Effect of Continuous Milking and Bovine Somatotropin Supplementation on Mammary Epithelial Cell Turnover

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ABSTRACT

Objectives were to determine effects of continuous milking (CM) and bovine somatotropin (bST) administration on 1) mammary epithelial cell (MEC) proliferation, apoptosis, and ultrastructure during late gestation and early lactation, 2) expression of genes associated with proliferation, and apoptosis in mammary epithelial cells, and 3) milk yield and composition. Second-gestation, first dry-period cows were randomly assigned to either continuous bST throughout late gestation and early lactation (+bST; $n = 4$) or no bST (–bST; $n = 4$) administration. Within each animal, udder halves were randomly assigned to CM or a 60-d dry period (control) treatment. Daily milk yield and weekly milk composition were measured during the last 60 d of gestation in CM halves and from 1 to 30 d postpartum for both halves. Mammary biopsies were obtained at -20 ± 7 , -8 ± 3 , $+1 \pm 0$, $+7 \pm 0$, and $+20 \pm 0$ d (mean $\pm$ standard error) relative to parturition. Prepartum half-udder milk yield was greater in +bST cows than in –bST cows (9.9 vs. 8.2 kg/d) and postpartum half-udder milk yields were dramatically reduced in CM halves compared with control halves (10.6 vs. 22.2 kg/d), regardless of bST treatment. Proliferation of MEC was reduced in CM halves at -8 d (2.7 vs. 5.4%). Apoptosis of MEC was elevated during early lactation for d +1 and +7 in control halves, but was only increased at d +1 in CM halves. Turnover of MEC was not affected by bST. Ultrastructure data indicated complete involution of the control half and lactation maintenance in CM glands (d -20). By d -8 , control tissue contained alveoli in an immature secretory state, but CM tissue contained both lactating and immature alveoli. Postpartum ultrastructure parameters were similar between halves until d 20 when control tissue was composed of a homogeneous population of lactating alveoli, but CM tissue contained lactating, engorged, and resting alveoli. Expression of

CCAAT/enhancer binding protein- β (CEBP- β), cyclin D1, and bcl₂ were up-regulated during late gestation, but did not differ between control and CM halves. Expression of α -lactalbumin was increased in CM halves during late gestation, but was not different in CM and control tissue after parturition. Other genes evaluated (bax, insulin-like growth factor binding protein 5, ATP-binding cassette 1, and p27) were not differentially expressed at any timepoints evaluated. Results indicate that CM reduced subsequent half-udder milk yield in primiparous cows through altered MEC turnover and secretory capacity. Negative effects of CM on the subsequent lactation were not alleviated by bST supplementation.

Key words: continuous lactation, bovine somatotropin, half udder, gene expression

INTRODUCTION

In dairy cows, a dry period less than 40 d results in reduced milk yield in the subsequent lactation (Remond et al., 1997). However, recent research indicates no negative effects on milk yield in multiparous cows provided a 30-d dry period with or without the use of bST (Bachman, 2002; Gulay et al., 2003; Rastani et al., 2003; Annen et al., 2004). Furthermore, continuously milked (CM, 0 d dry) multiparous cows supplemented with bST throughout late gestation produce as much milk as cows provided a 60-d dry period (Annen et al., 2004), but this appears to be bST-dependent because subsequent milk yield was reduced when bST was not utilized (Rastani et al., 2003). A study directly comparing CM vs. a 60-d dry period with and without bST has not been conducted. Furthermore, parity influences these results because substantial reductions in milk yield were reported for CM primiparous cows with (Annen et al., 2004) or without (Remond et al., 1992) bST during late gestation. Decreased production was also reported for bST-supplemented primiparous cows provided a 30-d dry period (Annen et al., 2004). This parity sensitivity to modified dry periods was observed previously in commercial trials (Remond et al., 1997) and in retrospective

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studies of dairy records (Wilton et al., 1967; Dias and Allaire, 1982). In contrast, others have reported no parity differences in future milk yield from cows subjected to a shortened dry period (Sorensen and Enevoldsen, 1991). Reasons for the discrepancies are not clear, but these results suggest that continued mammary development between the first and second lactations in dairy cows is impaired by maintaining lactation beyond 60 d before parturition.

Due to increased sensitivity of primiparous cows to modified dry periods and continued mammary development between the first and second lactations, we hypothesized that mammary epithelial cell (MEC) numbers may be reduced in CM primiparous cows. Additionally, this sensitivity to altered dry period lengths makes the primiparous cow a good model for evaluating the effects of short or omitted dry periods on MEC, as well as to evaluate potential methods for rescuing milk production in CM cows.

Study objectives were to evaluate the effects of CM and bST on: 1) MEC proliferation and apoptosis during late gestation and early lactation, 2) MEC ultrastructure, 3) expression of genes involved in mammary function, MEC proliferation, or apoptosis, and 4) milk yield and composition. The genes evaluated included: ATP-binding cassette 1 (**ABC1**, proposed stem cell marker); α -LA (lactose synthesis); bax (apoptosis); bcl₂ (survival); CCAAT/enhancer binding protein- β (**CEBP- β** , mammary growth); IGF-binding protein 5 (**IGFBP5**, apoptosis); and kinase inhibitor protein p27 (**p27**, cell cycle arrest). We hypothesized that MEC turnover and milk yield would be reduced by CM of primiparous cows and that bST would improve MEC turnover (through increased proliferation, decreased apoptosis, or both), and thus increase milk synthesis in the subsequent lactation.

MATERIALS AND METHODS

Animals and Experimental Design

The University of Arizona Institutional Animal Care and Use Committee approved all procedures involving animals. Cows were housed at the University of Arizona Dairy Research Center in individual pens with ad libitum access to feed and water. All diets used during the experiment were formulated to meet or exceed NRC (2001) requirements for the given physiological state (Table 1). As long as one udder-half was lactating, cows were fed a lactating cow ration (Table 1). If the CM half spontaneously dried (defined as <5 kg/d milk yield for 7 d), cows were fed a close-up dry-cow ration until parturition (Table 1). At parturition, the diet was switched back to the lactating ration. Cows were fed and lactating udder halves were milked at 0500 and 1700 h. Feed

Table 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	30.9 ⁴	34.4
Whole cottonseed	65.0	35.0

¹Diet was 51% DM and contained 18% CP, 1.74 Mcal NE/kg of DM, 25.2% ADF, and 40.3% NDF based upon analysis of the total mixed diet.

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

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

⁴Contained 2.62×10^5 IU/kg of vitamin A, 0.27×10^5 IU/kg of vitamin D, 0.09×10^5 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.

⁵Contained 4.98×10^5 IU/kg of vitamin A, 0.51×10^5 IU/kg of vitamin D, 0.07×10^5 IU/kg of vitamin E, 16.54% Cl, 0.28% K, 3.72% Mg, 0.15 % Na, 2.97% P, 3.42% S, 54 ppm Co, 512 ppm Cu, 1,039 ppm Fe, 40 ppm I, 1,463 ppm Mn, 5 ppm Mo, 10 ppm Se, 1,808 ppm Zn.

refusals were weighed and recorded before the 0500 h feeding. Ration DM percentage was determined twice daily and TMR samples, analyzed for nutrient composition (Dairy One, Ithaca, NY), were collected weekly. For statistical analyses, daily DMI and milk yield values for each cow were collapsed into a weekly mean representative of daily DMI or milk yield for that week of the experiment. Milk samples were obtained from successive a.m. and p.m. milkings, pooled (by volume), and analyzed for fat percentage, true protein percentage, lactose percentage, and SCC (Arizona DHIA, Tempe). Milk fat, protein, and lactose were analyzed using AOAC-approved infrared analysis (AOAC, 2000); SCC was analyzed using AOAC-approved cell-staining techniques (AOAC, 2000). The International Dairy Federation and the FDA certified all equipment used in the analyses.

The study design used 8 Holstein cows during the periparturient period between their first and second lactations in a half-udder model. Cows selected for the study were healthy and had SCC less than 500,000 cells/mL. Cows were assigned randomly to a systemic treatment of bST (Posilac, Monsanto Company, St. Louis, MO; +bST, n = 4) or no bST (−bST, n = 4). Within

each animal, udder halves were assigned randomly to CM or 60-d dry (control). The half-udder model was utilized so that the effects of both CM and bST could be examined with fewer animal numbers. All cows were supplemented with bST during their first lactation until 88 d before expected parturition. Cows in the +bST group continued to receive bST supplementation at 14-d intervals throughout late gestation and into the first 30 d of the next lactation. Due to off-label use of bST between parturition and 30 d postpartum, the study was conducted under an FDA investigational new animal drug protocol from Monsanto Company. Cows began the study at 67 d before expected parturition date and remained on the study until 30 d postpartum. Half-udder milk yields for both the right and left udder halves were measured from d 67 to 61 prepartum. Milk from each udder-half was collected into separate buckets using a unit designed to collect milk from all 4 quarters individually, but modified to collect milk from unilateral udder halves. At 60 d before expected parturition, the udder half assigned to the control treatment was dried by abrupt cessation of milking and treated with a long-acting, intramammary antibiotic (Quartermaster, Pfizer, La Jolla, CA). The CM udder half was milked throughout gestation and into the next lactation unless milk yield dropped below 5 kg/d for 7 consecutive days, at which time the udder-half was considered to have spontaneously dried, treated with a long-acting intramammary antibiotic, and milk removal ceased until parturition. At parturition, milking of both udder halves using the half-udder milker (described above) resumed until 30 d postpartum.

Tissue Samples

Mammary biopsy timepoints were planned for d -20, -7, 1, 7, and 20 relative to parturition. Actual biopsy timepoints were d -20 ± 7 (mean $\pm$ SEM), -8 ± 3 , 2, 7, and 20 relative to parturition. Because udder-half was the experimental unit, both udder halves were biopsied at each timepoint. Rear quarters were biopsied on d -20, 1, and 20, whereas front quarters were biopsied on d -8 and 7. All biopsies were conducted 1 to 2 h after the 0500 h milking. Biopsies were performed according to procedures by Farr et al. (1996) with modifications by Baumgard et al. (2000) to ensure the tissue core was mammary parenchyma. Potential surgical complications (i.e., infection at the surgery site, mastitis) were monitored by measuring whether milk yield, rectal temperature, and DMI were adversely affected by the biopsy procedure. A comparison of milk yield for 3 d before and 7 d after the d -20 (CM half only), and d 20 biopsies is shown in Figure 1. The milk yields at these timepoints were less affected by stage of lactation than were

Figure 1. Temporal pattern of milk yield for 3 d before and 7 d after mammary biopsy in continuously milked (CM) and 60-d dry (control) udder halves. Data are unadjusted means from all animals ($n = 8$) on the study. ○ = CM, d 20 biopsy, ■ = CM, d -20 biopsy, ● = control, d 20 biopsy.

milk yields around the d -8, 1, and 7 biopsies, which were rapidly decreasing (d -8) or increasing (d 1 and 7) regardless of biopsies. As demonstrated in Figure 1, milk yield was not adversely affected by the biopsy procedure. Additionally, there were no infections or mastitis caused by biopsies. Upon tissue harvesting, the sample core was divided for immunohistochemistry, electron microscopy, and gene expression analyses. Tissue for each of these analyses was always obtained from the same area of the tissue core. Tissue for immunohistochemistry was fixed in 10% neutral buffered formalin for 24 h at 4°C, and then transferred to 70% ethanol until further processing. Tissue was dehydrated in a series of ethanol concentrations, embedded in paraffin, and processed into 6- μ m sections on silanated slides according to standard techniques. Tissue for electron microscopy was fixed in half-strength Karnovsky's buffer for 2 h at 4°C, and then transferred to cacodylate buffer until further processing. Upon processing, the tissue was rinsed 3 times for 10 min in cacodylate buffer at 4°C, then incubated in 2% osmium tetroxide for 1 h at 4°C, dehydrated in a series of ethanol concentrations, and embedded in Spurr's plastic (Spurr, 1969). Tissue for gene expression analyses was placed in RNase-/DNase-free tubes, snap frozen in liquid nitrogen, and stored at -80°C until further processing.

Immunohistochemistry

Localization of Ki-67 Nuclear Proliferation Antigen. Proliferating MEC were identified as those cells expressing the Ki-67 nuclear proliferation (Ki67) antigen. The Ki67 antigen is expressed in proliferating cells (G_1 , S, G_2 , and M phase), but not in cells resting in G_0

phase (Duchrow et al., 2001). Slides were deparaffinized in xylene and rehydrated in a series of ethanol dilutions. Following dehydration and rehydration, tissue sections were quenched in 3% hydrogen peroxide for 10 min to eliminate endogenous peroxidase activity. Slides were then washed in distilled water (dH_2O ; 3×2 min). Antigen retrieval was accomplished by heating the slides in a covered glass container filled with 400 mL of citrate buffer (10 mM) for 5 min on a hot plate (boils at 3.5 min), leaving the slides undisturbed for 5 min, then heated again for 5 min (boils at 4.5 min). Slides remained in the covered glass container and cooled for 30 min. Slides were then washed in dH_2O (1×2 min) and Dulbecco's PBS (3×2 min). Tissue sections were blocked with 5% nonimmune goat serum (Zymed Laboratories Inc., San Francisco, CA) for 30 min at 37°C, followed by a 60-min incubation in primary antibody (MIB-I, mouse monoclonal antihuman Ki67; Zymed Laboratories Inc.) for 60 min at 37°C, and then incubated for 10 min at 37°C in a biotinylated secondary antibody that was conjugated to horseradish peroxidase. Slides were washed with PBS (3×2 min) followed by incubation of tissue sections with diaminobenzidine chromagen. The diaminobenzidine chromagen interacts with the horseradish peroxidase conjugate from the secondary antibody to form a brown deposit to indicate location of the Ki67 antigen. Slides were washed in dH_2O (3×1 min), counterstained with hematoxylin (Zymed Laboratories Inc.), dehydrated in a series of ethanol concentrations and xylene, and mounted with Permount (Fisher Scientific, Pittsburgh, PA).

Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) of the free 3'-OH DNA termini in situ enables visualization of cells that exhibit endonucleolytic degradation of DNA. These fragments of DNA are fundamental features of apoptotic cells (Wyllie et al., 1980). The TUNEL assay was performed using a commercial kit (ApopTag Peroxidase In Situ Apoptosis Detection Kit, Chemicon International, Temecula, CA). The manufacturer's protocol was followed with modifications; to reduce nonspecific binding of digoxigenin-conjugated nucleotides, the working concentration of terminal deoxynucleotidyl transferase enzyme was diluted to 4 parts reaction buffer (chemically labeled and unlabeled nucleotides) to 1 part terminal deoxynucleotidyl transferase compared with the 2:1 dilution recommended by the manufacturer. The diluted working terminal deoxynucleotidyl transferase concentration resulted in reduced nonspecific binding of digoxigenin-conjugated nucleotides. Additionally, after counterstaining with methyl green, cell morphology was improved by reducing the recommended 10 dips in one allotment of 100% butanol to 4 dips. Also, the

recommended 30-s incubation in a second allotment of butanol was reduced to 4 dips. This reduction in exposure of the tissue sections to butanol improved cell morphology and counterstaining for more accurate assessment of apoptotic cells and cell type. After butanol incubation slides were dehydrated and mounted with Permount.

Quantitation of Immunohistochemistry. Tissue sections were viewed with a light microscope to quantify cells expressing the Ki67 antigen and TUNEL positive (apoptotic) cells. Ten microscopic fields were quantified for each tissue sample. Each slide representing a sample from an individual cow contained 2 sections and cell quantification was divided equally between sections. At least 1,800 cells were counted for each tissue sample. Fields were defined by a 10×10 ocular grid at $500\times$ magnification. Counted fields were spread equally across tissue sections. To ensure random selection of fields and to reduce experimenter bias, fields were selected with the microscope out of focus and readers were blind to sample identification until all quantitation was complete.

Transmission Electron Microscopy

Thin sections for transmission electron microscopy were cut into approximately 0.08- μm sections using a diamond knife and stained with uranyl acetate (20 min) and lead citrate (3 min). Sections were viewed using a Philips 420 Transmission Electron Microscope (University of Arizona Electron Microscopy Core, Tucson). Reference sections for light microscopy were cut into 1- μm sections and stained with toluidine blue.

RNA Isolation

The RNeasy Mini Kit (Qiagen, Valencia, CA) was used to isolate total cellular RNA according to the manufacturer's protocol. Once isolated, RNA samples were divided into 3 aliquots; 2 were stored immediately at -80°C , and the third aliquot was used for measurement of RNA concentration and quality. Concentration of RNA was determined by photospectrometry. Integrity of 28S and 18S RNA bands was assessed by RNA 6000 Nano Chip (Agilent Technologies, Palo Alto, CA) according to the manufacturer's instructions on an Agilent 2100 bioanalyzer (Agilent Technologies) and samples reisolated if necessary.

Pools of RNA were made within cow by udder half for prepartum and for postpartum biopsy timepoints. No between-cow pooling of RNA samples occurred. Prepartum pools consisted of 1 μg of RNA from d -20 and -8 samples. Postpartum pools consisted of 1 μg of RNA from d 1, 7, and 20 samples. After pooling, each cow

Table 2. Summary of genes, primers, and between plate and sample variation

Gene of interest ¹	Primer sequence ²	Product size (bp)	CV, %	
			Interassay	Intraassay
Housekeeping gene 18S	F 5'AAACGGCTACCACATCCAAG3' R 5'TCGCGGAAGGATTTAAAGTG3'	200	0.60	0.53
Lactation gene α -lactalbumin	F 5'CTCTGCTCCTGGTAGGCATC3' R 5'ACAGACCCATTTCAGGCAAAC3'	125	0.61	0.75
Proliferation genes ABC1	F 5'GCCACCTTCCTCGTTGTC3' R 5'AAGTTGCTCACTGCCATCC3'	125	0.29	0.38
CEBP- β	F 5'CGTGTGTACACGGGACTGAC3' R 5'CCACATCAACAGCAACAACC3'	99	0.73	0.59
Cyclin D1	F 5'CTGGCCATGAACTACCTGGA3' R 5'CCACTTGAGCTTGTTACCA3'	213	0.37	0.33
p27	F 5'CGGGTTAGCGGAGCAGTG3' R 5'AGGCTTCTTGGGCGTCTG3'	142	0.32	0.45
Apoptosis genes bax	F 5'TTTGCTTCAGGGTTTCATCC3' R 5'CAGTTGAAGTTGCCGTGAGA3'	246	0.34	0.72
bcl ₂	F 5'ATGTGTGTGGAGAGCGTCAA3' R 5'CAGACTGAGCAGTGCCTTCA3'	201	0.59	0.50
IGFBP5	F 5'GAGCAAGCCAAGATCGAAAG3' R 5'GAAGATCTTGGGCGAGTAGG3'	90	0.43	0.63

¹Genes: 18S = ribosomal 18S; ABC1 = ATP-binding cassette 1; CEBP- β = CCAAT/enhancer binding protein- β ; IGFBP5 = IGF-binding protein 5; p27 = kinase inhibitor protein p27.

²F = Forward; R = reverse.

had a prepartum and postpartum RNA sample for each udder half.

Synthesis of cDNA and Real-Time PCR

Unless noted, manufacturer protocols were followed. Prior to cDNA synthesis, RNA was treated with deoxy-ribonuclease I (DNase; amplification grade, Invitrogen, Carlsbad, CA). The Superscript III First Strand Synthesis System (Invitrogen) for reverse transcription PCR (RT-PCR) was used for cDNA synthesis. Resulting cDNA was applied to QIAquick PCR purification kit columns (Qiagen) to clean cDNA with modifications to the manufacturer's protocol. Modifications included re-applying flow-through from the cDNA to the column for a second spin and changes to the elution step. Cleaned cDNA was eluted twice with 30 μ L of elution buffer, with a 5-min incubation each time. Cleaned cDNA was then used for real-time RT-PCR assays.

Real-Time RT-PCR

All primers and resulting products are described in Table 2. Primer sequences for bcl₂ were obtained from Colitti et al. (2004); ABC1 and p27 primer sequences were designed using beacon designer 3 software (Premier Biosoft International, Palo Alto, CA). All other primers were designed using Primer 3 (Rozen and Ska-

letsky, 2000). Bovine (if available) or human cDNA sequences used for primer design were obtained from GenBank (NIH, Bethesda, MD). Primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). A large, pooled cDNA sample was created to determine optimal PCR conditions for each primer set, to verify that each set amplified the gene of interest (GOI), for plate controls and for standard curves. Products synthesized by each primer pair were sequenced and determined homologous to the respective GOI.

Real time RT-PCR was run on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) in a 96-well format. Reaction mixtures contained 10 μ L of iTaq SYBR Green Supermix with ROX (BioRad Laboratories, Hercules, CA), 4 pmol of each primer, sample or standard cDNA, and RNase-/DNase-free water to a final volume of 20 μ L. Master mix (SYBR Green, primer, water) was pipetted into plate wells followed by addition of sample or standard curve cDNA. For all primers except bax, real-time RT-PCR conditions included 1 cycle of 2 min at 50°C, 1 cycle of 10 min at 95°C, and 40 cycles of 15 s at 95°C followed by 1 min at 58°C (annealing temperature). For bax, all conditions were the same except that the annealing temperature was 62°C. At the end of all real-time RT-PCR plate runs, a dissociation protocol was run to confirm that only one product was amplified by a primer pair. Thus, single product amplification was verified

Table 3. Effect of continuous milking (CM) and bST on half udder milk production and composition pre- and postpartum¹

Variable	Treatment				SE
	-bST		+bST		
	Control	CM	Control	CM	
Prepartum					
Half udder milk yield, kg/d	—	8.2 ^a	—	9.9 ^b	0.52
Fat, %	—	4.7 ^a	—	4.1 ^b	0.15
True protein, %	—	3.7	—	3.7	0.12
Lactose, %	—	4.9	—	4.9	0.09
Somatic cell linear score	—	2.7	—	3.2	0.36
Postpartum					
Half udder milk yield, kg/d	22.3 ^a	10.9 ^b	22.4 ^a	10.1 ^b	1.60
Fat, %	3.6	3.5	3.5	3.5	0.20
True protein, %	3.0	3.0	3.1	3.1	0.13
Lactose, %	4.8	4.8	4.9	4.8	0.10
Somatic cell linear score	3.7	3.1	2.9	2.3	0.50

^{a,b}Means within a row with different superscripts differ ($P < 0.05$).

¹Milk samples were collected weekly during the last 8 wk of gestation and wks 3 and 4 postpartum and composition evaluated. Data presented are least squares means. Udder half ($n = 16$) milk yield and composition data were collected during the last 60 d of gestation and first 30 d postpartum. Biweekly injections (500 mg/2 wk) of bST were given to bST-treated cows. Means were adjusted using data collected from 67 to 61 d prepartum as a covariate.

during primer optimization and again during actual real-time RT-PCR runs.

Real time RT-PCR was run on all samples for 8 GOI and a housekeeping gene, 18S rRNA, to correct for differences in RNA input. This normalization to the housekeeping gene resulted in a ΔCt (cycle threshold) value:

$$\Delta Ct = Ct_{GOI} - Ct_{18S}.$$

Statistical analyses were conducted using ΔCt values. Relative changes in expression levels were calculated using the following formula (Applied Biosystems, 2001):

$$\text{Relative change} = 2^{-\Delta\Delta Ct}$$

where 2 is based on an optimal PCR efficiency in which the PCR product is replicated every cycle; and $-\Delta\Delta Ct$ = least squares mean of ΔCt for a treatment – least squares mean of ΔCt for another treatment. For example, ΔCt for prepartum cyclin D1 expression in +bST CM tissue – ΔCt for prepartum cyclin D1 expression in +bST control tissue.

Differential expression was only declared if ΔCt values used in the equation were statistically different. All samples were run in duplicate and all standard curve points were run in triplicate. Each 96-well plate contained a standard curve and a pooled cDNA sample for 18S. These data were used to calculate an intra and interplate coefficient of variation (CV; Table 3). The

remaining wells were used for GOI samples. Each plate for a GOI contained a cDNA pool sample so that an interplate CV could be determined for each GOI (Table 2).

Statistical Analysis

All statistical analyses were performed using Proc Mixed procedures in SAS (v.8.2; SAS Institute, 1999). The level of significance was set at $P < 0.05$ for main effects and interactions. For milk yield and composition analyses, data collected from both udder halves from d 67 to 61 before expected parturition date were used as a covariate. The covariate was used due to yield and composition differences between udder halves within a cow before any treatments for the current study were applied. A covariate was not used for DMI, Ki67, TUNEL, and gene expression data analyses. Data from Ki67 and TUNEL assays were transformed (square root) to meet statistical assumptions for normal data distribution. Nontransformed data are presented in tables and figures, because the outcome of statistical tests with transformed and nontransformed data were equal. Additionally, SCS was calculated from SCC to achieve normal distribution of the data; the SCS values were used for all statistical comparisons. Statistical models for analysis of milk yield and composition, Ki67 index, and apoptotic index included main effects of bST, dry period length (CM or control), and time (week relative to parturition or day of biopsy relative to parturition), and their respective interactions. Time was fit as a

repeated measure using a spatial power covariance structure. The variability among udder halves (nested within cow) within experiment cells was used to test the whole-plot effects of bST, dry period length, and their interactions. The variability among data for dependent variables within udder half (nested within cow) was used to test for the effects of time and interactions involving time. Statistical analysis of ΔC_t values from real-time RT-PCR assays included independent variables of dry period length and gestation status (pre- vs. postpartum) and the interaction. Time (gestation status) was fit as a repeated measure using a first-order autoregressive covariance structure. Analysis of DMI included bST, time, and the interaction of bST by time as independent variables. Time was fit as a repeated measure using a first-order autoregressive covariance structure. The variability among cows (nested within treatment) within experiment cells was used to test the whole plot effects of bST. The variability among weekly DMI within animal was used to test for the effects of time and interactions involving time.

RESULTS

DMI

Dry matter intake was not affected by bST treatment; DMI decreased ($P < 0.05$) gradually during the final 4 wk of gestation, reaching nadir at 1 wk before parturition (data not shown). During the first 4 wk postpartum, DMI steadily increased ($P < 0.01$; data not shown).

Milk Yield and Composition

Prepartum half-udder milk yield in the CM halves was 21% greater ($P < 0.05$) in bST-treated cows compared with nontreated cows (Table 3 and Figure 2). Spontaneous dry-off occurred in both +bST and -bST CM halves and resulted in similar days dry for both treatments (5.6 ± 2.2 and 3.1 ± 1.5 d dry, respectively). Individual animal data for spontaneous dry-off are presented in Table 4. Postpartum half-udder milk yield was dramatically reduced (53%, $P < 0.001$) in CM halves compared with control halves, but was unaltered by bST treatment (Table 3 and Figure 2). The temporal pattern of early-lactation milk yield was similar in CM and control halves, except CM halves had decreased yields (Figure 2).

During late gestation, milk fat content decreased 12.8% ($P < 0.05$) in CM halves from cows treated with bST (Table 3). No other milk composition variables were altered by bST treatment. Milk protein content was increased ($P < 0.001$) throughout late gestation, reaching peak levels at 1 to 2 wk prepartum (data not shown). Postpartum milk composition was not affected by any

Figure 2. Temporal pattern of milk yield in continuously milked (CM, $n = 8$) and control (60-d dry; $n = 8$) udder halves during late gestation and early lactation. Milk yield during wk -8 to -1 was increased ($P < 0.05$) by bST. Postpartum milk yield was decreased ($P < 0.001$) by continuous milking and was unaltered ($P > 0.8$) by bST supplementation. ○ = 60-d dry, no bST; ■ = 60-d dry, bST; □ = CM, no bST; ● = CM, bST.

of the independent variables or their respective interactions (Table 3). Of particular interest, SCS was not different in CM and control halves, indicating similar mammary health.

Immunohistochemistry

Proliferation and apoptosis indices were not altered ($P > 0.1$) by bST administration. Proliferation of MEC was greater ($P < 0.01$) during late gestation (d -20 and -7) than during early lactation, regardless of dry period treatment (control vs. CM; Table 5). In control udder halves, MEC proliferation was increased during prepartum timepoints and peaked at 8 d prepartum with 5.4% of MEC expressing the Ki67 antigen (Table 5). In CM udder halves, MEC proliferation peaked at d -20 and declined thereafter. At d -8, MEC proliferation was enhanced 2-fold in control compared with CM glands (Table 5).

In both control and CM tissue, apoptosis was greater ($P < 0.05$) during the first week of the subsequent lactation (d 1 and 7) compared with prepartum timepoints and 20 d postpartum. In control udder halves, MEC apoptosis increased during late gestation and into early lactation, until peaking ($P < 0.01$) at d 7 (Table 5). At d 20, the apoptotic index had returned to levels observed at d -20 (Table 5). In CM udder halves, MEC apoptosis peaked at d 1 postpartum, but was unchanged at all other timepoints (Table 5). At d 7 postpartum, MEC apoptosis was 60% greater in tissue from control glands compared with CM glands (Table 5).

Table 4. Summary of spontaneous dry-off data for individual animals¹

Cow ID	bST	Half-udder milk yield at 60 d before expected calving date (kg/d)		DIM at 60 d before expected calving	Days dry in CM udder-half	Half-udder milk yield at spontaneous dry-off (kg/d)
		Control	CM			
338	+	11.4	10.9	444	9	0.27
359	+	10.2	9.1	387	4.5	0.32
377	+	11.9	11.5	332	0	—
401	+	15.1	14.9	285	9	0.36
409	—	12.3	15.1	353	1.5	1.86
412	—	10.0	9.1	315	0	—
413	—	10.0	10.7	291	4.5	0.10
416	—	10.7	8.9	342	6.5	0.45

¹Control = 60-d dry; CM = continuously milked.

Ultrastructure

There were no apparent changes in mammary ultrastructure as a result of bST supplementation at any of the timepoints evaluated. Alveoli and MEC within alveoli are shown and described in Figures 3 to 5.

At d -20, control tissue consisted of a homogeneous population of resting alveoli (not shown). The MEC within the alveoli were devoid of milk synthesis products except for an occasional lipid droplet. Nuclei were irregular in shape, centrally located, and occupied much of the intracellular area. Cell organelles [rough endoplasmic reticulum (**RER**) and mitochondria] were present in the cytoplasm, but were less abundant and organized than observed in lactating tissue. At this same timepoint, CM tissue consisted of both lactating alveoli and resting alveoli with features equal to those described for control tissue. Lactating (secretory) alveoli contained MEC with distinct polarization; basolaterally positioned nuclei and apically positioned lipid droplets and other secretory vesicles (not shown). The cytoplasm was densely populated with organelles associated with

milk synthesis; RER, mitochondria, and Golgi. Lumina contained lipid droplets and casein micelles.

By d -8, MEC in control tissue appeared to be responding to lactogenic signals and starting to differentiate toward a secretory phenotype (Figure 3, panels A and B). Cells lacked established polarity, but contained large lipid droplets and the lumina contained both lipid and casein. Cell organelles were more abundant than observed at d -20. Tissue from CM halves contained both lactating (Figure 3 C) and immature (Figure 3D) alveoli at d -8. Immature alveoli were more prevalent and with similar structures as those reported for control tissue. Also at this timepoint, macrophages and neutrophils were observed within the mammary epithelium for both dry period treatments (not shown).

Ultrastructure at 1 and 7 d was similar for control and CM treatments (Figure 4, panels A to D). Tissue from both treatments contained secretory and immature alveoli. Lactating alveoli (Figure 4, panels A and B) were densely packed with cellular organelles and apically located secretory vesicles and lipid droplets,

Table 5. Changes in mammary epithelial cell (MEC) proliferation and apoptosis during late gestation and early lactation in 60-d dry (control) and continuously milked (CM) udder halves¹

Variable	Day relative to parturition					SE
	-20	-8	1	7	20	
Proliferation, ² %						
Control	4.00 ^a	5.40 ^{by}	1.17 ^c	0.67 ^c	0.35 ^c	0.50
CM	3.74 ^a	2.70 ^{az}	1.04 ^b	0.46 ^b	0.27 ^b	0.49
Apoptosis, ² %						
Control	0.17 ^a	0.33 ^{ab}	0.56 ^b	0.67 ^{by}	0.17 ^a	0.09
CM	0.16 ^a	0.20 ^a	0.80 ^b	0.27 ^{az}	0.20 ^a	0.09

^{a-c}Within a treatment (row) differences over time are indicated by different superscripts ($P < 0.05$).

^{y,z}Differences between treatments (column) are indicated by different superscripts ($P < 0.05$).

¹Proliferation was determined by Ki67 antigen expression and apoptosis was determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. Data are means from 16 udder halves, 8 halves for each dry period treatment.

²Percentage of MEC counted that were proliferating or apoptotic.

Figure 3. Micrograph of an alveolus and cells (A, B) representative of tissue sampled 8 d prepartum from an udder half dried 52 d earlier and of lactating alveoli (C) and an immature alveolus (D) from a continuously milked udder half at 8 d prepartum. C = casein micelle; G = Golgi; LLD = large lipid droplet; M = mitochondria; N = nucleus; RER = rough endoplasmic reticulum; SLD = small lipid droplet.

which may be indicative of high synthetic capacity (Figure 4A). Rough endoplasmic reticulum was most prevalent in the perinuclear space, Golgi were apical of the nucleus and adjacent to lipid or secretory vesicles, and mitochondria were dispersed throughout the cytoplasm. The immature alveoli (Figure 4, panels C and D) were equivalent to those described at d -8. Some immature alveoli had spaces between cells indicating a lack of cell-to-cell junctions (tight junctions, desmosomes; Figure 4D). Lumina contained whole MEC, neutrophils with phagocytic vesicles, and cellular debris at d 1 and 7 (Figure 4B). Luminal structures in Figure 4D are neutrophils or macrophages engorged with casein micelles, lipid droplets, and cell debris.

By 20 d, there were marked differences in the alveolar types present in control and CM tissue. Control tissue largely consisted of fully secretory alveoli (Figure 5, panels A and B). The alveoli were composed primarily of MEC with distinct polarity, abundant lipid and secretory vesicles at the apical membrane, RER densely packed in the perinuclear space, mitochondria throughout the cytoplasm, and Golgi adjacent to both the nucleus and lipid or secretory vesicles. However, CM tissue was comprised of heterogeneous populations of se-

cretory, engorged, and resting alveoli (Figure 5, panels C to F). Secretory alveoli (Figure 5, panels A and B) were equal to those described in control tissue. Mammary epithelial cells within engorged alveoli were characterized by the presence of very large lipid droplets or vacuoles, loss of polarity, irregular shaped nuclei, and numerous migrating cells infiltrating the epithelium (Figure 5, panels E and F). Resting alveoli consisted of MEC with no structural evidence of milk synthesis or secretion and had lost polarity. Some resting alveoli contained apoptotic MEC (Figure 5, panels C and D). Lumina of resting alveoli were also devoid of secretory products.

Real-Time RT-PCR

The quantity of transcripts for the housekeeping gene, ribosomal 18S subunit, was not changed by any of the main effects or their respective interactions ($P \geq 0.12$); thus, data normalization to the 18S housekeeping gene was appropriate. There was no effect of bST or respective interactions on expression of any of the GOI evaluated. Therefore, data are summarized for dry period length and gestation status, but not by bST treat-

Figure 4. Micrograph of lactating (A), lactating with cellular debris in lumen (B), and immature (C, D) alveoli and cells representative of tissue from udder halves at 1 and 7 d postpartum. Dry period treatment had no apparent effect on histology at d 1 and 7 postpartum. CD = cellular debris; L = lipid; LLD = large lipid droplet; LJ = leaky cell-to-cell junctions; MP = macrophage engorged with cell debris and milk components; N = nucleus; NT = neutrophil with engulfed cell debris and milk components; SV = secretory vesicle; WMC = whole mammary epithelial cell.

ment. Expression of α -LA was dependent on ($P < 0.05$) dry period length, gestation status (prepartum vs. postpartum), and dry period length by gestation status (Table 6). Expression of α -LA was greater (27.7-fold; $P < 0.05$) in CM than in control tissue during the prepartum period (Figure 6, Table 6), but did not differ in the postpartum period (Figure 6, Table 6). Quantity of α -LA transcripts was less in prepartum CM tissue than postpartum control tissue, but similar to postpartum CM levels (Figure 6). Of the cell cycle or mammary development genes, ABC1 and p27 were not affected ($P > 0.1$) by dry period length, gestation status, or their interaction (Figures 6 and 7, Table 6). Cyclin D1 and CEBP- β expression were greater ($P < 0.03$) during the prepartum period than the postpartum period, but were not affected by dry period length (Figure 7, Table 6). Of the apoptosis-related genes evaluated, differential expression was only detected for bcl₂, which increased 2.6-fold in prepartum tissue compared with postpartum tissue (Figure 6, Table 6).

DISCUSSION

DMI

Dry matter intake was measured during the current study as a means of estimating the effect of eliminating or minimizing diet changes during late gestation on intake during the periparturient period. As long as the CM half was milking, cows were fed a lactating diet and cows that spontaneously dried the CM half before parturition were switched to a close-up, dry cow diet until parturition. During the last 3 wk of gestation the decline in DMI was only 19%, compared with 30 to 35% reductions regularly observed prepartum (Grummer, 1995). Furthermore, DMI was in excess of 15 kg/d during the last 4 wk of gestation and averaged 21.5 kg/d during the first 30 d of the subsequent lactation. Treatment with bST did not alter DMI during late gestation or early lactation. Others have reported improved DMI in both 28-d dry and CM cows compared with 56-d dry cows (Rastani et al., 2003) and in 30-d dry cows

Figure 5. Micrograph of a lactating alveolus and cells (A, B) prevalent in control (60-d dry) tissue at d 20. Lactating alveoli were present in continuously milked (CM) tissue, but resting (C, D) and engorged (E, F) alveoli were more prevalent structures in CM tissue at d 20 postpartum. AC = apoptotic mammary epithelial cell spilling cell debris into lumen; C = casein micelle; D = desmosome; G = Golgi; ES = endosome; L = lipid droplet; M = mitochondria; MV = microvilli; N = nucleus; RER = rough endoplasmic reticulum; V = vacuole.

compared with 60-d dry cows (Gulay et al., 2003). Data from these studies suggest that adjustments in nutritional management made to accompany a reduction in dry period length may increase DMI in transition cows, and may improve energy balance parameters during early lactation.

Milk Yield

Prepartum half-udder milk yield was increased by bST treatment and, when extrapolated to a whole udder, bST treatment during late gestation equated to a 3.4 kg/d increase in milk yield during the last 8 wk of

Table 6. Summary of Δ Ct (cycle threshold) values¹ in control (60-d dry) and continuously milked (CM) tissue during prepartum and postpartum periods²

Gene of interest	Prepartum			Postpartum		
	Control	CM	SEM	Control	CM	SEM
α -Lactalbumin	10.1 ^a	5.3 ^b	1.4	3.3 ^c	4.3 ^{bc}	0.52
ABC1	14.2	14.6	0.56	14.9	15.7	0.38
CEBP- β	10.6 ^x	10.8 ^x	0.62	11.7 ^y	12.5 ^y	0.50
Cyclin D1	7.9 ^x	8.5 ^x	0.50	9.5 ^y	9.6 ^y	0.44
p27	14.8	15.8	0.69	15.7	16.1	0.31
bax	10.9	11.5	0.70	11.3	12.6	0.57
bcl ₂	9.0 ^x	9.5 ^x	0.71	10.3 ^y	11.0 ^y	0.39
IGFBP5	12.0	12.3	0.51	12.1	12.7	0.43

^{a-c}Means within a row with different superscripts differ ($P < 0.05$).

^{x,y}Means within a row with different superscripts were different in prepartum vs. postpartum tissue.

¹ Δ Ct = Ct_{GOI} - Ct_{18S}, where GOI = gene of interest; a larger Ct value (more cycles to threshold) means less transcript abundance.

²Half-udders (n = 16) were sampled at 8 and 20 d prepartum and 1, 7, and 20 d postpartum. Gene expression was evaluated by real-time, reverse transcription-PCR using pre- and postpartum sample pools from each udder half. Genes: 18S = ribosomal 18S; ABC1 = ATP-binding cassette 1; CEBP- β = CCAAT/enhancer binding protein- β ; IGFBP5 = IGF-binding protein 5; p27 = kinase inhibitor protein p27.

Figure 6. Relative changes in prepartum and postpartum gene expression in control (60-d dry) tissue compared with continuously milked (CM) tissue. Half-udders ($n = 16$) were used to compare physiological state of the mammary gland with samples taken at 8 and 20 d prepartum and 1, 7, and 20 d postpartum. Gene expression was evaluated by real-time, reverse transcription-PCR using pre- and postpartum sample pools from each udder half. *Indicates a significant change in expression between control and CM tissue. Fold changes in expression were determined by $2^{-\Delta\Delta C_t}$ calculations. Genes: α -lac = α -lactalbumin; ABC1 = ATP-binding cassette 1; CEBP- β = CCAAT/enhancer binding protein- β ; IGFBP5 = IGF-binding protein 5; p27 = kinase inhibitor protein p27.

gestation. However, higher yields in +bST CM halves did not reduce the incidence of spontaneous dry-off in the final 2 wk of gestation, as days dry was similar for both treatments (5.6 ± 2.2 and 3.1 ± 1.5 d dry, respectively). Factors signaling spontaneous dry-off are unknown, but they appear to be regulated by maternal endocrine events near parturition. Estrogen and progesterone concentrations are elevated in the final weeks of gestation and are known inhibitors of milk secretion at high concentrations (Cowie, 1961). Production level or DIM at 60 d before expected parturition did not affect

the likelihood of spontaneous dry-off (Table 4). Additionally, neither CM of one udder-half nor bST administration influenced gestation length (277 ± 4 d).

Postpartum half-udder milk yield was reduced dramatically (53%) in CM compared with control halves, regardless of bST treatment. This is larger than the production losses (15 to 20%) reported in CM primiparous cows (Remond et al., 1992; Rastani et al., 2003; Annen et al., 2004), but similar reductions were reported in some, but not all CM udder-halves in a study by Smith et al. (1967). A reduction of this magnitude

Figure 7. Fold changes in gene expression in prepartum tissue compared with postpartum tissue regardless of dry period length or bST treatment. Half-udders ($n = 16$) were used to compare physiological state of the mammary gland with samples taken at 8 and 20 d prepartum and 1, 7, and 20 d postpartum. Gene expression was evaluated by real-time, reverse transcription-PCR using pre- and postpartum sample pools from each udder half. *Indicates a significant change in expression between control (60-d dry) and continuously milked tissue. Fold changes in expression were determined by $2^{-\Delta\Delta C_t}$ calculations. Genes: α -lac = α -lactalbumin; ABC1 = ATP-binding cassette 1; CEBP- β = CCAAT/enhancer binding protein- β ; IGFBP5 = IGF-binding protein 5; p27 = kinase inhibitor protein p27.

may be a result of decreased animal variability due to the half-udder model used in the current study or an increase in productive capacity of the control half due to some unknown aspect of the half-udder treatments. The control half had an average daily milk yield of 44.7 kg/d if extrapolated to a whole-udder yield. This production level is similar to those achieved by other second-lactation cows in the University of Arizona dairy herd during the first 30 d of lactation. Thus, the most likely cause of such a large decrease in milk yield in the current study compared with other CM studies is the removal of factors (e.g., DMI, genetic potential) responsible for between-animal variation.

MEC Proliferation

Proliferation, apoptosis, and ultrastructure of MEC were all affected by dry period length, and these results provide insight into the 53% reduction in milk yield of CM udder halves. Regardless of dry period length, MEC proliferation was higher 20 and 8 d before parturition compared with 1, 7, and 20 d postpartum. This result was expected because hormonal changes in progesterone, estrogen, growth hormone, prolactin, and glucocorticoids are associated with mammary development and differentiation into a secretory phenotype (Mayer and Klein, 1961; Anderson, 1974; Neville et al., 2002). In control glands, proliferation increased from d -20 and peaked at d -8. However, in CM glands, proliferation

peaked at d -20 and decreased thereafter. At d -20, MEC proliferation was similar in CM and control tissues, which was unexpected because data from Capuco et al. (1997) demonstrated a reduction in MEC proliferation in CM glands at this same time point. Localization of Ki67 methods used in the current study to identify proliferating MEC measure cells in all phases of the cell cycle except G_0 and does not have a rate component. Therefore, it is possible that equal numbers of MEC are progressing through the cell cycle, but the rate at which cells replicate may be enhanced in control glands and result in greater [3H]thymidine labeling (as detected by Capuco et al., 1997). A 50% reduction in MEC proliferation in CM halves at d -8 compared with control halves is greater than the reduction in proliferation in CM glands reported by Capuco et al. (1997) at d -7. Between these 2 experiments, data suggest a 30 to 50% reduction in MEC proliferation in CM glands during late gestation. In addition, CM may alter the number of MEC proliferating or the proliferation rate at d -20. When CM glands are compared with control glands of primiparous cows, the temporal pattern of MEC proliferation indicates that proliferation is decreasing in CM tissue, rather than peaking, at this critical time (d -8, the final phase of gestation) in mammary development.

After parturition, the number of MEC undergoing cell division decreased and was not altered by dry period length. A low proliferation state in the mammary gland following MEC differentiation and the onset of copious

secretion might be expected. Mitotic activity during lactation has previously been reported, but at a reduced frequency compared with nonlactating tissue during pregnancy (Altman, 1945; Mayer and Klein, 1961).

MEC Apoptosis

The other aspect of MEC turnover, apoptosis, has not previously been measured in CM bovine mammary glands. A low percentage of MEC were undergoing apoptosis during late gestation, and apoptotic indexes were similar in control and CM halves. Although both halves demonstrated an increase in MEC apoptosis during early lactation, the increased apoptosis persisted to d 7 in control halves, but decreased by d 7 in CM halves. 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 (Capuco et al., 2001; Hale et al., 2003; Sorensen and Sejrsen, 2003). Four hypotheses have been proposed to explain increased apoptosis during early lactation. They include: 1) an increase in apoptotic leukocytes that have migrated into the mammary epithelium and are indistinguishable from MEC due to morphological changes during apoptosis (Capuco et al., 2001; Hale et al., 2003); 2) shedding of old or dormant MEC from the mammary epithelium that were replaced with new MEC generated during late gestation, which may enable atrophy and increase milk synthesis in the remaining, new MEC (Sejrsen et al., 2003); 3) elimination of new cells that were generated during late gestation, but did not differentiate during the final stage of lactogenesis (Sejrsen et al., 2003); and 4) apoptosis of cells with errors incurred during DNA replication (Alberts et al., 2002). Prior to TUNEL assay methodology, increased cell loss during early lactation was indicated by other measures. Tucker (1969) reported high levels of MEC lost in milk during the first 1 to 2 wk of lactation, which then subsided until about wk 35 of lactation. Increased DNA degradation products (Kuretani, 1957, cited by Munford, 1964) and increased level of deoxyribonuclease (Griffith and Turner, 1958) in mammary tissue homogenates from the beginning of lactation compared with tissue taken later in lactation has also been reported.

In CM udder halves, a rapid decline in apoptosis after d 1 combined with reduced MEC proliferation at d -8 suggests that CM halves kept more old cells in the mammary epithelium and maintained total cell numbers. Total cell number, as measured by total parenchymal DNA, is not altered by lactation status during late gestation in multiparous cows (Swanson et al., 1967; Capuco et al., 1997). The latter authors proposed that a reduction in proliferation accompanied by a decrease

in apoptosis would maintain total MEC numbers, resulting in an increased proportion of old MEC in CM glands (Capuco et al., 1997). The bovine mammary gland may regulate apoptosis during early lactation in proportion to the number of new (replacement) MEC generated during late gestation, which may explain why CM has not been shown to reduce total MEC number.

Effect of bST on MEC Turnover

We did not detect an effect of bST or an interaction of dry period length and bST on MEC proliferation or apoptosis. Treating lactating cows with bST has been shown to increase MEC proliferation (Capuco et al., 2001), but this increase did not exceed mammary regression by apoptosis. This would agree with data showing no net growth of lactating bovine mammary tissue in bST-treated cows (Binelli et al., 1995; Capuco et al., 1995). Others have demonstrated that intramammary bST or IGF-I infusion in late-pregnant heifers was initially mammatogenic, but did not improve future milk yield (Collier et al., 2002). Furthermore, bST treatment during the dry period has been evaluated, but did not affect subsequent milk yield (Bachman et al., 1992; Simmons et al., 1994). A reduction in apoptosis as a result of bST treatment was expected due to reports of reduced mammary plasmin levels (Bauman and Vernon, 1993) and antiapoptotic effects of IGF-I (Hadsell et al., 2002), which is elevated in bST-supplemented animals (Vicini et al., 1991). Bovine somatotropin did not affect MEC survival in control or CM udder-halves in the current study, which is consistent with previous reports (Capuco et al., 2001). It is possible that antiapoptotic effects of IGF-I were not observed in tissue from +bST cows at d 1 and 7 due to reductions in plasma IGF-I concentration during early lactation regardless of bST treatment (Vicini et al., 1991).

Mammary Development in Late Gestation and Early Lactation

As previously mentioned, MEC proliferation and apoptosis data in the control udder-half provide insight into the timing of mammary growth and regression during a typical lactation cycle in dairy cows. Pregnancy, especially late pregnancy, has been well characterized as a period of extensive mammary growth and development (Munford, 1964; Tucker, 1969; Anderson, 1974). Anderson (1975) reported that 78% of ewe mammary growth occurred during pregnancy and 2% during early lactation. Previous research in nonlactating, pregnant animals (rats, mice, guinea pig, pig, goat) indicates that mammary DNA and number of alveolar cells con-

tinue to increase during early lactation (Munford, 1964; Tucker, 1966; Traurig, 1967; Knight and Peaker, 1984; Kim et al., 1999). Similar to the current study, Baldwin (1966) and Anderson (1975) reported minimal mammary growth during early lactation in cows and sheep. Formulas developed by Capuco et al. (2001) were used to calculate MEC turnover during the current study. Results from these calculations demonstrate that MEC accumulate during late gestation (d -20 and -8), but during early lactation (d 1 and 7), high apoptotic rates combined with low levels of proliferation support regression of MEC during the first 7 d postpartum and approximately static cell turnover at d 20. Accumulation of new MEC during late gestation and increased MEC shedding during early lactation support the hypothesis that increased milk yield during early lactation in dairy cows is a result of MEC atrophy and increased synthetic capacity (Capuco et al., 2001), rather than a combination of increased mammary development and secretory activity (Knight and Peaker, 1984). An increase in the RNA to DNA ratio during early lactation, combined with minimal mammary growth during early lactation (Anderson, 1975), supports the hypothesis of increased synthetic capacity of MEC being the primary driver of increasing milk yield during early lactation in cows and ewes.

Ultrastructure

Ultrastructure data from the current study provides evidence on the fate of MEC that are carried over to the subsequent lactation rather than replaced during gestation. Ultrastructure has not previously been evaluated in CM tissue. Descriptions and micrographs of nonlactating tissue and MEC (Larson, 1985; Holst et al., 1987) match nonlactating tissue from the control udder-half at d -20. Less abundant RER and mitochondria, rare Golgi, and an occasional lipid droplet were present in MEC from control tissue and lumina of resting alveoli were clear of secretion. In CM tissue, more abundant cell organelles, lipid droplets, secretory vesicles, basolaterally positioned nuclei, and apical orientation of secretory products were apparent and are common features of lactating MEC. Fewer lipid droplets and organelles in some CM alveoli are likely indicative of a lower secretory capacity and progression to a resting state. Decreasing milk yield throughout late gestation corresponds to increasing numbers of lower producing or resting alveoli.

At d -8, lactating alveoli were present in CM glands and immature alveoli were present in both control and CM glands. Immature alveoli appeared to be progressing toward lactating status. Large lipid droplets and increasing RER and mitochondria within MEC

with no established polarity, as well as the presence of secretory products (lipid, casein micelles) in expanded lumens are features of these immature alveoli and typical of the appearance of mammary tissue during lactogenesis (Akers and Heald, 1978; Sordillo and Nickerson, 1988). Lactating alveoli in CM tissue at d -8 appeared similar to lower producing alveoli at d -20, which coincided with lower milk yields reported during the last week of gestation and spontaneous dry-off of many CM halves. Additionally, migrating leukocytes appearing within the mammary epithelium became more frequent at this time. Macrophages and neutrophils have been reported to increase during the early dry period, decrease after involution, and increase again at parturition (Sordillo and Nickerson, 1988; Fetherston et al., 2001). Macrophages are thought to be the prevalent leukocyte in healthy mammary tissue, and intraepithelial neutrophils are thought to be triggered by an inflammatory response (Sordillo et al., 1997) and other associated factors (cytokines and prostaglandins; Sordillo et al., 1997). Both cell types can ingest accumulated milk components, cellular debris, and bacteria. Elevation of inflammatory factors at parturition (i.e., prostaglandins; Maule-Walker and Peaker, 1980) combined with accumulation of milk products within MEC and lumina of immature alveoli, permeability of the blood-milk barrier (Linzell and Peaker, 1973, 1974) and udder edema may facilitate intraepithelial infiltration of both macrophages and neutrophils at timepoints surrounding parturition. The biopsy procedure cannot be ruled out as a potential cause for increased neutrophils in mammary tissue. The mammary biopsy procedure likely induces an inflammatory response due to tissue insult at the biopsy site. Transient biopsies were obtained from all 4 quarters to enable a period of healing before a second or third biopsy was obtained from a given quarter. Additionally, careful attention was paid to biopsy site selection to avoid previously biopsied areas. Therefore, it is not likely that increased prevalence of neutrophils was due to biopsy. Rear quarter biopsies were taken at d -20, 1, and 20 (20 d between biopsies). Front quarter biopsies were obtained at d -8 and 7 (14 d between biopsies). Subcutaneous injections of recombinant colony-stimulating factor (cytokines) elevated milk neutrophils for 15 d compared with controls (Fetherston et al., 2001), indicating that repeat biopsy timing in the current study was within the ranges for an inflammatory response to occur and subside before the next biopsy. Increased prevalence of migrating cells at parturition and during early lactation has been reported by others (Sordillo and Nickerson, 1988; Fetherston et al., 2001) and these cells have also been referred to as colostrum cells or cells of Donn (Mayer and Klein, 1961).

During the postpartum period, the mammary epithelium had similar structural characteristics at d 1 and 7, but dramatic differences between control and CM tissues were apparent by d 20. At d 1 and 7, MEC from both control and CM glands appeared to be secretory or differentiating into fully secretory cells. The presence of immature alveoli and MEC during this first week of lactation suggests that secretory activation or stage II lactogenesis is not complete in cattle until after parturition. In humans, secretory activation does not occur until 4 d postpartum, but is complete at parturition in rodents (Neville et al., 2002). These ultrastructure data from d 1 and 7 suggest that increasing milk yield during early lactation is not only the result of increased secretory capacity of MEC, but also due to increasing numbers of alveoli completing activation and contributing to milk synthesis.

From the onset of copious milk secretion until the declining phase of lactation, maintenance of highly secretory alveoli is expected and increasing numbers of resting alveoli are expected during the decline phase of lactation (Knight and Peaker, 1984). Interestingly, at d 20 postpartum, control halves were mainly composed of secretory alveoli, but CM halves contained large populations of engorged and resting alveoli in addition to secretory alveoli. Due to the similarity of engorged alveoli in the current study and alveoli of involuting mammary tissue (Holst et al., 1987; E. L. Annen, A. V. Capuco, P. C. Gentry, and R. J. Collier; unpublished data), we hypothesized that MEC from these alveoli are entering a resting state and may undergo apoptosis. In the absence of an increase in MEC apoptosis at this time, it is more likely these engorged alveoli will enter a resting state and not undergo cell death, although some apoptotic cells were observed in resting alveoli (Figure 5, panels C and D). Additionally, we hypothesize that increased prevalence of engorged and resting alveoli as early as 20 d into the subsequent lactation are caused by the carryover of MEC from one lactation to the next in CM glands and these old cells enter a resting state during early lactation rather than during the decline phase of lactation. Migrating cells were still evident in mammary tissue from d 20, but the occurrence was more frequent in engorged and resting alveoli where phagocytosis of milk products and cellular debris was needed.

Mammary Gene Expression

To further investigate effects of CM on MEC turnover and function during late gestation and early lactation, expression of genes involved in milk synthesis, mammary growth, cell cycle regulation, and apoptosis were evaluated. α -Lactalbumin was evaluated because it is

a key enzyme in lactose synthesis and should be differentially expressed in lactating vs. nonlactating tissue. Accordingly, prepartum α -LA expression decreased 27-fold in control tissue compared with CM tissue. During the postpartum period, α -LA expression was not altered by dry period treatment. When evaluating the main effect of prepartum vs. postpartum, α -LA expression was less during the prepartum period, but this was largely due to the fact that α -LA expression was low in nonlactating, control tissue during the prepartum period.

A group of genes associated with MEC proliferation and mammary development were evaluated. Cyclin D1 synthesis increases in response to growth factor stimulation to initiate proliferation in quiescent cells. It begins to increase during the G₀ to G₁ transition and peaks during the mid-G₁ phase of the cell cycle (Donjerkovic and Scott, 2000). Cyclin D1 coordinates extracellular signals and cell cycle progression toward the G₁ to S phase transition by binding cyclin-dependent kinases or by binding histone acetylases and deacetylases to modify chromatin structure of other genes involved in cell proliferation and differentiation (Fu et al., 2004). We hypothesized that cyclin D1 expression during prepartum mammary development would be reduced by CM; however, dry period length did not affect cyclin D1 expression in either the prepartum or postpartum period. Cyclin D1 expression was higher during the prepartum period than the postpartum period. This increased transcription of cyclin D1 corresponds to a period of increased Ki67 expression during late gestation. Cyclin D1 is known to be responsive to estrogen and progesterone (Sutherland and Musgrove, 2004) and both of these hormones are known to be major regulators of mammary ductal and lobulo-alveolar development (Neville et al., 2002). Thus, it is not surprising that cyclin D1 expression was greater during late gestation than early lactation. Although data from the current study suggest that cyclin D1 mRNA expression was not limiting cell proliferation in CM tissue, it is possible that cyclin D1 function in CM tissue was altered by posttranscriptional modifications. Results from the current study demonstrate low levels of p27 expression [blocks cell cycle progression to S-phase (Musgrove et al., 2004)] in pre- and postpartum tissue regardless of dry period treatment. This gene is primarily regulated at the protein level (Musgrove et al., 2004), so changes in protein stability and resulting changes in p27 levels in CM and control tissue cannot be ruled out.

Knockout of all CEBP- β isoforms severely reduces lobulo-alveolar development, and partially impairs ductal growth and differentiation (Robinson et al., 1998; Seagroves et al., 1998). Due to suggestive evidence for impaired mammary development between first and sec-

and lactations in CM cows (Annen et al., 2004), it was believed that CEBP- β expression would be reduced in CM tissue. However, CEBP- β expression was not altered by CM, but was up-regulated in prepartum compared with postpartum tissue. This suggests that CEBP- β plays a role in bovine mammary development during late gestation, but is not limiting mammary development in CM cows. A recent study demonstrated that cyclin D1 may interact with CEBP- β (Lamb and Ewen, 2003); given the potential relationship between these 2 molecules, it is intriguing that both genes were up-regulated to a similar extent during late gestation.

A group of apoptosis-related genes was also evaluated. Bax is a proapoptotic member of the bcl₂ family of genes, of which bcl₂ is a prosurvival member (Haughn and Hockenbery, 2003). Bax is thought to induce apoptosis by translocating to the mitochondria and facilitating cytochrome c release by forming pores in the mitochondrial membrane (Breckenridge and Shore, 2003). Once cytochrome c is released it associates with procaspase-9 and APAF-1 to form an apoptosome that initiates a caspase cascade and commits cells to death (Breckenridge and Shore, 2003). Bcl₂ is thought to protect cells from apoptosis by preventing bax translocation to the mitochondria (Haughn and Hockenbery, 2003). Expression of bax was constant in pre- and postpartum tissue regardless of dry period treatment. However, bcl₂ expression was greater in prepartum tissue and may serve to protect MEC from apoptosis during late gestation mammogenesis. Apoptosis of MEC was increased in postpartum tissue, which corresponds to a period of lower expression of prosurvival bcl₂. Mammary development during late gestation involves a period of increased MEC proliferation and may require an increase in the bcl₂:bax ratio as another means of accumulating MEC in preparation for the ensuing lactation. Bcl₂ and bax expression have been measured in bovine mammary tissue at peak lactation, but have not been compared in pre- and postpartum tissues. Reports from early lactation are limited to the current study.

Insulin-like growth factor binding protein 5 is another factor associated with increased MEC apoptosis (Allan et al., 2004). Mammary epithelial cell IGFBP5 production is increased during mammary involution, a period of increased MEC apoptosis (Tonner et al., 1997). It is thought that elevated levels of IGFBP5 bind IGF-I and block its cell survival properties by inhibiting the serine threonine kinase (Akt) pathway (Tonner et al., 1997). In our study, IGFBP5 was not altered by gestation status (pre- vs. postpartum) or dry period treatment. We anticipated that IGFBP5 would increase during early lactation because this was a period of increased apoptosis. This suggests that during the first weeks of lactation, increased IGFBP5 expression is not

needed to attenuate the survival properties of IGF-I, because plasma IGF-I levels are reduced during early lactation (Vicini et al., 1991).

A stem cell population has been identified in mammary tissue, as transplant studies have demonstrated that a fully developed gland forms from progeny of a single cell (Kordon and Smith, 1998). The mammary gland contains 3 types of progenitor cells: those that are pluripotent and represent fully competent adult stem cells, those that form ductal structures, and those that form lobulo-alveolar structures (Smith, 1996). It has been proposed that the dry period may be required for replenishment of mammary stem and progenitor cells (Capuco and Akers, 1999). We evaluated the effects of omitting the dry period on expression of ABC1, a proposed marker for stem cells (Scotto, 2003). The ABC1 gene is in the same family as ATP-binding cassette G₂ (ABCG₂) and has the same ATP-binding cassette and presumably similar transport function (Scotto, 2003). Because ABCG₂ mRNA increases markedly (approximately 25-fold) during lactation, it was deemed unsuitable as a marker for stem cells during the periparturient period (A. V. Capuco, unpublished data). ABC1 and other genes that correlate with stem cell activity in mammary gland or other tissues (e.g., telomerase, Notch 3, CXCR) increased or tended to increase during the dry period (A. V. Capuco, unpublished data). As expected, ABC1 was expressed at very low levels at all timepoints studied, as side population cells make up less than 0.5% of the mammary epithelium (Alvi et al., 2003). There were no differences in ABC1 expression between CM and control tissue or between pre- and postpartum tissue. These data suggest that CM did not inhibit replenishment of mammary stem cells. It is also possible, that effects of stem cell replenishment during the dry period were specific to certain timepoints, had occurred before tissue sampling, or were limited to specific categories of progenitor cells. Pooling procedures used in the current study may have masked time-specific changes and measurement of steady-state levels of mRNA in whole-tissue RNA extracts may not have been sensitive enough to detect differences in a small subpopulation of cells.

CONCLUSIONS

This experiment was designed to study cell proliferation and apoptosis during the periparturient period (late gestation and early lactation) and the impact of CM on these processes. Additionally, bST was evaluated as a potential treatment to restore expected milk yield reductions in CM glands from primiparous cows. Continuously milked glands typically contained many resting and engorged alveoli rather than a majority of

secretory alveoli at d 20 postpartum. Negative effects of CM on MEC turnover and milk yield in primiparous cows were not overcome by bST supplementation. The cell turnover, ultrastructure, and production data support a hypothesis of increased carryover of old MEC to the subsequent lactation in CM glands. This study also supports the concept that the study of genes regulating MEC turnover may require methodology, such as laser capture dissection, that enables analysis of only specific cell types or categories of cells (proliferating, apoptotic, or stem cells) that may make up very small percentages of total mammary cells or MEC.

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