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Technical note: A noninvasive method for measuring mammary apoptosis and epithelial cell activation in dairy animals using microparticles extracted from milk

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ABSTRACT

Milk production from dairy animals has been described in terms of 3 processes: the increase in secretory cell numbers in late pregnancy and early lactation, secretion rate of milk per cell, and the decline in cell numbers as lactation progresses. This latter process is thought to be determined by the level of programmed cell death (apoptosis) found in the animal. Until now, apoptosis has been measured by taking udder biopsies, using magnetic resonance imaging scans, or using animals postmortem. This paper describes an alternative, noninvasive method for estimating apoptosis by measuring microparticles in milk samples. Microparticles are the product of several processes in dairy animals, including apoptosis. Milk samples from 12 Holstein cows, at or past peak lactation, were collected at 5 monthly samplings. The samples (n = 57) were used to measure the number of microparticles and calculate microparticle density for 4 metrics: annexin V positive and merocyanine 540 dye positive, for both and total particles, in both whole milk (WM) and spun milk. Various measures of milk production were also recorded for the 12 cows, including daily milk yield, fat and protein percentage in the milk, somatic cell count, and the days in milk when the samples were taken. A high correlation was found between the 4 WM microparticle densities and days in milk (0.46 to 0.64), and a moderate correlation between WM microparticle densities and daily milk yield (−0.33 to −0.44). No relationships were found involving spun milk samples, somatic cell count, or fat and protein percentage. General linear model analyses revealed differences between cows for both level of microparticle density and its rate of change in late lactation. Persistency of lactation was also found to be correlated with the WM microparticle traits (−0.65 to −0.32). As apoptosis is likely to be the major contributor to microparticle numbers in late lactation, this work found a noninvasive method for estimating apoptosis that gave promising results. Further investigation is required to find out the factors affecting microparticle production and how it changes throughout lactation.

Key words: apoptosis, lactation, microparticle, persistency

Technical Note

Dairy cows produce milk with a very characteristic pattern of a sharp yield increase in early lactation and a slower decline in later lactation. An exploration of the underlying biological basis of lactation as a way of describing the lactation curve of dairy animals has proposed 3 key processes (Pollott, 2000). These are secretion rate per active mammary cell, the rate of cell proliferation in early lactation, and the rate of cell death in late lactation. This decline in later lactation has been attributed to a net loss of active secretory cells rather than a decline in cell activity (Knight and Wilde, 1993; Knight et al., 1998; Capuco et al., 2003). Attempts to measure the underlying cause of this decline in milk yield have been somewhat problematic, with Knight et al. (1998) reporting the use of laddered DNA as a sign of mammary cell apoptosis (programmed cell death). Their approach required the collection of mammary tissue samples by slaughter or mammary biopsy. Capuco et al. (2001) came to similar conclusions using DNA collected from the whole udders of nonpregnant lactating cows slaughtered at 4 time points during lactation. In the current study, we took a noninvasive approach through the measurement of microparticles collected from milk, which may be indicative of apoptosis.

If the 3 components of milk yield can be definitively measured, then a better understanding of factors affecting lactation yield, length, and persistency can be developed. Knight et al. (1998) suggested that within any given milking regimen the secretion rate per cell remains constant throughout lactation. Assuming this is the case, then the number of secretory cells in the udder becomes even more important in determining

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productivity and persistence of lactation. Studies by Capuco et al. (2001) and Stefanon et al. (2002) suggested that following peak lactation, the decline in milk yield as lactation progresses is strongly influenced by the rate of apoptosis. Through biopsy, DNA measurement, and magnetic resonance imaging (MRI) of caprine mammary parenchyma it was found that differentiation and proliferation of mammary secretory tissue occurs during late gestation and the first few weeks of lactation (Knight and Peaker, 1984; Knight and Wilde, 1993). Similar studies have also shown decreases in the amount of DNA detected between peak lactation and drying off, suggestive of decreasing numbers of secretory cells, as DNA quantity is related to the number of cells (Wilde et al., 1997; Capuco et al., 2001). Knight and Wilde (1993) and Quarrie et al. (1995) used DNA laddering to identify cell death, whereas Capuco et al. (2001) used terminal deoxyuridine triphosphate nick-end labeling to estimate apoptosis. However, the factors involved in determining the rate of apoptosis are poorly understood, although it may be that minimizing the stimuli for apoptosis can lead to improved persistence and better lactation yields (Stefanon et al., 2002). Studies have suggested that numerous factors, both management related and physiological, influence the persistency of lactation (Stefanon et al., 2002; Capuco et al., 2003; Yart et al., 2012). Previously, it has been difficult to quantify the rate of apoptosis in the live animal, resulting in many lactation models being based on mathematical analysis of lactation records (Pollott, 2004; Albarrán-Portillo and Pollott, 2008).

Microparticles are membrane-bound vesicles of less than 1-μm diameter released from many different cell types, including erythrocytes, leucocytes, platelets, and endothelial cells (Nantakomol et al., 2008), and, importantly for this study, cells of epithelioid origin (Burnett et al., 2012). They are released, most commonly, at times of cell activation and apoptosis (Azevedo, 2012). Microparticles are formed by blebbing of the parent cell membrane (Orozco and Lewis, 2010), causing a loss of phospholipid asymmetry. Phosphatidylserine and phosphatidylethanolamine are negatively charged phospholipids found on the inner leaflet of the plasma membrane in healthy cells. During cell membrane blebbing and microparticle formation these phospholipids become exposed on the outer leaflet of the plasma membrane and the outer surface of the microparticle (Diamant et al., 2004). It is the presence of these normally hidden molecules that allow for the detection of microparticles. The phosphatidylserine specifically binds to annexin V, a calcium-dependent phospholipid-binding protein, and has been used to quantify microparticles in numerous studies (Théry et al., 2009; Azevedo, 2012; van der Pol et al., 2012), whereas the negatively charged lipophilic dye merocyanine (**MC**) 540 has also been used to detect the presence of disordered phospholipids on the membranes of microparticles (McCarthy et al., 2008). In addition to this, the outer membrane of the microparticle also contains membrane receptors and proteins originating from the parent cell, which can be detected using specific fluorochrome-labeled antibodies (Nantakomol et al., 2008; Azevedo, 2012). As microparticle formation could be the result of apoptosis (Diamant et al., 2004; Azevedo, 2012) and can be quantitatively measured (Nantakomol et al., 2008; Orozco and Lewis, 2010), we hypothesized that if microparticles can be detected in milk, then they may be able to give a quantitative value to the rate of apoptosis in the udder as lactation progresses.

The aim of this study was to identify the presence of microparticles in bovine milk and assess how their numbers changed with respect to stage of lactation and other factors, including milk production, indicators of mastitis, and protein and fat content of milk.

Sample Collection

All animals used in this study were part of the commercial dairy herd of the Royal Veterinary College (London, UK) and were not subjected to any extra experimental procedures over and above the normal day-to-day practices of such a herd and milk sampling during milking. Milk samples were taken from Holstein cows at, or after, peak lactation to follow the subsequent decline in yield. Samples were taken within a few days of the routine monthly milk sampling operated by National Milk Records Ltd. (Harrogate, UK). Data on SCC, fat percentage, protein percentage, and daily milk yield (**DMY**) were taken from these records and used in the analyses reported here. The DIM were calculated as the time between calving and milk sampling. The initial study population included all cows that were between DIM 40 and 200 at the time of the first sample collection. This also ensured that all cows in the study would still be milking at the time of the last sample. The sample population was determined by the first 12 viable cows to pass through the parlor at the time of the first sample collection. Following the preparation of the teat for milking, a 10-mL milk sample was collected from each of the 12 cows. Each sample contained milk from all 4 teats. Samples were taken monthly and were frozen before transport to the laboratory. Preliminary analyses (not shown) indicated that microparticle density was not altered by the freezing process compared with fresh milk taken from the same sample.