Animal (2011), **5:4**, pp 572–579 © The Animal Consortium 2010
doi:10.1017/S1751731110002284

animal

Use of milk epithelial cells to study regulation of cell activity and apoptosis during once-daily milking in goats

H. Ben Chedly[1,2a], P. Lacasse[3], P.-G. Marnet[1,2], M. Komara[1,2], S. Marion[1,2] and M. Boutinaud[1,2†]

[1]*INRA, UMR1080 Production du Lait, F-35590 Saint-Gilles, France;* [2]*Agrocampus Ouest, UMR1080 Production du Lait, F-35000 Rennes, France;* [3]*Dairy and Swine R&D Centre, Agriculture and Agri-Food Canada, PO Box 90 STN Lennoxville, Sherbrooke, Quebec, Canada J1M 1Z3*

(Received 5 June 2009; Accepted 12 August 2010; First published online 9 November 2010)

Generally, once-daily milking (ODM) decreases milk yield. This effect may be the consequence of a decrease in mammary epithelial cell (MEC) activity or a reduction in their number. The aim of this study was to determine the effect of ODM on the synthetic activity and rate of apoptosis of MEC using a non-invasive method. Eight Alpine goats were subjected to ODM or twice-daily milking for two 5-week periods. MECs were purified by centrifugation and immunocytochemical binding in milk after 1 and 5 weeks of each period. mRNA levels of some proteins involved in lactose and milk protein synthesis and in apoptosis were evaluated using real-time PCR. Isolation of MEC from milk was a useful method to investigate transcriptional regulation in a timeline study. ODM induced greater decreases in milk, lactose and protein yields after 1 week than after 5 weeks. This suggests an adaptation of the mammary gland to ODM, which reduces the inhibitory effect of this practice. Reductions in milk component yields were associated with lower α-lactalbumin transcripts, suggesting a transcriptional decrease of lactose synthesis during ODM. Glucose transporter GLUT1 transcripts were downregulated under ODM, suggesting that lactose precursor uptake by MEC might be involved in the regulation of lactose synthesis. κ-Casein mRNA levels tended to be lower during ODM. ODM increased levels of the pro-apoptotic transcript Bax after both 1 and 5 weeks, but no variation was observed in the Bax/Bcl-2 ratio. ODM affected cell synthetic activity through transcriptional regulation and may have induced apoptosis. The reduction of the negative effect of ODM on milk yield suggests that Alpine goats are able to adapt to ODM. Further studies are needed to investigate the effect of ODM on MEC turnover.

Keywords: milking frequency, mRNA, cell death, milk synthesis, mammary gland

Implications

The application of once-daily milking (ODM) in ruminants is a management practice that saves time and labour. However, this practice is associated with a negative impact on milk yield that limits its large-scale adoption. The understanding of the regulatory mechanisms involved in the negative impact on milk yield during ODM may give indications to minimise them.

Introduction

Once-daily milking (ODM) is a management practice adopted by some goat producers in order to simplify their dairying activities. However, adoption of this practice is hindered by the loss of milk yield that occurs when a farmer switches from twice-daily milking (TDM) to ODM. The loss of milk yield varies from 15% to 30% in small ruminants (Nudda *et al.*, 2002; Marnet and Komara, 2008). Milk yield declines when the number of mammary epithelial cells (MECs) and/or their synthetic activity decreases (Stelwagen, 2001; Capuco *et al.*, 2003). However, very few studies have investigated the effects of ODM on the cellular regulation pathways related to the observed loss of milk yield.

The regulation of lactose synthesis may be involved in the effects of ODM on milk yield. Indeed, lactose plays an important role in determining the milk volume secreted by the mammary gland because of its osmotic properties. Lactose synthesis may be modulated by the availability of glucose, its precursor and/or via regulation of the enzymes involved in its synthesis. Glucose transport in MEC is mainly ensured by the type 1 glucose transporter (GLUT1), which is the predominant glucose transporter in the mammary gland (Zhao and Keating, 2007). Several enzymes are involved in lactose synthesis,

[a] Present address: AgroSup Dijon, 26 boulevard docteur petitjean, BP 87999, 21079 Dijon Cedex, France.
† E-mail: marion.boutinaud@rennes.inra.fr

including galactosyltransferase (GT). This enzyme, associated with its co-factor α-lactalbumin (α-LA) to form lactose synthase, catalyses the final lactose synthesis reaction. For this reason, lactose synthesis should also be modulated by transcriptional regulation of these proteins.

After peak lactation, it is the loss of secretory cells, which accounts for the decrease in milk yield throughout declining lactation (Knight and Wilde, 1993). This decrease in cell numbers has been attributed to an increase in mammary apoptosis (Knight and Wilde, 1993). Similarly, the loss of milk yield during ODM may also be due to a decrease in the number of mammary cells. Indeed, ODM is known to induce apoptosis in the goat mammary gland (Li *et al*., 1999). The apoptotic state of a tissue can be evaluated by quantifying certain proteins involved in this death process. Previous studies carried out in mammary tissue or MEC used mRNA quantification of the pro-apoptotic proteins caspase-3 and Bax, together with the anti-apoptotic protein Bcl-2, as indicators of cell death (Wareski *et al*., 2001; Sorensen *et al*., 2006; Boutinaud *et al*., 2008).

Studying cellular regulation entails the collection of MEC. Sampling of the tissues after slaughter or by using a biopsy needle are ways of collecting MEC, but these techniques are invasive. The collection of MEC from milk is a non-invasive alternative, which was previously used to study mammary gene expression in cows (Hayashi *et al*., 2004; Murrieta *et al*., 2006) and in goats (Boutinaud *et al*., 2002). An improvement in the method was developed in cows, consisting of the specific purification of MEC from somatic milk cells using magnetic beads (Boutinaud *et al*., 2008). The use of milk MEC enables repeated sampling without carryover effects.

In this study, we used a non-invasive method for MEC recovery to analyse the transcriptional regulation of synthetic activity and apoptosis indicators occurring in the epithelium during ODM in an effort to explain goat mammary adaptation to ODM.

Material and methods

Animals and experimental design

Eight multiparous Alpine goats in mid-lactation producing 3.0 ± 0.7 kg/day of milk were assigned to two groups of four goats each. In the first group, goats were subjected to ODM for 5 weeks and switched to TDM for a further 5 weeks before returning to ODM for 5 weeks (Figure 1). The other group was subjected to the opposite order of milking frequency for the same periods of time, as shown in Figure 1. The two groups of goats were fed according to INRA's recommendations (INRA, 2007) in order to meet their TDM milk production needs. The goats thus received 800 g alfalfa, 1 kg energy concentrate and *ad libitum* hay and water, each day. The diet and the milking routines and equipment were previously described by Komara *et al*. (2009). The goats in the TDM were milked at 0645 h and 1645 h, whereas those in ODM were milked at 1645 h.

Milk samples

Milk production was recorded with a Waikato milk meter mk5 (Waikato, Hamilton, New Zealand) at each milking (5 days/week). Twice a week (Tuesday and Thursday), the fat and protein content and somatic cell count (SCC) in representative samples collected at morning and afternoon milkings were determined by a commercial laboratory using infra-red analysis (Lillab, Châteaugiron, France). Morning and afternoon samples were analysed separately and a proportional average was calculated. Milk lactose was analysed weekly using a colorimetric enzymatic reaction (kit for lactose/D-galactose, Roche, Meylan, France) and a multiparameter analyser (Kone Instrument Corporation, Espoo, Finland). Milk samples were analysed four times, as shown in Figure 1: after 1 and 5 weeks of each milking frequency (TDM or ODM) with regard to their total N, true protein N (precipitation at pH 4.6 with trichloroacetic acid and filtration) and casein (precipitation at pH 4.6 with 10% acetic acid and 1 M sodium acetate) content (Kjeldahl method, temporary norm FIL 20B).

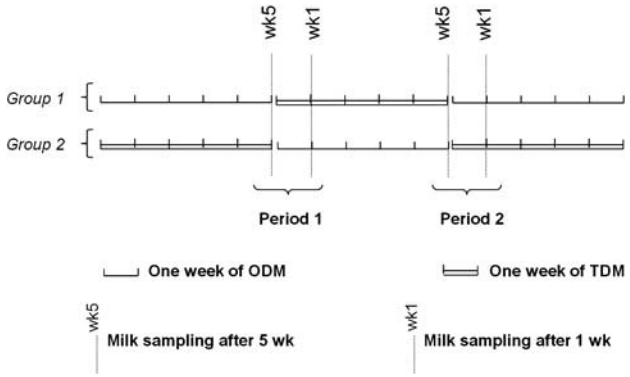

Figure 1 Experimental design: in group 1 ($n = 4$) goats were subjected to ODM for 5 weeks, then switched to TDM for further 5 weeks, returning thereafter to ODM for 5 weeks. Group 2 ($n = 4$) was subjected to the opposite order of milking frequency, for the same durations. Milk samples for MEC purification were collected at morning milking after 1 and 5 weeks of each milking frequency. Periods 1 and 2 were the periods used for the statistical model (ODM = once-daily milking; TDM = twice-daily milking; MEC = mammary epithelial cell).

Purification of MECs

Milk samples were collected at the morning milking after 1 and 5 weeks of each period (Figure 1) for MEC purification as described by Boutinaud *et al*. (2008). Briefly, 1.4 kg of fresh milk was centrifuged at 1500 **g** for 15 min at 4°C. The fat layer and the skimmed milk were discarded and the cell pellet was retained. The cell pellet was suspended in phosphate-buffered saline (PBS). After two washes in PBS, the cell pellet was resuspended in 1 ml of PBS containing 1% bovine serum albumin (BSA). Twenty microlitres of this cell suspension were counted with a haematocytometer (VWR International, Fontenay sous Bois, France) using light microscopy to determine the total milk cell count. A 200-μl volume of magnetic beads (Pan Mouse IgG, Dynal Biotech, Invitrogen, Cergy Pontoise, France) was incubated with 4 μl of anti-cytokeratin-8 antibody (clone K8.13, Sigma-Aldrich Chimie, Lyon, France) in 1 ml of 1% PBS–BSA for immunocytochemical binding of MEC. Each cell sample was incubated with 25 μl of the bead/antibody mix

described above. After 1 h of incubation, the samples were placed in a magnetic particle concentrator (MPC-S; Dynal Biotech, Invitrogen, Cergy Pontoise, France) and the supernatant containing non-selected cells was removed. The purified MECs were re-suspended in 1 ml of 1% PBS–BSA. A 20-μl aliquot of purified cell suspension was collected for haematocytometer cell count of purified MEC. The MECs were pelleted by centrifugation (5 min, 4°C, 5000 **g**) and 1 ml of Trizol (Invitrogen) was added. The cell samples were mixed and stored at −80°C until RNA extraction.

RNA extraction

The extraction of total RNA from MEC was done in Trizol, according to the manufacturer's recommendations. The RNA pellet was suspended in RNase-free water and the total quantity of RNA was determined with an Agilent 2100 bioanalyzer (Agilent Technologies, Massy, France). RNA quality was assessed using the RNA integrity number generated by version B.02 of Agilent 2100 Expert Software (Agilent Technologies).

Real-time reverse transcriptase-PCR (RT-PCR)

Complementary DNA was obtained using the First Strand cDNA kit (Roche Diagnostics, Meylan, France), according to the manufacturer's instructions, with 250 ng of total RNA. PCR amplifications of cDNA samples were carried out using the primers already described for cyclophilin, α-LA, κ-CN (κ-casein), GT, GLUT1, Bax and cytokeratin-8 (Boutinaud *et al*., 2008) and for *Bcl-2*, *r18S*, *caspase-3*, *β-actin* and *RPLP0* (Ben Chedly *et al*., 2009).

Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Briefly, 12.5 ng cDNA were mixed with 6 pmol of all forward and reverse primers and adjusted to 12.5 μl with SYBR Green PCR Master Mix (2×) and DNase-free water. The PCR amplification protocol consisted of 2 min of incubation at 50°C followed by 10 min at 95°C and 40 cycles of 15 s at 95°C and 60 s at 60°C. Finally, a dissociation protocol was carried out, involving a linear increase of 1°C/min from 60°C to 95°C with continuous fluorescence acquisition. The PCR reactions on each sample were performed in triplicate.

Quantification of mRNA

The number of amplified mRNA molecules was determined as described by Boutinaud *et al*. (2004) according to the following formula:

$$\text{Nb Mol} = 10^{(\text{Ct}-40)/\text{S}}$$

where, Nb Mol is the approximate number of mRNA molecules; Ct is the average cycle threshold for PCR in triplicate for a considered gene; and S is the slope of the calibration curve performed using serial cDNA dilutions of the same sample.

The calibration curves were generated for each target and housekeeping gene using serial dilutions of a mammary tissue cDNA sample (1 : 10, 1 : 20, 1 : 50, 1 : 100, 1 : 200, 1 : 1000 and 1 : 2000). A non-template negative control was incorporated in all PCR runs.

mRNA levels of the studied genes were expressed relative to a reference gene. The *cyclophilin*, *β-actin*, *r18S* and *glyceraldehyde-3-phosphate dehydrogenase* genes were evaluated as potential reference genes. The BestKeeper (Pfaffl *et al*., 2004), GeNorm (Vandesompele *et al*., 2002) and NormFinder (Andersen *et al*., 2004) programs were used to assess the variability of candidate reference genes. *Cyclophilin* was the gene with the most stable expression and was thus used as the reference gene during this study. The results for each target gene are expressed as a ratio using the selected reference gene.

Statistical analyses

Milk data, cell data and gene expression data were analysed by ANOVA using the SAS MIXED procedure (SAS Institute, 1999) with the REPEATED statements. The weeks were used as a repeated effect and the goats as the subject. The effect of milking frequencies (ODM and TDM), weeks (weeks 1 and 5), periods (periods 1 and 2), the goats and the interaction between milking frequencies and weeks were tested. Cell data were log10-transformed before analysis. Statistical analysis of real-time PCR data was carried out on the semi-absolute mRNA molecule number of the target gene/reference gene ratio multiplied by 10^4 and log10-transformed. The effects were considered significant at $P < 0.05$.

Results

Milk production and composition

Overall, ODM induced a decrease in milk yield compared to TDM ($P < 0.001$). As shown in Table 1, the effect of ODM declined with time (milking frequency × week, $P = 0.04$). The milk loss averaged 21% after 1 week of ODM and was reduced to 13% after 5 weeks.

Milking frequency did not affect the lactose content of milk. Therefore, lactose yield followed the same pattern as milk yield, with a reduction during ODM compared to TDM ($P < 0.001$) and a trend towards an interaction between milking frequency and weeks ($P = 0.06$). The loss of lactose averaged 23% after 1 week and 12% after 5 weeks.

As for lactose, the protein yield was lower during ODM than during TDM ($P < 0.001$), and an interaction between milking frequency and weeks was found ($P = 0.01$). Milk protein loss averaged 23% after 1 week and 10% after 5 weeks. There was no effect of milking frequency on protein content, but there was a trend towards a lower milk casein content ($P = 0.07$) during ODM than during TDM. Conversely, the milk whey protein content was greater during ODM than during TDM ($P = 0.01$), leading to a lower casein/whey protein ratio ($P < 0.01$). Nevertheless, the yields of casein and whey proteins were lower during ODM than during TDM ($P \leqslant 0.01$), which fell, respectively, by 24% and 18% after 1 week and 13.0% and 0.3% after 5 weeks.

ODM decreased the milk fat content ($P = 0.03$) and yield ($P < 0.001$) compared to TDM. The reduction in milk fat yield

Table 1 *Effect of MF after 1 and 5 weeks on milk yield and composition in dairy goats*[†]

	1 week		5 weeks			*P*-value	
Items	TDM	ODM	TDM	ODM	s.e.m.	MF	MF × weeks[‡]
Milk yield							
kg/day	3.24	2.55	3.03	2.65	0.19	<0.001	0.04
Milk protein							
g/day	100.2	76.8	90.9	81.5	5.9	<0.001	0.01
g/kg	31.0	30.1	29.9	30.8	0.6	0.96	0.008
Milk casein							
g/day	83.2	62.9	76.0	66.2	5.3	<0.001	0.04
g/kg	25.5	24.6	25.0	25.0	0.5	0.07	0.09
Milk whey protein							
g/day	17.6	14.4	14.5	14.5	1.1	0.01	0.02
g/kg	5.5	5.7	4.8	5.5	0.3	0.01	0.14
Casein/whey ratio	4.7	4.4	5.3	4.6	0.2	0.007	0.15
Milk fat							
g/day	126.4	93.9	112.0	92.1	13.5	<0.001	0.13
g/kg	38.1	35.8	36.1	33.9	2.6	0.03	0.96
Milk lactose							
g/day	144.4	111.3	137.6	121.0	9.9	<0.001	0.06
g/kg	44.2	43.6	45.1	45.3	0.8	0.56	0.37
Log10 SCC	5.3	5.5	5.3	5.1	0.1	0.90	0.10

MF = milking frequency; TDM = twice-daily milking; ODM = once-daily milking; MF × weeks = interaction between MF and weeks of treatment; SCC = somatic cell count.
[†]MF = ODM or TDM.
[‡]$n = 8$.

Table 2 *Effect of MF on milk cells and purified MEC recovered after 1 and 5 weeks in dairy goats*[†]

	1 week		5 weeks			*P*-value	
Items	TDM	ODM	TDM	ODM	s.e.m.	MF	MF × weeks
Total milk cells[‡]							
Cells/ml milk	5.44	5.65	5.45	5.69	0.15	0.05	0.85
Cells/day	8.32	8.63	8.27	8.65	0.15	0.01	0.76
Purified MEC[‡]							
Cells/ml milk	4.12	4.37	4.04	4.19	0.15	0.07	0.60
MEC yield (cells/day)	6.92	7.28	6.80	7.08	0.15	0.01	0.69

MF = milking frequency; MEC = mammary epithelium cell; TDM = twice-daily milking; ODM = once-daily milking; MF × weeks = interaction between MF and weeks of treatment.
[†]MF = ODM or TDM.
[‡]Log10-transformed data ($n = 8$).

averaged 26% after 1 week and 18% after 5 weeks. There was no interaction between milking frequency and week with regard to milk fat yield ($P = 0.13$) or content ($P = 0.96$), indicating that these parameters were not affected by the duration of ODM. Milking frequency did not affect the SCC.

Purification of milk MEC and gene quantification

On average, $570 \pm 112 \times 10^3$ cells/ml of total milk cells were recovered. The number of purified MEC averaged $23 \pm 4 \times 10^3$ cells/ml of milk, which led to $16.5 \pm 2.7 \times 10^6$ purified MEC recovered per sample. The milk MEC thus recovered represented an average of $4.6 \pm 0.6\%$ of total milk cells. As shown in Table 2, we observed a greater total milk cell yield and content per day ($P = 0.01$) associated with a greater yield of purified milk MEC per day ($P = 0.01$) in ODM than in TDM milk samples. Total RNA recovered from purified milk MEC averaged 31.8 ± 4.6 μg. Five samples were not analysed owing to the poor quality of their RNA (RNA integrity number <5).

mRNA levels of proteins involved in lactose and milk protein synthesis

ODM tended to reduce mRNA levels of κ-CN (Table 3). ODM downregulated the mRNA levels of α-LA and of the glucose transporter GLUT1 ($P = 0.02$) compared to TDM. No effect of milking frequency was observed with regard to GT mRNA

Table 3 *Effect of MF on log10-transformed mRNA levels in milk-purified MECs recovered after 1 and 5 weeks*[†]

	1 week		5 weeks			*P*-value	
Gene	TDM	ODM	TDM	ODM	s.e.m.	MF	MF × weeks[‡]
κ-Casein	6.58	6.25	6.37	6.28	0.20	0.06	0.27
α-Lactalbumin	6.29	5.40	5.91	4.89	0.46	0.02	0.86
Galactosyltransferase	4.15	4.46	4.75	4.98	0.35	0.49	0.92
GLUT1	3.47	3.02	3.53	2.80	0.24	0.02	0.50
Bax	3.72	4.37	3.99	4.30	0.29	0.04	0.42
Bcl-2	1.85	1.95	1.78	1.84	0.07	0.24	0.77
Caspase-3	2.85	3.20	2.98	3.21	0.18	0.07	0.68
Bax/Bcl-2 ratio	6.88	7.39	7.21	7.46	0.28	0.12	0.57

MF = milking frequency; MECs = mammary epithelium cells; TDM = twice-daily milking; ODM = once-daily milking; MF × weeks = interaction between MF and weeks of treatment; GLUT1 = type 1 glucose transporter.
[†]MF = ODM or TDM.
[‡]$n = 8$.

levels. No interaction was observed between milking frequency and weeks in terms of the mRNA levels of the different genes studied.

mRNA levels of apoptosis-related proteins

Bax mRNA levels were higher during ODM than during TDM ($P < 0.05$; Table 3). A tendency was also observed towards higher caspase-3 mRNA levels during ODM than TDM ($P = 0.07$). Milking frequency had no effect on Bcl-2 ($P = 0.24$) mRNA levels or the Bax/Bcl-2 ratio ($P = 0.12$). No interaction was observed between milking frequency and weeks relative to the mRNA levels of apoptosis-related proteins.

Discussion

In this study, we used a non-invasive method, purification of MEC from milk, to evaluate transcriptional regulation of some proteins involved in the synthesis of milk constituents and in apoptosis. Other methods are available for MEC recovery such as mammary sampling after slaughter or mammary biopsies. These techniques are invasive and are not adapted for timeline studies. A new non-invasive method using purified milk MEC developed by Boutinaud *et al.* (2008) was adapted to goats and consisted in the use of milk-purified MEC. The purification of MEC from goat milk permitted the recovery of a sufficient quantity of MEC for extracting total RNA and for carrying out RT-PCR analyses. A higher percentage of goat MEC (4%) was purified from the total milk somatic cell population compared to 2% in cows (Boutinaud *et al.*, 2008). Epithelial cells represent a higher proportion of milk somatic cells in goats than in cows (Boutinaud and Jammes, 2002). The use of milk cells presents several advantages compared to biopsies. This method enables repeated sampling without carryover effects. Thus, in this study, we were able to test the effect of weeks (weeks 1 and 5) during ODM in an inverted experimental design. Biopsies will be questioned in the future from an ethical point of view and alternative solutions should be envisaged. Milk cells are the most promising. This study is the first report that presents the purification of MEC from goat milk. This method provides the possibility to quantify gene expression, specifically in MEC. The tissue sampling methods, such as biopsy, permit the recovery of not only MEC but also other cells such as immune cells, myoepithelial cells and adipocytes. The heterogenicity in cell type can interfere with the result of gene expression, especially for a ubiquitously expressed gene such as housekeeping genes. The use of milk MEC raises a certain number of questions. It has been suggested that milk cells are dying cells and may already be committed to apoptosis. However, <10% of total milk cells exhibited DNA fragmentation, which usually characterises apoptosis in goat milk (Boutinaud and Jammes, 2002). We make the assumption that epithelial cells can be detached from mammary epithelium at milking due to the effect of pressure and contractions of mammary myoepithelial cells. It has been reported that milk somatic cells are viable (Feng *et al.*, 2007) and that more than 90% of MEC are viable (Thompson *et al.*, 1998). In addition, previous studies have shown that milk cell transcripts well represent the mammary tissue in bovine (Hayashi *et al.*, 2004; Murrieta *et al.*, 2006) and in caprine species (Boutinaud *et al.*, 2002). Moreover, in a recent experiment, we compared the use of milk MEC and biopsy during ODM and we found the same downregulation of genes involved in milk synthesis using both methods (Boutinaud *et al.*, 2009). Therefore, we suppose that the transcription profile of the milk-isolated MEC is representative of that of the MEC in the tissue.

The decline in the milk yield of goats associated with ODM compared to TDM ranges from 6% to 40% (Wilde and Knight, 1990; Salama *et al.*, 2003). Factors such as the duration of ODM application, the period of measurement, the stage of lactation and the breed of animals are probably responsible for much of this variability. Milk yield was reduced by ODM during this study, but the effect was less pronounced after 5 weeks of ODM (−12%) than after 1 week of the treatment (−22%). This observation was in line with the findings of Salama *et al.* (2003), who reported a greater loss of milk yield (19%) during the first 3 months of

ODM than at the end of lactation (14%). During that study, the difference in the rate of milk yield declined during ODM was attributed to either the stage of lactation or the duration of ODM, as they were confounded. During this study, the experimental design enabled a distinction between these effects and the results, thus confirming that the duration of ODM exerted an effect on loss of milk yield. Conversely, the duration of ODM does not alter the effect on milk yield and composition in dairy cows (Pomies *et al*., 2004). This suggests that in contrast with cows, the goat mammary gland could progressively adapt to ODM, thus gradually reducing the negative impacts of this management practice.

The decrease in milk lactose yield observed under ODM can be attributed to a reduction in lactose synthesis by MEC and a loss of lactose in the bloodstream due to the openings of tight junctions (Stelwagen *et al*., 1997). No indicators of tight junction openings were measured during this study. During a recent experiment, goats were milked at several successive 36-h milking intervals (Ben Chedly *et al*., 2009). This extended milking interval only induced tight junction openings at the first occurrence; but this effect had already disappeared at the second one. Thus, the loss of lactose due to tight junction openings is not very likely. Conversely, previous studies have shown that ODM decreased the activity of some key enzymes involved in lactose synthesis, such as GT (Wilde and Knight, 1990; Farr *et al*., 1995). The results of this study did not reveal an effect of ODM on GT transcripts, but rather a decrease in the levels of α-LA mRNA, the co-factor of GT during lactose synthase. These results agree with our previous observations in cows (Boutinaud *et al*., 2008) but differs from that of Bryson *et al*. (1993). However, in the latter experiment, goats were unilaterally subjected to ODM (no reduction in the systemic hormonal effect of milking stimulation), and Northern blotting (which is less sensitive than real-time RT-PCR) was used for mRNA quantification. ODM was also found to decrease GLUT1 transcripts during that experiment. Our results agree with the inhibitory effect of milk accumulation on GLUT1 protein and mRNA levels reported in rats (Camps *et al*., 1994). The ODM-induced downregulation of the GLUT1 observed could lead to a reduction in glucose availability for lactose synthesis, as previously observed by arteriovenous difference in cows (Delamaire and Guinard-Flament, 2006). Therefore, adaptation of the goat mammary gland to ODM seems to involve the negative regulation of lactose synthesis, due to the regulation of lactose synthase activity by way of transcriptional inhibition of its co-factor α-LA, and/or the reduction of glucose availability by way of a decrease in GLUT1 expression.

ODM reduced protein yield, with caseins being affected to a greater extent than whey proteins. This decrease in protein yield was of the same magnitude as the decline in milk volume, as also reported by Salama *et al*. (2003). As in the case of lactose, we observed an adaptation of the mammary gland to ODM so that the loss of milk casein yield was more pronounced after 1 week than after 5 weeks. The effect of ODM on milk caseins was associated with a trend towards a downregulation of κ-CN transcripts, a phenomenon that has already been reported after 7 days of ODM in cows (Boutinaud *et al*., 2008).

Milk whey protein secretions were also reduced by ODM, but to a lesser extent than milk volume, leading to an increase in the whey protein content. The disruption of tight junctions during ODM may permit the passage of plasma proteins, such as serum albumin into milk, thus increasing the milk whey protein content. As mentioned previously, leakage through tight junctions is an unlikely possibility, as this is a transient phenomenon in the goat mammary gland. Conversely, a relative reduction in milk whey protein synthesis by MEC may be the consequence of the decrease in α-LA transcripts.

Milking frequency affected both milk fat yield and content. A reduction in milk fat content under ODM was observed despite a decrease in milk volume. This indicates that ODM downregulates milk fat synthesis more than milk volume. Our results agree with those of Marnet and Komara (2008), but differ from those of Salama *et al*. (2003), who reported a 10% increase in milk fat content during ODM in goats. The mechanism by which milking frequency affects milk fat synthesis remains unknown, but recent study in unilaterally ODM cows has shown a reduction in the gene expression of enzymes involved in milk fat synthesis (Boutinaud *et al*., 2009). This suggests that the decrease in milk fat content can be attributed to transcriptional regulation.

The milk SCC was not significantly affected by ODM. This was consistent with the findings of Salama *et al*. (2003) in goats, Castillo *et al*. (2008) in ewes and Lacy-Hulbert *et al*. (1999) and Stelwagen *et al*. (1994) in cows. Other studies have reported an increase in the SCC in cows subjected to ODM (Stelwagen and Lacy-Hulbert, 1996; Kelly *et al*., 1998). Although the SCC is not a precise indicator of inflammation, our data suggest that ODM does not induce a severe inflammatory reaction in the goat mammary gland, and this is also consistent with the limited permeability of secretory epithelium and limited tight junction disruption that had been observed with 36-h milking intervals (Ben Chedly *et al*., 2009).

The decrease in milking frequency induced an increase in transcripts of the pro-apoptotic protein Bax. However, milking frequency exerted no effect on the Bax/Bcl-2 ratio, which is one of the measures used to estimate apoptosis. Boutinaud *et al*. (2008) also observed an increase in Bax mRNA levels during ODM, with no significant change to the Bax/Bcl-2 ratio in bovine milk MEC. In addition, ODM reduced the number of epithelial cells in the goat mammary gland (Boutinaud *et al*., 2003). Li *et al*. (1999) had previously demonstrated an increase in the apoptosis rate in goat udders milked once daily for 3 weeks. As previously reported, and in addition to the increase in the pro-apoptotic protein transcripts, we observed a tendency towards an increase in purified milk MEC collected from milk. An evaluation of the loss of epithelial cells in milk showed that more MEC were excreted in the milk each day during ODM than during TDM. This suggests a higher rate of epithelial cell exfoliation during ODM. In cows, reduction of milking frequency is associated with an increase of several matrix metalloproteinases in milk (Bernier-Dodier *et al*., 2010).

As their name implies, these enzymes can digest the matrix proteins that anchor the cells. If matrix metalloproteinase activity was enhanced in milk during ODM, they may have increased the release of MEC in milk. Further studies need to be carried out in order to access the mechanism by which ODM trigger exfoliation and apoptosis of MEC.

Conclusion

In this study, a non-invasive method was used (purification of milk MEC) to evaluate the transcriptional regulation of proteins involved in the synthesis of milk constituents and in apoptosis. This method enabled a specific recovery of MECs with a repeated sampling preventing any carryover effects. The results obtained showed that ODM induced a decrease in milk, lactose, protein and fat yields. The effect of ODM on milk secretion involved the transcriptional regulation of lactose enzymes and milk protein synthesis acting on α-LA, GLUT1 and κ-CN. ODM treatment also induced an upregulation of pro-apoptotic transcripts in milk MEC, suggesting an induction of apoptosis after 1 and 5 weeks. However, the ODM effect on MEC turnover should be investigated by further studies. The negative effects of ODM were less marked after 5 weeks of ODM, suggesting that the goat mammary gland adapts its secretory activity. Therefore, starting this management strategy in mid-lactation may not induce a long-term negative impact on the mammary gland and thus, might be useful to reduce the workload on a farm.

Acknowledgements

We thank Nicole Huchet and Isabelle Jicquel for their technical assistance. We are also grateful to Jean-Marc Aubry, Michel Chorho and Eric Siroux for taking care of the goats. Our thanks go to Luc Delaby and Steve Méthot for their assistance with the statistical analyses.

References

Andersen CL, Jensen JL and Orntoft TF 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Research 64, 5245–5250.

Ben Chedly H, Lacasse P, Marnet PG, Wiart-Letort S, Finot L and Boutinaud M 2009. Cell junction disruption after 36 h milk accumulation was associated with changes to mammary secretory tissue activity and dynamics in lacting dairy goats. Journal of Physiology and Pharmacology 60 (suppl. 3), 105–111.

Bernier-Dodier P, Delbecchi L, Wagner GF, Talbot BG and Lacasse P 2010. Effect of milking frequency on lactation persistency and mammary gland remodelling in mid-lactation cows. Journal of Dairy Science 93, 555–564.

Boutinaud M and Jammes H 2002. Potential uses of milk epithelial cells: a review. Reproduction Nutrition Development 42, 133–147.

Boutinaud M, Galio L and Devinoy E 2009. Transcripts in milk purified mammary epithelial cells can reveal the effect of once-daily milking. 6th International Symposium of Milk Genomics and Human Health, Paris, France.

Boutinaud M, Rousseau C, Keisler DH and Jammes H 2003. Growth hormone and milking frequency act differently on goat mammary gland in late lactation. Journal of Dairy Science 86, 509–520.

Boutinaud M, Ben Chedly MH, Delamaire E and Guinard-Flament J 2008. Milking and feed restriction regulate transcripts of mammary epithelial cells purified from milk. Journal of Dairy Science 91, 988–998.

Boutinaud M, Rulquin H, Keisler DH, Djiane J and Jammes H 2002. Use of somatic cells from goat milk for dynamic studies of gene expression in the mammary gland. Journal of Animal Science 80, 1258–1269.

Boutinaud M, Shand JH, Park MA, Phillips K, Beattie J, Flint DJ and Allan GJ 2004. A quantitative RT-PCR study of the mRNA expression profile of the IGF axis during mammary gland development. Journal of Molecular Endocrinology 33, 195–207.

Bryson JM, Wilde CJ and Addey CV 1993. Effect of unilateral changes in milking frequency on mammary mRNA concentrations in the lactating goat. Biochemical Society Transactions 21 (Part 3), 294S.

Camps M, Vilaro S, Testar X, Palacin M and Zorzano A 1994. High and polarized expression of Glut1 glucose transporters in epithelial-cells from mammary-gland – acute down-regulation of Glut1 carriers by weaning. Endocrinology 134, 924–934.

Capuco AV, Ellis SE, Hale SA, Long E, Erdman RA, Zhao X and Paape MJ 2003. Lactation persistency: insights from mammary cell proliferation studies. Journal of Animal Science 81, 18–31.

Castillo V, Such X, Caja G, Casals R, Albanell E and Salama AAK 2008. Effect of milking interval on milk secretion and mammary tight junction permeability in dairy ewes. Journal of Dairy Science 91, 2610–2619.

Delamaire E and Guinard-Flament J 2006. Increasing milking intervals decreases the mammary blood flow and mammary uptake of nutrients in dairy cows. Journal of Dairy Science 89, 3439–3446.

Farr V, Stelwagen K, Kerr M, Davis S and Eichler S 1995. Effect of once daily milking (ODM) on enzyme activities in the bovine mammary gland. Proceedings of the New Zealand Society of Animal Production 55, 12–13.

Feng S, Salter AM, Parr T and Garnsworthy PC 2007. Extraction and quantitative analysis of stearoyl-coenzyme A desaturase mRNA from dairy cow milk somatic cells. Journal of Dairy Science 90, 4128–4136.

Hayashi AA, McCoard SA, Roy NC, Barnett MPG, Mackenzie DDS and Mcnabb WC 2004. Gene expression in bovine mammary somatic cells isolated from milk. Journal of Animal and Feed Sciences 13, 401–404.

INRA 2007. Ruminant nutrition: recommended allowances and feed tables. Quae, Versailles, France.

Kelly AL, Reid S, Joyce P, Meaney WJ and Foley J 1998. Effect of decreased milking frequency of cows in late lactation on milk somatic cell count, polymorphonuclear leucocyte numbers, composition and proteolytic activity. Journal of Dairy Research 65, 365–373.

Knight CH and Wilde CJ 1993. Mammary cell changes during pregnancy and lactation. Livestock Production Science 35, 3–19.

Komara M, Boutinaud M, Ben Chedly H, Guinard-Flament J and Marnet PG 2009. Once daily milking effects in high yielding alpine dairy goats. Journal of Dairy Science 92, 5447–5455.

Lacy-Hulbert SJ, Woolford MW, Nicholas GD, Prosser CG and Stelwagen K 1999. Effect of milking frequency and pasture intake on milk yield and composition of late lactation cows. Journal of Dairy Science 82, 1232–1239.

Li P, Rudland PS, Fernig DG, Finch LM and Wilde CJ 1999. Modulation of mammary development and programmed cell death by the frequency of milk removal in lactating goats. Journal of Physiology 519 (Part 3), 885–900.

Marnet PG and Komara M 2008. Management systems with extended milking intervals in ruminants: regulation of production and quality of milk. Journal of Animal Science 86, 47–56.

Murrieta CM, Hess BW, Scholljegerdes EJ, Engle TE, Hossner KL, Moss GE and Rule DC 2006. Evaluation of milk somatic cells as a source of mRNA for study of lipogenesis in the mammary gland of lactating beef cows supplemented with dietary high-linoleate safflower seeds. Journal of Animal Science 84, 2399–2405.

Nudda A, Bencini R, Mijatovic S and Pullina G 2002. The yield and composition of milk in Sarda, Awassi, and Merino sheep milked unilaterally at different frequencies. Journal of Dairy Science 85, 2879–2884.

Pfaffl MW, Tichopad A, Prgomet C and Neuvians TP 2004. Determination of stable housekeeping genes, differentially regulated target genes and sample integrity: BestKeeper–Excel-based tool using pair-wise correlations. Biotechnology Letters 26, 509–515.

Pomies D, Remond B and Pradel P 2004. Performances and milk quality of dairy cows temporary milked once a day: incidence of once daily milking (ODM) duration and lactation stage. Rencontres Recherches Ruminants 11, 225–228.

Salama AA, Such X, Caja G, Rovai M, Casals R, Albanell E, Marin MP and Marti A 2003. Effects of once versus twice daily milking throughout lactation on milk

yield and milk composition in dairy goats. Journal of Dairy Science 86, 1673–1680.

SAS Institute 1999. Statistical Analysis System Release 8.01. Cary, NC, USA.

Sorensen MT, Norgaard JV, Theil PK, Vestergaard M and Sejrsen K 2006. Cell turnover and activity in mammary tissue during lactation and the dry period in dairy cows. Journal of Dairy Science 89, 4632–4639.

Stelwagen K 2001. Effect of milking frequency on mammary functioning and shape of the lactation curve. Journal of Dairy Science 84, E204–E211.

Stelwagen K and Lacy-Hulbert SJ 1996. Effect of milking frequency on milk somatic cell count characteristics and mammary secretory cell damage in cows. American Journal Veterinary Research 57, 902–905.

Stelwagen K, Davis SR, Farr VC, Eichler SJ and Politis I 1994. Effect of once-daily milking and concurrent somatotropin on mammary tight junction permeability and yield of cows. Journal of Dairy Science 77, 2994–3001.

Stelwagen K, Farr VC, McFadden HA, Prosser CG and Davis SR 1997. Time course of milk accumulation-induced opening of mammary tight junctions, and blood clearance of milk components. American Journal of Physiology 273, R379–R386.

Thompson PA, Kadlubar FF, Vena SM, Hill HL, McClure GHY, McDaniel LP and Ambrosone CB 1998. Exfoliated ductal epithelial cells in human breast milk: a source of target tissue DNA for molecular epidemiologic studies of breast cancer. Cancer Epidemiology Biomarkers & Prevention 7, 37–42.

Vandesompele J, De PK, Pattyn F, Poppe B, Van RN, De PA and Speleman F 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biology 3 (7): research0034.1–research0034.11.

Wareski P, Motyl T, Ryniewicz Z, Orzechowski A, Gajkowska B, Wojewodzka U and Ploszaj T 2001. Expression of apoptosis-related proteins in mammary gland of goat. Small Ruminant Research 40, 279–289.

Wilde CJ and Knight CH 1990. Milk yield and mammary function in goats during and after once-daily milking. Journal of Dairy Research 57, 441–447.

Zhao FQ and Keating AF 2007. Expression and regulation of glucose transporters in the bovine mammary gland. Journal of Dairy Science 90 (suppl. 1), E76–E86.