milk fat, protein and lactose contents in pregnant cows were greater than in non-pregnant cows after d126 of pregnancy and differences increased as pregnancy advanced until drying off (Roche, 2003). Olori et al., (1997) reported little effect of pregnancy on milk protein and lactose content but milk fat content increased significantly from the 6th month of pregnancy.

Apoptosis: definition

Apoptosis is a process of programmed cell death which involves a series of changes including loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (Kerr et al., 1972). Apoptosis is controlled by extrinsic or intrinsic inducers (Popov, et al., 2002). Two theories for the direct initiation of apoptotic mechanisms in mammals have been suggested: the TNF-induced (tumour necrosis factor) model (Santos et al., 2000) and Fas-Fas ligand-mediated model (Wajant, 2002), both involving receptors of the TNF receptor (TNFR) family coupled to extrinsic signals. TNF is the major extrinsic mediator of apoptosis and the binding of TNF to TNF- Receptor1 (TNF-R1) initiates the pathway that

leads to caspase activation by TNF receptor-associated death domain (TRADD) and Fas-associated death domain protein (FADD) (Chen et al., 2002). The Fas receptor binds the Fas ligand (FasL) resulting in the formation of death-inducing signaling complex (DISC), which contains the FADD, caspase-8 and caspase-10 (Wajant, 2002). Caspase-8 directly activates caspase-3 and triggers the execution of apoptosis. The detection of apoptosis by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) is based on the tagging of the DNA fragements. The duration of apoptosis has been estimated to be approximately 3h (Bursch et al., 1990) and TUNEL assay only marks fragmented DNA that is present during this short period (Capuco et al., 2001) which can be sited as a possible limitation of the TUNEL assay.

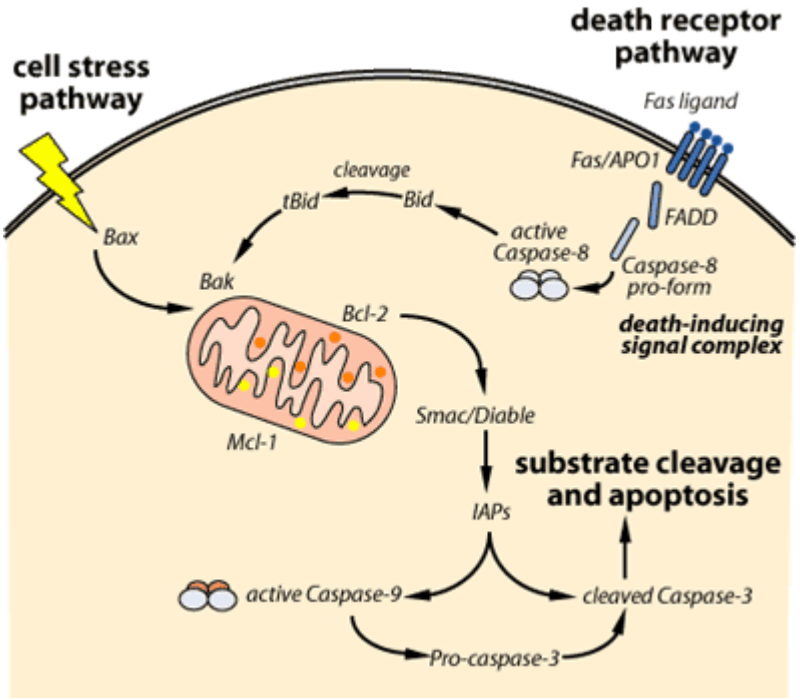

Figure 1.3 The intrinsic and extrinsic pathways leading to apoptosis. Apoptosis can be triggered in a cell through either the extrinsic pathway or the intrinsic pathway. The extrinsic pathway is initiated through the stimulation of the transmembrane death receptors, such as the Fas receptors and the intrinsic pathway is initiated through the release of signal factors by mitochondria within the cell (Adrain et al., 2002).

Mitochondrial regulation of apoptosis

Mitochondria represent the central point of apoptosis as it controls the extrinsic and intrinsic pathways of apoptosis. Cellular stress caused by factors such as DNA damage and heat shock (Desagher and Martinou, 2000) triggers apoptosis. Apoptotic proteins cause mitochondrial swelling through the formation of membrane pores causing apoptotic effectors to leak out (Werlen et al., 2003). Nitric oxide also induces apoptosis by dissipating the membrane potential of mitochondria and therefore makes it more permeable (Brune, 2003). Inhibitor of apoptosis proteins (IAPs) suppress the activity of caspases, which carry out the degradation of the cell, therefore the actual degradation enzymes can be seen to be indirectly regulated by mitochondrial permeability (Fesik, 2001). The release of cytochrome C from mitochondria leads to the activation of caspases that execute apoptosis (Roy, 2000). The release of cytochrome c is controlled by Bax protein, which undergoes a conformational change, leading to its translocation from the cytosol to mitochondria (Hou et al., 2003). Bax forms pores on mitochondrial outer membrane surface to cause the release of cytochrome c (Marti et al., 2000).

Caspases are a family of aspartate specific cysteine proteases that are believed to be major effectors of apoptosis (Mastrangelo and Betenbaugh, 1998). Once cytochrome c is released towards the cytosol, it binds to the apoptosis-activating factor-1 (Apaf-1). In the presence of ATP, this complex recruits and activates procaspase-9 to generate a functional apoptosome (Porter, 1999). Activated caspase-9 activates caspase-3 which is the final executionary arm of apoptosis (Desagher and Martinou, 2000). In addition to cytochrome c, another protein normally located in the mitochondrial intermembrane space, known as the apoptosis-inducing factor (AIF), is transported to the cytosol and the

nucleus during apoptosis causing DNA condensation and fragmentation (Susin et al., 2000). The release of AIF occurs before that of cytochrome c and before caspase activation; therefore, death induced by AIF is caspase-independent (Daugas et al., 2000).

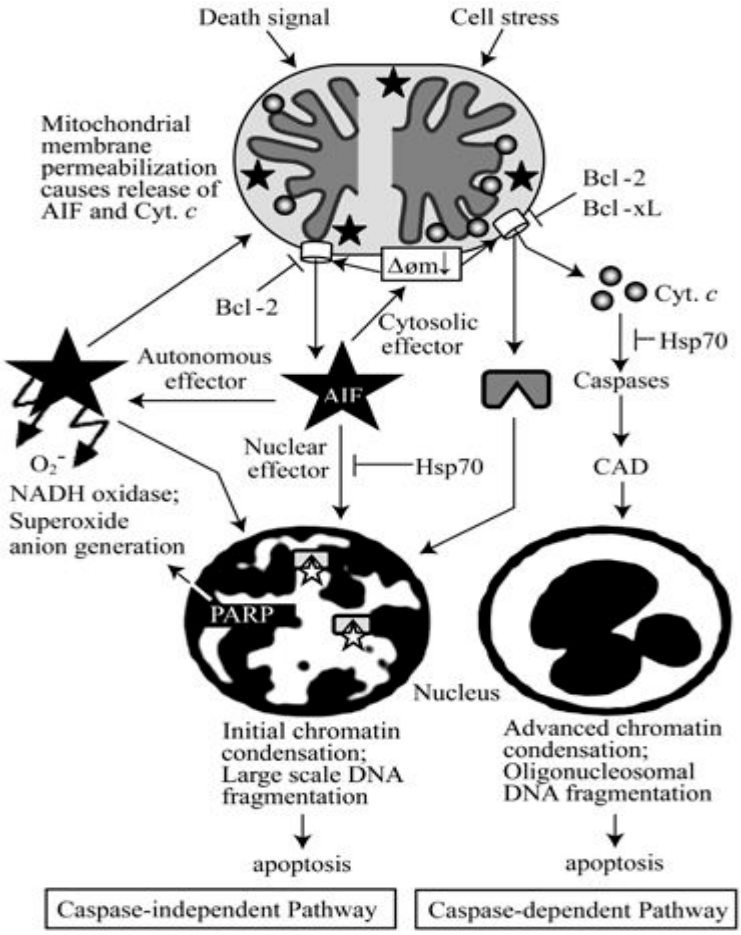

Fig. 1.4, Two pathways involved in the occurrence of apoptotic nuclear morphology. Left, caspase-independent apoptotic pathway mediated by mitochondrion released AIF. AIF plays three important roles in apoptosis: one is as an autonomous effector, the second as a cytosolic effector, the third as a nuclear effector. Right, caspase-dependent apoptotic pathway mediated by caspases and/or mitochondrion released cytochrome c (Cui-Xian et al., 2003).

Apoptosis is characterized by a series of morphological changes which include cell shrinkage and rounding because of the breakdown of the proteinaceous cytoskeleton by caspases (Santos, 2002). Chromatin undergoes condensation into compact patches

against the nuclear envelope in a process known as pyknosis, a hallmark of apoptosis (Madeleine, 2001). Nuclear envelope becomes discontinuous and DNA inside it is fragmented in a process referred to as karyorrhexis and eventually the nucleus breaks into several nucleosomal units due to the degradation of DNA (Nagata, 2002). Finally the cell membrane undergoes blebbing and the cell breaks apart into several vesicles called apoptotic bodies, which are eventually phagocytosed.

Effect of milking frequency on mammary epithelial cell apoptosis

Mammary epithelial cell (MEC) turnover is defined as rate of cell production in a lactating gland, calculated as the difference between proliferation and apoptosis percentages. Apoptotic cell death accounts for the 50% decline in the total population of secretory cells at the end of bovine lactation (Capuco et al., 1999). Previous experiments in cattle show that maintenance of persistency is heavily reliant upon the cellular dynamics of the mammary gland; especially, the rates of cell proliferation, apoptosis and the secretory capacity per cell (Tucker, 1981). Mammary apoptosis regulated through local mechanisms like intramammary pressure, blood supply and exposure to lactogenic hormones is sensitive to milking frequency (Li et al., 1999). Recent research done by Capuco et al., (2001) using TUNEL assay suggests that at 14 d, the apoptotic index was 0.27% of total cells, whereas at 90d, 120d and 240d of lactation, it was 0.05%, 0.1% and 0.03% respectively (Figure 1.4). But, Wilde et al., (1997) found mammary apoptosis rates as high as 2.4% for Holstein cows at 217d to 252d of lactation.

Changes in milking frequency can lead to changes in mammary cell turnover on the cellular level, altering the apoptotic state of the mammary gland in cattle (Hillerton et al., 1990) and goats (Wilde et al., 1987; Li et al., 1999). Carruthers et al., (1993), found

that reducing milking frequency from twice daily to once-daily milking increased the rate of apoptosis, while Norgaard et al., (2005), demonstrated that 3x or 4x milking of dairy cows in early lactation reduced apoptosis. Increased migration of polymorphonuclear leukocytes especially neutrophils into the mammary gland accounts for the elevated apoptosis percentages in early lactation stage (Concha, 1986; Sordillo et al., 1997). Li et al., (1999), suggests that the proportion of apoptotic cells was higher in 1x compared to 3x milked glands, suggesting a negative correlation of apoptosis with milking frequency

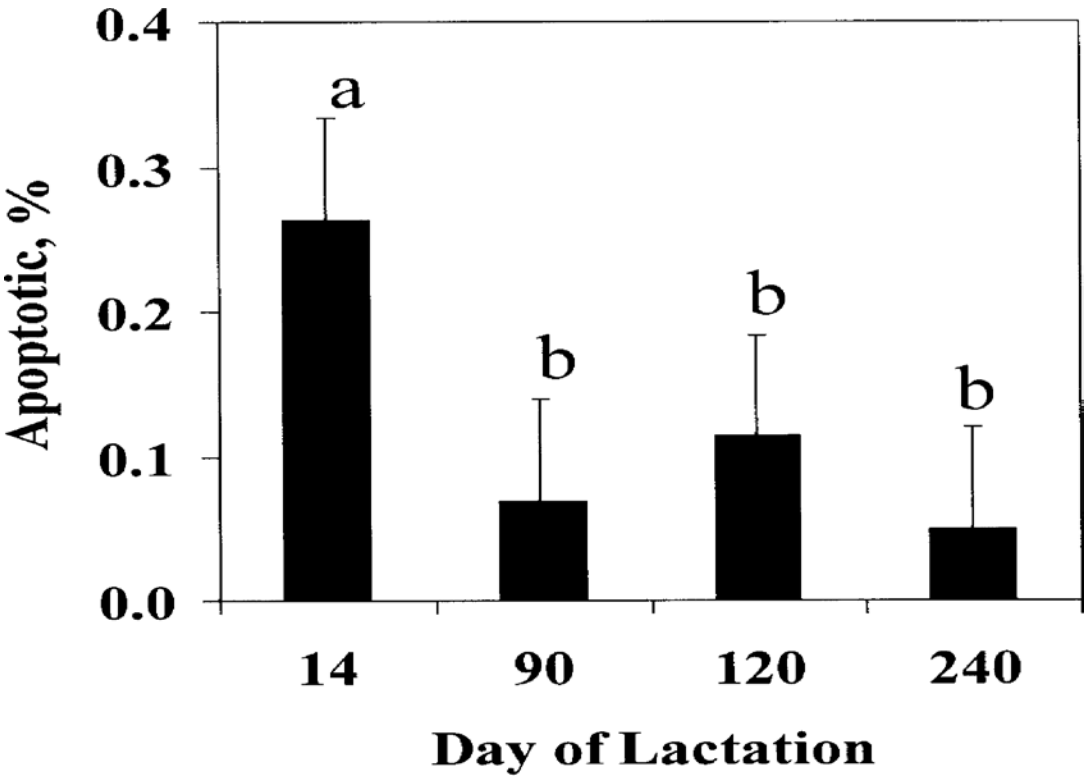

Figure 1.5, Mammary cell apoptotic index during lactation. Data expressed as a percentage of total cells. Each bar represents the mean ± SE for four to six cows. Apoptotic index was greatest during early lactation, but did not vary after peak lactation (Capuco et al., 2001).

Most studies on mammary gland remodeling suggested only apoptosis as a way of MEC death in mammary tissue. Recent research by Zarzynska et al., (2008) indicates that type II programmed cell death (PCD) or autophagic cell death is also observed in

mammary epithelial cells. Autophagy is a process of degradation and recycling of cellular constituents including long-lived proteins and organelles, which plays a role in bioenergetic management of starvation (Ferraro et al., 2007). Similarly to apoptosis, incidence of autophagy in bovine mammary gland is also highest in dry period, evidenced by higher expression of Beclin1 protein and higher number of cells with typical structures of auto phagososmes and auto phagolysosomes (Motyl et al., 2007). When autophagy fails in its function of natural cellular defense against starvation, a secondary response of apoptotic cell death can be triggered (Zarzynska et al., 2007). Ferraro et al., (2007) report that when autophagy is suppressed, apoptosis is initiated. Lemasters et al. (79) suggested that autophagy may also block apoptosis by preventing the release of pro-apoptotic mitochondrial factors to the cytoplasm which disrupts the post-mitochondrial cascade. Zarzynska et al., (2008) suggest possible regulation of apoptosis and autophagy by closely related family of beclin proteins (Figure 1.6).

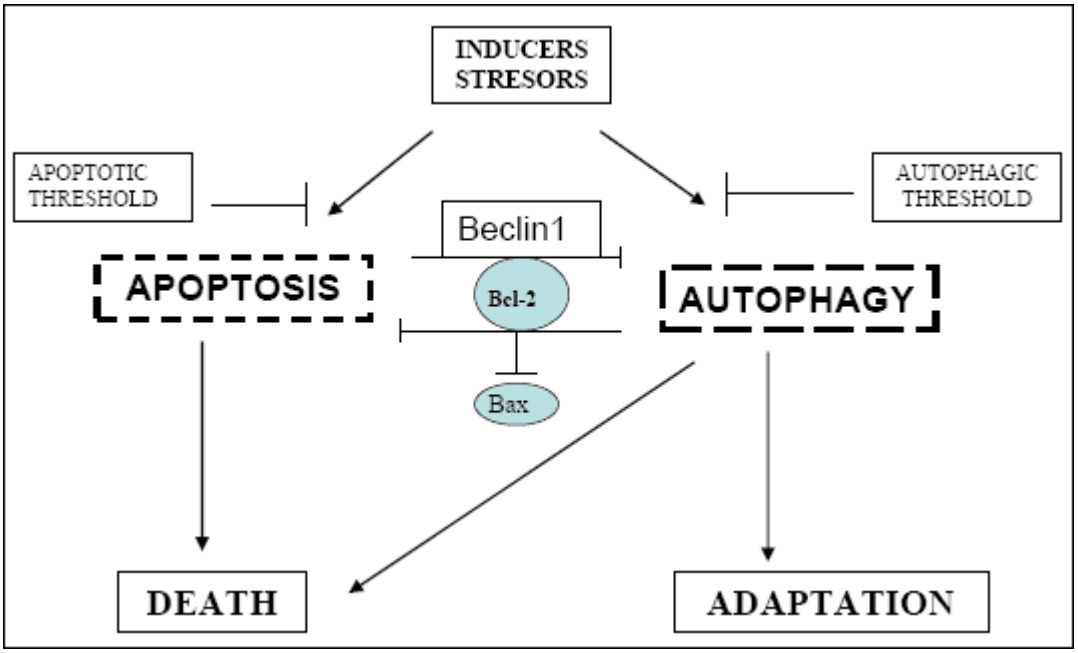

Figure 1.6 Inter relationships between apoptosis and autophagy. There are findings of a mixed phenotype of apoptosis and autophagy at the single-cell level. The mutual

inhibition of both processes could be regulated by Bcl-2, which inhibits autophagy by interacting with Beclin1 and could also inhibit apoptosis by blocking activation of Bax. Zarzynska et al., 2008; Journal of Physiol. and Pharm.2008, 59: 275-288.

Effects of milking frequency on serotonin levels in bovine milk

Serotonin is a hormone and neurotransmitter that is synthesized from the amino acid L-tryptophan and was initially identified as an active substance from brain extracts that produced peripheral vasoconstriction (Berger et al., 2009). Serotonin is a feedback inhibitor of lactation in the bovine specifically, decreasing α-lactalbumin and β-casein mRNA expression and milk yield in lactating dairy cows (Hernandez et al., 2008). Serotonin causes epithelial apoptosis in nursing mice (Matsuda et al., 2004). The mechanism by which serotonin down-regulates milk synthesis and secretion is by decreasing lactose production (Hernandez et al., 2008). Decrease in lactose production is mediated by the down regulation of α-lactalbumin, the key component of lactose synthetase, the rate limiting enzyme for lactose synthesis (Matsuda et al., 2004). The concentration of serotonin is analyzed by quantification of 5-HIAA (5-hydroxy indole acetic acid), which is the end product of serotonin metabolism (Timothy et al., 2003).

Effect of milking frequency on mitochondrial number

Mitochondrion, a membrane-enclosed organelle found in eukaryotic cells (Henze et al., 2003) generates the cell's supply of adenosine triphosphate (ATP) by oxidizing pyruvate and NADH, which are produced in the cytosol (Mc Bride et al., 2006). Mitochondria are also involved in signaling, cellular differentiation, cell death, as well as the control of the cell cycle and cell growth (Gardner et al., 2005). They are composed of compartments including the outer membrane, inner membrane, cristae and matrix (Zhang et al., 2008). The inner mitochondrial membrane is compartmentalized into numerous

cristae, which expand its surface area enhancing its ability to produce ATP (Voet et al., 2006). Mitochondrial matrix lodges the enzymes of citric acid cycle with the exception of succinate dehydrogenase, which is bound to inner mitochondrial membrane (King et al., 2006).

Mitochondrial biogenesis, mitophagy and mitoptosis are three important stages in mitochondrial cycle. Mitochondrial biogenesis is the process by which new mitochondria are formed in the cell which is activated by numerous different signals during times of cellular stress or in response to environmental stimuli (Vieira et al., 2009). Biogenesis of mitochondria usually occurs during the early lactation stage, when there is high energy demand in mammary cells (Rosano and Jones, 1976). Higher mitochondrial number is considered as a protective factor for the cell as it is a key regulator of the metabolic activity of the cell and plays an important role in the production and degradation of free radicals (Solange et al., 2000). Master regulator of mitochondrial biogenesis is peroxisome proliferator-activated receptor gamma-1α (PPARG-1α) which co-activates nuclear respiratory factor-1 (NRF-1) (Susin et al., 1998). The NRF-1 activates the mitochondrial transcription factor A, which transcribes nuclear-encoded mitochondrial proteins and those involved in mitochondrial DNA transcription, translation, and repair (Kroemer et al., 1998). In murine mammary cells, mitochondrial biogenesis involves two phases: replication during cell proliferation and maturation during cell differentiation. Wellings et al., (1960) suggests that mitochondrial numbers were less in virgin mammary gland, whereas the mitochondrial population in lactating gland was steadily increasing in number and size throughout the entire biogenesis stage. Jones and Rosano (1972), suggests that the period of greatest mitochondrial replication was during the epithelial

proliferation or mid to late pregnancy, since the number of organelles per cell increased while epithelial cells themselves were increasing maximally.

Estimation of mitochondrial numbers from different cell types from different species shows widely varying results. Allard et al., (1952) had shown that an average rat liver cell contains about 2500 mitochondria/ liver cell. Stemberger et al., (1984), observed that electron-microscopic sections (0.06 μm thick) of lactating mammary cells from rat and cow showed counts of 40 and 900 mitochondria/epithelial cell. Research done by Howe et al., (1956) in guinea -pig mammary gland sections (2μm thickness & 6000x magnification) found mitochondrial number/cell as high as 31 ± 12 and 12 ± 2 for lactating and non-lactating mammary cell respectively. Recent research by Hadsell et al., (2009) found mitochondrial numbers/cell as 20 ± 3 and 13 ± 2 on 8d and 35d respectively in nursing mice.

Mitochondrial biogenesis is followed by mitophagy which is the removal of damaged mitochondria from the cell before cell death (Lemasters, 2005). Mitophagy is a process of mitochondrial quality control which is independent of cellular ATP collapse (Priault et al, 2005). Mitoptosis, a form of cell death is mediated by reactive oxygen species, which cause the opening of the permeability transition pores in the inner mitochondrial membrane (Geminard et al., 2002). Mitoptosis causes cell death by releasing cell death proteins normally hidden within mitochondrial intermembrane spaces into the cytosol (Skulachev, 2006).

Effects of milking frequency on gene expressions in bovine milk

DNA and RNA of epithelial cells isolated from human milk has been known to

represent gene expression in the mammary gland (Linquist et al., 1994). Milk fat is secreted by an apocrine mechanism unique to mammary epithelial cells (Mc Manaman et al., 2004). During the secretion of milk fat globules, cytoplasmic lipid droplets which are enveloped in plasma membrane are also pinched off. Intracellular components of the mammary epithelial cells get entrapped within the fat globules as a cytoplasmic crescent containing most cell organelle except the nucleus (Huston and Patton, 1990). Heid et al., (1983) pointed out that bovine species have fewer cytoplasmic crescents in milk compared to other species as the space between apical plasma membrane and droplet is smaller in the case of bovine globule (10 nm) resulting in stronger attraction of membrane to droplet. Thus the milk fat globules represent an ideal source to sample the mammary epithelial cell cytoplasm. Changes in milk RNA should reflect overall changes in milk protein gene expression for different stages of lactation (Maningat et al., 2007). α-Lactalbumin (α-LA), regulatory subunit of the lactose synthetase enzyme complex, is abundantly expressed de novo only in the epithelial cells of the mammary gland and drives this enzyme complex to synthesize lactose, which is the major determinant of milk volume (Maningat et al., 2007).

SOCS (suppressor of cytokine signaling) proteins constitute a family of multi-domain proteins that have the capacity of inhibiting the JAK/STAT, prolactin and growth hormone signaling pathways (Aman and Leonard, 1997). SOCS proteins are induced in response to PRL, growth hormone, and leptin, as well as cytokines including interleukins (IL) and interferon (IFN-*γ)* (Larsen and Ropke, 2002). Expression of SOCS-3 mRNA in the mammary tissue of suckled rats is limited, but was shown to increase within 16 h of pup removal (Tam et al., 2001). In the bovine mammary gland, the expression of these

genes is induced by short-day photoperiod, a condition that induces prolactin secretion (Wall et al., 2005b). SOCS-1, -2, and -3 and cytokine-inducible SH2-containing protein are expressed in the bovine mammary gland and may play a role in mammary development and function during the transition period of dairy cows (Wall et al., 2005a). Mammary tissue expression of SOCS-1 is not affected by milking frequency; SOCS-2 mRNA is increased with frequent milking and SOCS-3 mRNA reduced by frequent milking (Wall et al., 2006).

CHAPTER 2

EFFECT OF MILKING FREQUENCY ON MILK YIELD, GENE EXPRESSION, MITOCHONDRIAL NUMBERS AND APOPTOSIS PERCENTAGES IN MAMMARY EPITHELIAL CELLS

Objectives of study I

Determine if mitochondrial function, SOCS gene expression, and rate of apoptosis are impacted by milking frequency in cows.

Hypothesis: Increased milking frequency in lactating cows will decrease mammary epithelial cell apoptosis, decrease SOCS gene expression, and increase mitochondrial number.

INTRODUCTION

In dairy animals, increased milking frequency enhances milk production and lactation persistence (Bauman et al., 1993). Milk production in dairy cows declines after peak lactation by a phenomenon referred as secretory diminution, which is associated with dramatic changes in mitochondrial oxidative damage within the mammary glands (Hadsell et al., 2005). Increased milking frequency increases milk production (Erdman and Varner, 1995), and in most cases (Bar-Peled et al., 1995; Hale et al., 2003) milk production remained elevated for a period even after the treatment was terminated. Changes in milking frequency in cattle lead to changes in mammary cell turnover and activity of key enzymes involved in milk synthesis (Hillerton et al., 1990; Hale et al., 2003). Recent research by Hadsell et al., (2006), found that mammary expression of SOCS proteins are elevated during prolonged lactation. The half-udder model is useful in demonstrating the effect of increased milking frequency on milk yield and mammary gene expression (Knight et al., 1990). Once daily milking in cows results in significant loss in milk production (Boutinaud et al., 2003). The effects of 4x milking frequency on milk yield and other lactation indices are poorly investigated. The endpoints were the effects of 1x vs. 4x milking frequency on milk yield and composition, mitochondrial numbers, mammary gene expression and epithelial cell apoptosis.

MATERIALS AND METHODS

Animals and Experimental design

Study protocol and procedures involving animals were approved and conducted in accordance with the University of Arizona Institutional Animal Care and Use Committee. Six pregnant primiparous dairy cows in good health with no previous history of mastitis were assigned to the study 40 days prior to expected calving. Animals received close-up ration until they calved and lactating ration from calving according to the management program of the University of Arizona Dairy. The close-up and lactating rations were formulated with 65% and 70% dry matter respectively. The animals were group housed until they calved. Following calving, the cows were housed in individual pens with access to shade and water and the udder halves of each animal were randomly assigned to 1x or 4x milking by coin flipping. The 1x udder halves were milked at 0400 h and 4x udder halves were milked at 0400 h, 1000h, 1600h and 2200h. Milk volume was recorded at each milking and samples were obtained from each udder half once weekly for composition analysis. Each udder half was biopsied on days 15, 60, 120 and 230 of lactation to collect tissue for evaluating mitochondrial and apoptosis percentages.

Milk composition

Weekly milk samples were taken and analyzed for fat percent, protein percent, lactose percent, solids not fat percent (SNF) and somatic cell count (SCC) by Arizona Dairy Herd Improvement Association, (AZ DHI) Tempe, AZ. Milk fat, protein, lactose and SNF were analyzed using AOAC approved infrared analysis and somatic cell count (SCC) by AOAC approved cell-staining techniques. All equipment used in the analyses

was certified by the International Dairy Federation and Food and Drug Administration (FDA).

Mammary tissue sampling

Since udder-half was the experimental unit, both udder halves were biopsied at each time point, according to procedures by Farr et al. (1996) with modifications by Baumgard et al., (2000) and Annen et al., (2007). Additional precautions were made to prevent infection and health events related to biopsies. After a skin incision, blunt dissection to the mammary capsule was performed to ensure tissue taken during the biopsy is mammary parenchyma. The biopsy instrument (Farr et al., 1996) was cored into the mammary capsule, and then retracted. Just prior to removing the instrument from the capsule a hemostat was clamped to the capsule just above the biopsy site. After removal of the biopsy instrument, the biopsy opening in the mammary capsule was clamped with hemostats and closed with absorbable suture Vicryl (Ethicon, Inc., Sommerville, NJ). The hemostat was then clamped to the capsule before removing the biopsy instrument by pulling gently toward the surgeon to improve visibility of the biopsy opening and hasten clamping of the opening to minimize blood loss. After the mammary capsule was closed, the surgery site was closed with a subcutaneous layer of dissolvable suture and a cutaneous layer of Braunamid suture (Jorgensen Laboratories, Loveland, CO). Incision sites were sprayed with topical antiseptic (Veterinus Derma Gel, Maximilian Zenho and Co., Brussels, Germany) and covered with a protective sealant spray (AluSpray®, Neogen Corporation, Lexington, KY). Biopsied quarters were treated with intramammary antibiotic (Amoxi-Mast®, Pfizer, La Jolla, CA) at the time of surgery and at the first two milkings after the biopsy. Systemic antibiotic, penicillin (6×10^6 IU; Agri-Cillin®,

AgriLabs, St. Joseph, MO), was initiated the day before a biopsy and continued until 3 d following the biopsy. Post surgery health was monitored by taking rectal temperature twice daily for 4 d following a biopsy and by continuous evaluation for any fluctuations in milk yield and dry matter intake. Previous research by Annen et al., (2007) demonstrated no adverse impact of multiple biopsies per quarter using this technique. Approximately 250mg tissue was isolated from each udder half, flash frozen and stored in liquid nitrogen for future use.

Mitochondrial staining-succinate dehydrogenase method

Mammary biopsy tissue was collected on the 15, 60, 120 and 230 days of lactation from both 1x and 4x udder halves. Mammary sections of 10µ thickness were made on each slide. The sections were stained with succinate dehydrogenase (SDH) with no counter stain. Tetrazolium solution (NBT) at 4mg/ml was made with nitro blue tetrazolium chloride, (Alfa Aesar, Alhambra, CA) distilled water, 0.2M tris buffer (ph-7.4), 0.05M MgCl2 and 0.83M succinate as the substrate. Mammary sections were incubated in incubating medium (NBT soln) for 1 hr at 37^{O}C. Slides were transferred to 15% formol saline and incubated for 15 mins. Sections were washed in distilled water for 3 mins and dehydrated in 93%, 95% and 100% alcohol respectively for 3mins. They were rinsed in xylene and mounted in 2 drops of Protocol secure mounting medium (Fisher scientific, Kalamazoo, MI) and cover slip. The positive stained slides were viewed under oil immersion objective of phase contrast microscope (Leica Microsystems, Wetzlar, Germany) at 100x magnification. Six fields were selected from each slide and the mitochondria which stained purple, were counted manually. The total epithelial cells from the corresponding fields were also counted manually. Mitochondria per cell section

were calculated as the total mitochondria from each field / total epithelial cells. The mitochondrial number from each cow for days 15, 60,120 and 230 were calculated and used for statistical analysis.

Cleaved caspase-3 staining procedure for apoptotic cells

Two slides per sample were fixed in 4% paraformaldehyde (Sigma-Aldrich St.Louis, MO) in PBS (ph 7.4) in the cold room for 15 mins. The slides were washed 3 times in PBS for 5 mins each and incubated in blocking buffer (5% Normal Goat Serum in PBS), (Sigma-Aldrich, St.Louis, MO) for 1.5 hrs at room temperature inside a humid chamber. The tissue sections on slides were circled using a pap pen before adding 50µl of the primary or secondary antibody and incubated for 5 mins at room temperature. A negative slide of each sample was incubated with only incubation buffer (1% BSA (Fisher Scientific, Carlsbad, CA) and 0.3% Triton X-100 (Fisher Scientific, Carlsbad, CA) in PBS). The second slide of each sample was incubated with 1:200 dilution of primary antibody (Cleaved Caspase-3; Cell signaling, Bethesda, MD) in incubation buffer. Both sets of slides were incubated overnight in the cold room in a humid chamber, after which the slides were washed three times in PBS for 5mins. All the fluorescent antibody procedures following were done in a dark room. The slides were incubated in secondary antibody (Alexa Flour 488 Goat anti rabbit; Invitrogen, Carlsbad, CA) for 2 hrs at room temperature, in a humid chamber. The slides were washed in PBS two times for ten mins and incubated in 0.1µm Sytox Orange in PBS (Molecular Probes, Carlsbad, CA). The slides were again washed two times for five mins in PBS and finally in water. The slides were mounted with glycerol mounting solution (Sigma-Aldrich, St.Louis, MO) and cover slip. Specimens were imaged at 40x magnification using a fluorescent

microscope *(*Leica Microsystems, Wetzlar, Germany) and digital images were captured using Image Pro5.1 software (Media cybernetics, Bethesda, MD) for quantitative analysis.

Quantitation of immunohistochemistry

Tissue sections were viewed under a fluorescent microscope (Leica Microsystems, Wetzlar, Germany) with a camera connected to the computer to quantify cells expressing the caspase-3 antigen positive (apoptotic) cells. For each slide, ten pictures were photographed at 40x magnification from the randomly selected fields. From each picture, the apoptotic cells were counted manually at 40x magnification and epithelial cells by the counting tool of Image-pro 5.1 software (Media cybernetics, Bethesda, MD). Caspase-3 stained apoptotic cells were stained green and normal epithelial cells red. Apoptosis percentage was calculated as the total number of caspase positive cells divided by total number of epithelial cells counted multiplied by 100.

Evaluation of serotonin levels in milk

Serotonin levels were analyzed in milk from 1x and 4x udder halves by determining the level of 5-hydroxy-3 Indole Acetic Acid, the end product of serotonin metabolism. Milk collected from each udder half, before biopsy was spun in 50ml conical tubes for 10 mins at 2700g (3300rpm) at 4^{O}C in Beckman J26 centrifuge (Beckman Coulter Inc, Fullerton, CA) using JS5.3 rotor .The fat layers were removed using a clean spatula and 10ml of milk was pipetted into two RNase free tubes and the defattened milk samples from day 15, 60 and 120, were stored at -80^{O}c until they were used for the assay. 5-HIAA ELISA kit (Genway Biotech Inc, San Diego, CA), commonly used for the

isolation of serotonin metabolite from human urine was used for our study following manufacturer's protocol.

Milk RNA isolation

Milk for RNA isolation was collected before mammary biopsy on days 15, 60,120 and 230 of lactation. About 40 ml of milk from each udder half was collected into sterile 50ml RNase- free centrifuge tubes. Four ml of milk was decanted into a tube containing 8ml TRIzol LS (Invitrogen, Carlsbad, CA) containing RNase, shaken for about 15 sec, stored on ice immediately, transferred to the lab and stored at -80^{O}c. The isolation procedure was performed using manufacturer's recommendations for fat containing samples and the sample was stored finally in 20ul of RNA solution at -80^{O}c.

Complementary deoxyribonucleic acid (cDNA) synthesis

Prior to cDNA synthesis, RNA was treated with Deoxyribonuclease I (amplification grade, Invitrogen, Carlsbad, CA) according to manufacturer's recommendations. iScript III First Strand Synthesis System (Bio-Rad, Irvine, CA) for reverse transcription polymerase chain reaction (RT-PCR) was used for cDNA synthesis according to the manufacturer's protocol.

Quantitative RT- PCR

Gene sequences used for primer design were obtained from GenBank (NIH, Bethesda, MD). The abundance of mRNAs for SOCS-1, SOCS-2, SOCS-3, CIS, α-lactalbumin and β-casein were analyzed using primer sequences for the bovine genes. Optimal PCR conditions for each primer set were determined by running RT-PCR reactions across a temperature gradient. Products were then separated by gel (2% agarose) electrophoresis, stained with ethidium bromide, and visualized with ultraviolet

light. Reactions, which correspond to the annealing temperature resulting in the most abundant single product of desired base pair length, were selected for q-PCR. Real time RT-PCR was run on a Bio-Rad Prism (Irvine, CA) in a 96-well format. Reaction mixtures contained 10 µl of ABsolute BlueSYBR Green Fluorescein (Thermo Scientific, Surrey, UK). Master mix (Syber Green, primer, water) was pipetted into wells followed by addition of sample or standard curve cDNA. Plates were then assayed on the Bio-Rad iQ5 (Irvine, CA). Quantitative RT-PCR was run on all samples for six genes of interest (GOI) and a housekeeping gene, (RPS15A) (Table 2.9). The genes evaluated for selecting the housekeeping genes were 18S, GapDH (Robinson et al., 2006), HPRT (Goossens et al., 2005), ACTB, UXT, RPS15, RPS9 (Bionaz et al., 2007), and PPP1R11, RPS15A (Piantoni, 2007). RPS15A was selected from the nine genes evaluated because it showed consistency with all genes of interest. All samples were run in triplicate.

Statistical Analysis

All statistical analysis were performed using Proc Mixed procedures in SAS (SAS Institute, Cary, NC). For milk yield and composition analyses, data collected from both udder halves from month 1 to 8 of lactation was used. Data from the caspase-3 assay was transformed into Arc-cosine value to meet statistical assumptions for normal data distribution. Statistical models for analysis of milk yield, mitochondrial and apoptotic percentages included main effects of milking frequency, biopsy dates and their respective interactions. Statistical analysis of ΔCt values from qRT-PCR assays included independent variables of biopsy dates, udder halves and their interaction.

RESULTS

Dry matter Intake (DMI)

Mean dry matter intake for early (1-45d), mid (46-150d) and late (151-230d) lactation stages for six cows were 22.2, 24.1 and 25.1 kgs respectively, indicating an increase in feed intake from early to late lactation (Figure 2.3).

Milk yield

Increasing the milking frequency elevated the 4x udder half milk yield ($P<0.0001$) compared to the 1x udder half (Table 2.2; Figure 2.2). The mean 4x udder half milk production was also different from 1x udder half at early ($P<0.0001$), mid ($P<0.0001$), and late ($P<0.0001$) lactation stages (Figure 2.1, Table 2.2). The lactation curve of the 1x udder halves formed a flat graph, instead of sigmoid, with the peak yield extended for a prolonged period (Figure 2.2).

Milk composition

Milk composition variables from 4x udder halves, protein percent ($P= 0.013$), lactose percent ($P=0.004$) and SNF percent ($P=0.006$) were different from 1x udder half variables (Table 2.3; Table 2.4). However, mean fat% was not affected ($P=0.25$) by increased milking frequency (Figure 2.4). Increased milking frequency also had an overall effect on yield of milk components like fat ($P=0.001$), protein ($P=0.005$), lactose ($P=0.03$) and SNF ($P=0.001$) (Table 2.3). Mean somatic cell count (SCC) for 1x and 4x udder halves was 580,000 cell/ml and 799,000 cell/ml for the whole lactation. The 4x udder halves showed higher SCC for the whole lactation except for the 6^{th} and 8th month of lactation (Figure 2.8).

Mitochondrial number

Increased milking frequency had an overall effect on mitochondrial numbers in 4x udder half (P=0.002) compared to 1x udder half (Figure 2.9, Table 2.5) for the whole study. Mean mitochondrial numbers were numerically higher in 4x udder halves for the complete lactation (Table 2.5).

Serotonin metabolite

We did not detect an effect of milking frequency on serotonin metabolite, 5-HIAA in the 1x and 4x udder halves as the amount of metabolite was below detectable concentrations (data not shown). The assay kit used was for isolating serotonin metabolite from human urine and it failed to detect 5-HIAA in bovine milk.

Apoptosis percentage

Increased milking frequency had no overall effect (P=0.70) on mammary apoptosis percentage for the entire lactation (Table 2.6; Figure 2.12). There was also no significant difference between the 1x and 4x udder half apoptosis percentages at d15 (P=0.95), d60 (P=0.57), d120 (P=0.72) and d230 (P=0.93). But we found a difference between the d15 and d230 (P = 0.04), d120 and d230 (P = 0.006) apoptosis percentages within the 1x udder half. Also, the apoptosis percentage within the 4x udder halves showed difference between d15 and d230 (P = 0.038).

Gene expression

Fold change in SOCS-1 gene expression from milk was higher in 4x udder halves for throughout the lactation (Figure 2.10). There was no difference between the 1x and 4x udder half gene transcripts of SOCS1 (P=0.31), SOCS2 (P=0.14), SOCS3 (P=0.08) and CIS (P=0.26). α- lactalbumin and β-casein gene expressions (ΔCt values) had significant difference between the 1x and 4x udder halves (P=0.04) and (P=0.001) respectively for

the entire lactation (Table 2.7). Fold changes of α-lactalbumin and β-casein gene expressions were also higher for 4x udder halves at d15, d60, d120 and d230 of lactation (Figure 2.11).

DISCUSSION

We used the half-udder model in our study because of some apparent advantages. Comparison of milk production from 1x and 4x udder halves in the same cow reduces the between-animal variation and the number of animals needed to detect statistical relevance because the genetics, animal management, nutrients ingested and the concentrations of hormones and growth factors in the peripheral circulation are identical (Gulay et al., 2005). However, we cannot assume that perfusion and exposure of tissues of both half udders in the same cow to blood-borne factors will be identical when one udder half is milked only once and the other half, four times daily. We also observed that the 1x udder halves were under-developed and small in size compared to the 4x halves and assume that this difference in gross morphology is likely due to the differences in mammary tissue response to local or systemic factors.

Cows milked twice daily consume slightly more feed but produce less milk than cows milked 3x (Pearson et al., 1979). Also, Holmes et al., (1992) reported an 8% decrease in feed intake in peak lactation in 2x milked cows compared to 1x milked cows. But recent research by Van Baale et al., (2005) suggest that milking cows 6x does not improve milk yield over 3x because of time away from feed in the 6x milked group. In our study, this was not a major constraint as the cows were away from the feed for only 15 minutes for all milkings, except the 400 am milking which was carried out in 40 minutes. The average dry matter intake for early, late and mid lactation was 22.2, 24.1 and 25.1 kgs respectively, suggesting that there was only slight difference in feed intake for the different stages of lactation. Our experimental model could not detect a difference in feed intake and feeding pattern between control and treatment groups, as the same cow

was used in a half udder design. In our study, environment seems to be the only factor controlling the feed intake as the mean dry matter intake gradually increased from early towards late lactation (cows were exposed to summer through fall months in Arizona) (Figure 2.3).

When milking frequency was reduced, the udders became firmer and cows had milk leakage and took shorter strides when walking towards the milking parlor. The udder distension associated with reduced milking frequency also resulted in discomfort to cows leading to less time for lying down (Tucker et al., 2007). Previous work also found that reducing the milking frequency to 1x caused increased pressure in the mammary gland leading to increased permeability of the tight junctions and lactose leakage into the circulation (Stelwagen et al., 1997). In our study, 4x milking was very successful in relieving the discomfort for cows caused by udder distension as the milk was removed continuously every 6 hours. Because of the half udder model, we could not observe a clear difference in the behavioral patterns of cows like the lying time in pens, and walking styles towards parlor caused by reduced milking frequencies.

Milk yield in dairy cows can be increased by a variety of means like improving the genetic background, environment, hormone treatment, milking and feeding methods (Bar Peled et al., 1995). But one of the most fundamental methods to increase milk yield is by increasing milking frequency (Collier and Romagnolo, 2002). The average milk yields in our study from the 1x udder halves for early, mid and late lactation were 8.2, 6.4 and 4.2 kgs respectively. The major disadvantage of once-daily milking is reduced milk production with losses ranging from 7 to 38% for periods of once-daily milking compared to twice-daily milking (Davis et al., 1998). We also found more than 50% loss

in milk yield from 1x, compared to the 4x udder halves. When milking interval is prolonged, milk yield is reduced by sequential developmental adaptations, initially as a down-regulation of cellular differentiation (Wilde et al., 1987) and later as a net loss in mammary cell number via apoptosis (Li et al., 1999). In the present study, the mean milk production for early, mid and late lactation for 4x udder halves were 20, 18.8 and 13.6 kgs respectively. The mean milk production from the 4x udder halves was almost three times higher (2.6 fold) than 1x udder halves during the three stages of lactation, suggesting an overall enhanced performance of the 4x udder halves. The increase in the concentration of the Feedback Inhibitor of Lactation (FIL) synthesized by the mammary gland (Wilde et al., 1995) and the intramammary pressure (Peaker, 1980) are supposed to be the reasons for the drop in milk synthesis in 1x udder halves. Also, the loss of tight junction (TJ) integrity after about 20 h of milk accumulation may play a role in the milk yield losses in 1x udder halves (Stelwagen et al., 1994b).

Two recent studies have reported no effect of frequent milking during early lactation on milk production (Fernandes et al., 2004; VanBaale et al., 2005). These findings conflict with the results of our study, as well as with the results of previous studies (Bar-Peled et al., 1995; Hale et al., 2003; Dahl et al., 2004). We found both an acute and long-term increase in milk production from the 4x udder halves in response to increased milking frequency. Total lactation production for all six cows was greater ($P<0.0001$) for 4x udder halves (22,966 kg) than 1x udder halves(7,948 kg) which accounted for a 2.89 fold increase. Milk production declined steadily after d86 of lactation for 4x udder halves and d69 for 1x udder halves (Figure 2. 2). At peak milk production, 4x udder halves were producing 2.1 fold more milk ($P<0.0001$) than their 1x

counter parts. The total milk production from all six cows for early, mid and late lactation stages averaged 2038 kg, 3972 kg and 1983 kg for 1x udder halves and 5260 kg, 11351 kgs and 6353 kg for 4x udder halves respectively.

Several possible explanations can be given for the increase in milk production with 4x milking. One possibility is that there is an increase in blood flow to the 4x side of the mammary gland, which would allow for increased exposure to the blood supply and to lactogenic hormones in circulation. Wall et al., (2007) suggests an argument against this theory, which states that although milk yield and blood flow are sometimes positively correlated, an increase in blood flow to the mammary gland does not always enhance milk production (Prosser et al., 1996). Another possibility is that the capacity of the 4x side of the udder was increased with frequent milking, which could support a higher level of milk production. A more realistic reason for the increased production of the 4x udder halves would be related to decreasing udder pressure more frequently. Increase in surges of prolactin, decrease in cell stretch and up regulation of parathyroid hormone-related peptide (PTHrP) can also be suggested as possible explanation for increase in milk yield from 4x udder halves (Stelwagen et al., 2001). Parity of cows seems to be another factor elevating the milk yield in the cows in our study. Even though primiparous cows have less udder size and capacity than older cows, emptying the treatment udders four times daily might permit rate of milk secretion to reach a maximum and increase production (Amos et al., 1985). We conclude that at calving, total number of cells were same in the 1x and 4x udder halves, but mammary cells in 4x glands had undergone hypertrophy whereas cells in 1x glands atrophied during the course of lactation. In other words, we speculate that increased milk production in 4x udder halves was due to greater numbers

of cells that had differentiated and gained functionality rather than accretion of additional mammary epithelial cells.

The lactation curve for both udder halves were not sigmoid but formed a flatter graph, with the lactation persistence extended for a longer period during the 230 days of lactation. Primiparous cows are reported to have a proportionately greater response to increased milking frequency than multiparous cows (Amos et al., 1985), which was true in our study also. Lactation persistency is also greater in primiparous cows and is negatively affected by concurrent pregnancy (Bar Peled et al., 1995). The mechanism by which pregnancy influences persistence is believed to be caused by the estradiol and progesterone hormones that maintain pregnancy (Stefanon et al., 2002). Cows in the present study were also primiparous and this might have contributed to the elevated persistency noticed in both udder halves. When cows are concomitantly lactating and pregnant, it is possible that a decline in secretory capacity per mammary cell accompanies advanced pregnancy, due to the conflicting metabolic demands of gestation and lactation. This factor is readily apparent during late pregnancy, when the number of mammary epithelial cells actually increases simultaneous with a rapid decline in milk production (Capuco et al., 1997). Cows that conceive in the 1^{st}, 2^{nd}, or 3^{rd} month of lactation suffered more milk losses during the 300d lactation than non-pregnant cows or cows becoming pregnant in mid or late lactation (Brotherstone et al., 2004). Similarly, Tekerli et al., (2000) demonstrated that cows conceived shortly after calving had lower lactation persistency and that the decline in the slope of the lactation curve after peak yield decreased with increased days open. In our study, the cows were not inseminated during the whole lactation, which might have contributed to the elevated persistency and

the flatter lactation curve. Thus, the increase in milk production from parturition to peak lactation can be attributed to increased synthetic capacity per cell (hypertrophy) rather than increased cell proliferation (hyperplasia).

Mammary gland is the only tissue in cows that produces lactose (Stelwagen et al., 1994), which is the major osmotic determinant in milk that accounts for half of milk osmotic pressure and milk volume. Glucose is the main precursor for lactose synthesis and quantity of glucose taken up by mammary gland from the glucose arterial pool is a determinant of milk synthesis (Boutinaud et al., 2008). Dairy cows milked 1x daily produce more concentrated milk with less lactose (Carruthers et al., 1993). In the present study also, the mean lactose percentage from the 1x udder half was lower (4.0%) compared to 4x udder halves (4.3%). Milk of cows milked 1x had a lower percentage of protein than milk of 2x milked cows (Knight & Dewhurst, 1994). In our experiment also, protein percentage was lower on the 1x udder halves (2.72%) compared to 4x udder halves (2.83%). Total protein in milk is the result of the proteins synthesized in the mammary gland and the serum proteins entering the milk when mammary tight junctions are disrupted. The elevated lactose and protein percentages in the 4x udder halves may be attributed to the fact that the increased blood flow towards the 4x udder halves might have enhanced the influx of their precursors into the mammary cells. This might have resulted in enhanced production of lactose and protein in the golgi apparatus and endoplasmic reticulum respectively, which ultimately entered the milk. The mean fat percentage was higher in the 1x udder halves (3.58%). We assume that changes observed in milk fat concentration between udder halves may be attributed to the differing regulatory mechanisms for milk fat globule secretion and transfer between the alveolar

and cisternal compartments in the mammary gland (Davis et al., 1999). The mean SNF percentage was also higher on 4x udder halves (8.28%) compared to 1x udder halves (7.58%). The 4x udder halves produced 82.8 kg of fat, 73.6 kg of protein and 112.7kg of lactose than 1x udder halves for the whole lactation. Tight junctions regulate milk component concentration only in the 1x udder halves as recent research by Delamaire and Guinard, (2007) suggests that the opening of tight junctions would not affect milk yield in cows milked more than twice daily. So we conclude that increased mammary blood flow and nutrient mobilization are the regulating factors in the 4x udder halves, whereas tight junctions control the milk component concentration in 1x udder halves.

The use of Somatic Cell Count (SCC) to monitor udder health has proven to be a valuable tool for the past few decades for the identification of cows with major contagious pathogenic infections and is being proposed as useful for selection decisions for breeding programs of dairy cattle (Shook, 1985). The capacity of mammary cells to produce milk is damaged during an infection in a mammary gland, resulting in increase in the somatic cell count in milk (Harmon, 1994). The current legal limit for somatic cell count in US market is 750,000 cells/ ml of milk (Eberhart et al., 1982). In our study, the mean SCC for 1x udder halves was 580,000 cell/ml and 4x udder halves was 799,000 cells/ml. Our results were similar to previous research done by Hale et al., (2003) that milking frequency did not produce any difference in somatic cell count. The effect of environment on milk quality was crucial in our study as the cows were exposed to the summer months in Arizona, which might have elevated the SCC for both udder halves during the months 3-7 (May- September) of the study (Figure 2.9). The reasons for the rise in SCC as lactation progresses may be a concentration effect (as less milk is

produced) and the presence of chemostatic cytokines that draw polymorphnuclear leucocytes into milk in late lactation (Manlongat et al., 1998). Harmon, (1994) suggests that several factors like infection status, lactation number, stage of lactation, estrus, exercise, heat stress, stray voltage and day-to-day variation may affect SCC in milk. An uninfected udder typically has SCC less than 100,000 cells/ml and when the SCC is 100,000 to 199,999 cells/ml, the presence of infection is ruled out by bacteriological testing (Smith et al., 2001).

We could not detect any pathogens from the milk samples sent to microbiology lab for bacterial culture. This opens other mechanisms for explaining the elevated SCC. In many reports (Lynch et al., 1991; Stelwagen et al., 1997), once-daily milking increased SCC in milk, and as milk output decreased, the cells were more concentrated (Kamote et al., 1994). However, in a recent study it was reported that an increase in polymorph nuclear cells appeared in milk (Kelly et al., 2000) by infiltration from blood due to the opening of tight junctions in once-daily milked glands. It seems that in our study, an increase in tight junction opening better explains the elevated levels of SCC from both udder halves (Colditz, 1988). Somatic cell counts are often used in dairy industry to estimate milk production losses, as they reflect infection in the gland. Eberhart et al, (1982) suggest that SCC of 500,000 cells/ml and 1,000,000 cells/ml cause production losses of 6% and 18% respectively. According to this scale, we estimate a total loss of 7% (560 kg) and 12% (2760 kg) in milk production for 1x and 4x udder halves, as their mean SCC were 580,000 cell/ml and 799,000 cells/ml respectively.

Regulation of milk secretion is controlled by systemic and local feedback mechanisms that include local hormonal factors like prolactin (Peaker et al., 1998).

Previous research by Linzell and Peaker, (1971) suggests the possibility of a milk yield being regulated by a chemical in the milk called as Feedback Inhibitor of Lactation (FIL). But recent research by Collier et al., (2008) suggests that this FIL can be serotonin, which acts as an autocrine/paracrine regulator of lactation in bovine species (Hernandez et al., 2008). We analyzed the effect of milking frequency on serotonin levels in both udder halves, expecting lower levels of serotonin metabolite (5-HIAA) in the 4x udder halves. The concentration of serotonin metabolite was too low to be detected. We conclude that the assay kit, which was originally for human urine could not detect the serotonin levels in bovine milk.

Little is known about the regulation of mammary cell mitochondria and their contribution to the process of milk biosynthesis during lactation. Because of the large increase in mitochondrial numbers and activity during the pre partum and early lactation periods in mice, the mammary gland appears to provide an excellent opportunity to delineate the mechanism of mitochondrial biogenesis in a mammalian system. In a lactating mammary gland, previous research has pointed the role of mammary mitochondria in the supply of citrate for milk lipid biosynthesis (Watson and Lowenstein, 1970). Previous research has pointed out that mitochondrial numbers in mice increases dramatically in early lactation when mitochondrial biogenesis takes place simultaneously along with epithelial cell development. We also found an elevated mitochondrial numbers per epithelial cell from both udder halves in early lactation. Mean mitochondrial numbers from the 4x udder halves were higher through out the study. Also, the milk yield from the 4x udder halves were positively correlated with the mitochondrial numbers throughout the lactation. This suggests an increased mitochondrial proliferation and an elevated

metabolic activity in the 4x udder halves. Mitochondrial numbers from both udder halves were lower at d230 indicating that mammary metabolic activity was reduced dramatically in late lactation. Although the early phase of lactation is associated with an increased mitochondrial number, loss of mitochondrial function during prolonged lactation cannot be attributed to a loss of mammary epithelial cell mitochondrial numbers, but to mitoptosis and oxidative damage. We assume that in response to the lactogenic hormones initiating and maintaining secretion, mammary cells increase in volume and also the mitochondria elongate and increase in diameter. Mitochondrial numbers does not decrease with the declining phase of lactation, but there is a definite loss of mitochondrial ATP synthesis activity with prolonged lactation. Mammary sections from bovine gland has more fat in the cytoplasm of its secretory cells compared to rat, mice and guinea-pig sections which will reduce the number of epithelial cells and there by mitochondrial counts in a given field. This species specific histological factor can be sited as a possible explanation for the reduced mitochondrial number/cell in our experiment compared to previous studies done in laboratory animals. Another important aspect to be discussed in this context is the elongation of the mitochondria which is occurring in the cell as it increases its volume, taken in conjunction with the thickness of the section. Counting the number of filamentous mitochondria from mammary sections is liable to be inaccurate because some of them will be cut more than once in thinner sections of 0.05μm and 2μm. We speculate that, in previous studies of stained lactating cells, the mitochondrial counts might have been exaggerated. This suggests a probable argument for our lower mitochondrial numbers. We assume that the mitochondrial numbers from stained sections at 10μm conflict with those from previous researches probably because there is

experimental cell overlapping of mitochondrial filaments within the thickness of the section.

To further investigate the effects of increased milking frequency on epithelial cell turnover and function during the lactation cycle, expression of genes involved in milk synthesis, mammary growth, cell cycle regulation, and apoptosis were evaluated. SOCS-1, -2, -3 and CIS proteins play an important role in the bovine mammary gland development and function (Wall et al., 2005). SOCS gene expression in the mammary tissue has only recently been investigated and is still not fully understood. Mammary gene expression of SOCS-1, SOCS-2 and CIS mRNA was not affected by 4x milking; whereas expression of SOCS-3 mRNA tended ($P = 0.08$) to increase with frequent milking. Our results on the effect of frequent milking on SOCS-1, -2 and -3 mRNA expression in milk of dairy cows are consistent with the observation by Wall et al., (2005) that milk removal had no effect on expression of SOCS-1,but was associated with decreased expression of SOCS-3 in the mammary gland. Changes in SOCS-3 mRNA in response to 4x milking frequency indicate that expression of these genes is regulated locally by milk accumulation. Previous research by Tam et al., (2001), suggests that SOCS-3 may be involved in the inhibition of milk secretion between milkings in the bovine mammary gland which contributed to the enhanced milk yield in the continuously milked glands compared to 1x milked glands. We also found a reduced expression of SOCS-3 in 4x udder half milk compared to 1x. SOCS-3 expression is enhanced by milk accumulation (Tam et al., 2001) and in our study, since the 4x udder halves were evacuated more frequently, the action of SOCS-3 was limited to the 1x udder halves only. Although the regulation of SOCS mRNA expression in the bovine milk is evident from

our results, we could not find uniformity in action across the different SOCS genes. Previous research has indicated that SOCS-1 and -3 share common functions and regulation, where as SOCS-2 and cytokine-inducible SH2-containing protein (CIS) are similar in their functions and regulation (Larsen & Ropke, 2002). Differences in the response of SOCS gene expressions in milk to 1x and 4x milking frequency clearly indicate that there are no common factors governing the expression of these genes, but each of them have to be analyzed and studied separately.

Recent studies show that milk is a non invasive source of viable mammary epithelial cells (Boutinaud & Jammes, 2002) which can be used to analyze mammary mRNA levels of milk protein (Boutinaud et al., 2002). The effect of milking frequency on cellular mechanisms governing regulation of a-lactalbumin gene expression and mammary regulation of glucose uptake has little been investigated. α- Lactalbumin and β-casein genes were analyzed because they produce the key enzymes involved in lactose and protein synthesis respectively. Decreasing the milking frequency (1x daily milking) reduces gene expression of α- Lactalbumin, which in turn decreases total milk yield and amount of glucose taken up by mammary gland. We found higher gene transcripts in milk for α-lactalbumin and β-casein in the 4x udder halves. Previous research also suggests higher mammary expression of α-lactalbumin mRNA in 4x milked cows compared to 2x cows (Wall et al., 2006). Mammary glucose uptake and lactose production are regulated at three levels, which may be responsible for variation in lactose yield and milk yields from udder halves (Boutinaud et al., 2008). The first is transport of glucose into mammary epithelial cells by a passive mechanism by type-1 glucose transporter (GLUT-1); (Zhao et al., 1996) and active mechanisms including type-1

sodium glucose co-transporter (SGLT-1); (Zhao et al., 2005) and galactosyl transferase [GAT-(1,4)] which catalyses formation of lactose from glucose. Baldwin et al., (1994) suggests that prolactin maintains GLUT-1 protein levels in rat mammary gland. We assume that the 1x milking frequency causes a decline in prolactin release into plasma, there by decreasing GLUT-1 protein levels. We conclude that the increase in mammary glucose uptake, lactose production and milk yield from 4x udder halves were associated at cellular level with a clear up-regulation of α-Lactalbumin expression.

Apoptosis occurs in the bovine mammary glands during involution (Wilde et al., 1997). It may be reasonable to speculate that during the extended milking interval in the 1x udder halves, the process of apoptosis is initiated every time and the galactopoietic process is rescued each time the gland is milked after 24 h (Stelwagen, 2001). Changes occurring in 1x udder halves during milk accumulation fits well with other changes such as tight junction leakiness, reduced mammary blood flow and milk secretion starting after approximately 18 to 20 h of milk stasis (Stelwagen et al. 1994b, 1997). As expected, in the present study both udder halves demonstrated an increase in mammary epithelial cell (MEC) apoptosis during early lactation (0.14%). Elevated levels of apoptosis are expected during the mammary renewal process in the early dry period (Capuco and Akers, 1999), but have only recently been reported during early lactation by TUNEL assay (Capuco et al., 2001; Hale et al., 2003; Sorensen and Sejrsen, 2003). Annen et al., (2007) proposes four hypotheses to explain increased apoptosis during early lactation which include: 1) an increase in migration of apoptotic leukocytes (morphologically similar to mammary epithelial cell, MEC) into mammary epithelium (Capuco et al., 2001; Hale et al., 2003); 2) expulsion of old cells from mammary epithelium during late

gestation; 3) removal of cells that were not fully differentiated and generated during late gestation, 4) apoptosis of cells with mistakes incurred during DNA replication (Alberts et al., 2002). We noticed a difference in apoptosis percentage in the 1x udder halves between d15 and d120 (P = 0.04) and d15 and d230 (P = 0.02). Four times milking in dairy cows in early lactation reduces mammary apoptosis (Norgaard et al., 2005). But in our study, we found little difference between the apoptosis percentages in 4x (0.1%), compared to 1x udder halves (0.09%) with no overall change (P=0.70) between treatments for whole lactation. We found difference (P = 0.02) in apoptosis percentages the between d15 and d230 samples with in 4x halves. A similar study by Capuco et al., (2001), by TUNEL assay, also found almost similar apoptosis percentages at d14 (0.27%), d90 (0.05%), d120 (0.1%) and d240 (0.03%). Recent study by Duan et al., (2003), compared TUNEL, caspase-3 and cytokeratin -18 assays and suggested that caspase-3 immunohistochemistry is best suited for detection and quantification of apoptosis in mammary sections.

Previous research suggested only apoptosis as the cause for cell death in mammary cells. Recent research by Zarzynska et al., (2008) indicates that type II programmed cell death (PCD) or autophagic cell death is also observed in mammary epithelial cells. Overholtzer et al., (2007), also reported a new type of cell death in mammary epithelial cell lines in suspension: non-apoptotic cell elimination called “entosis”, which resembles cell cannibalism and cell-in-cell phenotype; it is initiated by detachment of mammary epithelial cells from extracellular matrix (ECM). Mitochondrial regulation is also involved in the autophagic cell death; evidenced by the regulatory proteins Bax-Bcl2 and their interactions. These interactions at the molecular level suggest

that autophagic and apoptotic cell deaths are closely intermingled and cross-reacting at cellular level. Even though caspase-3 assay is an improvisation over the previously followed methods for quantifying apoptotic cells, it does not give an estimate of the total cell death incurring in a mammary cell. We conclude that reason why we could not find a difference in apoptosis percentages between udder halves is that apoptosis percentages measured by caspase-3 is only estimate a portion of the actual cell death in a mammary cell. Also another important factor to be mentioned in this context is that duration of apoptosis in mammary cells has been estimated as only 3hrs (Capuco et al., 2001) and little is known about the duration of other cell death mechanisms in the cell. This also can be a limiting factor when we are estimating the total cell death in a cell for a particular day of lactation.

We conclude that increasing the milking frequency from 1x to 4x increases the overall production performance in Holstein dairy cows. Total milk yield, yield of components, gene expression and mitochondrial numbers were found to be higher from 4x udder halves, indicating an enhanced performance of 4x milked udder halves.

CHAPTER 3

SUMMARY AND CONCLUSIONS

The experiment was designed to study the effects of increased milking frequency on milk yield and composition, mammary gene expressions, mammary epithelial cell apoptosis and mitochondrial number for a complete lactation. Most previous works elucidating the effects of milking frequency on mammary functioning were based on short-term experiments ranging in length from only days to several weeks and so it was difficult to deduce any permanent effects on mammary functioning from such studies. Our experiment is unique in that it is the first study conducted to analyze the effect of milking frequency on mammary indices for a whole lactation period. Half udder model proved to be a reinforcing factor to our study since the same cow was exposed to similar environmental and systemic factors. Time away from the feed is often considered as a crucial factor in milking frequency studies, but was not a limiting factor in our study as the cow pens were in close proximity to milking parlor. We found that 4x milking frequency proved highly successful in increasing milk production in dairy cows. In this context, we strongly recommend augmenting the traditional milking frequency of 2x to 3x or 4x. This practice of increasing the milking frequency without increasing cow numbers can also be extended to countries like Canada, where the use of somatotropin is prohibited. Assessing the energy production rates in mammary mitochondria is an indicator of mammary metabolism and function in a lactating dairy cow. Our attempt to measure mitochondrial numbers paves way to a new arena for analyzing the mammary metabolic rates. But mere mitochondrial numbers does not provide a vivid idea of mammary metabolic rate as the ATP synthesis rates varies dramatically among mammary mitochondria. In this regard, we speculate that assessing the mitochondrial ATP synthesis rates would be a better follow-up for this study.

Increased milking frequency elevated mammary gene expressions of SOCS, alpha- lactalbumin and beta-casein as measured by transcript numbers for these genes in milk. This experiment also supports the idea that the study of genes regulating milk components may require more specific methods as gene expressions shown by epithelial cells make only twenty percent of total milk cells. Mammary apoptosis percentages were also not changed by 4x milking frequency. Recent research suggests possibility of mechanisms like autophagy and entosis other than apoptosis, governing mammary cell death. We suggest that future research should focus on developing techniques that measure the total cell death mechanisms in a mammary cell. Based on the fact that measures of synthetic rate (mitochondria number and gene transcript numbers in milk) are increased in 4x udder halves, we conclude that increased synthetic activity per cell is the primary cause for the increase in milk yield during increased milking frequency. We also conclude that decline in milk yield related to 1x milking is primarily due to decreased synthetic activity per cell rather than massive cell loss. We speculate that short-term alterations of milking frequency can transiently increase milk yield, whereas long-term changes in milking frequency may lead to sustained changes in milk yield as a result of changes in the secretory activity of mammary cells.

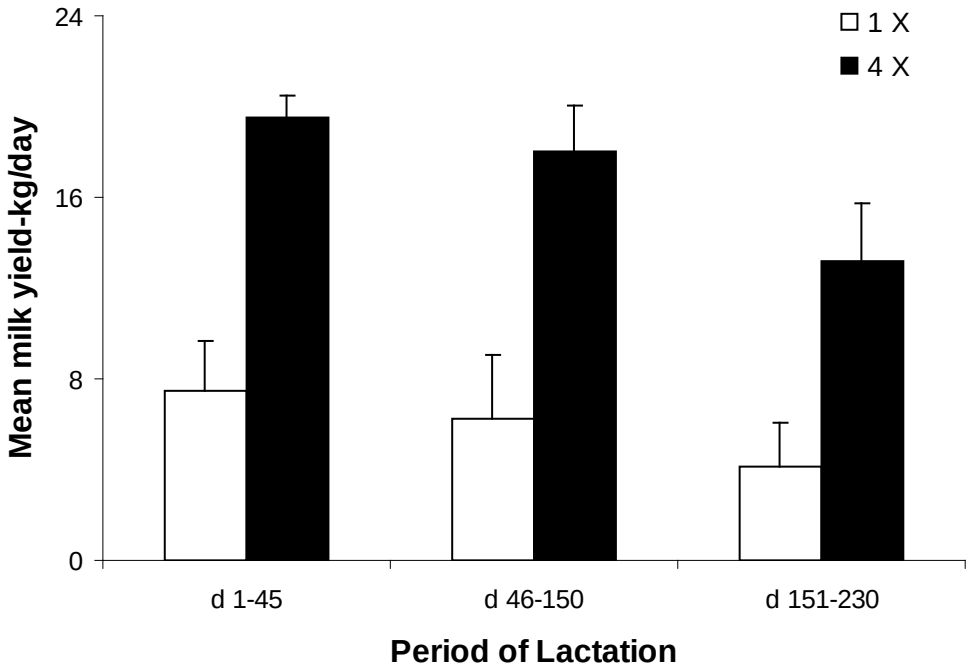

Figure 2.1 Effect of increased milking frequency on mean half udder milk yield in lactating Holstein dairy cows during three periods (1-45, 46-120 and 151-230) of lactation. Mean milk yield from the 4x udder halves was increased in all three stages of lactation. The figure shows the mean ± SD, n=6/treatment; Means differ, P <0.0001.

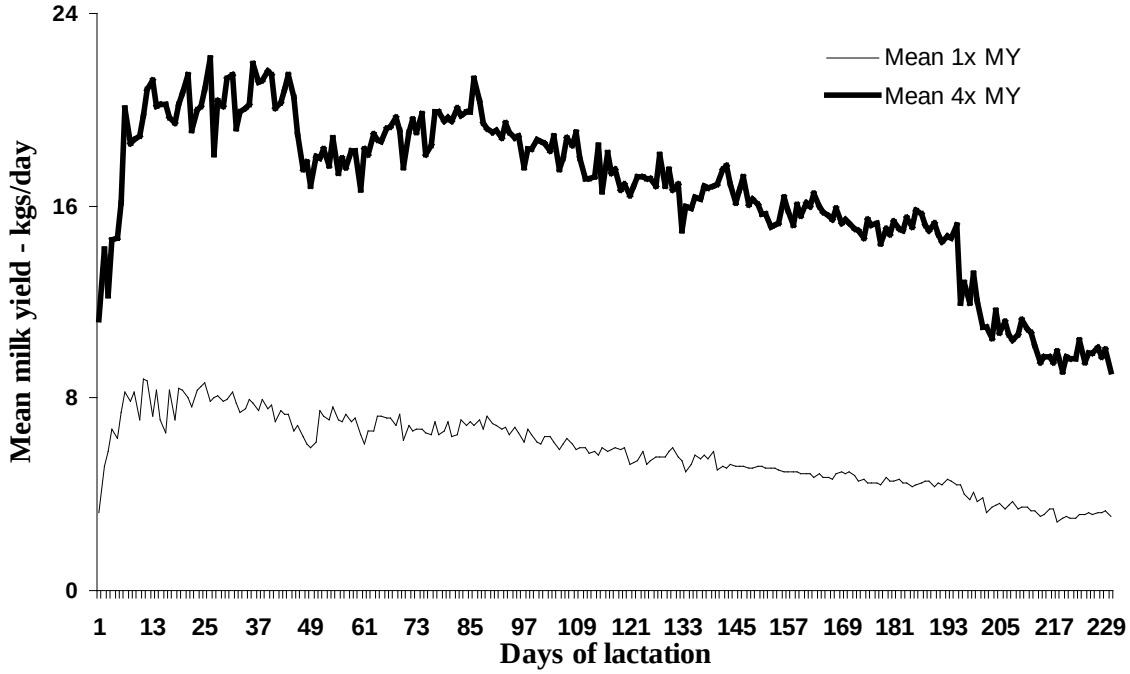

Figure 2.2 Effect of increased milking frequency on mean half udder milk yield in lactating Holstein dairy cows for 230 days. Mean milk yield from the 4x udder halves was higher for the whole lactation. The figure shows the mean, n=6/treatment; Means differ, P <0.0001.

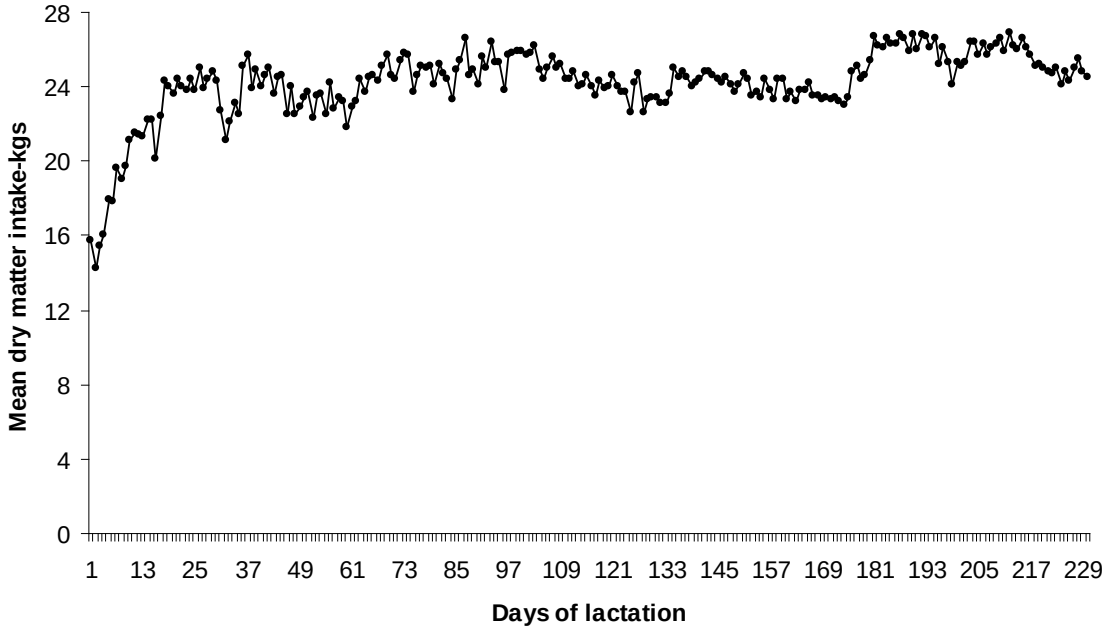

Figure 2.3, Effect of increased milking frequency on mean dry matter intake (DMI) for 230 days of lactation for Holstein dairy cows. Mean DMI increases from early through mid and late lactation stage. The figure shows the mean, n=6/treatment.

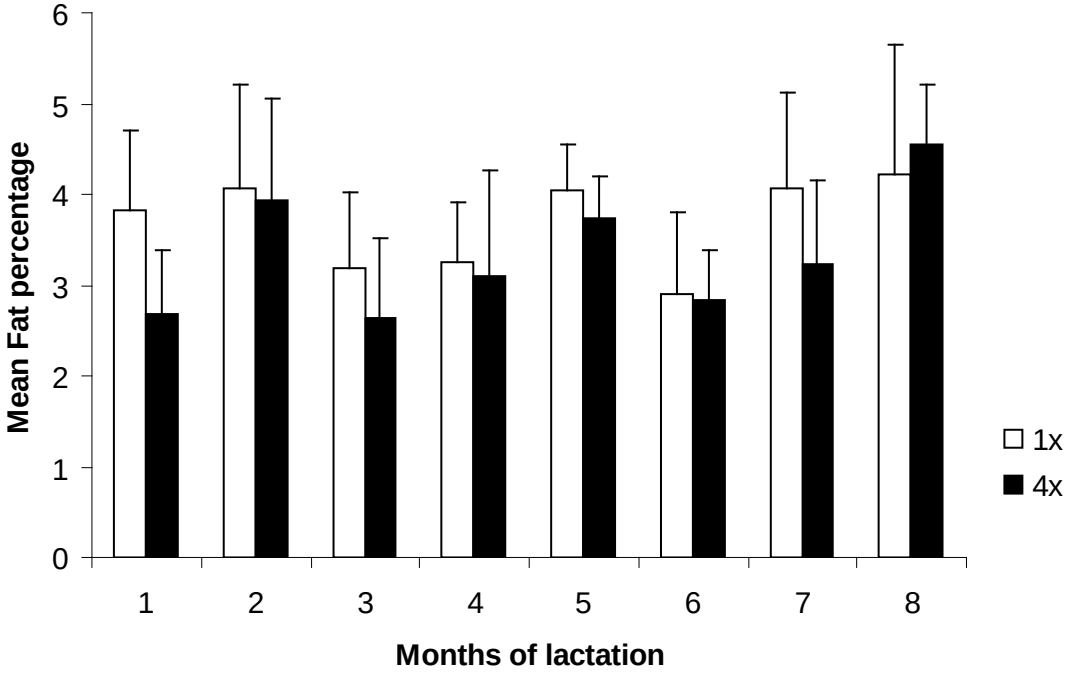

Figure 2.4 Effect of increased milking frequency on mean half udder milk fat percentage in lactating Holstein dairy cows. Mean fat percentage from the 1x udder halves were higher throughout the period. The figure shows the mean, n=6/treatment. Means differ, P=0.25.

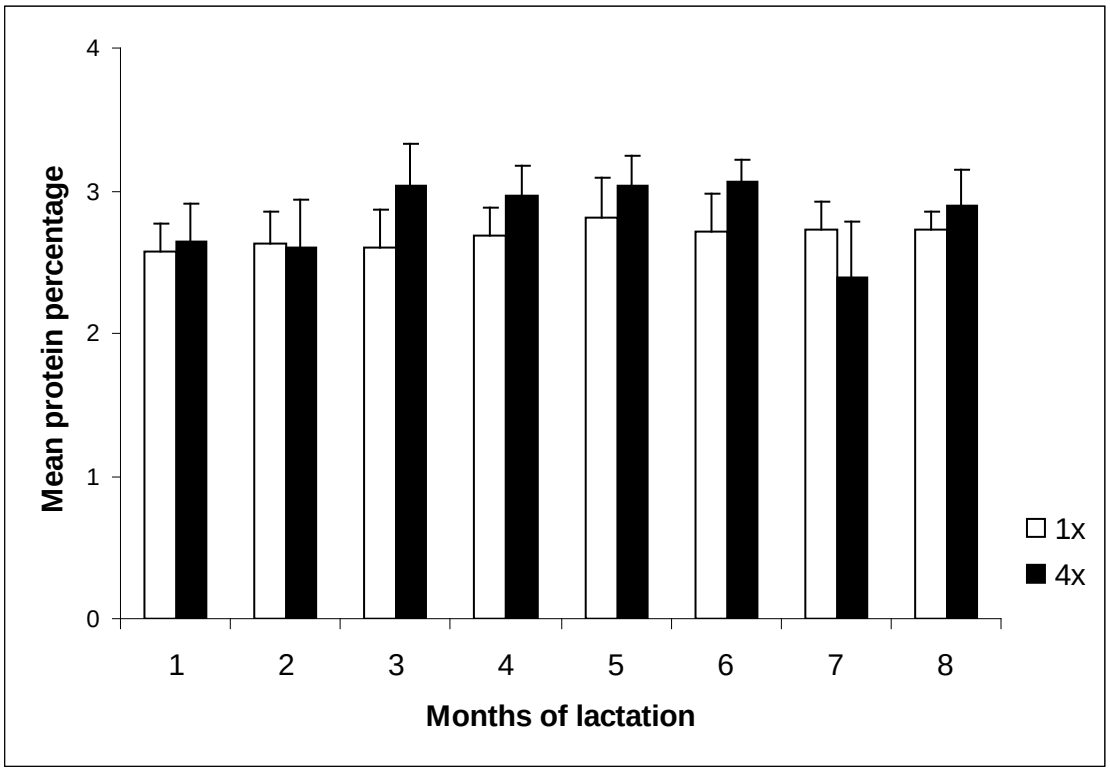

Figure 2.5 Effect of increased milking frequency on mean half udder milk protein percentage in lactating Holstein dairy cows. Mean protein percentage from the 4x udder halves were higher throughout the lactation. The figure shows the mean, n=6/treatment. Means differ, P=0.013.

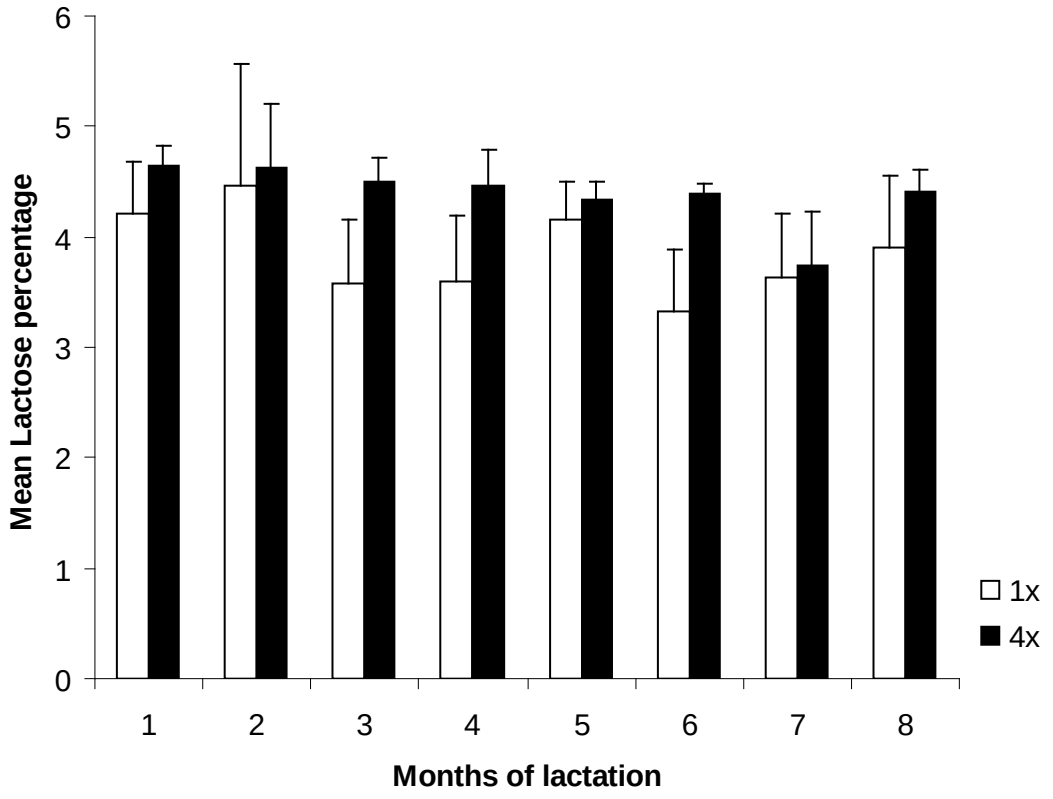

Figure 2.6 Effect of increased milking frequency on mean half udder lactose percentage in lactating Holstein dairy cows. Mean lactose percentage from 4x udder half was higher for the entire lactation. The figure shows the mean, n=6/treatment. Means differ, P=0.03.

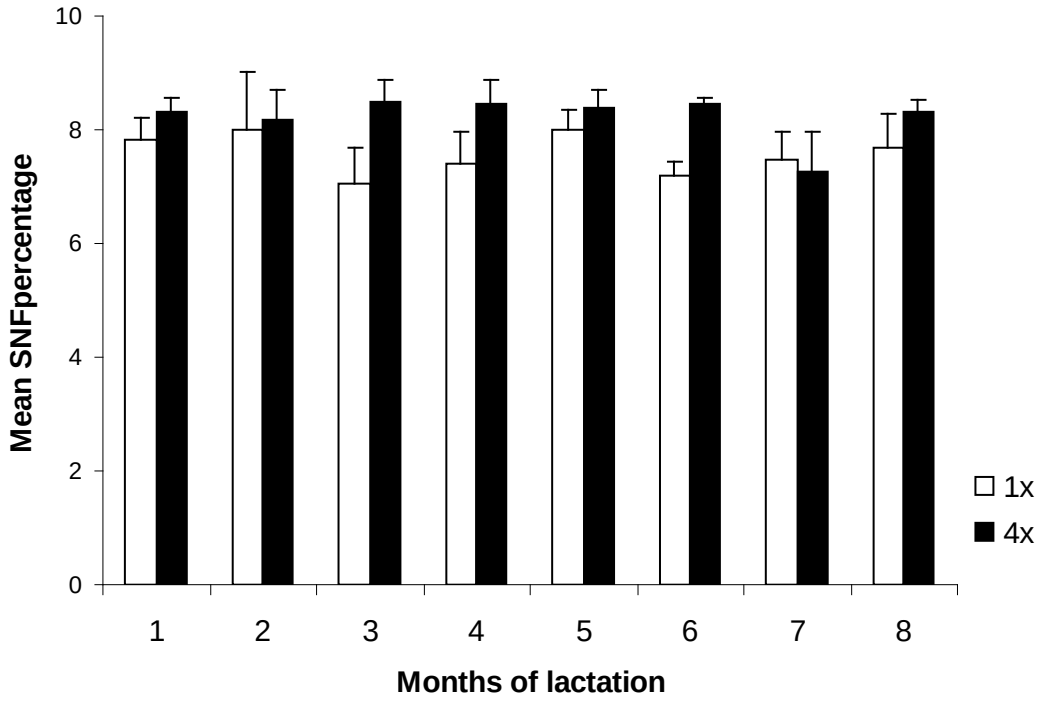

Figure 2.7 Effect of increased milking frequency on mean half udder SNF percentage in lactating Holstein dairy cows. Mean SNF percentage was higher in the 4x udder half for the entire lactation. The figure shows the mean, n=6/treatment. Means differ, P=0.001.

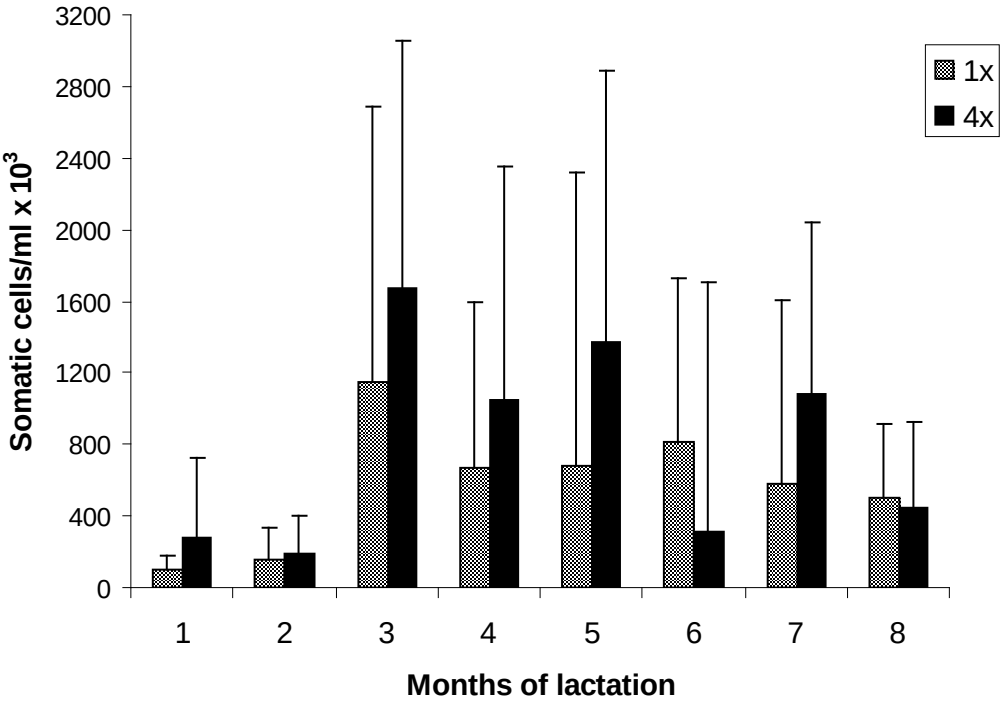

Figure 2.8 Effect of increased milking frequency on mean half udder somatic cell count (SCC) percentage in lactating Holstein dairy cows. Mean SCC percentage was higher in the 4x udder half (799,000 cells/ml of milk) for the entire lactation (except months 6 & 8) compared to 1x udder halves (580, 000 cells/ml). The current legal limit is 750,000 cells/ml of milk for somatic cell count (SCC) in US market. The figure shows the mean, n=6/treatment.

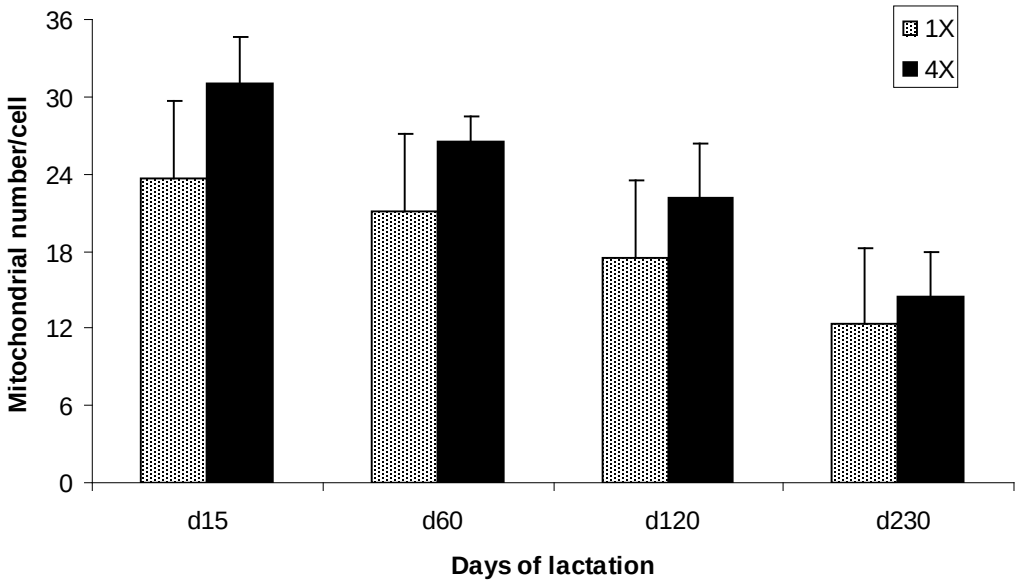

Figure 2.9 Effect of increased milking frequency on mean half udder mitochondrial numbers/epithelial cell in lactating Holstein dairy cows. Increased milking frequency had an overall effect on mitochondrial numbers (P=). Mammary sections (10μ thick) were stained with succinate dehydrogenase and counted at 100 x magnification. Mitochondrial number is ratio of total mitochondria to total epithelial cells from ten fields (calculated manually). The figure shows the mean, n=6/treatment.

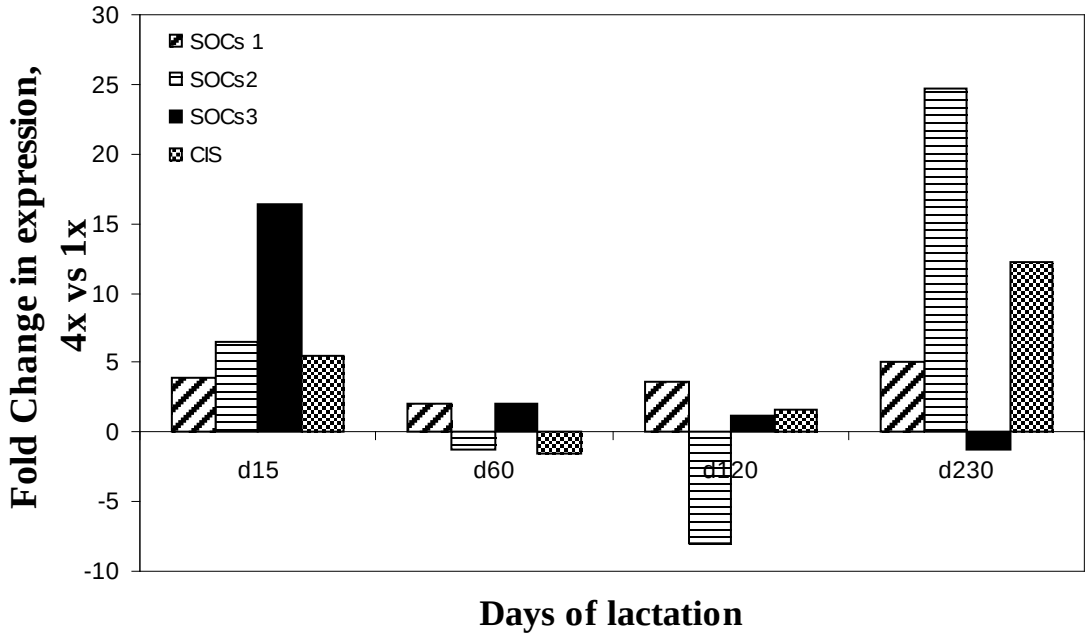

Figure 2.10 Fold changes in gene expression in 4x udder half milk compared with 1x udder half. Half-udders (n = 12) were used to compare physiological state of the mammary gland with samples taken at days 15, 60, 120 and 230 days of lactation. Gene expression was evaluated by real-time, reverse transcription-PCR using 1x and 4x udder half milk samples from each udder half. Cycle threshold (Ct) values were calculated using RPS15A as the housekeeping gene. Fold changes in expression were determined by $2^{-\Delta\Delta Ct}$ calculations. Genes: SOCS (1=3) = Suppressor of cytokine signaling genes 1, 2 and 3. CIS = cytokine-inducible SH2-containing protein gene.

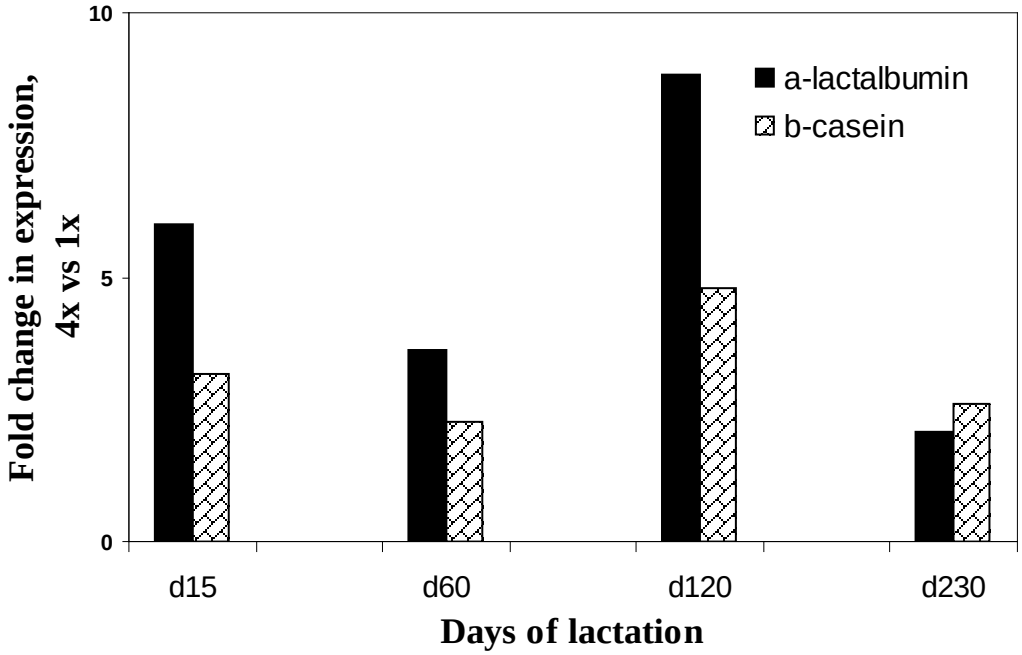

Figure 2.11 Fold changes in a-lactalbumin and b-casein gene expressions in 4x udder half milk compared with 1x udder half. Half-udders (n = 12) were used to compare physiological state of the mammary gland with samples taken at days 15, 60, 120 and 230 days of lactation. Gene expression was evaluated by real-time, reverse transcription-PCR using 1x and 4x udder half milk samples from each udder half. Cycle threshold (Ct) values were calculated using RPS15A as the housekeeping gene. Fold changes in expression were determined by $2^{-\Delta\Delta Ct}$ calculations. Genes = Alpha-lactalbumin and Beta-casein.

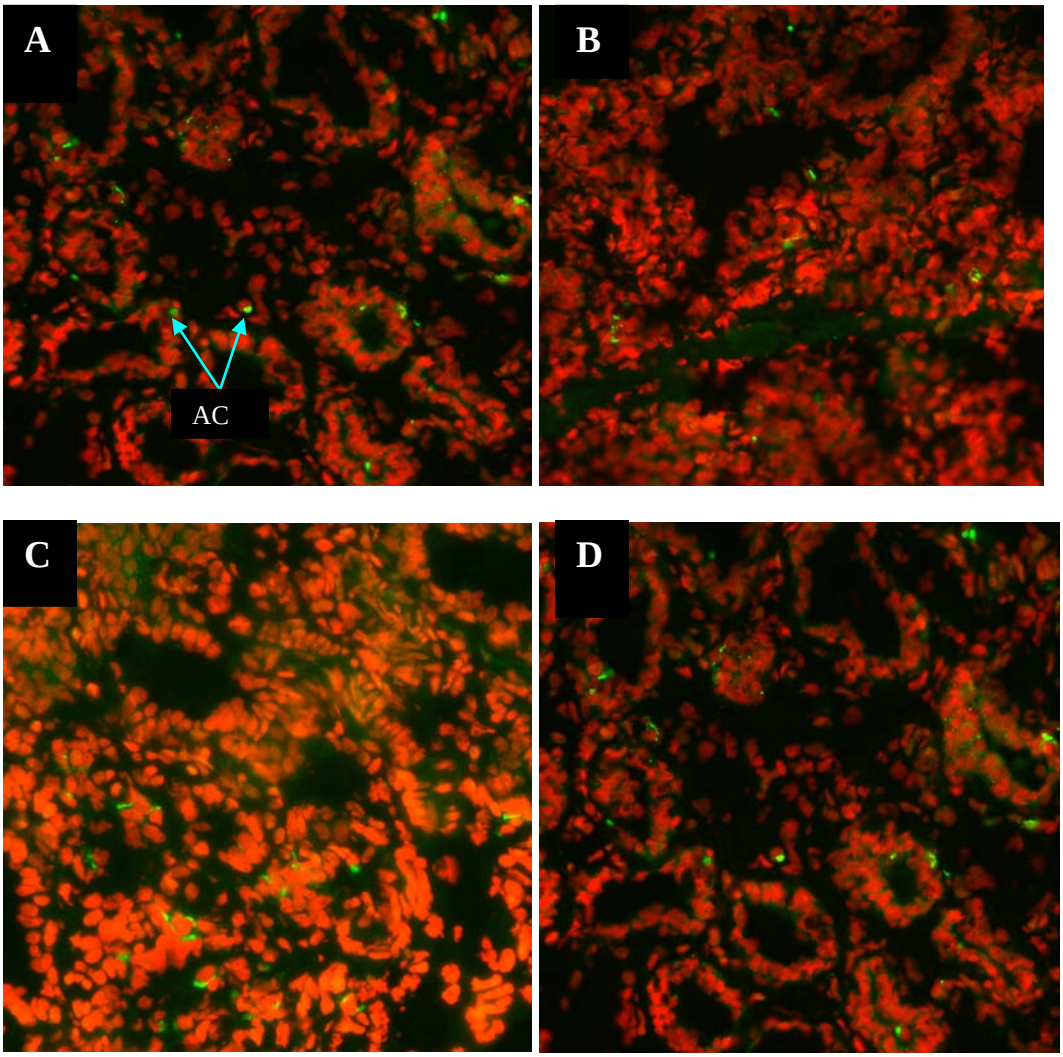

Figure 2.12 Fluorescent micrograph of lactating alveolus with normal and apoptotic epithelial cells. A, B = 1x udder half at d15 and d60 of lactation. C, D = 4x udder half at d 120 and d 230. Apoptotic cells stained green (caspase-3) and normal epithelial cells red. AC= apoptotic mammary epithelial cell.

Table 2.1 Composition of the total mixed diet fed to lactating and close-up dry cows

Ingredient, g/kg of DM	Lactating cows[1]	Close-up cows[2]
Alfalfa hay	532.9	601.6
Almond hulls	6.2	—
AminoPlus[3]	16.5	—
Bermudagrass hay	—	18.2
Corn (steam-flaked)	163.6	205.3
Corn distillers grain	21.8	—
Dry citrus pulp	78.6	105.6
EnerG II	14.4	—
Fat Bran (15% tallow)	29.5	—
Molasses (cane)	40.8	—
Supplement[4]	30.94	34.4
Whole cottonseed	65.0	35.0

[1]Diet was 70% DM and contained 18% CP, 1.74 Mcal NEl/kg of DM, 25.2% ADF, and 40.3% NDF based upon analysis of the total mixed diet.

[2]Diet was 65% DM and contained 15.3% CP, 1.63 Mcal NEl/kg of DM, 43.9% ADF, and 56.7% NDF based upon analysis of the total mixed diet.

[3]AminoPlus (Soy Best, West Point, NE); EnerG II (Bioproduct, Fairlawn, OH).

[4]Contained 2.62 × 105 IU/kg of vitamin A, 0.27 × 105 IU/kg of vitamin D, 0.09 × 105 IU/kg of vitamin E, 0.36% Cl, 0.31% K, 4.61% Mg, 10.34 % Na, 3.54% P, 0.47% S, 59 ppm Co, 408 ppm Cu, 1,736 ppm Fe, 28 ppm I, 1,447 ppm Mn, 6 ppm Mo, 10 ppm Se, 1,810 ppm Zn.

Table 2.2 Effect of increased milking frequency on half udder milk yield in lactating Holstein dairy cows.

	Milk yield, kg/d			
	1x	4x	SEM	P-value
Entire lactation	8.0	19.0	0.90	<0.0001
M1	7.5	18.3	1.7	<0.0001
M2	8.4	17.1	1.7	<0.0001
M3	5.7	17.7	1.4	<0.0001
M4	6.4	17.5	1.6	<0.0001
M5	10.2	22.1	1.8	<0.0001
M6	10.0	21.8	1.7	<0.0001
M7	7.4	17.8	2.6	<0.0001
M8	8.2	19.5	2.6	<0.0001

M1-M8 = Months 1-8 of lactation. 1x, 4x = Mean half udder milk yield for the 1x and 4x milked quarters for eight months of lactation. Mean udder half milk yields differ, $P<0.0001$

Table 2.3 Effect of increased milking frequency on mean half udder milk composition for 230days of lactation in Holstein dairy cows

Variable	1x	4x	SEM	P-value
Fat %	3.58	3.40	0.11	0.250
Protein%	2.72	2.83	0.03	0.013
Lactose%	4.00	4.30	0.07	0.004
SNF%	7.80	8.10	0.07	0.0006
Fat yield, kg/d	0.29	0.65	0.02	0.001
Protein yield, kg/d	0.22	0.54	0.01	0.005
Lactose yield, kg/d	0.32	0.81	0.70	0.033
SNF, kg/d	0.60	1.53	0.06	0.0001

1x, 4x = Mean half udder milk composition for 230 days of lactation. Milk samples from each udder halves were collected each week for 8 months of lactation and analyzed by Arizona Dairy Herd Improvement Association, (AZ DHI). Milk fat, protein, lactose and SNF were analyzed using AOAC approved infrared analysis. Yield of variable was calculated by multiplying the mean milk yield with composition of variable for 230d.

Table 2.4 Effect of increased milking frequency on half udder milk composition for eight months of lactation in Holstein dairy cows

Variable	M1	M2	M3	M4	M5	M6	M7	M8
Fat %	**	0.91	0.14	0.91	0.40	0.65	0.12	0.40
Protein%	0.40	0.10	0.80	0.59	0.22	0.11	0.09	0.35
Lactose%	0.86	0.36	0.68	0.30	0.06	0.67	***	0.12
SNF%	0.85	0.89	0.89	0.89	**	*	***	0.09
Fat yield, kg/d	***	***	***	***	***	***	***	**
Protein yield, kg/d	0.97	0.09	0.46	0.15	0.38	0.37	0.28	***
Lactose yield, kg/d	0.89	0.92	0.80	0.86	0.13	0.14	**	0.38
SNF yield, kg/d	0.97	0.97	0.86	0.62	**	**	***	*

M1-M8 = P values for the level of significance between mean 1x and 4x udder half milk composition variables for eight months of lactation. Milk samples from udder halves were collected each week for 8 months of lactation and analyzed by Arizona Dairy Herd Improvement Association, (AZ DHI). Milk fat, protein, lactose and SNF were analyzed using AOAC approved infrared analysis.
*** = <0.001, ** = <0.01, * = <0.05

Table 2.5 Effect of increased milking frequency on mean half udder mitochondrial numbers in lactating Holstein dairy cows

	1x	4x	SEM	P-value
Entire lactation	18.6	23.6	0.8	0.002
d15	23.6	31.1	1.7	0.001
d60	21.1	26.5	1.9	0.017
d120	17.5	22.2	1.6	0.012
d230	12.3	14.5	1.6	0.207

1x, 4x = Mean mitochondrial numbers for 1x and 4x udder halves at days15, 60 120 and 230 of lactation. Mitochondria were stained by succinate dehydrogenase and numbers from ten fields were calculated as (Total mitochondria counted/Total epithelial cell). The figure shows the mean, n=6/treatment; Means differ, P=0.002.

Table 2.6 Effect of increased milking frequency on mean half udder epithelial cell apoptosis percentage in lactating Holstein dairy cows

	1x	4x	SEM	P-value
Entire lactation	0.09	0.10	0.01	0.70
D15	0.14	0.14	0.03	0.95
D60	0.09	0.11	0.03	0.57
D120	0.07	0.08	0.03	0.72
D230	0.06	0.06	0.03	0.93

1x, 4x = Mean apoptotic percentages for 1x and 4x udder halves at days15, 60 120 and 230 of lactation. Apoptosis was determined by activated caspase-3 assay. Apoptotic cells were counted manually and normal cells by counting tool of Image Pro 5.1 (in split watershed mode) from the fluorescent micrograph pictures. Apoptosis percentage was calculated as (Total number of apoptotic cells counted/Total epithelial cells) *100. The table shows the mean, n=6/treatment; Means did not differ, P=0.70.

Table 2.7 Effect of increased milking frequency on mean half udder gene expressions in lactating Holstein dairy cows

Gene of interest	1x	4x	SEM	P-value
SOCS1	4.1	3.6	0.4	0.31
SOCS2	9.4	10.8	0.9	0.14
SOCS3	5.1	4.3	0.4	0.08
CIS	7.8	8.7	0.6	0.26
α-Lactalbumin	6.0	4.4	0.5	0.04
β-Casein	1.1	0.4	0.2	0.001

1x, 4x = Mean ΔCt values for 1x and 4x udder half milk. ΔCt = CtGOI− CtRPS15A, (where GOI = gene of interest and RPS15A = housekeeping gene) a larger Ct value (more cycles to threshold) means less transcript abundance. Half-udders (n = 12) were sampled at days 15, 60, 120 and 230 of lactation. Gene expression was evaluated by real-time, reverse transcription-PCR from 1x and 4x udder half milk sample pools. Genes = Suppressor of cytokine signaling; Cytokine inducible SH2 containing protein, alpha-lactalbumin, beta-casein

Gene	Accession #	Primer sequence 5' to3'	Amplicon size (bp)
SOCS-1	CB460055	L-CACAGCAGAAAATAAAGCCAGAGA R-CTCGTACCTCCTACCTCTTCATGTT	94
SOCS-2	AY183452	L-CTGACTTTCTGAGGTTCGTGGT R-TTTCGTTCCTTCCACTTCTTCAG	100
SOCS-3	NM 174466	L-GGCCACTCTCCAACATCTCTGT R-TCCAGGAACTCCCGAATGG	99
CIS	BC113307	L-ATGTACTGGCACTAACCCATCCA R-ATGTTGGCTCCATHTTCTTGTTT	103
a- LA	BT025469	L-CTCTGCTCCTGGTAGGCATC R-ACAGACCCATTCAGGCAAAC	125
b- casein	X147111	L-GCTATGGCTCCTAAGCACAAAGA R-GGAAACATGACAGTTGGAGGAAG	173
RPS15A	BC108231	L- GAATGGTGCGCATGAATATC R- GACTTTGGAGCACGGCCTAA	101

Table 2. 8 Primer sequences for bovine suppressors of cytokine signaling (SOCS) -1, -2 and -3; CIS, a-lactalbumin and b-Casein genes. Gene-specific primers were designed based on the available bovine sequences in GenBank (accession numbers indicated). Amplicons were purified and sequenced, and product sequences were then BLAST searched against the National Center for Biotechnology Information database to confirm identity of the product.

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