The effect of milking reinitiation following extended nonmilking periods on lactation in primiparous dairy cows

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ABSTRACT

In dairy cows, extended periods of nonmilking results in reduced milk secretion, modifications in milk composition, and eventually involution of the mammary glands. The aim of this study was to determine the effect of extended nonmilking periods on the recovery of milk yield and composition, and levels of prolactin and insulin-like growth factor-I in pasture-fed cows after resuming milking. Pasture-fed, primiparous, non-pregnant, Friesian dairy cows at mid lactation (mean $\pm$ standard deviation, 97 ± 2 d in milk, 14.0 ± 2.5 L/d) were divided into 3 groups ($n = 6$ per group). The cows were subjected to nonmilking periods of 7, 14, or 28 d. Twice-daily milking was resumed for 7 d following the nonmilking periods. Milk yield recoveries at the end of the 7-d remilking period were 91, 51, and 29% for the 7, 14, and 28-d nonmilking groups, respectively. The somatic cell count declined to less than 400,000 cells/mL by d 3 and 6 of remilking for the 7- and 14-d-nonmilking groups, respectively, but remained greater than 800,000 cells/mL in the 28-d-nonmilking group through the 7-d remilking period. By d 7 of remilking, the somatic cell count for the 7-d-nonmilking group was not different from pretrial values. Upon remilking, the milk fat content returned to pretrial values for the 7- and 14-d-nonmilking groups, although it remained lower than pretrial for the 28-d-nonmilking group. All 3 nonmilking groups had a higher milk protein content following 7 d of remilking, compared with pretrial values. The lactose content returned to pretrial values for the 7-d-nonmilking group but remained lower for the 14- and 28-d-nonmilking groups. Circulating prolactin concentrations increased once remilking was resumed, compared with the pretrial and nonmilking periods. Prolactin concentrations did not majorly differ between the groups, with the levels upon 7 d of remilking remaining higher than the pretrial concentrations and the nonmilking periods. Plasma concentrations of insulin-

like growth factor-I increased during the nonmilking period and were greater in all 3 nonmilking groups on d 1 of remilking than pretrial values and returned to pretrial concentrations following remilking for the 7-d-nonmilking group, whereas the 14- and 28-d-nonmilking groups remained higher than the pretrial values. These data indicate that the process of involution is fully reversed after remilking following 7 d of milk stasis but more extended periods of nonmilking prevent the complete recovery of lactation. However, even after 28 d of milk stasis, the milk synthesis capacity of the mammary gland could still be partially recovered.

Key words: milk production, involution, mammary gland, dairy cow

INTRODUCTION

In dairy cows, the cessation of milk removal for extended periods, such as while drying off, initiates the process of mammary gland involution, resulting in a decline of mammary secretions and changes in the composition of the secretion (Hurley, 1989). This may be reversible upon remilking following extended nonmilking periods (Noble and Hurley, 1999). Because of the dairy management system in New Zealand (seasonal, pasture based), farms are vulnerable to adverse weather conditions and calamities such as flooding, preventing cows from being milked for extended periods of time, sometimes for up to several days, and thus, it is of interest to understand the duration of nonmilking and reversibility of mammary gland involution. The extent to which mammary gland involution is reversible varies among species. In rodents, the cessation of milk removal results in involution of the mammary gland in 2 distinct stages. During the first stage, mammary engorgement leads to a rapid reduction of milk synthesis and secretion and the initiation of apoptosis. The second stage begins 72 to 96 h after mammary engorgement and involves further apoptosis, degradation of the extracellular matrix, and structural degradation of alveoli with almost complete loss of mammary epithelial cells (Jaggi et al., 1996; Lund et al., 1996; Li et al., 1997). This regression results ultimately in irreversible involution

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during the second stage. However, this process is reversible within the first 48 h (Jaggi et al., 1996; Li et al., 1997; McMahon et al., 2004). The reversible nature of the initial stages of involution in the rodent mammary gland has been demonstrated following resuckling of engorged rat mammary glands (McMahon et al., 2004).

In contrast to the rodent mammary gland, involution in cows does not occur to the same extent (Capuco and Akers, 1999; Wilde et al., 1999). The cessation of milk removal is followed by changes occurring within 16 to 18 h of milk accumulation, which includes distension of the mammary gland, a decline in the rate of milk secretion, an increase in intramammary pressure, a decrease in mammary blood flow, increased tight-junction permeability and lactose efflux, and an inflammatory response (Hurley, 1989; Davis et al., 1999). Milk secretion completely ceases by approximately 30 h after milking (Hurley, 1989). Milk protein gene expression declines (Goodman and Schanbacher, 1991) as early as 24 h after milking (Singh et al., 2008), and apoptosis of mammary epithelial cells increases (Wilde et al., 1997), which may occur by 72 h after milking (Singh et al., 2005), although not to the same extent as in rodent mammary glands. Morphological changes in the mammary gland occur within 7 d of nonmilking, demonstrated by a reduction in luminal alveolar area and an increase in the stromal area, as secretions are absorbed (Holst et al., 1987; Hurley, 1989). The rate of involution becomes more gradual, and by d 21 to 30 of nonmilking extensive remodeling of the mammary gland has occurred in preparation for the next lactation (Holst et al., 1987; Hurley, 1989; Capuco and Akers, 1999). However, unlike in rodents, many alveolar structures are retained (Holst et al., 1987; Hurley, 1989), which may allow for reversibility of involution following extended nonmilking periods in cows (Noble and Hurley, 1999). Milk yield can be fully restored upon remilking following nonmilking periods of up to 7 d (Farr et al., 1998; Dalley and Davis, 2006). However, only a partial recovery of milk yield was observed following an 11-d-nonmilking interval (Noble and Hurley, 1999) although a longer remilking period may have allowed for a greater milk yield recovery. Local intramammary signals are responsible for initiating involution and the loss of mammary epithelial cells by apoptosis (Li et al., 1997). The primary signal initiating the involution process is unknown but several mechanisms have been postulated including the accumulation in milk of regulatory factors such as putative feed-back inhibitory factor (Wilde et al., 1999), milk casein fractions (Shamay et al., 2003) and serotonin (Collier et al., 2012). Alternatively, physical distension of the mammary gland that results in changes in cell shape may activate mechanotransduction pathways, resulting in

loss of tight junction integrity and cell-extracellular survival signaling (Davis et al., 1999; Stelwagen, 2001). Furthermore, hormonal regulation through the interaction of prolactin (**PRL**) and growth hormone via IGF-I may play a role in regulating mammary function and mammary epithelial cell survival (Flint and Knight, 1997; Accorsi et al., 2002). The aim of this study was to determine the effect of extended nonmilking periods in pasture-fed cows on recovery of milk yield and composition, and PRL and IGF-I levels.

MATERIALS AND METHODS

Animal Experiment

Eighteen nonpregnant, primiparous, Friesian dairy cows in mid lactation (97 ± 2 DIM), during spring and early summer, producing 14.0 ± 2.5 L/d of milk were used in the study. Cows selected for the study were determined to be free of intramammary infection as indicated by negative bacterial cultures and low SCC (mean: 48,980 cells/mL, range: 17,000 to 134,000). Prior to commencement of the experiment, all cows were solely pasture fed and milked twice daily. The experiment consisted of a 7-d pretrial period, a nonmilking treatment period of 3 specified lengths, and a 7-d milking reinitiation period. Cows were randomly assigned to 1 of 3 treatments ($n = 6$ per group) consisting of 7, 14, or 28 d of nonmilking during the nonmilking treatment period. Following cessation of milk removal, all cows were managed as a single herd away from the milking shed and had restricted pasture and additional bulk filler (straw) to facilitate cessation of milk synthesis. Upon reinitiation of milking, cows were monitored for signs of mastitis. To prevent mastitis, a systemic administration antibiotic [Mamyzin, Boehringer Ingelheim (NZ) Ltd., Manukau City, Auckland, New Zealand] was administered to all cows for 3 consecutive days before the nonmilking intervals and to remilking. Secretions were hand stripped from the involuting glands as required and before the cups were placed on the teats to further stimulate reinitiation of lactation. Following the nonmilking period, twice daily milking was reinitiated, and milk production and milk composition was measured for 7 d. The average milk yield in the reference herd of 45 primiparous cows, which were of similar DIM to the trial cows and milked during the trial period, ranged from 14.1 to 17.3 L/d.

Data and Sample Collection

Daily milk yields during the pretrial and milking reinitiation period were electronically recorded (Alfa Laval Cow Management System, Ruakura Dairy,

AgResearch Limited, Hamilton, New Zealand). Milk samples were collected at the morning milking on d 5 to 7 of the pretrial period and at both the morning and evening milkings during d 1 to 7 of the remilking period. Samples were analyzed for fat, protein, and lactose by infrared spectrometry and for SCC by flow cytometry (Fossomatic equipment, LIC Herd Testing Station, Hamilton, New Zealand).

Plasma Hormone Analysis

PRL Analysis. Plasma PRL concentrations were measured in duplicate by RIA as previously described (Pearson et al., 1996). Briefly, bovine PRL was assayed using ovine PRL (NIDDK-oPRL-1–2) for standards and radioiodination, and ovine PRL antiserum (NIDDK-anti-oPRL-2). The PRL was iodinated by the Iodogen method (Pierce, Rockford, IL) using 125 Iodide (New England Nuclear NEZ0033A). The assay method is essentially as prescribed for the NIDDK reagents. Separation of antibody-bound label from free-label was by second antibody precipitation using an excess sheep anti-rabbit serum (generated at the Ruakura Research Centre, Hamilton, New Zealand). Sensitivity was 0.6 ng/mL, and interassay and intraassay coefficients of variation at 10 ng/mL were 11.96 and 8.03%, respectively.

IGF-I Analysis. The IGF-I was extracted from plasma samples as previously described (Breier et al., 1991). Following extraction, IGF-I concentrations were measured by RIA. Bovine IGF-I was assayed using GroPep IGF-I (CM005, Novozymes Biopharma AU Ltd., Thebarton, Australia) and GroPep anti-human IGF-I antiserum. GroPep IGF-I was radioiodinated with 125 I (NEN, NEZ0033A) in a Pierce IODO-GEN Pre-Coated Iodination Tube (Pierce) and purified on a reversed-phase Sep-Pak column (Waters Corporation, Milford, MA). Assays were performed by incubation of standard (recombinant human IGF-I from Ciba-Geigy Ltd., Basel, Switzerland) or sample extracts with tracer and primary antibody [GroPep anti-human IGF-I (Rabbit) antiserum; KAG-PAA2, Novozymes Biopharma AU Ltd.] for 16 h at 4°C. Quality-control samples were included after every 15 samples, and all samples were assayed in duplicate. Separation of bound and free antigen was achieved using a solid-phase second antibody coated cellulose suspension, SacCel anti rabbit IgG (AA-SAC 1, IDS, Bolden, Tyne and Wear, UK). Following 30 min of incubation at 4°C, bound antigen was separated by centrifugation, and the soluble free fraction was removed by aspiration. Radioactivity in the pellet was counted in an LKB 1260 gamma counter (Wallac, Turku, Finland), and calculations were performed using 4-parameter logistics curve-fitting soft-

ware. The sensitivity was 0.37 ng/mL and interassay and intraassay coefficients of variation were 10.4 and 6.1%, respectively.

Statistical Analysis

Pretrial milk production, milk composition, and SCC (log transformed) were used as covariates in the analysis of covariance for reinitiation milk production, milk composition, and SCC (log transformed) data.

Pretrial milk yield for each cow was calculated as the average of 7 d before nonmilking intervals. Pretrial milk composition and SCC (log transformed) were calculated as the average of d 5 to 7 just before nonmilking intervals. Data were analyzed by ANOVA, using pretrial values (described above) as covariates (GenStat, 2011) and reported as means adjusted for covariate with standard errors of difference, unless otherwise stated. In each group, a paired *t*-test was used to compare one time with another. Statistical significance between groups were denoted as **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

RESULTS

Average milk yields and SCC (log transformed), pretrial and throughout the remilking interval, are presented in Figure 1. Milk yield was negligible for some cows on d 1 of remilking and thus not shown. By d 2, the 7-d-nonmilked group had a greater milk yield recovery (*P* < 0.05) than the 14- and 28-d-nonmilked groups. From d 3, all groups were different from each other (*P* < 0.05). By d 6 of remilking, the milk yield recoveries were 91, 51, and 29% for the 7-, 14-, and 28-d-nonmilked groups, respectively, with only the 7-d-nonmilked group not significantly different from pretrial milk yields (Figure 1A). Milk SCC was high and similar for the 3 groups on d 1 of remilking, but by d 2 of remilking all groups were different from each other (*P* < 0.05). The SCC declined to less than 400,000 cells/mL by d 3 and 6 of remilking for the 7- and 14-d-nonmilked groups, respectively, but remained greater than 800,000 cells/mL in the 28-d-nonmilked group. By d 7 remilking, SCC for the 7-d-nonmilked group was not different from pretrial values (Figure 1B). Milk composition for pretrial and remilked samples are presented in Figure 2. The mean $\pm$ standard deviation (SD) pretrial fat content was $3.93 \pm 0.53\%$. For the 7-d-nonmilked group the fat content was higher (*P* < 0.001) than pretrial values only on d 1 of remilking and was similar for the remaining days of remilking. The 14-d-nonmilked group had a similar fat content on d 1 compared with pretrial, was lower on d 2 to 4 (*P* < 0.01), and was again similar to pretrial on d 5 to 7 of remilking. The 28-d-nonmilked

Figure 1. Milk yield (A) and SCC (B) in cows before milk stasis (pretrial, $\blacklozenge$) and during remilking for 7 d following extended nonmilking intervals of 7 (—●—), 14 (---■---), and 28 d (---▲---) ($n = 6$ per group) at mid lactation. Data for milk yields are expressed as means adjusted for pretrial milk yields of 7 d prior, and data for SCC are expressed as back-transformed means of mean log SCC adjusted for pretrial log SCC values of 3 d before the nonmilking period, with bars for standard error of the difference.

group had lower fat content than pretrial on all 7 d of remilking ($P < 0.01$). At d 7 of remilking the fat content for the 28-d-nonmilked group was lower ($P < 0.05$) than for the 7- and 14-d-nonmilked groups (Figure 2A).

The mean $\pm$ SD pretrial protein content was $3.5 \pm 0.2\%$. On d 1 of remilking, compared with pretrial values, the protein content was higher for the 7- ($P < 0.01$) and 14-d-nonmilked groups ($P < 0.05$) and tended to be higher for the 28-d group ($P = 0.06$). At d 2 of remilking, all 3 nonmilked groups had similar protein content to pretrial values. At d 6 of remilking, all groups had higher protein content compared with pretrial ($P < 0.01$), although for the 7- ($P < 0.001$) and 28-d ($P < 0.01$) nonmilked groups, this occurred as early as d 4 of remilking. At d 7 of remilking, the protein content for the 14- and 28-d-nonmilked groups was higher ($P < 0.05$) than the 7-d-nonmilked group (Figure 2B).

The mean $\pm$ SD pretrial lactose content was $5.0 \pm 0.15\%$. The 7-d-nonmilked group had a similar lactose content to pretrial at d 6 and 7 of remilking. However, for all other time points for the 7-d-nonmilked group, and all remilking time points of the 14- and

Figure 2. Milk composition in cows before milk stasis (pretrial, $\blacklozenge$) and during remilking for 7 d following extended nonmilking intervals of 7 (—●—), 14 (---■---), and 28 d (---▲---) ($n = 6$ per group) at mid lactation. Data are expressed as means adjusted for pretrial values of 3 d before the nonmilking period, with bars for standard error of the difference.

28-d-nonmilked groups, the lactose content was lower than pretrial ($P < 0.05$). At d 7 of remilking, the lactose content for the 7- and 14-d-nonmilked groups were similar, although the 28-d-nonmilked group remained lower ($P < 0.05$, Figure 2C).

Plasma PRL concentrations during the pretrial and remilking periods are presented in Figure 3. The mean $\pm$ SD pretrial PRL concentration was 7.8 ± 2.6 ng/mL, which remained the same following 7 d of nonmilking (9.4 ± 3.1 ng/mL) but was increased by 14 and 28 d of nonmilking to 9.5 ± 1.4 ng/mL ($P < 0.01$) and 13.2 ± 3.2 ng/mL ($P < 0.05$), respectively. At d 1 of remilking, all 3 nonmilked groups had higher ($P < 0.01$) PRL concentrations than pretrial (Figure 3) and the nonmilked

period ($P < 0.05$). Prolactin did not differ between the groups at d 1 to 6 of remilking. For all 3 groups, by d 5 of remilking, the PRL concentration had returned to the levels detected during the nonmilked periods. At d 7 of remilking, although the 14-d-nonmilked group had lower ($P < 0.05$) concentrations than the other 2 groups, all 3 groups had higher PRL ($P < 0.01$) than the pretrial values (Figure 3) and the nonmilked periods ($P < 0.05$). Plasma PRL concentrations were variable, and for the 7-d-nonmilked groups, the values were the same as pretrial on d 2 and d 4 to 6 of remilking. The 28-d-nonmilked group PRL values were the same as pretrial values on d 4 and 5 during remilking.

Plasma IGF-I concentrations pretrial and during the 7-d remilking period are presented in Figure 4. The mean $\pm$ SD pretrial IGF-I concentration was 119.9 ± 19.3 ng/mL and increased during nonmilking for the 7-, 14-, and 28-d-nonmilked periods to 143.9 ± 20.9 ng/mL ($P < 0.01$), 143.8 ± 18.6 ng/mL ($P < 0.01$), and 193.0 ± 25.3 ng/mL ($P < 0.05$), respectively. At this time, the IGF-I concentration was the same following 7 and 14 d of nonmilking, although after 28 d of nonmilking, the IGF-I concentration was higher ($P < 0.05$) than both of the shorter nonmilking periods. On d 1 of remilking, all 3 groups had higher IGF-I concentrations than pretrial values ($P < 0.05$, Figure 4), although these values were the same as the final nonmilked day. Plasma IGF-I concentrations returned to pretrial values by d 4 of remilking for the 7-d-nonmilked group. However, for the 14-d-nonmilked and 28-d-nonmilked groups, the IGF-I concentration remained higher than pretrial except d 3 and 7 of remilking, respectively.

DISCUSSION

This study demonstrates that a complete recovery of milk yield is possible following a 7-d-nonmilking period and that even partial recoveries can occur following 14- and 28-d nonmilking, with the loss of milk production increasing with the duration of the nonmilking interval. It must be noted that in the present study the cessation of milking was during mid lactation. Normally, cows are dried off during late lactation following gradual post-peak lactation mammary involution, a time when cows are also pregnant and have increased levels of estrogen, which is well known to have a milk inhibitory effect (Capuco et al., 2003). The fact that the cows in the present study were not pregnant and not in the declining phase of lactation may have influenced milk yield recovery upon remilking.

It has been previously shown that milk yields can be fully recovered following shorter than a 7-d period of nonmilking of cows in early to mid lactation (Farr et al., 1998; Dalley and Davis, 2006). Following a 7-d-

Figure 3. Plasma prolactin (PRL) concentrations in cows before milk stasis (pretrial, ◆) and during remilking for 7 d following extended nonmilking intervals of 7 d (—●—), 14 d (---■---), and 28 d (---▲---) ($n = 6$ per group) at mid lactation. Data are expressed as means adjusted for pretrial plasma PRL of 2 d before the nonmilking period, with bars for standard error of the difference.

nonmilking period, the present study supports the earlier finding by Dalley and Davis (2006) that milk yields upon remilking recover close to pretrial values. Moreover, an 11-d-nonmilking period in multiparous cows during late lactation, that is, during the declining phase of lactation, resulted in very low milk yield recoveries following 3 d of twice daily remilking of half-udders (Noble and Hurley, 1999). In agreement, in the present study, although in primiparous cows during mid lactation, 3 d of remilking of whole udders following a greater than 7-d-nonmilking period is insufficient to measure the full extent of lactation recovery (Figure 1). For example, in the 14-d-nonmilking group, milk recovery was only 38% after 3 d of remilking. Recovery increased to approximately 50% recovery by d 5 of

Figure 4. Plasma IGF-I concentrations in cows before milk stasis (pretrial, ◆) and during remilking for 7 d following extended nonmilking intervals of 7 d (—●—), 14 d (---■---), and 28 d (---▲---) ($n = 6$ per group) at mid lactation. Data are expressed as means adjusted for pretrial values of 3 d before the nonmilking period, with bars for standard error of the difference.

remilking and remained at this level for d 6 and 7 of remilking. Although a greater milk yield recovery was possible following the 11-d-nonmilking period as postulated by Noble and Hurley (1999), the present study suggests the recovery was likely to be only partial. It is possible that some alveoli may have already begun to involute, and therefore, full recovery of lactation may not have been possible (Noble and Hurley, 1999).

Regulation at an alveolar level has been demonstrated in mammary tissue from lactating cows where the majority of alveoli are active and expressing milk proteins but a small population of alveoli are inactive and involuting (Molenaar et al., 1992, 1996) or may be in a quiescent state (Vetharaniam et al., 2003). Although minimal apoptosis is detected during lactation (Wilde et al., 1997; Capuco et al., 2001; Singh et al., 2005), some alveoli may be in the early phases of involution but not yet committed to cell death. It is proposed that cells in the regressing alveoli may be quiescent and have not progressed to the senescent state. Quiescent alveoli may be capable of reactivation to their active state (Davis et al., 1999, Vetharaniam et al., 2003). During milk stasis there is retention of regions of alveoli that are actively expressing milk proteins (Molenaar et al., 1992, 1996). This heterogeneity and the quiescent state of regressing alveoli may play a role in the reversibility following extended nonmilking intervals in bovine mammary function. In the rodent mammary gland, involution is proposed as a 2-stage process (Lund et al., 1996), with limited apoptosis occurring during the first stage, which is reversible up to 48 h of pup removal, and the second phase, which is irreversible from 72 h, is indicated by loss of the majority of epithelial cells by apoptosis and extensive restructuring of the gland to a virgin-like state (Jaggi et al., 1996; Lund et al., 1996; Li et al., 1997). Although the cessation of milking in dairy cows results in an increase in apoptosis of mammary epithelial cells (Wilde et al., 1997) by 72 h after milking (Singh et al., 2005), the number of apoptotic products in involuting mammary tissue is only slightly increased (Wilde et al., 1997; Singh et al., 2005) and the massive cell death that occurs in rodents at involution (Walker et al., 1989) does not occur. Thus, involution in ruminants occurs to a lesser extent than in rodents, only replacing senescent or damaged cells (Capuco et al., 2001), and the retention of alveolar structures (Capuco and Akers, 1999; Wilde et al., 1999) may allow lactation to be re-initiated after extended nonmilking periods. The degree of lactation recovery appears to be inversely related to the length of the nonmilking interval. This may be due to the proportion of alveoli or mammary epithelial cells that have entered the irreversible or senescent phase of involution in the bovine mammary gland.

The cellular events that occur in the reversibility of mammary gland involution have been previously reported for the trial described in the present study (Mallah et al., 2013). A further 2 groups, following 7- and 28-d-nonmilking periods, were also examined. In these latter 2 nonmilked groups there was an increase in both the apoptotic and proliferating nuclei of mammary tissue compared with lactating tissue (Mallah et al., 2013). Earlier studies report that during lactation, cell proliferation is low (Capuco et al., 2001; Sorensen et al., 2006), and in pregnant dry cows proliferation increases from early to late in the dry period (Capuco et al., 1997; Sorensen et al., 2006). However, the cows in the present study were not pregnant, but results support an earlier study in nonpregnant beef cows that showed an increase in cell proliferation in nonsuckled glands 5 to 7 d following the cessation of suckling (Capuco and Akers, 1990). It was postulated that the rate of mammary involution may be influenced by a concurrent cell proliferation (Capuco and Akers, 1999). The recovery of lactation function upon remilking, either full or partial, was indicated by a decline in both apoptotic and proliferating nuclei (Mallah et al., 2013). That is, the apoptotic frequency in mammary tissue after 7 and 28 d of nonmilking was 2 and 1.5%, respectively, and following 7 d of remilking, it was reduced to 0.6 and 0.5%, respectively, although remaining higher than control mammary tissue from lactating cows (Mallah et al., 2013). The decline in proliferating nuclei following remilking of 7- and 14-d-nonmilked groups indicated a return to the levels of control lactating mammary samples. However, remilking following a 28-d-nonmilking interval did not influence proliferation and levels remained high. Thus, both cell apoptosis and proliferation are associated with the reinitiation of milk production in dairy cows (Mallah et al., 2013). The competition between the recruitment of new mammary epithelial cells through proliferation and the death of existing ones may have an important bearing on the reestablishment of lactation function following milk stasis.

Future studies will focus on understanding the cell signaling pathways that regulate the balance between cell death and proliferation at the onset and in the reversibility of mammary gland involution in dairy cows. Preliminary results suggest the balance between signal transducer and activator of transcription-5 and transcription-3 may play a role in the transition from lactation to involution in mammary gland (Singh et al., 2009). This supports studies in rodents that indicate that the reciprocal activation of these transcription factors at the onset of involution may regulate the balance between cell survival and death (Clarkson et al., 2006).

The present study demonstrated that upon remilking following 7 d of nonmilking the milk composition returns or almost returns to pretrial composition. In comparison, following remilking of the 14- and 28-d-nonmilking groups, milk composition did not return to pretrial composition. For example, the lactose content, although depressed following 7 d of nonmilking, recovered to pretrial levels upon remilking but remained lower than pretrial in the 14- and 28-d-nonmilking groups. A decrease in milk secretion following an extended period of milk accumulation coincides with the loss of integrity of the tight junctions (Stelwagen et al., 1997). Farr et al. (1998) suggest that following remilking, the sustained low levels of milk lactose of 32- to 40-h-nonmilking periods may indicate that the integrity and permeability of the tight junctions has been modified and that the recovery of tightness takes several hours. The present study indicates a nonmilking interval greater than 7 d may not allow complete recovery of tight junction integrity, and thus, only partial recovery of milk yield is achieved. Maintaining the 3-dimensional structure of epithelia is crucial to biological function, and one possible trigger of involution postulated is the change in cell shape as the gland becomes engorged with milk. The epithelia become stretched, and this may activate mechanotransduction pathways resulting in loss of tight junction integrity and cell-extracellular matrix survival signaling (Davis et al., 1999; Stelwagen, 2001). In support of a role for mechanotransduction, mammary cell stretch *in vitro* was associated with the induction of apoptotic pathways (Quaglini et al., 2009). Furthermore, studies *in vivo* demonstrated that acute physical distension of rat mammary glands with sucrose solution accelerated the induction of apoptosis, upregulation of the apoptotic factor activated signal transducer and activator of transcription-3, and loss of the extracellular matrix factor β 1-integrin and tight junction factor occludin (Phyn et al., 2007). Recently, the distribution and morphological changes in the mechanosensors primary cilia during lactation and involution implicate a role in initiating involution of the mammary gland (Millier et al., 2013).

In the present study, the milk protein content for all 3 nonmilked groups increased during remilking compared with pretrial values, and by d 7 of remilking, the 14- and 28-d-nonmilked groups had higher protein content than the 7-d-nonmilked group. Whereas milk protein levels decrease during involution, mammary protein concentration increases because of an increase in serum-derived proteins and *de novo* synthesis of proteins such as lactoferrin (Hurley, 1989). Changes in fat content following remilking were most evident in the 28-d-nonmilked group, with the level being lower than pretrial and the 14- and 28-d-nonmilked groups.

The present study supports the earlier findings that lactose rapidly declines and fat concentration more slowly declines in secretions during involution (Hurley, 1989; Farr et al., 1998). Furthermore, enzymes involved in lactose and fat synthesis in mammary tissue during involution decrease in activity (Hurley, 1989).

Prolactin not only enhances mammary cell activity and differentiation (Akers et al., 1981a,b) but also stimulates proliferation of mammary epithelial cells (Olazabal et al., 2000) and has been associated with enhanced mammary cell survival (Accorsi et al., 2002; Boutinaud et al., 2012). The reestablishment of lactation in dairy cows has been reported in a previous study in which extended nonmilking periods of 12 d demonstrated recovery of milk yield to pretrial levels after remilking (Hamann and Reichmuth, 1990). However, in that study cessation of milking was in one udder half while the contralateral udder half was continually milked. Therefore, the presence of hormones such as PRL may have delayed the onset of involution in the unmilked glands (Akers and Keys, 1985; Feng et al., 1995; Noble and Hurley, 1999). In support, in the present study PRL increased during the reestablishment of lactation for all 3 nonmilked groups. It has been reported that PRL concentrations were lower during the dry period compared with cows that were still being milked (Bernier-Dodier et al., 2011). In contrast, in the present study, during the nonmilked periods, the PRL levels remained similar to pretrial lactating values for the 7-d-nonmilked group, although the 14- and 28-d-nonmilked groups had a small increase. The reason for the discrepancy between the 2 studies is unclear but may be related to differences in the stage of lactation and pregnancy status of the cows used in the different studies. Previous studies report an increase in PRL concentrations during the dry period due to long-day photoperiod and heat stress (Auchtung et al., 2005; do Amaral et al., 2009, 2010); however, in the present study, these factors are unlikely to have an effect on PRL because heat stress was not an issue during the current trial. Nonetheless, as expected, remilking following the nonmilking periods enhanced PRL levels to those described previously in pasture-fed lactating cows (Bluett et al., 2005).

Circulating IGF-I is a mitogen for the mammary epithelium from prepubertal heifers (Weber et al., 2000) and a survival factor for mammary epithelial cells (Flint and Knight, 1997). Circulating IGF-I increased during the nonmilking periods to similar levels observed upon remilking. For the 7-d-nonmilked group, the IGF-I returned to pretrial concentration by 7 d of remilking but remained higher for the 14- and 28-d nonmilked groups. This supports earlier observations that IGF-I concentration is low during lactation and increased in

the dry period (Sorensen et al., 2006). Furthermore, results indicated that systemic IGF-I, rather than local IGF-I, plays a major role in cell proliferation (Sorensen et al., 2006). Thus, in the present study, at the end of the nonmilked periods, the higher IGF-I concentration for the 28-d-nonmilked group compared with the shorter nonmilked periods may explain the prolonged mammary epithelial proliferation observed upon remilking for this group.

The SCC, an indicator of udder health and milk quality, remained higher in all remilked groups than pretrial values. The SCC values were below the New Zealand dairy industry milk processor penalty limit of 400,000 cells/mL in the 7- and 14-d-nonmilked groups by d 3 and 6 of remilking, respectively. Dalley and Davis (2006) have demonstrated similar recovery times with 7 d of nonmilking where SCC had declined to less than 400,000 cells/mL by d 5 of remilking. However, SCC values were greater than 400,000 for the entire remilking period in the 28-d-nonmilked group. Elevated SCC are likely due to an enhanced influx of white blood cells from the blood, and mammary epithelial cells as the alveolar structures break down, as well as a concentrating effect from low milk volume in that treatment group.

CONCLUSIONS

The results suggest that the process of involution is almost fully reversible following 7 d of milk stasis, but more extended periods of nonmilking prevent the complete recovery of lactation, which is probably through a decrease in milk-secreting mammary epithelial cell number or activity. Further studies are required to understand the local molecular mechanisms of the mammary gland for regulating function in response to extended nonmilking periods.

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