

Milk somatic cells are not suitable biomarkers of lactating ruminant mammary gland function

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Abstract

Understanding the molecular regulation of milk production by the ruminant mammary gland may help farmers meet increasing production milestones. Investigating these mechanisms currently relies upon invasive sampling by post-mortem or biopsy. The objective of this study was to determine if milk somatic cells, shed by the gland during milking, could be used as a non-invasive source of cells to measure these mechanisms. To answer this we employed two widely used molecular techniques, biochemical indices and quantitative polymerase chain reaction (qPCR). *Experiment 1*: Biochemical indices as measures of cell size, protein production capacity and efficiency were determined in milk somatic cells and mammary tissue harvested from six lactating dairy goats. Results showed protein production capacity ($P = 0.03$) and cell size ($P = 0.03$) were higher in mammary tissue compared to somatic cells while protein production efficiency was unaffected ($P = 0.55$). *Experiment 2*: Milk protein and ribosomal RNA gene expression were measured using qPCR in milk somatic cells and mammary tissue collected from three lactating dairy cows treated with growth hormone and four treated with saline. Results showed expression of all genes differed between milk somatic cells and mammary tissue. Taken together, these results indicate milk somatic cells are not suitable for measuring biochemical indices or gene expression in the lactating ruminant mammary gland.

Keywords: ruminant lactation; milk somatic cells; biochemical indices; gene expression

Introduction

The growing nutritional significance of ruminant milk has increased global demand, placing pressure on New Zealand farmers to make constant productivity increases through the application of novel environmental, genetic and nutritional intervention strategies (Lopez-Villalobos et al. 2000). Understanding how the mammary gland regulates milk production at the molecular level may be crucial to helping farmers meet increasing performance milestones.

Currently, the majority of studies investigating molecular regulation of milk production rely upon the collection of tissue harvested from biopsies or at slaughter (Hayashi et al. 2009; Soberon et al. 2010). Collection at slaughter has the advantage of harvesting large samples; however the requirement to euthanise animals provides samples from only one time point during lactation. Biopsies partially address this deficiency by allowing multiple sample collections during lactation; however they are invasive and can require long recovery times between sample collections (Soberon et al. 2010).

A potential solution lies in the mammary epithelial cells that, along with immune cells, are shed into milk during lactation. Previous studies in a wide range of species show that milk somatic cells are viable and exhibit characteristics of fully differentiated alveolar cells (Taylor-Papadimitriou et al. 1977; Smith-Kirwin et al. 1998). Ruminant studies using milk somatic cells collected from goats

(Boutinaud et al. 2002) and purified mammary epithelial cells from bovine milk (Boutinaud et al. 2008), show they can be used to determine the expression of key genes within the mammary gland.

Two techniques used to study molecular regulation of milk production are biochemical indices and quantitative polymerase chain reaction (qPCR). Biochemical indices are measured using the DNA, RNA and protein extracted from cells or tissue (Rao & Rao 1982). When they are quantified, the ratio of nucleic acids and protein can be used to indirectly measure cell size, using the ratio of protein to DNA; protein production capacity, the ratio of RNA to DNA; and protein production efficiency, the ratio of protein to RNA (Rao & Rao 1982). To date, no studies have been conducted to determine if milk somatic cells can be used to determine biochemical indices of the lactating ruminant mammary gland. Measurement of gene expression is routinely performed using qPCR (Chiang et al. 1996). This sensitive technique is employed throughout the dairy industry to determine changes in a wide range of factors affecting milk production, such as the presence of mastitis causing bacteria to the level of milk gene expression (Boss et al. 2011).

The objective of this study was to determine if milk somatic cells could be used to measure biochemical indices and gene expression in the lactating ruminant mammary gland.

Materials and methods

Animal trial procedures

All procedures involving animals were carried out in compliance with the guidelines of the AgResearch Grasslands Animal Ethics Committee, Palmerston North, New Zealand.

Experiment 1: Six dairy goats in the mid to late stages of their second-lactation were milked and cells harvested from the milk collected immediately prior to slaughter. At slaughter lobular-alveolar mammary tissue was collected and snap frozen in liquid nitrogen for subsequent analysis.

Experiment 2: The trial design and sample collection methodology has been described in detail elsewhere (Hayashi et al. 2009). Briefly, eight non-pregnant second parity spring-calves Jersey cows were treated with either a single 500 mg subcutaneous injection of a slow-release dose of commercially available growth hormone (Lactatropin, Elanco Animal Health, Bryanston, South Africa) or isotonic saline (Control). Cows were housed indoors, offered an *ad libitum* total mixed ration diet three times daily and milked twice daily. Six days following injection, milk somatic cells were collected and the cows euthanized. Mammary tissues were collected, and both sets of samples snap frozen in liquid nitrogen. RNA from the milk somatic cells was extracted and stored at -80°C at the time of the trial for use in future experiments, therefore biochemical indices could not be measured using these samples.

Collection of milk somatic cells

Milk somatic cells were harvested from goat's and cow's milk as follows. The mammary gland was first milked using a milking machine until no further milk was recovered. Between 50 mL and 150 mL of residual milk was removed by hand milking, collected into sterile 50 mL conical tubes and centrifuged at 600 *g* for 10 minutes at 4°C . Preparations from preliminary samples indicated that the collection of cells from residual milk yielded a much greater proportion of viable milk cells of between 60% and 70%, based on trypan blue viability staining. Cream and skim milk were removed and cell pellets were washed twice in 5 mL of ice-cold phosphate buffer solution (pH = 7.2) and centrifuged

at 600 *g* at 4°C for 10 minutes. All the supernatant was discarded and the pellet was snap frozen in liquid nitrogen and stored at -80°C .

Biochemical indices

Experiment 1. Milk somatic cells and mammary tissue, that had been harvested from lactating goats and stored at -80°C , were used to extract total DNA, RNA and protein using TRIzol® reagent (Invitrogen, Auckland, New Zealand), according to the manufacturer's instructions. DNA and RNA were quantified using a NanoDrop Spectrophotometer ND-1000 (Nanodrop Technologies, Wilmington, DE, USA). Protein was quantified using the Bradford method (Bradford 1976). The ratio of protein to DNA, a measure of cell size; the ratio of RNA to DNA, a measure of protein production capacity; and ratio of protein to RNA, a measure of protein production efficiency, were calculated. To compensate for variations in cell number, total DNA which is a measure of cell number, was used to normalise between milk somatic cells and mammary tissue. Differences between milk somatic cells harvested from lactating goats and mammary tissue were analysed using MIXED procedure (SAS 2006), with a linear model that included the fixed effects of mammary tissue. Log transformed data was back-transformed and presented with 95% confidence intervals (CI). Differences between means were considered significant at $P < 0.05$.

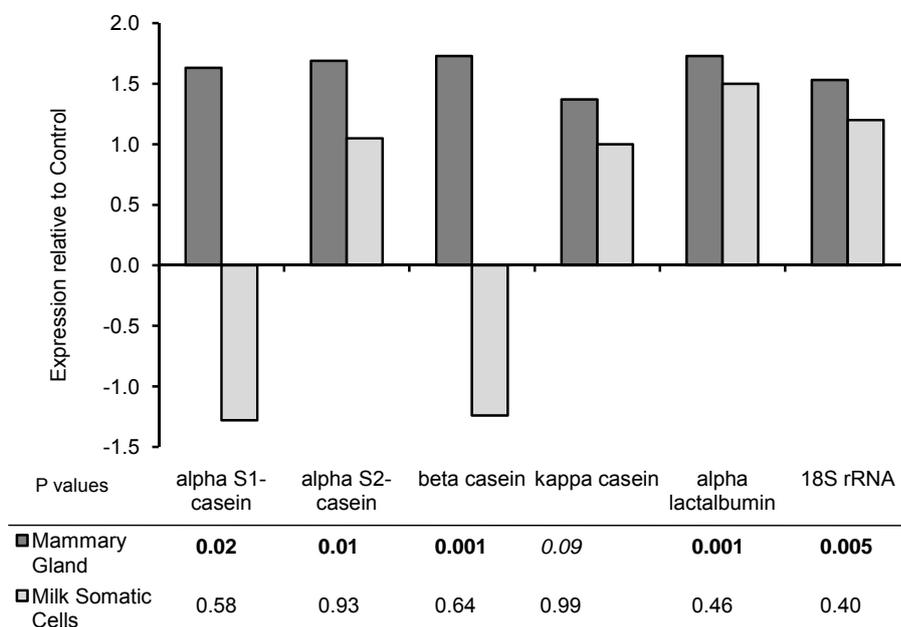
Quantitative PCR

Experiment 2. Stored bovine somatic cell RNA extracted from a previously published trial was used for this study (Hayashi et al. 2009). Mammary tissue RNA was extracted from the same cows using an RNeasy mini kit (Qiagen, San Diego, CA, USA), with on-column DNase I treatment (Qiagen, San Diego, CA, USA), according to the manufacturer's instructions. RNA integrity was assessed using gel electrophoresis in a 1% non-denaturing agarose gel, stained with SYBR® Safe (Invitrogen, Auckland, New Zealand). All milk somatic cell RNA samples for one GH treated cow were degraded so it was excluded from the analysis, leaving three treated cows and four control cows. The remaining milk somatic cell samples were purified using an RNeasy

Table 1 Biochemical indices of cell size (protein to DNA ratio), protein production capacity (RNA to DNA ratio) and protein production efficiency (protein to RNA ratio) from milk somatic cells and mammary tissue collected from six lactating goats. Back transformed data are represented as least squared means (95% confidence interval (CI)). Bolding of P value indicates significance ($P < 0.05$).

Index	Milk somatic cells	95% CI	Mammary tissue	95% CI	P value
Cell size	15.7	13.2–18.6	34.9	29.4–41.5	0.03
Protein production capacity	0.17	0.14–0.22	0.50	0.39–0.63	0.03
Protein production efficiency	90.1	71.9–113.0	70.4	56.2–88.3	0.55

Figure 1 Gene expression profiles of the major milk proteins and 18S ribosomal RNA in somatic cells and mammary tissue collected from four lactating dairy cows treated with growth hormone and three treated with saline. Columns represent relative expression in GH-treated animals compared to saline-treated controls, with P values noted below. Values in bold indicate significance at ($P < 0.05$) while values in italics indicate approaching significance with a P value between 0.05 and 0.10.



mini kit with on-column DNase I treatment (Qiagen, San Diego, CA, USA), to remove any residual DNA contamination. Purified RNA (500-ng) was reverse transcribed to make complementary DNA (cDNA) using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Auckland, New Zealand). Quantitative PCR was performed using the cDNA as a template and the SensiFAST™ SYBR No-ROX Kit (Bioline, London, UK) as per the manufacturer's instructions in a Rotor-Gene™ 6000 (Qiagen, San Diego, CA, USA). All primers used have been previously published (McCoard et al. 2010). The PCR efficiency and quantification cycle (Cq) values were obtained for each sample using LinRegPCR (Ramakers et al. 2003). Reactions were performed in triplicate and all values entered into REST 2009 (Pfaffl et al. 2002) and the fold change in expression determined.

Results

Experiment 1. Results showed protein production capacity was 294% ($P = 0.03$) and cell size was 223% ($P = 0.03$) higher in mammary tissue compared to milk somatic cells collected from goats, while no difference ($P = 0.55$) in protein production efficiency was observed (Table 1).

Experiment 2. Results showed that milk somatic cell expression of the major milk protein genes, alpha-S1-casein, alpha-S2-casein, beta-casein, kappa-casein, alpha-lactalbumin and the ribosomal RNA gene 18S, between GH treated and Control animals (Fig. 1) was not significantly different ($P > 0.05$). While expression significance was not detected in milk somatic cells, the direction of alpha-S2-casein,

kappa-casein, alpha-lactalbumin and 18S ribosomal RNA matched that of mammary tissue. The expression of alpha-S1-casein (-1.28-fold) and beta-casein (-1.24-fold) decreased in milk somatic cells from GH-treated animals, where expression in mammary tissue increased 1.63- and 1.73-fold, respectively. Overall, milk somatic cell expression of the major milk proteins and 18S ribosomal RNA was lower than that of mammary tissue.

Discussion

This study was conducted to determine if milk somatic cells could be used as non-invasive measures of the lactating ruminant mammary gland. To answer this question we employed two techniques widely used in molecular biology, biochemical indices and qPCR.

Biochemical indices are a quick and relatively inexpensive set of measurements that are made using the ratios of DNA, RNA and protein extracted from milk somatic cells and mammary tissue. Analysis of these ratios can be used to determine if a treatment, or nutritional regime, has altered levels of the components necessary to support milk production in the mammary gland (Toerien & Cant 2007). Our research showed that the biochemical indices measures of cell size, protein production capacity and protein production efficiency differed between milk somatic cells and mammary tissue harvested from lactating goats.

Gene expression analysis using qPCR is a method routinely employed to study how the lactating mammary gland controls and supports milk

production (Rius et al. 2010). It amplifies the RNA template used by milk secreting cells to manufacture milk components and the factors necessary to support their production (Chiang et al. 1996). This study shows that milk somatic cell expression of the major milk proteins and 18S ribosomal RNA was lower than that of mammary tissue. This result is consistent with previous research where northern blots were used to measure the expression of three milk genes in milk cells harvested from goat's milk. The authors of that study were able to show that when compared to mammary tissue, milk somatic cell hybridisation signals for alpha-S1-casein, kappa-casein and alpha-lactalbumin were always lower (Boutinaud et al. 2002).

We propose two potential explanations for why the biochemical indices and gene expression profiles differed between somatic cells and mammary tissue in the lactating ruminant. First, studies show that during lactation the ruminant mammary gland expresses high levels of the apoptosis markers BCL-2 and BAX around the acinar cell lining (Kim 2000) and present fragmented DNA laddering consistent with on-going apoptosis (Wilde et al. 1997). This suggests that milk somatic cells from this study may be entering apoptosis resulting in the observed decreases in protein production capacity, cell size and gene expression. Second, cells harvested from ruminant milk are a heterogeneous population of immune cells and milk secreting cells, with goat's milk also containing cytoplasmic particles (Boutinaud et al. 2002). While the residual milk collected in both experiments increased the percentage of milk somatic cells, studies show that residual milk still contains immune cells, albeit at a lower percentage compared to other fractions. This suggests that the remaining immune cells and cytoplasmic particles may contribute to the differences found in the biochemical indices measures and gene expression profiles between the milk somatic cells and the mammary tissue. To prevent this, it has been shown in several studies that milk secreting cells should be purified from milk using immuno-magnetic separation (Alcorn et al. 2002; Feng et al. 2007; Boutinaud et al. 2008; Boutinaud et al. 2012). This technique exploits the presence of cytokeratin 8 and other unique markers found on the surface of milk secreting cells (Gomm et al. 1995). Once purified, the milk secreting cells can be used as measures of changes occurring within the mammary gland.

Conclusion

This study has shown milk somatic cells harvested from residual milk cannot be used to assess biochemical indices or gene expression changes in the lactating ruminant mammary gland. Results from the literature suggest this may be due to apoptosis or the presence of immune cells and cytoplasmic particles. These studies advocate the purification of

milk secreting cells from milk before any molecular analysis is undertaken. This is important as milk somatic cells may be an inexpensive and non-invasive tool that can be used to understand the molecular regulation of milk production, and provide novel production technologies to dairy farmers. Therefore, the use of milk somatic cells to measure molecular changes within the lactating mammary gland warrants further investigation.

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References

- Alcorn J, Lu X, Moscow JA, McNamara PJ 2002. Transporter gene expression in lactating and nonlactating human mammary epithelial cells using real-time reverse transcription-polymerase chain reaction. *Journal of Pharmacology and Experimental Therapeutics* 303: 487–496.
- Boss R, Naskova J, Steiner A, Graber HU 2011. Mastitis diagnostics: quantitative PCR for *Staphylococcus aureus* genotype B in bulk tank milk. *Journal of Dairy Science* 94: 128–137.
- Boutinaud M, Rulquin H, Keisler DH, Djiane J, 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, Ben Chedly MH, Delamaire E, 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, Lollivier V, Finot L, Bruckmaier RM, Lacasse P 2012. Mammary cell activity and turnover in dairy cows treated with the prolactin-release inhibitor quinagolide and milked once daily. *Journal of Dairy Science* 95: 177–187.
- Bradford MM 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248–254.
- Chiang PW, Song WJ, Wu KY, Korenberg JR, Fogel EJ, Van Keuren ML, Lashkari D, Kurnit DM 1996. Use of a fluorescent-PCR reaction to detect genomic sequence copy number and transcriptional abundance. *Genome Research* 6: 1013–1026.
- Feng S, Salter AM, Parr T, 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.
- Gomm JJ, Browne PJ, Coope RC, Liu QY, Buluwela L, Coombes RC 1995. Isolation of pure populations of epithelial and myoepithelial cells from the

- normal human mammary gland using immunomagnetic separation with Dynabeads. *Analytical Biochemistry* 226: 91–99.
- Hayashi AA, Nones K, Roy NC, McNabb WC, Mackenzie DS, Pacheco D, McCoard S 2009. Initiation and elongation steps of mRNA translation are involved in the increase in milk protein yield caused by growth hormone administration during lactation. *Journal of Dairy Science* 92: 1889–1899.
- Kim SH 2000. Regulation of apoptosis and functional activity in bovine mammary Acini. *Korean Journal of Biological Sciences* 4: 347–352.
- Lopez-Villalobos N, Garrick DJ, Blair HT, Holmes CW 2000. Possible effects of 25 years of selection and crossbreeding on the genetic merit and productivity of New Zealand dairy cattle. *Journal of Dairy Science* 83: 154–163.
- McCoard S, Hayashi A, Pacheco D, Sciascia Q, Nones K, Rounce J, Sinclair BR, McNabb WC, Roy NC 2010. Effect of growth hormone on the mammary transcriptome profile during established lactation in the dairy cow. *Proceedings of the New Zealand Society of Animal Production* 70: 39–42.
- Pfaffl MW, Horgan GW, Dempfle L 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. *Nucleic Acids Research* 30: e36.
- Ramakers C, Ruijter JM, Deprez RH, Moorman AF 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. *Neuroscience Letters* 339: 62–66.
- Rao KV, Rao KS 1982. Changes in DNA, RNA and protein and the activities of acid and alkaline deoxyribonucleases in grey and white matter regions of developing and aging rat cerebrum. *Mechanisms of Ageing and Development* 18: 225–238.
- Rius AG, Appuhamy JA, Cyriac J, Kirovski D, Becvar O, Escobar J, McGilliard ML, Bequette BJ, Akers RM, Hanigan MD 2010. Regulation of protein synthesis in mammary glands of lactating dairy cows by starch and amino acids. *Journal of Dairy Science* 93: 3114–3127.
- SAS 2006. SAS/STAT 9.3 User's guide. SAS Institute Inc., NC, USA.
- Smith-Kirwin SM, O'Connor DM, De Johnston J, Lancey ED, Hassink SG, Funanage VL 1998. Leptin expression in human mammary epithelial cells and breast milk. *The Journal of Clinical Endocrinology and Metabolism* 83: 1810–1813.
- Soberon F, Lukas JL, Van Amburgh ME, Capuco AV, Galton DM, Overton TR 2010. Effects of increased milking frequency on metabolism and mammary cell proliferation in Holstein dairy cows. *Journal of Dairy Science* 93: 565–573.
- Taylor-Papadimitriou J, Shearer M, Tilly R 1977. Some properties of cells cultured from early-lactation human milk. *Journal of the National Cancer Institute* 58: 1563–1571.
- Toerien CA, Cant JP 2007. Abundance and phosphorylation state of translation initiation factors in mammary glands of lactating and nonlactating dairy cows. *Journal of Dairy Science* 90: 2726–2734.
- Wilde CJ, Quarrie LH, Tonner E, Flint DJ, Peaker M 1997. Mammary apoptosis. *Livestock Production Science* 50: 29–37.