BRIEF COMMUNICATION: The effect of re-milking following extended non-milking periods on the proliferation and apoptosis of mammary epithelial cells in dairy cows

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Introduction

In dairy cows, the increase in milk yield from parturition to peak lactation is attributed to an increase in the secretory activity of mammary epithelial cells (MECs) (Capuco et al. 2001). However, following peak lactation, there is a decline in milk production, which is attributed to the loss of MECs by programmed cell death or apoptosis (Capuco et al. 2001). The role of involution and the factors that regulate it in the bovine mammary gland are not well understood. Previous studies have characterised the process of involution in the bovine mammary gland by determining whether involution is reversible following an extended milk stasis. Involution was fully reversible following seven days of non-milking (Dalley & Davis 2006), but was only partially reversible following longer non-milking intervals (Noble & Hurley 1999; Singh et al. 2012).

The aim of this study was to determine if the apoptosis and proliferation of MECs is reversible with resumption of milking following extended non-milking intervals in pasture-fed cows.

Materials and methods

The study used 30 Friesian primiparous non-pregnant dairy cows that had been in milk for 97 ± 2 days, were pasture-fed, milked twice daily and free of any intramammary infection (Singh et al. 2012). The cows were divided into six groups of five cows each. Mammary alveolar tissue was obtained at slaughter from lactating cows six hours post-milking (Control), cows with non-milking intervals of 7- or 28- days, and cows where milking was resumed for seven days following non-milking intervals of 7-, 14-, or 28-days, as described previously (Singh et al. 2012). All procedures were approved by the Ruakura Animal Ethics Committee.

Mammary gland tissue sections were prepared and *in situ* end labelling (ISEL) performed to detect cell death as described previously (Singh et al. 2005). The sections were also used for immunohistochemistry using an anti-Ki-67 antibody [SP6] (Sapphire Bioscience, Auckland, New Zealand) to detect cell proliferation using standard procedures. For both ISEL and Ki-67 assays, six fields per animal were photographed at 125x magnification using a ProgRes C14 digital camera (JENOPTIK Laser) and Corel PaintShop Pro X1 software. Automated cell counting was optimised using ImageJ (US National Institute of Health, http://rsb.info.nih.gov/nih-image/) and the total number of epithelial nuclei and positively-labelled nuclei were then counted in each field using ImageJ macros. An average of 8,224 and 12,365 total epithelial nuclei were counted per animal for ISEL and Ki-67, respectively. The percentages of ISEL- and Ki-67- positive nuclei were calculated as the number of labelled nuclei divided by the total number of epithelial nuclei counted. The values were transformed on a logit scale using the following formula where the logit of the percentage of labelled nuclei = $\log_e$ (Percentage of labelled nuclei / (100 - Percentage of labelled nuclei)). Transformed data were analysed by ANOVA in GenStat (Payne et al. 2009).

Results

Cows with 7- and 28-day non-milking intervals had a greater percentage of mammary apoptotic nuclei than the Control group at the start of re-milking (1.6- and 1.5-fold respectively) ($P < 0.001$) (Figure 1). Re-milking for seven days following both 7- and 28-day non-milking intervals reduced apoptosis by 1.3-fold ($P < 0.05$) (Figure 1) compared with the respective non-milked groups. However, after seven days of re-milking, apoptosis in both 7- and 14-day non-milked groups remained greater (1.2-fold) ($P < 0.05$) (Figure 1) than the lactating Control group, and there was a trend for greater apoptosis in the 28-day non-milked group (1.1-fold) ($P < 0.1$; Figure 1). Seven days re-milking reduced the extent of apoptosis to the same level across all three groups ($P > 0.05$) (Figure 1).

Cessation of milking for both seven and 28 days resulted in an increase in cell proliferation by 1.2 fold compared with the Control group ($P < 0.05$ and $P = 0.06$, respectively) (Figure 2). After seven days of re-milking, proliferation for the 7-day non-milked group declined by 1.2 fold ($P < 0.05$) and was the same as the Control group ($P > 0.05$) (Figure 2). However, seven days re-milking following a 28-day non-milking interval had no effect on cell proliferation compared with the 28-day non-milked group ($P > 0.05$) (Figure 2). There was considerable between-animal variation in this group, with cell proliferation completely reverting back to the control level in some cows but not others (GS Mallah, Unpublished data). After seven days of re-milking,

Figure 1 Logit transformation of the percentage of apoptotic nuclei identified by an *in situ* end-labelling procedure in bovine mammary glands following re-milking for seven days after extended non-milking intervals of seven, 14 and 28 days during mid-lactation. Control glands were milked twice-daily throughout the trial. Coding for treatments = Integer before back-slash represents non-milking interval (days) and integer after back-slash represents the day after remilking on which the sample was taken. Error bars represent the standard error of the mean. Significance is indicated as *** P <0.001, * P <0.05, † P <0.1. Single measurements are relative to the Control group. Other comparisons indicated by overarching brackets.

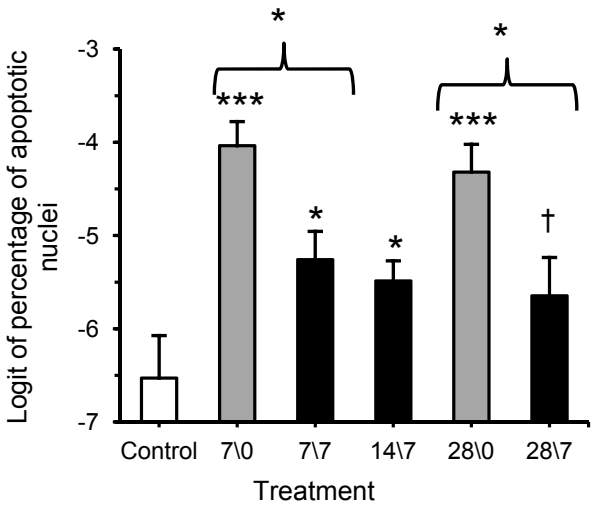
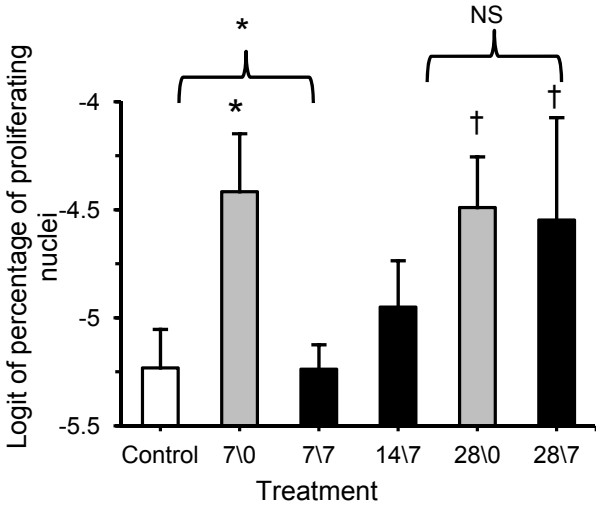


Figure 2 Logit transformation of the percentage of proliferating nuclei identified by a Ki-67 labelling procedure in bovine mammary glands following re-milking for seven days after extended non-milking intervals of seven, 14 and 28 days during mid-lactation. Control glands were milked twice-daily throughout the trial. Coding for treatments = Integer before back-slash represents non-milking interval (days) and integer after back-slash represents the day after remilking on which the sample was taken. Error bars represent the standard error of the mean. Significance is indicated as *** P <0.001, * P <0.05, † P <0.1. Single measurements are relative to the Control group. Other comparisons indicated by overarching brackets.



14-day non-milked glands had a similar percentage of proliferating nuclei as glands in the Control cows (P >0.05) (Figure 2). Hence, seven days of re-milking following a 7- and 14-day non-milking interval reduced cell proliferation to the control levels, but re-milking did not have the same effect after 28 days non-milking (Figure 2).

Discussion

Previously, our milk yield data indicated that lactation can be fully restored after a non-milking interval of seven days, but is only partially restored after extended non-milking intervals of 14 or 28 days (Singh et al. 2012). In the present study, we provide further evidence to support this response by investigating mammary cell apoptosis and proliferation. Cessation of milking increased the number of apoptotic and proliferating nuclei, indicating that involution was triggered within seven days of milk stasis, consistent with the previous studies (Singh et al. 2005; Wilde et al. 1997). Recovery of lactation function, either full or partial, was indicated by reduction in the percentages of apoptotic and proliferating nuclei upon re-milking.

In dairy cows, the decline in milk production during involution is due to the loss of MECs through the process of apoptosis (Capuco et al. 2001; Wilde et al. 1997). Wilde and co-workers (1997) reported that about 4% of MECs become apoptotic after seven days of milk stasis in dairy cows. In the present study, we report an apoptotic frequency of 2 and 1.5% after seven and 28 days of non-milking, respectively. However, after a 7-day re-milking period, the apoptotic frequency was reduced to 0.6 and 0.5%, respectively. Therefore, our data indicates that the re-initiation of lactation function upon re-milking may be attributable, at least in part, to the survival of a population of MECs.

The re-establishment of lactation after extended periods of non-milking is further supported by changes in cell proliferation. An increase in cell proliferation occurs at the onset of involution in both pregnant (Capuco et al. 1997) and non-pregnant cows (Capuco and Akers 1990). It has been hypothesised that the increase in cell proliferation during involution may either provide a means to re-initiate lactation on restoration of secretion removal (Capuco & Akers 1999) or cause increased cell renewal or turnover prior to the next lactation (Capuco et al. 1997). Our results support the former hypothesis, as

an increase in cell proliferation during non-milking provided a means to restore lactation function on re-milking.

In conclusion, our results demonstrate that cell proliferation and cell apoptosis are associated with the re-initiation of milk production in dairy cows. The competition between the recruitment of new MECs through proliferation and the death of existing ones may have an important bearing on the re-establishment of lactation function following milk stasis.

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