Journal of Cell Signaling
ISSN: 2576-1471

Phyn et al., J Cell Signal 2017, 2:3

Research Article Open Access

Induced Physical Distension of Rat Mammary Glands Accelerates the Onset of Apoptosis and Involution

Claire V. C. Phyn[1,2], **Stephen R. Davis**[1,3], **Joanne M. Dobson**[1,4], **Kerst Stelwagen**[1,5*] **and Kuljeet Singh**[1,6]

[1]*AgResearch Ltd., Ruakura Research Centre, Hamilton 3214, New Zealand*

[2]*DairyNZ Ltd., Hamilton 3286, New Zealand*

[3]*LIC, Hamilton 3286, New Zealand*

[4]*Carne Technologies Ltd, Cambridge 3450, New Zealand*

[5]*SciLactis Ltd., Hamilton 3214, New Zealand*

[6]*Science Consultancy, Hamilton 3200, New Zealand*

[*]**Corresponding author:** Kerst Stelwagen, SciLactis Ltd, Waikato Innovation Park, Ruakura Lane, Hamilton 3240, New Zealand, Tel: +64211535152; E-mail: kerst.stelwagen@scilactis.co.nz

Received date: July 03, 2017; **Accepted date:** August 22, 2017; **Published date:** August 29, 2017

Abstract

Objective: Weaning is a process that results in a reduction in milk secretion, an increase in mammary epithelial cell (MEC) apoptosis, and involution of the mammary gland. The local mechanisms initiating MEC apoptosis and involution are unclear, although the physical morphology of the MEC may influence cell-cell and cell-extracellular matrix communication and thus may alter function. This study examined the effect of physical distension of alveoli on the early molecular events that occur at the onset of involution of rat mammary glands.

Methods: Mammary tissue was collected post-mortem from lactating Sprague-Dawley rats at 0, 1, 3, and 6 h (n=6 per time point) following acute physical distension of an inguinal gland with isosmotic sucrose solution (0.8 ml; equivalent to ~6 h worth of milk accumulation) *via* the teat canal followed by sealing with adhesive (infused). The remaining teats on each rat were either left unsealed for pups to suckle (control), or were sealed to induce milk accumulation and mammary engorgement (engorged).

Results: There was a low number of positive *in situ*-end labeled (ISEL) nuclei in suckled control glands indicating they had a low number of cells with fragmented DNA or were apoptotic. However, there was a greater number of ISEL nuclei in sucrose-infused and milk-engorged teat-sealed glands by 1 and 6 h, respectively, such that these changes were accelerated by sucrose infusion, compared with milk accumulation alone. The timing of the decline in abundance of β1-integrin (cell-extracellular matrix protein) and occludin (tight junction protein), and increase in abundance of the activated apoptotic marker signal transducer and activator of transcription factor-3 (pSTAT3) protein were also accelerated by sucrose infusion.

Conclusion: Physical distension by sucrose infusion accelerated the onset of the first stage of mammary apoptosis and involution, supporting a role for mechanotransduction during these processes.

Keywords: Mammary involution; Dairy cow; Mechanotransduction; Tight junctions; Rat

Abbreviations: EMC: Extracellular Matrix; ISEL: *In-situ* End Labelling; MEC: Mammary Epithelial Cell; pSTAT3: Phosphorylated Signal Transducer and Activator of Transcription Factor-3; SED: Standard Error of the Difference; TJ: Tight Junction; ZO-1: Zonula Occludens-1.

Introduction

Post-lactational involution in the rodent mammary gland requires the coordination of several processes including cessation of milk production, elimination of secretory mammary epithelial cells (MEC) by apoptosis, and extensive proteolytic degradation of the basement and extracellular matrix (ECM) membranes. At the onset of involution, milk accumulates within alveolar lumens. This locally-derived signal is sufficient to down-regulate prolactin receptors thereby down-regulating milk synthesis [1,2], milk protein genes [3], and cell survival factors, and up-regulating pro-apoptotic signals [4] and apoptosis [5-7]. It is uncertain whether milk accumulation activates these processes *via* an autocrine mechanism or stretch-sensitive pathways due to physical alveolar engorgement.

Studies indicate that the increased milk production response to more frequent milk removal was due to removal of a chemical inhibitor in milk of lactating goats [8,9] or casein-derived phosphopeptides causing local disruption of tight junction (TJ) integrity and inhibition of milk secretion in goats and cows [10,11]. The local synthesis of serotonin has also been implicated to disrupt TJ and regulate mammary function in cows [12,13]. However, other studies in lactating goats and cows have reported that during extended periods of milk accumulation, physical distension of the mammary gland rather than chemical inhibition is responsible for reductions in TJ integrity and milk secretion [14-16].

It has been proposed that physical distension causes changes in MEC shape, which may activate mechanotransduction pathways, resulting in loss of TJ integrity and ECM survival signalling [17,18]. To maintain secretory activity, the MEC require communication with the ECM *via* integrins and adjacent MEC through junctional complexes that consist of desmosomes, intermediate junctions and TJ [19]. Integrins have been shown to be mechanosensors in other cell types [20], and occludin, an integral transmembrane TJ protein, was down-regulated during stretch in lung alveolar epithelial cells [21]. Thus, the down-regulation of β1-integrin [22,23] and TJ proteins [24,25] during mammary engorgement could be implicated in this mechanotransduction cascade. Furthermore, the stretching of MEC *in vitro*, results in the induction of apoptotic pathways [26], and morphological changes in the mechanosensors primary cilia occur during involution of the mammary gland [27,28].

In the rodent mammary gland, processes initiating the progressive reduction in milk secretion, the loss of survival signals and the gain of pro-apoptotic signals occur within the first 6-12 h of milk accumulation [6,22,29,30]. Therefore, in this study, rat mammary glands were acutely distended to the equivalent of approximately 6 h milk accumulation, using an isosmotic sucrose solution, with the aim of accelerating involution and, at the same time, diluting any potential chemical feed-back factors in the milk. The role of mechanotransduction will be supported if early mammary involution events, such as acute changes in β1-integrin, TJ components (occludin, claudin-1 and ZO-1), pro-apoptotic marker signal transducer and activator of transcription factor-3 (pSTAT3), and the initiation of apoptosis are observed following induced physical distension.

Materials and Methods

Animals and tissue collection

All animal manipulations were conducted in compliance with the rules and guidelines of the Ruakura Animal Ethics Committee (Hamilton, New Zealand). Litter size of female Sprague-Dawley rats was adjusted to 10-12 pups at parturition (d 1 lactation). At peak lactation (d 13-17), twenty-four rats were randomly allocated post-suckling (i.e. with emptied mammary glands) to four groups (n=6 per group) and all rats had two teats (a caudal thoracic and an abdominal gland) sealed with surgical adhesive (Loctite 454 gel PRISM, Loctite Australia Pty. Ltd., Caringbah, NSW, Australia) to induce mammary engorgement. At the same time, an inguinal gland was acutely distended by injecting 0.8 ml of sterile isosmotic (i.e., to milk, measured as 300 mOsm) sucrose solution (BDH Laboratory Supplies, Poole, England), *via* the teat canal into the mammary gland and the teat sealed with surgical adhesive. Nine pups of each litter were returned to their dam and allowed to suckle the remaining unsealed teats, which served as controls.

The infusion volume of 0.8 ml isosmotic sucrose solution was calculated as follows using raw mammary weight data kindly provided by Dr C. McMahon from previous mammary engorgement studies [22,25]. Milk secretion rate was measured from the increase in mammary mass of three abdominal inguinal glands after 6 h of teat-sealing (mean 8.17 ± 0.52 g) compared with three suckled contralateral glands (mean 5.65 ± 0.23 g) from rats (n=6) at peak lactation (d 16). The mean 6 h milk secretion rate was calculated to be 0.84 ± 0.11 g per gland, which is in agreement with values of 0.84 to 1.30 g calculated from previously reported milk secretion data [29,30-33]. Therefore, a volume of 0.8 ml was chosen to induce distension of rat mammary glands equivalent to approximately 6 h of milk accumulation. Rat mammary glands were previously reported to be compliant to a volume of 1.0-1.2 ml during incremental (0.2 ml) intra-mammary infusion of skim milk, with greater volumes resulting in rapid rises in intra-mammary pressure and possible gland rupture [34].

Dams were killed by carbon dioxide asphyxiation and subsequent cervical dislocation either immediately or 1, 3, and 6 h after sealing teats (n=6 rats per time point). Mammary tissue was collected post-mortem for each treatment (control, engorged, infused) per rat and snap-frozen in liquid nitrogen before storage at -80°C for subsequent protein extraction. A 5 mm thick slice through a mammary gland was also collected for each treatment per rat and fixed for 24 h in 4% paraformaldehyde for histological analysis and *in situ* end-labelling (ISEL).

Intra-mammary infusion procedure

Animals were restrained in a plastic rat cone and anaesthetized with propofol (15 mg/kg bodyweight i.v. Rapinovet, Schering-Plough Animal Health Ltd., Upper Hutt, New Zealand) for the duration of the procedure (10-15 mins). To assist visualization of the teat orifice and facilitate even distribution of infused sucrose in the mammary gland, 100 mu i.v. oxytocin (Oxytocin V, Vetpharm (NZ) Ltd., Glenfield, Auckland, New Zealand) was also administered. The rat was placed on a warmed platform (37°C) beneath a dissecting microscope and an inguinal gland illuminated using a fiber-optic light source. Any excess fur was clipped to expose the teat area, which was then washed with 70% ethanol. The teat was gently grasped between thumb and forefinger and a small amount of milk ejected to visualize the opening of the teat canal. The fine tip of a glass capillary tube (1.0 mm OD, Fisher Scientific, Pittsburgh, Pennsylvania, USA) that had been drawn out over a flame and fire-polished to ~0.2-0.3 mm was gently inserted into the teat orifice. The sucrose solution (0.8 ml) was slowly injected into the mammary gland lumen from a syringe attached to the glass capillary tube *via* polyvinyl tubing (Dural Plastics and Engineering, Auburn, New South Wales, Australia). The teat was then sealed using surgical adhesive to prevent pups from removing the gland's contents.

In situ end-labelling (ISEL) analysis

Haematoxylin and eosin stained sections of alveolar mammary tissue from each time point (n=6 per group) were prepared from fixed tissue, examined under a microscope, and representative serial sections were chosen for ISEL studies described previously [23,25]. The ISEL was performed on suckled (control), teat-sealed (engorged) and acutely distended (infused) mammary sections, as described previously [23]. The Klenow fragment of a DNA polymerase was used to incorporate digoxigenin-11-2'-deoxy-uridine-5'-triphosphate (alkali stable, DIG-11-dUTP; Roche Applied Science, Mannheim, Germany), into fragmented or damaged DNA, a characteristic of apoptosis. Randomly selected fields (n=10; 100X and 200X magnification) were photographed per sample using a ProgRes C14 digital camera (JENOPTIK Laser, Optik, Germany) and visualized using Paint Shop Pro 7.02 software (Jasc Software Inc., Minnesota, USA). Quantitative analysis of the number of positive ISEL nuclei was carried out for each section using 100X magnification, as described previously [23]. Briefly, the numbers of ISEL nuclei and alveoli in each field were counted in ImageJ (US National Institute of Health, http://rsb.info.nih.gov/nih-image). The ISEL nuclei were identified as either located within the secretory epithelial layer or the lumen of mammary alveoli. Each count of ISEL nuclei per 100X magnification field was incremented by one

(to correct for counts of zero during $\log_{10}$-transformation) followed by a correction for the number of alveoli per field to obtain the mean number of ISEL nuclei per alveolus.

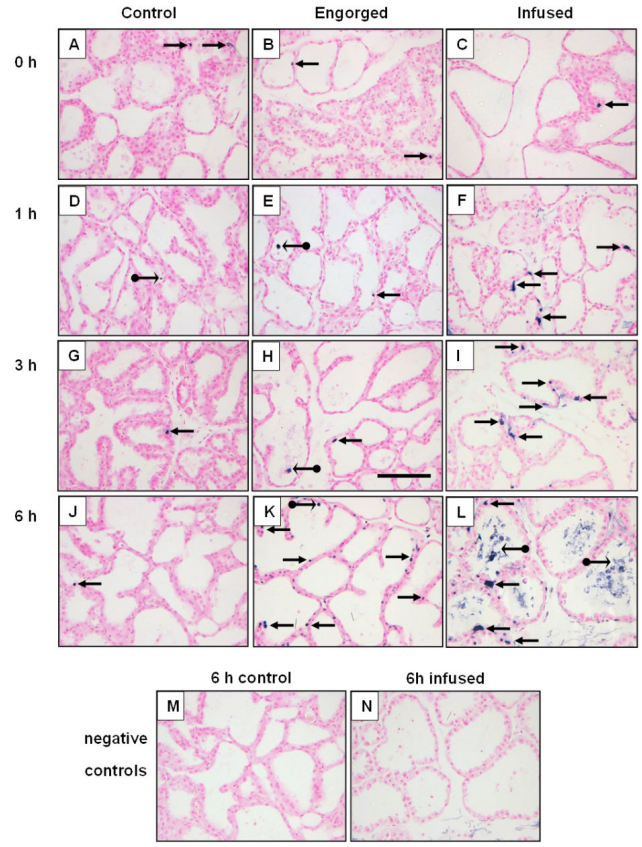

Figure 1: *In situ* end-labelling (ISEL) of apoptotic nuclei in rat mammary tissue following acute physical distension. Representative sections are shown at 200X magnification for suckled (control) glands and corresponding teat-sealed (engorged) and acutely distended (infused) glands at 0 h (A-C); 1 h (D-F); 3 h (G-I); and 6 h (J-L), respectively. Positive ISEL nuclei are labelled blue-black and indicate apoptosis. The omission of Klenow enzyme during ISEL was used to provide concurrent negative controls, with representative sections shown for 6 h control (M) and 6 h infused (N) glands. Sections are counterstained with nuclear fast red. Examples of epithelial ISEL nuclei (→), and luminal ISEL nuclei (single or regions →) are shown. Scale bars are at 100 μm.

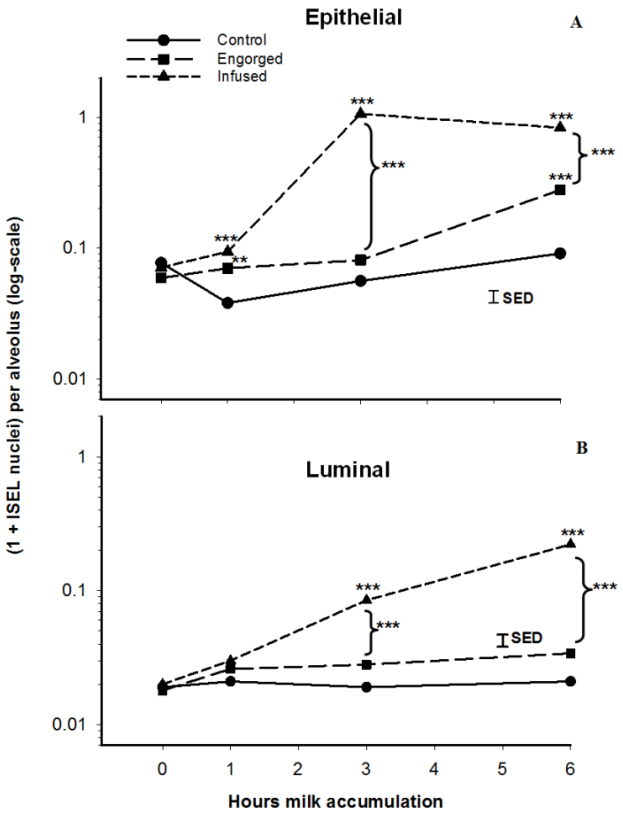

Figure 2: Quantitative analysis of the number of *in situ* end-labelled (ISEL) apoptotic nuclei located within alveolar epithelia or lumina following the acute physical distension of rat mammary glands. Data are expressed as the back-transformed mean number of epithelial (A) and luminal 1+ISEL nuclei per alveolus (B) for the suckled (control), teat-sealed (engorged) and acutely distended (infused) glands of each animal at 0, 1, 3 and 6 h (n=3 per time point) following teat sealing. The SED is shown for comparing numbers of ISEL nuclei between control, engorged and infused glands within a type (i.e., epithelial or luminal; **P<0.01, ***P<0.001 relative to control glands, }=P-value significant for infused glands relative to engorged glands), and for comparing type (i.e., epithelial *vs.* luminal) within a treatment (i.e., control or engorged or infused) at each time point.

Western blot analyses

Protein was isolated from 100 mg aliquots of ground mammary tissue and the concentration determined as described previously [22,25]. Western analyses were performed according to Laemmli [35] using 8%, 15% and 7% SDS-PAGE separating gels for occludin, claudin-1 or ZO-1 detection, respectively. For β1-integrin, STAT3 and pSTAT3 detection, samples were electrophoresed on 7.5% SDS-PAGE separating gels as described previously [36]. Twenty μg protein was loaded in each lane for all analyses except for claudin-1 where 40 μg was used. Separated proteins were then transferred, using either semi-dry (occludin and claudin-1 detection) or wet (β1-integrin, STAT3, pSTAT3 and ZO-1 detection) blotting systems, onto nitrocellulose membranes. Duplicate gels were used to demonstrate even loading using Coomassie blue staining.

The membranes were probed with primary antibodies to rabbit anti-human occludin (1:50,000 dilution), claudin-1 (1:30,000 dilution), and ZO-1 (1:50,000 dilution), all from Zymed Laboratories Inc. (California, USA); and with rabbit anti-human β1-integrin (1:3,000 dilution), and pSTAT3 (Tyr 705; 1:2,000 dilution), and rabbit anti-mouse STAT3 (1:10,000 dilution), all from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Membranes were then incubated with goat anti-rabbit secondary antibody conjugated to horseradish peroxidase (Sigma Chemical company, St. Louis, MO), followed by enhanced chemiluminescence, as described by McMahon et al. [22]. Developed films were scanned and immunoreactive bands subjected to

densitometric analyses using a GS-800 densitometer (Bio-Rad Laboratories) and Quantity One software (Bio-Rad Laboratories).

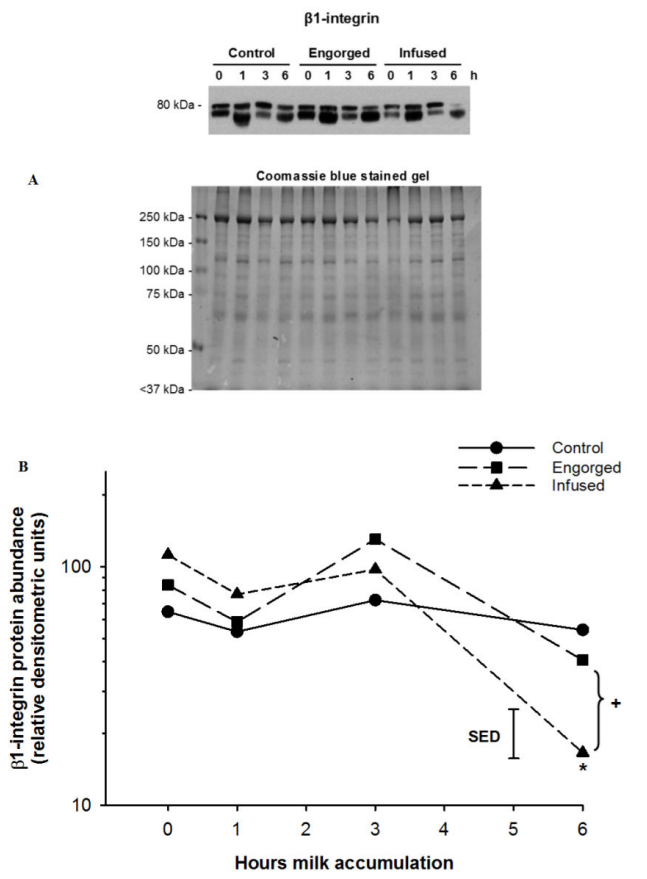

Figure 3: The pattern of abundance of β1-integrin protein following the acute physical distension of rat mammary glands. Densitometric analyses of western blots are shown for the abundance of β1-integrin in NP-40-soluble protein fractions in suckled (control), teat-sealed (engorged) and acutely distended (infused) mammary glands of animals at 0, 1, 3, and 6 h (n=6 per time point) following teat sealing. Densitometry for β1-integrin was performed on the top immunoreactive band at ~80 kDa. Results are presented as: (A) a representative western blot and duplicate gel stained with Coomassie blue to indicate uniformity of loading with molecular masses (kDa) indicated to the left of the panel and (B) back-transformed mean relative densitometric units with the SED to compare control, engorged and infused glands at each time point (+P<0.1, *P<0.05 relative to control glands, }=P-value significant for infused glands relative to engorged glands).

Statistical analyses

Data were analyzed by ANOVA in GenStat [37], with blocking on animal to detect differences between control, engorged and infused glands at each time point. For the quantitative analysis of ISEL apoptotic nuclei, data were $\log_{10}$-transformed, analysed by ANOVA and then expressed as the back-transformed mean (1+ISEL nuclei) per alveolus and per 100X magnification field. Densitometry results from western blotting were also $\log_{10}$-transformed and adjusted for between gel variations. The abundance of protein was then expressed as back-transformed mean relative units. Data are presented as means for the control, engorged and infused glands at each time point with the SED between means. The least significant differences identify the means significantly different from each other (*P<0.05, **P<0.01, or ***P<0.001).

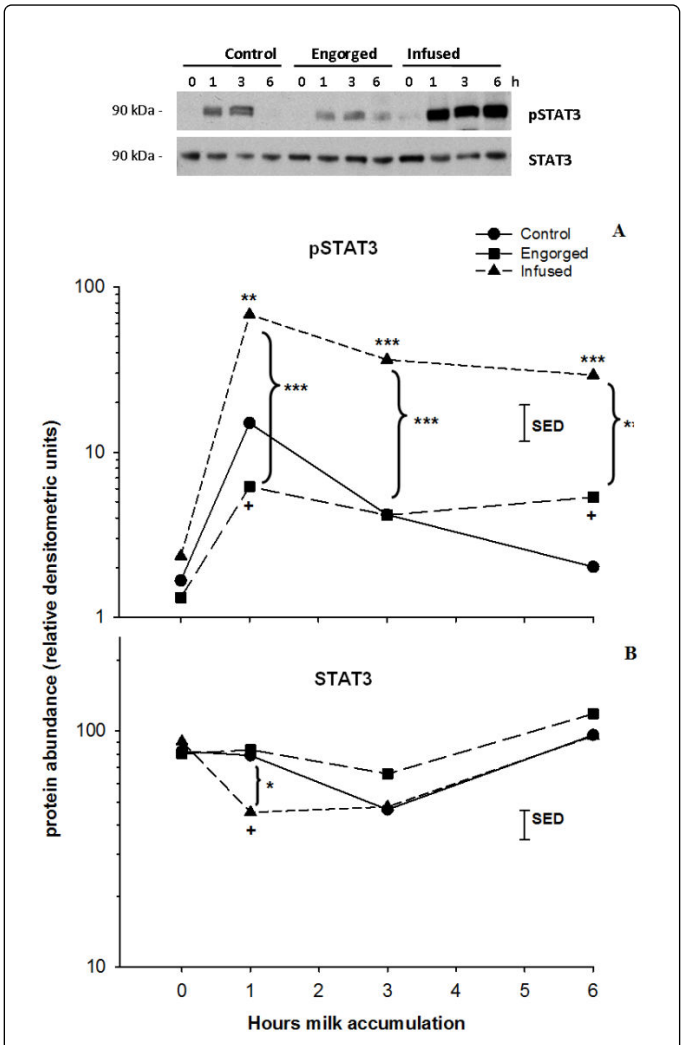

Figure 4: The pattern of abundance of pSTAT3 and STAT3 protein following the acute physical distension of rat mammary glands. Densitometric analyses of western blots are shown for the abundance of pSTAT3 and STAT3 in NP-40-soluble protein fractions in suckled (control), teat-sealed (engorged) and acutely distended (infused) mammary glands of animals at 0, 1, 3, and 6 h (n=6 per time point) following teat sealing. Results are presented as: (A) representative western blots and (B) back- transformed mean relative densitometric units with the SED to compare control, engorged and infused glands at each time point (+P<0.1, *P<0.05, **P<0.01, ***P<0.001 relative to control glands, }=P-value significant for infused glands relative to engorged glands). Duplicate gels were stained with Coomassie blue to indicate uniformity of loading per lane and a representative example is shown in Figure 3.

Results

Time course of onset of apoptosis following the acute physical distension of rat mammary glands

Representative sections of ISEL used to detect apoptosis in rat mammary tissue following acute physical distension are presented in Figure 1 at 200X magnification. Apoptotic nuclei were not labelled in negative controls (Figure 1M, 6 h control and N, 6 h infused). Numbers of positive ISEL nuclei, analyzed using 100X magnification are presented in Figure 2. A low frequency of apoptotic cells was detected in suckled (control) glands (Figures 1A, D, G and J and 2), with no changes in the number of total ISEL nuclei per alveolus (epithelial and luminal) during the course of the experiment. However, by 1 h, there was an increase in the total number of ISEL nuclei per alveolus ($P<0.001$) for infused glands compared with control glands. By 3 h and 6 h this difference was dramatic, with 17.7-fold and 11.2-fold increases ($P<0.001$) for infused glands compared with control glands. The total number of ISEL nuclei per alveolus was also increased for engorged glands compared with controls by 6 h ($P<0.001$). Furthermore, by 3 h and 6 h the numbers of total ISEL nuclei per alveolus in infused glands compared with engorged glands were increased by 12.4-fold and 3.7-fold ($P<0.001$), respectively, indicating that the acute physical distension procedure had accelerated the induction of apoptosis in rat mammary glands.

Within epithelia, the number of ISEL nuclei increased ($P<0.001$) at 1, 3 and 6 h for infused glands, while luminal ISEL nuclei increased ($P<0.001$) at 3 and 6 h, compared with control glands (Figure 2A). The number of epithelial ISEL nuclei per alveolus was also greater in engorged glands compared with control glands at 1 ($P<0.01$) and 6 h ($P<0.001$, Figure 2A), but there were no significant differences for luminal ISEL nuclei (Figure 2B). Moreover, by 3 and 6 h there were a greater ($P<0.001$) number of epithelial and luminal ISEL nuclei per alveolus in infused glands compared with engorged glands (Figure 2). For all treatments, the number of apoptotic cells was greater ($P<0.001$) in the epithelial layer than within alveolar lumens. Luminal ISEL apoptotic nuclei in infused glands at 6 h (Figure 2B) were associated with a small number of neutrophils (Figure 1L) and sloughed epithelial cells, although we were unable to distinguish between the apoptosis of these cell types.

The number of alveoli per 100X magnification field (data not shown) was used to correct counts of ISEL nuclei per field for changes in alveolar lumen size following induced physical distension and teat sealing. There was a significant decrease in the number of alveoli per field in infused glands compared with control glands at 3 ($P<0.001$) and 6 h ($P<0.05$), and compared with engorged glands at 0 and 3 h ($P<0.05$). A reduced number of alveoli per field were detected in engorged glands compared with control glands at 3 h only ($P<0.001$). However, the number of alveoli per field was greater ($P<0.01$) in control glands at 3 h compared with 0, 1 and 6 h, suggesting that the differences between treatments at 3 h should be interpreted carefully. The lower number of alveoli per field in infused glands at 0, 3 and 6 h reflects the accelerated distension of mammary glands following an infusion of sucrose equivalent to 6 h worth of milk secretion, which was visualized as an increased alveolar lumen size and flattening of the secretory epithelium in stained histological sections (Figure 1).

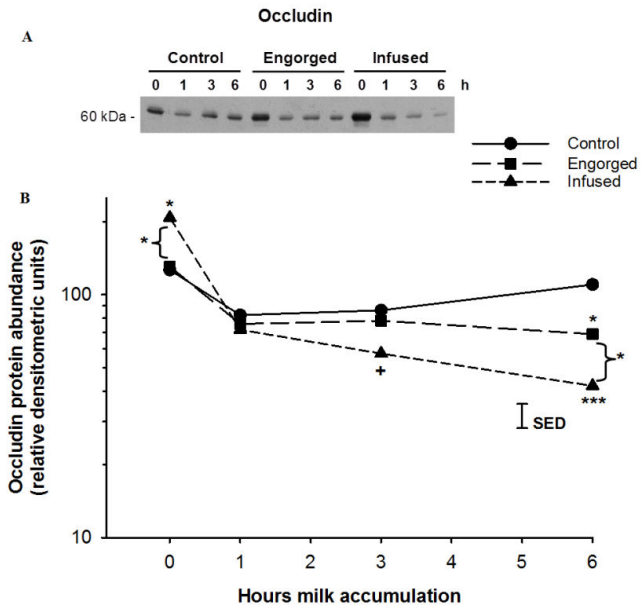

Figure 5: The pattern of abundance of occludin protein following the acute physical distension of rat mammary glands. Densitometric analyses of western blots are shown for the abundance of occludin in NP-40-soluble protein fractions in suckled (control), teat-sealed (engorged) and acutely distended (infused) mammary glands of animals at 0, 1, 3, and 6 h (n=6 per time point) following teat sealing. Results are presented as: (A) a representative western blot and (B) back-transformed mean relative densitometric units with the SED to compare control, engorged and infused glands at each time point (+$P<0.1$, *$P<0.05$, **$P<0.01$, ***$P<0.001$ relative to control glands, {=P-value significant for infused glands relative to engorged glands). Duplicate gels were stained with Coomassie blue to indicate uniformity of loading per lane and a representative example is shown in Figure 3.

Time course of changes in β1-integrin, STAT3 and TJ protein expression following the acute physical distension of rat mammary glands

The abundance of β1-integrin (Figure 3) and pSTAT3 (Figure 4) following induced physical distension of rat mammary glands were examined as examples of proteins well known to be down-regulated and up-regulated, respectively, during mammary apoptosis and involution. A band for β1-integrin was detected at ~80 kDa (Figure 3A). A lower MW band was also detected in, although only the ~80 kDa band of β1-integrin altered consistently with treatment as reported previously (McMahon et al. [22]). Antibodies against pSTAT3 detected multiple bands at ~90 kDa consistent with phosphorylation, while a single immunoreactive band was detected at ~90 kDa for total STAT3 (Figure 4A). The abundance of β1-integrin was reduced in infused glands compared with control (3.3-fold, $P<0.05$) and engorged (2.5-fold, $P<0.1$) glands by 6 h (Figure 3B). In contrast, pSTAT3 was increased in infused glands compared with control and engorged glands by 1 h (4.5-fold, $P<0.01$ and 11.0-fold, $P<0.001$; respectively), 3 h (8.7-fold, $P<0.001$ each) and 6 h (14.5-fold, $P<0.001$ and 5.5-fold, $P<0.01$; respectively) (Figure 4B). However, abundance of total STAT3

was down-regulated at 1 h in infused glands compared with control (1.7-fold, P<0.1) and engorged (1.8-fold, P<0.05) glands, with no other differences between treatments during the course of the experiment (Figure 4B). The STAT3 abundance in control glands was lower (P<0.05) at 3 h compared with 6 h and pSTAT3 expression was higher (P<0.01) at 1 h than 0 and 6 h, although no other differences were detected between time points (Figure 4B).

Immunoreactive bands for TJ proteins occludin (~60 kDa), claudin-1 (~22 kDa and ~28 kDa) and ZO-1 (~225 kDa) are shown in Figures 5A-7A, respectively. The abundance of occludin protein was up-regulated (1.6-fold, P<0.05) in infused glands compared with control and engorged glands immediately following physical distension at 0 h (Figure 5B). The abundance then declined in infused (2.6-fold, P<0.001) and engorged (1.6-fold, P<0.05) glands to be down-regulated relative to controls within 6 h of teat-sealing (Figure 5B). Furthermore, abundance was decreased (1.6-fold, P<0.05) in infused glands compared with engorged glands at 6 h. The abundance of claudin-1 ~28 kDa band decreased in engorged glands (4.2-fold, P<0.05), but not in infused glands, compared with controls by 6 h. However, there were no changes in abundance of the claudin-1 ~22 kDa band between treatments (Figure 6B). The ZO-1 protein abundance was decreased by 1.6-fold (P<0.05) in engorged glands, but not in infused glands, compared with controls by 6 h (Figure 7B).

In control glands, there were no significant differences detected between time points for β1-integrin, occludin and ZO-1 proteins (Figures 5B-7B, respectively). However, the abundance of claudin-1 ~28 kDa band was lower (P<0.01) at 3 h than 0 and 1 h for control glands, with no other differences between time points (Figure 6B). The abundance of the claudin-1 ~22 kDa band declined between 0 and 6 h time points for all treatments (Figure 6B).

Discussion

Overall, this study demonstrated that acute physical distension of rat mammary glands with an infusion of isosmotic sucrose solution accelerated the onset of apoptosis, the loss of abundance of β1-integrin and occludin protein, and the increase in pro-apoptotic pSTAT3 protein, compared with milk accumulation alone. This procedure would have diluted any chemical inhibitor(s) in milk and the intra-mammary volume would have been sufficient to distend the mammary epithelium to the equivalent of 6 h worth of milk accumulation following the cessation of milk removal. Therefore, these results support the hypothesis that physical distension of the mammary epithelium during extended periods of milk accumulation is a trigger of mammary apoptosis and involution [17-18]. This outcome is consistent with earlier studies in goats, in which progressive induction of distension without milk stasis (by isosmotic lactose replacement of milk removed at successive milking's) stopped milk secretion within 1-2 days [14], and acute distension of the mammary gland, with isosmotic sucrose solution to increase the intra-mammary pressure to levels normally observed following the cessation of milking, reduced the rate of milk secretion within 6 h [15]. Furthermore, in cows where milk removal was reduced to once daily, infusion of an intra-mammary solution of sucrose and lactose equivalent to "5 hours-worth" of milk secretion resulted in mammary TJ becoming leaky much earlier (after 7 h instead of 17 h post-milking) and a greater inhibition of milk secretion [16]. Again, in these experiments, dilution of potential chemical inhibitor(s) in milk would have occurred and these results would argue against a chemical feedback mechanism and, instead, suggest that physical distension plays a major role in regulating mammary function during infrequent milking or the cessation of milk removal.

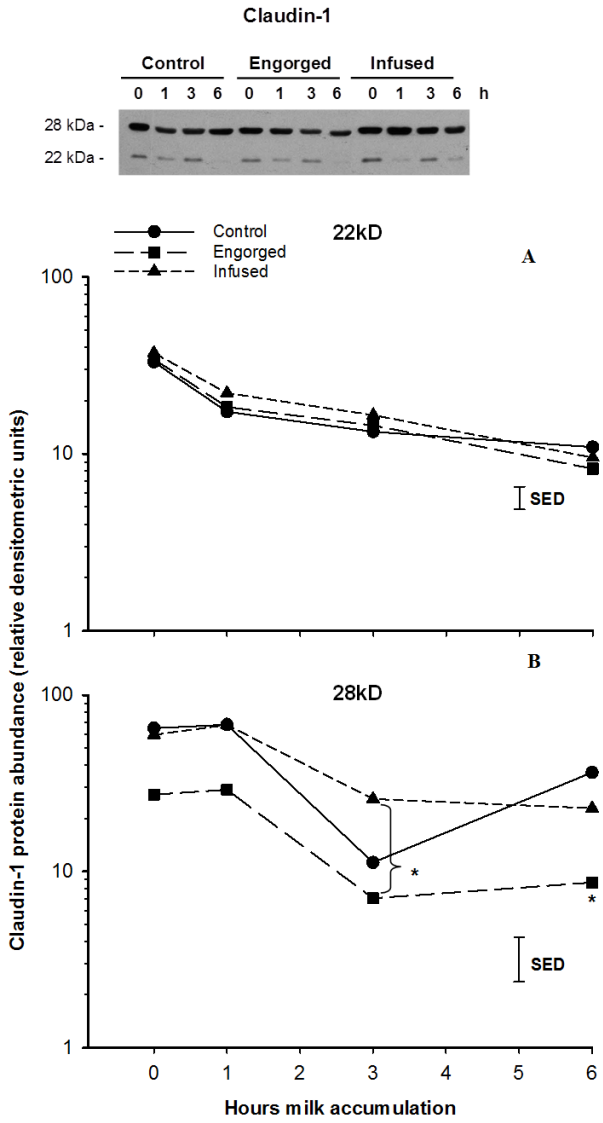

Figure 6: The pattern of abundance of claudin-1 protein following the acute physical distension of rat mammary glands. Densitometric analyses of western blots are shown for claudin-1 in NP-40-soluble protein fractions for the ~28 kDa and ~22 kDa bands, in suckled (control), teat-sealed (engorged) and acutely distended (infused) mammary glands of animals at 0, 1, 3, and 6 h (n=6 per time point) following teat sealing. Results are presented as: (A) a representative western blot and (B) back-transformed mean relative densitometric units with the SED to compare control, engorged and infused glands at each time point (*P<0.05 relative to control glands, }=P-value significant for infused glands relative to engorged glands). Duplicate gels were stained with Coomassie blue to indicate uniformity of loading per lane and a representative example is shown in Figure 3.

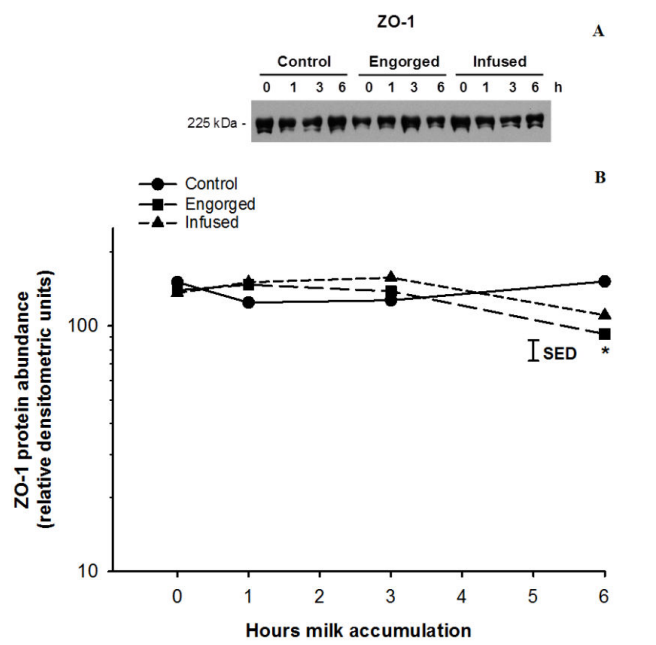

Figure 7: The pattern of abundance of ZO-1 protein following the acute physical distension of rat mammary glands. Densitometric analyses of western blots are shown for ZO-1 in NP-40-soluble protein fractions in suckled (control), teat-sealed (engorged) and acutely distended (infused) mammary glands of animals at 0, 1, 3, and 6 h (n=6 per time point) following teat sealing. Results are presented as: (A) a representative western blot and (B) back-transformed mean relative densitometric units with the SED to compare control, engorged and infused glands at each time point (+P<0.1, *P<0.05 relative to control glands). Duplicate gels were stained with Coomassie blue to indicate uniformity of loading per lane and a representative example is shown in Figure 3.

The validity of using intra-mammary infusions of isosmotic sucrose or lactose solutions to replace milk secretions and test chemical inhibition or physical distension hypotheses has been discussed previously [14,15]. It is unlikely that either sucrose or lactose themselves act as chemical inhibitors, as neither crosses the apical membrane into MEC [14,38]. Although the dilution of milk components in the alveolar lumen may cause some movement of ions (along concentration gradients) across the apical membrane, the use of an isosmotic solution should minimize osmotic water movements [39,40]. Furthermore, it is also unlikely that the observed changes in abundance of protein and degree of apoptosis in the present study were caused by mechanical rupture of the mammary epithelium, as no evidence of this was observed during post-mortem mammary tissue collection. Previous studies have also shown that the rat mammary gland is compliant to a volume of 1.0-1.2 ml [34].

Instead, these changes in abundance of TJ and ECM proteins and apoptosis were most likely induced by physical stretching of the mammary epithelium, which is confirmed by histological observations that most alveoli were distended, and, accordingly, there were reduced numbers of alveoli per field, and MEC were flattened after isosmotic sucrose infusion. Furthermore, in the hours following the infusion procedure there was reduced staining of milk components within alveolar lumens compared with teat-sealed glands, indicating that dilution of milk components, and possibly a reduction in milk secretion, had occurred. This histology was supported by gross observations during tissue collection that teat-sealed glands were pale pink in colour and engorged with milk secretion, whereas infused glands were very pale and contained a clear solution, presumably the infused sucrose along with diluted milk. Moreover, within 6 h of the infusion procedure, obvious gaps were visible between MEC, and some alveoli had started to collapse with large milk vesicles (containing coalescing fat droplets and proteins) apparent within alveolar lumens, indicating that a loss of TJ integrity had occurred consistent with the gradual decrease in occludin protein abundance within 3-6 h post-infusion.

In contrast, the immediate up-regulation of occludin protein in infused glands following the acute physical distension procedure may reflect an initial attempt to maintain mammary TJ integrity before the system was overwhelmed. Rapid increases in occludin protein, presumably to enhance TJ synthesis and repair, have also been reported in response to TJ breakdown induced by low-calcium conditions in mouse MEC *in vitro* [41]. Furthermore, TJ have previously been reported to respond to mechanical tension as alterations in TJ strand arrangement and orientation occur when MEC are stretched *in vitro* [42]. In lung alveolar epithelial cells increased TJ permeability occurs during cyclic stretch [21,43,44]. The latter was associated with a decline in abundance of occludin protein, but no differences were detected in ZO-1 protein [21]. Although there was a transient increase and then decline in occludin protein in the current study, there were no changes in the abundance of either ZO-1 or claudin-1 within 6 h following acute mammary physical distension.

Instead, there were decreases in occludin, claudin-1 ~28 kDa band and ZO-1 by 6 h in engorged teat-sealed glands compared with suckled controls, which is similar, albeit earlier, to the recently reported gradual decrease in these proteins in response to mammary engorgement [25]. However, only changes in the protein abundance of occludin, but not claudin-1 and ZO-1, appear to be accelerated by physical distension compared with milk accumulation alone. Although both claudin-1 and occludin are transmembrane TJ proteins and play a role in maintaining epithelial barrier function, occludin may also have a protective function and its disruption may play a role in initiating apoptosis and cell death signaling [45].

A rapid loss of cell-ECM survival signaling through decreased expression of integrins (β1, α6 and α5), and down-stream signal transduction factors FAK and 14-3-3, also occurs during the initial phase of mammary apoptosis and involution [22,23,36]. The present study indicates that in the rat mammary gland, the loss of β1-integrin protein is accelerated by induced physical distension of the secretory epithelium. Cell-ECM interactions mediated by integrins are essential for MEC survival, control of milk protein mRNA expression and maintenance of differentiated state [46-48]. Moreover, integrins can function as mechanotransducers in response to mechanical stress in other cell/tissue types (reviewed by [20,49]); with β1-integrin-signaling pathways mediating mechanical stretch-induced apoptosis in vascular smooth muscle cells [50].

Therefore, the changes in occludin and β1-integrin following physical distension of the mammary epithelium support the theory that cell-cell and cell-ECM contacts act as mechanosensors and/or participate in mechanotransduction pathways. Furthermore, these mechanisms may be indirectly linked *via* a close association with the cytoskeleton of the cell. F-actin (the polymerized, fibrous form of

actin) is a component of the cytoskeleton protein framework which gives the cell its shape, and is also thought to be an important sensor of mechanical stress in many cell types, including epithelia [21,51,52]. In this regard, cyclic stretch in lung alveolar epithelial cells alters F-actin distribution and increases TJ permeability [21,43,53]. These mechanisms are related as disruption of the F-actin cytoskeleton perturbs TJ structure and function [21,54], and stabilizing F-actin during cell stretching reduces the stretch-induced permeability increase [43]. In agreement, the abundance of ZO-1, a scaffolding protein linking occludin to the cytoskeleton [55], was also down-regulated in the current study, at 6 h, albeit that the effect was only statistically significant in the engorged group.

This study also indicated that mammary apoptosis can be induced by physical stretching of the mammary epithelium as increases in the number of epithelial, and to a lesser extent, luminal, apoptotic nuclei were observed by 3 and 6 h in infused glands compared with engorged and control glands. While the effect was very rapid, the ISEL technique can detect apoptosis in cells at a very early stage, before they have entered the actual point of cell death [56], and a similar induction in apoptosis in thymus glands has been documented within 2 h following dexamethasone treatment of adrenalectomized rats [57].

The active, phosphorylated, form of STAT3, but not total STAT3, was up-regulated within 1 h following the acute physical distension procedure. Phosphorylated STAT3 is a marker of apoptosis and the acute phase immune response, and is greatly increased during the early stages of both rodent [6,58,59] and bovine [60] mammary involution. In the present study, STAT3 is activated in response to induced physical distension of the mammary epithelium, which is in agreement with Quaglino et al. [26] who reported that mammary cell stretching *in vitro* also resulted in the activation of STAT3. Taken together, these studies indicate that STAT3 participates in mechanotransduction pathways during extended periods of milk accumulation.

In the present study, physical distension accelerated the onset of the first stage of mammary apoptosis and involution, compared with milk accumulation alone; however, an adjunct role for autocrine inhibitory mechanism(s) in the control of local mammary function cannot be excluded. In lactating goats, the milk secretion rate was increased when milk was removed at an extra daily milking, even when the milk was replaced immediately by an equal volume of an inert sucrose solution to maintain the gland's distension [8]. This suggests that the response was not due to relief from the physical presence of accumulated milk. Similarly, when milk stored in the mammary gland was diluted with an isosmotic sucrose solution, the milk secretion rate increased suggesting that dilution of a chemical inhibitor occurred [9]. Casein phosphopeptides derived from activation of the milk protease plasmin disrupt TJ and inhibit milk secretion during extended periods of milk accumulation in goats and cows [10,11,61], although the exact mechanism by which this occurs remains to be determined.

However, β-casein (fraction 1-28), a phosphopeptide peptide derived from mild activation of plasmin activity, reduces milk secretion by a process associated with its ability to block potassium channels in the apical membranes of MEC [62]. Furthermore, a separate mechanism that regulates TJ during milk stasis implicates local synthesis of serotonin by the mammary epithelium in a negative feedback, autocrine-paracrine loop that opposes endocrine stimulation of milk production and secretion in lactating mice [63] and dairy cows [12,13]. Hence, while the current study supports physical distension (i.e. mechanotransduction) as a regulator of mammary function during extended periods of milk accumulation, it is likely that both, chemical and physical mechanisms, acting either independently or in concert, finely control mammary responses to the frequency and completeness of milk removal. Although the results from our *in vivo* study are in agreement with those from an *in vitro* study showing that mechanical stress resulted in the induction of other mammary involution-associated factors, such as c-FOS, ERK1/2, AKT and leukemia inhibitory factor [26], it is unclear how physical distension would impact on the various involution-associated cell signaling pathway.

Acknowledgement

The authors gratefully acknowledge Rick Broadhurst and the staff at the Ruakura Small Animal Colony (AgResearch Ltd) for animal care and maintenance, and Harold Henderson (AgResearch Ltd) for providing statistical advice. This study was funded by Ministry of Business, Innovation and Employment (MBIE).

Financial Support

The research was supported by a grant from the Business, Innovation and Employment (MBIE) Ministry of New Zealand (Contract grant number: FRST C10X0702).

References

1. Hayden TJ, Smith SV (1981) Effects of bromocriptine and occlusion of nipples on prolactin receptor and lactose synthetase activity in the mammary gland of the lactating rat. J Endocrinol 91: 225-232.
2. Kim JY, Mizoguchi Y, Yamaguchi H, Enami J, Sakai S (1997) Removal of milk by suckling acutely increasis the prolactin receptor gene expression in the lactating mouse mammary gland. Mol Cell Endocrinol 131: 31-38.
3. Strange R, Li F, Saurer S, Burkhardt A, Friis RR (1992) Apoptic cell death and tissue remodeling during mouse mammary gland involution. Development 115: 49-58.
4. Green KA, Streuli CH (2004) Apoptosis regulation in the mammary gland. Cell Mol Life Sci 61: 1867-1883.
5. Quarrie LH, Addey CV, Wilde CJ (1996) Programmed cell death during mammary tissue involution induced by weaning, litter removal, and milk stasis. J Cell Physiol 168: 559-569.
6. Li M, Liu X, Robinson G, Bar-Peled U, Wagner KU, et al. (1997) Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. Proc Natl Acad Sci USA 94: 3425-3430.
7. Marti A, Feng Z, Altermatt HJ, Jaggi R (1997) Milk accumulation triggers apoptosis of mammary epithelial cells. Eur J Cell Biol 73: 158-165.
8. Henderson AJ, Peaker M (1984) Feed-back control of milk secretion in the goat by a chemical in milk. J Physiol 351: 39-45.
9. Henderson AJ, Peaker M (1987) Effects of removing milk from the mammary ducts and alveoli, or of diluting stored milk, on the rate of milk secretion in the goat. Q J Exp Physiol 72: 13-19.
10. Shamay A, Shapiro F, Leitner G, Silanikove N (2003) Infusions of casein hydrolyzates into the mammary gland disrupt tight junction integrity and induce involution in cows. J Dairy Sci 86: 1250-1258.
11. Shamay A, Shapiro F, Mabjeesh SJ, Silanikove N (2002) Casein-derived phosphopeptides disrupt tight junction integrity, and precipitously dry up milk secretion in goats. Life Sci 70: 2707-2719.
12. Hernandez LL, Collier JL, Vomachka AJ, Collier RJ, Horseman ND (2011) Suppression of lactation and acceleration of involution in the bovine mammary gland by a selective serotonin reuptake inhibitor. J Endocrinol 209: 45-54.
13. Collier RJ, Hernandez LL, Horseman ND (2012) Serotonin as a homeostatic regulator of lactation. Domest Anim Endocrinol 43: 161-170.

14. Fleet IR, Peaker M (1978) Mammary function and its control at the cessation of lactation in the goat. J Physiol 279: 491-507.
15. Peaker M (1980) The effect of raised intramammary pressure on mammary function in the goat in relation to the cessation of lactation. J Physiol 301: 415-428.
16. Stelwagen K, Farr VC, Davis SR, McFadden HA (1998) Inhibition of milk secretion and the extent of filling of the bovine mammary gland. J Dairy Sci (Suppl 1) 81: 376.
17. Davis SR, Farr VC, Stelwagen K (1999) Regulation of yield loss and milk composition during once-daily milking: a review. Livest Prod Sci 59: 77-94.
18. Stelwagen K (2001) Effect of milking frequency on mammary functioning and shape of the lactation curve. J Dairy Sci 84: E204-E211.
19. Mepham TB (1987) Physiology of lactatioo. Open University Press, Milton Keynes, England.
20. Geiger B, Bershadsky A (2002) Exploring the neighborhood: adhesion-coupled cell mechanosensors. Cell 110: 139-142.
21. Cavanaugh KJ, Oswari Jr. J, Margulies SS (2001) Role of stretch on tight junction structure in alveolar epithelial cells. Am J Respir Cell Mol Biol 25: 584-591.
22. McMahon CD, Farr VC, Singh K, Wheeler TT, Davis SR (2004) Decreased expression of ß1-integrin and focal adhesion kinase in epithelial cells may initiate involution of mammary glands. J Cell Physiol 200: 318-325.
23. Singh K, Dobson J, Phyn CVC, Davis SR, Farr VC, et al. (2005) Milk accumulation decreases expression of genes involved in cell-extracellular matrix communication and is associated with induction of apoptosis in the bovine mammary gland. Livest Prod Sci 98: 67-78.
24. Markov AG, Kruglova NM, Fomina YA, Fromm M, Amasheh S (2012) Altered expression of tight junction proteins in mammary epithelium after discontinued suckling in mice. Pflugers Arch-Eur J Physiol 463: 391-398.
25. Phyn CV, Stelwagen K, Davis SR, McMahon CD, Dobson JM, et al. (2017) Tight junction protein abundance and apoptosis during involution of rat mammary glands. J Cell Physiol 232: 2075-2082.
26. Quaglino A, Salierno M, Pellegrotti J, Rubinstein N, Kordon EC (2009) Mechanical strain induces involution-associated events in mammary cells. BMC Cell Biol 10: 55e.
27. Millier MJ, Singh K, Poole CA (2013) Characterization of primary cilia distribution and morphology during lactation, stasis, and involution in the bovine mammary gland. Anat Rec (Hoboken) 296: 1943-1953.
28. Biet J, Poole CA, Stelwagen K, Margerison JK, Singh K (2016) Primary cilia distribution and morphology during involution of the bovine mammary gland. J Dairy Sci 99: 3966-3978.
29. Hanwell A, Linzell JL (1972) A simple technique for measuring the rate of milk secretion in the rat. Comp Biochem Physiol A Comp Physiol 43: 259-270.
30. Nguyen AV, Pollard JW (2000) Transforming growth factor beta3 induces cell death during the first stage of mammary gland involution. Development 127: 3107-3118.
31. Grigor MR, Allan JE, Carrington JM, Carne A, Geursen A, et al. (1987) Effect of dietary protein and food restriction on milk production and composition, maternal tissues and enzymes in lactating rats. J Nutr 117: 1247-1258.
32. Grigor MR, Poczwa Z, Arthur PG (1986) Milk lipid synthesis and secretion during milk stasis in the rat. J Nutr 116: 1789-1797.
33. Grigor MR, Thompson MP (1987) Diurnal regulation of milk lipid production and milk secretion in the rat: effect of dietary protein and energy restriction. J Nutr 117: 748-753.
34. DeNuccio DJ, Grosvenor CE (1971) Effects of volume and distribution of milk on the oxytocin-induced contraction of the lactating rat mammary gland in vivo. J Endocrinol 51: 437-446.
35. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 227: 680-685.
36. Singh K, Davis SR, Dobson JM, Molenaar AJ, Wheeler TT, et al. (2008) cDNA microarray analysis reveals that antioxidant and immune genes are upregulated during involution of the bovine mammary gland. J Dairy Sci 91: 2236-2246.
37. Lawes Agricultural Trust (2005) GenStat for Windows, Version 8.1.
38. Peaker M (1978) Ion and water transport in the mammary gland. Larson BL, editor. New York: Academic Press. pp: 437-462.
39. Linzell JL, Mepham TB, Peaker M (1976) The secretion of citrate into milk. J Physiol 260: 739-750.
40. Peaker M (1977) Mechanism of milk secretion: milk composition in relation to potential difference across the mammary epithelium. J Physiol 270: 489-505.
41. Stelwagen K, Callaghan MR (2003) Regulation of mammary tight junctions through parathyroid hormone-related peptide-induced activation of apical calcium channels. J Endocrinol 178: 257-264.
42. Pitelka DR, Taggart BN (1983) Mechanical tension induces lateral movement of intramembrane components of the tight junction: studies on mouse mammary cells in culture. J Cell Biol 96: 606-612.
43. Cavanaugh KJ, Cohen TS, Margulies SS (2006) Stretch increases alveolar epithelial permeability to uncharged micromolecules. Am J Physiol Cell Physiol 290: C1179-C1188.
44. Cavanaugh KJ, Jr Margulies SS (2002) Measurement of stretch-induced loss of alveolar epithelial barrier integrity with a novel in vitro method. Am J Physiol Cell Physiol 283: C1801-C1808.
45. Beeman N1, Webb PG, Baumgartner HK (2012) Occludin is required for apoptosis when claudin-claudin interactions are disrupted. Cell Death Dis 3: e273.
46. Boudreau N, Sympson CJ, Werb Z, Bissell MJ (1995) Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. Science 267: 891-893.
47. Pullan S, Wilson J, Metcalfe A, Edwards GM, Goberdhan N, et al. (1996) Requirement of basement membrane for the suppression of programmed cell death in mammary epithelium. J Cell Sci 109: 596 631-642.
48. Faraldo MM, Deugnier MA, Tlouzeau S, Thiery JP, Glukhova MA (2002) Perturbation of beta1-integrin function in involuting mammary gland results in premature dedifferentiation of secretory epithelial cells. Mol Biol Cell 13: 3521-600 3531.
49. Shyy JY, Chien S (1997) Role of integrins in cellular responses to mechanical stress and adhesion. Curr Opin Cell Biol 9: 707-713.
50. Wernig F, Mayr M, Xu Q (2003) Mechanical stretch-induced apoptosis in smooth muscle cells is mediated by beta1-integrin signaling pathways. Hypertension 41: 903-911.
51. Dewey CF Jr (1984) Effects of fluid flow on living vascular cells. J Biomech Eng 106: 31-35.
52. Wechezak AR, Viggers RF, Sauvage LR (1985) Fibronectin and F-actin redistribution in cultured endothelial cells exposed to shear stress. Lab Invest 53: 639-647.
53. Margulies SS, Oswari J, Matthay MA, Tschumperlin DJ (1999) Alveolar epithelial cytoskeleton and cell vulnerability to stretch. Proc Bioengen Conf 42: 517-518.
54. Madara JL (1992) Relationships between the tight junctions and ther cytoskeleton. In: Cereijido M, editor. Tight Junctions, CRC Press, Boca Raton. pp: 105-120.
55. Fanning AS, Jameson BJ, Jesaitis LA, Anderson JM (1998) The tight junction protein ZO-1 establishes a link between the transmembrane protein occludin and the actin cytoskeleton. J Biol Chem 273: 29745-29753.
56. Migheli A, Attanasio A, Schiffer D (1995) Ultrastructural detection of DNA strand breaks in apoptotic neural cells by in situ end-labelling techniques. J Pathol 176: 27-35.
57. Compton MM, Cidlowski JA (1986) Rapid in vivo effects of glucocorticoids on the integrity of rat lymphocyte genomic deoxyribonucleic acid. Endocrinology 118: 38-45.
58. Philp JA, Burdon TG, Watson CJ (1996) Differential activation of STATs 3 and 5 during mammary gland development. FEBS Lett 396: 77-80.

59. Chapman RS, Lourenco PC, Tonner E, Flint DJ, Selbert S, et al. (1999) Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. Genes Dev 13: 2604-2416.

60. Singh K, Vetharaniam I, Dobson JM, Prewitz M, Oden K, et al. (2016) Cell survival signaling in the bovine mammary gland during the transition from lactation to involution. J Dairy Sci 99: 1-21.

61. Silanikove N, Merin U, Leitner G (2006) Physiological role of indiginous milk enzymes: an overview of an evolving picture. Int Dairy J 16: 533-545.

62. Silanikove N, Shamay A, Shinder D, Moran A (2000) Stress down regulates milk yield in cows by plasmin induced beta-casein product that blocks K+ channels on the apical membranes. Life Sci 67: 2201-2212.

63. Matsuda M, Imaoka T, Vomachka AJ, Gudelsky GA, Hou Z, et al. (2004) Serotonin regulates mammary gland development via an autocrine-paracrine loop. Dev Cell 6: 193-203.