Regulation of tight junction proteins during engorgement of the mammary gland

Claire Vanessa Cooper Phyn

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Regulation of tight junction proteins during engorgement of the mammary gland

A thesis presented in partial fulfilment of the requirements for the degree of

Doctor of Philosophy
in Animal Science

at

Massey University
Palmerston North, New Zealand.

Claire Vanessa Cooper Phyn

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Abstract

Extended periods of milk accumulation result in loss of secretory activity, increased apoptosis and eventually, involution of mammary glands. This process is associated with increased permeability of the tight junction (TJ) complexes between adjacent mammary epithelial cells (MECs). The change in cell shape during mammary engorgement from a cuboidal to a flattened morphology may initiate changes in protein and gene expression (mechanotransduction) that trigger these processes. Therefore, this study examined the regulation of the major TJ protein components during mammary engorgement, and in particular the role of physical distension of the mammary epithelium in the regulatory process. Expression of the integral transmembrane TJ proteins, occludin and claudin-1, and the cytoplasmic TJ protein, ZO-1, were down-regulated in both bovine and rat mammary glands during the early stages of mammary apoptosis and involution following the abrupt cessation of milk removal. In the rat, these responses were locally regulated as they occurred only in teat-sealed glands in a hemi-suckled model. Furthermore, the down-regulation of TJ proteins is consistent with a loss of TJ integrity during mammary engorgement. Induced physical distension of rat mammary glands *in vivo* transiently up-regulated the expression levels of occludin protein and mRNA, and ZO-1 mRNA, followed by an accelerated decrease in expression compared with the effects of milk accumulation alone. This was associated with the initiation of apoptosis, the up-regulation of the pro-apoptotic factor pSTAT3, and the down-regulation of the cell-ECM survival factor β1-integrin. An *in vitro* model was also developed to stretch MECs, mimicking the flattening in cell shape during mammary engorgement *in vivo*. While stretching MECs *in vitro* did not conclusively alter TJ protein expression, the overall results of this project support further investigation into the role of the TJ complex in mechanotransduction pathways. In addition, the results point to crosstalk between cell-ECM survival signalling and STAT3 death signalling as a candidate for regulation by physical distension of the mammary epithelium. In conclusion, this study supports the hypothesis that physical distension during engorgement of the mammary glands with milk is a primary trigger initiating apoptosis of MECs through changes in the regulation of gene pathways controlling cell survival and death, and the disruption of TJ function.

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To my husband Derek,

even though he may not understand a word of it…

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List of Abbreviations

S.I. (Système International d'Unités) abbreviations for units, and standard notations for chemical elements, formulae and chemical abbreviations are used in the text. Other abbreviations are listed below.

A_{260}	absorbance at 260 nm
A_{280}	absorbance at 280 nm
A_{260}/A_{280}	ratio of absorbance at 260 nm to absorbance at 280 nm
ABHA	p-aminobenzoyl-gly-pro-$_D$-leu-$_D$-ala hydroxamic acid
ANOVA	analysis of variance
APS	ammonium persulphate
bp	base pairs
BCA	bicinchoninic acid
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt
BME	β-mercaptoethanol
BSA	bovine serum albumin
CAPS	3-[cyclohexylamino]-1-propanesulfonic acid
cDNA	complementary DNA
contig	contiguous sequence
C_T	threshold cycle
DAB	3, 3'-diaminobenzidine tetrahydrochloride
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ΔMSA	change in membrane surface area
DEPC	diethylpyrocarbonate
dGTP	deoxyguanosine triphosphate
DIG-11-dUTP	digoxigenin-11-2'-deoxy-uridine-5'-triphosphate
DMEM	Dulbecco's modified Eagle's medium
DMEM:F12	Dulbecco's modified Eagle's medium F12 nutrient mix
DMSO	dimethyl sulphoxide

DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
DPX	DePeX mounting agent
dsDNA	double-stranded DNA
DTT	1, 4-dithiothreitol
dTTP	deoxythymidine triphosphate
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EST	expressed sequence tag
FAK	focal adhesion kinase
FCS	foetal calf serum
FIL	feedback inhibitor of lactation
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GAR	goat anti-rabbit
H&E	haematoxylin and eosin
HEPES	4-[2-hydroxyethyl]-1-piperazineethanesulphonic acid
IAA	isoamyl alcohol
i.d.	internal diameter
IgA	immunoglobulin A
IGF-1	insulin-like growth factor 1
IgG	immunoglobulin G
ISEL	*in situ* end labelling
i.v.	intravenous
Kb	kilobase
kDa	kiloDaltons
kHz	kilohertz
LB	Luria broth
mA	milliamperes
MDCK	Madin-Darby canine kidney cells
MEC	mammary epithelial cell
MOPS	3-[N-morpholino]propane-sulphonic acid

MQ	Milli-Q filter-purified water
mRNA	messenger RNA
MSA	membrane surface area
MSAA	mammary serum amyloid A3
MW	molecular weight
NBT	4-Nitro-Blue tetrazolium chloride
NFR	nuclear fast red
NP-40	Nonidet P-40
ODM	once-daily milking
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
polyA	polyadenylated
PRL	prolactin
PVP	polyvinylpyrolidone
rep.	replicates
RmT	room temperature
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT	reverse transcriptase
RT-PCR	reverse transcription-polymerase chain reaction
rRNA	ribosomal RNA
SCC	somatic cell count
SED	standard error of the difference
SEM	standard error of the mean
SDS	sodium dodecyl sulphate
SSC	salt/sodium citrate
ssDNA	single-stranded DNA
TAE	Tris-acetate EDTA
TB	Terrific broth
TBS	Tris buffered saline
TBST	Tris buffered saline containing Tween-20

TDM	twice-daily milking
TE	Tris/EDTA
TEMED	N, N, N', N'-tetra-methylenediamine
TER	transepithelial resistance
TGF	transforming growth factor
TJ	tight junction
T_m	melting temperature
TNF	tumor necrosis factor
Tris	Trizma Base or Tris[hydroxymethyl]aminomethane
Tris HCl	2-amino-2-(hydroxymethyl)-1,3-propanediol
U	Units
UV	ultraviolet
V	volts
VEA	vesicle-engorged alveoli
v/v	volume per volume
w/v	weight per volume
ZO	zonnula occludens

CHAPTER ONE

Review of Literature

1.1 INTRODUCTION

Understanding the physiological and molecular mechanisms regulating the mammary responses that occur at the cessation of milking and the subsequent initiation of mammary involution may provide insights that improve lactation traits such as persistency and milk production responses to milking frequency (e.g., once-daily milking), and lead to the development of novel strategies for improving milk production, especially in later lactation (Stelwagen, 2001; Vetharaniam *et al.*, 2003).

Both extended milking intervals and the abrupt cessation of milk removal result in extended periods of milk accumulation within the mammary gland. Local intra-mammary signals are rapidly initiated which progressively lead to reduced milk secretion, increased mammary secretory cell death (i.e., by apoptosis) and ultimately, mammary involution, if the gland remains unmilked (Quarrie *et al.*, 1994, 1996; Li *et al.*, 1997a; Marti *et al.*, 1997). This process is associated with an increase in the permeability of the tight junction (TJ) complexes between adjacent secretory cells in the mammary epithelium (Fleet & Peaker, 1978; Stelwagen *et al*, 1994b, 1997; Yamamuro & Sensui, 1994; Kim *et al.*, 1997; Stelwagen, 2001). While the timing of the initial rise in TJ permeability approximately coincides with the start of a decrease in milk secretion rate (Stelwagen *et al.*, 1994b, 1997), the role of TJs during mammary engorgement is not well understood and further research may provide insights into the molecular mechanisms regulating this process.

The primary signal triggering the loss of TJ integrity and the onset of mammary apoptosis and involution in response to local milk accumulation is unknown. The build up of a chemical inhibitory factor(s) in milk is postulated to regulate mammary function (Linzell & Peaker, 1971b; Henderson & Peaker, 1984, 1987; Wilde *et al.*, 1995; Shamay *et al.*, 2002, 2003). Alternatively, physical distension of the secretory

epithelium, to accommodate secreted milk, results in changes in cell shape (Richardson, 1947) that have been proposed to initiate stretch-sensitive pathways (mechanotransduction), leading to loss of secretory activity and activation of pro-apoptotic signals (Davis *et al.*, 1999; Stelwagen, 2001). This thesis investigates the regulation of TJ proteins during the early stages of mammary apoptosis and involution, and the role of physical distension of the mammary epithelium in triggering this process.

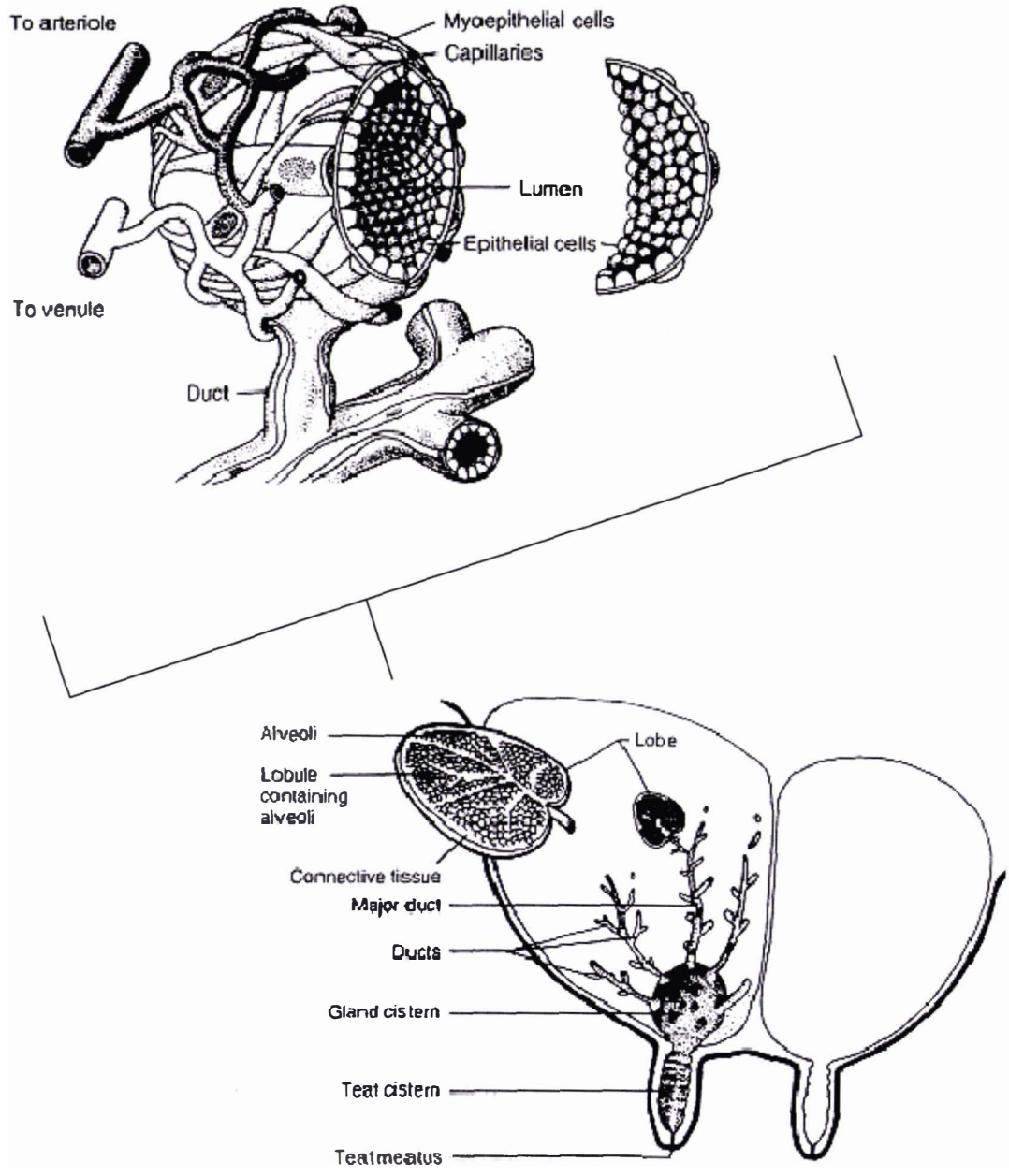


Figure 1.1. Illustration of the structure of the bovine mammary gland.
The diagram depicts the duct system in one quarter of the bovine mammary gland with a single lobe illustrated. In the bovine mammary gland four individual quarters are fused into a single gland complex. An enlargement of an alveolus showing the lumen, secretory epithelial cells, myoepithelial cells, and the capillary network is also shown. Adapted from http://nongae.gsnu.ac.kr/~cspark/teaching/chap10.html.

This review includes a brief overview of the basic physiology of the mammary gland and the role of TJs, before leading onto the physiological mechanisms regulating the response to milk accumulation during extended milking intervals and the early stages of mammary apoptosis and involution.

1.2 MAMMARY GLAND STRUCTURE AND PHYSIOLOGY

1.2.1 Morphology of the lactating mammary gland

The lactating mammary gland is composed of a branching network of ducts formed of epithelial cells, ending in extensive lobulo-alveolar clusters that are the sites of milk secretion (Mepham, 1987; McManaman & Neville, 2003; Fig. 1.1). There are large differences in the number, size and position of mammary glands between species, yet their histological structure is remarkably similar. An individual alveolus consists of up to about 300 polarised secretory epithelial cells that are arranged in a single layer lining a hollow lumen into which milk products are secreted (Fig. 1.1). Alveoli are surrounded by myoepithelial cells that contract in response to oxytocin, forcing the milk out of the alveolar lumen and into the duct system. Outside of the myoepithelial cells the alveolus is surrounded by a basement membrane, and then a connective tissue stroma, which contains blood vessels that supply the cells with nutrients, oxygen and hormonal signals. Groups of up to 200 alveoli that are drained via a common duct are encapsulated by thin sheets of connective tissue and are known as lobules.

Milk products drain into a succession of larger ducts and eventually into collecting ducts, the arrangement of which differs between species. For example, in ruminants the large ducts empty into a voluminous cistern which in a fully distended gland in dairy cows accommodates half of the total stored milk (Davis *et al.*, 1998). However, after a normal milking interval (i.e., 8-16 h) dairy cows store <30% of the total milk yield in the cistern, with the majority of milk held in the alveolar compartment (Bruckmaier *et al.*, 1994; Knight *et al.*, 1994; Davis *et al.*, 1998; Ayadi *et al.*, 2003, 2004). In other species (such as rats, mice, guinea-pigs), while the ducts empty into a common sinus at the base of the teat, its capacity is small and most of the milk is therefore stored in the

lobulo-alveolar tissue (Mepham, 1987). In both these types of duct system milk removal from the glands is via a single teat orifice. By contrast, in rabbits and women each of the ducts draining separate lobes terminates at separate nipple orifices (or galactophores), rather than supplying a common reservoir.

1.2.2 The lactating mammary secretory cell and tight junctions

Lactating mammary epithelial cells (MECs) are highly active metabolically and, when viewed under an electron microscope, show obvious signs of milk synthesis and secretion (Linzell & Peaker, 1971a; Mepham, 1987; McManaman & Neville, 2003; Fig. 1.2). These secretory cells are cuboidal in shape and have a polarised structure containing a well-developed rough endoplasmic reticulum and Golgi apparatus, a spherical nucleus, a large number of mitochondria and free ribosomes, lysosomes, and the products of synthesis (e.g., milk fat globules and protein granules). There are a moderate number of microvilli on the apical surface and the basal cell membrane is folded, which contributes to the efficiency of removing metabolic precursors from the interstitial fluid (Linzell & Peaker, 1971a; Mepham, 1987).

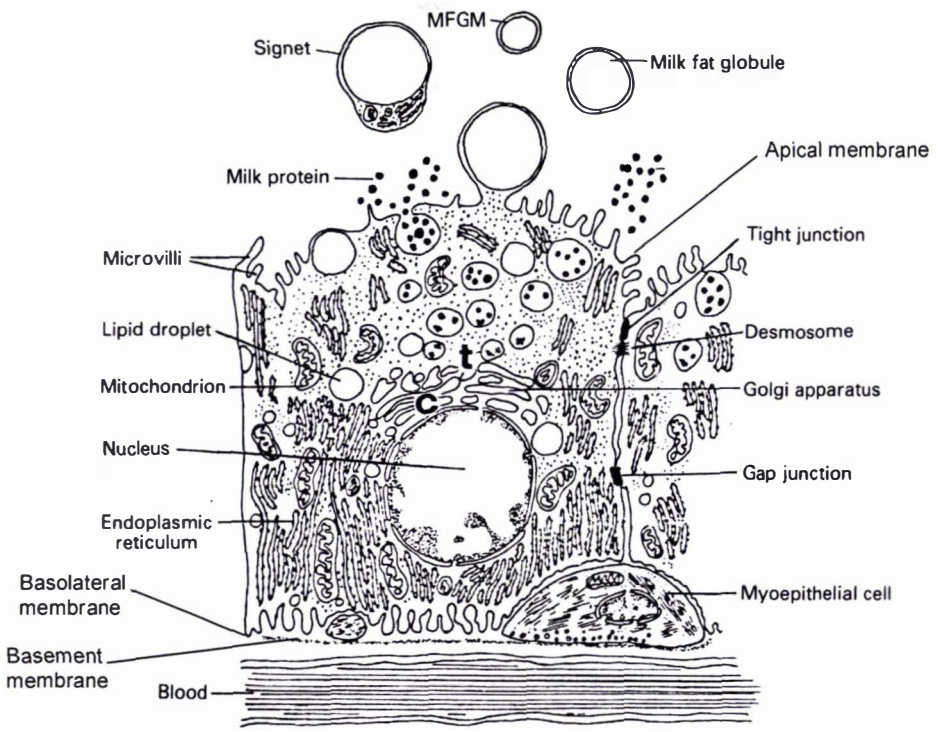


Figure 1.2. Schematic representation of a mammary secretory cell.
This figure shows the internal structure of a mammary secretory cell as interpreted from electron micrographs. **c**, *cis* face of Golgi apparatus; **t**, *trans* face of Golgi apparatus; **MFGM**, milk fat globule membrane. Adapted from Mepham (1987).

MECs are joined to each other through an apical junctional complex composed of TJs and adherens junctions, and by desmosomes and gap junctions (Linzell & Peaker, 1971a; Mepham, 1987; McManaman & Neville, 2003; Fig. 1.2). These junctional complexes facilitate cell-cell contact and communication, and play a critical role in maintaining MEC survival and function. The most apical member is the TJs, also called zonula occludens, which form a narrow, continuous seal that surrounds each MEC. The TJs act to 1) regulate the paracellular exchange of substances between interstitial and milk compartments ('barrier function'), and 2) define cell polarity (and thereby, vectorial milk secretion) by separating the plasma membrane into apical and basolateral domains of distinct protein and lipid composition ('fence function') (Schneeberger & Lynch, 1992; Nguyen & Neville, 1998; Tsukita *et al.*, 2001; Itoh & Bissell, 2003). Freeze-fracture and thin section electron microscope studies have shown that the TJ is composed of a belt-like network of strands in which the membranes of adjacent cells come into close proximity, forming intercellular contact points (or 'kisses') (Pickett *et al.*, 1975).

The adherens junctions encircle the MECs just below the TJ and provide cell-cell adhesion by linking the actin cytoskeletons of adjacent cells through the transmembrane proteins, cadherins (Mepham, 1987; Takeichi, 1991). In addition, desmosomes confer spot-like adhesion to adjacent MECs, whereas gap junctions appear to be a route by which ions, small molecules and electrical currents spread between adjacent cells, a factor that may be significant in the synchronous behaviour of the MECs constituting a single alveolus (Mepham, 1987).

During lactation, the TJs of the alveolar MECs are highly impermeable or "tight", allowing milk to be stored between milkings while restricting the paracellular movement of milk components from the lumen into the interstitial fluid and visa versa (Nguyen & Neville, 1998; Itoh & Bissell, 2003). By preventing the mixing of the constituents of milk and interstitial fluid, the TJs help maintain the composition of milk, which contains lactose, milk proteins, milk fat and low concentrations of sodium and chloride, whereas the interstitial fluid contains plasma proteins and high concentrations of sodium and chloride. During pregnancy mammary TJs are "leaky", undergoing closure around parturition to become the impermeable TJs of the lactating gland

(Linzell & Peaker, 1973, 1974; Nguyen & Neville, 1998). This coincides with the onset of copious milk secretion (lactogenesis stage II), and as such the TJs contribute to the change in composition of the mammary secretions into that of mature milk.

The closure of TJs around parturition appears to be controlled by the same hormonal signals, progesterone withdrawal and the presence of glucocorticoids and prolactin, which initiate lactogenesis (Nguyen & Neville, 1998; Nguyen *et al.*, 2001). The mechanism for TJ closure is not completely understood, but appears to correlate with certain ultra-structural changes in the TJ network (Nguyen & Neville, 1998). The importance of hormones has also been demonstrated *in vitro* using mammary epithelial cell lines, with glucocorticoids and/or prolactin inducing TJ formation and increased expression of TJ protein components (Singer *et al.*, 1994; Stelwagen *et al.*, 1999).

Conversely, an increase in the permeability of mammary TJs occurs during reduced milking frequencies (Stelwagen *et al.*, 1994a & b, 1997; Delamaire & Guinard-Flament, 2006b), cessation of lactation (Fleet & Peaker, 1978), mastitis (Linzell & Peaker, 1972), and following supra-physiological doses of oxytocin (Linzell *et al.*, 1975; Yamamuro & Sensui, 1994). These physiological states are associated with decreased milk secretion. Therefore, the TJ state appears to be closely related to milk secretion (Nguyen & Neville, 1998; Itoh & Bissell, 2003). An increase in TJ permeability is accompanied by a decrease in the milk secretion rate, whereas a decrease in TJ permeability is accompanied by an increase in the milk secretion rate. It is not known whether 1) the maintenance of impermeable TJs is part of normal MEC secretory activity, such as the synthesis of milk constituents; or 2) TJ disruption is followed by partial depolarisation of the cell which affects secretory activity; or 3) TJs can signal to the synthetic and secretory pathways to regulate milk production (Nguyen & Neville, 1998).

1.2.3 Mechanisms of milk synthesis and secretion

A detailed account of milk synthesis and secretion is outside the scope of this literature review and the reader is referred to comprehensive reviews by Linzell and Peaker (1971a), Mepham (1987), Shennan and Peaker (2000), and McManaman and Neville (2003). However, a summary of the main mechanisms of milk secretion is given below

to illustrate the role of TJs in this process. Milk precursors and substrates leave the blood and are taken up from the interstitial fluid through the basolateral membrane of the MEC. Once inside the cell the precursors enter the appropriate synthetic pathway or are transported intact through the cell (e.g., immunoglobulins) and finally, milk components are secreted into the alveolar lumen through the apical membrane. There are four known pathways using these transcellular routes. However, there also exists a further, paracellular, route, which is regulated by TJs. These five pathways utilised by the mammary epithelium in the secretion of milk components are summarised below:

1) In the transmembrane pathway, substances cross the apical cell membrane (and for those directly derived from blood, the basolateral membrane as well) into the milk. Examples include water, ions (Na+, K+ and Cl-), urea, glucose and therapeutic drugs.

2) In the Golgi pathway, secretory products are transported to or sequestered by the Golgi apparatus and then transported in secretory vesicles to the apical membrane where they fuse and release their contents into the milk space by exocytosis. Examples are aqueous solutes such as casein, whey proteins, lactose, citrate and calcium.

3) In the milk fat pathway, coalesced milk fat droplets are extruded from the secretory cell completely enveloped in apical membrane (milk fat globule membrane) and a small portion of cytoplasm is occasionally included. Lipid-soluble hormones and drugs, and lipid-associated proteins are also secreted by this route.

4) In transcytosis, a wide range of substances derived from serum or stromal cells are transferred across the cell and into the milk space. Examples include immunoglobulins (particularly during colostrum formation), serum albumin, transferrin, lipoprotein lipase, hormones and growth factors (e.g., insulin, prolactin and oestrogen).

5) The paracellular pathway provides a direct passage of interstitial fluid and serum components into milk between the cells via "leaky" TJs. There is little or no "flow" through the paracellular pathway during lactation because the TJs are highly impermeable (Nguyen & Neville, 1998; Itoh & Bissell, 2003). However, as discussed in section 1.2.2 above, TJs are dynamic structures and can be regulated by numerous stimuli, some of which result in loss of integrity. In these cases the paracellular pathway is present and the concentrations of lactose and K+ in milk decrease (moving down their concentration gradients), whereas those of Na+ and Cl- increase. Therefore, a common

way of assessing TJ permeability is to measure the rate of appearance of lactose in plasma (Stelwagen *et al.*, 1997). The paracellular pathway is useful during pregnancy, during mastitis, and after involution, in that inflammatory cells and protective molecules can enter the milk space, while secretory products and cells can be cleared from the gland during mammary regression. It is also important to note that leukocytes can also force their way (or diapedese) between MECs to enter the milk space, and that the TJs seal tightly behind them leaving no permanent gap (McManaman & Neville, 2003).

1.3 THE ROLE OF MILK REMOVAL IN LACTATION

1.3.1 Maintenance of lactation

Lactation is the result of two separate processes: 1) the synthesis and secretion of milk (refer to section 1.2.3 above) into the alveolar lumen, and 2) the subsequent removal of milk from the gland (Holmes *et al.*, 2002). The level of milk production is a function of the number of mammary secretory cells present and their activity, with daily milk production approximately 1 – 2 ml per gram mammary tissue across all species (Holmes *et al.*, 2002). The term, galactopoiesis, is given to the maintenance and/or enhancement of established lactation. The major factors affecting milk yield and composition include the stage of lactation, genotype, nutrient supply, hormone and growth factor signals, age, pregnancy, environmental temperature, disease and mastitis, milking practices, and the frequency or completeness of milk removal (Holmes *et al.*, 2002). However, the two key components regulating the maintenance of lactation are galactopoietic hormones and the removal of accumulated milk. A hormonal complex controls lactation, but unless milk is removed frequently from the gland, synthesis of milk will not continue despite an adequate hormonal status. Conversely, lactation will not persist indefinitely despite regular milk removal. Thus, the suckling/milking stimulus and the actual removal of milk from the gland are required to maintain milk synthesis and secretion.

The hormones required for maintenance of lactation include prolactin, growth hormone, glucocorticoids, thyroid hormones, insulin, oxytocin and parathyroid hormone (for reviews see Delouis *et al.*, 1980; Tucker, 1985, 1994; Rillema, 1994). Growth hormone

has a central role in the maintenance of lactation in the dairy cow, whereas prolactin fills this role in other species, especially rodents (Bauman, 1992; Flint *et al.*, 1992; Tucker, 1994). Prolactin is released from the anterior pituitary gland in response to the milking/suckling stimulus in both ruminants and non-ruminants, but prolactin does not limit established milk secretion in the dairy cow (Karg & Schams, 1974; Plaut *et al.*, 1987; Tucker, 1994). Nevertheless, there is increasing evidence to suggest that growth hormone and prolactin influence milk production in both ruminants and non-ruminant species by separate, yet additive, mechanisms (Flint *et al.*, 1992; Tucker, 1994).

1.3.2 Milk removal and the local regulation of milk secretion

The milk production response to the frequency or completeness of milk removal is regulated by a local intra-mammary mechanism. For example, a unilateral increase in milking frequency, from twice-daily milking (TDM) to either hourly or three times a day, increased milk yield in that gland only (Linzell & Peaker, 1971b; Blatchford & Peaker, 1982; Henderson *et al.*, 1983). Clearly, this result could not be attributed to systemic control, such as the action of galactopoietic hormones. Furthermore, a similar response was obtained by frequent milking of a denervated auto-transplanted mammary gland that was incapable of stimulating the release of hormones (Linzell & Peaker, 1971b). Milk removal was essential for the response to frequent milking, as hourly massage of the gland without milk removal had no effect (Linzell & Peaker, 1971b).

In a similar manner, when one udder side was milked twice daily and the other side was milked only once daily, milk yield was decreased only in the less frequently milked gland (Stelwagen & Knight, 1997). Unilateral cessation of milk removal in ruminants (Quarrie *et al.*, 1994), or teat-sealing in rodents (Quarrie *et al.*, 1996; Li *et al.*, 1997a; Marti *et al.*, 1997), induced mammary engorgement and involution in the treated glands only. The mechanisms by which local intra-mammary signals regulate mammary function in response to extended periods of milk accumulation are discussed in section 1.6.

1.3.3 Mechanisms of milk removal and the milk ejection reflex

Milk is stored within the lumen of the gland, either in the secretory alveoli or duct system, until it is removed at intervals by either sucking by the young, hand milking or machine milking. All three processes increase the pressure gradient between the inside and the outside of the teat sufficiently to overcome the forces holding the teat canal closed (Holmes *et al.*, 2002). The first phase of milk removal involves the passive withdrawal of the milk present in the gland and teat cisterns and the large ducts through the teat without milk ejection (i.e., foremilk or cisternal milk) (Bruckmaier *et al.*, 1994; Knight *et al.*, 1994; Davis *et al.*, 1998). However, the bulk of the milk secreted between milkings is stored in the alveoli and small ducts, so that active expulsion is required to overcome the resistance to milk flow presented by these small apertures (i.e., alveolar milk) (Mepham, 1987; Bruckmaier *et al.*, 1994; Knight *et al.*, 1994; Davis *et al.*, 1998; Ayadi *et al.*, 2003, 2004).

Active expulsion of alveolar milk is brought about by the milk ejection reflex, which (as described by Mepham, 1987; Holmes *et al.*, 2002) is a neuroendocrine reflex consisting of an afferent pathway (neural) and an efferent pathway (hormonal, blood-borne) where stimulation of the mammary gland, particularly the teats or nipples, results in the release of the hormone oxytocin from the posterior pituitary into the bloodstream. Oxytocin then travels via the cardiovascular system to the mammary gland where it binds to receptors on the myoepithelial cells surrounding the alveoli causing them to contract and squeeze the alveoli. This results in an increase in intra-luminal pressure and the expulsion of milk from the alveoli through the duct system and into the gland and teat cisterns, which enables rapid milk removal. The milk ejection reflex takes about 30-60 seconds, and the release of oxytocin can also be a conditioned response to the various stimuli associated with the normal milking process in dairy animals. Following milk ejection, oxytocin concentrations in blood rapidly decline, halving concentration every 60-90 seconds, so that the myoepithelial cells gradually relax and milk can no longer be removed from the gland.

1.3.4 Completeness of milk removal

In all mammals the gland is never emptied completely following milking. A small amount of "residual milk" remains in the alveoli and small ducts, and can vary from 5 – 20% of the total volume of milk present depending upon the individual (Holmes *et al.*, 2002). Residual milk occurs due to a decline in the milk ejection pressure as the effect of endogenous oxytocin wears off and because the milking machine (or young) is unable to achieve complete harvest of the gland (Woolford *et al.*, 1982). Cows with greater quantities of residual milk tend to have shorter lactations and lower total lactation yields (Schmidt, 1971). Residual milk can only be removed by injection of a large dose of oxytocin (e.g., Davis & Hughson, 1988).

High levels of residual milk are also associated with greater milk yield losses during once-daily milking (ODM) (Carruthers *et al.*, 1993a). Treatments which increase efficiency of milk removal by either machine stripping or oxytocin injection reduce the milk yield loss on ODM (Woolford *et al.*, 1982; Carruthers *et al.*, 1993b). Furthermore, incomplete milking, when milk is left in the gland in addition to the normal residual milk, can result in large declines in subsequent milk yields (Wheelock *et al.*, 1965; Schmidt, 1971). These results emphasise the importance of efficient milk removal in maintaining milk production. In the next section the effect of the frequency of milk removal on milk production in dairy cows will be discussed.

1.3.5 Milking interval and the frequency of milk removal

1.3.5.1 Twice-daily milking

Even though more-frequent milking results in greater production, TDM of dairy cows is the most common milking practice in New Zealand and many other countries. While equal intervals of 12 h are considered optimal for milk production under TDM, unequal intervals of 8 to 16 h have very little (<3% difference) or no effect on milk or milk fat yields (e.g., Hansson *et al.*, 1958; Ormiston *et al.*, 1967; Bartsch *et al.*, 1981). However, TDM with the longest milking interval greater than 16 h (e.g., 6/18 h or 3/21 h etc.) will

result in a loss of milk production (Bartsch *et al.*, 1981). In New Zealand, it is common practice to use milking intervals of approximately 10 and 14 h.

1.3.5.2 Milking more frequently than twice daily

Increasing the frequency of milk removal results in an increased daily milk yield, although it is usually uneconomic to milk dairy cows more than thrice daily. The increases in milk production under thrice-daily milking are variable, but in early-mid lactation range from 5 – 20%, with the level of response tending to be greater during longer periods of increased milking frequency (e.g., Pelissier, 1978; Pearson *et al.*, 1979; Phillips, 1979; Poole, 1982; Waterman, 1983; Klei *et al.*, 1997). Similarly, milking thrice-daily over a whole lactation increases milk production by 6 – 26% (e.g., Amos *et al.*, 1985; DePeters *et al.*, 1985; Barnes *et al.*, 1990; Klei *et al.*, 1997). However, Klei *et al.* (1997) reported that milking thrice daily for 100 d in late lactation resulted in no increase in total milk production for the whole lactation. Nonetheless, the absolute increases in daily yield to thrice-daily milking are remarkably constant and range between three to four litres (Erdman & Varner, 1995).

Milking more frequently than thrice daily increases milk production even further, for example, increasing milking frequency from thrice daily to six times daily increased milk yield by another 21% (Bar-Peled *et al.*, 1995). Furthermore, frequent milking and bovine somatotropin separately and additively increase milk production (Speicher *et al.*, 1994; Knight *et al.*, 1992). The increase in milk production when cows are milked more frequently arises from: 1) an increase in daily production, 2) a higher peak milk production and 3) increased persistency of milk production (Pearson *et al.*, 1979; Amos *et al.*, 1985; DePeters *et al.*, 1985). The latter is due to a carryover effect of frequent milking early in lactation on the remainder of lactation (Pearson *et al.*, 1979; Poole, 1982; Bar-Peled *et al.*, 1995), which may be the result of increased mammary cell activity and mammary cell proliferation (Wilde *et al.*, 1987b; Knight *et al.*, 1990; Hale *et al.*, 2003). Milking more frequently may also result in improved milk composition and quality, partially due to reduced exposure to proteolytic enzymes as a result of decreased storage time in the mammary gland and partially due to better maintenance of TJ integrity (Sorensen *et al.*, 2001).

1.3.5.3 Milking less frequently than twice daily

Reducing the milking frequency from TDM to ODM (i.e., 24 h milking interval) results in a loss of milk production, and the topic has been reviewed in depth by Davis *et al.* (1999). The size of the milk production loss under ODM has been investigated in numerous part-lactation studies, but relatively few full-lactation trials, with a summary of these results given in Table 1.1. Yield losses from part-lactation studies ranged from 7 – 40% and the loss tended to be lower in late lactation (Carruthers *et al.*, 1993a, Stelwagen & Knight, 1997), but greater in cows with high initial somatic cell counts (Kamote *et al.*, 1994). In the full-lactation studies, average milk production losses were much larger, ranging from 22 – 50% (Table 1.1). Claesson *et al.* (1959) reported a milk production loss of 50% in the first lactation and 40% in the second lactation for ODM compared with TDM. In more recent work, production losses of 30 – 35% (Holmes *et al.*, 1992; Remond *et al.*, 2004; Clark *et al.*, 2006) have been reported for Friesian cows on ODM compared with those on TDM during an entire lactation, although Jersey cows had a smaller milk production loss of 22% (Clark *et al.*, 2006). Furthermore, while milk yield losses under ODM were usually unrelated to the level of TDM production, most studies have reported that the yield loss is extremely variable between individual animals, suggesting the possibility of selecting ODM tolerant cows.

The decrease in milk production under ODM is due to a decrease in daily production as well as shorter lactations (Claesson *et al.*, 1959; Remond *et al.*, 2004; Clark *et al.*, 2006). During ODM there was an increase in yield loss as lactation progressed, i.e., poorer persistency, in some studies (Claesson *et al.*, 1959; Hickson *et al.*, 2006) but not all (Remond *et al.*, 2004).

Several other experiments have indicated that long-term ODM results in loss of secretory potential (Davis & Hughson, 1988; Carruthers *et al.*, 1993a; Remond *et al.*, 1999; Hickson *et al.*, 2006). For example, Carruthers *et al.* (1993a) noted that short-term ODM (1 – 2 weeks) did not result in any measurable carry-over effects. However, following a 12-week period of ODM, cows returning to TDM showed poor yield recovery with milk yields still 16 – 21% below controls after 3 weeks back on TDM. Furthermore, ODM induced a more rapid decline in functional udder capacity. Since

functional udder capacity is determined by the internal surface area of the udder (Davis & Hughson, 1988), this result suggests a greater loss of secretory cells during ODM. A similar conclusion was drawn by Wilde & Knight (1990), who reported that ODM of goats for 4.5 weeks resulted in a milk production loss of 26% with a 6 – 7% carry-over of yield loss when returned to TDM. Li *et al.* (1999) demonstrated that a decrease in milk yield after 4 weeks of ODM in goats was associated with reduced alveolar size due to increased loss of secretory cells by apoptosis, and after 10 weeks a heterogeneous population of secretory, resting and involuting alveoli which contributed to a faster rate of udder regression.

A series of experiments carried out at Ruakura examined the effect of breed (Friesian vs. Jersey) and strain (high vs. low protein content) on the response to ODM (Carruthers *et al.*, 1993a). During short-term periods of ODM (2 weeks) in mid- and late-lactation, there were no significant differences in % yield loss between Friesians, and two strains of Jerseys, with either high or low protein concentrations in milk. However, smaller protein losses were observed in high-protein Jerseys in late lactation. Longer-term trials of ODM (12 weeks) during weeks 6 to 18 of lactation resulted in % yield losses of 10, 21 and 27 % for high-protein and low-protein Jerseys and Friesians, respectively. Only the differences between high-protein Jerseys and Friesians were significant. Nevertheless, the authors indicated that Jerseys may be more tolerant to ODM over a full-lactation, as Friesians showed a faster rate of udder regression during ODM. Recent experiments comparing ODM of Friesian cows with Jersey cows over up to four consecutive lactations have suggested that this is indeed the case (Clark *et al.* 2006; Hickson *et al.* 2006).

Prediction of the yield loss using udder characteristics, milk yield and composition, age of cow or other factors have not proved successful so far (Carruthers *et al.*, 1993a), although heifers have been shown to have a greater production loss during ODM than mature cows in some reports (Carruthers *et al.*, 1993a; Clark *et al.*, 2006). However, it has been suggested that cows can adapt to less-frequent milking. Heifers milked once daily in their first lactation had an improved response in the second lactation (Claesson *et al*, 1959). The increased tolerance with prolonged periods of infrequent milking may be due to stretching of the cisterns and ducts, allowing more milk to be stored. There is

evidence of a positive relationship between cisternal volume and milk production response to ODM (Stelwagen & Knight, 1997; Knight & Dewhurst, 1994).

A detailed discussion of the factors regulating the initial (acute) milk production loss and altered mammary function to ODM and other extended periods of milk accumulation is covered in section 1.6. Furthermore, a reduction in milking frequency from TDM to ODM is usually accompanied by significant changes in milk composition (Table 1.2; reviewed by Davis *et al.*, 1999). Typically these involve an increase in concentrations of milk protein, milk fat, serum albumin, sodium, chloride and somatic cell count, and a decrease in the concentrations of lactose and potassium. Most of these changes can be attributed to an increased exchange of milk and interstitial fluid that occurs through an increase in TJ permeability during ODM (Stelwagen *et al.*, 1994a, 1997). The role of TJ permeability during extended periods of milk accumulation will be discussed in section 1.6.4.

Overall, ODM reduces daily milk yield but there are a number of specific situations where ODM can be useful in low cost dairying systems (Davis *et al.*, 1999). For example, milking cows once daily at an increased stocking rate reduces the milk yield loss per hectare compared with TDM at a moderate stocking rate, and a number of savings can be made on labour and milking-associated expenses (Clark *et al.*, 2006). Other "compromise" systems include: milking three times every two days (e.g., Woolford *et al.*, 1985), which can result in lower milk production losses compared with ODM, depending upon the length of the milking intervals used; and missing one milking per week, which has little effect on milk production and composition (e.g., O'Brien *et al.*, 2002).

Table 1.1. Summary of the levels of milk production loss (relative to twice-daily yields) during once-daily milking (ODM) of dairy cows.

Reference	Control yield (kg milk/day)	% loss under ODM	Stage of lactation	ODM duration	Breed of dairy cow
Claesson *et al.* (1959)	-	50% 40%	All All	Whole 1st lactation Whole 2nd lactation	Swedish Red and White
Carruthers *et al.* (1993a)	12-15 20 9-11 14 12-15 21	14-18% 18% 9-12% 13% 10-21% 27%	Mid Mid Late Late Mid Mid	2 weeks 2 weeks 2 weeks 2 weeks 12 weeks 12 weeks	Jersey (high and low protein) Friesian Jersey (high and low protein) Friesian Jersey (high and low protein) Friesian
Carruthers *et al.* (1991)	9-10	11%	Late	2 weeks	Jersey and Friesian
Holmes *et al.* (1992)	-	35% (range 10-50%)	All	Whole lactation	Jersey, Friesian and X-bred
Kamote *et al.* (1994)	14 14	26% 14%	Late Late	4 weeks 4 weeks	Friesian (high SCC) Friesian (low SCC)
Lynch *et al.* (1991)	-	11-15%	Late	5-13 weeks	Friesian and X-bred
Stelwagen & Knight (1997)	35 16	38% 28%	Early Late	3 weeks 3 weeks	Friesian (British) Friesian (British)
Stelwagen & Lacy-Hulbert (1996)	13.5	15%	Mid	6 days	Jersey
Stelwagen *et al.* (1994a)	13	7%	Late	2 weeks	Friesian
Knight & Dewhurst (1994)	21	23%	Mid	1-2 weeks	Friesian (British)
Wilson (1965)	8	27%	Late	5-7 weeks	Unknown breed
Parker (1965)	8	34%	Late	12 weeks	Unknown breed
Knutson *et al.* (1993)	-	27%	Late	1 week	Friesian
O'Brien *et al.* (2000)	13	29%	Late	10 weeks	Friesian (Irish)
Auldist & Prosser (1998)	21 16	19% 13%	Early Early	2 days 2 days	Friesian (*ad lib* feeding) Friesian (restricted feeding)
Lacy-Hulbert *et al.* (1999)	6.5	13%	Late	4 weeks	Friesian
Remond *et al.* (1999)	21-30 27-37	25% 40%	From calving From calving	3 weeks 6 weeks	Holstein Friesian
Remond *et al.* (2004)	-	30%	From calving	Whole lactation	Holstein Friesian
O'Brien *et al.* (2002)	13	29%	Late	10 weeks	Friesian (Irish)
Clark *et al.* (2006)	-	22% 31%	All	Whole lactation	Jersey Friesian

Table 1.2. Changes in milk composition during once-daily milking (ODM) of dairy cows.

Milk component	Change	Reference
Fat	Increase	4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 22, 23, 24
	No change	1, 3, 13, 19
Total protein	Increase	2, 4, 5, 6, 7, 8, 11, 12, 13, 22, 23
	No change	1, 3, 10, 19
Casein	Increase	4, 8, 15
	No change	3, 5, 10, 22, 24
Whey protein	Increase	3, 4, 8, 10, 15, 22
Casein:whey ratio	Decrease	8, 10
Protein:fat ratio	Decrease	10
β-lactoglobulin	Increase	7
	No change	20
α-lactalbumin	Increase	7, 20
Serum albumin	Increase	4, 8, 10, 13, 14
Immunoglobulin G	Increase	4, 8, 10
Lactoferrin	Increase	21
Lactose	Decrease	1, 3, 4, 6, 7, 8, 9, 10, 11, 13, 15, 16, 20, 23
	No change	5, 19, 24
Sodium	Increase	8, 12, 15
Chloride	Increase	3, 12, 15
Potassium	Decrease	8, 12, 15
Calcium	Increase	8
Plasmin	Increase	17, 20
	No change	5, 7, 8, 22, 24
Plasminogen	Increase	7, 17, 20, 22
	No change	8
Plasminogen activator	Increase	17
pH	Increase	8, 20
Somatic Cell Count	Increase	1, 2, 9, 10, 14, 20, 23
	No change	4, 5, 8, 13, 19, 22, 24

Changes are identified to be significantly different from twice-daily milking (TDM). Reference key: (1) Holmes *et al.* (1992); (2) Lynch *et al.* (1991); (3) Claesson *et al.* (1959); (4) Remond *et al.* (1999); (5) O'Brien *et al.* (2000); (6) Carruthers *et al.* (1991); (7) Knutson *et al.* (1993); (8) Lacy-Hulbert *et al.* (1999); (9) Kamote *et al.* (1994); (10) Auldist & Prosser (1998); (11) Carruthers *et al.* (1993a); (12) de Villiers & Smith (1976); (13) Stelwagen *et al.* (1994a); (14) Stelwagen & Lacy-Hulbert (1996); (15) Wheelock *et al.* (1965); (16) Stelwagen *et al.* (1998a); (17) Stelwagen *et al.* (1994c); (18) Farr *et al.* (1995); (19) Remond *et al.* (2002); (20) Kelly *et al.* (1998); (21) Farr *et al.* (2002); (22); O'Brien *et al.* (2002) ; (23) Clark *et al.* (2006); (24) Remond *et al.* (2004).

1.4 MILK ACCUMULATION AND THE MILK SECRETION RATE

1.4.1 Milk secretion rate

The milk production responses to changes in the frequency of milk removal, or the length of the interval between milkings, are a function of the rate of milk secretion following milk removal. Originally it was believed that milk and milk fat secretion rates progressively declined as milk accumulated in the gland, while fat percentage tended to increase (reviewed by Elliott, 1959). However, a long preceding interval has a detrimental effect on secretion rate (Bailey *et al.*, 1954; Turner, 1955a & b; Elliott *et al.*, 1960; Schmidt, 1960; Farr *et al.*, 1998), and, in early experiments, no allowance was made for variations in residual milk content from the preceding milking interval. It has now been established that milk secretion rate, corrected for residual milk and the effect of the length of the preceding interval, is linear up to 12 – 16 h and milk secretion does not stop until 24 – 40 h after the last milking in dairy cows (Turner, 1955a; Elliott *et al.*, 1960; Schmidt, 1960; Wheelock *et al.*, 1966) – although there is considerable variation between individual animals (Elliott *et al.*, 1960; Wheelock *et al.*, 1966; Davis *et al.*, 1999). It is worth noting that, in rats, a decline in milk secretion rate begins 4 – 8 h after the last milk removal (Hanwell & Linzell, 1972).

Therefore, during a single 24 h milking interval (i.e., the first day of ODM in cows), a decline in the rate of milk secretion occurs during the last 8 – 12 h, resulting in a drop in the total daily milk yield compared with two milking intervals of 8 – 16 h. However, a decline in milk secretion rate was not observed when residual milk was removed at the start of the milk accumulation period (Davis & Hughson, 1988). Farr *et al.* (1998) measured the milk secretion rate in the 24 h immediately after extended periods of milk accumulation (following removal of residual milk) and reported that there was a marked sensitivity of secretion to mammary engorgement over the period 24 – 32 h with milk secretion rate being 50-60% of the previous TDM rate in the 4 – 8 h following a 32 h interval. Similarly, milk yield in an 8 h milking interval following a 24 h milking interval was 25% less than that after an 8 h milking interval (Elliott *et al.*, 1960). Therefore, a long milking interval (> 16 h) will not only reduce the milk yield over that

interval but will also affect the milk yield in the subsequent intervals. In this project, the focus will be on the initial changes induced by an extended period of milk accumulation.

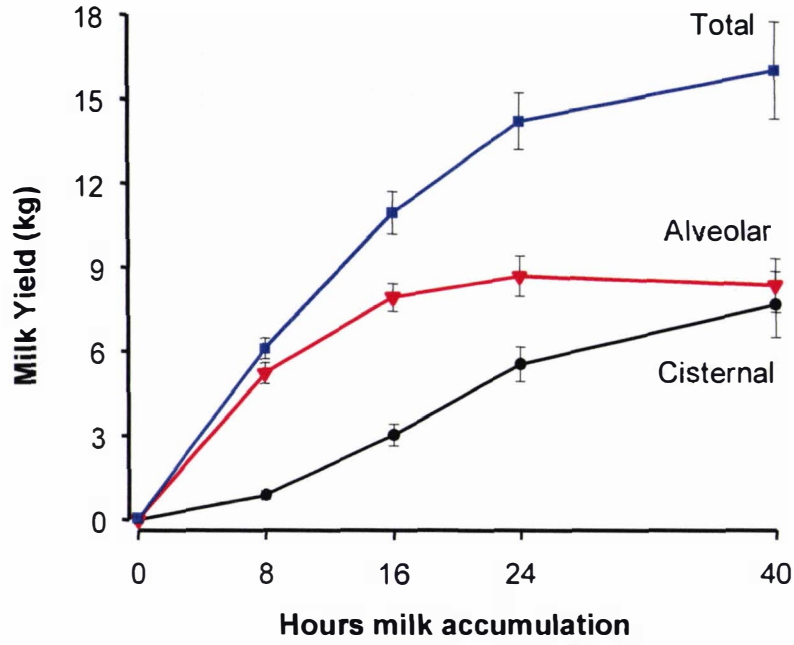


Figure 1.3. The accumulation of milk in the alveolar and cisternal compartments (and in total) in the udders of cows (n=12) at four time intervals after milking.
Cisternal fractions were measured after injection of adrenaline and alveolar fractions after subsequent injection of oxytocin. Redrawn from Davis *et al.* (1998).

1.4.2 Pattern of milk accumulation

The pattern of accumulation of milk in the alveolar and cisternal compartments of the bovine mammary gland is illustrated in Figure 1.3 (Davis *et al.*, 1998). Similar patterns have been reported by other authors (e.g., Knight *et al.*, 1994; Stelwagen *et al.*, 1996; Ayadi *et al.*, 2003, 2004). The alveolar and cisternal compartments begin to fill shortly after milking, although the rate of filling of the cistern is relatively low during the first 8 h post-milking (Stelwagen *et al.*, 1996). The alveolar compartment is rapidly filled with milk such that its capacity begins to be exceeded 6 – 8 h after milking when there is a marked increase in the rate of milk accumulation in the cisternal compartment. While the alveolar compartment is over 90% full at 16 h post-milking, milk continues to drain and accumulate in the cisterns. The rate of total milk accumulation slowed slightly between 16 and 24 h and considerably between 24 and 40 h as the milk secretion rate declines. Depending upon the individual animal, milk may continue to accumulate for

up to 40 h after the last milking, by which time net accumulation of milk stops. At full udder capacity (between 30 – 40 h post-milking), the volumes of milk contained in the cisternal and alveolar compartments were similar. A similar pattern of milk accumulation has been reported in dairy goats, except that 75% of the total milk stored in the udder at 24 h was cisternal milk (Salama *et al.*, 2004).

1.4.3 Anatomical factors affecting milk accumulation

Davis *et al.* (1999) proposed that the primary requirement to avoid (or delay) milk production decreases during extended periods of milk accumulation is for the mammary gland to possess sufficient capacity for milk secretion to continue for 24 h or more. There are several different strategies by which mammals may increase storage capacity. These include an increase in milksolids concentration, development of large milk storage cisterns, or alternatively, an increase in the size or distensibility of the alveoli. However, there is no evidence that alveolar diameter varies substantially between species (Munford, 1964).

Animals which have a natural nursing interval of greater than 24 h (e.g., rabbits, seals) have more concentrated milk than that of animals which have much shorter intervals (e.g., primates, rodents; Davis *et al.*, 1999). For these mammals, the ability to secrete and store concentrated milk provides the young with a nutrient rich diet. The production of concentrated milk is usually achieved through a reduction in lactose synthesis and, as lactose is the major osmole in milk (see Linzell & Peaker, 1971a), a subsequent reduction in water content.

Carruthers *et al.* (1993a) examined the effect of milk protein content on tolerance to ODM. While Jersey cows producing relatively concentrated milk (4.3% protein and 6.6% milk fat) could store up to 5 hours-worth of milk secretion more than Friesian cows with relatively dilute milk, there was no significant difference in the milk production response to ODM. However, Jersey cows have better tolerance during long-term ODM than Friesians (Clark *et al.*, 2006), although this advantage might not be due to differences in milk solids content as there were no differences in milk production

between Friesians with either high or low milksolids content in a long-term trial (D. Clark, pers. comm., unpublished observations).

The gland cistern represents approximately half of the functional udder capacity in cows (Davis *et al.*, 1998), and hence its size impacts on the amount of milk the gland can accumulate before milk secretion stops. Lactating goats (Knight *et al.*, 1989) and cows (Dewhurst & Knight, 1992; Knight & Dewhurst, 1994) that had large cisterns were less responsive to frequent milking and more tolerant of infrequent milking than those with small cisterns. Others have also reported that the proportion of milk stored in the cistern or total udder capacity (as hours-worth of milk secretion) was positively correlated with increasing tolerance of ODM (Davis *et al.*, 1987; Stelwagen & Knight, 1997), although this relationship was not as strong in some studies (Carruthers *et al.*, 1993a). Continuous drainage of the cistern during ODM, representing cisterns with infinite capacity, increased milk production by 7% (Stelwagen *et al.*, 1996). However, simply removing cisternal milk 9 h after normal milking did not alter the milk yield of cows on ODM (Farr *et al.*, 1997), highlighting the importance of alveolar milk accumulation in the yield response to ODM.

There was a positive correlation between cisternal milk at 8 h post-milking (Knight & Dewhurst, 1994; Stelwagen & Knight, 1997), and cisternal milk at 24 h post-milking as a proportion of total cisternal capacity (Davis *et al.*, 1998), with yield loss under ODM. These measurements reflect the ability of the alveoli to drain freely into the cistern (refer to Fig. 1.3) and suggest that this characteristic is also important in determining the yield loss under ODM, or the length of time for which milk secretion will continue unabated during an extended period of milk accumulation. The mechanisms by which alveolar drainage impacts on the response to milk accumulation will be discussed in sections 1.6.2 and 1.6.3.

1.5 MAMMARY APOPTOSIS AND INVOLUTION

In the previous sections the importance of the completeness and frequency of milk removal in regulating milk production and mammary function were discussed. In this section the role of milk accumulation in initiating mammary apoptosis and involution is examined. Mammary involution can be induced at any stage of lactation by complete cessation of milk removal in dairy animals, or by litter removal in rodents, where upon the mammary gland becomes distended with milk in a process known as mammary engorgement. As the intra-mammary pressure increases in the gland, milk secretion, and then milk synthesis, decreases with no initial change in cell morphology. However, if engorgement persists, the secretory cells die by apoptosis and the alveoli regress to a less differentiated state. This process is termed involution and results in a gland with very small alveoli containing only a few MECs and an increased area of interalveolar stroma (see Holst *et al.*, 1987; Hurley, 1989; Capuco & Akers, 1999; Wilde *et al.*, 1999).

The process of induced mammary involution is well-characterised in rodents and can be divided into several distinct stages that involve cessation of milk production, apoptosis of MECs and tissue remodelling (Lund *et al.*, 1996). The mechanisms which act in one stage may be different to those which are activated during the next stage (Furth, 1999). The first phase of mammary involution begins within 12 h of pup removal and lasts for approximately 72 h (Lund *et al.*, 1996; Li *et al.*, 1997a). The levels of systemic galactopoietic hormones fall and as milk accumulates locally within alveolar lumens the gland becomes engorged. The result is a rapid decline in milk synthesis, cessation of milk secretion, down-regulation of differentiated gene expression and the initiation of MEC apoptosis (Walker *et al.*, 1989; Strange *et al.*, 1992; Marti *et al.*, 1997). The first phase of involution is reversible in that lactation can be re-established if pups are returned within 48 h (Jaggi *et al.*, 1996; Li *et al.*, 1997a; McMahon *et al.*, 2004, Appendix VII). MECs which have entered the initial non-committed stage of apoptosis are able to return to an active secretory state in response to milk removal. However, individual MECs already committed to the terminal stage of apoptosis at this time will continue the cell death process and be removed from the secretory epithelium.

The first phase of secretory de-activation and apoptosis during mammary involution is initiated by local mammary-derived signals since artificial addition of galactopoietic hormones (e.g., glucocorticoids) to MECs does not affect apoptosis, although it does prevent the second stage of mammary involution in which matrix degradation and gland remodelling occurs (Li *et al.*, 1997a). Furthermore, it is well established that teat-sealing in rodents, which prevents milk removal but maintains the stimuli of suckling and levels of systemic galactopoietic hormones, induces mammary apoptosis in the treated glands only (Quarrie *et al.*, 1996; Li *et al.*, 1997a; Marti *et al.*, 1997). Therefore, these local intra-mammary 'death' signals are dominant over protective effects related to systemic galactopoietic hormone stimulation. However, maintenance of systemic hormone levels by suckling does delay the entry of teat-sealed glands into the second stage of involution, suggesting that they act as survival factors (Li *et al.*, 1997a).

During the second stage of mammary involution, extracellular matrix (ECM)-degrading proteases are produced by the gland, resulting in collapse of lobulo-alveolar structures and extensive remodelling, such that the involuted gland strongly resembles that of the mature virgin gland, in preparation for the next lactation (Talhouk *et al.*, 1992; Lund *et al.*, 1996; Li *et al.*, 1997a). Apoptosis continues during this stage until 50 to 80 % of epithelial cells have been cleared from the gland (Walker *et al.*, 1989). Consequently, this stage of involution is irreversible with only very limited recovery of lactation after 72 h following litter removal (Jaggi *et al.*, 1996; Lund *et al.*, 1996; Li *et al.*, 1997a; Sorensen & Knight, 1997).

However, the role of apoptosis in mammary involution in ruminants is not well understood. Ruminants appear to tolerate the effects of milk accumulation better than rodents and, by delaying commitment to apoptosis, retain the ability to re-initiate lactation if circumstances alter. In dairy cows, lactation can be almost fully restored after 7 d (Dalley & Davis, 2006) and partially reinstated after 11 d (Noble & Hurley, 1999) of involution following the abrupt cessation of milk removal. The rapid widespread apoptosis and extensive tissue remodelling that is a feature of rodent mammary involution (Walker *et al.*, 1989; Strange *et al.*, 1992; Quarrie *et al.*, 1996; Marti *et al.*, 1997) is not evident in the bovine mammary gland (Holst *et al.*, 1987; Molenaar *et al.*, 1996b; Wilde *et al.*, 1997). However, increased apoptosis has been demonstrated in cows after 7 d of involution in late lactation (Wilde *et al.*, 1997). In

goats, unilateral cessation of milk removal in one mammary gland in late lactation has little effect on MEC apoptosis after 2 – 3 days, but apoptosis was evident after 1 week and maximal 2 – 3 weeks after induction of mammary engorgement in that gland (Quarrie *et al.*, 1994; Li *et al.*, 1999). These latter studies also confirmed that the induction of mammary apoptosis and involution in ruminants is locally regulated, occurring only in unmilked glands, whereas milked glands from the same animal continued to lactate with no observed effects on mammary apoptosis or secretory alveolar structure (Quarrie *et al.*, 1994; Li *et al.*, 1999).

Based on magnetic resonance imaging, 60% of parenchymal (i.e., lobulo-alveolar) tissue is lost during goat mammary involution (Fowler *et al.*, 1990), whereas in beef cows, tissue weight and cell number (as indicated by DNA content) were reduced by about 50% after 6 weeks of involution (Akers *et al.*, 1990). These findings suggest that apoptosis is simply delayed in ruminants, in comparison with rodents, but that ultimately the extent of MEC turnover between lactations is comparable to that in rodents (Wilde *et al.*, 1999). However, the concurrent state of pregnancy during normal bovine mammary involution at the end of lactation may account for at least part of the maintenance of alveolar structure during the dry period in dairy cows (Capuco & Akers, 1999).

In dairy animals, the decline in milk yield following peak lactation is also due to loss of MECs by apoptosis (Capuco *et al.*, 2001) in a process termed gradual involution. Decreasing the frequency of milk removal to ODM results in a decline in daily milk yield and poorer persistency of lactation, as discussed in section 1.3.5.3 earlier (e.g., Hickson *et al.*, 2006). The latter reflects an increased loss of secretory cells by apoptosis during ODM compared with TDM (Li *et al.*, 1999). Therefore, the initiation of pro-apoptotic processes during an extended period of milk accumulation is a key regulator in reducing the long-term milk production under ODM.

1.6 LOCAL REGULATION OF MAMMARY RESPONSES TO MILK ACCUMULATION

1.6.1 Overview of the local regulation of mammary function

Local mammary-derived signals are responsible for initiating the decline in milk secretion and the progressive induction of mammary apoptosis and involution in response to an extended period of milk accumulation (refer to sections 1.3.2 and 1.5). A number of physiological changes occur in the bovine mammary gland during the first 24 h of milk accumulation as shown in Table 1.3 (Davis *et al.*, 1999). These include a decline in the rate of milk secretion, a decrease in mammary blood flow, increased TJ permeability and lactose efflux, an inflammatory response and an increase in intra-mammary pressure. These changes are initiated around 16-18 h post-milking. However, if mammary engorgement is not relieved, these physiological changes are intensified leading to the progressive induction of apoptosis and involution of mammary glands. Similar changes occur in the rodent mammary gland during the initial phase of involution – albeit at a much faster rate (Hanwell & Linzell, 1972; Kim *et al.*, 1997; Wilde *et al.*, 1999).

Several mechanisms are suspected to act as primary regulators in triggering this sequence of events, and these are illustrated in Figure 1.4 (Green & Streuli, 2004). The first mechanism is related to the accumulation in milk of chemical inhibitory and/or pro-apoptotic factors that are normally removed by milking, such as casein phosphopeptides (Shamay *et al.*, 2002, 2003). Another mechanism is that stretch-sensitive pathways may become activated as the alveolar lumen distends with secreted milk. When the adhesion receptors which link the basal MEC surface with the basement membrane become stretched, or the cell-cell adhesion junctions (e.g., adherens junctions or TJs) become stretched or compromised, this may lead to pro-apoptotic signals (Davis *et al.*, 1999; Stelwagen, 2001; Green & Streuli, 2004). Furthermore, the loss of TJ integrity may result in pro-apoptotic factors relocating from the apical to basolateral side of MECs to either induce apoptosis directly or antagonise survival signals (Green & Streuli, 2004). The roles of these physiological changes and mechanisms in regulating the local

mammary responses to extended periods of milk accumulation will be discussed in the following sections.

Table 1.3. Physiological changes in the bovine mammary gland during the first 24 h of milk accumulation.
Adapted and updated from Davis *et al.* (1999).

Parameter	Observation	Comment
Milk secretion	Slows after 16 h	Rate maintained if residual milk removed at start
Mammary blood flow	Begins to decrease at 16 h	Probably response to reduced mammary metabolism Slow recovery (> 24 h)
Alveolar distension	Complete by 16 h	Considerable size variation among alveoli. Smaller alveoli likely to have been fully distended for several hours by 16 h time point
Intra-mammary pressure	Rises rapidly after 16-18 h	See Turner (1955a). Limited data in literature
Cisternal fill	Begins to accelerate after 8 h	Cisterns are 40-50% full at 16-18 h when intra-mammary pressure begins to rise rapidly
Lactose efflux	Begins at 17-18 h and sustained until milk removal	Efflux a consequence of increased mammary permeability and/or pressure differential between milk and interstitial fluid. Efflux much reduced in succeeding intervals
Tight junction permeability	Enhanced and slow to recover	Signal triggering increased permeability unknown
Changes in gene expression	Changes within 24 h	Limited data available for ruminants. Refer to Molenaar *et al.* (2004), Singh *et al.* (2004a&b, 2005)
Inflammation	Onset after 16 h	Evidence includes transient increase in blood flow, increased neutrophil numbers in milk and tight junction changes

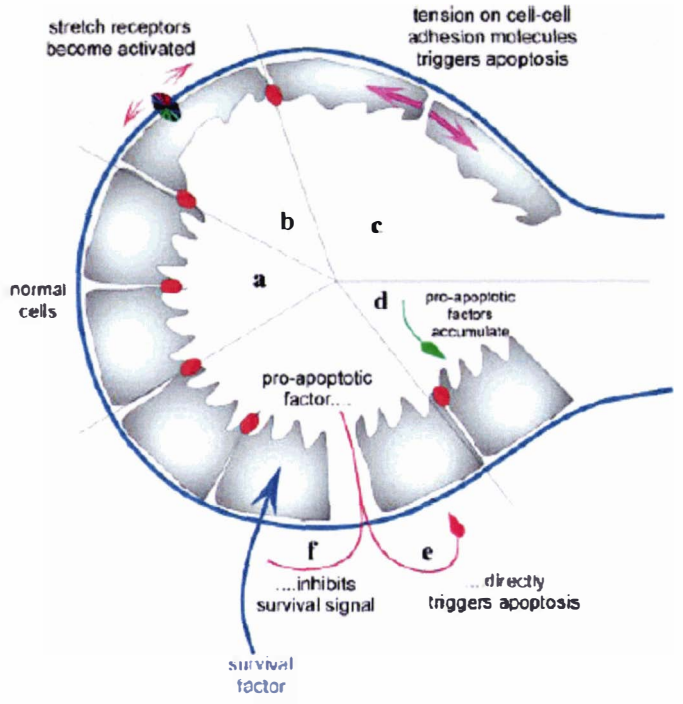


Figure 1.4. Possible apoptotic triggers at the onset of involution.
(a) Normal cells are columnar, tight junctions (red ovals) seal their apical interfaces, and they interact with basally located myoepithelial cells (not shown) as well as the basement membrane (blue line). (b,c) If milk is not removed the alveoli expand leading to luminal cell stretching. This may activate stretch receptors (b) or cause sufficient tension on cell-cell adhesion molecules (c) to induce apoptosis. (d) Pro-apoptotic factors that are normally excreted may accumulate (green arrow). (e,f) An unresolved mechanism may deregulate tight junction, leading to the relocation of pro-apoptotic factors to the basal cell surface, which could either trigger apoptosis directly (e) or inhibit survival factors (f). Figure reproduced from Green & Streuli (2004).

1.6.2 Alveolar distension and cell stretching

The degree and duration of alveolar distension has been suggested as the most important primary signal triggering a local regulatory response to milk accumulation in the mammary gland (Davis *et al.*, 1999; Stelwagen, 2001). Evidence supporting the role of physical distension of the mammary epithelium in regulating mammary function comes from several experiments in lactating goats and cows. In the goat studies, progressive induction of distension of the mammary epithelium without mammary engorgement (by isosmotic lactose replacement of milk removed at successive milkings) stopped milk secretion within 1-2 days (Fleet & Peaker, 1978). Acute distension of the mammary gland, with isosmotic sucrose solution to raise the intra-mammary pressure to levels normally observed following the cessation of milking, reduced the rate of milk secretion within 6 h (Peaker, 1980). In an experiment with cows which were milked once daily, and were also infused with an intra-mammary solution of sucrose and lactose equivalent to "5 hours worth" of milk, TJs became leaky much earlier (after 7 h instead of 17 h) and milk secretion was inhibited more than in ODM control cows (Stelwagen *et al.*, 1998a). In these experiments, dilution of potential chemical inhibitor(s) in milk (refer to section 1.6.3) would have occurred. Thus, these results indicate that physical distension of the mammary epithelium could play a major role in regulating mammary function during infrequent milking or the cessation of milk removal.

This mechanism is likely an outcome of the substantial changes in cell shape that occur as the alveoli distend to accommodate secreted milk products. Milk accumulates in the alveoli due to the tone of the alveolar milk ducts providing resistance to milk flow into the ducts/cisternal system. Valve-like structures have been identified where the milk duct exits the alveolus, and these may have a role in generating resistance to alveolar drainage (Caruolo, 1980). The mammary secretory cells are elongated or cuboidal after milk removal and become increasingly flattened and stretched with milk accumulation (Richardson, 1947). The intra-mammary pressure, an indicator of the degree of mammary distension, begins to rise rapidly after 16 – 18 h post-milking (Turner, 1955a; Davis *et al.*, 1999). Interestingly, this is about the same time that the alveolar compartment reaches maximal capacity and the cisternal compartment is about 40 – 50% full (Davis *et al.*, 1998; Fig. 1.3). Changes in mammary function (Table 1.3), such

as reduction in mammary blood flow and milk secretion, are apparent well before the total capacity of the udder is reached. Therefore, the induced changes are triggered by milk accumulation in the secretory alveoli occurring within 16 h post-milking, rather than the rapid rise in hydrostatic pressure after 16 h post-milking.

Further evidence of a role of alveolar distension in regulating mammary function is suggested by data showing that animals with higher proportions of residual milk and, therefore, relatively distended alveoli, have lower alveolar productivity (Peaker & Blatchford, 1988) and higher yield loss under ODM (Carruthers *et al.*, 1993a). In lactating ruminant udders, empty actively secreting alveoli are situated among lobules of distended non-secretory alveoli which express the immune-associated protein, lactoferrin, but not the major milk proteins, α-S1-casein or α-lactalbumin (Molenaar *et al.*, 1992). It is likely that high levels of residual milk reduce secretory activity by increasing the proportion of distended non-secretory alveoli rather than being evenly distributed across alveoli (Davis *et al.*, 1999; Vetharaniam *et al.*, 2003).

In a similar manner, prolonged engorgement of alveoli with milk, during the cessation of milk removal, leads to a progressive increase in the number of quiescent (distended, non-secretory) alveoli which will undergo apoptosis and eventually become senescent (dedifferentiated), and can no longer contribute to lactation (Davis *et al.*, 1999; Vetharaniam *et al.*, 2003). Experiments which demonstrate that milk yield can be recovered fully after 7 d (Dalley & Davis, 2006) or partially after 11 d (Noble & Hurley, 1999) of involution in dairy cows suggest that the quiescent alveoli can be re-activated to a secretory state if the engorgement can be relieved.

The cellular responses to alveolar distension are probably initiated about 10-16 h post-milking in cows (i.e., shortly before the changes in milk secretion and blood flow become apparent; Davis *et al.*, 1999). Changes in cell shape have been shown to induce intracellular signalling events (mechanotransduction) in lung, uterine and bladder tissues (Wirtz & Dobbs, 1990; Daifotis *et al.*, 1992; Yamamoto *et al.*, 1992). Mechanotransduction is the process by which physical stimuli, such as pressure or cell stretching, induce intracellular signalling events that culminate in changes in gene expression and modification of cellular function. Similar mechanisms have also been proposed in mammary tissue in response to milk accumulation during ODM or the

cessation of milk removal (Stelwagen *et al.*, 1997; Davis *et al.*, 1999; Stelwagen, 2001). The possible mechanisms by which mechanotransduction results in the loss of secretory activity and the induction of mammary apoptosis and involution are discussed in section 1.7.

1.6.3 Chemical regulation of mammary function

1.6.3.1 Evidence supporting chemical regulation

Linzell and Peaker (1971b) suggested that the increase in milk yield under more frequent milking in goats was due to more frequent removal of a constituent of milk, which limits milk secretion by negative feedback on the secretory cells. Several experiments were then conducted which supported this hypothesis. When glands were milked thrice daily, but the milk removed at the extra milking was replaced by an equal volume of inert sucrose solution to maintain the gland's distension the same as TDM, the rate of milk secretion still increased (Henderson & Peaker, 1984). These results led to the suggestion that the response to local frequent milking was not due to relief from the physical presence of stored milk. Henderson and Peaker (1987) diluted milk in the udder with isosmotic sucrose solution and milk yield increased, indicating that dilution of a chemical inhibitor occurred. Furthermore, this study also provided evidence that the secretory alveoli are the site of action of the chemical inhibitor *in vivo*, as an oxytocin injection plus hourly drainage of milk to evacuate milk from secretory alveoli increased milk yield whereas hourly drainage of cisternal milk did not. Successive studies by this research group then focussed on the identity and mode of action of the chemical inhibitor (section 1.6.3.2).

1.6.3.2 Feedback inhibitor of lactation (FIL)

Wilde *et al.* (1987a) tested the ability of goat milk fractions to inhibit lactose or casein synthesis in mammary tissue explants from mid-pregnant rabbits, which synthesise milk constituents when cultured in the presence of lactogenic hormones. They reported that synthesis of milk protein and carbohydrate was inhibited by a milk fraction containing

whey proteins with a molecular weight of 10 – 30 kDa. When this fraction was injected via the teat canal into the mammary glands of lactating rabbits (Wilde *et al.*, 1987a) and goats (Wilde *et al.*, 1988) it inhibited milk secretion in a dose-dependent and reversible manner. The effect was specific to the 10 – 30 kDa whey protein fractions as neither carrier solution nor large quantities of other whey proteins had any effect on milk yield. A single bioactive protein was subsequently isolated from this fraction by a combination of anion exchange chromatography and chromatofocusing, and was named the feedback inhibitor of lactation (FIL) (Wilde *et al.*, 1995). FIL is a 7.6 kDa glycoprotein with an N-terminal sequence which contains no homology with other milk proteins or recorded sequence tags. FIL has also been identified in fractions from cows' milk and was reported to have strong structural and functional similarity to caprine FIL (Addey *et al.*, 1991).

The site of FIL synthesis was identified by immunohistochemistry using anti-bovine FIL antiserum to probe mammospheres (Wilde *et al.*, 1995). Mammospheres were formed from MECs from late-pregnant goats that were cultured on a reconstituted basement membrane termed EHS matrix. Mammospheres are multicellular structures enshrouded in matrix material, with cells arranged peripherally around a central luminal space, into which they secrete milk proteins, reminiscent of lactating alveoli *in vivo*. Casein, α-lactalbumin and FIL were found to be localized predominantly in the lumen and could be extracted by treating the mammospheres with EGTA to break down the intercellular TJs (Wilde *et al.*, 1995). The synthesis of FIL within mammary secretory cells was also demonstrated using [^{35}S]methionine (Rennison *et al.*, 1993). These results suggest that the ability of the mammary gland to produce and secrete FIL into milk, where it confers an additional local level of control on the rate of milk secretion, is an example of autocrine control.

Wilde *et al.* (1995) showed that when purified FIL was injected into one gland through the teat canal it reduced milk yield ipsilaterally in a concentration-dependent manner. High doses of FIL (500-750μg) reduced milk yield by 12 – 17% over 3 days, whereas smaller doses (100-250μg) depressed milk yield by only 1 – 4% for 1- 2 days. FIL reduced milk yield *in vivo* without affecting gross milk composition, i.e., protein, lactose or milk fat concentrations, suggesting that it does not open TJs. Furthermore, the

inhibition of milk secretion was specific to FIL as injection of other whey proteins had no effect.

FIL's ability to regulate milk secretion was examined in other experiments *in vivo* that immunised goats against FIL (Wilde *et al.*, 1996). The theory behind this approach was that, if antibody against FIL (produced by active immunisation) was secreted into milk, it would act to prevent autocrine inhibition by FIL and increase milk yield. Goats were immunised against FIL three times at 8-weekly intervals after peak lactation to produce a circulating titre of antibody against FIL. The immune response was evident after the second immunisation and increased after the third, at which time anti-FIL antibody was detected in milk. Immunisation against FIL prevented the decline in milk yield after peak lactation in goats under TDM. Furthermore, immunised goats milked once a day had a smaller production drop than non-immunised ODM controls. Therefore, it appeared that antibodies against FIL in milk partly prevented autocrine inhibition.

However, the mechanism of autocrine inhibition of milk secretion by FIL is still unclear. FIL has been demonstrated to inhibit milk protein secretion *in vitro* by blocking the early stages of the constitutive secretory pathway at the level of ER-Golgi transport (Rennison *et al.*, 1993), but its exact mode of action and kinetics of feedback inhibition *in vivo* are unknown. The mechanism does not simply involve an increase in concentrations of FIL as milk accumulates, as this does not take into account the presence of residual milk in the gland following milk removal. Clearly, some form of processing of FIL must occur during milk accumulation to explain how the concentration of FIL decreases in residual milk soon after milking, otherwise milk secretion would continue to be inhibited. This has been postulated to be either an activation step leading to the formation of FIL from a precursor, or a catabolic step in which a clearance mechanism is overwhelmed by secretion of FIL in milk (Peaker, 1995; Wilde *et al.*, 1995, 1996, 1998; Knight *et al.*, 1998). However, no evidence has been published supporting either of these processes.

Another potential mechanism of action is that as the mammary epithelium becomes stretched during milk accumulation, cell-surface receptors for FIL might become exposed (Peaker, 1995). However, the results of Stelwagen *et al.* (1998a) discussed earlier (section 1.6.2) have indicated that this mechanism is unlikely. Furthermore,

while the site of action of FIL has been demonstrated to be located on the apical membrane (Blatchford *et al.*, 1998), specific receptors for FIL have yet to be determined.

While a role for FIL in inhibiting milk secretion in response to extended periods of milk accumulation has been presented, it is unclear at present whether FIL actually has a role in activating mammary apoptosis and involution. However, it has been suggested that FIL contributes to the increased rate of loss of mammary secretory cells during long-term infrequent milking by decreasing the sensitivity of the secretory cell to circulating galactopoietic hormones through a down-regulation of prolactin (and possibly IGF-1) receptors (Bennett *et al.*, 1990, 1992; Knight *et al.*, 1998). Therefore, more information, including the protein sequence and structure of FIL, measurement of FIL concentrations in milk and the identification of the gene coding FIL, is required to establish the mechanisms of action of FIL and its kinetics in relation to milk removal in order to verify FIL as a primary regulator of mammary function. The failure of 15 years of research at the Hannah Institute to isolate and characterize FIL calls into question the evidence of its existence as a discrete entity. However, there is no doubt that some bovine milk protein fractions are inhibitory to milk secretion in primary mouse mammary cells in culture (S. R. Davis pers. comm.; Wilde *et al.*, 1995). The functional significance of these observations remains to be established.

1.6.3.3 Other chemical regulators of mammary function

Casein phosphopeptides derived from massive activation of plasmin protease activity have recently been shown to disrupt TJs and inhibit milk secretion during extended periods of milk accumulation in goats and cows (Shamay *et al.*, 2002, 2003; Silanikove *et al.*, 2006). While the exact mechanism by which this occurs remains to be determined, β-casein (fraction 1-28), a phosphopeptide derived from mild activation of plasmin activity, was demonstrated to reduce milk secretion by a process associated with its ability to block potassium channels in the apical membranes of MECs (Silanikove *et al.*, 2000). Direct evidence for a role of casein phosphopeptides in stimulating apoptosis during milk accumulation is yet to be provided. However, the plasmin system has previously been implicated in the onset of involution (Politis *et al.*,

1996) and increases in plasmin, plasminogen and plasminogen activator activity observed in the milk of ODM cows are consistent with this role (Knutson *et al.*, 1993; Stelwagen *et al.*, 1994c; Kelly *et al.*, 1998; refer to Table 1.2). In contrast, milk plasmin did not increase with thrice daily milking compared with TDM (Klei *et al.*, 1997) and very frequent milk removal (i.e., every 2 h) resulted even in a reduction of plasmin activity in milk (Kaartinen *et al.*, 1990). Therefore, degradation of milk proteins by plasmin protease activity during extended periods of milk accumulation may induce inhibitory signals leading to loss of secretory activity and mammary apoptosis and involution (Shamay *et al.*, 2002, 2003; Silanikove *et al.*, 2006).

A separate mechanism 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 (Matsuda *et al.*, 2004). While this homeostatic regulation appears important for suppression of milk secretion in response to milk accumulation within alveolar lumens, the specific mechanisms governing this process are not yet established. Furthermore, it is currently unknown whether a role exists for serotonin in mammary apoptosis and involution in the bovine mammary gland.

Other potential chemical regulators of mammary function include factors which accumulate in milk during involution, and which have been shown to stimulate apoptosis under certain conditions. These include the major milk protein α-lactalbumin (Hakansson *et al.*, 1995, 1999), IGFBP-5 (Tonner *et al.*, 1997, 2002; Marshman *et al.*, 2003), and TGF-β1 (Letterio *et al.*, 1994; Atwood *et al.*, 1995; Kordon *et al.*, 1995).

1.6.4 Tight junction permeability

The TJ complex (as described in section 1.2.2) plays an important role in the regulatory response to changes in milking frequency and milk accumulation. In the mammary gland, the TJs between alveolar epithelial cells are relatively impermeable during lactation, and thus allow milk to be stored in the alveolar lumen without leakage into the interstitial fluid (Nguyen & Neville, 1998). However, during extended periods of milk accumulation, in both ruminants and rodents, the TJs start to become "leaky" (Fleet &

Peaker, 1978; Stelwagen *et al.*, 1994a & b, 1997; Yamamuro & Sensui, 1994; Delamaire & Guinard-Flament, 2006b; section 1.2.2), allowing the mixing of milk and plasma components (section 1.2.3).

In both goats and cows, some TJ integrity is lost after approximately 18 h of milk accumulation which coincides with the start of a decline in the rate of milk secretion (Stelwagen *et al*, 1994a & b, Stelwagen *et al.*, 1997; Fig. 1.5). The increase in TJ permeability is typically measured as a rise in plasma concentrations of lactose (e.g., Stelwagen *et al.*, 1997; Fig. 1.5). Since lactose is synthesised only by the mammary gland and is not secreted through the basolateral membrane, the plasma concentration of lactose provides a measure of the leakage rate of milk components from the lumen of the mammary gland into the blood stream.

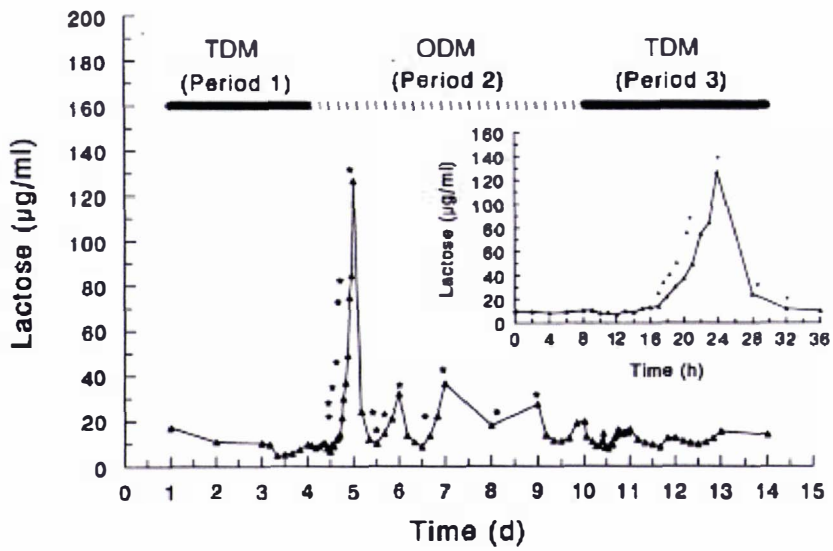


Figure 1.5. Average concentrations of lactose in blood during transition from twice-daily milking (TDM) to once-daily milking (ODM) and reverse.
Inset figure shows the time course of the surge in plasma lactose during the first 36 h of ODM. Adapted from Stelwagen *et al.* (1997).

Plasma concentrations of lactose and α-lactalbumin and those of sodium and serum albumin in milk rose after 18 h of milk accumulation during the first day of ODM in dairy cows (Stelwagen *et al.*, 1997; Fig. 1.5). TJs reverted to the closed state soon after milking at 24 h, showing that the increase in TJ permeability was transient. After the first day TJ permeability increased and decreased with each cycle of milk accumulation and milk removal, but leakage became smaller with each cycle, suggesting that the

barrier function of TJs in the mammary gland was partially restored during ODM. However, increased duration of milk accumulation (up to 32 h or 40 h) enhanced the reduction in lactose concentrations in milk and the rates of recovery of lactose concentration (and of milk yield) were relatively slow, taking > 24 h (Farr *et al.*, 1998), demonstrating that resealing of TJs is not a trivial task for the secretory cell epithelium. These observations indicate that the effect of milk accumulation on mammary TJs is not merely a pressure-mediated effect leading to efflux of lactose from the gland and is corroborated by findings in cows based on the kinetics of lactose disappearance from blood (Stelwagen *et al.*, 1997).

Nevertheless, there is a large increase in intra-mammary pressure after 16-18 h post-milking (Turner, 1955a; Davis *et al.*, 1999), which does not dramatically decline, along with mammary volume, until 3 to 7 d following the abrupt cessation of milk removal (Mackenzie, 1968; Fleet & Peaker, 1978; Hurley, 1989). This decline suggests a significant loss of TJ integrity at that time. Therefore, the increase in plasma lactose and milk albumin concentrations at 18 h may at least partly reflect an intra-mammary pressure differential where small molecules move by conductance between milk and intercellular fluid compartments (Davis *et al.*, 1999).

The mechanism by which TJ integrity is disrupted in response to extended periods of milk accumulation and the role of TJs in the loss of secretory activity and the induction of mammary apoptosis and involution are unknown. The mechanism is, however, related to the local accumulation in milk of chemical inhibitory factors (section 1.6.3) and/or the duration of physical distension of the alveolar epithelium (section 1.6.2) (Stelwagen *et al.*, 1997; Davis *et al.*, 1999; Stelwagen, 2001). A potential role for TJs in the mechanotransduction events in the mammary gland is discussed in section 1.7. In rodents, the loss of systemic stimuli, such as prolactin, on withdrawal of the suckling stimulus may also mediate the loss of TJ integrity during cessation of milking, but whether this mechanism is important in ruminants is less clear (Stelwagen, 2001).

The exact mechanism by which leaky TJs affect milk production also remains uncertain. Stelwagen *et al.* (1997) reported that only one-fifth of the 15% milk yield loss associated with ODM in dairy cows was caused by milk "leakage" into blood, indicating that milk secretion was actively reduced in response to milk accumulation.

However, a direct link between TJ breakdown and milk secretion is suggested by experiments showing that milk yield can be reduced by a similar amount to that observed during ODM by disrupting TJs using the calcium chelators, EGTA (Stelwagen *et al.*, 1995) or citrate (Neville & Peaker, 1981). Therefore, these observations suggest that TJs play a major role in the mammary response to extended periods of milk accumulation.

1.6.5 Mammary blood flow

In addition to local and systemic stimuli, the lactating mammary gland is dependent upon the provision of nutrients from the blood to sustain milk production. Mammary blood flow is closely related to milk yield (Linzell, 1974) and declines with advancing lactation (Nielsen *et al.*, 1990). However, the close relationship between blood flow and milk yield does not always hold. For example, Maltz *et al.* (1984) found no effect of hourly milking on mammary blood flow in goats, despite a 15% increase in the rate of milk secretion. Furthermore, the ratio of mammary blood flow to milk yield declined as milk yield increased in goats (Nielsen *et al.*, 1990). Colchicine treatment reduced milk yield but was not associated with a decline in mammary blood flow (Henderson & Peaker, 1980).

Other studies have reported changes in mammary blood flow with differing milking intervals. Frequent milking with oxytocin resulted in higher blood flow to the mammary gland (Prosser & Davis, 1992), while Shinde (1978) observed that blood flow velocity in a mammary vein declined with increasing milking interval. ODM reduced milk yield by 28% and mammary blood flow by 10% compared with TDM in Holstein Friesian cows (Guinard-Flament & Rulquin, 2001). During extended periods of milk accumulation, mammary blood flow has been shown to decline around 21 h in goats (Stelwagen *et al.*, 1994b), within 16 h in cows (Delamaire & Guinard-Flament, 2006a), and within 8 h in rats (Hanwell & Linzell, 1973). Milk secretion rate starts to decline at around the same time – 19 h in goats (Stelwagen *et al.*, 1994b), 16 h in cows (Davis *et al.*, 1998), and by 4 – 8 h in rats (Hanwell & Linzell, 1973). Furthermore, decreases in mammary blood flow were shown to reduce the uptake of precursor nutrients in the mammary gland during milking intervals of greater than 16 h in cows, which directly

contributed to the reduction in milk yield as the efficiency of the mammary gland in synthesising milk components remained relatively unchanged after 7 d of treatment (Delamaire & Guinard-Flament, 2006a).

Interestingly, a transient increase in mammary blood flow was observed during the period 12 -16 h post-milking in goats (Stelwagen *et al.*, 1994b), and may reflect a mild inflammatory response induced by milk accumulation. Elevated numbers of neutrophils are found during milk accumulation in cows (Stelwagen & Lacy-Hulbert, 1996) and in sheep (Colditz, 1988). Furthermore, disruption of mammary TJs by EGTA also increased mammary blood flow to levels seen during 12 – 16 h of milk accumulation (Stelwagen *et al.*, 1994b, 1995).

Several pieces of evidence indicate that the decline in mammary blood flow associated with milk accumulation is not simply due to intra-mammary pressure restricting the flow of blood through the tissue. Firstly, rats appear to be more sensitive to the suckling stimulus rather than milk removal *per se*, as mammary blood flow was maintained in sealed but still-suckled glands to 18 h, as opposed to 8 h in sealed non-suckled glands (Hanwell & Linzell, 1973). Secondly, the decline in mammary blood flow and milk secretion rate occur before maximum udder capacity is reached at ~25 h in goats (Stelwagen *et al.*, 1994b) and ~30 h in cows (Davis *et al.*, 1999). Furthermore, removing milk after a period of mammary engorgement did not immediately restore arterial flow (Hanwell & Linzell, 1973; Farr *et al.*, 2000).

Milk accumulation has been suggested to be associated with closure of capillaries resulting in a reduction in blood flow, probably by the production of local vasoactive compounds (Silver, 1956; Farr *et al.*, 2000). Locally produced vasoactive compounds that have been shown to alter mammary blood flow include; parathyroid hormone-related protein, IGF-1, prostacyclin, nitric oxide, endothelin (for review see Prosser *et al.*, 1996). The production of these compounds by mammary epithelial cells provides a mechanism for the gland to control its own blood supply and, hence, nutrient flow for milk synthesis in response to changes in milking frequency and the cessation of milk removal.

1.7 SIGNALLING PATHWAYS: MECHANOTRANSDUCTION IN RESPONSE TO MILK ACCUMULATION

A scheme integrating the concepts regulating the local intra-mammary response to an extended period of milk accumulation (as discussed in section 1.6) was proposed by Davis *et al.* (1999) and is shown in Figure 1.6. In this scheme the primary signal initiating these mammary responses is the change in MEC shape during alveolar distension with milk (section 1.6.2). While this scheme shows the events occurring within the initial 24 h of the first day of ODM, these same events will eventually lead to mammary apoptosis and involution if the gland remains unmilked. Physical distension of the mammary epithelium results in flattening and stretching of the MECs (Richardson, 1947), and is postulated to induce changes in gene expression that culminate in the modification of cellular function in a process known as mechanotransduction (section 1.6.2).

Wide-spread, dramatic, changes in gene expression have been well-documented in response to milk accumulation in rodents, and an excellent review of the various signalling pathways that these genes participate in regulating the initiation of mammary apoptosis and involution is provided by Green and Streuli (2004). Expression of a number of genes are differentially regulated less than 24 h after milk removal has stopped, and include the up-regulation of pro-apoptotic genes (such as caspases, Bax, c-fos, sulphated glycoprotein, p53), and the down-regulation of cell survival genes (such as Akt, β1-integrin) and milk protein genes (e.g., Strange *et al.*, 1992; Jaggi *et al.*, 1996; McMahon *et al.*, 2004, Appendix VII). Other key changes include the down-regulation of the prolactin signal transduction pathway through STAT5a and STAT5b and activation of the pro-apoptotic and acute phase immune response marker, STAT3 (Li *et al.*, 1997a). The net result is an induction of processes leading to a loss of secretory activity and the initiation of apoptosis (Walker *et al.*, 1989; Strange *et al.*, 1992; Marti *et al.*, 1997).

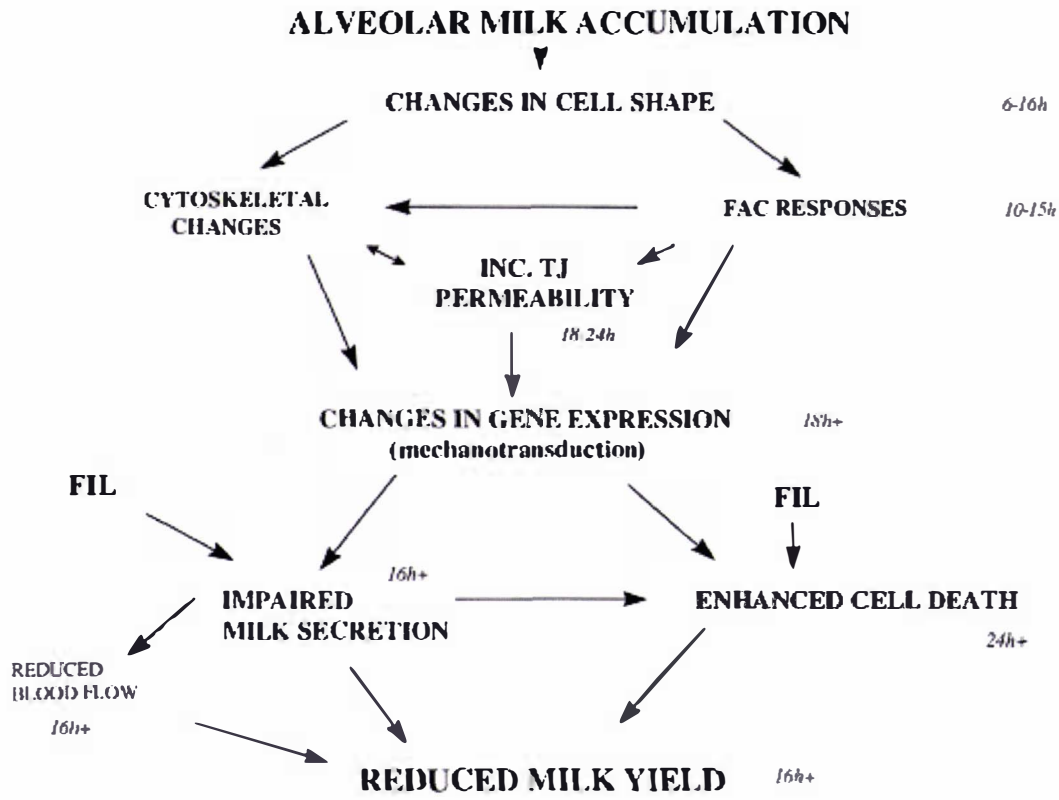


Figure 1.6. Scheme showing events leading to changes in gene expression and reduced milk yield during extended milking intervals.
FAC, focal adhesion complex; FIL, feedback inhibitor of lactation. The trigger for FIL action has yet to be defined, but effects on milk secretion and cell death have been described (see text). Figures in italics mark the approximate time scale of events described, relative to the last milking in dairy cows. A similar sequence of events occurs in rodents, except at a much faster rate and includes a contribution from loss of the galactopoietic hormone signalling following weaning (see text). Associated with these changes is a mild inflammatory response leading to increased neutrophils in milk and increased mammary permeability (see text). Adapted from Davis *et al.* (1999).

While there is limited data in ruminants, parallel studies by colleagues using the same bovine mammary involution model described in Chapter 3 of this thesis have recently demonstrated that dramatic changes in gene expression occur within the first 24 – 48 h following the abrupt cessation of milk removal (Molenaar *et al.*, 2004; Singh *et al.*, 2004a & b; 2005, Appendix VI). Expression of the major milk proteins, α-lactalbumin and α-S1-casein, were decreased by 24 h post-milking, while the immune-associated genes, mammary serum amyloid A3 and lactoferrin, were markedly increased between 36 h and 8 d after last milking (Molenaar *et al.*, 2004; Singh *et al.*, 2004b). Furthermore, microarray analysis revealed that widespread changes in bovine mammary gene expression occurred within 36 h post-milking (Singh *et al.*, 2004b). Many of the genes that were differentially expressed at 36 h post-milking were involved in cellular

processes such as cell communication, death, differentiation, growth and/or maintenance. Genes involved in metabolic functions that were differentially expressed were mostly from both biosynthetic and catabolic pathways, as well as nucleic acid metabolism. These changes in gene expression reflect the loss of secretory activity and induction of mammary apoptosis and involution occurring in response to extended periods of milk accumulation. However, the challenge will be in deciphering which changes in gene expression are crucial in triggering these processes.

While a primary role for mechanotransduction in regulating the mammary responses to extended periods of milk accumulation has been proposed (e.g., Davis *et al.*, 1999; Stelwagen, 2001), it has not been investigated to date. Furthermore, the relationship between this process and the dramatic changes in gene expression induced during mammary apoptosis and involution (reviewed by Green & Streuli, 2004) are not yet understood. The trigger and mediator of changes in TJ permeability are unknown, as is the impact of this change on secretory cell function, although increased TJ permeability is associated with reduced milk secretion (Stelwagen *et al.*, 1995).

Physical distension of the mammary epithelium is likely to result in cytoskeletal changes and subsequent mechanical tension on both cell-cell and cell-ECM interactions, which may lead to loss of survival signals and initiate pro-apoptotic signals (Fig. 1.4 and 1.6). Therefore, the roles of the focal adhesion complexes (FACs) which mediate cell-ECM adhesion, and the TJs complexes which mediate cell-cell integrity, are principal candidates for investigation of mechanotransduction events in the mammary gland. A scheme illustrating the effects of stretching of the mammary secretory cells on FACs and TJs is depicted in Figure 1.7. These two mechanisms may be indirectly linked via close associations with the cytoskeleton.

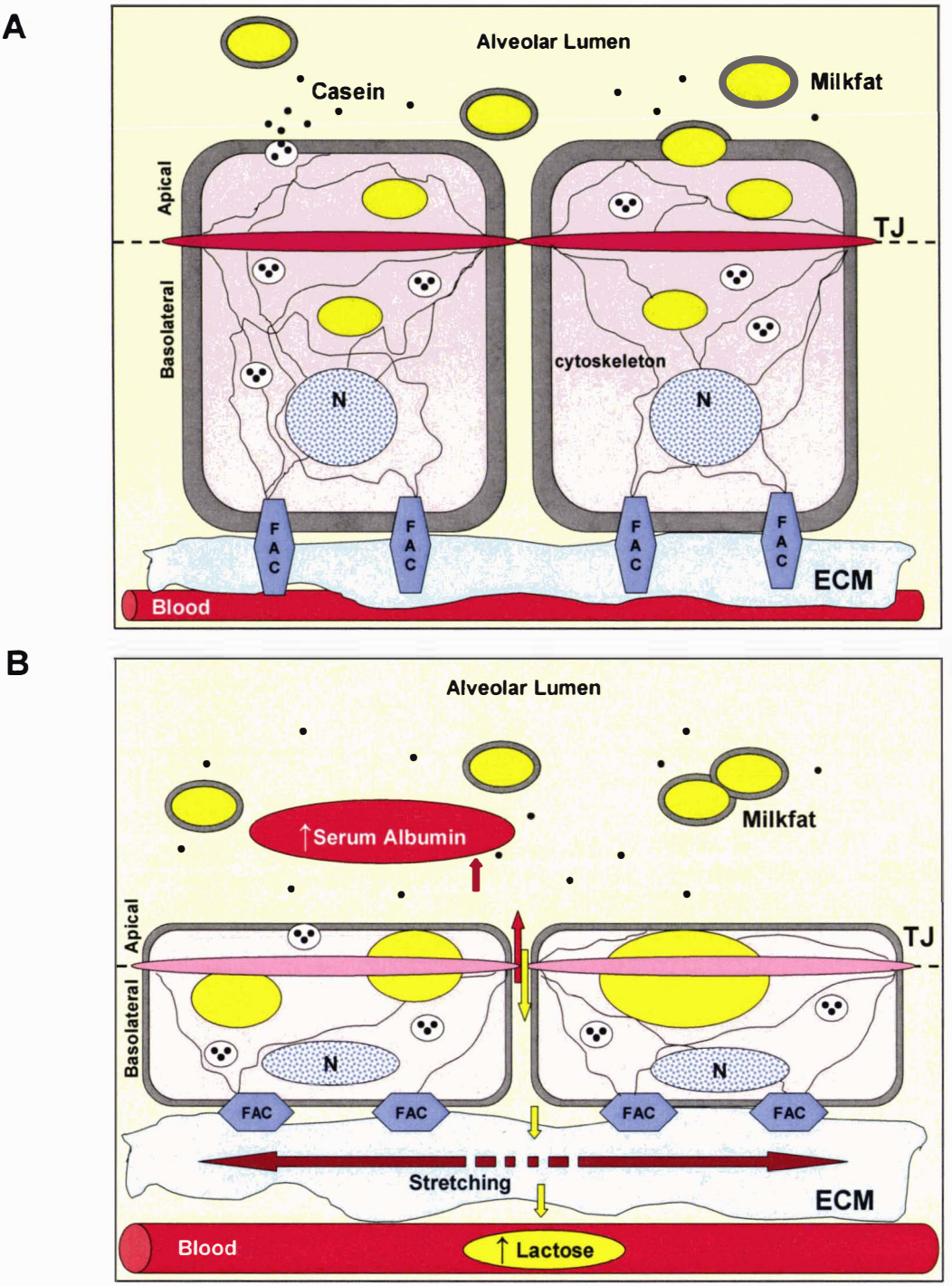


Figure 1.7. Scheme of the events occurring during stretching of the mammary epithelium in response to alveolar milk accumulation.

(**A**) Adjacent mammary epithelial cells recently after milk removal are cuboidal in shape and are actively secreting milk components (milk fat and protein) via the apical membrane into the alveolar lumen. The secretory cells are attached at their basolateral membranes to an extracellular matrix (ECM) via focal adhesion complexes (FAC). The tight junction (TJ) complexes seal the paracellular pathway preventing the mixing of milk with interstitial fluid. (**B**) The alveoli distend to accommodate secreted milk products resulting in stretching of the mammary epithelium and consequently a flattening of the mammary epithelial cell shape. Mechanical tension is sensed by the cytoskeleton, FACs and TJs resulting in mechanotransduction events. TJ complexes become "leaky" allowing the paracellular pathway to open and the efflux of lactose and potassium (down their concentration gradients) from milk into the blood, and the influx of serum albumin, sodium and chlorine into milk. Milk secretion is inhibited and the mammary epithelial cells contain large vacuoles (fat droplets and secretory vesicles). Finally, signals are initiated which progressively lead to mammary apoptosis and involution (not shown).

It is known that FACs predominately mediate cell-ECM adhesion through integrins, which are cell surface receptors that bridge the intra- and extra-cellular compartments to enable direct communication between adaptor molecules in the cytoplasm and specific receptor motifs in the ECM (Hynes, 1992). In the mammary gland, integrin-mediated cell adhesions play essential roles in maintaining MEC survival and differentiated function (Boudreau *et al.*, 1995; Streuli *et al.*, 1995; Pullan *et al.*, 1996; Faraldo *et al.*, 1998, 2002; Naylor *et al.*, 2005). Concurrent studies by colleagues, McMahon *et al.* (2004, Appendix VII) and Singh *et al.* (2005, Appendix VI), have focussed on the role of the cell-ECM survival mechanism during mammary engorgement. Their results demonstrate that cell-ECM survival signalling through β1-integrin and its down-stream signal transduction factor, focal adhesion kinase (FAK), is rapidly down-regulated during the first, reversible, phase of mammary apoptosis and involution. This indicates that communication between MECs and their basement membrane is lost during mammary engorgement, which is thought to contribute to the induction of apoptosis. Integrins and the FAK pathway have been shown to participate in mechanosensitive events in other cell types (Shyy & Chien, 1997; Geiger *et al.*, 2001; Geiger & Bershadsky, 2002) and therefore, this process may also operate in the mammary gland.

While a loss of TJ integrity occurs during mammary engorgement, the mechanism by which TJ complexes revert from a "tight" to a "leaky" state is unknown, but most likely involves a change in the function and/or composition of molecules present in the TJ complex. The basic structure and key components of the TJ complex are illustrated in Figure 1.8. Occludin (Furuse *et al.*, 1993) and claudins (Furuse *et al.*, 1998; Morita *et al.*, 1999; Tsukita & Furuse, 1999) are integral transmembrane TJ proteins that bind to the cytoplasmic TJ proteins zonula occludens (ZO-1, ZO-2 and ZO-3), providing the TJ with structural and signalling links to the cell interior (Furuse *et al.*, 1994; Haskins *et al.*, 1998; Itoh *et al.*, 1999a & b). Importantly, ZO-1 links occludin and claudins to the actin cytoskeleton (Fanning *et al.*, 1998; Itoh *et al.*, 1999a) and is involved in signal transduction, regulation of gene expression, suppression of proliferation and stimulation of differentiation (Yamamoto *et al.*, 1997; Balda & Matter, 2000; Meyer *et al.*, 2002; Matter & Balda, 2003).

The roles of these TJ proteins in the mammary gland are largely unexplored. The major transmembrane TJ protein, occludin, appears to be required for mammary TJ formation

and closure during lactogenesis *in vivo* (Saitou *et al.*, 2000) and is up-regulated in response to lactogenic hormones *in vitro* (Stelwagen *et al.*, 1999). Occludin may also act as a cell survival signal as artificial truncation of occludin induces MEC apoptosis both *in vitro* and *in vivo* (Beeman & Neville, 2001). In addition, TJ proteins could play a role in mechanotransduction events in the mammary gland, as they participate in cell signalling (Aijaz *et al.*, 2006) and have been demonstrated to be mechanosensitive in other epithelial cells. For example, occludin protein expression was down-regulated during an increase in TJ permeability in response to *in vitro* stretching of lung alveolar epithelial cells (Cavanaugh *et al.*, 2001). Therefore, the role of the TJ protein complex in regulating mammary function requires consideration.

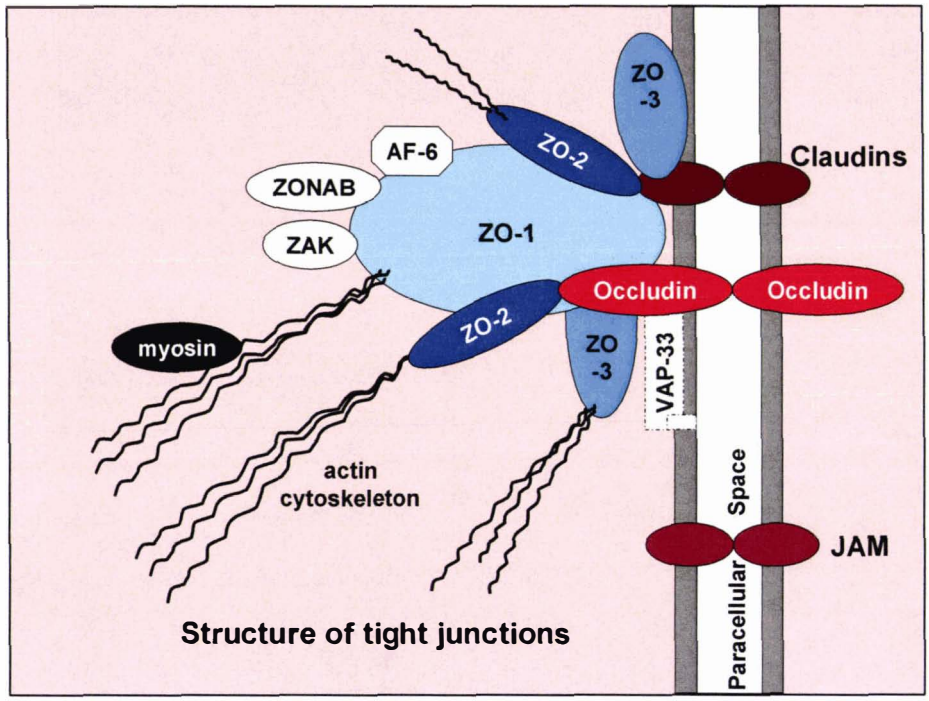


Figure 1.8. Basic structure and components of the tight junction complex.
Tight junctions consist of transmembrane proteins (occludin, claudins and junctional adhesion molecule (JAM)) and the cytoplasmic proteins zonula occludens (ZO-1, ZO-2, ZO-3). The ZO family link the tight junction to the actin cytoskeleton. Also associated with the tight junction complex are the signalling proteins ZONAB, ZAK, AF-6 and VAP-33, amongst others.

1.8 OBJECTIVES OF THIS RESEARCH

The overall aim of this project is to investigate the regulation of TJ protein expression during mammary engorgement, and in particular the role of physical distension of the mammary epithelium in the regulatory process. The hypotheses to be examined are 1)

that TJ protein expression is down-regulated during mammary engorgement, and 2) that the change in MEC shape from a cuboidal to a flattened/stretched morphology triggers the decrease in TJ protein expression and the onset of mammary apoptosis and involution. The integral transmembrane TJ proteins, occludin and claudin-1, and the cytoplasmic TJ protein, ZO-1, will be examined as they are the most well-characterised key components of the TJ complex.

In the first study, the temporal expression of TJ proteins will be determined relative to the onset of MEC apoptosis and involution in bovine mammary glands. A similar investigation will then be carried out in rats, which provide an excellent model of tightly co-ordinated control of mammary apoptosis and involution. In this study the effect of teat-sealing to induce local mammary engorgement, whilst maintaining the suckling stimulus and systemic galactopoietic hormone levels, on the temporal expression of TJ proteins will be investigated. Bovine and rat models of mammary engorgement will be compared to provide a detailed time course post-milking of the changes in histological morphology, the initiation of apoptosis, the decline in secretory activity and the induction of mammary involution, relative to changes in TJ protein expression.

The role of physical distension of the mammary epithelium in triggering the disruption of TJ function and the initiation of mammary apoptosis and involution will then be examined using two approaches. In an *in vivo* study, rat mammary glands will be artificially distended, using isosmotic sucrose solution, with the aim of initiating the process of involution. The effect on TJ protein expression and the instigation of pro-apoptotic processes will be determined. The final experiment will use an *in vitro* approach, the objective being to develop a model which clearly separates the effects of physical stretching of MECs from the actions of possible chemical inhibitors in milk. The effect of stretching MECs *in vitro* on the expression of TJ proteins and markers of cell survival and cell death will then be determined.

Therefore, this project aims to improve our understanding and knowledge of the role of TJ proteins and mechanotransduction events in the regulation of local mammary function in response to extended periods of milk accumulation.

CHAPTER TWO

General Materials and Methods

This chapter contains descriptions of general materials and methods used in two or more chapters in this thesis. The 'Methods' section within individual chapters contains detailed descriptions of methods pertaining to specific experiments.

2.1 MATERIALS

2.1.1 Chemicals and biological compounds

Manufacturers of brand products not specifically mentioned within 'Methods' sections are detailed below. All other reagents used were of analytical grade, unless stated otherwise. The contact addresses of manufacturers and companies whose products and/or services were used in this thesis are listed in Appendix I.

Acrylamide, ammonium persulphate (APS), bromophenol blue, coomassie brilliant blue G-250, glycine, N, N'-methylene-bis-acrylamide, Tween-20 detergent, and N, N, N', N'-tetra-methylenediamine (TEMED) were all electrophoresis purity and from Bio-Rad Laboratories. β-mercaptoethanol (BME), dimethyl sulphoxide (DMSO), Trizma Base (Tris), diethylpyrocarbonate (DEPC), 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS) and 3, 3'-diaminobenzidine tetrahydrochloride (DAB) were obtained from the Sigma Chemical Company. Polyvinylpyrolidone (PVP)-25 was purchased from Serva Electrophoresis GmbH. Sodium dodecyl sulphate (SDS), Nonidet P-40 (NP-40) detergent, 30% H_2O_2 and glycerol were supplied by BDH Laboratory Supplies.

Agarose and bovine serum albumin (BSA)-fraction V, used in blocking and incubation buffers for western blotting and immunohistochemistry (sections 2.2.8.2 and 2.2.9, respectively), were bought from GIBCO Products, Invitrogen.

2.2 METHODS

2.2.1 Animal maintenance and tissue collection protocols

2.2.1.1 Bovine mammary tissue collection

Dairy cows were milked at AgResearch's Ruakura dairy farm and were maintained on grazed pasture with supplementary grass silage as required. Following experimental protocols (described in section 3.2.1; approved by the Ruakura Animal Ethics Committee) animals were killed by captive bolt and exsanguination at the Ruakura abbatoir. Tissue samples were collected post-mortem from the major anatomical areas of the bovine mammary gland (refer to Fig. 1.1), but only the secretory alveolar samples were used in this thesis. Slices of alveolar tissue of approximately 50 mm in length and 10 mm thick (approximately 30 g total) were collected from the middle of the upper one-third of a rear quarter of each animal. These were immediately frozen in liquid nitrogen and stored at -80 °C. Frozen tissue samples were ground with a custom-made mortar and pestle tissue grinder (Molenaar, McCracken and Farr, http://www.agresearch.co.nz/agr/agrsci/crusher/tissue_crush.htm) under liquid nitrogen to obtain a homogenous sample before analysis. Additional tissue samples, collected for histological analyses, were cut into strips (approximately 30 x 30 x 5 mm) and placed directly into either 4% paraformaldehyde fixative (4% (w/v) paraformaldehyde, 0.1 M sodium phosphate buffer pH 7.4) or 10% formalin fixative (10% (v/v) formalin, 0.075 M sodium phosphate buffer pH 7.4).

2.2.1.2 Rat mammary tissue collection

Female Sprague-Dawley rats were obtained from, and maintained in, the Small Animal Colony at AgResearch Ruakura. Animals were fed a standard laboratory chow (Diet 86; Sharps Grain and Seed) and water *ad libitum*, and were maintained in rooms at 25 °C with a 14 h light: 10 h dark daily photoperiod. Each female was mated at approximately 13 weeks of age. Experimental animals were used only in protocols approved by the Ruakura Animal Ethics Committee. Following treatments, animals were euthanised

with carbon dioxide followed by cervical dislocation. Whole mammary glands were separated from the skin and surrounding tissues for collection (Fig. 2.1). Glands intended for protein and mRNA extraction were sliced into 1 ml cryovials, immediately frozen in liquid nitrogen and stored at -80 °C. Frozen tissue samples (approximately 1 to 5 g) were ground with a mortar and pestle under liquid nitrogen to obtain a homogeneous sample before analysis. For histological analyses, approximately one-third of the gland (20 x 20 x 5 mm) was placed directly into 4% paraformaldehyde.

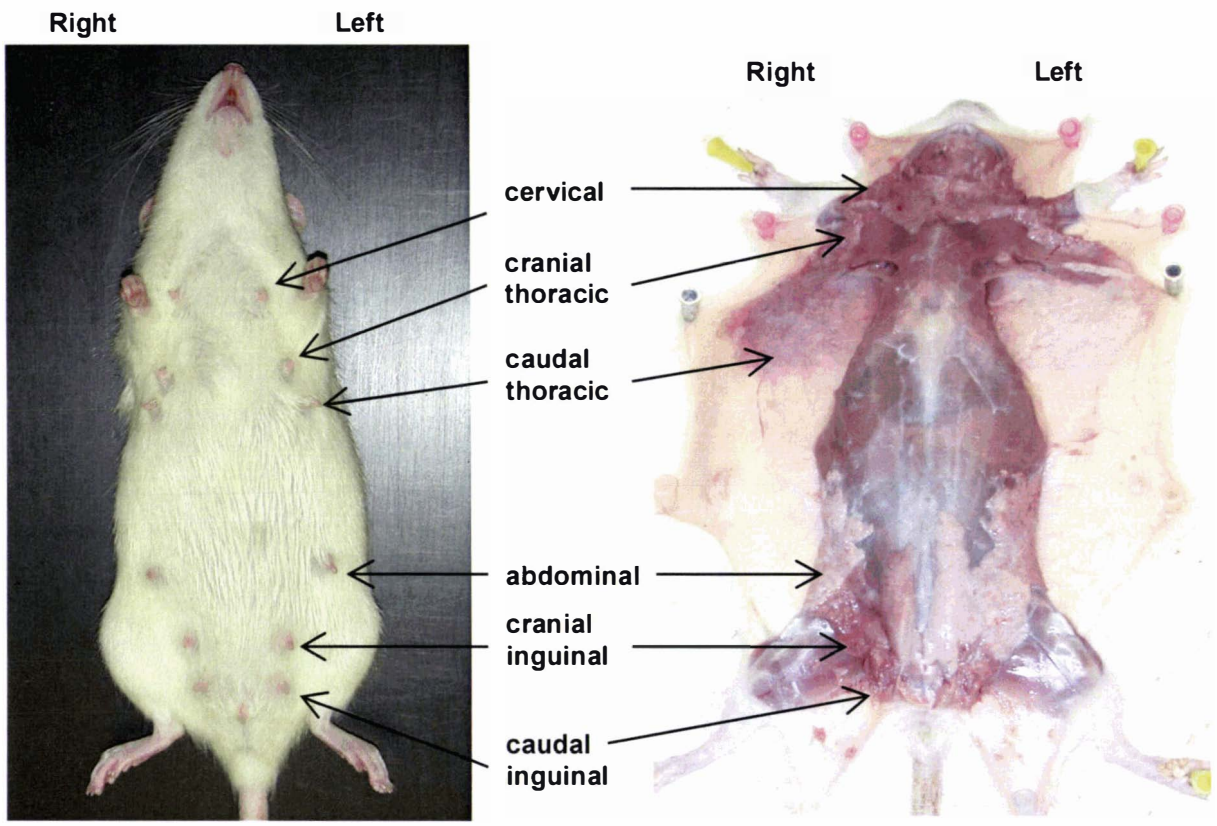


Figure 2.1. The position of the six pairs of mammary glands on female Sprague-Dawley rats. For the rat in the right-hand image, the right abdominal teat and the left caudal thoracic and cranial inguinal teats were sealed to engorge these glands with milk prior to rat euthanasia and dissection. This helps to distinguish the boundaries between each gland. The six pairs of mammary glands on the rat were exposed by making a long incision along the ventral midline and four short incisions along the legs. Whole mammary glands were then carefully dissected away from the skin and surrounding tissues.

2.2.2 Histological processing and analysis

2.2.2.1 Histological processing

Mammary tissue slices were placed in TissueTek cassettes (Sakura-Finetek Europe BV) and fixed for 18-24 h in either 4% paraformaldehyde or 10% formalin, to prevent degradation and to stabilise the tissue for processing and sectioning. They were then washed and stored in 70% ethanol prior to automatic processing (Leica JUNGTP1050 Tissue Processor; Leica Microsystems) to remove water from the tissue and replace it with solvents and finally paraffin wax. Briefly, tissues were dehydrated through a range of alcohol washes (70% ethanol (2 x 1 h), 95% ethanol (2 x 1 h), and 100% ethanol (4 x 45 min)), cleared in toluene (2 x 1 h) and infiltrated with paraffin wax at 60 °C (1 x 1 h and 2 x 2 h). Finally, processed tissues were embedded in paraffin wax (BDH Laboratory Supplies) and mounted on the back of cassettes. Serial sections of embedded tissue were cut 8 μm thick on a microtome, and floated onto polylysine-coated microscope slides (Biolab Scientific) in a heated water bath (45-50 °C). Slides were dried at 37-50 °C, and stored at room temperature (RmT) until required.

2.2.2.2 Histological analysis of alveolar morphology

To visualise the morphology of mammary tissue sections, slides were stained in haematoxylin and eosin (H&E) solutions using the following procedure. Slides were de-waxed in 2 x 5 min washes of xylene and hydrated in 100% ethanol (2 min), 95% ethanol (2 min), 70% ethanol (1 min) and 50% ethanol (1 min) followed by a 1 min wash in water. Nuclei were stained blue/black in Gill's haematoxylin (0.4% (w/v) haematoxylin, 1.85 M sodium iodate, 53 mM aluminium sulphate, 25% (v/v) ethylene glycol, 4% (v/v) acetic acid) for 4 min and then rinsed under running water to remove excess dye. The blue colour was intensified by bathing slides in Scott's tap water (24 mM sodium bicarbonate, 81 mM magnesium sulphate) for 2-3 min, followed by a further rinse under running water. To stain the cytoplasm pink/red, sections were bathed in eosin (1% (w/v) eosin, 2% (v/v) acetic acid) for 2 min and rinsed under running water. They were then dehydrated in increasing concentrations of ethanol (50%, 70%,

95% (30 s), 100% (30 s) and 100% (1 min)) before being cleared in 2 x 5 min washes of xylene. Coverslips were mounted onto the slides using DePeX (DPX) mounting agent (BDH Laboratory Supplies) and left to set. Stained sections were viewed under a binocular microscope (Olympus BH2; Olympus Optical Co., Inc.) and the key morphological features noted under both low and high magnification.

2.2.2.3 In situ end-labelling (ISEL) of apoptotic nuclei

ISEL was performed using the method of Ansari *et al.* (1993) with modifications suggested by Molenaar *et al.* (1996b). Slides were warmed at 55 °C for 30 min, then de-waxed in 2 x 10 min washes of xylene and hydrated in 100% ethanol (2 x 5 min), 95% ethanol (2 min), 70% ethanol (2 min) and Milli-Q (MQ) water (Millipore) (2 min). Tissue sections were permeabilised with 10 µg/ml proteinase K (Invitrogen) in 0.2 M Tris pH 7.2, 2 mM calcium chloride for 15 min at 37 °C and dehydrated in 2 x SSC (0.3 M sodium chloride, 30 mM sodium citrate) (5 min), 70% ethanol (2 min), 95% ethanol (2 min) and 100% ethanol (2 min). Air-dried sections were then outlined with PAP pen (Zymed Laboratories Inc.) to contain solutions during the subsequent ISEL reactions.

ISEL was performed directly on the slide in a 50 µl volume per section using the Klenow fragment of a DNA polymerase to incorporate digoxigenin-11-2'-deoxy-uridine-5'-triphosphate (alkali stable, DIG-11-dUTP; Roche Applied Science), into fragmented or damaged DNA, a characteristic of apoptosis. Each reaction consisted of; 1 U Klenow enzyme (labelling grade, Roche Applied Science), 0.2 mM dGTP, dCTP and dATP (PCR grade, Roche Applied Science), 20 µg/ml BSA (Molecular Biology Grade, Roche Applied Science), 1 mM dithiothreitol (DTT) (Invitrogen) and 1 µM DIG-11-dUTP in a final concentration of 1 x React 2 Buffer (100 mM Tris HCl pH 7.6, 10 mM magnesium chloride, 150 mM sodium chloride; Invitrogen). Klenow enzyme was omitted from the reactions of negative controls, which were performed concurrently on a serial section. The reactions were allowed to proceed for 2 h at 37 °C in a humidified chamber then rinsed several times in water, before blocking non-specific binding sites with blocking buffer (buffer 1; 100 mM Tris pH 7.5 and 150 mM sodium chloride, containing 2% blocking solution (Roche Applied Science)), for 1 h at 37 °C. The slides were washed with buffer 1 (3 x 5 min) then incubated with anti-Digoxigenin-

AP-Fab fragments (Roche Applied Science) diluted 500-fold in blocking buffer for 1 h at 37 °C. The slides were washed with buffer 1 (3 x 5 min), then buffer 3 (100 mM Tris pH 9.5, 100 mM sodium chloride, 50 mM magnesium chloride) before addition of the substrates 450 µg/ml 4-nitro-blue tetrazolium chloride (NBT) (Roche Applied Science) and 175 µg/ml 5-bromo-4-chloro-3-indolyl-phosphate, 4-toluidine salt (BCIP) (Roche Applied Science) in buffer 4 (100 mM Tris pH 9.5, 100 mM sodium chloride containing 1 mM levamisole (Sigma Chemical Company) to inhibit endogenous peroxidase activity). The alkaline phosphatase reaction was allowed to proceed for 1.5 h and then stopped by rinsing the sections with water followed by washes in 70% ethanol (2 min), 95% ethanol (30 min), 70% ethanol (2 min) and MQ water (2 min) to darken the ISEL signal from brown to blue/black. Sections were counterstained with Nuclear fast red (NFR) solution (0.1% (w/v) NFR, 7.5 mM aluminium sulphate) for 2-4 min and rinsed with water. They were then dehydrated in increasing concentrations of ethanol (70% (2 min), 95% (2 min), and 100% (2 x 2 min)) before being cleared in 2 x 2 min washes of xylene and sealed with a cover slip using DPX (BDH Laboratory Supplies).

Sections were examined "blind" under light microscopy (Olympus BH-2) and given a qualitative score based upon the overall number of positive ISEL nuclei, where 1 = none to low, 2 = low to moderate, 3 = moderate to high, 4 = high and 5 = very high. Ten randomly selected fields (100 x magnifications) were photographed per sample using a ProgRes C14 digital camera (JENOPTIK Laser) and Paint Shop Pro 7.02 software (Jasc Software Inc.). The numbers of ISEL nuclei and alveoli in each field were counted using the mark and count analysis tool in ImageJ (US National Institute of Health, http://rsb.info.nih.gov/nih-image). ISEL nuclei were identified as either located within the secretory epithelial layer or the lumen of mammary alveoli. Each count of ISEL nuclei per field was incremented by 1 (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.

The use of both qualitative and quantitative analyses provided independent, alternative, measures of the level of apoptosis in bovine and rat mammary sections. In all experimental conditions studied (refer to results sections 3.3.3.1, 3.3.3.2, 4.3.2.1 and 5.3.2.1) there were strong, highly significant, correlations between the quantitative and

qualitative datasets and similar results were obtained using either measurement. This helped to substantiate the results and indicated that either method was suitable. However, while the quantitative measures of the level of apoptosis were more accurate than the qualitative scores in that ten randomly selected fields were measured per section rather than one score for the whole section, and that the location (epithelial vs. luminal) of ISEL apoptotic nuclei were identified, they were far more labour intensive to perform. Therefore, qualitative measurements can be a reasonable alternative where highly accurate data are not required. For completeness, both qualitative and quantitative datasets are presented in this thesis.

2.2.3 RNA extraction from mammary tissue

During experimental procedures that involved isolating or working with RNA, precautions were undertaken to prevent undesired contamination with RNases that may have lead to RNA degradation. All solutions were prepared with DEPC-treated MQ water or treated with DEPC. To treat water or solutions, 0.1% (v/v) DEPC was added and mixed vigorously for 30 min, then autoclaved to destroy the DEPC. Solutions that could not be autoclaved were incubated at 65 °C overnight. RNase AWAY (Molecular BioProducts) was used to wipe down hard surfaces, while glassware was baked at 180 °C for 2 h.

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. A 150 mg aliquot of ground tissue was homogenised in 1.5 ml TRIzol using a mechanical homogeniser. Bovine tissue was homogenised with an Ultra Turrax T25 (Janke & Kunkel), and rat tissue with an Omni TH Tissue Homogeniser (Omni International). To remove insoluble material (extracellular membranes, polysaccharides and high molecular weight DNA), the homogenate was centrifuged at 10,000 x *g* for 10 min at 4 °C. The supernatant was collected avoiding the fat layer, mixed vigorously with 0.3 ml chloroform and incubated for 2-3 min at RmT. The mixture was centrifuged at 10,000 x *g* for 15 min at 4 °C to induce separation into three phases, with the RNA remaining exclusively in the top aqueous phase. The aqueous phase was transferred to a clean tube, mixed with 0.75 ml isopropanol and incubated for 10 min at RmT to precipitate the RNA. The RNA was pelleted by centrifugation at 10,000 x *g* for 10 min

at 4 °C and the supernatant removed. The resulting pellet was washed once in 75% ethanol, vortexed and spun at 8,000 x *g* for 5 min, aspirated and allowed to air dry for 10 min. Depending upon the size of the RNA pellet, it was resuspended in 100-300 µl of DEPC water, with solubilisation facilitated by incubation at 65 °C for 10 min. RNA was stored at -80 °C. The amount of RNA in each sample was measured by absorbance at 260 nm (A_{260}) using a spectrometer (BioPhotometer; Eppendorf) so that 1.0 absorbance unit = 40 µg/ml RNA. A ratio of the absorbance readings at 260 nm to 280 nm (A_{260}/A_{280}) of >1.6 to ~2.0 provided an estimate of RNA purity, with values below 1.6 indicating contamination with protein, DNA or poor solubilisation of the RNA sample.

2.2.3.1 Agarose/formaldehyde gel electrophoresis for RNA

The integrity of isolated RNA was verified by agarose/formaldehyde gel electrophoresis. Equivalent amounts of RNA (2.5 µg) were mixed with RNA loading buffer containing 50% (v/v) deionised formamide, 17.5% (v/v) deionised formaldehyde, 0.5 x MOPS buffer (10 mM 3-[N-morpholino]propane-sulphonic acid (MOPS), 4 mM sodium acetate anhydrous pH 7.0, 0.5 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0), 1.7% (v/v) gel loading dye (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 1 mM EDTA pH 8.0, 50% (v/v) glycerol), and 30 µg/ml ethidium bromide in a total volume of 12 µl. RNA samples were heat denatured at 65 °C for 15 min and incubated immediately on ice before electrophoresis in a 1.2% (w/v) agarose gel, containing 1.8% (v/v) formaldehyde and 1 x MOPS buffer (20 mM MOPS, 8 mM sodium acetate anhydrous pH 7.0, 1 mM EDTA pH 8.0). The gel was electrophoresed at constant voltage, 5-7.5 V/cm of gel width (i.e., 70-100 V), in running buffer (1.8% (v/v) formaldehyde, 1 x MOPS buffer) for approximately 1 hour. The ethidium bromide allowed the RNA bands to be observed under ultraviolet (UV) transillumination using a Gel Doc and Quantity One software (Bio-Rad Laboratories).

2.2.4 First strand cDNA synthesis

To avoid false positives arising from amplification of genomic DNA during reverse transcription-polymerase chain reaction (RT-PCR), RNA was treated with the DNaseI, Amplification Grade Kit (Invitrogen) following the manufacturer's instructions. Briefly, 1 μg RNA was incubated with 1 U DNaseI in 10 μl 1 x DNaseI reaction buffer at RmT for 15 min. RNA samples were then incubated with 1 μl 25 mM EDTA at 65 °C for 10 min to inactivate the DNase and rapidly chilled on ice for 10 min.

DNase-treated RNA samples were column-purified using a QIAGEN RNeasy Mini Kit (QIAGEN Sciences) in accordance with the manufacturer's instructions. Following the wash steps, RNA was eluted from the QIAGEN RNeasy column in 50 μl of DEPC water. The RNA was then precipitated by vigorous mixing with $^{1}/_{10}{}^{th}$ volume 3 M sodium acetate and 3 x total volume 100% ethanol and incubation for either 30 min at -70 °C or overnight at -20 °C. Samples were centrifuged at 10,000 x *g* for 15 min at 4 °C. The resulting RNA pellet was washed with 250 μl 75% ethanol and centrifuged at 10,000 x *g* for 10 min at 4 °C. The supernatant was removed and the pellet allowed to air dry for 5 min before resuspending in 9 μl DEPC water.

An aliquot of purified RNA (1 μl) was saved for use as the reverse transcriptase (RT)-negative control during real-time polymerase chain reaction (PCR). The remaining 8 μl of RNA were used to generate first strand cDNA with the SuperScript II Reverse Transcriptase First-Strand Synthesis System kit (Invitrogen). Briefly, 8 μl of purified RNA was denatured with 1 μl each of 10 mM dNTP mix and 0.5 μg/μl $Oligo(dT)_{12-18}$ primer at 65 °C for 5 min and then rapidly chilled on ice for 2-3 min. Master mixture (9 μl) prepared on ice and containing 2 μl 10 x RT buffer (200 mM Tris HCl pH 8.4, 500 mM potassium chloride), 4 μl 25 mM magnesium chloride, 2 μl 0.1 M DTT, and 1 μl 40 U/μl RNaseOUT RNase Inhibitor was added to the RNA and the sample heated to 42 °C for 2 min. One microlitre of 50 U/μl SuperScript II RT enzyme was added to each sample, making a total volume of 20 μl, and cDNA synthesis performed at 42 °C for 50 min. The reaction was terminated at 70 °C for 15 min and cDNA samples were immediately chilled on ice before storage at -20 °C. RT reactions yielded a maximum of 50 ng/μl of reversely transcribed RNA from a starting amount of 1 μg RNA per sample.

Prior to use as templates in PCR amplification, cDNA samples were diluted in TE (10 mM Tris pH 8.0, 1 mM EDTA pH 8.0).

2.2.5 Real-time RT-PCR

2.2.5.1 Oligonucleotide primers

Oligonucleotide primers to genes of interest were designed from either published nucleic acid sequences or from orthologous contiguous sequences (contig) constructed from expressed sequence tags (EST) in the AgResearch EST Database. Forward and reverse primers were designed, where possible, so that amplicons spanned intron sequences to prevent amplification of genomic DNA during real-time PCR. Custom forward and reverse primers were synthesised by Invitrogen (NZ) from sequences provided to them. The primers were reconstituted in 200 µl TE and stored at -20 °C. The concentration of each stock primer was quantified at A_{260} using a spectrometer (BioPhotometer; Eppendorf) so that 1.0 absorbance unit = 30 µg/ml of ssDNA. Primers were diluted in sterile MQ water to a working concentration of 10 µM. Forward and reverse primers were combined and diluted in MQ water to make primer pairs, which were optimised at a range of final concentrations between 50-600 nM for each real-time PCR reaction.

2.2.5.2 Real-time PCR amplification conditions

Quantitative real-time RT-PCR using the relative standard curve method with SYBR Green I Chemistry was carried out in either the ABI PRISM 7700 or 7900HT Sequence Detection System (Applied Biosystems) as directed by the manufacturer. Samples were assayed in duplicate, and RT-negative and 'no-template' (i.e., omission of the cDNA template) controls were included in each assay to detect false positives and background fluorescence, respectively. Each plate included an assay for an endogenous control gene as well as primers for target gene(s) of interest. The former was a housekeeping gene with constant expression (refer to Appendix IV), regardless of experimental treatments, and was used to normalise for the initial amount of cDNA present in each sample.

Standard curves of serial dilutions of mammary cDNA template were assayed for each primer pair, in order to calculate the relative amounts of each gene in cDNA samples.

Each real-time reaction (15 µl) contained 7.5 µl SYBR Green Master Mix (Applied Biosystems), 5.5 µl sterile deionised water, 1 µl cDNA template, and 1 µl primer pair. An initial denaturation step of 95 °C for 10 min was used to ensure complete denaturation of cDNA and to activate the hot-start AmpliTaq Gold polymerase (Applied Biosystems) present in the SYBR Green Master Mix (Applied Biosystems). Real-time PCR was then performed for 40 cycles of 95 °C for 15 s (denaturing), 56 °C for 30 s (annealing), 72 °C for 30 s (extending) and 78 °C for 10 s (measuring fluorescence). Fluorescence was measured at an elevated temperature after elongation to remove non-specific signals (e.g., primer dimers) and ensure that only specific PCR products were quantified. A further run of 95 °C for 15 s, then 60 °C for 15 s followed by a slow ramp up to 95 °C for 15 s was performed after the last cycle to provide a dissociation curve of the amplified products. Analysis of the melting temperature (T_m) of amplified products allowed for the detection of the target product as well as any non-specific products, including primer dimers which typically had a lower T_m of between ~70-75 °C (Fig. 2.2). Optimisation of cDNA template concentration, primer pair concentration, annealing temperature, and real-time PCR cycling conditions was performed in preliminary experiments for each gene of interest. This process determined the linear range of PCR efficiency, maximised the amplification of a single specific product and prevented primer dimer formation during the final PCR reactions.

Agarose gel electrophoresis (section 2.2.5.3) and sequencing of amplified products also confirmed the specificity of each real-time reaction. For sequencing, PCR products were firstly column-purified using the QIAGEN QIAquick PCR Purification Kit (QIAGEN Sciences) and eluted in 30 µl of T.1E (10 mM Tris pH 8.0, 0.1 mM EDTA pH 8.0) following the manufacturer's instructions. The concentration of PCR products was measured at A_{260} using a spectrometer (BioPhotometer; Eppendorf) so that 1.0 absorbance unit = 50 µg/ml dsDNA. PCR products were then either cloned into a plasmid vector (section 2.2.6), or submitted directly, for DNA sequencing (Waikato DNA Sequencing Facility). The DNA sequences of PCR products were aligned with

those of the sequences they were designed from to confirm homology (i.e., > 90% identical).

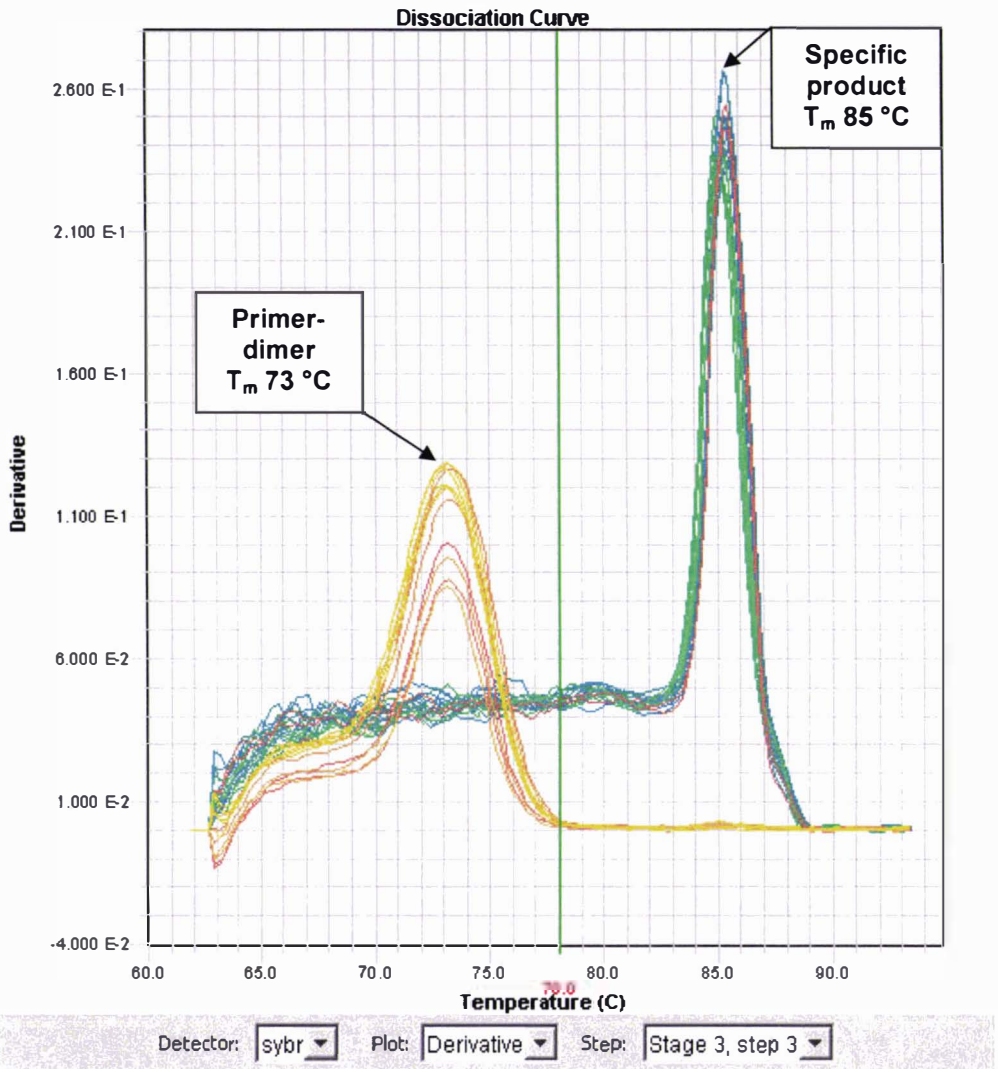


Figure 2.2. Example of results from a dissociation curve analysis.
The dissociation curve above shows a typical amplification peak of a specific polymerase chain reaction (PCR) product with a melting temperature (T_m) of 85 °C, while an example of a non-specific primer-dimer has a characteristically lower T_m of 73 °C. Wells containing both specific and non-specific products would have dual amplification peaks. Primer-dimer formation is most prevalent in no template control wells and sample wells containing low concentrations of the specific cDNA template. During a real-time PCR run, the fluorescence is measured at an elevated temperature (e.g., 78 °C) after elongation to ensure that only specific PCR products are quantified. Primer pair concentration, cDNA template concentration and real-time PCR cycling conditions are optimised for genes of interest in preliminary experiments to maximise amplification of a single specific product and prevent non-specific products, including primer dimer formation, in final experiments.

2.2.5.3 Agarose gel electrophoresis for DNA

DNA samples were visualised by electrophoresis on a 1% or 2% (w/v) agarose gel, containing 1 x TAE (40 mM Tris, 0.1% (v/v) glacial acetic acid, 1 mM EDTA pH 8.0) and 0.5 mg/l ethidium bromide. Wells were loaded with DNA (e.g., approx. 2 µl plasmid DNA or 5 µl PCR product) mixed with DNA loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 30% (v/v) glycerol) in 12 µl total volume. The gel was run at a constant 100 V in electrophoresis buffer (1 x TAE, 0.5 mg/l ethidium bromide) for approximately 1 h. The DNA was then visualised under UV transillumination using a Gel Doc and Quantity One software (Bio-Rad Laboratories) to confirm the size and number of bands present.

2.2.5.4 Analysis of real-time PCR data

Following real-time PCR, average threshold cycle (C_T) values for target and normaliser genes were generated for each sample. The C_T value was defined as the cycle number at which the fluorescence of each reaction crossed the threshold, which was placed above background fluorescence and within the log-linear phase of exponential product amplification. Therefore, the lower the C_T value the higher the amplification of PCR products. Relative quantification of gene expression was performed using the standard curve method as described in "User Bulletin #2: ABI PRISM 7700 Sequence Detection System" (Applied Biosystems, 2001). Briefly, amounts of target and endogenous control genes were calculated for each sample from standard curves of the amount of cDNA template (ng) plotted against C_T value. The amount of target gene in each sample was normalised by dividing by the amount of endogenous control gene and the resulting values $\log_{10}$-transformed before statistical analysis. Averages for each treatment group were back-transformed and results expressed as the fold change relative to a calibrator sample, usually the control group.

2.2.6 Cloning

2.2.6.1 pGEM-T Easy Vector

The pGEM-T Easy Vector (Promega; Fig 10.1 and 10.2, Appendix II) was selected as a vector for cloning PCR products as it has been specifically constructed to allow efficient ligation of adenosine-tailed amplicons, typically produced by *Taq* polymerase during PCR. The vector was linearised using *Eco*R V, and a thymidine added onto the 3' terminal ends by the manufacturer to provide an overhang complementary to the PCR product ends.

2.2.6.2 Ligation, transformation and screening

Approximately 15-20 ng purified PCR product (insert) was ligated with 50 ng pGEM-T Easy Vector (Promega) using 3 U of T4 DNA Ligase (Promega) and 2 x Rapid Ligation Buffer (Promega) in 10 µl total volume. The reaction mixture was incubated for 1 h at RmT (or overnight at 4 °C). Ligation products were visualised by agarose gel electrophoresis (section 2.2.5.3) before transformation into MAX Efficiency DH5α Competent Cells (Invitrogen), which have an efficiency of > 1 x 10^9 transformants/µg pUC19 DNA. Two microlitres of ligation mixture was added to 50 µl of cells, incubated on ice for 20 min and then heat shocked at 42 °C for 2 min, followed by a cold shock on ice for 2 min. The cells were then transferred to 950 µl of Terrific broth (TB) (4.7% (w/v) TB broth base; GIBCO Products, Invitrogen) on ice and brought to RmT before incubation for 1.5 h at 37 °C with shaking at 300 rpm. Three different volumes (i.e., 20, 200 and 780 µl) of the transformation culture were plated onto Luria broth (LB) agar plates (3.2% (w/v) LB agar powder; GIBCO Products, Invitrogen), containing 100 µg/ml ampicillin (Applichem), 80 µg/ml IPTG (GIBCO Products, Invitrogen) and 50 µg/ml X-Gal (GIBCO Products, Invitrogen), and incubated overnight at 37 °C.

Confirmation of a successful ligation was determined by colony growth comparisons between experimental plates and control plates that omitted ligase or insert DNA. Blue/white screening was used to identify recombinant clones, where white colonies

generally contain inserts due to interruption of the coding sequence of β-galactosidase in the pGEM-T Easy vector, which would otherwise produce blue colonies. To ensure subsequent isolation of a single colony of recombinant plasmid DNA, one blue colony, one pale blue colony and ten white colonies were restreaked onto LB agar plates containing 100 μg/ml ampicillin and incubated overnight at 37 °C. Eight of the resulting white colonies and one each of the blue and pale blue colonies were used to inoculate 5 ml LB broth (2% (w/v) LB broth base; GIBCO Products, Invitrogen), containing 100 μg/ml of ampicillin, each. These were allowed to grow overnight at 37 °C, with shaking at 300 rpm.

2.2.6.3 Alkaline lysis extraction of plasmid DNA

The recombinant plasmid DNA was extracted by alkaline lysis (Sambrook and Russell, 2001). Firstly, a 500 μl aliquot of each overnight culture was mixed with 75 μl glycerol, to produce a 15% solution, which was snap frozen in liquid nitrogen and stored at -80 °C until required. Then cells were harvested from the remaining culture by centrifuging at 3000 x *g* for 10 min. The supernatant was removed and the pellet resuspended in 100 μl of chilled solution I (25 mM Tris pH 8.0, 10 mM EDTA, 50 mM glucose, 0.1 mg/ml RNase A (Roche Applied Science)) and vortexed to ensure complete dispersion. The cells were mixed with 200 μl solution II (1% (w/v) SDS, 0.2 N NaOH), incubated for 5 min at RmT, then mixed with 150 μl of chilled solution III (3 M potassium, 5 M acetate) and incubated for a further 5 min on ice. The cell mixture was then centrifuged at 20,000 x *g* for 10 min to remove the cellular debris.

The DNA was extracted from the supernatant and purified by phenol chloroform extraction. Briefly, an equal volume of phenol:chloroform:isoamyl alcohol (IAA) (25:24:1, v/v) (Invitrogen) was added to the supernatant, the solution was mixed vigorously and centrifuged at 20,000 x *g* for 10 min. The upper aqueous phase was retained and the DNA precipitated by mixing with 2 volumes of 100% ethanol and centrifuging at 20,000 x *g* for 30 min. The resulting DNA pellet was washed in 1 ml 80% ethanol and air-dried for 10-15 min before resuspending the DNA in 50 μl TE.

The presence of the insert in plasmid DNA was confirmed by digestion with restriction endonucleases (Roche Applied Science). The insert was excised from the vector, or the insert and/or vector sequences were specifically cut so as to linearise the plasmid into 1 or more fragments of predicted lengths. A restriction digest included 4 µl (approx. 5-10 µg) purified plasmid DNA, 1 µl 10 x restriction enzyme buffer, 1 µl 10 x acetylated BSA (1 mg/ml; New England Biolabs) and between 5-10 U of each enzyme, made up to 10 µl total volume in MQ water. The digestion mixture was incubated at 37 °C for at least 1 h. Then 2 µl DNA loading dye was added to each sample, which was then analysed by agarose gel electrophoresis (section 2.2.5.3).

If RNase A was not included in solution I during cell lysis and/or RNA contamination was observed during agarose gel electrophoresis, the DNA was treated with the RNase Cocktail kit (0.125 U RNase A, 5 U RNase T1; Ambion) at 37 °C for 1 h. The DNA was then further purified prior to sequencing using phenol chloroform extraction and Phase Lock Gel Heavy columns (Eppendorf) following the manufacturer's instructions and ethanol re-precipitation. The DNA was finally resuspended in 30 µl DEPC water and submitted for sequencing (Waikato DNA Sequencing Facility) using primers for both T7 and SP6 DNA promoter sequences, as these sites flank the insertion region in the pGEM-T Easy vector (Fig. 10.1 and 10.2, Appendix II).

2.2.7 Protein extraction from mammary tissue

A 100 mg aliquot of ground tissue was homogenised using an Omni TH Tissue Homogeniser (Omni International) in 1 ml low salt buffer (10 mM 4-[2-hydroxyethyl]-1-piperazineethanesulphonic acid (HEPES) pH 7.9, 1.5 mM magnesium chloride, 10 mM potassium chloride) containing 1% (v/v) NP-40 detergent and protease inhibitors (1 mM sodium orthovanadate, 0.5 mM DTT, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 1 µg/ml aprotinin, 1 µg/ml p-aminobenzoyl-gly-pro-$_D$-leu-$_D$-ala hydroxamic acid (ABHA), 1 µg/ml pepstatin A, 1 µg/ml leupeptin; all from Sigma Chemical Company) and rotated for 30 min at 4 °C. A 100 µl aliquot was saved for analysis of the total homogenate fraction. The remaining homogenate was centrifuged at 10,000 x *g* for 30 min at 4 °C and the supernatant collected as the NP-40-soluble protein fraction. To collect the NP-40-insoluble protein fraction, the remaining pellet was resuspended by

sonication in 250 µl low salt buffer (containing 1% (v/v) NP-40 detergent, 1% (w/v) SDS and protease inhibitors) and rotated for 30 min at 4 °C. Samples were mixed with loading buffer (62.5 mM Tris pH 6.8, 2% (w/v) SDS, 5% (v/v) BME, 10% (v/v) glycerol), boiled for 5 min, and stored at -20 °C until required for subsequent western analysis (Laemmli, 1970). The amount of protein present in each sample was determined in a separate aliquot, prior to mixing in loading buffer, using the bicinchoninic acid (BCA) method as per the manufacturer's instructions (Sigma Chemical Company).

2.2.8 Western blot analysis

2.2.8.1 SDS-polyacrylamide gel electrophoresis (PAGE)

Discontinuous one-dimensional SDS-PAGE was performed according to Laemmli (1970). Briefly, polyacrylamide separating gels (80 x 50 x 1.5 mm) were prepared containing between 7-15% acrylamide-bis (consisting of 97.3% (w/v) acrylamide and 2.7% (w/v) N, N'-methylene-bis-acrylamide), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate, and 0.1% (v/v) TEMED in 0.375 M Tris pH 8.8. Stacking gels contained 4 % acrylamide-bis, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate, and 0.2% (v/v) TEMED in 0.125 M Tris pH 6.8. Stacking gels were loaded with up to 40 µg protein per well and electrophoresis was carried out in the Mini-PROTEAN II apparatus (Bio-Rad Laboratories) at 150 V in electrode buffer (0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS, pH 8.3) for approximately 1 h to separate protein samples. The gel was then either stained with Coomassie blue or used for western analysis (section 2.2.8.2).

Gels were stained in Coomassie blue solution (0.08% (w/v) Coomassie brilliant blue G-250, 1.6% (w/v) phosphoric acid, 8% (w/v) ammonium sulphate, 20% (v/v) methanol; Neuhoff *et al.*, 1988) for 18 h at RmT with gentle agitation. They were then de-stained in several changes of 25% (v/v) methanol until the optimal colour intensity was obtained. Stained gels were scanned using a GS-800 densitometer (Bio-Rad Laboratories) and Quantity One software (Bio-Rad Laboratories) and stored in 20%

(w/v) ammonium sulphate. The blue stained protein bands allowed assessment of the banding pattern between different samples and showed whether an even protein loading was obtained.

2.2.8.2 Immunoblotting

Separated proteins were electrophoretically transferred from SDS-PAGE gels onto BioTrace NT nitrocellulose membrane (Gelman Laboratory) using either a semi-dry or wet blotting system. Semi-dry transfers were carried out as described by McLaren *et al.* (1994) using a LKB MuiltiphorII Electrophoresis Unit (LKB-Produkter). Briefly, gels were equilibrated for 15 min in Tris-glycine transfer buffer (48 mM Tris, 39 mM glycine, 20% (v/v) methanol, pH 9.2). The semi-dry transfer was then performed at 35 mA per gel (0.8 mA/cm^2) for 1 h. Alternatively, the Trans-Blot Cell (Bio-Rad Laboratories) wet blotting system was used to transfer proteins in a solution containing 10 mM CAPS, 10% (v/v) methanol, pH 11 at 20 V overnight (or at 50 V for 2 h).

Following transfer, membranes were immersed in Ponceau S stain (0.1% (w/v) Ponceau S, 1% acetic acid) for 5 min and de-stained briefly in MQ water. They were then scanned using a GS-800 densitometer (Bio-Rad Laboratories) and Quantity One software (Bio-Rad Laboratories). Protein bands stained red, showing the effectiveness of the transfer and whether an equivalent protein loading was obtained. In some cases the transferred gel was stained concurrently with Coomassie Blue. This also allowed assessment of the effectiveness of the transfer.

Membranes were briefly rinsed in MQ water, and then TBST (0.05 M Tris, 0.15 M sodium chloride, 0.05% (v/v) Tween-20, pH 7.6), before blocking non-specific binding sites in blocking buffer (TBST containing 1% PVP-25, 0.1% BSA) for 2 h at RmT, with agitation. Blocked membranes were then probed with primary antibodies diluted in incubation buffer (TBST containing 0.1% PVP-25, 0.1% BSA) for 18 h at 4 °C. The concentration of the primary antibody was optimised by using half log serial dilutions (e.g., 1:1000, 1:3000, 1:10,000, 1:30,000, 1:100,000) in incubation buffer for each protein extraction. The protein loading (i.e., between 10-40 μg protein) was also optimised for selected primary antibody concentrations.

Membranes were then washed for 3 x 10 min in TBST at RmT with agitation, and exposed to goat anti-rabbit (GAR) secondary antibody conjugated to horseradish peroxidase (Sigma Chemical Company) at a dilution of 1:10,000 in incubation buffer for 2 h at RmT with agitation. After incubation, membranes were washed for 3 x 10 min in TBST and rinsed twice in TBS (0.05 M Tris, 0.15 M sodium chloride, pH 7.6) to remove the detergent. They were then immersed for 5 min in enhanced chemiluminescence (ECL) solution (20 mM Tris pH 8.6 containing 0.01% H_2O_2, 1.2 mM 5-amino-2,3-dihydro-1,4-phthalazinedione (Luminol; Sigma Chemical Company) diluted from a 0.5 M stock solution in DMSO, and 0.068 mM p-Coumaric acid (Sigma Chemical Company) diluted from a 0.17 M stock solution in DMSO) and the signal visualised by exposure to X Omat AR film (Eastman Kodak Company). Developed films were scanned and the density of immunoreactive bands quantified (within a linear range) using a GS-800 densitometer (Bio-Rad Laboratories) and Quantity One software (Bio-Rad Laboratories).

2.2.8.3 Stripping membranes

Stripping solution (0.05 M Tris, 2% (w/v) SDS, 0.7% (v/v) BME, pH 7.6) was pre-heated to 50 °C for 30 min. Membranes were submerged in the warmed stripping solution in a closed container and incubated for 30 min at 50 °C with gentle agitation. Membranes were then washed for at least 3 x 10 min in large volumes of TBST. If required, some membranes were checked for residual antibody binding by incubating with ECL solution and exposing to film. They were then washed for a further 30 min in TBST with several changes of wash buffer. Membranes were subsequently either re-used for immunoblotting, or stored dry and enclosed in plastic at RmT.

2.2.9 Immunohistochemistry

Immunohistochemistry was performed on mammary tissue slides that had been prepared as described in section 2.2.2.1. Slides were heated at 55 °C for 30 min, then de-waxed in 3 x 5 min washes of xylene and hydrated in 100% ethanol (2 x 5 min), 90% ethanol (5 min), and 70% ethanol (5 min) followed by a 5 min wash in TBS (0.1 M Tris,

0.19 M sodium chloride, pH 7.5). It was then necessary to subject the sections to antigen retrieval to expose proteins and allow antibody detection. This was achieved by heating the slides to 90-95 °C in 10 mM sodium citrate for 30 min. After cooling for 20 min, each section was outlined using a PAP pen (Zymed Laboratories Inc.) to contain solutions on the section during subsequent incubations. Slides were then rinsed for 3 x 10 min in TBS and placed on racks in an air-tight container lined with moist paper towels. Sections were overlaid with 3% H_2O_2 in TBS for 30 min to inactivate endogenous peroxidase activity followed by 3 x 10 min washes in TBS.

Avidin and biotin binding sites were blocked for 15 min each using the DAKO pre-mixed kit (Dako Cytomation) with 10 min washes in TBS containing 0.1% Tween-20 (TBST) between each block. Any remaining non-specific binding sites were blocked with normal swine serum (Dako Cytomation; 1:10 dilution in incubation buffer, i.e., TBST containing 0.1% BSA) for 2 h at RmT. Excess serum was removed by washing the slides for 3 x 10 min in incubation buffer before probing with primary antibodies (diluted in incubation buffer containing 1.5% normal swine serum) overnight at 4 °C. The concentration of primary antibody was optimised for bovine and rat mammary sections using half-log serial dilutions (e.g., 1:100, 1:300, 1:1000, 1:3000) in preliminary experiments. Incubations in diluent alone (i.e., no primary antibody), or in rabbit immunoglobulin G (IgG; Sigma Chemical Company) at the same molar concentration as the primary antibody, were used as negative controls. After incubating, the slides were rinsed for 3 x 10 min in TBST before being exposed to biotinylated GAR (Biogenex) for 1 h at a dilution of 1:40 in incubation buffer. After a 10 min rinse in TBST, slides were incubated in strepavidin-conjugated horseradish peroxidase (Biogenex) for 1 h at a dilution of 1:40 in incubation buffer. Slides were again washed for 10 min in TBST, then for 2 x 10 min in TBS and finally for 3 x 10 min in 0.175 M sodium acetate pH 7.7 before detection using DAB (0.175 M sodium acetate pH 7.7 containing 0.5 mg/ml DAB, 0.01% H_2O_2, 25 mg/ml nickel sulphate) for up to 30 min.

To preserve the image, slides were lightly counterstained in 0.5% (w/v) eosin, 2% (v/v) acetic acid for 2-3 min and then rinsed in 70% ethanol until optimal colour was achieved. Slides were then dehydrated in 90% ethanol (5 min) and 100% ethanol (2 x 5 min) before being cleared in 2 x 5 min washes of xylene. Coverslips were mounted onto

the slides using DPX (BDH Laboratory Supplies) and left to set. Sections were viewed under a binocular microscope (Olympus BH2) and the detection signal compared with negative controls and serial H&E stained sections. Photographs were taken using a ProgRes C14 digital camera (JENOPTIK Laser) and Paint Shop Pro 7.02 software (Jasc Software Inc.).

CHAPTER THREE

Tight junction protein expression and apoptosis in engorged bovine mammary glands

3.1 INTRODUCTION

TJs (zonula occludens) are the most apical of the junctional complexes (e.g., gap, adherens and desmosomes) found between adjacent epithelial cells. These junctional complexes facilitate cell-cell contact and communication, and play a critical role in maintaining cell survival and function. In the mammary gland TJs surround each cell, forming gasket-like seals to restrict the paracellular movement of ions and small molecules between interstitial fluid and milk ('barrier function'). In addition, the TJ defines cell polarity (and vectorial milk secretion) by separating the plasma membrane into apical and basolateral domains of distinct protein and lipid composition ('fence function'; Schneeberger & Lynch, 1992). In the past decade, much insight has been gained into the molecular basis of these properties in epithelia with the discovery of a growing list of proteins which are either direct components of the TJ or localise to the junctional complex. Occludin (Furuse *et al.*, 1993) and claudins (Furuse *et al.*, 1998; Morita *et al.*, 1999; Tsukita & Furuse, 1999) are integral transmembrane TJ proteins that bind to the cytoplasmic TJ proteins ZO-1, ZO-2 and ZO-3, providing the TJ with structural and signalling links to the cell interior (Furuse *et al.*, 1994; Haskins *et al.*, 1998; Itoh *et al.*, 1999a & b). Importantly, ZO-1 links occludin and claudins to the actin cytoskeleton (Fanning *et al.*, 1998; Itoh *et al.*, 1999a) and is involved in signal transduction, regulation of gene expression, suppression of proliferation and stimulation of differentiation (Yamamoto *et al.*, 1997; Balda & Matter, 2000; Meyer *et al.*, 2002; Matter & Balda, 2003).

In accordance with a role in cell functioning, mammary TJs are formed during the onset of copious milk secretion (or lactogenesis stage II) around parturition, when the gland switches from a developing, non-lactating state to a differentiated, lactating state (Linzell & Peaker, 1973, 1974). The same hormonal milieu required for lactogenesis,

progesterone withdrawal and the presence of glucocorticoids and prolactin, is required for TJ closure at this time (Nguyen & Neville, 1998; Nguyen *et al.*, 2001). Furthermore, increased expression of occludin and ZO-1 proteins during TJ formation in response to glucocorticoids and/or prolactin have been reported in mouse mammary cells *in vitro* (Singer *et al.*, 1994; Stelwagen *et al.*, 1999), indicating a role for these proteins in the mammary gland *in vivo.*

In support of a close relationship between TJ function and milk secretion, loss of TJ integrity is well documented during conditions which cause a sudden decrease in the rate of milk removal from the mammary gland (Stelwagen *et al.*, 1994a & b, 1997; Delamaire & Guinard-Flament, 2006b), or complete cessation of milk removal leading to involution (Fleet & Peaker, 1978). These extended periods of milk accumulation result in a decline in milk secretion rate coinciding with the rise in TJ permeability after approximately 18 h in dairy cattle (Stelwagen *et al.*, 1997) and 21 h in goats (Stelwagen *et al.*, 1994b), as assessed by the start of an exponential increase in blood lactose. Only a small proportion of the reduction in milk yield can be accounted for by milk 'leakage' into blood, indicating that milk secretion rate is actively reduced (Stelwagen *et al.*, 1997). The exact mechanism through which leaky TJs affect milk production remains unclear, although a direct link is suggested by experiments showing that milk secretion was reduced by disrupting mammary TJs using the calcium chelators, EGTA (Stelwagen *et al.*, 1995) or citrate (Neville & Peaker, 1981).

The changes in TJ permeability and milk secretion associated with milk accumulation and increased milking intervals are initiated by a local intra-mammary mechanism. This was illustrated by experiments whereby a unilateral increase or decrease in milking frequency resulted in changes in milk yield in that gland only (Linzell & Peaker, 1971b; Henderson *et al.*, 1983; Stelwagen & Knight, 1997). Furthermore, unilateral cessation of milk removal in ruminants (Quarrie *et al.*, 1994), or teat-sealing in rodents (Quarrie *et al.*, 1996; Li *et al.*, 1997a; Marti *et al.*, 1997), induced mammary engorgement and involution in the treated glands only. Therefore, accumulation of milk within alveolar lumens triggers the first stage of involution, which is characterised by loss of TJ integrity (Stelwagen, 2001), reduced milk synthesis and secretion, progressive gain of death signals and loss of survival factors leading to the induction of apoptosis (Strange

et al., 1992; Lund *et al.*, 1996; Li *et al.*, 1997a). These changes are reversible in that lactation can be re-initiated upon milk removal (Jaggi *et al.*, 1996; McMahon *et al.*, 2004, Appendix VII). However, the identity of the signal initiating this process is unknown.

The accumulation in milk of local chemical regulatory factors has been postulated to control mammary function (Linzell & Peaker, 1971b; Henderson & Peaker, 1984, 1987). In support of this mechanism, a negative feedback inhibitor (FIL) found in milk decreases milk secretion both *in vitro* and *in vivo* (Wilde *et al.*, 1987a, 1988, 1995), although its exact mode of action is still uncertain. Moreover, FIL does not alter milk composition (Wilde *et al.*, 1995), suggesting that it does not open TJs. More recently, local synthesis of serotonin by the mammary epithelium has been implicated in a negative feedback, autocrine-paracrine loop that opposes endocrine stimulation of milk production and secretion in lactating mice (Matsuda *et al.*, 2004). Other studies have reported that intra-mammary injections of casein phosphopeptides, containing fractions derived from plasmin protease activity, locally disrupted mammary TJ integrity and reduced milk secretion in goats (Shamay *et al.*, 2002) and cows (Shamay *et al.*, 2003). Although repeated doses of the casein-derived phosphopeptides caused rapid irreversible cessation of milk secretion (Shamay *et al.*, 2002, 2003), the precise mechanism by which this occurred and its effect on mammary apoptosis are, as yet, unknown.

An alternative explanation to chemical regulation is that the flattening in cell shape (Richardson, 1947), as the gland becomes engorged with milk, may initiate signalling events (mechanotransduction) which culminate in disrupted TJ function, reduced milk secretion and induction of apoptotic pathways (Stelwagen *et al.*, 1997; Davis *et al.*, 1999; Stelwagen, 2001). Two distinct, but possibly related, mechanisms may be influenced by physical distension of the mammary epithelium. Firstly, TJ and adherens junctions may become stretched or lose integrity, inducing pro-apoptotic signals. Breakdown of specialised cell-cell junctions is the first visible stage of MEC apoptosis during mammary involution prior to cytoplasmic and nuclear condensation (Walker *et al.*, 1989; Strange *et al.*, 1992; Tenniswood *et al.*, 1992), and truncation of the adherens junction protein E-cadherin (Vallorosi *et al.*, 2000), or the TJ protein occludin (Beeman

& Neville, 2001), induces apoptosis both *in vitro* and *in vivo*. Furthermore, the loss of TJ integrity may result in pro-apoptotic factors relocating from the apical to the basolateral side of MECs to either trigger apoptosis directly or inhibit survival factors (Green & Streuli, 2004). The second system potentially affected by cell shape results from mechanical tension on focal adhesion complexes which link MECs with their basement membrane. Cell-ECM interactions mediated by integrins are essential for MEC survival, control of milk protein mRNA expression and maintenance of differentiated state (Boudreau *et al.*, 1995; Streuli *et al.*, 1995; Pullan *et al.*, 1996; Faraldo *et al.*, 1998, 2002; Naylor *et al.*, 2005). Concurrent studies within our group have recently reported that mammary engorgement results in loss of cell-ECM survival signalling through β1-integrin and induction of pro-apoptotic markers (McMahon *et al.*, 2004, Appendix VII; Singh *et al.*, 2004a; 2005, Appendix VI). Furthermore, control of cell-cell and cell-ECM signalling during mammary engorgement may be related via a common link to the intracellular cytoskeleton, which plays a vital role during milk secretion (Patton, 1976a & b; Guerin & Loizzi, 1978; Nickerson *et al.*, 1980).

Mechanisms regulating the loss of cell-cell integrity are likely to affect the function and composition of molecules present in the TJ complex. A down-regulation of TJ proteins may occur during milk accumulation in a mechanotransduction cascade that initiates increased TJ permeability. The objective of the present study was, therefore, to investigate the effect of mammary engorgement on the expression of the major TJ proteins occludin, claudin-1 and ZO-1, in relation to the decline in secretory activity and the induction of apoptosis in bovine mammary glands. The first model of mammary engorgement examined the abrupt cessation of milk removal during peak lactation, and a second group of tissues was available to study the effect of short-term unilateral ODM in late lactation.

3.2 MATERIALS AND METHODS

3.2.1 Animals and tissue collection protocols

All tissues and raw milk production data were kindly provided by the Dairy Science and Technology Group, AgResearch Ruakura using the following collection protocols.

3.2.1.1 Experiment 1 – Time course following the abrupt cessation of milking in mid-lactation

Primiparous, NZ Friesian, dairy heifers were slaughtered to provide tissues for analysis. The heifers grazed rye-grass/white clover pasture and were milked twice-daily from parturition. In mid-lactation (mean 89.1 ± 2.2 days in milk), forty-two, non-pregnant animals were allocated at random to seven groups balanced for milk yield and milking ceased after two consecutive 12 h intervals. Animals were slaughtered (between 1000 h and 1100 h) at 0, 6, 12, 18, 24, 36 and 72 h (n = 6 cows per time point) following the last milking and mammary tissue was collected post-mortem. Large variations in the rate of loss of milk protein (α-lactalbumin and α-S1-casein) mRNA expression were observed between animals by 36 to 72 h post-milking (Molenaar *et al.*, 2004). Therefore, further mammary tissue samples were collected from non-pregnant NZ Friesian dairy heifers slaughtered either 72 h (n=4) or 192 h (i.e., 8 d) (n=6) following the last morning milking in mid-lactation (mean ± SEM, 116.9 ± 6.0 days in milk). A summary of milk production data combined across both tissue collections is provided in Table 3.1.

Table 3.1. Average (± SEM) milk yield, days in milk and somatic cell count (SCC) for NZ Friesian dairy heifers prior to the abrupt cessation of milking in mid-lactation.

	Milk yield (kg/cow/day)	Days in milk	SCC (x 1000 cells/ml)
Means (± SEM)	14.3 ± 0.3	94 ± 3	165 ± 30

3.2.1.2 Experiment 2 – Short-term unilateral ODM in late lactation

Four, non-pregnant, multiparous, Jersey or Jersey x Friesian dairy cows (average age ± SEM, 5.3 ± 0.5 years) in late lactation (mean ± SEM, 12.5 ± 2.1 kg milk/cow/day; 185.5 ± 20.6 days in milk; mean SCC ± SEM, 110 ± 42 x 1000 cells/ml) were used in this study. These animals were part of a larger trial and had been immunised to produce immunoglobulin A (IgA) in their milk, according to Patent WO98/54226 (Dr. Colin Prosser, Dairy Science and Technology Group, AgResearch Ruakura). The left and right udder halves of each cow were randomly assigned to two milking frequencies; either TDM at 0700 and 1500 h (control) or ODM at 0700 h only, for 4 days. Animals were not milked on the fourth morning and were killed for mammary tissue collection at approximately 26 h and 18 h after the final milking for ODM and TDM udder halves, respectively. Milk yields were measured separately for each udder half at day 0 of a control period (i.e., 1 d before the treatment period) where both glands were milked twice a day to determine pre-treatment differences. During the treatment period, unilateral milk yields were measured on day 3 for each ODM and TDM udder half.

All experimental procedures were approved by the Ruakura Animal Ethics Committee under the following numbers; RAEC #3700 (experiment 1; 0 to 72 h collection), RAEC #4328 (experiment 1; extra 72 h and 8 d collection) and RAEC #4264 (experiment 2). Animals were slaughtered at the Ruakura Abattoir using standard commercial methods (i.e., electrical stunning followed by exsanguination). Alveolar mammary tissue was collected post-mortem and immediately frozen on liquid nitrogen before storage at -80 °C as outlined in section 2.2.1.1. A further sample was fixed for 18-24 h in either 4% paraformaldehyde (experiment 1) or 10% formalin (experiment 2) for histological analyses as described in section 2.2.1.1.

3.2.2 Histological analysis

Formalin-fixed tissue slices were processed through a range of alcohol washes and then embedded in paraffin wax before mounting onto slides and staining with H&E for histological analyses (see sections 2.2.2.1 and 2.2.2.2). Experiment 1 H&E sections were professionally cut, mounted and stained by Mr. Basil Young, Hamilton, NZ. The

histological features were noted for each slide and for experiment 1, changes in morphology were related to relative changes in α-lactalbumin mRNA expression during mammary engorgement (Molenaar *et al.*, 2004; Singh *et al.*, 2004b). A representative area of each section was photographed under 25 x and either 200 x (experiment 1) or 100 x (experiment 2) magnifications using a ProgRes C14 digital camera (JENOPTIK Laser) and Paint Shop Pro 7.02 software (Jasc Software Inc.).

3.2.3 ISEL of apoptotic nuclei

For experiment 1, ISEL was performed on mammary sections from 3 representative cows from each of the early time points (0, 6, 18, and 24 h post-milking) and 6 representative cows from each of the later time points (36, 72 and 192 h post-milking) as described in section 2.2.2.3. For experiment 2, mammary sections from each cow (n=8) were examined for apoptosis using ISEL.

Qualitative and quantitative analysis of the number of positive ISEL nuclei was carried out for each section as described in section 2.2.2.3. Briefly, ISEL nuclei were identified as either located within the secretory epithelial layer or the lumen of mammary alveoli. Each count of ISEL apoptotic nuclei per 100 x magnification field was incremented by 1 (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 apoptotic nuclei per alveolus.

3.2.4 Real-time RT-PCR

Total RNA was extracted from a 150 mg aliquot of ground alveolar mammary tissue using TRIzol (Invitrogen) and 1 μg was treated with 1 U DNaseI (Invitrogen) (sections 2.2.3 and 2.2.4). Samples were purified through RNeasy gel columns (QIAGEN Sciences) and converted to cDNA using the SuperScript II Reverse Transcriptase First-Strand Synthesis System kit (Invitrogen) (section 2.2.4). cDNA products were diluted 5-fold in TE (equivalent to approximately 10 ng/μl reversely transcribed total RNA) and then samples (1 μl) were assayed in duplicate, by quantitative real-time RT-PCR with

SYBR Green I Chemistry using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as described in section 2.2.5. For experiment 1, cDNA samples were randomly allocated to one of three 96-well optical plates (Applied Biosystems) so that each time point was represented evenly across the three plates. For experiment 2, cDNA samples were assayed on one plate only. Each plate included an assay for an endogenous control gene (ubiquitin) as well as primers for the target gene of interest (i.e., occludin, claudin-1 or ZO-1). Ubiquitin was selected as a more suitable endogenous control than β-actin during bovine mammary engorgement, as described in section 12.1.1.1, Appendix IV. The sequences of the primer sets and other relevant details are outlined in Table 3.2. Primers were used at a final concentration of 300 nM, except for ZO-1 primers which were used at 100 nM, in each 15 µl real-time reaction. A standard curve of serial dilutions of bovine mammary cDNA template, as well as RT-negative and 'no-template' control reactions were included on each plate for each primer pair.

Real-time PCR was performed under the following conditions: 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, 56 °C for 30 s, 72 °C for 30 s and 78 °C (except for claudin-1 detection at 83 °C) for 10 s. The C_T values generated for each real-time PCR reaction were used to quantify the relative abundance of each gene using the relative standard curve method (Applied Biosystems) as described in section 2.2.5.4. Real-time PCR efficiencies (E) for each primer pair were determined from the slopes of relative standard curves according to the equation: $E = 10^{(-1/slope)}$. The maximal efficiency of PCR is E = 2 where every PCR product is replicated every cycle and the minimal value is E = 1, corresponding to no replication. The efficiency rates per cycle for investigated genes were: occludin (E = 1.81), claudin-1 (E = 1.89), ZO-1 (E = 1.65), β-actin (E = 1.86) and ubiquitin (E = 1.86). Dissociation curve analysis and gel electrophoresis (section 2.2.5.3) of amplified products confirmed the specificity of real-time reactions. PCR products were then QIAquick column-purified (QIAGEN Sciences) and either submitted directly (ubiquitin), or firstly cloned into the pGEM-T Easy vector (claudin-1, occludin, β-actin and ZO-1) as detailed in section 2.2.6, for DNA sequencing which verified their authenticity (Waikato DNA Sequencing Facility; results not shown).

Table 3.2. Sequences of PCR primers (forward and reverse), primer position and PCR product sizes of bovine nucleic acid sequences used for investigating gene expression by real-time RT-PCR.

Gene	Nucleic acid sequence[1]	Primer	Primer sequence[2] (5'→3')	Primer position[3] (bp)	Product size (bp)
β-actin	Genbank accession no. K00622	Forward	CGC ACC ACT GGC ATT GTC AT	211	206
		Reverse	TTC TCC TTG ATG TCA CGC AC	416	
Ubiquitin	Genbank accession no. Z18245	Forward	GGC AAG ACC ATC ACC CTG GAA	798	201
		Reverse	GCC ACC CCT CAG ACG AAG GA	998	
Occludin	Contig	Forward	GAC CTG ATG AAT TCA AAC CTA ATC	170	183
		Reverse	CGA TAC CAA GCA TAG ACA GGA T	352	
Claudin-1	Contig	Forward	GCT AGT GAC AAC ATC GTG AC	342	395
		Reverse	ATG AAG AGA GCC TGA CCA AA	736	
ZO-1	Contig	Forward	GAT TCA CAC CAA AAC CAT ACA C	23	303
		Reverse	CGT CTT CGT CTT CAT CTT CCT	325	

[1] Nucleic acid sequences for bovine occludin, claudin-1 and ZO-1 were not publicly available when these primers were designed. Therefore, bovine contiguous sequences (contigs) significantly similar (i.e., orthologous) to published human and rat sequences were obtained from the AgResearch EST Database and are presented in Appendix III.

[2] Bovine β-actin primer sequences were kindly provided by Dr. Rita Lee (AgResearch Ruakura). Primer sequences for detection of ubiquitin and occludin were designed using the VectorNTI Suite 7 software package (InforMax Inc.), while the GCG Wisconsin Package (Version 10.3; Accelrys Inc) was used for claudin-1 and ZO-1.

[3] Refers to the 5' position of the primers in the nucleic acid sequence.

3.2.5 Western immunoblotting

Protein extracts were prepared from aliquots of ground mammary tissue as described in section 2.2.7, and proteins separated by electrophoresis on either 8%, 15% or 7% SDS-PAGE gels for occludin, claudin-1 or ZO-1 detection, respectively (refer to section 2.2.8.1). For experiment 1, samples were randomly allocated to one of six gels so that each time point was represented once on each gel. Samples from experiment 2 (n=8) were loaded on the same SDS-PAGE gel for each western analysis. Separated proteins were then transferred, using either semi-dry (occludin and claudin-1 detection) or wet (ZO-1 detection) blotting systems, onto nitrocellulose membranes and probed with primary antibodies to rabbit anti-human occludin, claudin-1 and ZO-1 (obtained from

Zymed Laboratories Inc.) (section 2.2.8.2). 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).

3.2.6 Immunohistochemistry

Immunohistochemistry with primary antibodies to rabbit anti-human occludin (1: 1000 dilutions; Zymed Laboratories Inc.) was performed on representative bovine mammary sections as described in section 2.2.9.

3.2.7 Data and statistical analyses

Differences between means were analysed by ANOVA in GenStat (releases 6.1, 7.1 and 8.1; Lawes Agricultural Trust, 2002, 2003 and 2005). For experiment 2, treatment differences between unilateral ODM and TDM glands were analysed within cow. 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 100 x magnification field. Relative quantification of mRNA expression following real-time PCR was performed using the standard curve method (Applied Biosystems) as described in section 2.2.5.4. The amount of target gene in each sample was normalised to the amount of ubiquitin to control for the initial concentration of cDNA, and the resulting values $\log_{10}$-transformed for statistical analysis. Densitometry results from western blotting were also $\log_{10}$-transformed and for experiment 1, adjusted for between gel variations. The levels of mRNA and protein expression were then expressed as back-transformed mean fold changes relative to the 6 h time point mean (experiment 1) or the TDM mean (experiment 2). In experiment 1, 6 h post-milking was designated as the calibrator sample as it was the most representative of actively lactating mammary glands. The 6 h time point was used in preference to the 0 h time point because 0 h represents mammary tissue taken after a 12 h milking interval (i.e. the regular milking interval immediately prior to the start of the experiment was 12 h) whereas the 6 h sample was the sample taken after the shortest milking interval. Data are presented as the means at each time point post-milking (experiment

1), and the means for ODM and TDM glands (experiment 2), with either the standard error of the mean (SEM) or the standard error of the difference (SED) between means. The least significant differences identify the means significantly different from each other (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

Correlations between the qualitative and quantitative scores of ISEL apoptotic nuclei and with the mRNA expression of milk proteins and immune-associated genes were performed in Minitab (Minitab Release 14.20, 2005; Minitab Inc., USA). The dataset for the mRNA expression of milk protein (α-lactalbumin and α-S1-casein) and immune-associated genes (mammary serum amyloid A3 (MSAA) and lactoferrin) during bovine mammary engorgement (i.e., experiment 1) was determined by real-time RT-PCR and was kindly supplied by Dr. Kuljeet Singh (Dairy Science and Technology Group, AgResearch). A further dataset, determined by northern blot analysis, was kindly supplied by Dr. Adrian Molenaar (Dairy Science and Technology Group, AgResearch) with correlations presented in Appendix V.

3.3 RESULTS

3.3.1 Milk production

3.3.1.1 Experiment 2 – Decreases in milk yield during short-term ODM

The udder halves of mixed-age dairy cows in late lactation were milked unilaterally either once- or twice-daily for 4 days, with individual cow and mean milk yield data shown in Table 3.3. The total daily milk yield during TDM prior to the treatment period was strongly negatively correlated with days in milk ($r = -0.98$, $P<0.05$). An average milk yield loss of 1.4 ± 0.4 kg (24%) was observed between control and treatment periods for glands milked once-daily for 4 days ($P=0.05$). No significant changes in milk yield were observed for TDM glands after 4 days. Milk yields, adjusted for pre-treatment TDM differences between udder halves, were decreased by an average 1.5 kg ± 0.5 kg (24%) in ODM compared with TDM glands ($P=0.05$). The small number of replicates used in this experiment do not allow for significant associations to be detected between the milk yield loss under ODM and prior daily milk yield under TDM or days in milk (results not shown). However, there tended to be greater milk yield losses with ODM for glands with higher milk yields during TDM ($r = -0.87$, $P=0.135$).

Table 3.3. Daily milk yields and milk yield losses for dairy cows during short-term, unilateral, once-daily milking (ODM) in late lactation.
Udder halves within each cow were randomly allocated to two groups, either ODM or twice-daily milking (TDM) for 4 days in late lactation with milk yields measured on day 3 of this treatment period. During the control period (day 0) both ODM and TDM glands were milked twice a day to determine pre-treatment differences.

Cow	Breed[1]	Days in milk[2]	Day 0 Control Period		Day 3 Treatment Period	Difference between control and treatment periods (kg and %)		Milk yield loss under ODM (kg and %)[3]
			Total daily milk yield (kg)	Unilateral milk yield ODM vs TDM glands (kg and %)	Unilateral milk yield ODM vs TDM glands (kg and %)	ODM glands	TDM glands	
1	J	157	14.2	7.2 vs 7.0 (3%)	5.4 vs 6.2 (-13%)	-1.8 (-25%)	-0.8 (-11%)	-1.0 (-14%)
2	J x F	213	10.0	4.4 vs 5.6 (-21%)	4.1 vs 5.7 (-28%)	-0.3 (-7%)	0.1 (2%)	-0.4 (-8%)
3	F x J	144	17.6	6.9 vs 10.7 (-36%)	4.6 vs 10.9 (-58%)	-2.3 (-33%)	0.2 (2%)	-2.5 (-28%)
4	J	228	8.3	4.3 vs 4.0 (8%)	3.2 vs 4.9 (-35%)	-1.1 (-26%)	0.9 (23%)	-2.0 (-48%)
Mean (± SEM)		**185.5 ± 20.6**	**12.5 ± 2.1**	**5.7 ± 0.8 vs 6.8 ± 1.4 (-16%)**	**4.3 ± 0.5 vs 6.9 ± 1.4 (-38%)**	**-1.4 ± 0.4 (-24%)**	**0.1 ± 0.4 (1%)**	**-1.5 ± 0.5 (-24%)**
SED (P-value)				**0.96 (P=0.324)**	**1.25 (P=0.129)**	**0.43 (P=0.051)**	**0.35 (P=0.793)**	**0.48 (P=0.053)**

[1] J = Jersey, F = Friesian
[2] Days in milk from calving to slaughter
[3] Absolute (kg) decreases in milk yield from ODM glands compared with TDM glands during the treatment period corrected for pre-treatment (i.e. control period) differences. Relative milk yield losses are expressed as a percentage (%) of the mean unilateral daily milk yield during the control period.

3.3.2 Histological morphology

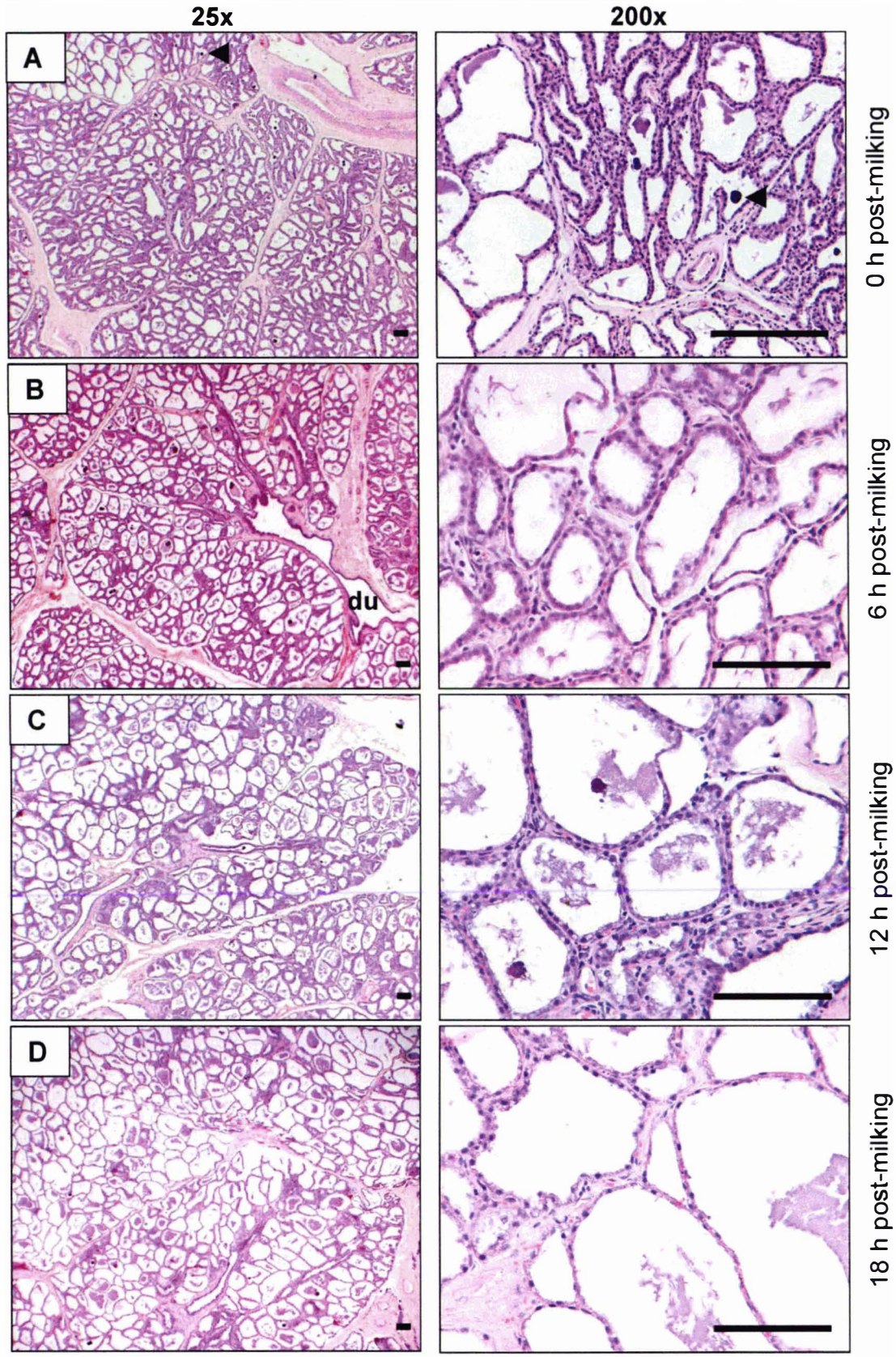
3.3.2.1 Experiment 1 – Time course of morphological changes post-milking

Representative alveolar tissue sections following the abrupt cessation of milking in mid-lactation are shown in Figure 3.1. Mammary tissue that was collected immediately following the last morning milking (0 h post-milking) consisted of small to moderately sized alveoli that were relaxed with an irregular-shaped edge, indicative of recent milk removal (Fig. 3.1A). An occasional lobule contained large distended (or stretched) alveoli suggesting that they had not been milked out. A single layer of cuboidal MECs lined the alveolar lumen, except for those in distended lobules, where cells were flattened and stretched laterally. There was little milk staining within alveolar lumina and the occasional amyloid body was present.

The 6 h time point was the most representative of actively lactating mammary tissue. It was characterised by large uniform areas of moderately-sized open alveoli surrounded by cuboidal MECs, and with only a small amount of inter-alveolar stromal tissue visible (Fig. 3.1B). At a high magnification, it was observed that many small vesicles were associated with the apical side of secretory MECs in the alveolar lumen. By 12 h and 18 h, milk was accumulated in the alveolar lumen which resulted in larger and engorged alveoli with stretched, flattened MECs and the presence of large milk vesicles (i.e., coalescing milk fat globules and proteins) within some lumina (Fig. 3.1C and D). A decreased number of secretory vesicles were associated with the apical side of cells with a flattened morphology compared to those with a cuboidal shape. However, while most alveoli were still large with a stretched morphology at 24 h post-milking (Fig. 3.1E), a few localised, collapsed areas were apparent. These were characterised by smaller alveoli with a "ruffled" appearance due to vesicle accumulation within the epithelial cells.

Although there was very little difference in the morphology of the tissue between cows at the early time points, considerable variation in the degree of involution was evident between animals by 36 h (Fig. 3.1F, G and H). Samples with low relative α-lactalbumin

mRNA expression (as identified by Molenaar *et al.*, 2004 and Singh *et al.*, 2004b) showed an earlier response to involution than samples with moderate or high relative α-lactalbumin expression. There was also heterogeneity present within the tissue samples, with lobules of involuting alveoli in some areas, while in others alveoli retained the appearance of lactation. By 72 h (Fig. 3.1I to L) and 8 d (Fig. 3.1M to P) this relationship was even more obvious. Tissue from cows with high relative α-lactalbumin mRNA expression still had open, stretched alveoli with a few areas of involuting, vesicle-engorged alveoli (VEA) (Fig. 3.1F, I and M). VEA (also called vacuole-containing alveoli) are defined as alveoli in which large vacuoles containing fat droplets and material from secretory vesicles are present within MECs and high numbers of vesicles containing coalescing fat droplets and proteins exist in the alveolar lumen (Holst *et al.*, 1987; Hurley, 1989; Oliver & Sordillo, 1989; Molenaar *et al.*, 1996a). The lumina of such alveoli typically stain deeply with eosin. Samples from cows with a moderate α-lactalbumin mRNA level showed more heterogeneity, with areas of open, stretched, lactating alveoli and collapsed VEA (Fig. 3.1G, J, K, N and O). The tissue of low-α-lactalbumin cows exhibited a non-lactating phenotype and was characterised by collapsed alveoli, abundance of large vesicles within MECs and alveolar lumens, thickened areas of stromal tissue between alveoli and broad bands of supportive connective tissue containing adipocytes (Fig. 3.1H, L and P). A large number of leukocytes were present inside the alveolar lumen of highly involuted tissue (Fig. 3.1O and P), causing it to take on a highly cellular appearance and making it difficult to distinguish alveolar boundaries.



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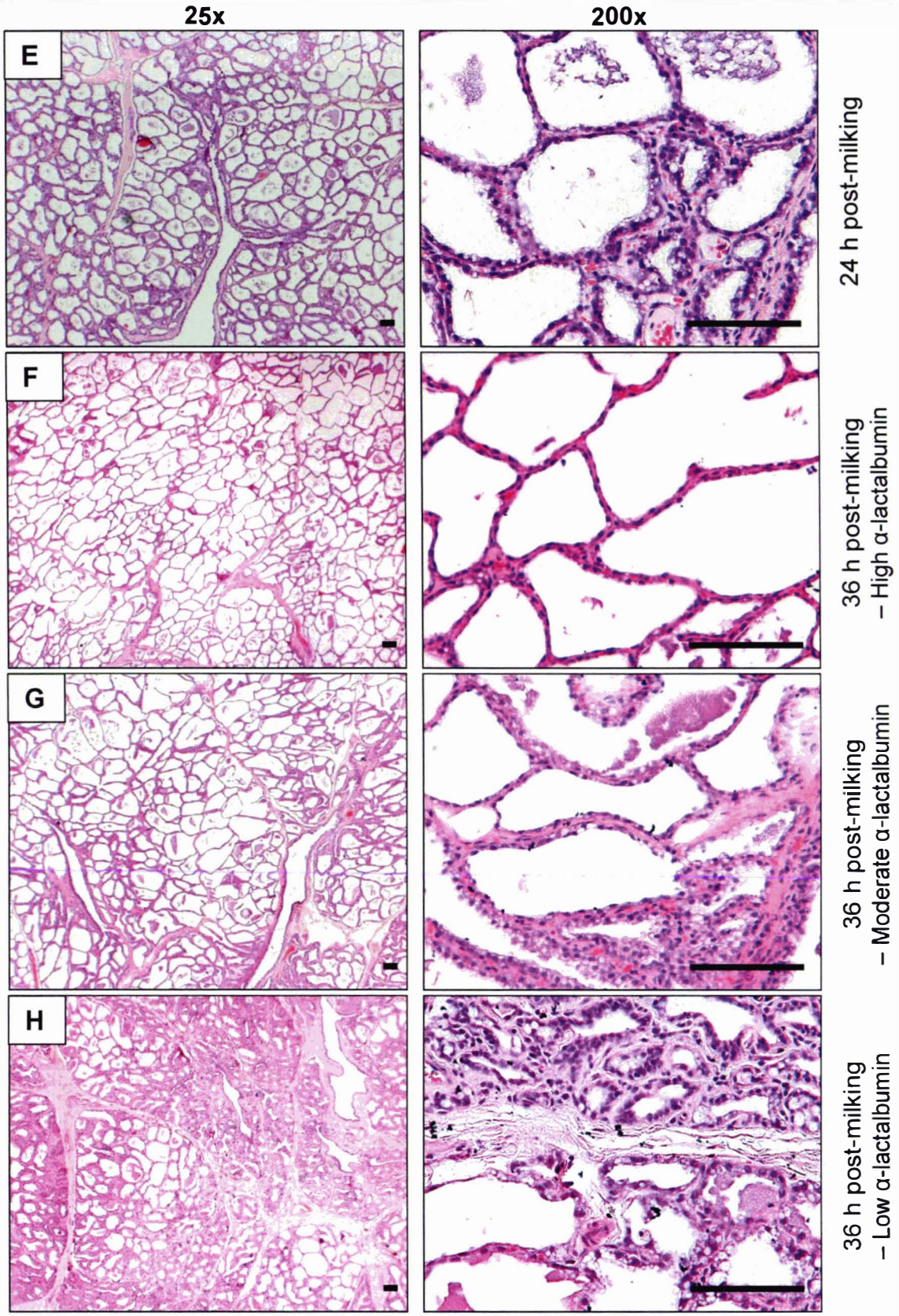

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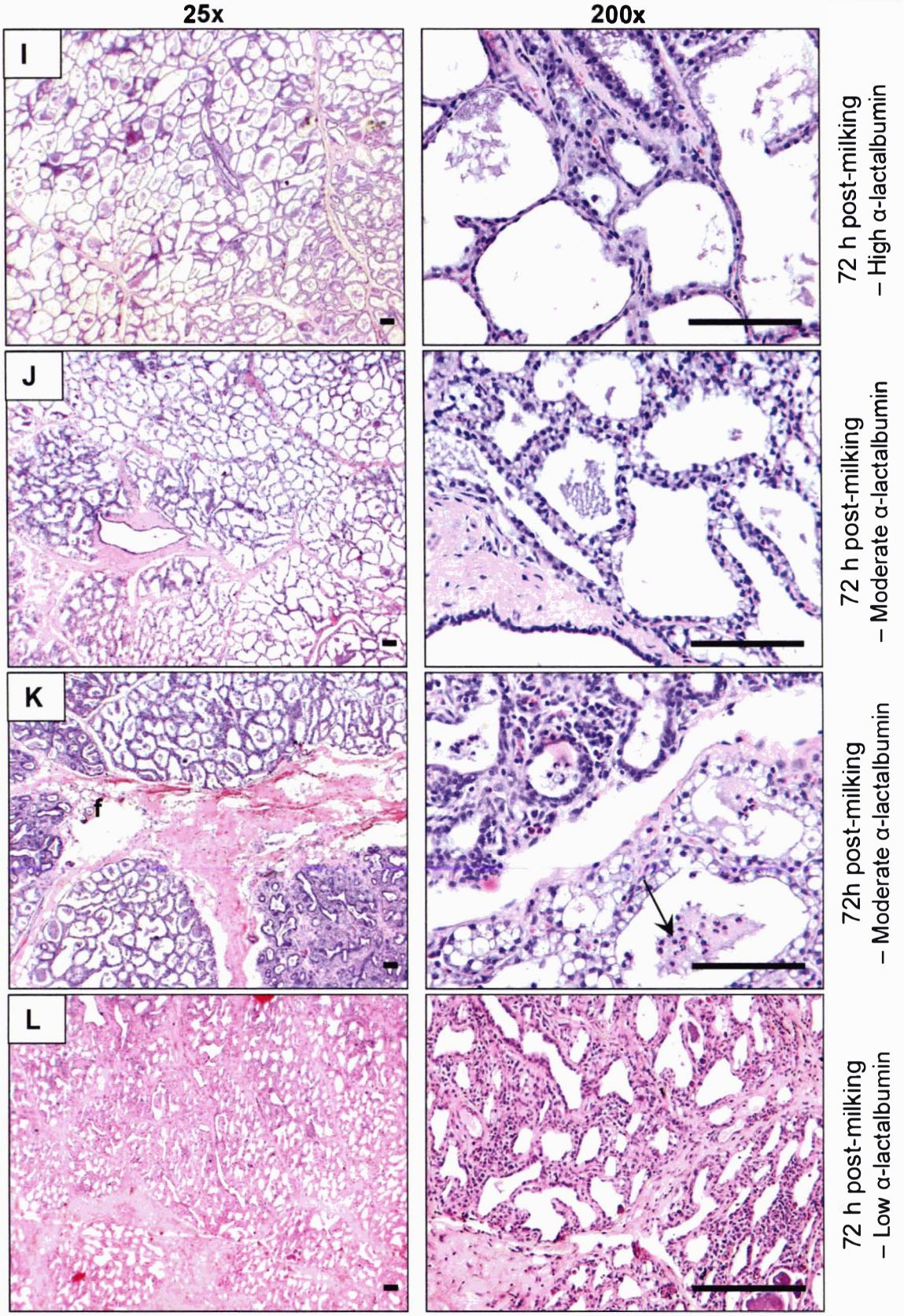

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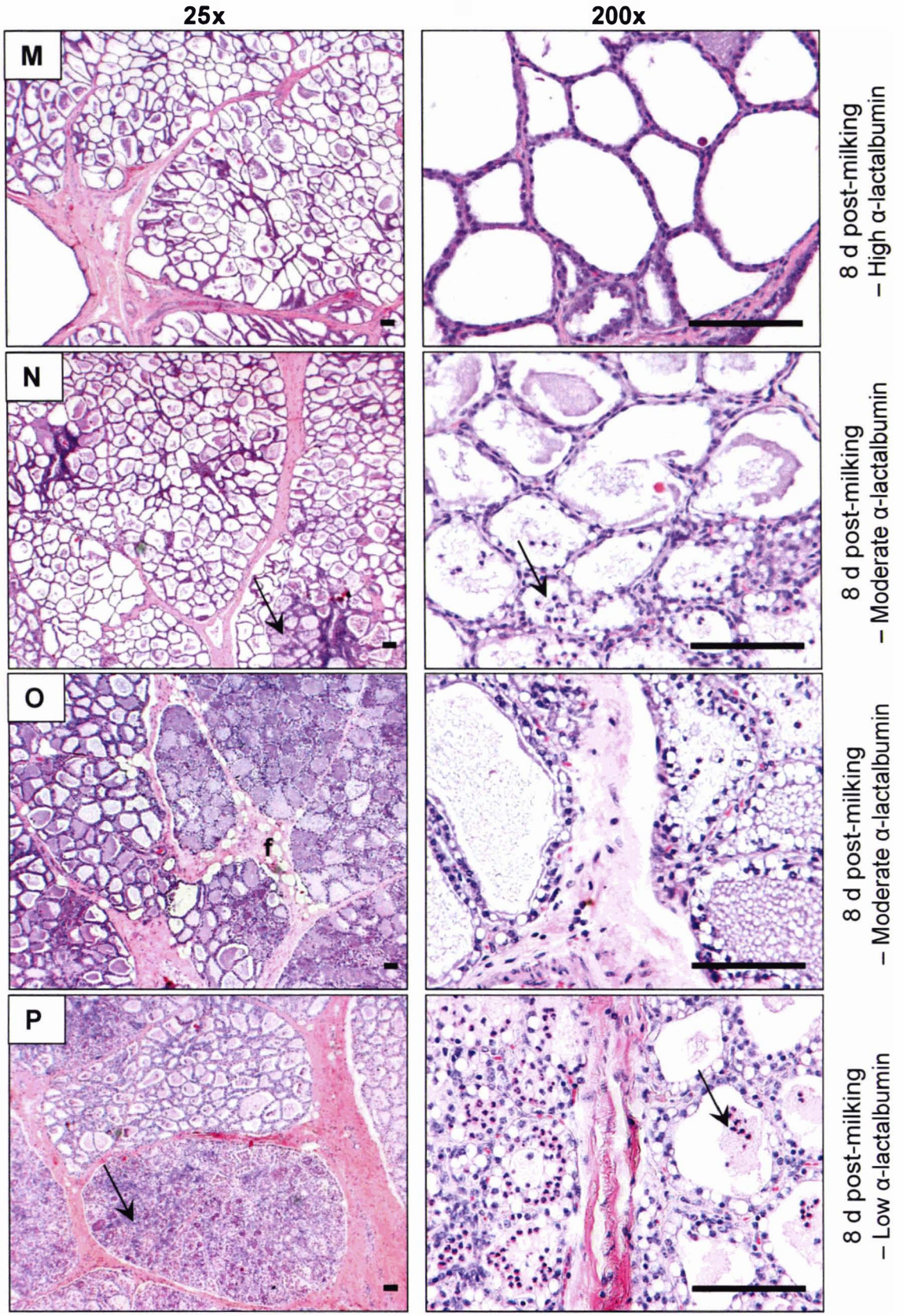
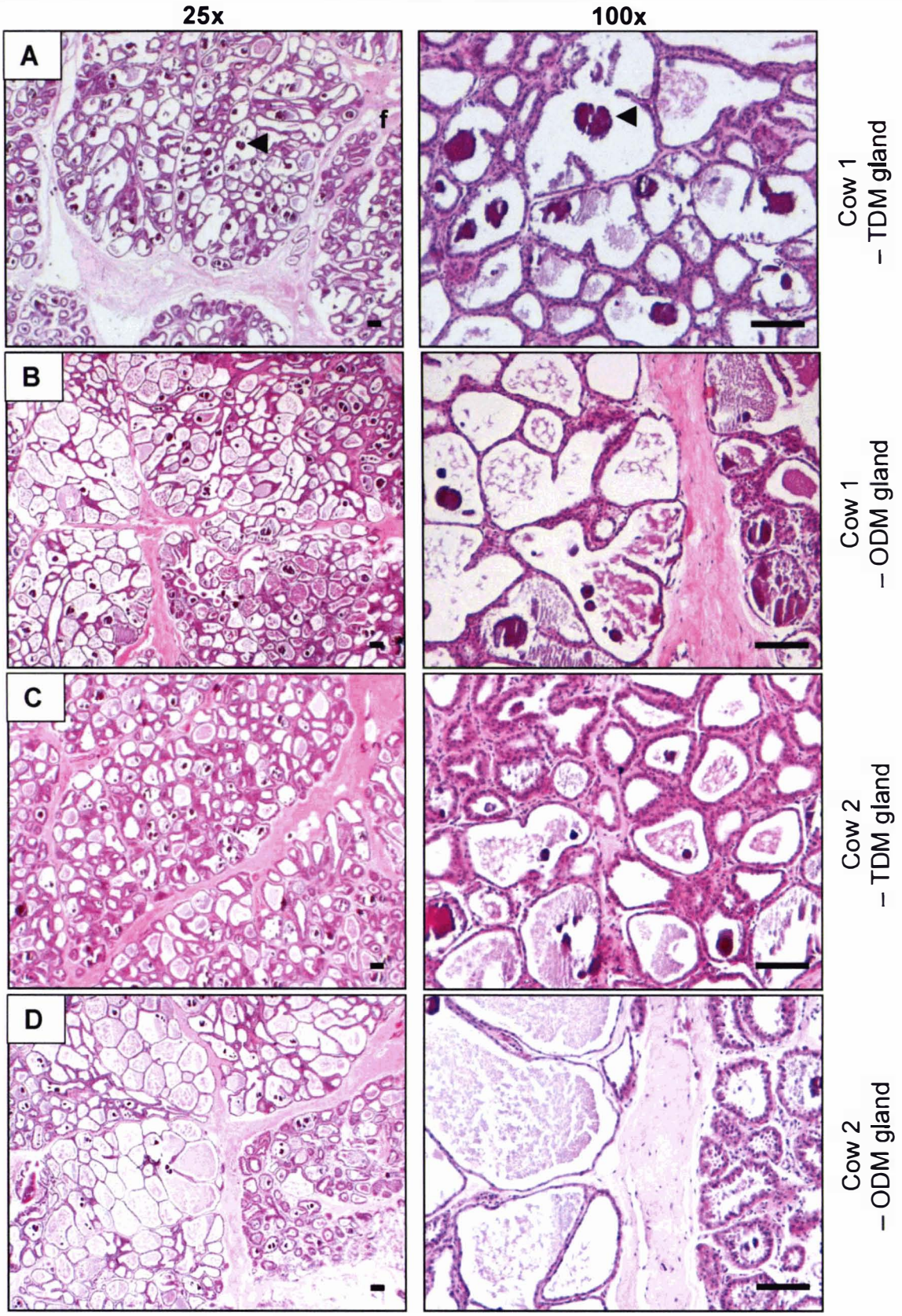


Figure 3.1. Morphological changes in bovine alveolar mammary tissue following the abrupt cessation of milking in mid-lactation.

Representative H&E stained sections are shown at 25 x and 200 x magnification for glands at 0 h (**A**); 6 h (**B**); 12 h (**C**); 18 h (**D**); 24 h (**E**); 36 h (**F**, **G**, **H**); 72 h (**I**, **J**, **K**, **L**) and 192 h (i.e., 8 d) (**M**, **N**, **O**, **P**) post-milking. Histological variation was observed between animals with high (**F**, **I**, **M**), moderate (**G**, **J**, **K**, **N**, **O**) and low (**H**, **L**, **P**) relative α-lactalbumin mRNA expression at the later time points. Examples of amyloid bodies (arrowheads), ducts (du), fat cells (f) and leukocytes (arrows) are shown. Refer to the text for a detailed description of histological features. Scale bars are at 100 μm.

3.3.2.2 Experiment 2 – Morphological changes following short-term ODM

Bovine mammary glands milked unilaterally either once-daily or twice-daily for a short period in late lactation showed differences in histological morphology as illustrated in Figure 3.2. The tissue of ODM glands (Fig. 3.2B, D, F and H) had large areas containing stretched alveoli with a lot of milk accumulated in the alveolar lumen. However, there were also areas of moderately-open alveoli with increased inter-alveolar stroma and areas with smaller, collapsed alveoli indicative of reduced secretory activity associated with the gradual involution of mammary glands following peak lactation. The alveolar mammary tissue of cows 2 and 4 showed more signs of gradual involution than cows 1 and 3, which were 10 weeks earlier in lactation (see Table 3.3) (Fig. 3.2D and H). TDM tissue (Fig. 3.2A, C, E and G) was characterised by mostly moderately open, and a few stretched, alveoli. Overall, the alveoli were smaller than in mid-lactation (compared with 6, 12 and 18 h post-milking Fig. 3.1B, C and D, respectively) and had more surrounding inter-alveolar stromal tissue. Heterogeneity between lobules, while not as striking as that observed in ODM glands, was also present in TDM glands. Some areas were already collapsed with small alveoli, while others had open and moderately-stretched alveoli. Other signs of gradual involution were also observed, with increased numbers of larger sized amyloid bodies and thick bands of connective tissue, containing adipocytes, around the lobules of both ODM and TDM glands (Fig. 3.2).



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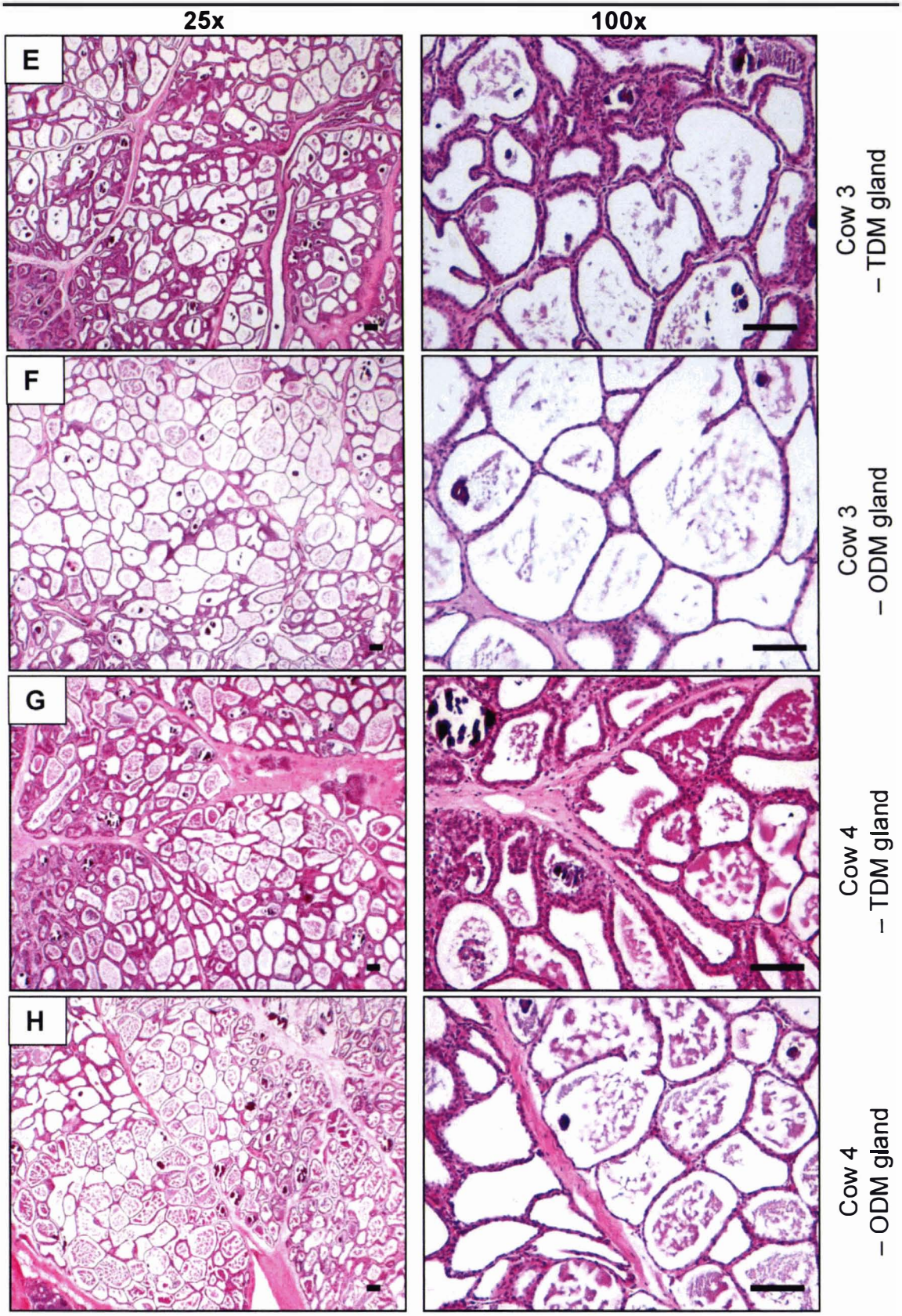


Figure 3.2. Morphological changes in bovine alveolar mammary tissue following short-term unilateral once-daily milking (ODM) in late lactation.

H&E stained sections are shown at 25 x and 100 x magnification. Pictures (**A** & **B**) cow 1 (arrowheads point to examples of amyloid bodies, f = fat cells); (**C** & **D**) cow 2; (**E** & **F**) cow 3; and (**G** & **H**) cow 4; depict the twice-daily milked (TDM) and ODM glands of each animal, respectively. Mammary samples were collected at approximately 26 h and 18 h after the final milking for ODM and TDM udder halves, respectively. Refer to the text for a detailed description of histological features. Scale bars are at 100 μm.

3.3.3 ISEL of apoptotic nuclei

3.3.3.1 Experiment 1 – Time course of the onset of apoptosis post-milking

ISEL was used to detect the degree of apoptosis in bovine mammary glands following the abrupt cessation of milking. Representative sections following the last milking are shown in Figure 3.3. Apoptotic nuclei were not labelled in negative controls (Fig. 3.3N). Sections were given a qualitative score between 1 (lowest) to 5 (highest) of the level of apoptosis with means for each time point post-milking presented in Fig. 3.4. Numbers of positive ISEL nuclei were then counted to provide a quantitative measurement of apoptosis (Fig. 3.5). There was a strong positive correlation ($r = 0.86$, $P<0.001$) between the quantitative and qualitative scores of apoptosis (Fig. 3.6), and similar results were obtained from both datasets, substantiating the findings discussed below.

The mammary tissue sections from 0 to 36 h post-milking had low numbers of positive ISEL nuclei and hence, a low level of apoptosis (Fig. 3.3, 3.4 and 3.5). By 72 h and 8 d, the total number of ISEL apoptotic nuclei per alveolus was significantly increased (3.4-fold, $P<0.01$ and 5.5-fold, $P<0.001$; respectively) compared with 6 h post-milking (Fig. 3.3 and 3.5A). A significant linear increase ($P<0.01$) from low to moderately high levels of ISEL nuclei was also observed for the mean qualitative score of apoptosis by 8 d relative to 6 h post-milking (Fig. 3.4). The number of ISEL nuclei located within the secretory epithelial cell layer surrounding the alveoli followed a similar pattern with significantly more per alveolus by 72 h ($P<0.01$) and 8 d ($P<0.001$), compared with 6 h post-milking (Fig. 3.5A). There were greater numbers of ISEL nuclei per alveolus within the epithelial cell layer than the alveolar lumens at 36 h ($P<0.05$) and 72 h ($P<0.001$) post-milking. However, by 8 d a dramatically increased ($P<0.001$) number of ISEL nuclei located within the lumen accounted for the majority ($P<0.001$) of ISEL nuclei detected at that time (Fig. 3.3 and 3.5A).

ISEL apoptotic nuclei present in the alveolar lumen at the later time points were associated with leukocyte (e.g., macrophages and neutrophils) invasion (Fig. 3.3).

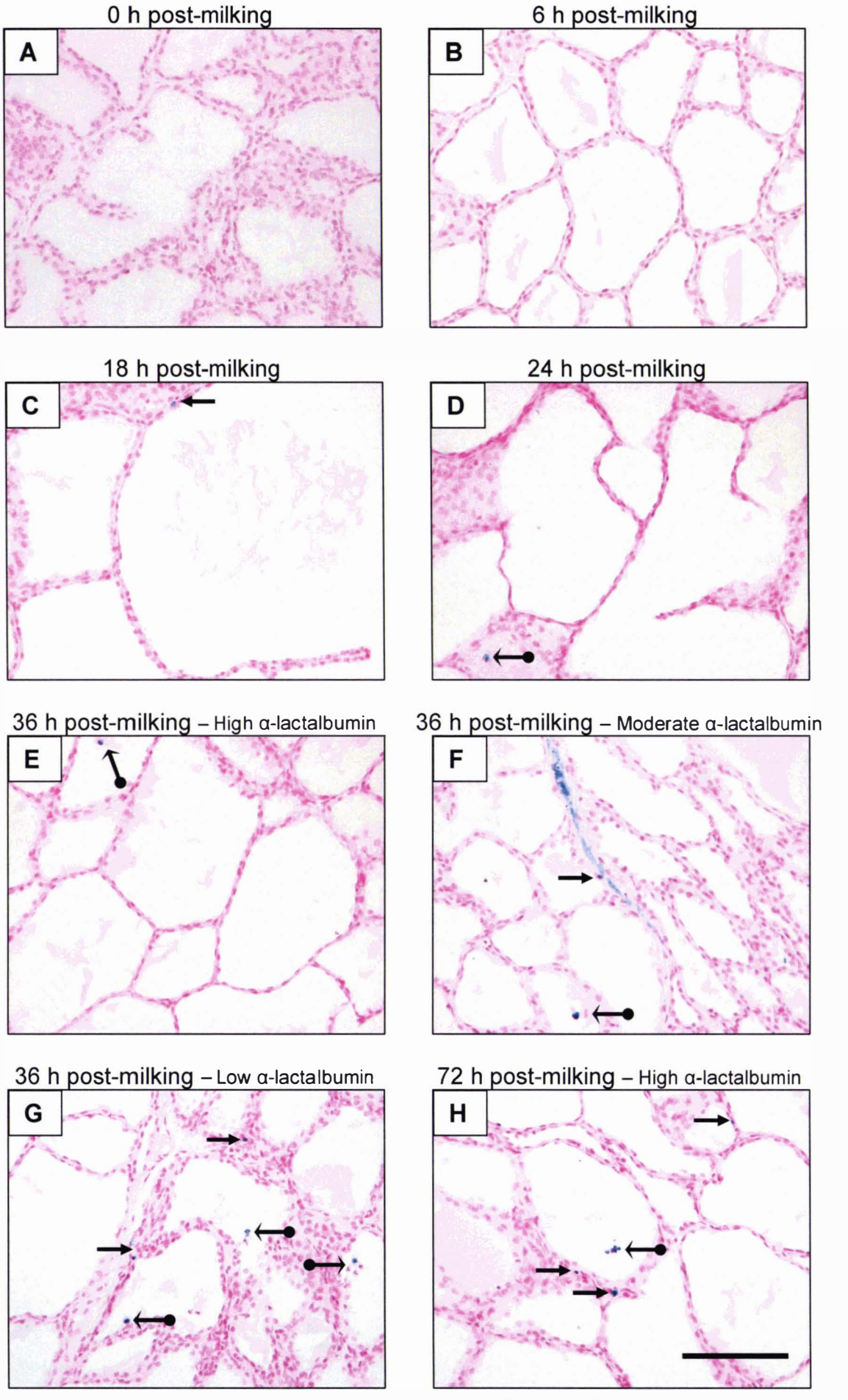
However, it was not possible to distinguish between the apoptosis of epithelial cells and leukocytes in the alveolar lumen. Milk vesicles accumulated within engorged alveolar lumens after 8 d were sometimes diffusely labelled, but were clearly distinguishable from the intense, specific-labelling of apoptotic bodies (Fig. 3.3L).

The number of alveoli per 100 x magnification field was used to correct counts of ISEL nuclei per field for changes in alveolar lumen size occurring during mammary engorgement. A similar pattern of apoptosis was observed for the uncorrected number of ISEL nuclei per field (Fig. 3.5B) and the corrected number of ISEL nuclei per alveolus (Fig. 3.5A) during mammary engorgement, and there was a strong positive correlation ($r = 0.98$, $P<0.001$) between the datasets (Fig. 3.7). This is because the overall numbers of alveoli per field did not significantly change following the last milking (Fig. 3.8). However, there was a small linear decrease from 0 to 24 h post-milking ($P<0.05$), followed by increased numbers at 36 h before declining again by 8 d relative to 0 h post-milking ($P<0.05$) (Fig. 3.8). This pattern is indicative of an increased alveolar lumen size due to the accumulation of secreted milk during the early stages of induced involution, followed by re-absorption of milk secretion and finally regression of alveolar tissue relative to stromal tissue (Fig. 3.1), as discussed in section 3.3.2.1 above.

There was considerable variation in the extent of apoptosis between cows at the later time points (36 h to 8 d), which was associated with the morphology of the tissue section and milk protein mRNA expression. Animals that were at a more advanced stage of involution, with lower relative α-lactalbumin mRNA expression, had increased numbers of apoptotic nuclei (compare 8 d cows Fig. 3.3K, L, and M). Therefore, the total number of ISEL nuclei per alveolus was associated with reduced mRNA expression of major milk proteins (Fig. 3.9) and increased expression of immune-associated genes (Fig. 3.10) during bovine mammary engorgement.

There were strong negative correlations between the level of α-lactalbumin mRNA expression and the number of ISEL nuclei per alveolus ($r = -0.88$, $P<0.001$) and the qualitative score of apoptosis ($r = -0.78$, $P<0.001$) (Fig. 3.9A). A similar pattern was observed for α-S1-casein mRNA expression, which was inversely associated with the

number of ISEL nuclei per alveolus ($r = -0.71$, $P<0.001$) and the qualitative score of apoptosis ($r = -0.70$, $P<0.001$) (Fig. 3.9B). Gene expression of MSAA was strongly positively correlated to the number of ISEL nuclei per alveolus ($r = 0.84$, $P<0.001$) and the qualitative score of apoptosis ($r = 0.74$, $P<0.001$) (Fig. 3.10A). Lactoferrin mRNA expression tended to be positively associated with the number of ISEL nuclei per alveolus ($r = 0.31$, $P<0.1$) and the qualitative score of apoptosis ($r = 0.29$, $P=0.1$) (Fig. 3.10B). Correlations using the northern blot generated mRNA expression of these genes gave similar results (refer to Fig. 13.1 and 13.2, Appendix V).



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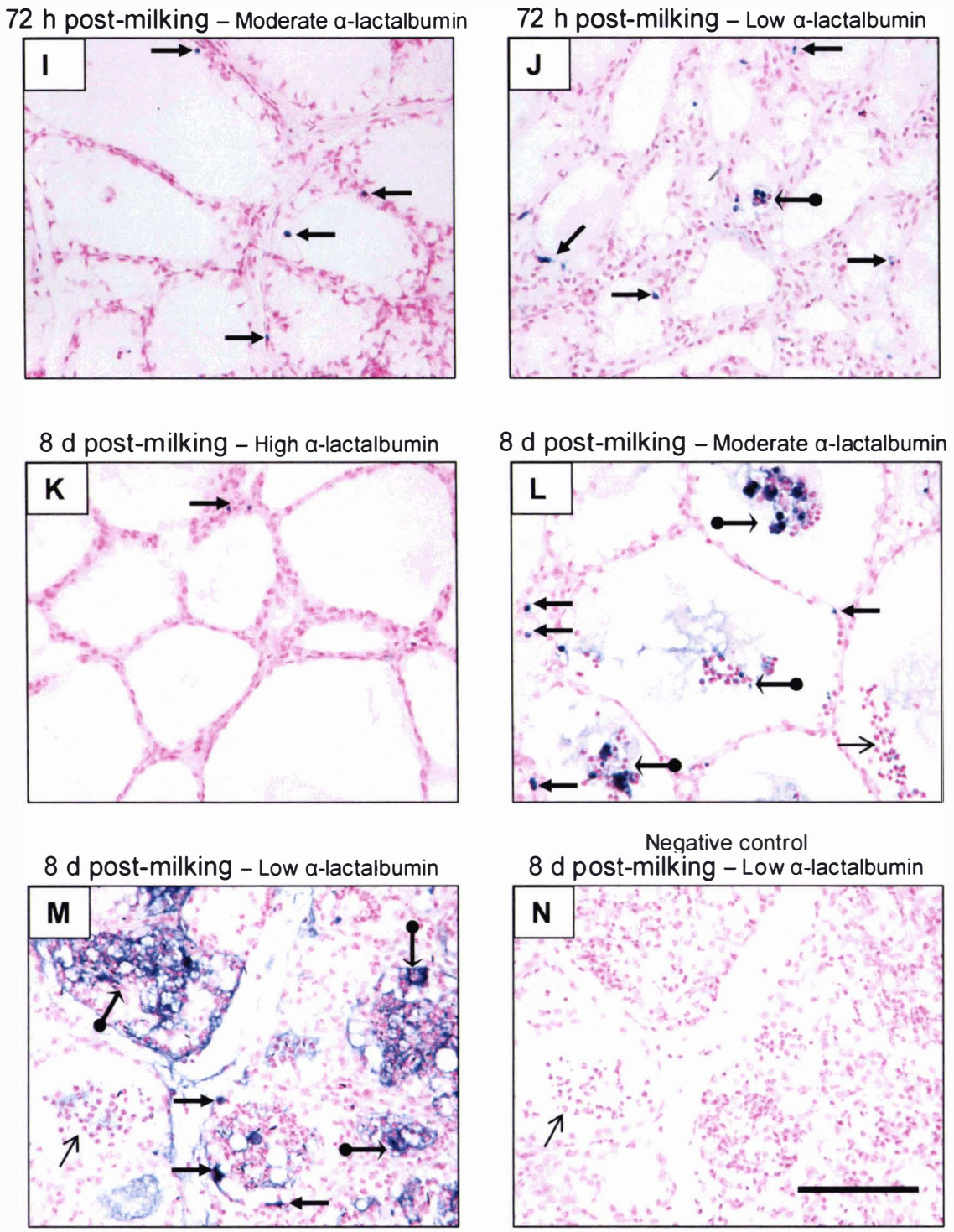


Figure 3.3. *In situ* end-labelling (ISEL) of apoptotic nuclei in bovine alveolar mammary tissue following the abrupt cessation of milking in mid-lactation.
Representative sections are shown at 200 x magnification for glands at 0 h (**A**); 6 h (**B**); 18 h (**C**); 24 h (**D**); 36 h (**E**, **F**, **G**); 72 h (**H**, **I**, **J**) and 192 h (i.e., 8 d) (**K**, **L**, **M**, and **N** – negative control) post-milking. Variation was observed between animals with high (**E**, **H**, **K**), moderate (**F**, **I**, **L**) and low (**G**, **J**, **M**) relative α-lactalbumin mRNA expression at the later time points. Positive ISEL nuclei are labelled blue-black and indicate apoptosis. The omission of Klenow enzyme during ISEL was used to provide concurrent negative controls. Sections are counterstained with nuclear fast red. Examples of epithelial ISEL nuclei (⟶), luminal ISEL nuclei (single or regions; ⟶), and regions of leukocyte invasion (⟶) are shown. Scale bars are at 100 μm.

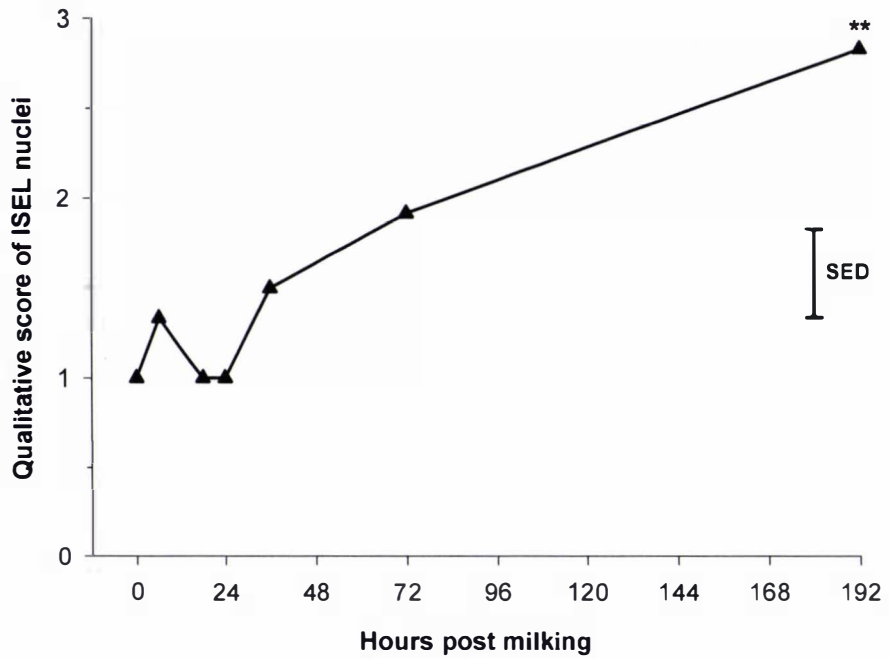
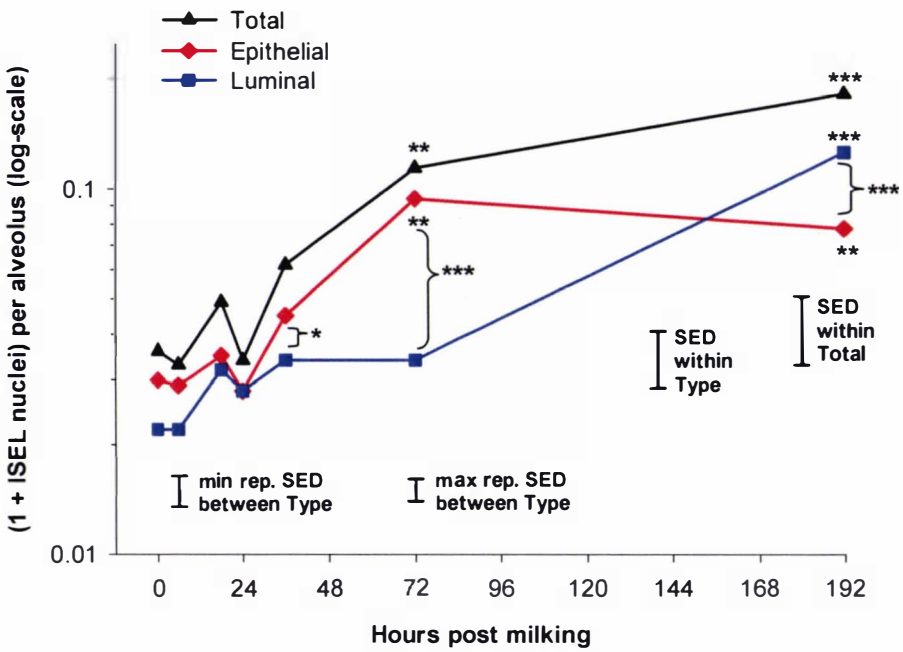


Figure 3.4. Qualitative analysis of *in situ* end-labelled (ISEL) apoptotic nuclei in bovine mammary glands, at 0, 6, 18, 24 h (n=3 per time point) and at 36, 72 and 192 h (n=6 per time point) following the last milking.

Data are expressed as the mean qualitative score of the level of apoptosis on a scale of 1 to 5 where; 1 = none to low, 2 = low to moderate, 3 = moderate to high, 4 = high and 5 = very high numbers of positive ISEL nuclei. The max-min replicates (rep.) (n=3 vs n=6) SED is shown for comparing different time points to the 6 h time point (** $P<0.01$).

A



B

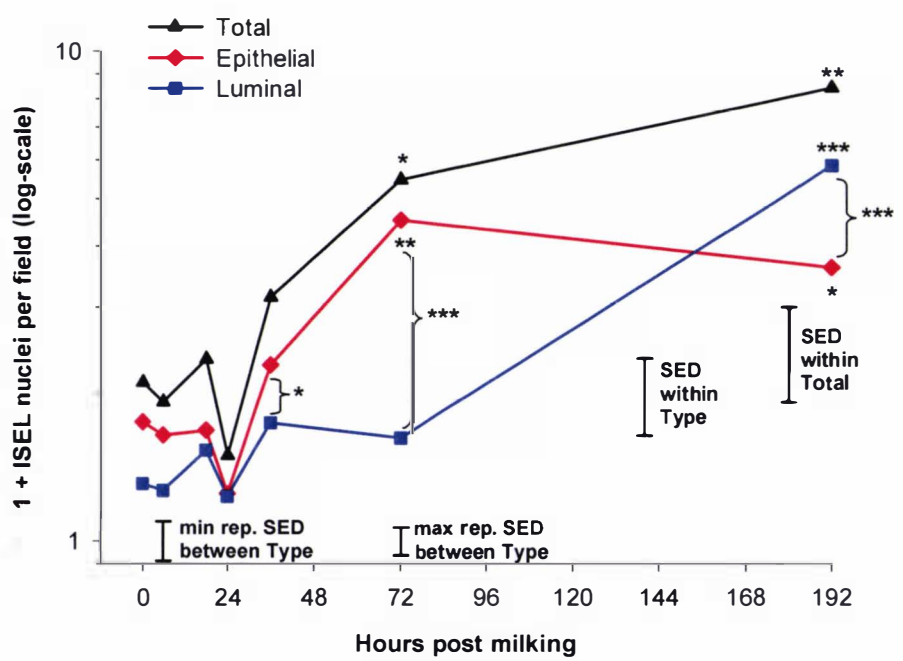


Figure 3.5. Quantitative analysis of *in situ* end-labelled (ISEL) apoptotic nuclei in bovine mammary glands, at 0, 6, 18, 24 h (n=3 per time point) and at 36, 72 and 192 h (n=6 per time point) following the last milking.

Data are expressed as the back-transformed mean number of total, epithelial and luminal 1+ ISEL nuclei per alveolus (**A**) and per 100 x magnification field (**B**), with the max-min replicates (rep.) (n=3 vs n=6) SED within total and SED within type (epithelial or luminal) for comparing different time points to the 6 h time point. The between type SEDs for comparing type at each time point (i.e. epithelial vs luminal) are shown for time points with either the min rep. (n=3) or the max rep. (n=6). (* P<0.05, ** P<0.01, *** P<0.001).

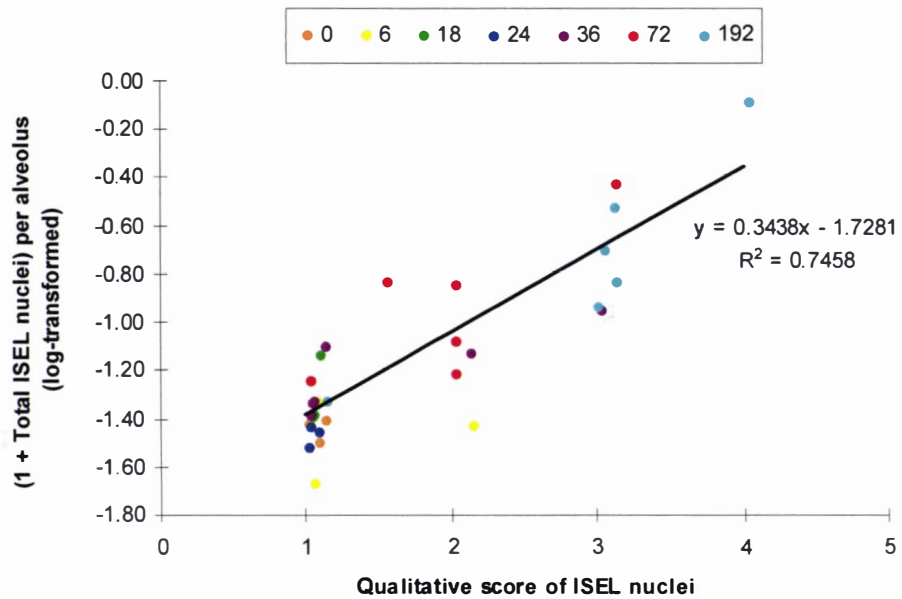


Figure 3.6. The relationship between qualitative and quantitative analyses of *in situ* end-labelled (ISEL) apoptotic nuclei in bovine mammary glands, at 0, 6, 18, 24 h (n=3 per time point) and at 36, 72 and 192 h (n=6 per time point) following the last milking.

Data are expressed as the qualitative score of the level of apoptosis on a scale of 1 to 5 where; 1 = none to low, 2 = low to moderate, 3 = moderate to high, 4 = high and 5 = very high numbers of positive ISEL nuclei and for the quantitative analysis; as the $\log_{10}$-transformed mean number of total 1+ ISEL nuclei per alveolus for each animal. The qualitative score of ISEL nuclei are graphed with jitter to distinguish between data points.

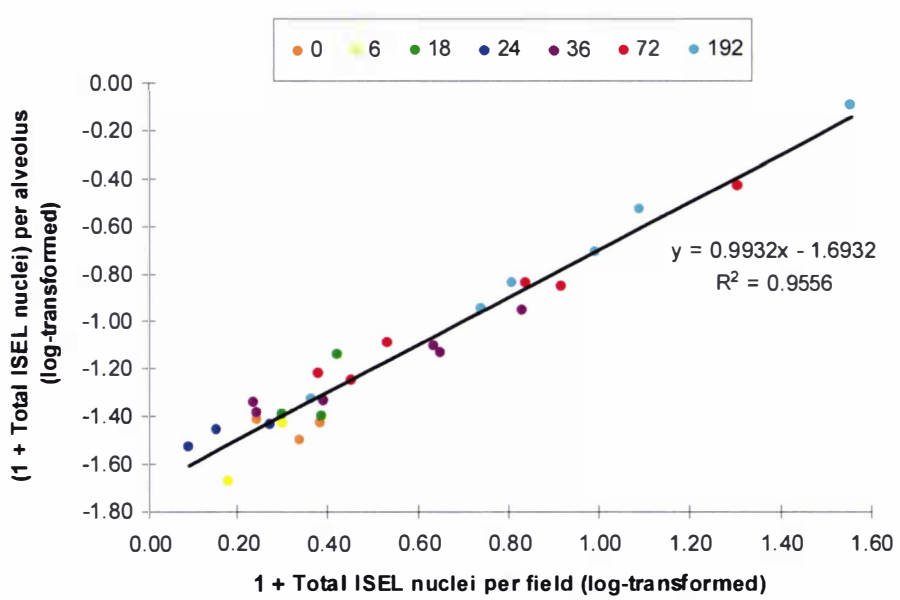


Figure 3.7. The relationship between the number of *in situ* end-labelled (ISEL) apoptotic nuclei per field and per alveolus during the engorgement of bovine mammary glands.

Data are expressed as the $\log_{10}$-transformed mean number of total 1+ ISEL nuclei per 100 x magnification field and per alveolus for each animal at 0, 6, 18, 24 h (n=3 per time point) and at 36, 72 and 192 h (n=6 per time point) following the last milking.

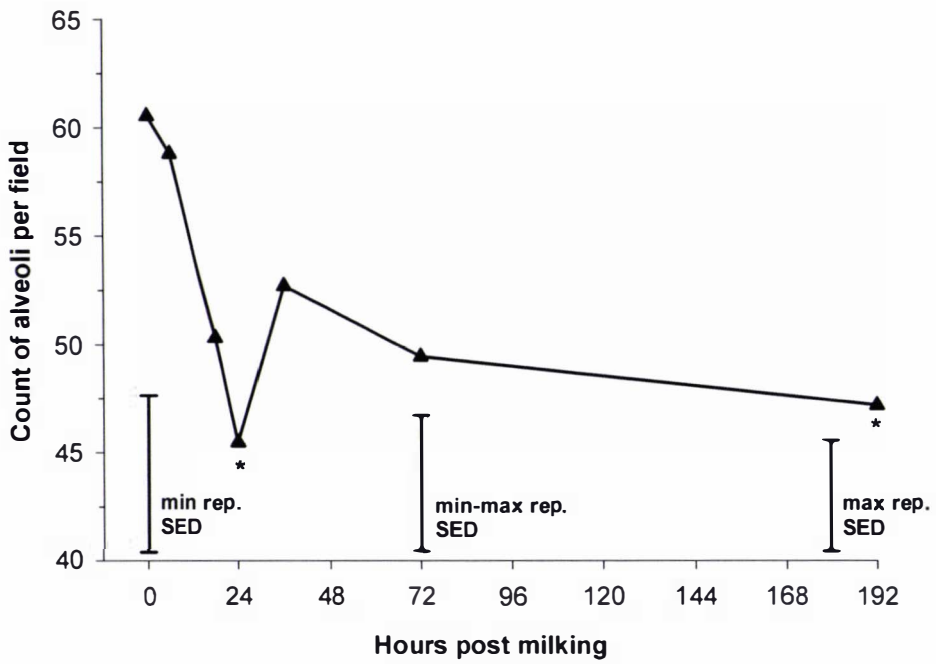


Figure 3.8. The number of alveoli per field during the engorgement of bovine mammary glands. Data are expressed as the mean number of alveoli per 100 x magnification field in bovine mammary glands at 0, 6, 18, 24 h (n=3 per time point) and at 36, 72 and 192 h (n=6 per time point) following the last milking. SEDs are shown for comparing time points with either the min replicates (rep.) (n=3) or the max rep. (n=6) (* $P<0.05$ relative to the 0 h time point).

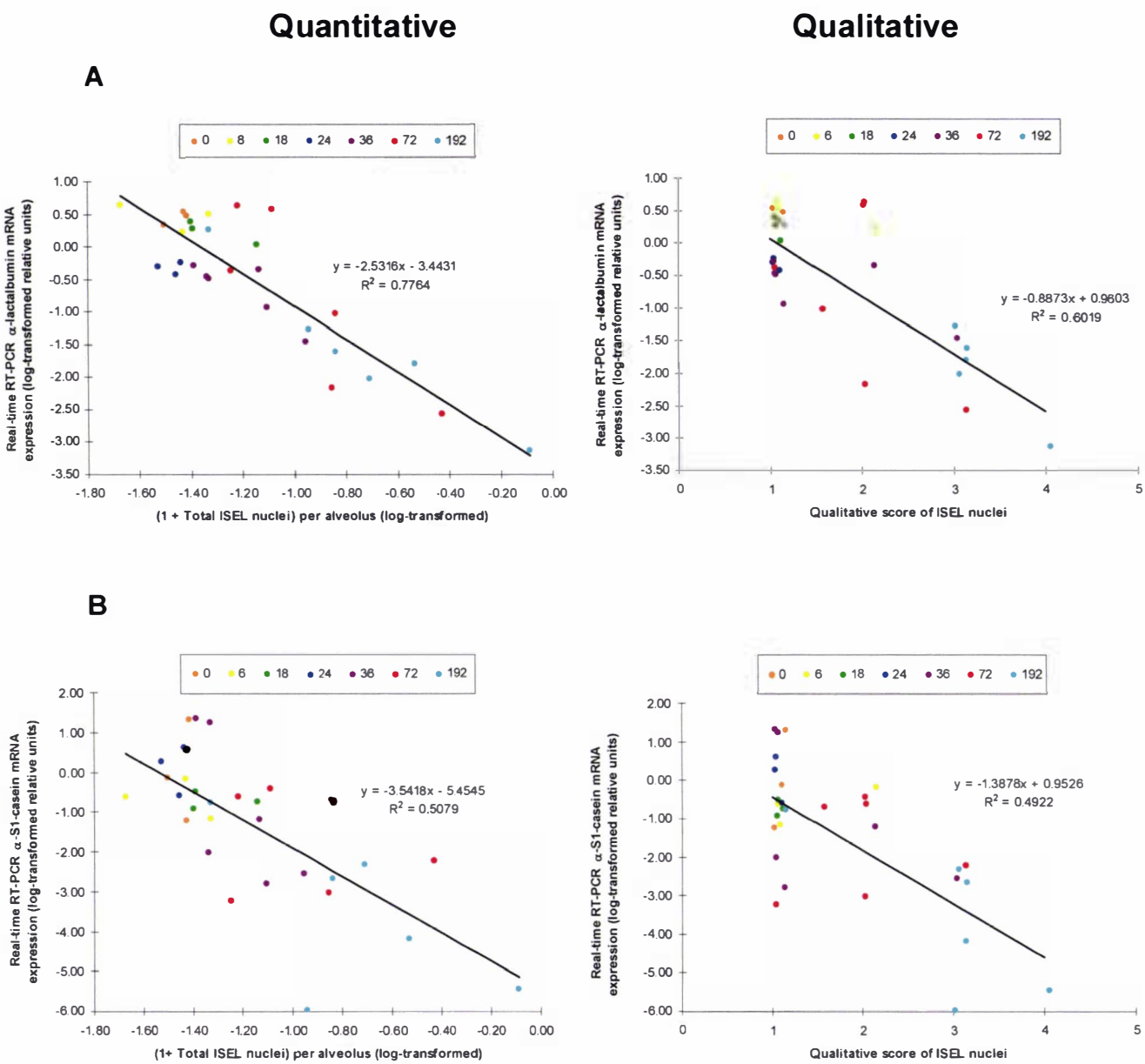


Figure 3.9. The relationship between the level of apoptosis and real-time RT-PCR milk protein mRNA expression during the engorgement of bovine mammary glands.
(**A**) α-lactalbumin and (**B**) α-S1-casein mRNA expression levels are negatively correlated with quantitative and qualitative analyses of apoptosis. Milk protein mRNA expression data were analysed by real-time RT-PCR and are expressed as $\log_{10}$-transformed relative units for each animal at 0, 6, 18, 24 h (n=3 per time point) and at 36, 72 and 192 h (n=6 per time point) following the last milking. For analysis of *in situ* end-labelling (ISEL), data for each animal are expressed as either: the qualitative score of the level of apoptosis on a scale of 1 to 5 where; 1 = none to low, 2 = low to moderate, 3 = moderate to high, 4 = high and 5 = very high numbers of positive ISEL nuclei, or the $\log_{10}$-transformed mean number of total 1+ ISEL nuclei per alveolus. The qualitative score of ISEL nuclei are graphed with jitter to distinguish between data points.

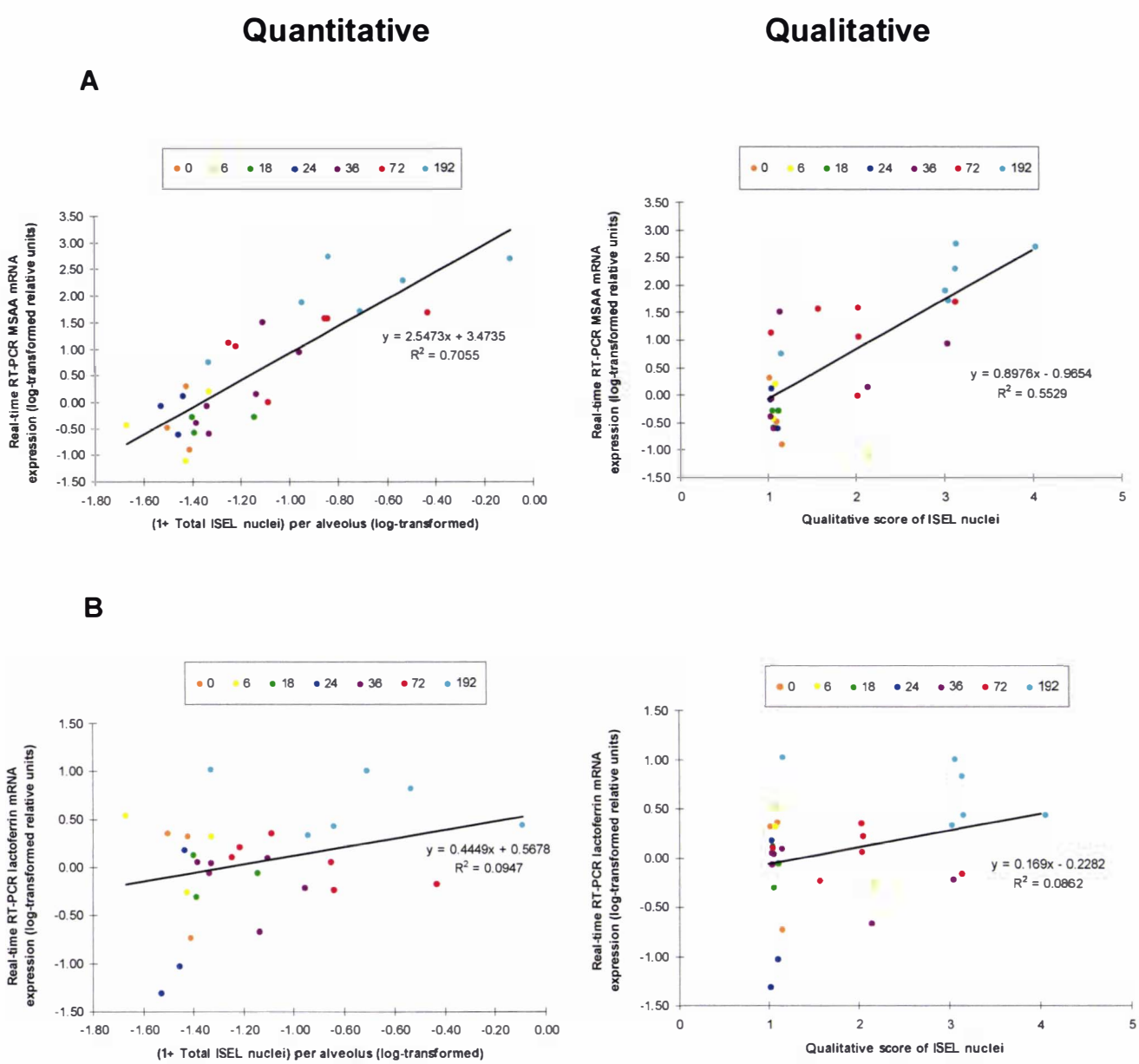


Figure 3.10. The relationship between the level of apoptosis and real-time RT-PCR immune-associated gene expression during the engorgement of bovine mammary glands.
(**A**) mammary serum amyloid A3 (MSAA) and (**B**) lactoferrin mRNA expression levels are positively correlated with quantitative and qualitative analyses of apoptosis. Immune-associated gene expression data were analysed by real-time RT-PCR and are expressed as $\log_{10}$-transformed relative units for each animal at 0, 6, 18, 24 h (n=3 per time point) and at 36, 72 and 192 h (n=6 per time point) following the last milking. For analysis of *in situ* end-labelling (ISEL), data for each animal are expressed as either: the qualitative score of the level of apoptosis on a scale of 1 to 5 where; 1 = none to low, 2 = low to moderate, 3 = moderate to high, 4 = high and 5 = very high numbers of positive ISEL nuclei, or the $\log_{10}$-transformed mean number of total 1+ ISEL nuclei per alveolus. The qualitative score of ISEL nuclei are graphed with jitter to distinguish between data points.

3.3.3.2 Experiment 2 – Detection of apoptosis following short-term ODM

ISEL was also used to compare the degree of apoptosis between ODM and TDM glands during late lactation, with representative sections shown in Figure 3.11A to D. A qualitative score of 1 was given to each section (Fig. 3.11E) indicating that very low numbers of positive ISEL nuclei, and hence apoptosis, were detected in both TDM and ODM glands (Fig. 3.11). Apoptotic nuclei were not labelled in negative controls (data not shown).

In agreement with the qualitative analysis of apoptosis (Fig. 3.11E), there were no differences ($P>0.05$) between ODM and TDM glands in the numbers of total, epithelial and luminal ISEL nuclei per alveolus (Fig. 3.12A). A greater proportion of ISEL nuclei were located within the secretory epithelial layer rather than within alveolar lumens for TDM glands ($P<0.01$), but not ODM glands ($P>0.05$) (Fig. 3.12A). A similar pattern was observed when the data were expressed as ISEL nuclei per field (Fig. 3.12B), and there was a positive association between the number of total ISEL nuclei per alveolus and per field ($r = 0.74$, $P<0.05$; Fig. 3.13). However, there were significantly greater numbers of epithelial ISEL nuclei per field, but not per alveolus, in TDM glands compared with ODM glands (Fig. 3.12B and A, respectively). There were also fewer ($P<0.05$) alveoli per field in the ODM glands compared with their corresponding TDM glands (Fig. 3.14). This is indicative of the increased alveolar lumen size in many of the ODM lobules, which was discussed in section 3.3.2.2 above (Fig. 3.2).

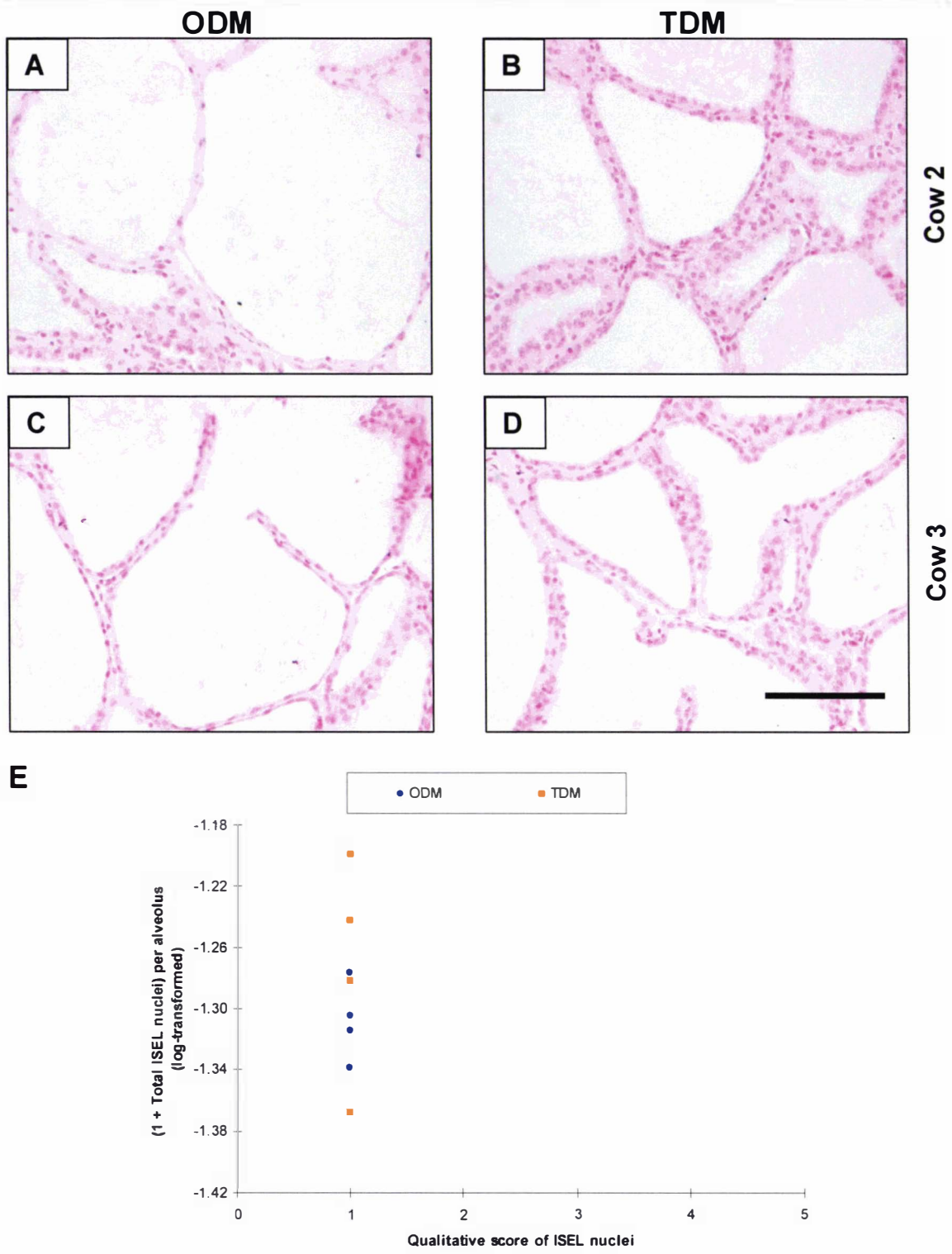
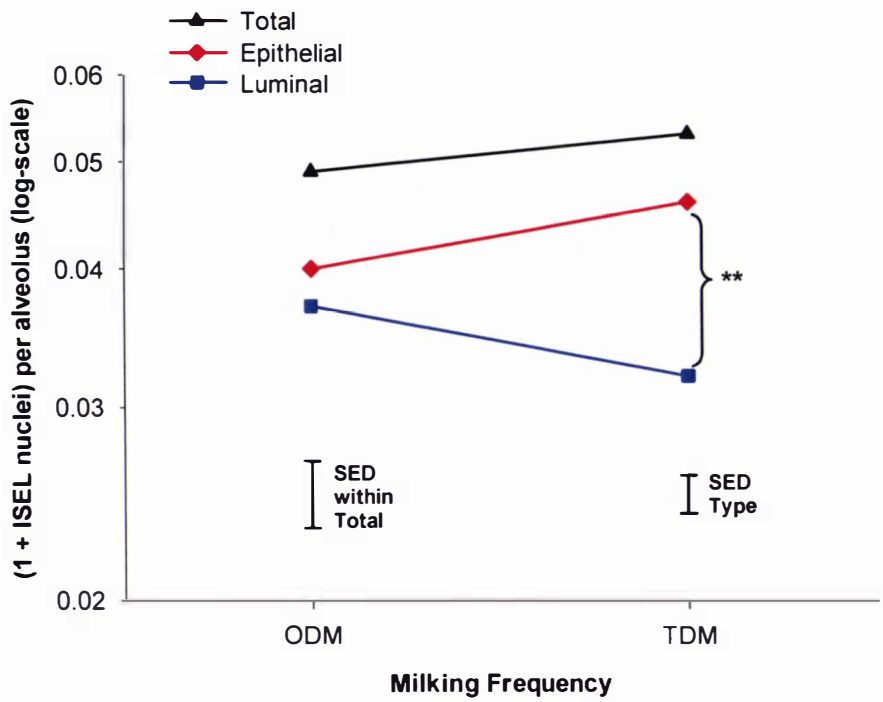


Figure 3.11. *In situ* end-labelling (ISEL) of apoptotic nuclei in bovine alveolar mammary tissue following short-term, unilateral, once-daily milking (ODM) in late lactation.
Representative sections are shown at 200 x magnification for ODM (**A** and **C**) and their corresponding twice-daily milked (TDM) glands (**B** and **D**, respectively). Sections are counterstained with nuclear fast red and the scale bar equals 100 μm. **E**) The relationship between the qualitative and quantitative analyses of apoptosis. Data are expressed as the qualitative score of the level of apoptosis on a scale of 1 to 5 where; 1 = none to low, 2 = low to moderate, 3 = moderate to high, 4 = high and 5 = very high numbers of positive ISEL nuclei and for the quantitative analysis; as the $\log_{10}$-transformed mean number of total 1+ ISEL nuclei per alveolus for each animal. None or very few blue-black positive ISEL nuclei were present, indicating a low level of apoptosis in both ODM and TDM glands.

A



B

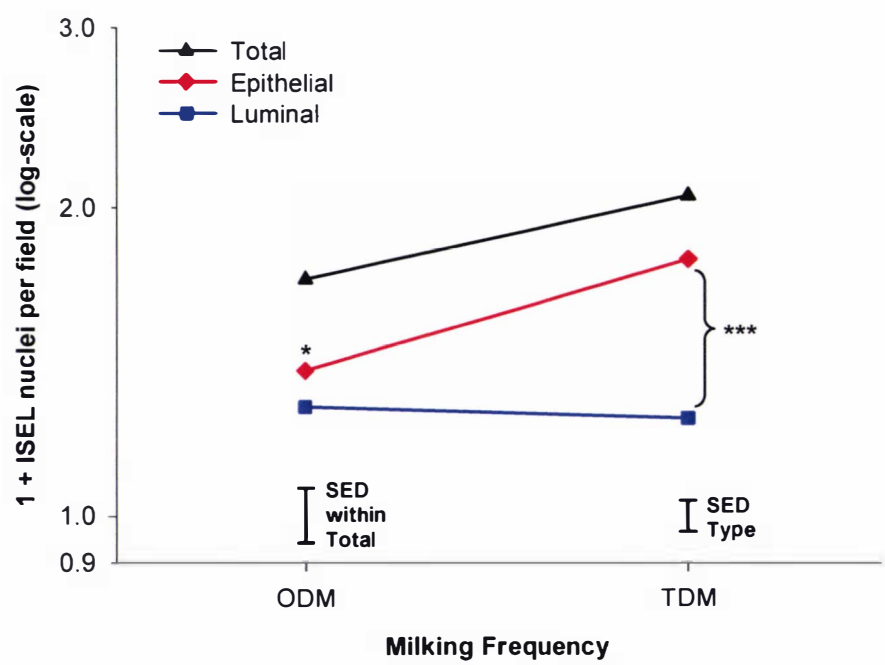


Figure 3.12. Quantitative analysis of *in situ* end-labelled (ISEL) apoptotic nuclei in bovine mammary glands following short-term, unilateral, once-daily milking (ODM) in late lactation.
Data are expressed as the back-transformed mean number of total, epithelial and luminal 1+ ISEL nuclei per alveolus (**A**) and per 100 x magnification field (**B**). The SED within total is shown for comparing the total number of ISEL nuclei between ODM glands (n=4) and their corresponding twice-daily milked (TDM) glands (n=4). The SED type is shown for comparing numbers of ISEL nuclei between ODM and TDM glands within a type (i.e., epithelial or luminal), and for comparing type (i.e., epithelial vs luminal) within a milking frequency (i.e., ODM or TDM). (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

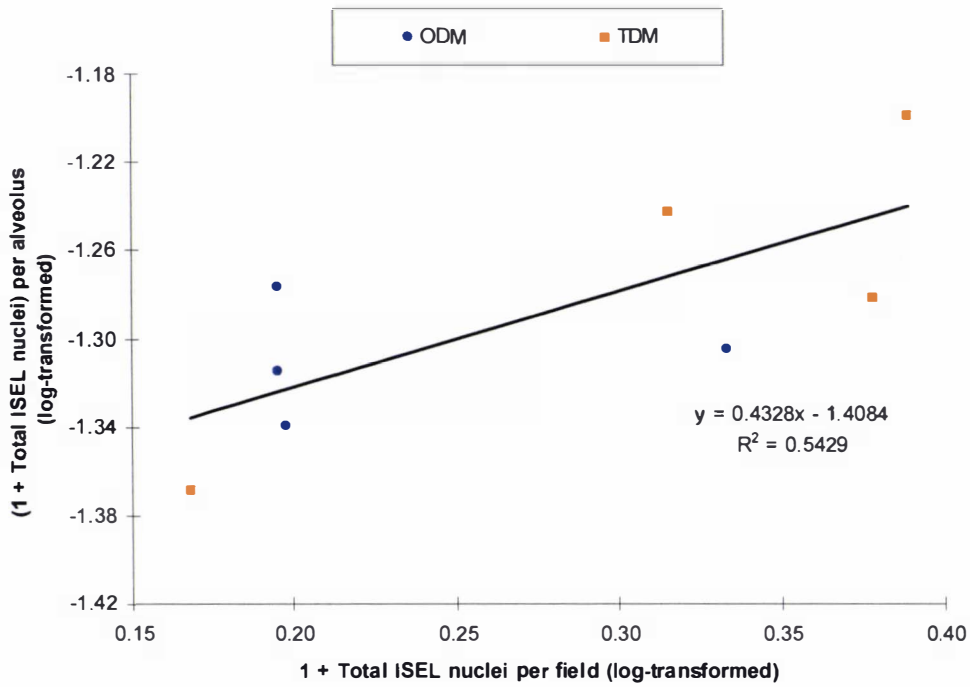


Figure 3.13. The relationship between the number of *in situ* end-labelled (ISEL) apoptotic nuclei per field and per alveolus in bovine mammary glands following short-term, unilateral, once-daily milking (ODM) in late lactation.
Data are expressed as the $\log_{10}$-transformed mean number of total 1+ ISEL nuclei per 100 x magnification field and per alveolus for the ODM glands (n=4) and their corresponding twice-daily milked (TDM) glands (n=4).

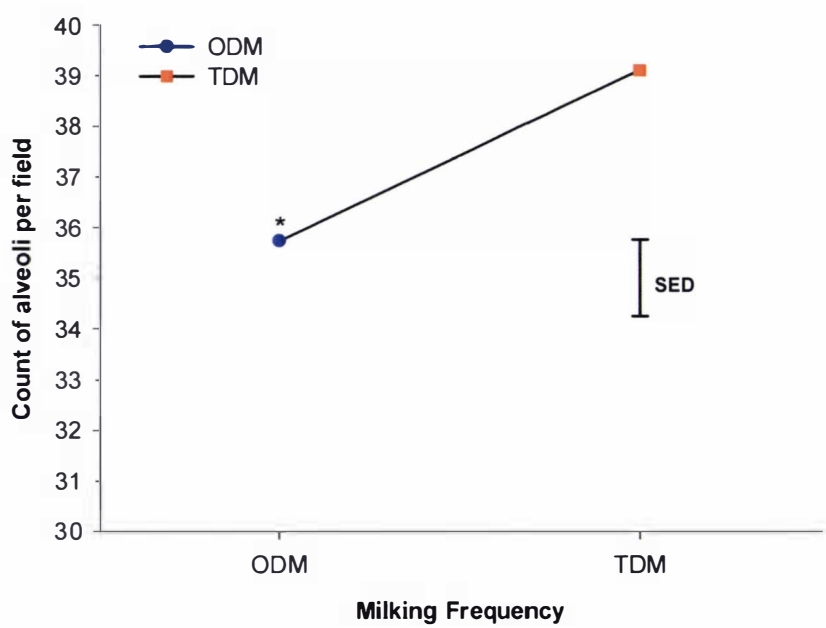


Figure 3.14. The number of alveoli per field in bovine mammary glands following short-term, unilateral, once-daily milking (ODM) in late lactation.
Data are expressed as the mean number of alveoli per 100 x magnification field. The SED is shown for comparing the ODM glands (n=4) with their corresponding twice-daily milked (TDM) glands (n=4) (* $P<0.05$).

3.3.4 Gene expression of TJ proteins

3.3.4.1 Experiment 1 – Time course of changes in mRNA expression of TJ proteins post-milking

Quantitative real-time RT-PCR was used to determine temporal changes in mammary mRNA expression of TJ proteins for up to 8 d after the abrupt cessation of milking (Fig. 3.15). The pattern of change was similar among occludin, claudin-1 and ZO-1, with mRNA expression highest at 6 h and 18 h post-milking before declining to a nadir by 36 h post-milking. There was a 3.5-fold and 1.9-fold decrease ($P<0.05$) in occludin and ZO-1 mRNA expression, respectively, by 36 h after the last milking relative to 6 h. However, while claudin-1 mRNA expression was reduced 2-fold by 36 h (and up to 8 d) after the last milking relative to 6 h, this decrease was not significant due to large variations between animals at this time. For all TJ proteins, there were no further decreases in mRNA expression from 36 h to 8 d involution.

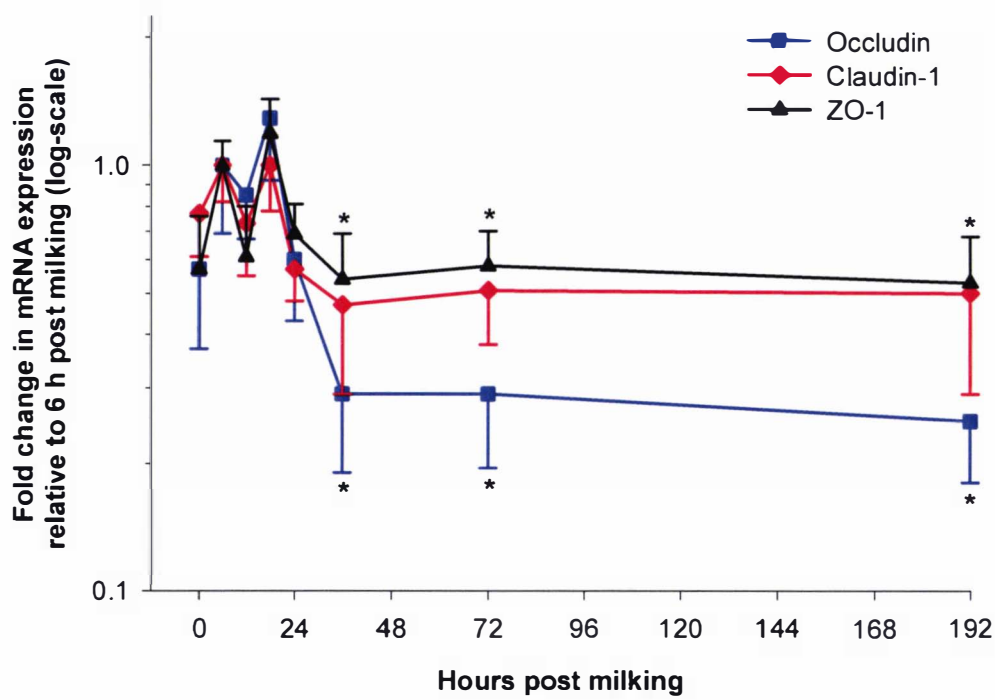


Figure 3.15. The pattern of gene expression of tight junction proteins during the engorgement of bovine mammary glands.
Changes in mRNA expression levels of occludin, claudin-1, and ZO-1 in alveolar mammary tissue of lactating cows at 0, 6, 12, 18, 24, 36, 72 and 192 h following the last milking (n=≥6 per time point). Data are expressed as back-transformed mean (± SEM) fold changes for each gene relative to the 6 h time point (* $P<0.05$).

3.3.4.2 Experiment 2 –mRNA expression of TJ proteins following short-term ODM

Gene expression of the TJ proteins: occludin, claudin-1 and ZO-1, were compared in alveolar mammary tissue taken from unilateral ODM and TDM glands (Fig. 3.16). There were no significant differences in occludin mRNA expression between ODM and TDM glands (Fig. 3.16B). However, expression of claudin-1 and ZO-1 genes increased ($P<0.05$) by 1.5-fold and 1.7-fold, respectively, in ODM glands compared with TDM glands (Fig. 3.16B). The individual cow responses to ODM were similar irrespective of the number of days in milk (Fig. 3.16A).

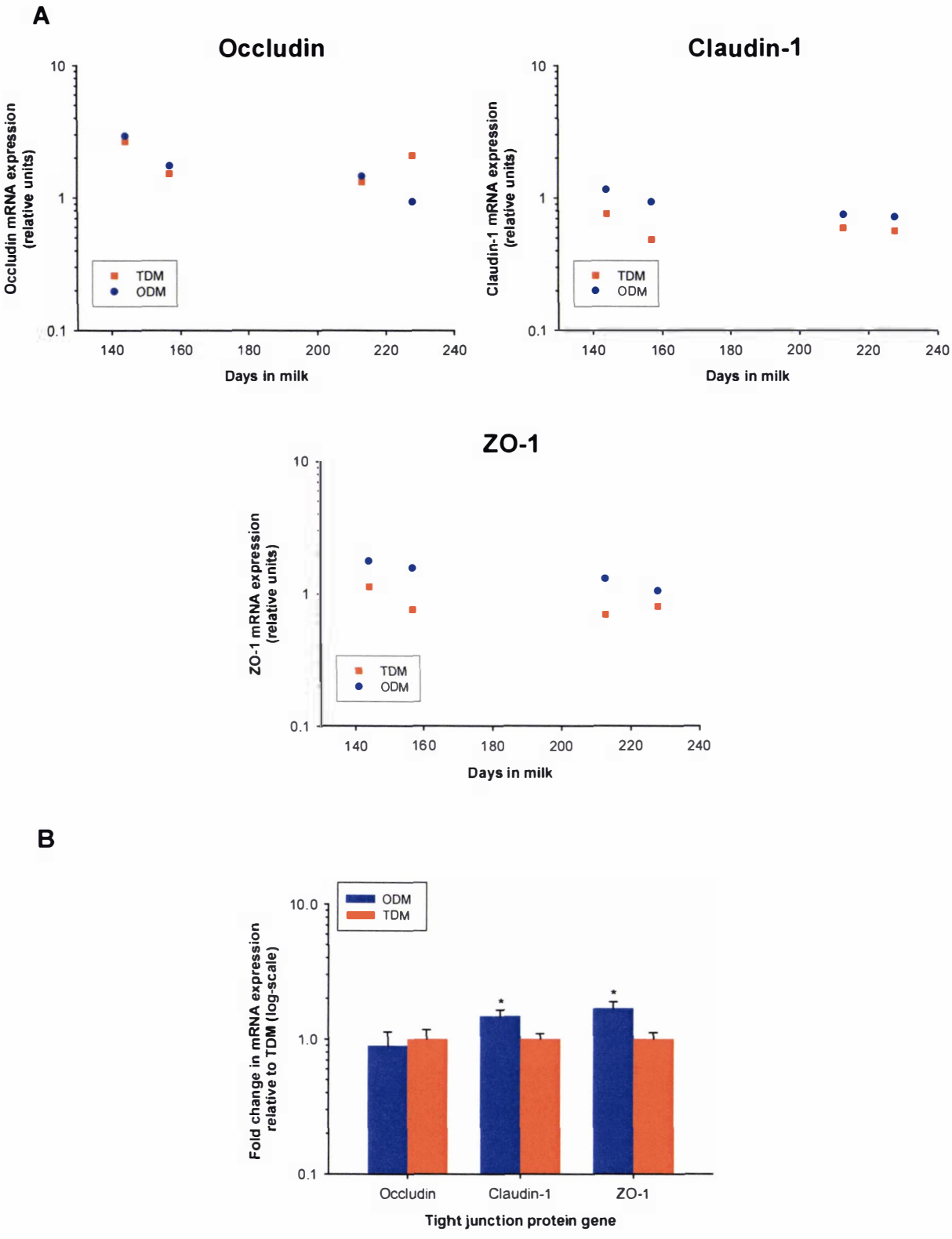


Figure 3.16. Gene expression of tight junction proteins in bovine mammary glands following short-term, unilateral, once-daily milking (ODM) in late lactation.
The mRNA expression of occludin, claudin-1 and ZO-1 was determined in alveolar mammary tissue of lactating cows milked unilaterally either once or twice daily. Data are presented as: (**A**) back-transformed relative mRNA expression levels of each gene for ODM glands and their corresponding twice-daily milked (TDM) glands for individual cows (n=4) compared with days in milk, and (**B**) back-transformed mean (+ SEM) fold changes for ODM glands (n=4) relative to TDM glands (n=4) (* $P<0.05$).

3.3.5 Western blot analysis of TJ protein expression

3.3.5.1 Location and solubility of TJ proteins

TJ proteins are partially resistant to detergent extraction due to their association with large protein complexes (i.e., TJ-actin cytoskeleton complex) and with the cell membrane. Therefore, the solubilities of bovine alveolar mammary occludin, claudin-1 and ZO-1 and their protein subcellular locations were examined (Fig. 3.17). The use of NP-40 detergent during protein extraction improved the solubility of TJ proteins. Occludin exhibited characteristic multiple-banding patterns between ~60 and ~75 kDa, consistent with phosphorylation. The higher molecular weight (MW) occludin bands were partly resistant to detergent extraction and a portion remained in the membrane rather than the cytosolic fraction. Claudin-1 migrated as bands at ~22 kDa (a doublet) and ~28 kDa. Both bands were detergent soluble, but the ~28 kDa band was less soluble than the ~22 kDa bands and remained in the membrane fraction. A weak immunoreactive signal for claudin-1 was also detected at ~60 kDa, and assumed to be the claudin-1 homodimer (results not shown). Stronger expression of the ~60 kDa band was observed in samples extracted without detergent for the soluble supernatant and cytosol rather than the insoluble pellet and membrane fractions. For ZO-1, immunoreactive bands were detected at ~225 kDa, with higher MW bands resistant to detergent extraction and primarily located in the membrane fraction, while lower MW bands were also found in the cytosolic fraction. Overall, these results confirmed that TJ proteins were partially insoluble under NP-40 detergent extraction. In particular the higher MW bands were of limited solubility and tended to be preferentially located in the membrane fraction, indicating stronger incorporation with the TJ complex.

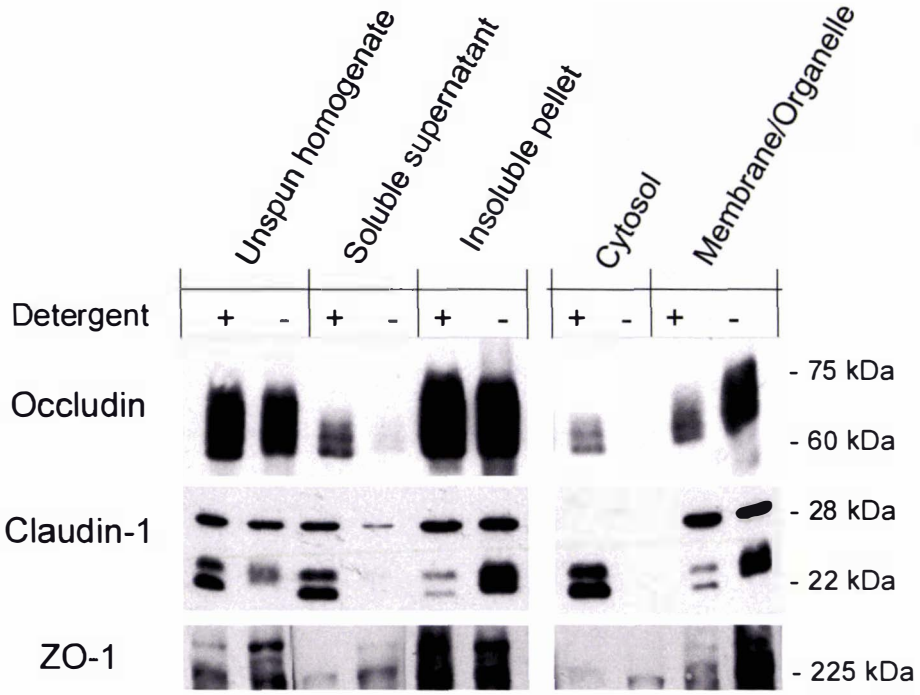


Figure 3.17. Western blots showing the solubilities of tight junction proteins to detergent extraction and their subcellular location in bovine alveolar mammary tissue.
Lactating alveolar mammary tissue was homogenised in low salt buffer with (+) or without (-) 0.5% NP-40 detergent (unspun homogenate). Aliquots of each of these homogenates were centrifuged at 10,000 x g for 5 min at 4 °C and the soluble supernatant collected. The remaining insoluble pellets were resuspended in the respective original buffer solutions. To obtain the subcellular fractions, another aliquot of each of the original homogenates was centrifuged at 400 x g for 10 min at 4 °C. The supernatants were collected and centrifuged at 100,000 x g for 1 h at 4 °C with the resulting supernatants containing the cytosolic fraction (cytosol), while the pellets were resuspended in the respective original buffer solutions, for the membrane/organelle fraction. Twenty micrograms of protein was loaded into each lane. The primary antibodies used were rabbit anti-human occludin (1:10,000 dilution), claudin-1 (1:1000 dilution) and ZO-1 (1:1000 dilution) (obtained from Zymed Laboratories Inc.).

3.3.5.2 Experiment 1 – Time course of changes in TJ protein expression post-milking

Western blotting was performed to determine the pattern of TJ protein expression in NP-40-soluble and NP-40-insoluble fractions during the early stages of mammary engorgement (up to 72 h post-milking). There were no differences ($P>0.05$) in the level of occludin expression in the soluble fraction during mammary engorgement (Fig. 3.18A). In contrast, occludin expression in the insoluble fraction had decreased by 1.5-fold ($P<0.01$) and 1.7-fold ($P<0.001$) by 36 h and 72 h post-milking, respectively, relative to 6 h (Fig. 3.18A).

Claudin-1 protein expression in the soluble fraction for the ~28 kDa band was decreased 2.8-fold by 36 h ($P<0.05$) and 3.0-fold by 72 h ($P<0.01$) relative to 6 h post-milking (Fig. 3.18B). There were no significant differences in the expression of the claudin-1 ~22 kDa band relative to 6 h post-milking (Fig. 3.18B). However, expression of the ~22 kDa band was significantly higher ($P<0.05$) at 0 h post-milking than that at 12 and 18 h post-milking. A weak signal was detected for the ~60 kDa claudin-1 homodimer, which was insufficient for densitometric analysis (results not shown).

There was a marked decline in ZO-1 protein expression in the soluble fraction by 36 h (2.0-fold decrease; $P<0.10$) and 72 h (2.7-fold decrease; $P<0.05$) of mammary engorgement relative to the 6 h time point (Fig. 3.19). The decline in ZO-1 expression in the insoluble protein fraction was variable and not significant compared with the 6 h time point, although a 2.6-fold reduction was still apparent by 72 h post-milking (Fig. 3.19). Nevertheless, ZO-1 insoluble protein expression was significantly decreased at 18 h (3.3-fold, $P<0.1$), 24 h (4.2-fold, $P<0.05$) and 72 h (5.7-fold, $P<0.05$) compared with the 0 h time point.

Even sample loading of SDS-PAGE gels was verified by Coomassie Blue staining of untransferred gels for soluble and insoluble protein fractions (Fig. 3.20), while the effective transfer of proteins onto a nitrocellulose membrane was confirmed by Ponceau S staining (results not shown).

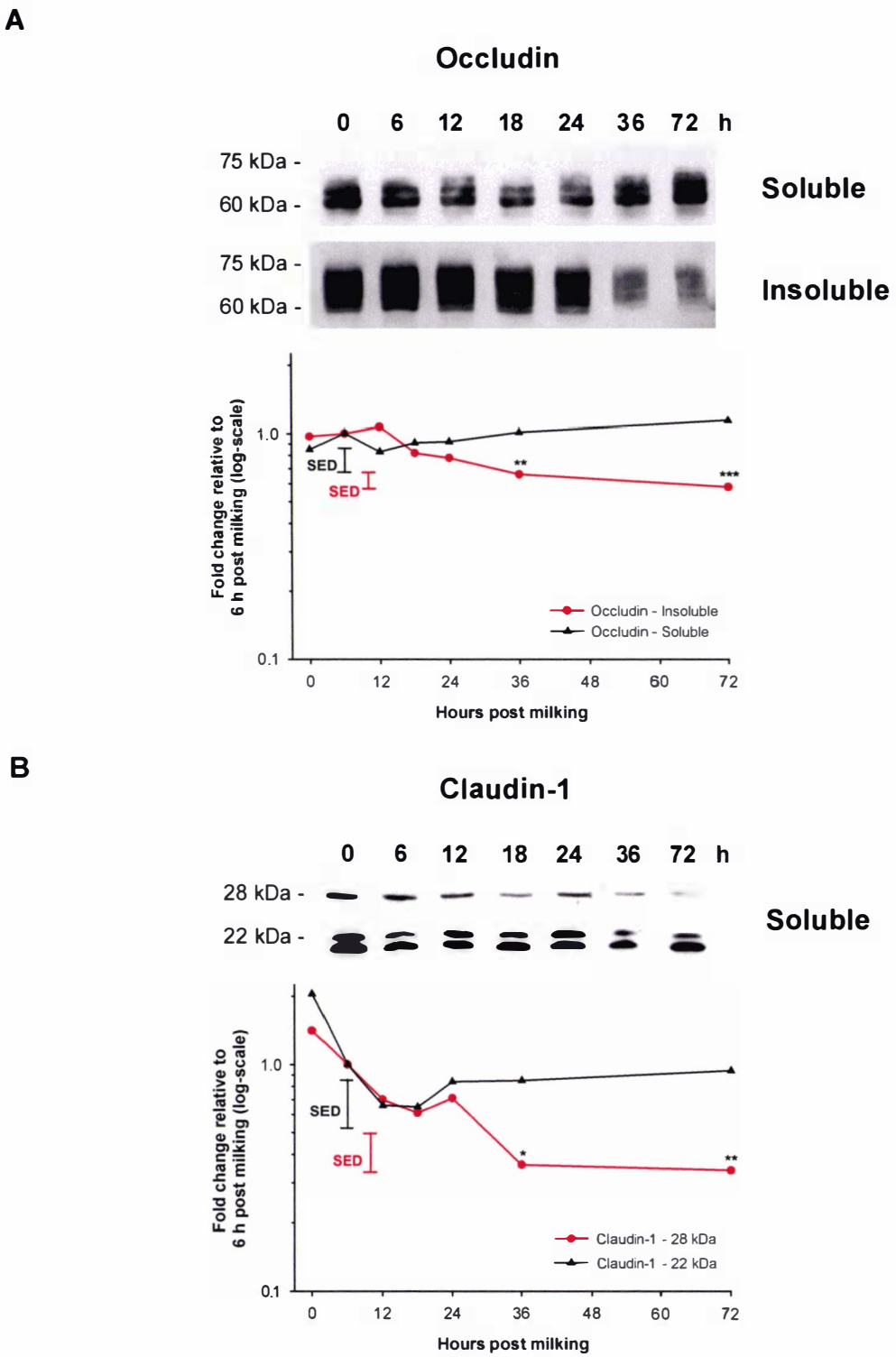


Figure 3.18. The pattern of occludin and claudin-1 protein expression during the engorgement of bovine mammary glands.

Densitometric analyses of Western blots are shown for the expression levels of: (**A**) occludin in NP-40-soluble and –insoluble protein fractions, and (**B**) claudin-1 ~28 kDa and ~22 kDa bands in NP-40-soluble protein fractions, from alveolar mammary tissue of lactating cows at 0, 6, 12, 18, 24, 36, and 72 h following the last milking (n=6 per time point). Results are graphed as back-transformed mean fold changes relative to the 6 h time point with the SED, (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). Representative Western blots are also shown, where twenty (occludin detection) or forty (claudin-1 detection) micrograms of protein was loaded into each lane. The primary antibodies used were rabbit anti-human occludin (1:50,000 dilution) and claudin-1 (1:3000 dilution) (obtained from Zymed Laboratories Inc.).

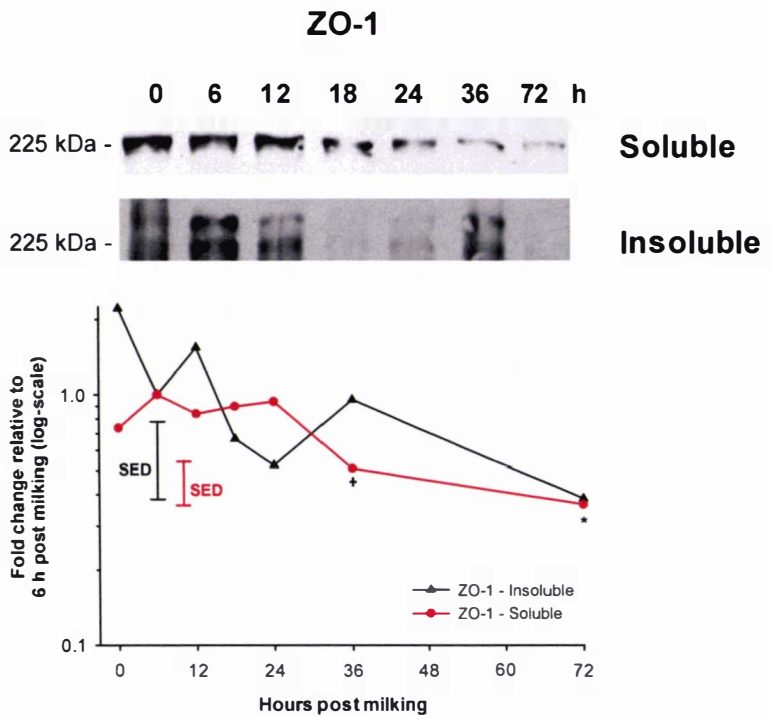


Figure 3.19. The pattern of ZO-1 protein expression during the engorgement of bovine mammary glands.

Densitometric analyses of Western blots are shown for the expression levels of ZO-1 in NP-40-soluble and –insoluble protein fractions from alveolar mammary tissue of lactating cows at 0, 6, 12, 18, 24, 36, and 72 h following the last milking (n=6 per time point). Results are graphed as back-transformed mean fold changes relative to the 6 h time point with the SED, ($^{+}$ $P<0.10$, * $P<0.05$). Representative Western blots are also shown, where twenty micrograms of protein was loaded into each lane. The primary antibody used was rabbit anti-human ZO-1 (1:1000 and 1:5000 dilutions for soluble and insoluble fractions, respectively; obtained from Zymed Laboratories Inc.).

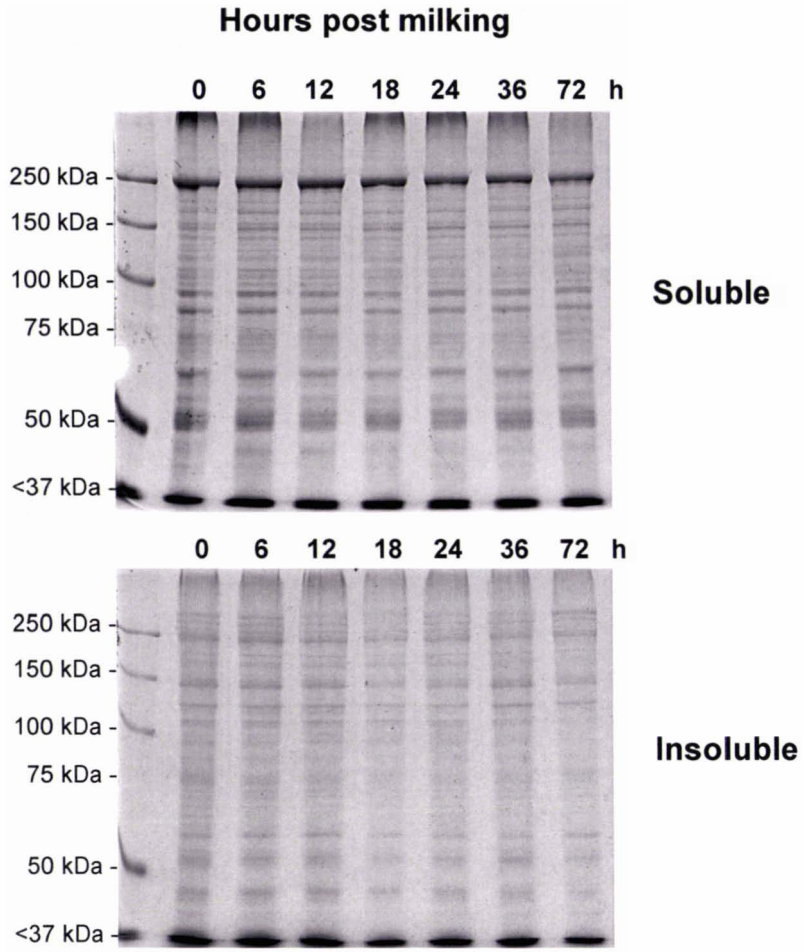
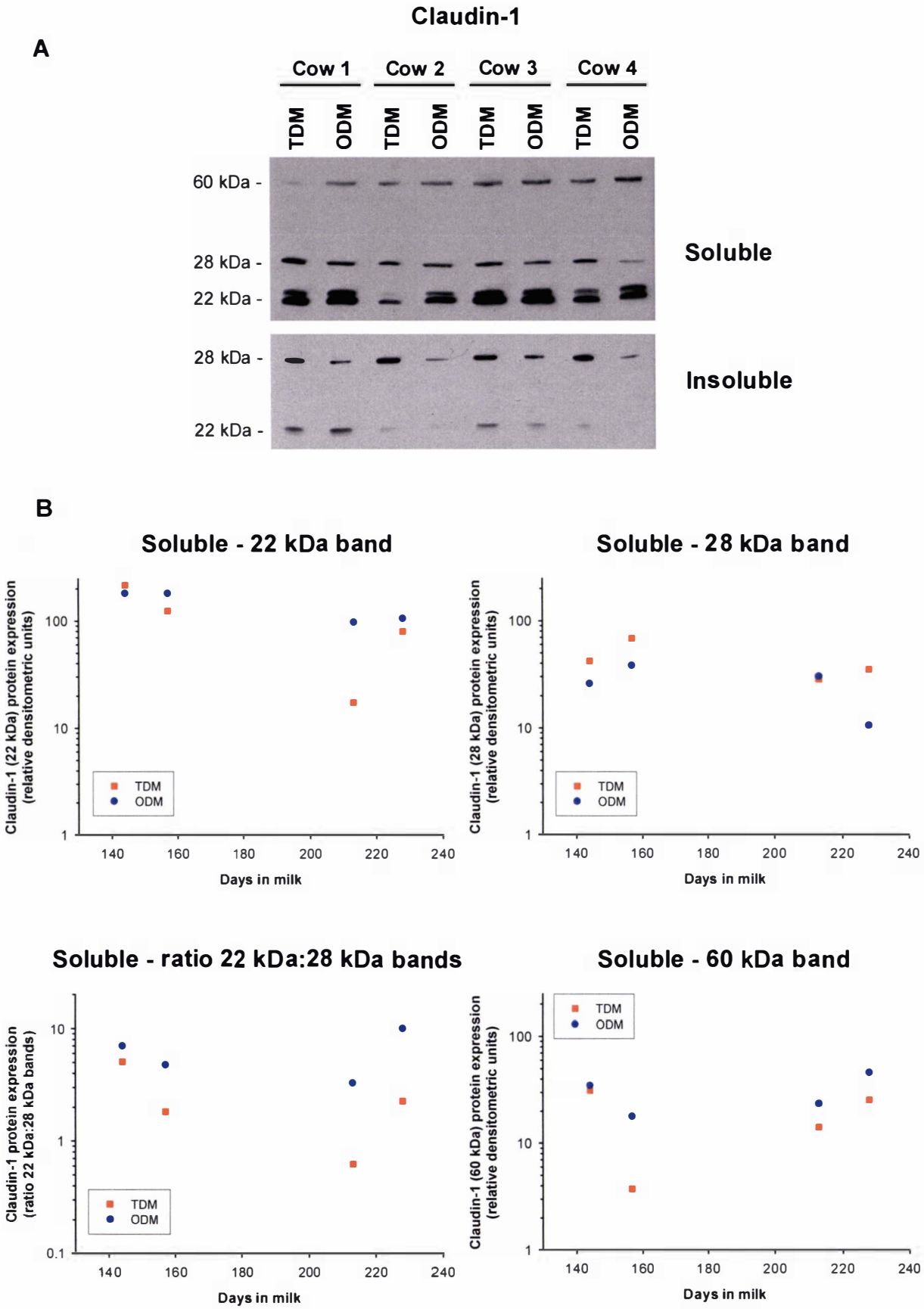


Figure 3.20. Coomassie blue stained gels loaded with protein samples from bovine mammary glands following the abrupt cessation of milking in mid-lactation.
Representative 8% SDS-PAGE gels containing NP-40-soluble and –insoluble protein fractions from alveolar mammary tissue of lactating cows at 0, 6, 12, 18, 24, 36, and 72 h following the last milking (n=6 per time point). Twenty micrograms of protein was loaded into each lane, except lane 1 which contains 5 µl of the Precision Plus Protein Standard (Bio-Rad Laboratories). Gels were not transferred and were stained with Coomassie blue for 18 h then de-stained in 25% methanol until optimal colour intensity was obtained.

3.3.5.3 Experiment 2 – TJ protein expression following short-term ODM

Western blotting was used to compare the expression of the TJ proteins, claudin-1, occludin and ZO-1, in alveolar mammary tissue taken from unilateral ODM and TDM glands (Fig 3.21, 3.22 and 3.23, respectively). Claudin-1 protein expression of the ~22 kDa band relative to the ~28 kDa band (ratio 22 kDa:28 kDa) was increased ($P<0.05$) in both soluble (3.0-fold) and insoluble (3.8-fold) fractions for ODM glands compared with TDM glands (Fig 3.21). This occurred because expression of the claudin-1 ~28 kDa band was decreased in the insoluble fraction (4.3-fold, $P<0.05$), and tended to be decreased in the soluble fraction (1.7-fold, $P=0.118$), for ODM compared with TDM glands. In comparison, expression of the ~22 kDa band was either unchanged (insoluble fraction) or slightly increased (soluble fraction) for ODM glands compared with TDM glands. Expression of the ~60 kDa homodimer followed a similar trend to the ~22 kDa band in the soluble fraction, but was not detected in the insoluble fraction (results not shown). There were no significant differences in occludin and ZO-1 protein expression between ODM and TDM glands for both soluble and insoluble fractions (Fig. 3.22 and 3.23, respectively). However, while expression of claudin-1, occludin, and ZO-1 proteins was generally lower for animals later in lactation (i.e., cows 2 and 4 compared with cows 1 and 3), differences between ODM and TDM glands tended to be greater indicating possible variability in response to ODM (Fig. 3.21A, B and C; Fig. 3.22A and B; and Fig. 3.23A and B, respectively).

Even sample loading of SDS-PAGE gels was verified by Coomassie Blue staining of untransferred gels for soluble and insoluble protein fractions (Fig. 3.24), while the effective transfer of proteins onto a nitrocellulose membrane was confirmed by Ponceau S staining (results not shown).



(Figure 3.21 is continued overleaf)

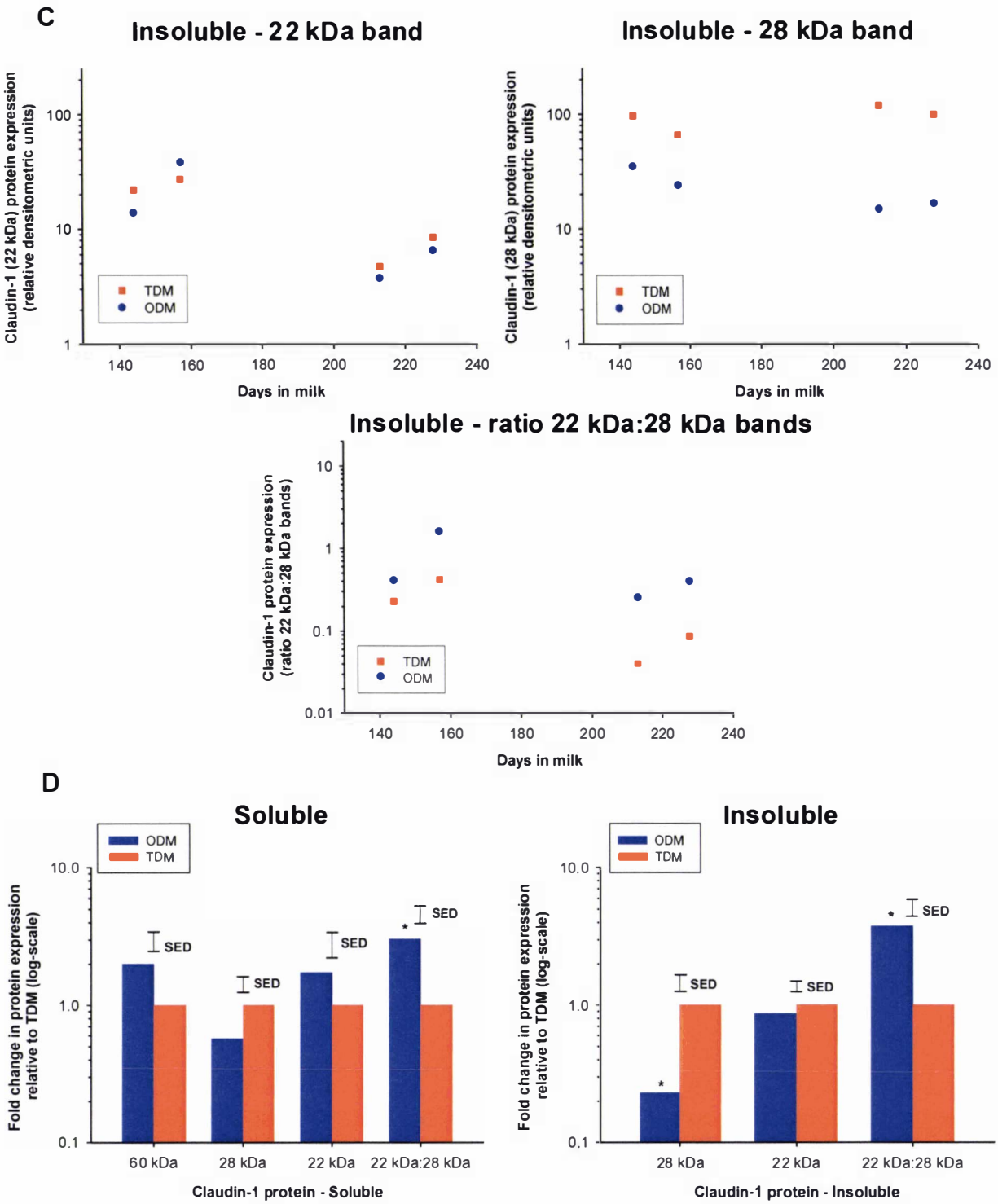


Figure 3.21. Claudin-1 protein expression in bovine mammary glands following short-term, unilateral, once-daily milking (ODM) in late lactation.

Densitometric analyses of Western blots are shown for the expression levels of claudin-1 ~60 kDa, ~28 kDa and ~22 kDa bands in NP-40-soluble and -insoluble fractions from alveolar mammary tissue of lactating cows milked unilaterally either once or twice daily. Results are presented as: (**A**) representative Western blots where twenty micrograms of protein was loaded into each lane, (**B**) back-transformed relative densitometric units for ODM glands and their corresponding twice-daily milked (TDM) glands for individual cows (n=4) compared with days in milk for soluble and (**C**) insoluble fractions, and (**D**) back-transformed mean fold changes for ODM glands (n=4) relative to TDM glands (n=4) with the SED (* $P<0.05$). The primary antibody used was rabbit anti-human claudin-1 (1:5000 and 1:10,000 dilutions for soluble and insoluble fractions, respectively; obtained from Zymed Laboratories Inc.).

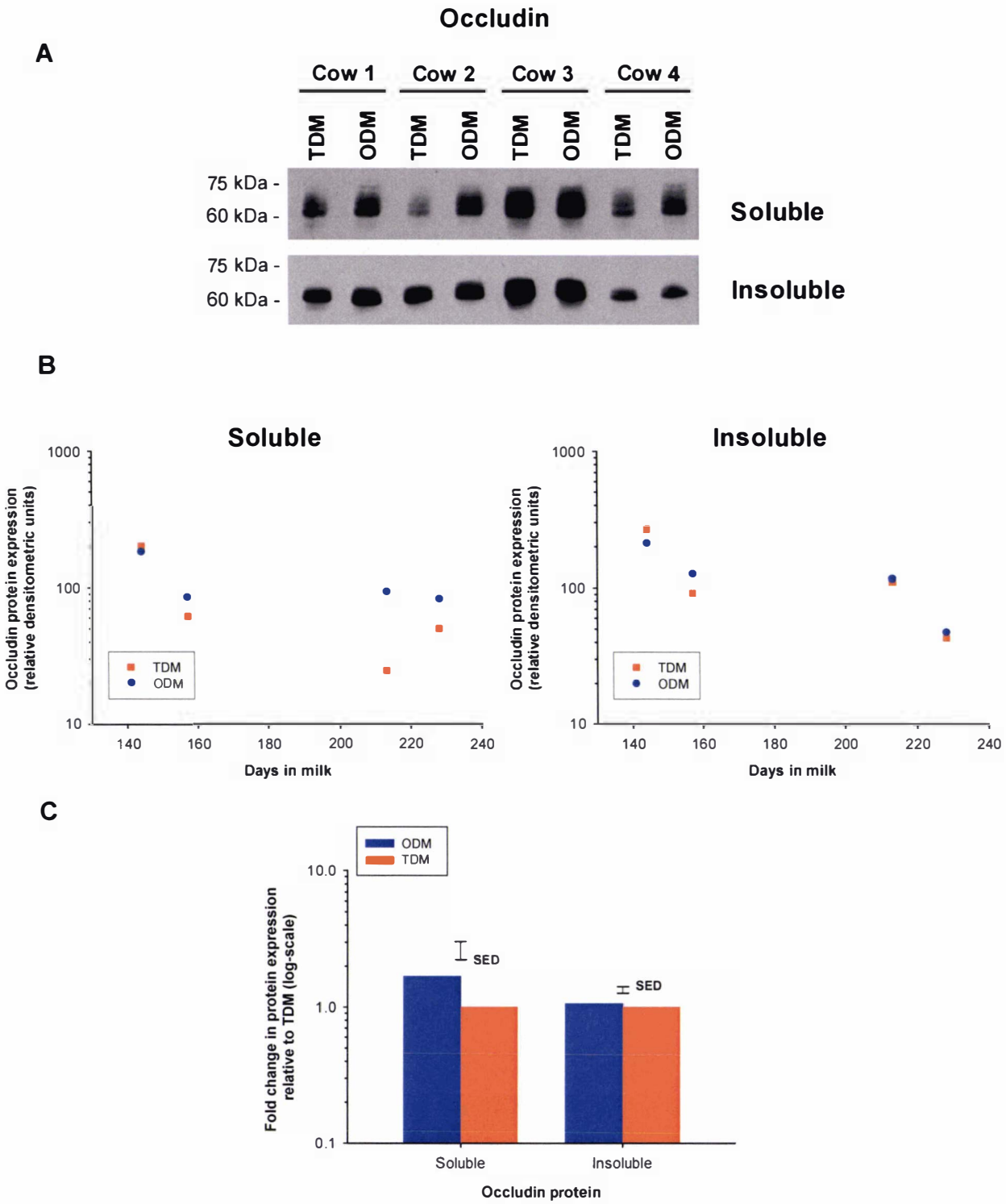


Figure 3.22. Occludin protein expression in bovine mammary glands following short-term, unilateral, once-daily milking (ODM) in late lactation.
Densitometric analyses of Western blots are shown for the expression levels of occludin in NP-40-soluble and –insoluble protein fractions from alveolar mammary tissue of lactating cows milked unilaterally either once or twice daily. Results are presented as: (**A**) representative Western blots where twenty micrograms of protein was loaded into each lane, (**B**) back-transformed relative densitometric units for ODM glands and their corresponding twice-daily milked (TDM) glands for individual cows (n=4) compared with days in milk, and (**C**) back-transformed mean fold changes for ODM glands (n=4) relative to TDM glands (n=4) with the SED. The primary antibody used was rabbit anti-human occludin (1:50,000 dilution; obtained from Zymed Laboratories Inc.).

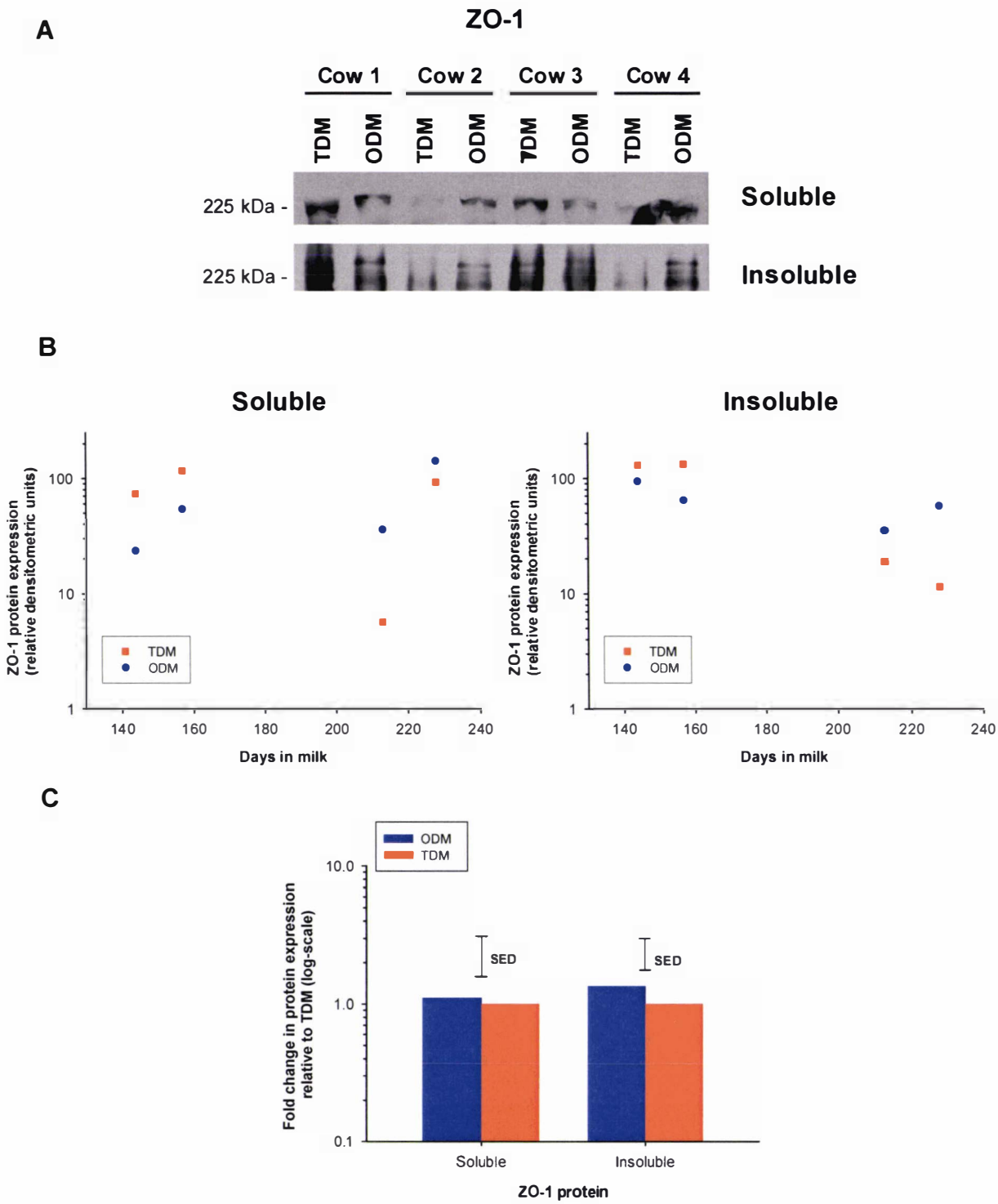


Figure 3.23. ZO-1 protein expression in bovine mammary glands following short-term, unilateral, once-daily milking (ODM) in late lactation.

Densitometric analyses of Western blots are shown for the expression levels of ZO-1 in NP-40-soluble and –insoluble protein fractions from alveolar mammary tissue of lactating cows milked unilaterally either once or twice daily. Results are presented as: (**A**) representative Western blots where twenty micrograms of protein was loaded into each lane, (**B**) back-transformed relative densitometric units for ODM glands and their corresponding twice-daily milked (TDM) glands for individual cows (n=4) compared with days in milk, and (**C**) back-transformed mean fold changes for ODM glands (n=4) relative to TDM glands (n=4) with the SED. The primary antibody used was rabbit anti-human ZO-1 (1:15,000 and 1:1000 dilutions for soluble and insoluble fractions, respectively; obtained from Zymed Laboratories Inc.).

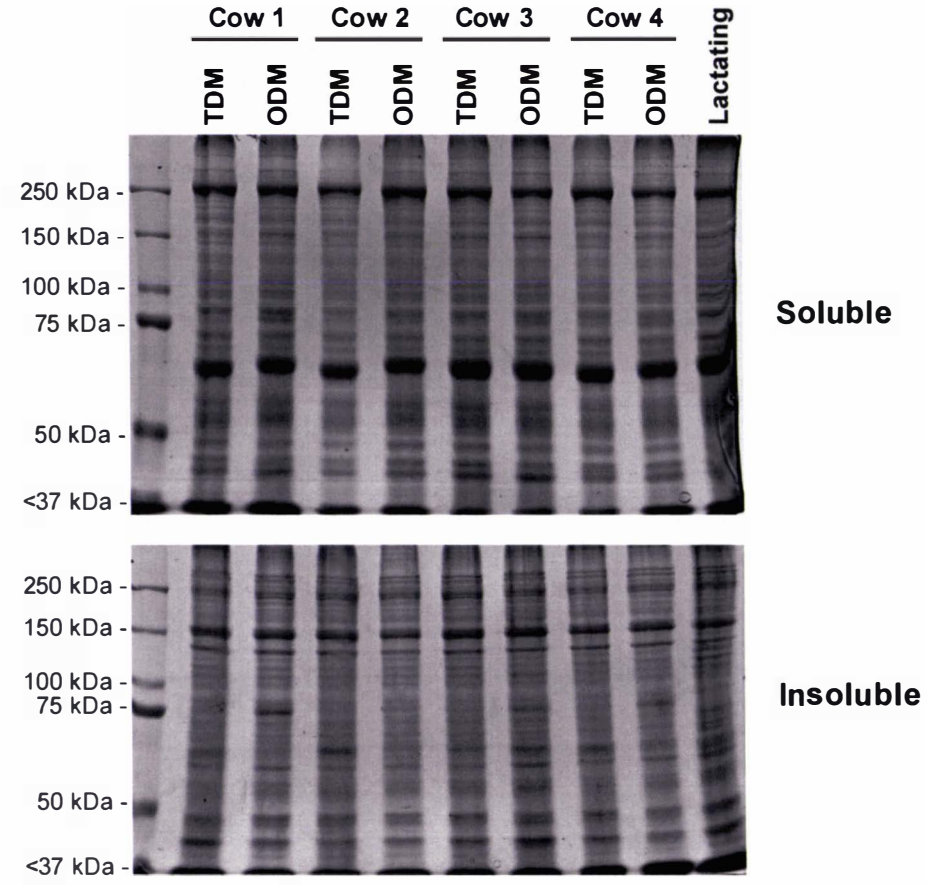


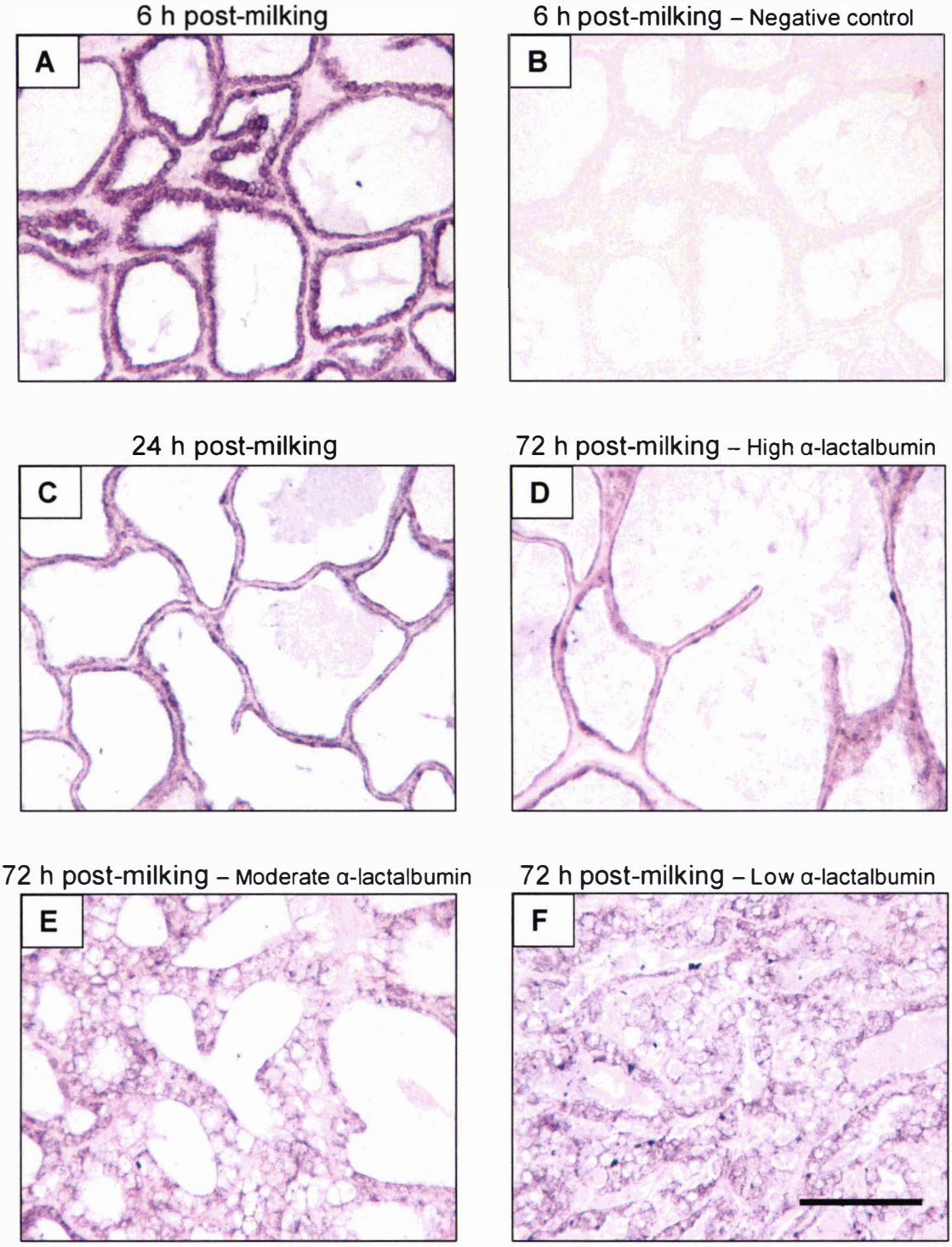
Figure 3.24. Coomassie blue stained gels loaded with protein samples from bovine mammary glands following short-term, unilateral, once-daily milking (ODM) in late lactation.
Representative 8% SDS-PAGE gels containing NP-40-soluble and –insoluble protein fractions from the alveolar tissue of ODM glands (n=4), and their corresponding twice-daily milked (TDM) glands (n=4), of each animal. A lactating mammary control sample from an animal at 6 h post milking during mid-lactation was also loaded in lane 10. Twenty micrograms of protein was loaded into each lane, except lane 1 which contains 5 µl of the Precision Plus Protein Standard (Bio-Rad Laboratories). Gels were not transferred and were stained with Coomassie blue for 18 h then de-stained in 25% methanol until optimal colour intensity was obtained.

3.3.6 Immunohistochemistry of occludin protein expression

3.3.6.1 Experiment 1 – Time course of changes in occludin protein expression and localisation post-milking

Immunohistochemistry was used for localisation of occludin protein expression in bovine alveolar mammary tissue following the abrupt cessation of milking. Lactating alveoli at 6 h post-milking (Fig. 3.25A) were strongly labelled for occludin protein on the apical and lateral membranes of epithelial cells, consistent with the location of TJ. A signal was also detected on the basal membrane, while the cytoplasm of epithelial cells was diffusely labelled. The occludin signal appeared reduced for stretched, flattened epithelial cells as alveoli became distended and engorged with milk over time (Fig. 3.25C, D and G), although a weak signal remained on the cell membranes of involuting VEA with low and moderate α-lactalbumin mRNA expression at 72 h and 8 d post-milking (Fig. 3.25E, F, H and I). While it was difficult to assess whether or not this was simply due to the distortion of cell shape during mammary engorgement, a reduction in signal is consistent with down-regulated occludin protein expression reported in section 3.3.5.2.

The cell membranes of leukocytes and sloughed epithelial cells present within alveolar lumina after 72 h to 8 d were not labelled. Furthermore, there was no signal in concurrent negative control sections where immunohistochemistry was performed either without primary antibodies to occludin (Fig. 3.25B and J), or substituted IgG at the same molar concentration (results not shown).



(Figure 3.25 is continued overleaf)

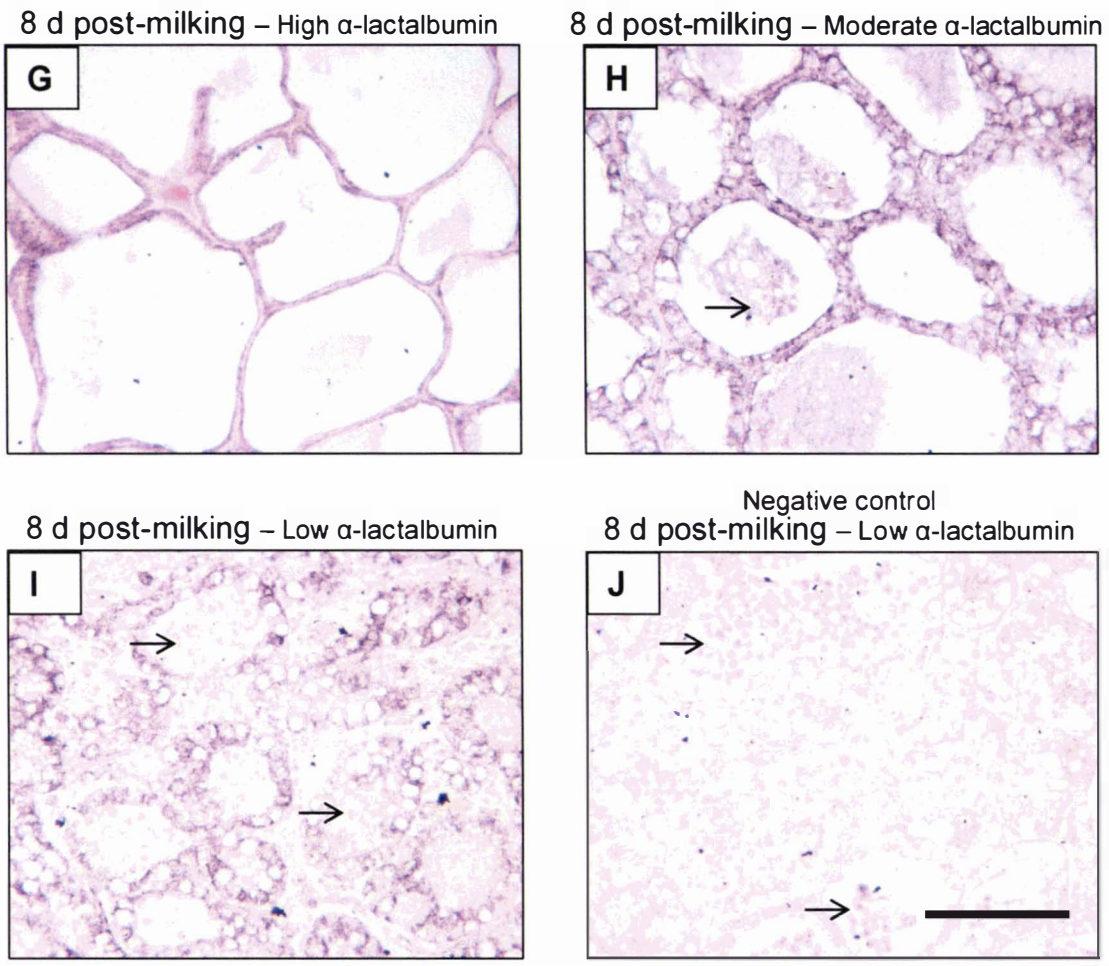


Figure 3.25. Occludin protein expression and localisation in bovine alveolar mammary tissue following the abrupt cessation of milking in mid-lactation.
Representative sections labelled with antibodies to occludin are shown at 200 x magnification for glands at 6 h (**A**, and **B** - negative control), 24 h (**C**); 72 h (**D**, **E**, **F**) and 192 h (i.e., 8 d) (**G**, **H**, **I**, and **J** - negative control) post-milking. Variation was observed between animals with high (**D**, **G**), moderate (**E**, **H**) and low (**F**, **I**) relative α-lactalbumin mRNA expression at the later time points. The omission of primary antibodies to occludin was used to provide concurrent negative controls. Sections are lightly counterstained with eosin and regions of leukocyte invasion (⟶) are shown. Scale bars are at 100 μm.

3.3.6.2 Experiment 2 – Occludin protein expression and localisation following short-term ODM

A strong signal for occludin protein was detected on epithelial cell membranes, and to a lesser extent cytoplasm, of alveoli from TDM glands (Fig. 3.26B and D). In comparison, the more heterogeneous tissue of ODM glands was strongly labelled on small to moderately open alveoli, but reduced on stretched, distended alveoli (Fig. 3.26A and C, respectively). Negative control sections had no signal for occludin protein (results not shown).

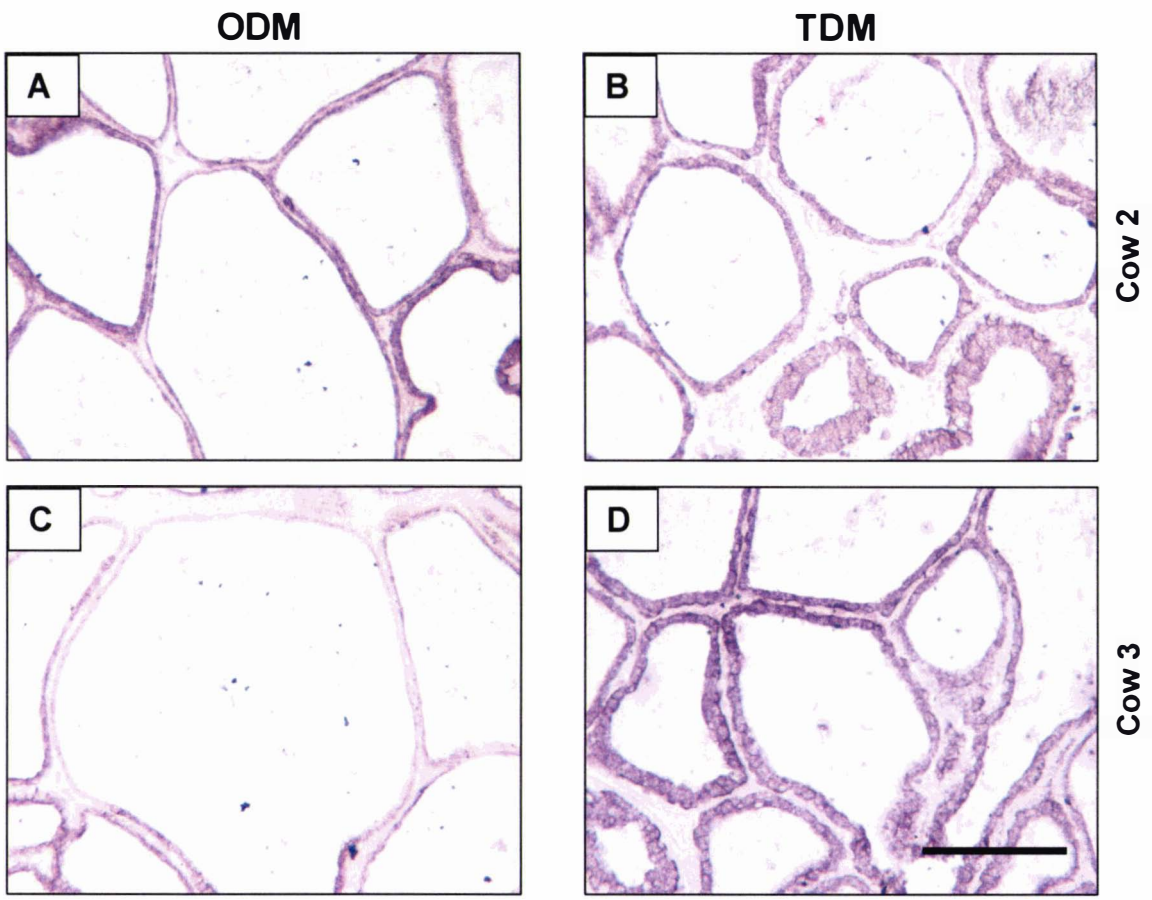


Figure 3.26. Occludin protein expression and localisation in bovine alveolar mammary tissue following short-term, unilateral, once-daily milking (ODM) in late lactation.
Representative sections labelled with antibodies to occludin are shown at 200 x magnification for ODM (**A** and **C**) and their corresponding twice-daily milked (TDM) glands (**B** and **D**, respectively). The omission of primary antibodies to occludin was used to provide concurrent negative controls (results not shown). Sections are lightly counterstained with eosin and the scale bar equals 100 µm.

3.4 DISCUSSION

In ruminant mammary glands, an increase in TJ permeability occurs during extended periods of milk accumulation and is associated with reduced milk secretion (Stelwagen *et al.*, 1994b, 1997). If the gland remains unmilked, locally-derived signals ultimately lead to the onset of mammary apoptosis and involution (Quarrie *et al.*, 1994; Wilde *et al.*, 1997). The current investigation reports temporal changes in expression of the major TJ proteins: occludin, claudin-1 and ZO-1 relative to the initiation of these pro-apoptotic processes following the abrupt cessation of milk removal. By 18 h post-milking, the alveoli were engorged with milk and evidence of reduced secretory activity was apparent as vesicles accumulated within alveolar lumens and epithelial cells from 24 h. These changes were accompanied by decreases in TJ mRNA and protein expression by approximately 36 h following the last milking. This occurred slightly after reported rises in TJ permeability at 18 h (Stelwagen *et al.*, 1997) and decreases in milk protein mRNA expression at 24 h (Molenaar *et al.*, 2004; Singh *et al.*, 2004b). However, the changes in TJ protein expression preceded the increase in MEC apoptosis at 72 h, suggesting a role for TJs during the early stages of involution.

In this regard, expression levels of mammary TJ proteins were more than halved after 36 h post-milking and these levels were maintained for at least 8 d of involution. Immunohistochemistry also showed decreased labelling of occludin protein on MEC membranes during engorgement, although a weak signal still remained for involuting alveoli at 8 d. This agrees with the results of Holst *et al.* (1987) who reported that, although TJs were still present during involution, they were less densely stained and harder to identify. Collectively these results indicate that the bovine mammary gland retains some TJ structure during the reversible phase of involution, and is supported by reports that lactation can be almost fully restored after 7 d (Dalley & Davis, 2006) and partially reinstated after 11 d (Noble & Hurley, 1999) of involution. The latter may also be explained by observations in the current study, and by others (Holst *et al.*, 1987; Molenaar *et al.*, 1996b; Wilde *et al.*, 1997), that the rapid widespread remodelling and massive apoptosis that occurs during rodent mammary involution (Strange *et al.*, 1992; Quarrie *et al.*, 1995, 1996; Marti *et al.*, 1997) is not evident in the bovine mammary gland.

However, there was considerable variation between animals in terms of the onset of apoptosis and loss of secretory activity during involution. There was very little difference in the morphology of alveolar tissue between cows, and little or no apoptosis was detected between 0 and 24 h post-milking. However, significant variation in the degree of involution was evident between cows by 36 h and even more so at 72 h and 8 d. These changes were closely related to milk protein mRNA expression and the number of ISEL apoptotic cells present. Overall, the data demonstrated that animals with a high level of milk protein mRNA expression (i.e., α-lactalbumin and α-S1-casein) exhibited the lactating phenotype and had low levels of apoptosis, while those with higher numbers of apoptotic nuclei had an engorged or involuting phenotype with decreased milk protein mRNA expression and increased expression of immune-associated genes (i.e., lactoferrin and MSAA). Inverse relationships between α-lactalbumin and lactoferrin mRNA expression have previously been identified by *in situ* hybridisation on lactating alveoli and involuting VEA (Molenaar *et al.*, 1992, 1996a).

The differences at 36 h, 72 h and 8 d indicate that individual cows differ in their rates of involution following the abrupt cessation of milk removal. Differences were also apparent within animals in the stage of involution of individual lobules and alveoli, suggesting that alveoli enter involution asynchronously. This agrees with previous reports of heterogeneity of milk protein gene expression and secretory activity among alveoli during lactation and induced or gradual involution (Mosimann, 1969; Molenaar *et al.*, 1992, 1996a; Li *et al.*, 1999). The local actions of survival factors (e.g., IGF-1), or alternatively reduced receptivity to death factors (e.g., TNF), may prevent some alveoli from progressing from a quiescent to a senescent state. These alveoli may therefore become activated upon reinstatement of milk removal (Davis *et al.*, 1999; Shorten *et al.*, 2002; Vetharaniam *et al.*, 2003).

Relationships for individual animals between the rate of involution, as measured by α-lactalbumin mRNA expression and the number of ISEL apoptotic cells, and the expression of TJ proteins were not detected in this study. However, there was an overall temporal decline in both TJ protein expression and milk protein mRNA expression (Molenaar *et al.*, 2004; Singh *et al.*, 2004b) prior to the increase in apoptosis suggesting

that although TJ protein expression may not relate to the individual rate of involution it is associated with this process.

To further examine temporal patterns of TJ expression during milk accumulation, mammary protein samples were separated into NP-40 detergent-soluble and -insoluble fractions. The resistance of TJ proteins to solubilisation in detergent-salt extractions indicates their incorporation into the TJ-cytoskeleton complex and is associated with reduced TJ permeability (Sakakibara *et al.*, 1997; Wong, 1997; Chen *et al.*, 2000). In the bovine mammary gland, occludin, claudin-1 and ZO-1 were detected in both detergent-soluble and -insoluble fractions. The higher molecular mass of the insoluble protein fractions for occludin and ZO-1 is consistent with their phosphorylated forms enriched in TJs (Sakakibara *et al.*, 1997; Wong, 1997; Chen *et al.*, 2000; Li & Mrsny, 2000). Occludin protein expression declined 1.7-fold in the insoluble fraction, but there was no significant change in the soluble fraction during mammary engorgement. A decline (~2.5-fold) in ZO-1 protein expression was detected in both fractions during mammary engorgement, while the ~28 kDa claudin-1 band was reduced by 3-fold in the soluble fraction. Furthermore, reduced levels of TJ protein expression are associated with TJ disruption in other cell types (Li & Mrsny, 2000; Tian & Phillips, 2002; Ma *et al.*, 2004). Conversely, increased levels of occludin and ZO-1 proteins have been reported during decreased TJ permeability in response to glucocorticoids and/or prolactin in mouse mammary epithelial cells *in vitro* (Singer *et al.*, 1994; Woo *et al.*, 1996; Stelwagen *et al.*, 1999).

However, the down-regulation of TJ proteins by 36 h of milk accumulation in the present study does not coincide with the reported rise in TJ permeability at 18 h (Stelwagen *et al.*, 1997). The evidence for impaired TJ integrity at this time comes from an increase in concentrations of milk components (e.g., lactose and α-lactalbumin) in plasma, coupled with a reciprocal increase in concentrations of blood components (e.g., serum albumin and sodium) in milk (Stelwagen *et al.*, 1997). It is assumed that these changes are caused via an increased permeability of the paracellular pathway which is regulated by TJs. However, a rapid increase in intramammary pressure also occurs within 16-18 h of milk accumulation (Davis *et al.*, 1999) and does not dramatically decline, along with mammary volume, until 3 to 7 d following the abrupt cessation of

milk removal (Mackenzie, 1968; Fleet & Peaker, 1978; Hurley, 1989), suggesting a massive loss of TJ integrity at that time. Therefore, the increase in plasma lactose and milk albumin concentrations at 18 h may reflect an intramammary pressure differential where small molecules move by conductance between the milk and intercellular fluid compartments. In this regard, TJs are selectively permeable to certain solutes on the basis of charge and size (reviewed by Aijaz *et al.*, 2006). While this study does not provide direct evidence that TJ breakdown occurs at 18 h, the changes in TJ protein expression preceded the massive drop in intramammary pressure and volume at 3 to 7 d (Mackenzie, 1968; Fleet & Peaker, 1978; Hurley, 1989), indicating that some loss of TJ integrity may occur before this time point.

Furthermore, other TJ components may be down-regulated earlier than the proteins that were studied here. There are over 20 members of the claudin family (Tsukita *et al.*, 2001), and numerous other scaffolding and signalling proteins linked to the TJ (reviewed by Matter & Balda, 2003; Schneeberger & Lynch, 2004; Aijaz *et al.*, 2006) which may fulfil this function. However, occludin, claudin-1 and ZO-1 are the best characterised, with key roles in the formation and maintenance of TJ complexes attributed to these proteins (reviewed by Schneeberger & Lynch, 2004; Aijaz *et al.*, 2006). An alternative explanation is that changes in phosphorylation states and/or the cellular location of TJ components are more important than protein levels *per se*. It is possible that during mammary engorgement decreased TJ protein and mRNA levels are the next step following initial changes in signal transduction occurring within 18 h post-milking.

Therefore, the down-regulation, at both gene and protein levels, of occludin, claudin-1 and ZO-1 after ~36 h of milk accumulation would contribute to the loss of TJ integrity, postulated to be initiated by stretch-induced mechanotransduction in the MECs. The response also appears to be dependent upon the duration and magnitude of engorgement as, in contrast to extended milk accumulation, short-term ODM, where alveolar distension is relieved by milk removal every 24 h, caused increases in claudin-1 and ZO-1 mRNA expression. While the differences between ODM and TDM glands in this experiment are potentially confounded with time since last milking (26 h vs 18 h, respectively), the increase in claudin-1 and ZO-1 mRNA expression in ODM glands

suggests evidence of TJ repair as the mammary gland adjusts to the reduced milking frequency. Indeed, the dramatic increases in plasma lactose and α-lactalbumin concentrations that indicate loss of TJ integrity during the initial 24 h of ODM are reduced significantly with each successive milking, implying that the barrier function may be partially restored (Stelwagen *et al.*, 1997) or that the intramammary pressure differential is reduced during ODM. Increased occludin protein expression, presumably to enhance TJ synthesis and repair, was also reported in response to TJ breakdown by low-calcium conditions in mouse mammary cell lines *in vitro* (Stelwagen & Callaghan, 2003).

The mechanisms driving these changes in TJ protein expression have yet to be determined. However, evidence of a local mechanism is supported by experiment 2, where changes in TJ protein expression were accompanied by a 24% reduction in milk yield in the ODM glands only. This milk production loss is typical of those reported previously in late lactation (Davis *et al.*, 1999). A local mechanotransduction pathway may involve signalling to and from the TJ complex by ZO-1. This protein is known to participate in signal transduction in other epithelia (Balda & Matter, 2000; Meyer *et al.*, 2002), and has been demonstrated to participate in signalling events initiated by glucocorticoid-mediated decreases in TJ permeability in mouse MECs (Singer *et al.*, 1994) and rat mammary epithelial tumor cells (Woo *et al.*, 1999; Rubenstein *et al.*, 2003). Furthermore, this mechanism may be related to loss of the integrin survival signal from the ECM during rat (McMahon *et al.*, 2004, Appendix VII) and bovine (Singh *et al.*, 2004a; 2005, Appendix VI) mammary involution, through a common link via the actin cytoskeleton.

In conclusion, this study demonstrated that milk accumulation in bovine mammary glands resulted in down-regulated expression of the transmembrane TJ proteins occludin and claudin-1, as well as the structural TJ protein, ZO-1. However, the temporal relationship between these changes and loss of TJ integrity is unclear. Therefore, further work is required to determine the mechanisms by which occludin, claudin-1 and ZO-1 are regulated during mammary engorgement. This is the focus of subsequent chapters in this thesis.

CHAPTER FOUR

Tight junction protein expression and apoptosis in engorged rat mammary glands

4.1 INTRODUCTION

Extended periods of milk accumulation result in reduced milk secretion, increased apoptosis and eventually, involution of mammary glands (as described in Chapter 3). This process is associated with increased TJ permeability between MECs (Stelwagen *et al.*, 1997; Stelwagen, 2001) and down-regulated expression of the major TJ proteins: occludin, claudin-1 and ZO-1 in bovine mammary glands (refer to Chapter 3). Recent studies within our group have reported loss of cell-ECM survival signalling through β1-integrin and FAK during bovine (Singh *et al.*, 2004a; 2005, Appendix VI) and rat (McMahon *et al.*, 2004, Appendix VII) mammary engorgement. However, the role of cell-cell communication by TJ proteins is not well understood during mammary involution and further research may provide insights into the molecular mechanisms regulating these processes.

The rodent mammary gland provides an excellent model of tightly co-ordinated control of mammary apoptosis and involution. Induced involution of rodent mammary glands is well-characterised and occurs in two distinct stages; an initial apoptotic stage that begins within 12 h, and a second stage commencing from 72 to 96 h involving further apoptosis, ECM degradation and remodelling of lobular-alveolar structures (Jaggi *et al.*, 1996; Lund *et al.*, 1996; Li *et al.*, 1997a). During the first stage, local mammary-derived signals induce rapid reductions in milk synthesis and secretion and initiate apoptosis even in the presence of systemic galactopoietic hormones (Li *et al.*, 1997a). However, maintenance of systemic hormone levels by suckling prevents teat-sealed glands from entering the second stage of involution, suggesting that they act as survival factors (Li *et al.*, 1997a). The first stage lasts up to 72 h in rodents, but is described as being reversible up to 48 h and is irreversible thereafter due to the commitment of MEC to secretory de-activation, terminal apoptosis and remodelling as the mammary gland

enters the second stage of involution (Jaggi *et al.*, 1996; Li *et al.*, 1997a; McMahon *et al.*, 2004, Appendix VII).

The primary signal triggering this involution process is unknown but may relate to the presence in milk of regulatory factors, such as FIL (Wilde *et al.*, 1995), serotonin (Matsuda *et al.*, 2004), or casein hydrolysates (Shamay *et al.*, 2002, 2003). Alternatively, physical distension of the mammary gland results in changes in cell shape (Richardson, 1947) which may activate mechanotransduction pathways, resulting in loss of TJ integrity and cell-ECM survival signalling (Davis *et al.*, 1999; Stelwagen, 2001).

Dramatic changes in gene expression occur during mammary involution resulting in the progressive loss of cell survival factors and gain of pro-apoptotic signals (reviewed by Green & Streuli, 2004; Singh *et al.*, 2004b). However, the relationship of these changes to the disruption of TJ integrity during involution and the role of TJ proteins in the mammary gland is unclear.

The major transmembrane TJ protein, occludin, may play a vital role in TJ formation and closure during lactogenesis as occludin -/- mice have impaired lactation and fail to suckle their young (Saitou *et al.*, 2000). This is supported by observations *in vitro* that occludin and ZO-1 proteins are up-regulated during TJ formation in response to lactogenic hormones (glucocorticoids and/or prolactin) (Singer *et al.*, 1994; Stelwagen *et al.*, 1999). The roles of these proteins during the TJ breakdown associated with milk accumulation and involution warrant further investigation, as truncation of occludin induces MEC apoptosis both *in vitro* and *in vivo* (Beeman & Neville, 2001). Therefore, this study investigated the temporal expression of three major TJ proteins; occludin, claudin-1 and ZO-1 relative to the onset of apoptosis during the engorgement of rat mammary glands.

4.2 MATERIALS AND METHODS

4.2.1 Animals and tissue collection protocols

Rat mammary tissues were collected and kindly provided by the Dairy Science and Technology Group, AgResearch Ruakura using the following protocol described by McMahon *et al.* (2004), Appendix VII. Briefly, litter sizes of female Sprague-Dawley rats were restricted to nine pups at parturition (d 1 lactation). At peak lactation (d 16), thirty-six rats were randomly allocated to six groups (n = 6 per group). Each rat had three adjacent teats (one abdominal and two inguinal) sealed with surgical adhesive (Loctite 454 gel PRISM, Loctite Australia Pty. Ltd.) to induce mammary engorgement. Three unsealed abdominal and inguinal teats on the contralateral side were suckled by pups and served as controls. Dams were killed by carbon dioxide gas and cervical dislocation at 0, 6, 12, 18, 24, and 36 h after sealing teats. Mammary tissue was collected post-mortem for each treatment and frozen on liquid nitrogen before storage at -80 °C as outlined in section 2.2.1.2. A 5 mm thick slice through an abdominal gland was also collected for each treatment and fixed for 24 h in 4% paraformaldehyde for histological analyses as described in section 2.2.1.2.

4.2.2 Histological analysis

Formalin-fixed tissue slices were processed through a range of alcohol washes and then embedded in paraffin wax before mounting onto slides and staining with H&E for histological analyses (see sections 2.2.2.1 and 2.2.2.2). The histological features were noted for each slide and a representative area was photographed under 100 x and 400 x magnifications using a ProgRes C14 digital camera (JENOPTIK Laser) and Paint Shop Pro 7.02 software (Jasc Software Inc.).

4.2.3 ISEL of apoptotic nuclei

ISEL was performed on suckled (control) and teat-sealed (engorged) mammary sections from 3 representative animals as described in section 2.2.2.3. Qualitative and quantitative analysis of the number of positive ISEL nuclei was carried out for each section as described in section 2.2.2.3. Briefly, ISEL nuclei were identified as either located within the secretory epithelial layer or the lumen of mammary alveoli. Each count of ISEL apoptotic nuclei per 100 x magnification field was incremented by 1 (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 apoptotic nuclei per alveolus.

4.2.4 Real-time RT-PCR

Total RNA was extracted from a 150 mg aliquot of ground mammary tissue using TRIzol (Invitrogen) and 1 μg was treated with 1 U DNaseI (Invitrogen) (sections 2.2.3 and 2.2.4). Samples were purified through RNeasy gel columns (QIAGEN Sciences) and converted to cDNA using the SuperScript II Reverse Transcriptase First-Strand Synthesis System kit (Invitrogen) (section 2.2.4). cDNA products were diluted 5-fold in TE (equivalent to approximately 10 ng/μl reversely transcribed total RNA) and then samples (1 μl) were assayed in duplicate, by quantitative real-time RT-PCR with SYBR Green I Chemistry using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) as described in section 2.2.5. cDNA samples were assayed on a 384-well optical plate (Applied Biosystems), which included an assay for an endogenous control gene (ubiquitin) as well as primers for the target gene of interest (i.e., occludin or ZO-1). Ubiquitin was selected as a more suitable endogenous control than β-actin during rat mammary engorgement, as described in section 12.1.2.1, Appendix IV. The sequences of the primer sets and other relevant details are outlined in Table 4.1. Primers were used at a final concentration of 300 nM in each 15 μl real-time reaction. A standard curve of serial dilutions of rat mammary cDNA template, as well as RT-negative and 'no-template' control reactions were included on each plate for each primer pair.

Real-time PCR was performed under the following conditions: 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, 56 °C for 30 s, 72 °C for 30 s and 78 °C for 10 s. The C_T values generated for each real-time PCR reaction were used to quantify the relative abundance of each gene using the relative standard curve method (Applied Biosystems) as described in section 2.2.5.4. The real-time PCR efficiency rates per cycle for investigated genes were: occludin (E = 1.81), ZO-1 (E = 1.89), β-actin (E = 1.76) and ubiquitin (E = 1.85). Dissociation curve analysis and gel electrophoresis (section 2.2.5.3) of amplified products confirmed the specificity of real-time reactions. PCR products were then QIAquick column-purified (QIAGEN Sciences) and either submitted directly (ubiquitin, occludin and ZO-1), or firstly cloned into the pGEM-T Easy vector (β-actin) as detailed in section 2.2.6, for DNA sequencing which verified their authenticity (Waikato DNA Sequencing Facility) (results not shown).

Table 4.1. Sequences of PCR primers (forward and reverse), primer position and PCR product sizes of rat nucleic acid sequences used for investigating gene expression by real-time RT-PCR.

Gene	Nucleic acid sequence	Primer	Primer sequence[1] (5'→3')	Primer position[2] (bp)	Product size (bp)
β-actin	Genbank accession no. NM_031144	Forward	CGT ACC ACT GGC ATT GTG AT	439	206
		Reverse	TTC TCT TTA ATG TCA CGC AC	644	
Ubiquitin	Genbank accession no. NM_138895	Forward	CCG GCA AGA CCA TCA CCC TA	89	170
		Reverse	TGA CTC TTT CTG GAT GTT GTA G	258	
Occludin	Genbank accession no. AB016425	Forward	CGG GAA TGT CAA GAA CGA GAA	1056	595
		Reverse	ACG GAC AAG GTC AGA GGA A	1650	
ZO-1	Genbank accession no. XM_218747	Forward	GAG TGA AGG CAA TTC CGT ATC	4863	295
		Reverse	TGG CAG AAG ATT ATG GTT GAA C	5157	

[1] Primer sequences for detection of ubiquitin, β-actin and ZO-1 were designed using the VectorNTI Suite 7 software package (InforMax Inc.), while the GCG Wisconsin Package (Version 10.3; Accelrys Inc) was used for occludin.
[2] Refers to the 5' position of the primers in the nucleic acid sequence.

4.2.5 Western immunoblotting

Protein extracts were prepared from aliquots of ground mammary tissue as described in section 2.2.7, and proteins separated by electrophoresis on either 8%, 15% or 7% SDS-PAGE gels for occludin, claudin-1 or ZO-1 detection, respectively (refer to section 2.2.8.1). Samples from the control and engorged glands of each animal were randomly allocated to one of six gels so that each time point was represented once on each gel. Separated proteins were then transferred, using either semi-dry (occludin and claudin-1 detection) or wet (ZO-1 detection) blotting systems, onto nitrocellulose membranes and probed with primary antibodies to rabbit anti-human occludin, claudin-1 and ZO-1 (obtained from Zymed Laboratories Inc.) (section 2.2.8.2). 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).

4.2.6 Immunohistochemistry

Immunohistochemistry with primary antibodies to rabbit anti-human occludin (1: 1000 dilutions; Zymed Laboratories Inc.) was performed on representative control and engorged mammary sections as described in section 2.2.9.

4.2.7 Data and statistical analyses

Data were analysed by ANOVA in GenStat (releases 6.1, 7.1 and 8.1; Lawes Agricultural Trust, 2002, 2003 and 2005), with blocking on animal to detect differences between control and engorged 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 100 x magnification field. Relative quantification of mRNA expression following real-time PCR was performed using the standard curve method (Applied Biosystems) as described in section 2.2.5.4. The amount of target gene in each sample was normalised to the amount of ubiquitin to control for the initial concentration of cDNA, and the resulting

values $\log_{10}$-transformed for statistical analysis. Densitometry results from western blotting were also $\log_{10}$-transformed and adjusted for between gel variations. The levels of mRNA and protein expression were then expressed as back-transformed mean relative units. Data are presented as means for the control and engorged glands at each time point with the SED between means. The least significant differences identify the means significantly different from each other (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). Correlations between the qualitative and quantitative scores of ISEL apoptotic nuclei were performed in Minitab (Minitab Release 14.20, 2005; Minitab Inc., USA).

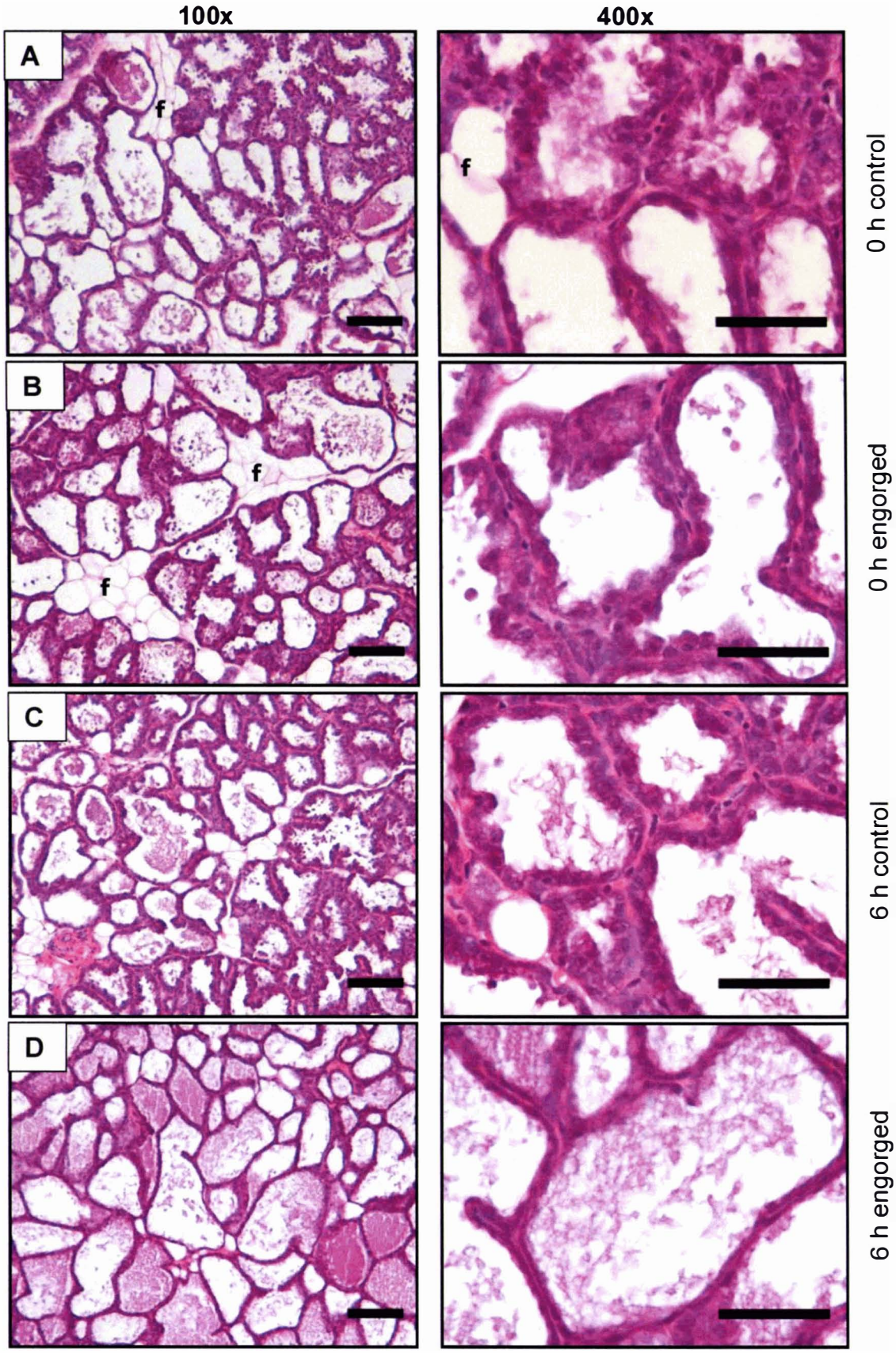
4.3 RESULTS

4.3.1 Histological morphology

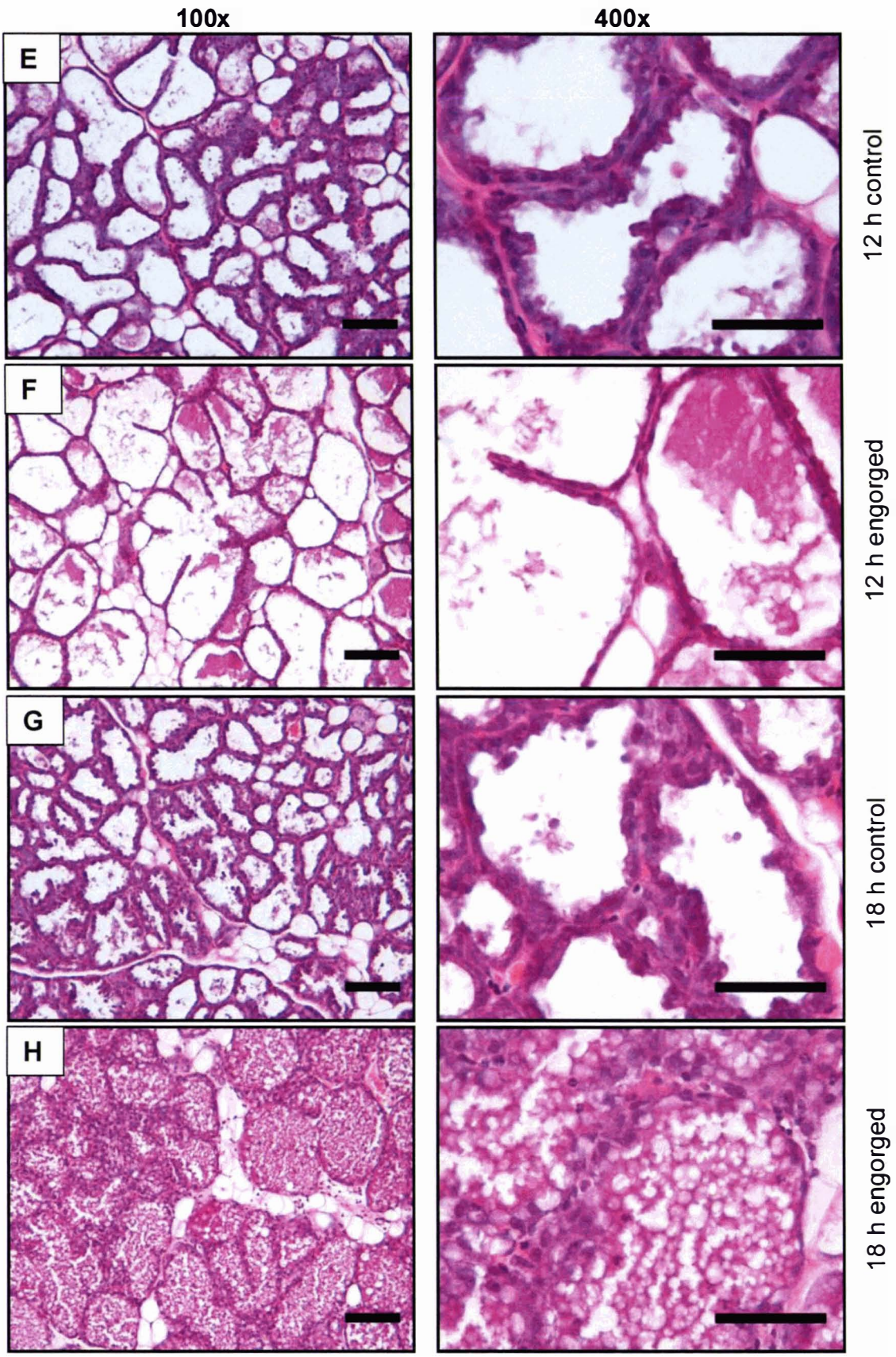
4.3.1.1 Time course of morphological changes during rat mammary engorgement

Representative H&E stained tissue sections from control and engorged rat mammary glands are shown in Figure 4.1. The suckled mammary glands (Fig. 4.1A, B, C, E, G, I, and K) at peak lactation were dominated by lobules of secretory alveolar epithelium, interspersed with a few small areas of adipocytes. Alveoli consisted of a single layer of cuboidal epithelial cells attached to the basement membrane. The alveolar lumina were lightly stained for milk secretions and a few sloughed epithelial cells were also present in some lumina. The alveolar size and shape depended upon the degree of luminal milk accumulation and varied both within and between lobules. Furthermore, the histological and gross morphology of non-sealed controls did not change during the course of the experiment, confirming that they were suckled by pups.

In contrast, the alveoli of teat-sealed glands were distended with milk secretion within 6 h (Fig. 4.1D) and reached a maximal luminal size by 12 h (Fig. 4.1F), resulting in flattening of the secretory epithelium. By 18 h (Fig. 4.1H) following teat-sealing, alveolar lumina were heavily engorged with milk vesicles (i.e., coalescing milk fat globules and proteins) and contained increased numbers of leukocytes and sloughed epithelial cells. However, it was difficult to discriminate between leukocytes and apoptotic bodies in these sections under light microscopy. The accumulation of vesicles within epithelial cells was also apparent by 24 and 36 h following teat-sealing (Fig. 4.1J and L) and in some areas alveoli were starting to collapse. These histological changes are compatible with increased mammary gland weight following teat-sealing where a maximum mass was reached by 12 h, after which time the mass of glands declined although they were still heavier than control glands at 36 h (as reported by McMahon *et al.*, 2004; Appendix VII).



(Figure 4.1 is continued overleaf)



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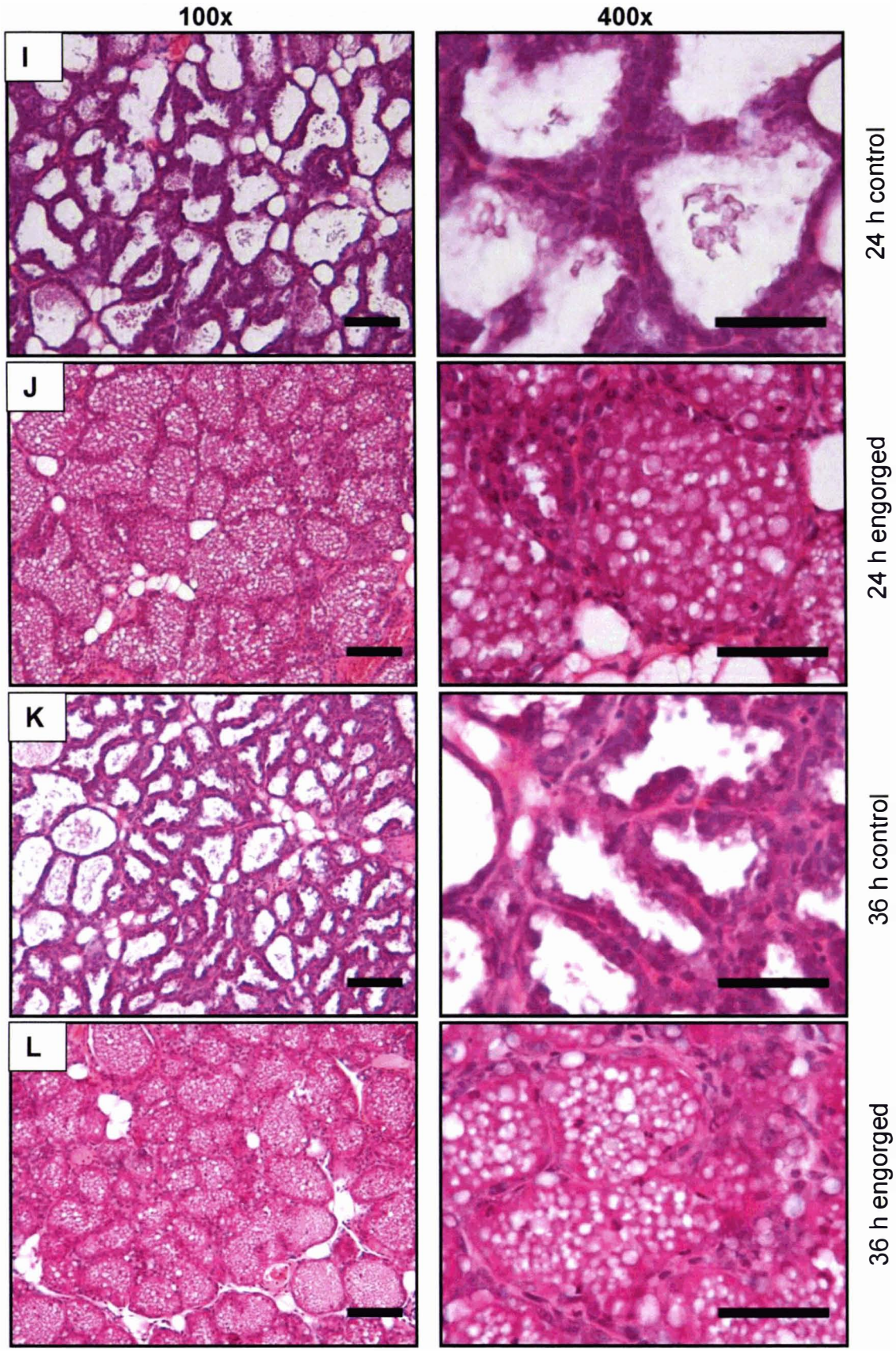


Figure 4.1. Morphological changes in rat mammary glands following teat-sealing induced mammary engorgement.

Representative H&E stained sections are shown for suckled (control) glands and corresponding teat-sealed (engorged) glands at 0 h (**A, B** – examples of fat cells (f) are shown); 6 h (**C, D**); 12 h (**E, F**); 18 h (**G, H**); 24 h (**I, J**); 36 h (**K, L**), respectively. Refer to the text for a detailed description of histological features. Scale bars are at 100 μm and 50 μm for 100 x and 400 x magnification, respectively.

4.3.2 ISEL of apoptotic nuclei

4.3.2.1 Time course of the onset of apoptosis during rat mammary engorgement

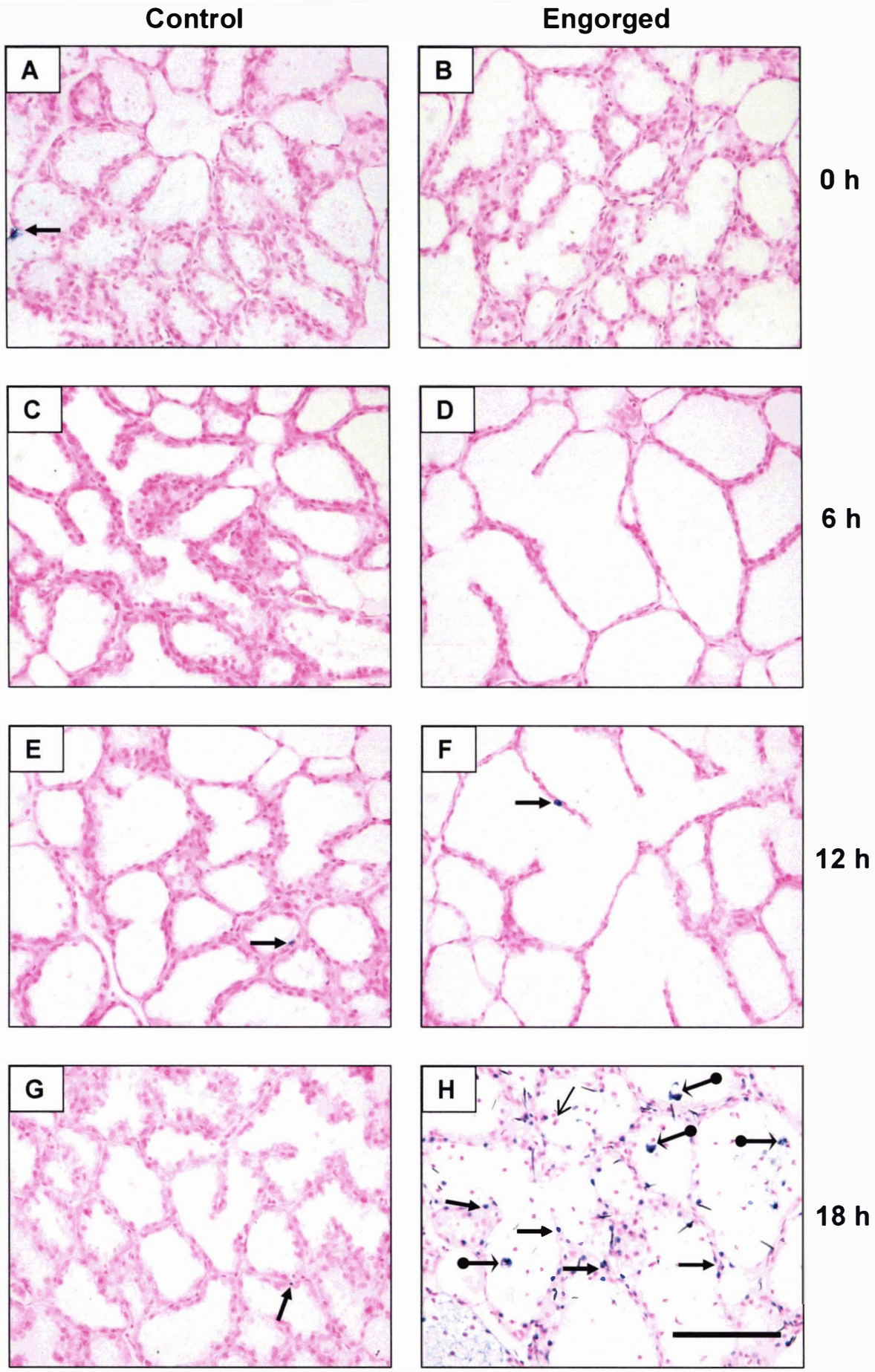
ISEL was used to detect increased apoptosis during the engorgement of rat mammary glands, with representative sections shown in Figure 4.2. Apoptotic nuclei were not labelled in negative controls (Fig. 4.2M and N). Sections were given a qualitative score between 1 (lowest) to 5 (highest) of the level of apoptosis with means for the control and engorged glands at each time point presented in Fig. 4.3. Numbers of positive ISEL nuclei were then counted to provide a quantitative measurement of apoptosis (Fig. 4.4 and 4.5).

Qualitative analysis of each section showed that a low to moderate amount of apoptosis occurred in suckled (control) glands, indicating that apoptosis is a normal part of cell turnover in rat mammary glands during peak lactation. However, high numbers of ISEL nuclei were present in engorged glands by 18, 24 and 36 h following teat-sealing (Fig. 4.2 and 4.3). The quantitative analysis showed similar results (Fig. 4.4), and there was a strong positive correlation between the quantitative and qualitative scores of apoptosis ($r = 0.94$, $P<0.001$; Fig. 4.6). The numbers of total, epithelial and luminal ISEL nuclei detected per alveolus remained constant in the control glands during the course of the experiment (Fig. 4.4A and 4.5A). A dramatic increase ($P<0.001$) in the total number of apoptotic nuclei per alveolus was observed by 18, 24 and 36 h following teat-sealing in the engorged glands compared with their suckled controls (Fig 4.4A). A significant increase ($P<0.001$) was also detected for ISEL apoptotic nuclei located within either epithelia or lumina by 18 h (Fig 4.5A). However, apoptosis was generally greater ($P<0.001$) in the epithelial layer than within alveolar lumens for both control and engorged glands, except at 36 h when a similar number was detected in both locations for engorged glands (Fig 4.5A).

ISEL apoptotic nuclei present in the alveolar lumen of engorged glands at 18, 24 and 36 h were associated with the presence of small numbers of leukocytes (e.g., macrophages and neutrophils) and sloughed epithelial cells (Fig. 4.2H, J, L and N). It was not possible

to distinguish between the apoptosis of epithelial cells and leukocytes in the alveolar lumen, although macrophages often contained distinct positive ISEL granules, consistent with the engulfment of apoptotic bodies. Furthermore, while milk accumulated within engorged alveolar lumens after 18 to 36 h was sometimes diffusely labelled, this was clearly distinguishable from the intense specific labelling of apoptotic bodies (Fig. 4.2H, J and L).

The number of alveoli per 100 x magnification field was used to correct counts of ISEL nuclei per field for changes in alveolar lumen size occurring during mammary engorgement. A similar pattern of apoptosis was observed for the uncorrected number of ISEL nuclei per field and the corrected number of ISEL nuclei per alveolus (Fig. 4.4 and 4.5), and there was a strong positive correlation between the datasets ($r = 0.99$, $P<0.001$; Fig. 4.7). The numbers of alveoli per field did not significantly change in control glands during the experiment. However, the numbers of alveoli per field were decreased in engorged glands at 6 h ($P<0.01$) and at 12, 18, 24 and 36 h ($P<0.001$ each) compared with control glands (Fig. 4.8). This pattern is indicative of an increased alveolar lumen size as the mammary gland accumulates secreted milk during the early stages of forced involution following teat-sealing, as discussed in section 4.3.1.1 above.



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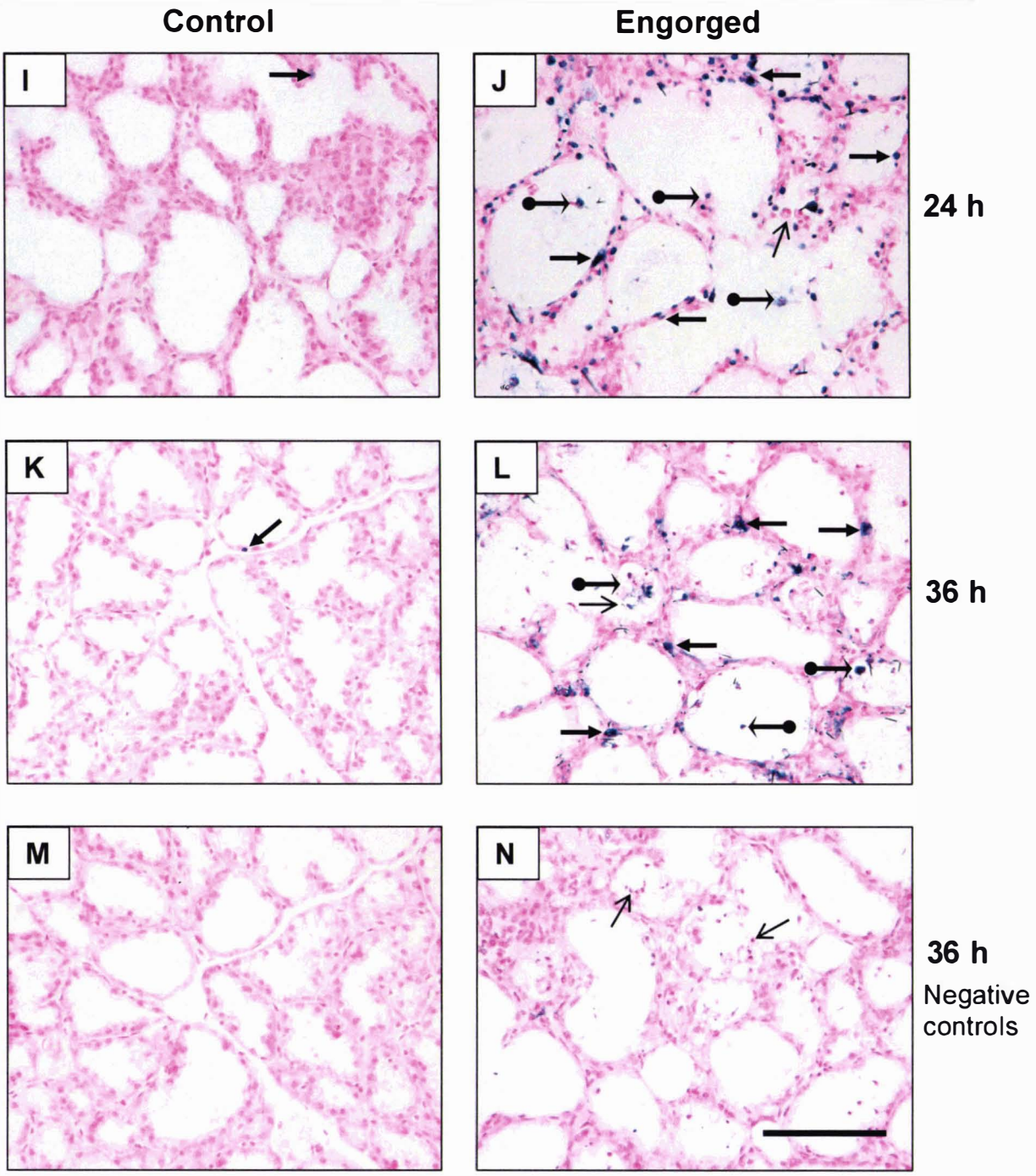


Figure 4.2. *In situ* end-labelling (ISEL) of apoptotic nuclei in rat mammary tissue following teat-sealing induced mammary engorgement.
Representative sections are shown at 200 x magnification for suckled (control) glands and corresponding teat-sealed (engorged) glands at 0 h (**A, B**); 6 h (**C, D**); 12 h (**E, F**); 18 h (**G, H**); 24 h (**I, J**) and 36 h (**K, L,** and **M, N** – negative controls), 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. Sections are counterstained with nuclear fast red. Examples of epithelial ISEL nuclei (⟶), luminal ISEL nuclei (⟶), and regions of leukocyte invasion (⟶) are shown. Scale bars are at 100 μm.

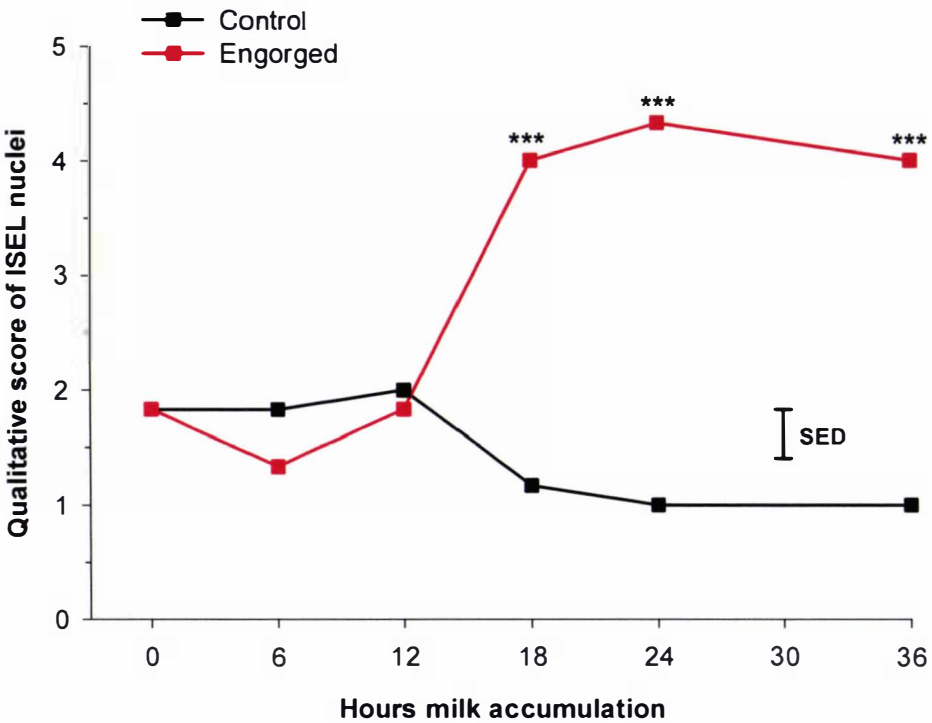
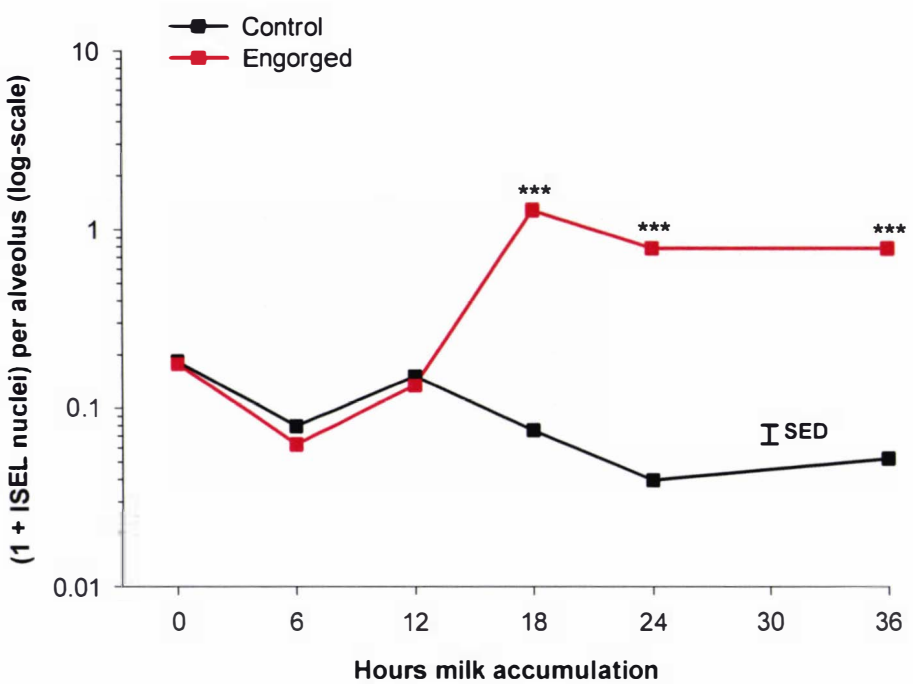


Figure 4.3. Qualitative analysis of *in situ* end-labelled (ISEL) apoptotic nuclei during the engorgement of rat mammary glands.
Data are expressed as the mean qualitative score of the level of apoptosis on a scale of 1 to 5 where; 1 = none to low, 2 = low to moderate, 3 = moderate to high, 4 = high and 5 = very high numbers of positive ISEL nuclei for the suckled (control) and engorged glands of each animal at 0, 6, 12, 18, 24 and 36 h (n=3 per time point) following teat-sealing. The SED is shown for comparing control and engorged glands at each time point (*** $P<0.001$). There were no differences ($P>0.05$) in the qualitative score of apoptosis between time points for control glands.

A



B

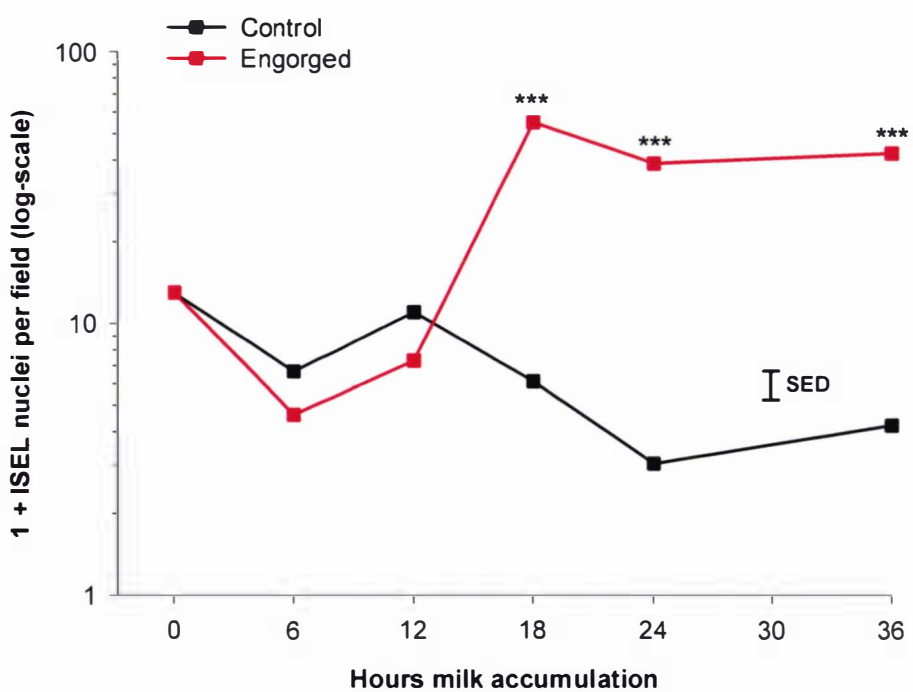


Figure 4.4. Quantitative analysis of the total number of *in situ* end-labelled (ISEL) apoptotic nuclei during the engorgement of rat mammary glands.
Data are expressed as the back-transformed mean number of total 1+ ISEL nuclei per alveolus (**A**) and per 100 x magnification field (**B**) for the suckled (control) and engorged glands of each animal at 0, 6, 12, 18, 24 and 36 h (n=3 per time point) following teat-sealing. The SED is shown for comparing control and engorged glands at each time point (*** $P<0.001$). There were no differences ($P>0.05$) in numbers of ISEL apoptotic nuclei per alveolus and per field between time points for control glands.

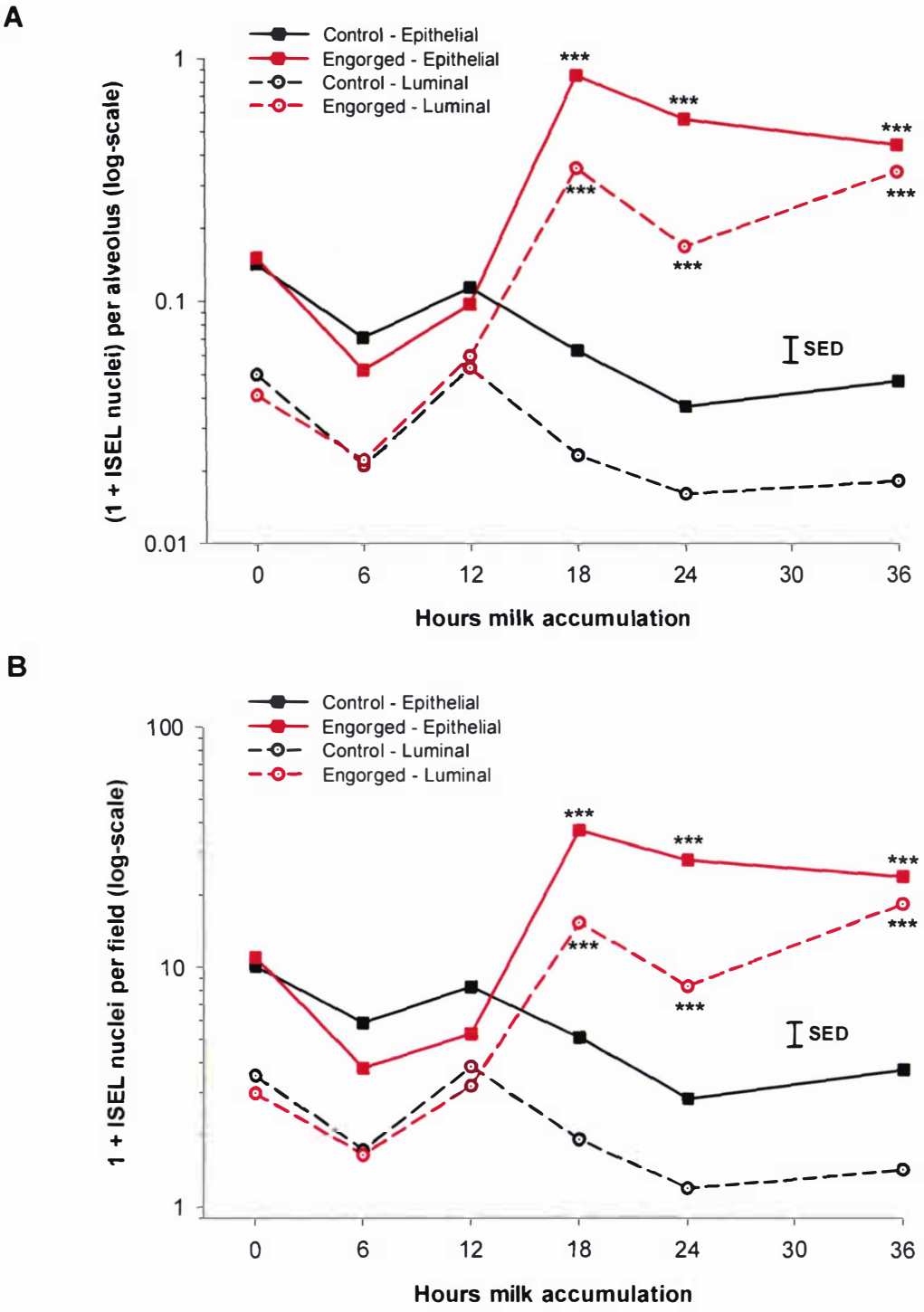


Figure 4.5. Quantitative analysis of the number of *in situ* end-labelled (ISEL) apoptotic nuclei located within alveolar epithelia or lumina during the engorgement of rat mammary glands.
Data are expressed as the back-transformed mean number of epithelial and luminal 1+ ISEL nuclei per alveolus (**A**) and per 100 x magnification field (**B**) for the suckled (control) and engorged glands of each animal at 0, 6, 12, 18, 24 and 36 h (n=3 per time point) following teat-sealing. The SED is shown for comparing numbers of ISEL nuclei between control and engorged glands within a type (i.e., epithelial or luminal; *** $P<0.001$), and for comparing type (i.e., epithelial vs. luminal) within a treatment (i.e., control or engorged) at each time point. There were no differences ($P>0.05$) in numbers of epithelial or luminal ISEL nuclei per alveolus and per field between time points for control glands, except for a small difference in numbers of epithelial ISEL nuclei between the 0 h and 24 h time points ($P<0.05$).

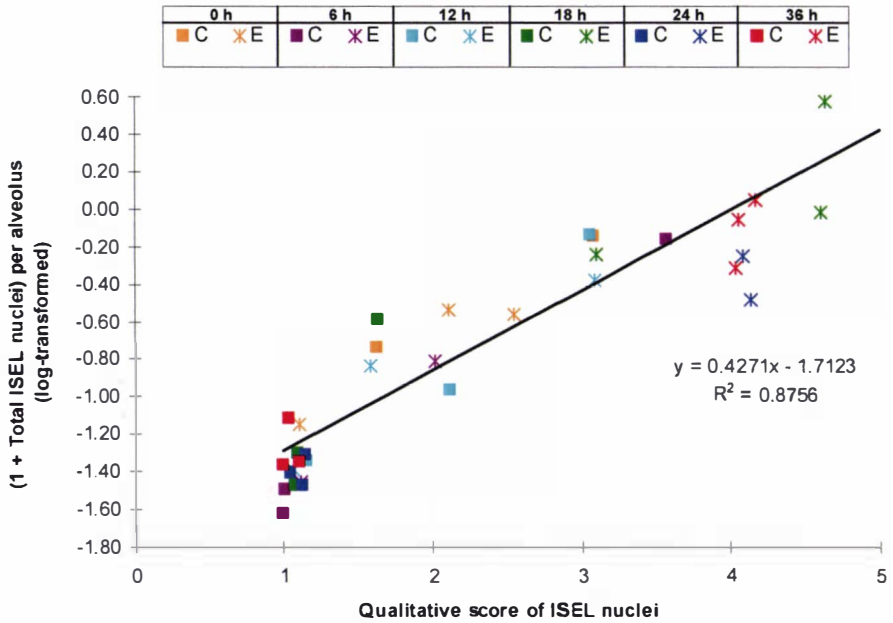


Figure 4.6. The relationship between qualitative and quantitative analyses of *in situ* end-labelled (ISEL) apoptotic nuclei during the engorgement of rat mammary glands.
Data are expressed as the qualitative score of the level of apoptosis on a scale of 1 to 5 where; 1 = none to low, 2 = low to moderate, 3 = moderate to high, 4 = high and 5 = very high numbers of positive ISEL nuclei, and as the log-transformed mean number of total 1+ ISEL nuclei per alveolus, for the suckled (control) and engorged glands of each animal at 0, 6, 12, 18, 24 and 36 h (n=3 per time point) following teat-sealing.

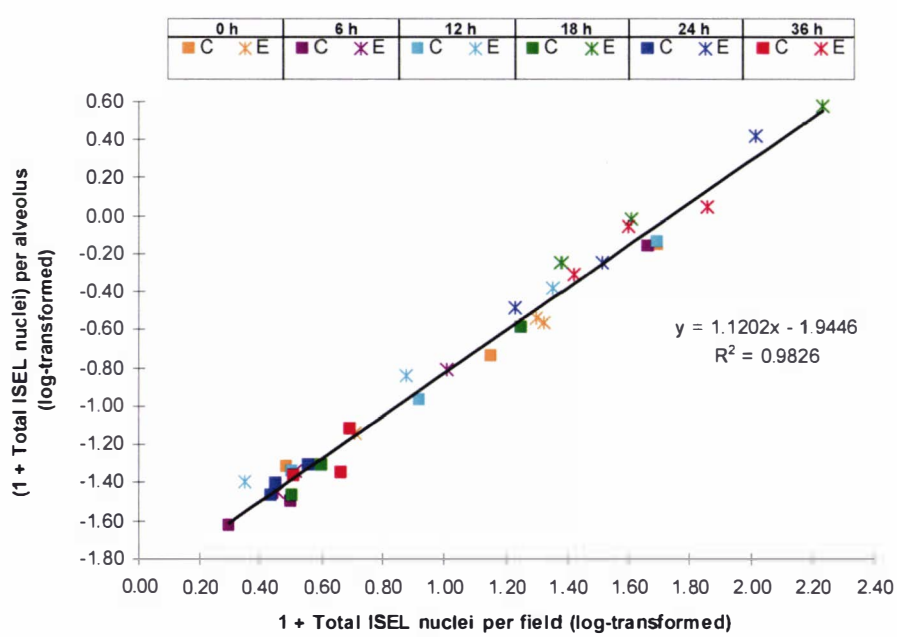


Figure 4.7. The relationship between the number of *in situ* end-labelled (ISEL) apoptotic nuclei per field and per alveolus during the engorgement of rat mammary glands.
Data are expressed as the log-transformed mean number of total 1+ ISEL nuclei per 100 x magnification field and per alveolus for the suckled (control) and engorged glands of each animal at 0, 6, 12, 18, 24 and 36 h (n=3 per time point) following teat-sealing.

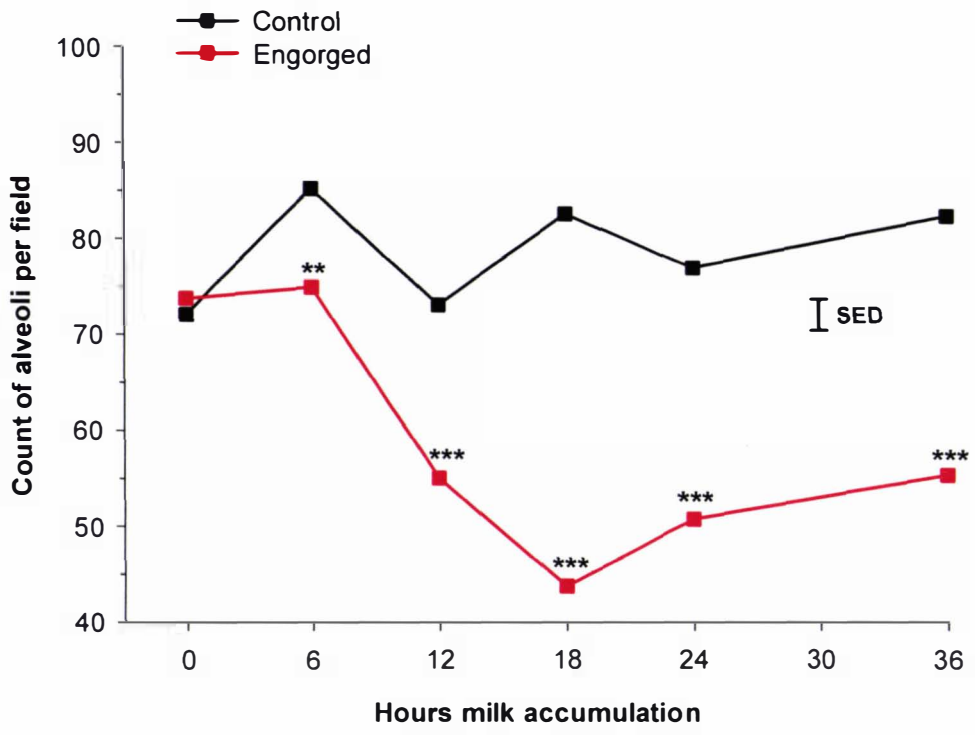


Figure 4.8. The number of alveoli per field during the engorgement of rat mammary glands.
Data are expressed as the mean number of alveoli per 100 x magnification field for the suckled (control) and engorged glands of each animal at 0, 6, 12, 18, 24 and 36 h (n=3 per time point) following teat-sealing. The SED is shown for comparing control and engorged glands at each time point (** $P<0.01$, *** $P<0.001$). There were no differences ($P>0.05$) in numbers of alveoli per field between time points for control glands.

4.3.3 Gene expression of TJ proteins

4.3.3.1 Time course of changes in mRNA expression of TJ proteins during rat mammary engorgement

Quantitative real-time RT-PCR was used to determine temporal changes in mammary mRNA expression of TJ proteins up to 36 h following teat-sealing (Fig. 4.9). Occludin mRNA expression was not significantly changed during mammary engorgement. However, there was a significant decrease in ZO-1 mRNA expression in engorged glands compared with their suckled controls by 12 h (1.8-fold, $P<0.05$) and at 24 h (2.1-fold, $P<0.01$) following teat-sealing.

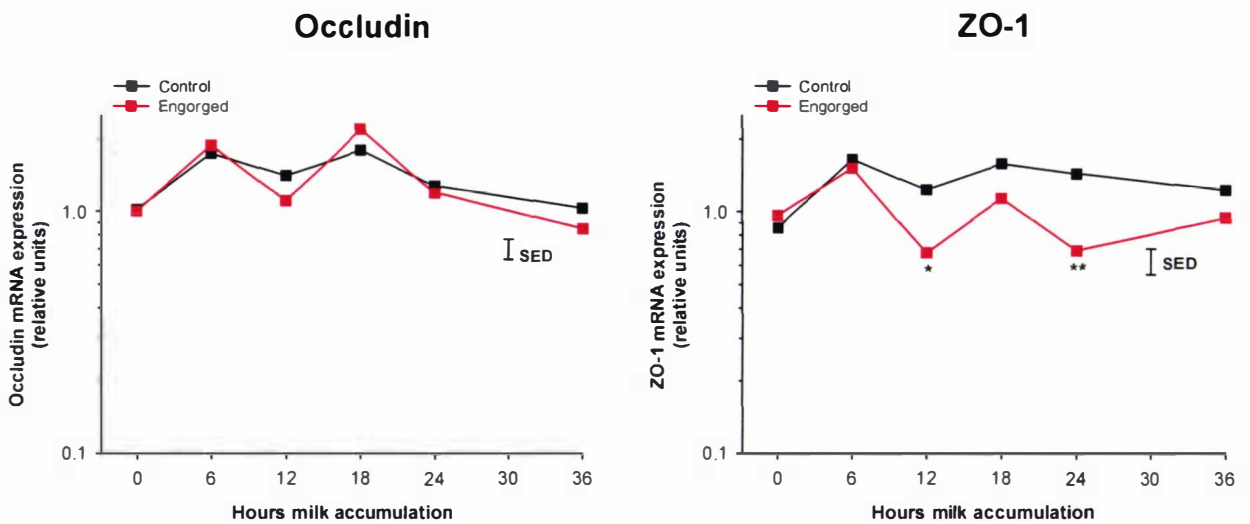


Figure 4.9. The pattern of gene expression of tight junction proteins during the engorgement of rat mammary glands.
Changes in mRNA levels of occludin and ZO-1 in suckled (control) and engorged glands of animals at 0, 6, 12, 18, 24, and 36 h following teat-sealing (n=6 per time point). Data are expressed as back-transformed mean relative units with the SED to compare control and engorged glands at each time point (* $P<0.05$, ** $P<0.01$). There were no differences ($P>0.05$) in occludin and ZO-1 mRNA expression between time points for control glands, except for slightly lower ($P<0.05$) ZO-1 levels at 0 h compared with 6 and 18 h.

4.3.4 Western blot analysis of TJ protein expression

4.3.4.1 Location and solubility of TJ proteins

The solubilities of occludin, claudin-1 and ZO-1 to detergent extraction and their subcellular locations were examined in samples of rat mammary protein (Fig. 4.10). Results were similar to those reported in section 3.3.5.1 for bovine alveolar mammary protein samples, with the use of NP-40 detergent improving the solubilities of the TJ proteins examined. Occludin exhibited characteristic multiple-banding patterns between ~60 and ~65 kDa consistent with phosphorylation. The higher MW occludin bands were partially resistant to detergent extraction and primarily located in the membrane rather than the cytosolic fraction. Claudin-1 migrated as bands at ~22 kDa and ~28 kDa. Both bands were detergent soluble, but the ~28 kDa band was less soluble than the ~22 kDa band and remained in the membrane fraction. However, unlike bovine alveolar mammary proteins, the ~60 kDa claudin-1 homodimer was not detected in rat samples. Immunoreactive bands to ZO-1 antibody were detected at ~225 kDa, with higher MW bands resistant to detergent extraction and located in the membrane fraction, while lower MW bands were predominantly found in the cytosolic fraction. Overall, these results confirmed that TJ proteins were partially insoluble under NP-40 detergent extraction. In particular the higher MW bands were of limited solubility and tended to be preferentially located in the membrane fraction, indicating stronger incorporation with the TJ complex.

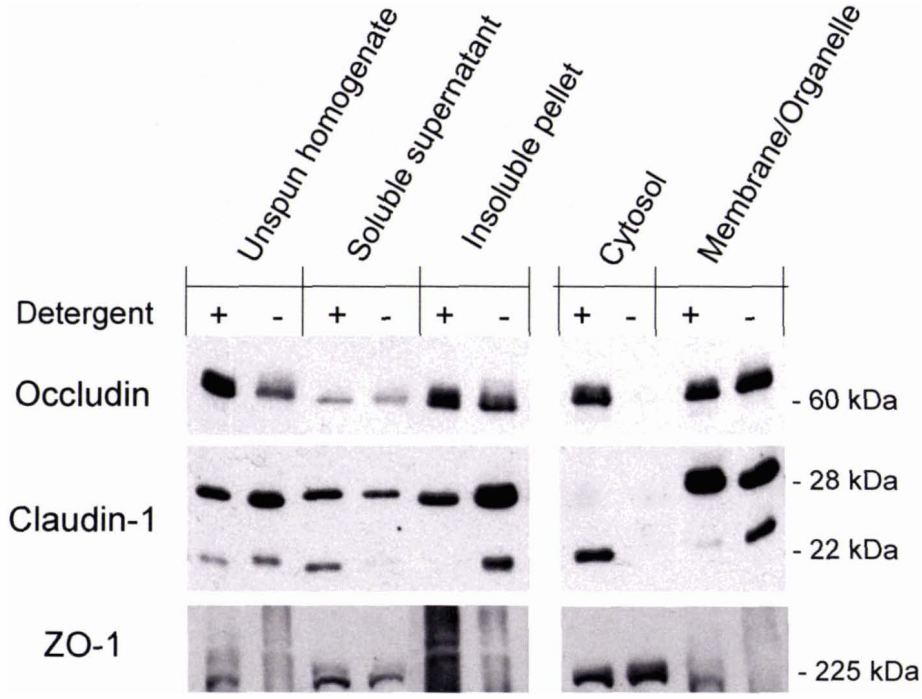


Figure 4.10. Western blots showing the solubilities of tight junction proteins to detergent extraction and their subcellular location in rat mammary tissue.

Lactating mammary tissue was homogenised in low salt buffer with (+) or without (-) 0.5% NP-40 detergent (unspun homogenate). Aliquots of each of these homogenates were centrifuged at 10,000 x g for 5 min at 4 °C and the soluble supernatant collected. The remaining insoluble pellets were resuspended in the respective original buffer solutions. To obtain the subcellular fractions, another aliquot of each of the original homogenates was centrifuged at 400 x g for 10 min at 4 °C. The supernatants were collected and centrifuged at 100,000 x g for 1 h at 4 °C with the resulting supernatants containing the cytosolic fraction (cytosol), while the pellets were resuspended in the respective original buffer solutions, for the membrane/organelle fraction. Twenty micrograms of protein was loaded into each lane. The primary antibodies used were rabbit anti-human occludin (1:10,000 dilution), claudin-1 (1:1000 dilution) and ZO-1 (1:1000 dilution) (obtained from Zymed Laboratories Inc.).

4.3.4.2 Time course of changes in TJ protein expression during rat mammary engorgement

Western blotting was used to determine the pattern of TJ protein expression during the engorgement of rat mammary glands. Occludin protein expression was decreased in the NP-40-soluble fraction of engorged glands by 18 h (2.2-fold, $P<0.01$), 24 h (2.5-fold, $P<0.01$) and 36 h (2.9-fold, $P<0.001$) following teat-sealing, compared with their suckled control glands (Fig. 4.11A and B). A large reduction ($P<0.001$) in occludin expression in the NP-40-insoluble fraction was also observed in engorged glands compared with control glands by 18 h (6.6-fold), 24 h (14.7-fold) and 36 h (9.8-fold) (Fig. 4.11A and B). Expression of the ~28 kDa and ~22 kDa bands of claudin-1 protein were examined in the soluble fraction (Fig. 4.12A and B). The claudin-1 ~28 kDa band significantly declined during mammary engorgement, with a 3.4-fold decrease from 0 h to 36 h ($P<0.01$) and a 5.1-fold reduction relative to controls at 36 h ($P<0.001$). Conversely, expression of the claudin-1 ~22 kDa band was greatly increased by 6 h (3.5-fold, $P<0.01$), and peaked at 12-18 h (21.7-fold, $P<0.001$), after which time expression declined to lower levels although remaining increased compared with controls at 36 h (2.9-fold, $P<0.05$). A dramatic reduction ($P<0.001$) in ZO-1 protein expression in the soluble fraction was detected in engorged glands compared with their respective control glands by 12 h (4.2-fold), 18 h (5.0-fold), 24 h (7.8-fold) and 36 h (26.2-fold) following teat-sealing (Fig. 4.13A and B). Expression of ZO-1 in the insoluble fraction was also decreased in engorged glands compared with control glands by 12 h (2.9-fold, $P<0.05$), 18 h (6.5-fold, $P<0.001$), 24 h (3.9-fold, $P<0.01$) and 36 h (3.5-fold, $P<0.05$) (Fig. 4.13A and B). There were no significant changes in occludin and ZO-1 protein expression within control glands during the course of the experiment (Fig 4.11 and 4.13, respectively). Variations in claudin-1 protein expression were detected between time points for control glands, but these were minor compared with differences between control and engorged glands at each time point (Fig. 4.12A and B).

Even sample loading of SDS-PAGE gels was verified by Coomassie Blue staining of untransferred gels for soluble and insoluble protein fractions (Fig. 4.14), while the effective transfer of proteins onto a nitrocellulose membrane was confirmed by Ponceau S staining (results not shown).

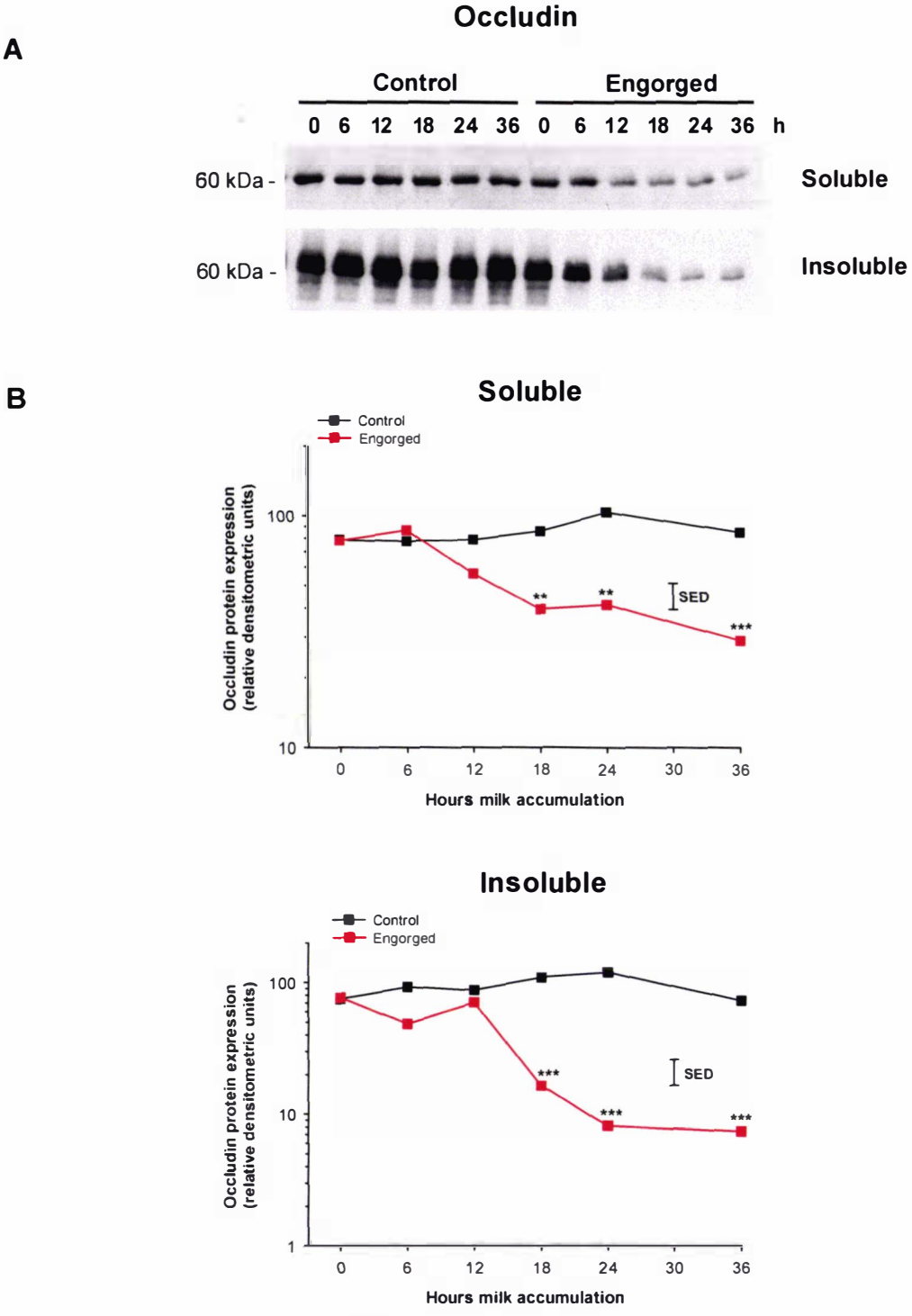


Figure 4.11. The pattern of occludin protein expression during the engorgement of rat mammary glands.

Densitometric analyses of Western blots are shown for the expression levels of occludin in NP-40-soluble and –insoluble protein fractions in suckled (control) and engorged mammary glands of animals at 0, 6, 12, 18, 24, and 36 h following teat-sealing (n=6 per time point). Results are presented as: (**A**) representative Western blots where twenty micrograms of protein was loaded into each lane, and (**B**) back-transformed mean relative densitometric units with the SED to compare control and engorged glands at each time point (** $P<0.01$, *** $P<0.001$). There were no differences ($P>0.05$) in occludin expression between time points for control glands. The primary antibody used was rabbit anti-human occludin (1:50,000 and 1:30,000 dilutions for soluble and insoluble fractions, respectively; obtained from Zymed Laboratories Inc.).

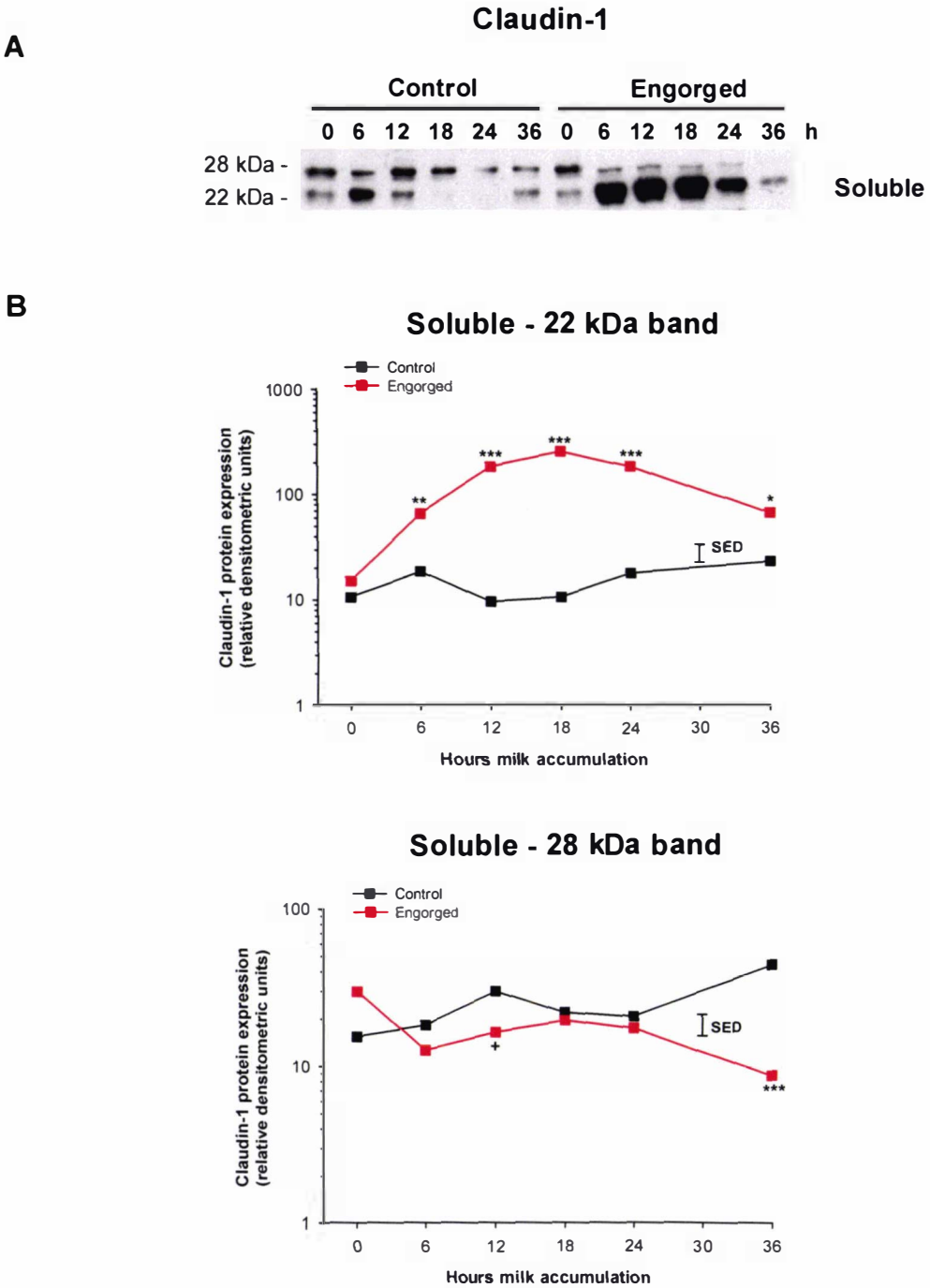


Figure 4.12. The pattern of claudin-1 protein expression during the engorgement of rat mammary glands.

Densitometric analyses of Western blots are shown for the expression levels of claudin-1 in NP-40-soluble protein fractions for the ~28 kDa and ~22 kDa bands, in suckled (control) and engorged mammary glands of animals at 0, 6, 12, 18, 24, and 36 h following teat-sealing (n=6 per time point). Results are presented as: (**A**) a representative Western blot where forty micrograms of protein was loaded into each lane, and (**B**) back-transformed mean relative densitometric units with the SED to compare control and engorged glands at each time point ($^{+}$ $P<0.10$, * $P<0.05$, ** $P<0.01$, *** $P<0.001$). There were no differences ($P>0.05$) in claudin-1 ~22 kDa band expression between time points for control glands, except between 12 h and 36 h ($P<0.05$). Claudin-1 ~28 kDa band expression in control glands was greater at 36 h compared with 0 h ($P<0.01$), 6 and 24 h ($P<0.05$), with no other differences ($P>0.05$) between time points. The primary antibody used was rabbit anti-human claudin-1 (1:3000 dilution; obtained from Zymed Laboratories Inc.).

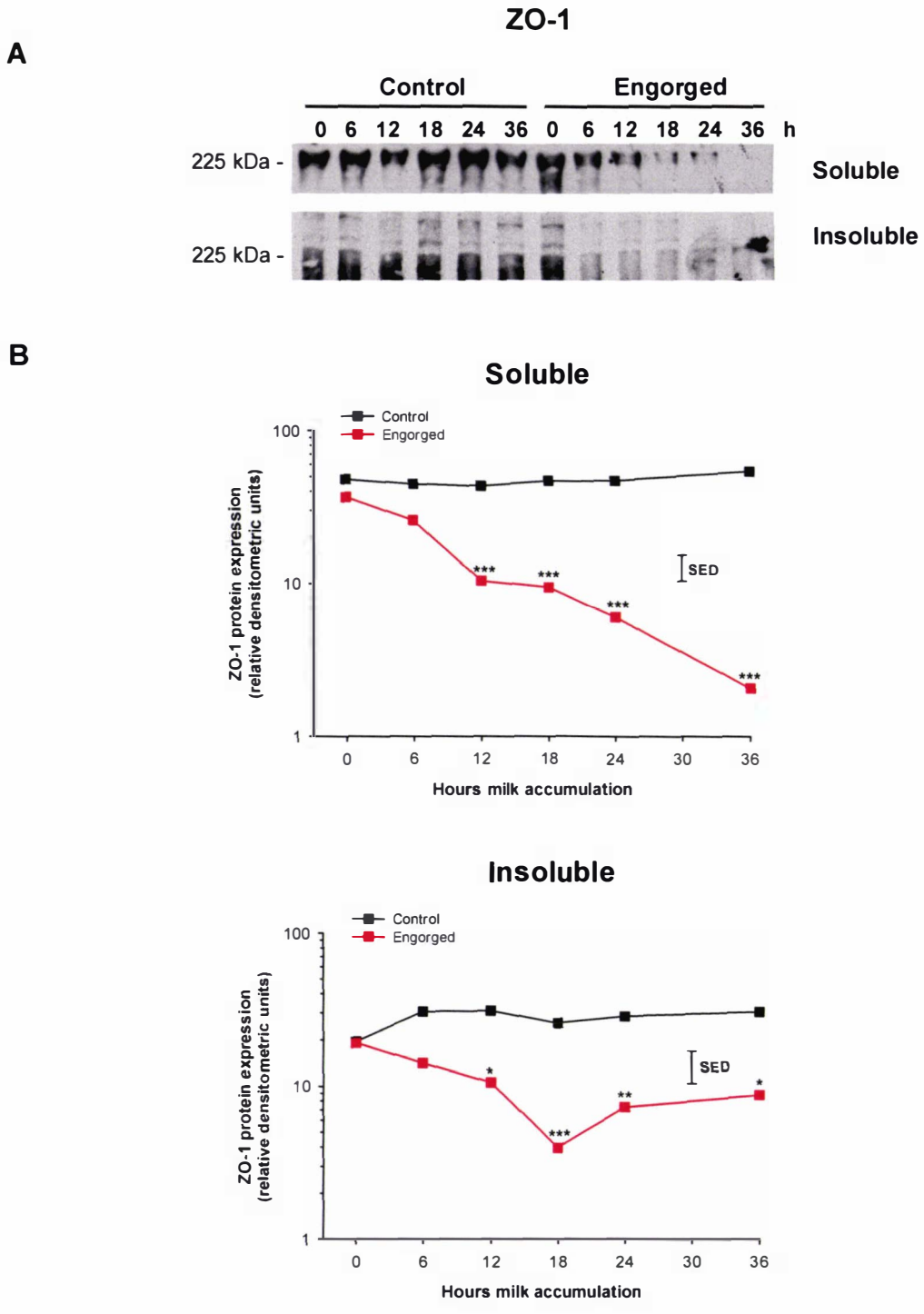


Figure 4.13. The pattern of ZO-1 protein expression during the engorgement of rat mammary glands.

Densitometric analyses of Western blots are shown for the expression levels of ZO-1 in NP-40-soluble and –insoluble protein fractions in suckled (control) and engorged mammary glands of animals at 0, 6, 12, 18, 24, and 36 h following teat-sealing (n=6 per time point). Results are presented as: (**A**) a representative Western blot where twenty micrograms of protein was loaded into each lane, and (**B**) back-transformed mean relative densitometric units with the SED to compare control and engorged glands at each time point (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). There were no differences ($P>0.05$) in ZO-1 expression between time points for control glands. The primary antibody used was rabbit anti-human ZO-1 (1:50,000 and 1:1000 dilutions for soluble and insoluble fractions, respectively; obtained from Zymed Laboratories Inc.).

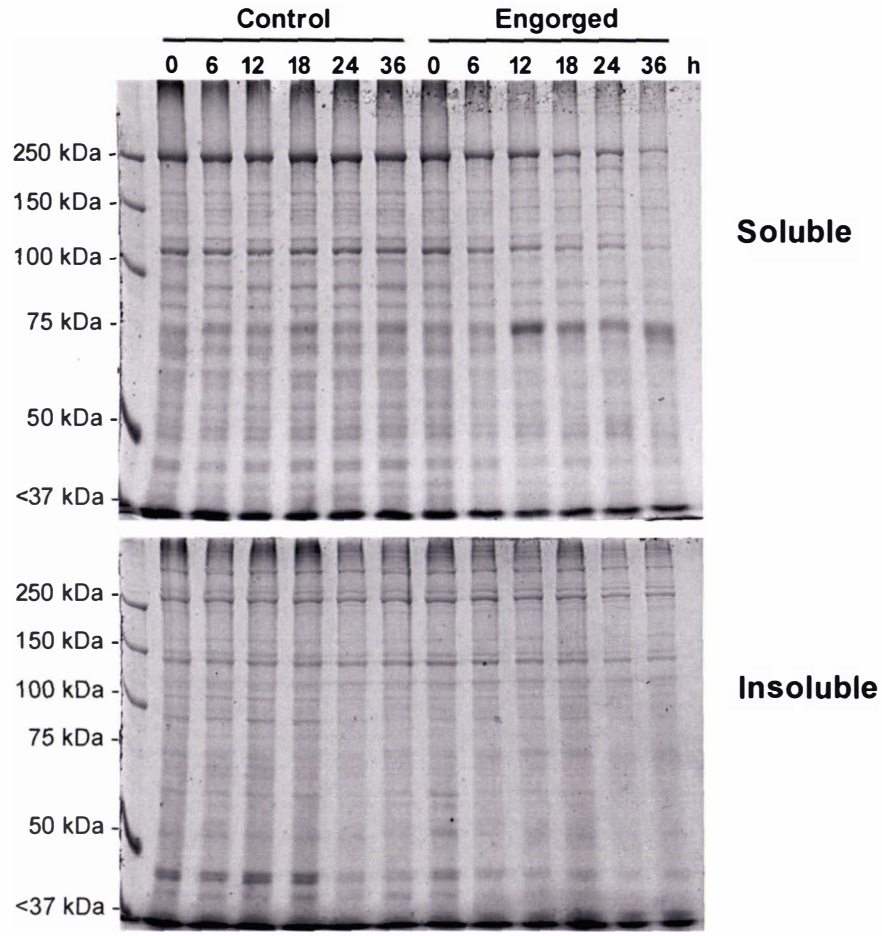


Figure 4.14. Coomassie blue stained gels loaded with protein samples from control and engorged rat mammary glands following teat-sealing.
Representative 8% SDS-PAGE gels containing NP-40-soluble and –insoluble protein fractions extracted from suckled (control) and engorged mammary glands of animals at 0, 6, 12, 18, 24, and 36 h following teat-sealing. Twenty micrograms of protein was loaded into each lane, except lane 1 which contains 5 µl of the Precision Plus Protein Standard (Bio-Rad Laboratories). Gels were not transferred and were stained with Coomassie blue for 18 h then de-stained in 25% methanol until optimal colour intensity was obtained.

4.3.5 Immunohistochemistry of occludin protein expression

4.3.5.1 Time course of changes in occludin protein expression and localisation during rat mammary engorgement

Immunohistochemistry was used for localisation of occludin protein expression in suckled and engorged rat mammary tissue. The alveoli of suckled control glands (Fig. 4.15A, C and E) were surrounded by a single layer of cuboidal epithelial cells strongly labelled for occludin protein on the apical, basal and especially lateral membranes and diffusely labelled within the cytoplasm. In contrast, occludin labelling was dramatically reduced as alveoli in teat-sealed glands became engorged and distended with milk (Fig. 4.15D and F). By 36 h after teat-sealing only a faint signal was detected on epithelial cell membranes (Fig. 4.15F), consistent with the down-regulation of occludin protein expression reported in section 4.3.4.2. Furthermore, there was no labelling on the cell membranes of leukocytes and sloughed epithelial cells present within engorged alveolar lumina after 18 to 36 h (Fig. 4.15D and F), or on concurrent negative control sections where primary antibodies to occludin were either omitted (Fig. 4.15G and H), or substituted with IgG at the same molar concentration (results not shown).

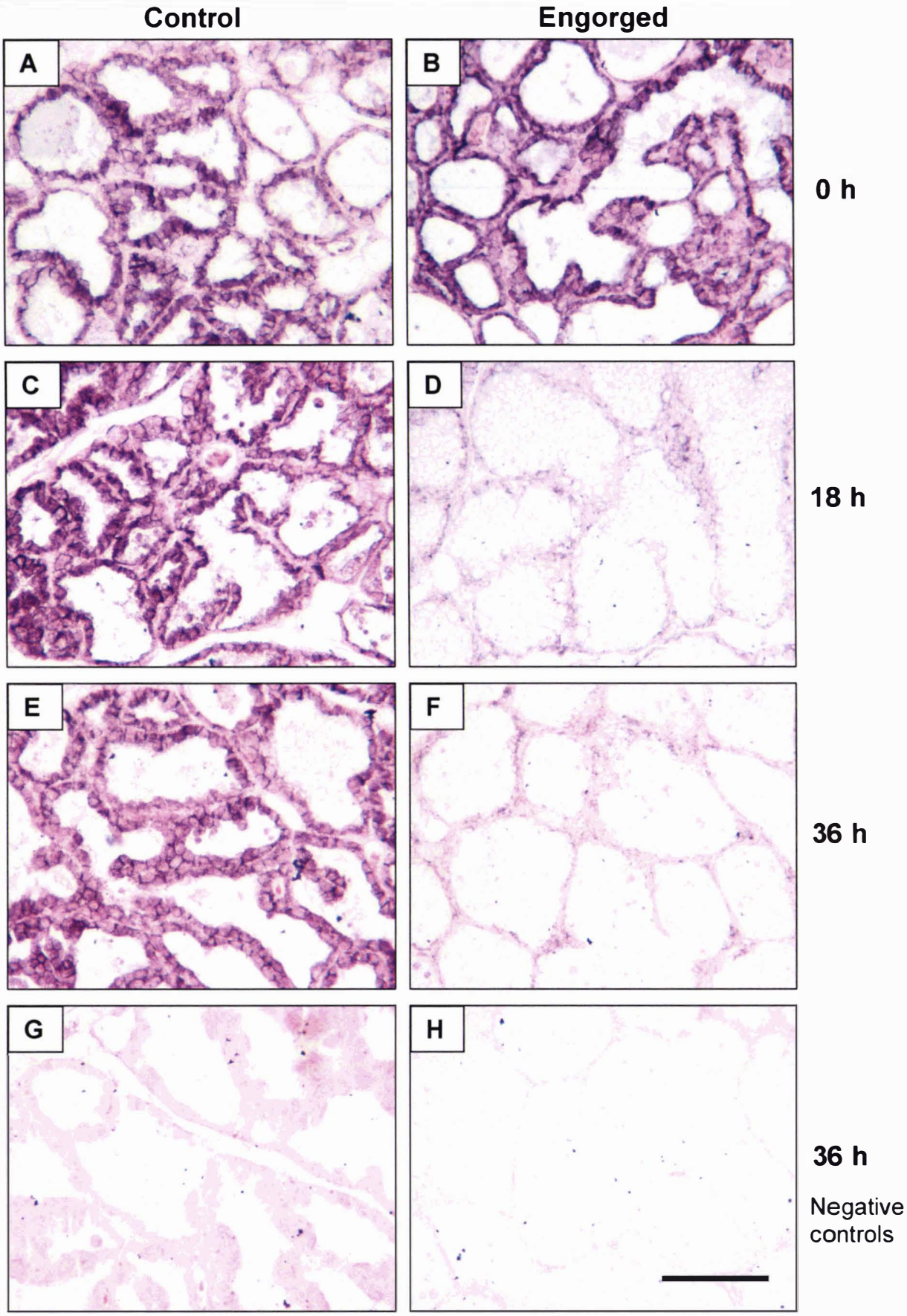


Figure 4.15. Occludin protein expression and localisation in rat mammary tissue following teat-sealing induced mammary engorgement.

Representative sections labelled with antibodies to occludin are shown at 200 x magnification for suckled (control) glands and corresponding teat-sealed (engorged) glands at 0 h (**A, B**); 18 h (**C, D**); and 36 h (**E, F,** and **G, H** - negative controls), respectively. The omission of primary antibodies to occludin was used to provide concurrent negative controls. Sections are lightly counterstained with eosin and the scale bar equals 100 µm.

4.4 DISCUSSION

This study demonstrated that in this rat model, cessation of milk removal by teat-sealing resulted in a rapid down-regulation of TJ protein expression and the initiation of apoptosis in MECs. These responses are consistent with the local regulation of mammary involution as no changes were detected in suckled glands on the contralateral side to teat-sealed glands. The apoptosis of MECs was observed to be dramatically increased within 18 h following teat-sealing. This was earlier than previous reports where the induction of mammary apoptosis was shown from 24 to 48 h after teat-sealing in mice (Quarrie *et al.*, 1995, 1996; Marti *et al.*, 1997). Furthermore, changes in expression of TJ proteins were detected before (i.e., claudin-1 and ZO-1), or at the same time (i.e., occludin) as the increase in MEC apoptosis, indicating that TJ breakdown is a relatively early event during mammary involution. However, while the question remains as to whether changes in TJ proteins are causal to the onset of apoptosis, the breakdown of specialised cell-cell junctions is the first visible stage of MEC apoptosis during mammary involution (Walker *et al.*, 1989; Strange *et al.*, 1992; Tenniswood *et al.*, 1992).

In rats, a rise in plasma lactose occurs by 7 h after litter removal, also supporting disruption of TJ integrity as an early process during involution (Yamamuro & Sensui, 1994). However, a significant increase in plasma lactose was not reported until 12 to 18 h of involution in mice when maximal lumen size and MEC flattening were seen by 12 h post-weaning (Kim *et al.*, 1997). Plasma lactose was not measured in the current study, nonetheless maximal gland weight was reached at 12 h (McMahon *et al.*, 2004; Appendix VII), after which time the mass of glands declined indicating reabsorption of secreted milk through a massive loss of TJ integrity. This was supported by the presence of very large numbers of milk vesicles, containing coalescing fat droplets and proteins which would have been too large to pass through leaky TJs (Molenaar *et al.*, 1996a), within engorged alveolar lumens by 18 h, and interestingly this coincided with the induction of apoptosis.

However, this does not rule out an initial rise in TJ permeability by 7 h as reported by Yamamuro & Sensui (1994), and earlier events are supported by changes in the

expression of claudin-1, an integral transmembrane TJ protein, within 6 h following teat-sealing. Furthermore, large reductions in the expression of the TJ proteins, ZO-1 and occludin, were observed in both soluble and insoluble fractions by 12 h and 18 h, respectively. The reduction in occludin protein expression was also confirmed by a rapid loss of occludin signal on the epithelial cell membranes of engorged mammary sections. However, while ZO-1 mRNA was decreased at 12 and 24 h, occludin mRNA was unchanged indicating that the observed decrease in occludin protein expression is a post-transcriptional event during the initial stages of mammary involution, or due to induction of protease activity at the TJ. In support of increased proteolytic activity at the TJ, the ~28 kDa claudin-1 band was decreased during engorgement, while the ~22 kDa claudin-1 band was initially increased to 18 h before decreasing by 36 h. This could have been the result of protease degradation of the ~28 kDa to the ~22 kDa fragment, although a change in the glycosylation state during the down-regulation of claudin-1 protein expression cannot be ruled out.

Proteolytic degradation of specialised cell-cell junction proteins has been previously demonstrated in MECs. Truncation of the adherens junction protein, E-cadherin was shown during rodent mammary apoptosis and involution *in vivo* (Vallorosi *et al.*, 2000). Furthermore, the TJ proteins, occludin, ZO-1 and ZO-2, were reported to be cleaved by caspases and/or metalloproteinases during apoptosis of human breast epithelial cells *in vitro* (Bojarski *et al.*, 2004). The activation and expression of proteases including caspases, plasmin/plasminogen, cathepsins and metalloproteinases increase dramatically during mammary involution (Strange *et al.*, 1992; Talhouk *et al.*, 1992; Lund *et al.*, 1996; Marti *et al.*, 1999; 2000; 2001; Wiesen & Werb, 2000; Burke *et al.*, 2003), and they may be involved in the degradation of cell-cell junction proteins in addition to ECM proteins. No evidence of protease cleavage of occludin or ZO-1 proteins was detected in the current study. However, experimentally induced truncation of occludin caused MEC apoptosis both *in vivo* and *in vitro* (Beeman & Neville, 2001), suggesting that intact occludin protein is necessary as a cell survival 'signal'.

Loss of cell-ECM survival signalling was demonstrated in the same experimental model as the current study by colleagues, McMahon *et al.* (2004), Appendix VII. They observed a rapid decrease (within 6 h) in the abundance of β1-integrin and FAK proteins

in engorged rat mammary glands. Therefore, decreased cell-ECM communication by integrins and cell-cell contact by TJ proteins were initiated prior to the onset of apoptosis between 12 and 18 h. In addition, the timing of apoptosis agrees with reduced levels of cytochrome C protein in the mitochondrial fraction observed by 12 h following teat-sealing (McMahon *et al.*, 2004; Appendix VII). Relocation of cytochrome C from the mitochondria to the cytoplasm results in activation of pro-apoptotic caspases (Liu *et al.*, 1996; Li *et al.*, 1997b; Zou *et al.*, 1997) and is regulated by anti-apoptotic Bcl-2 family members which prevent the pro-apoptotic Bax family members from opening voltage-dependent anion channels in the mitochondrial membrane (Shimizu *et al.*, 1999; 2001). An increase in the relative levels of cell death promoter to cell survival promoter Bcl-2 family members (Heermeier *et al.*, 1996; Schorr *et al.*, 1999) and in the abundance of activated caspases (Marti *et al.*, 1999, 2000, 2001; Prince *et al.*, 2002; McMahon *et al.*, 2004, Appendix VII) occur in the early stages of mammary apoptosis and involution.

In rodents, the losses of cell-ECM and cell-cell communication and the induction of apoptosis during mammary involution (McMahon *et al.*, 2004, Appendix VII; the present study) occur more rapidly and more extensively than those observed during bovine involution (Singh *et al.*, 2004a; 2005, Appendix VI; Chapter 3). Species differences in the time course of the reversible and irreversible stages of involution are probably evolutionary in nature, and may reflect the reproductive strategies/timescales of each species. The rat has large numbers of offspring from many litters in a short lifespan, whereas the modern dairy cow typically has a single offspring once per year and is genetically selected for high milk yield and persistent lactations. Furthermore, the ability of dairy cows to store milk in a large cisternal compartment is also likely to delay the onset of inhibitory signals relative to rodents, which store the majority of milk products within the secretory alveoli. Despite these differences in time course, the responses to mammary engorgement are locally regulated in both species and exhibit a similar cascade of events (Wilde *et al.*, 1999; McMahon *et al.*, 2004, Appendix VII; Singh *et al.*, 2004a; 2005, Appendix VI; Chapter 3), supporting the use of rodent models for the study of mammary involution.

However, prolactin signalling is more important in rodents, with prolactin and other systemic galactopoietic hormones acting as survival factors both during lactation and through the first stage of mammary involution (Li *et al.*, 1997a). While local signals (i.e., milk accumulation) were sufficient to down-regulate prolactin signal transduction through STAT5 and initiate apoptosis, teat-sealed glands did not proceed into the second, lobular-alveolar remodelling, stage of involution in the presence of systemic galactopoietic hormones (Li *et al.*, 1997a). Closure of the prolactin signalling pathway in response to local milk accumulation was also indicated by a rapid down-regulation of the long-form of the prolactin receptor mRNA, which was reversible upon milk removal (Kim *et al.*, 1997). The loss of prolactin signalling during mammary engorgement may relate to the disruption of TJs as prolactin and glucocorticoids are reported to increase mammary TJ integrity *in vivo* (Nguyen & Neville, 1998; Stelwagen *et al.*, 1998b; Nguyen *et al.*, 2001) and *in vitro* (Singer *et al.*, 1994; Stelwagen *et al.*, 1999). Furthermore, the local actions of cytokines and growth factors, such as the up-regulation of TGF-β1 and TGF-β3 expression (Strange *et al.*, 1992; Faure *et al.*, 2000; Nguyen & Pollard, 2000), may also play a role in loss of TJ integrity during mammary apoptosis and involution. TGF-β1 has been shown to antagonise glucocorticoid-mediated TJ formation in mouse MECs *in vitro* (Woo *et al.*, 1996), and induce disassembly of both adherens and TJ complexes in proximal tubular HK-2 cells (Tian & Phillips, 2002), while TGF-β3 perturbed the TJ permeability barrier in Sertoli cells (Lui *et al.*, 2001).

However, the primary local control of mammary function in response to milk accumulation is related to either the presence of inhibitory factors in milk (Wilde *et al.*, 1995; Shamay *et al.*, 2002; 2003; Matsuda *et al.*, 2004), or to physical distension of MECs and consequent changes in gene expression by mechanotransduction (Davis *et al.*, 1999; Stelwagen, 2001). It is unclear whether the observed changes in TJ protein expression and the onset of apoptosis are initiated by one or both of these mechanisms. However, changes in cell shape during mammary engorgement are likely to result in mechanical tension on both cell-cell and cell-ECM interactions, which may result in loss of survival signals and initiate pro-apoptotic signals. In conclusion, the rapid down-regulation of TJ proteins demonstrated in this study is consistent with a loss of epithelial cell-cell integrity and communication during the early stages of mammary apoptosis and

involution. The hypothesis that physical distension of the mammary gland initiates these processes is examined in the next chapters.

CHAPTER FIVE

Tight junction protein expression and apoptosis in acutely distended rat mammary glands

5.1 INTRODUCTION

Involution in the rodent mammary gland requires the coordination of several processes including cessation of milk production, elimination of the majority of secretory MECs by apoptosis, and extensive proteolytic degradation of the basement membrane and ECM. The remodelling of lobular-alveolar structures returns the mammary gland to a virgin-like state in preparation for the next lactation. During the first, reversible, stage of involution, milk accumulates within alveolar lumens and this locally-derived signal is sufficient to trigger apoptosis (Quarrie *et al.*, 1996; Li *et al.*, 1997a; Marti *et al.*, 1997; Chapter 4). However, a fall in the levels of systemic galactopoietic hormones, such as prolactin and glucocorticoids, is required for progression into the second, irreversible, stage of involution (Li *et al.*, 1997a).

Dramatic changes in gene expression or activity are rapidly initiated during mammary involution and result in the down-regulation of milk protein genes (Strange *et al.*, 1992; Li *et al.*, 1996; 1997a; Lund *et al.*, 1996; Travers *et al.*, 1996), and the progressive loss of cell survival factors and increase of pro-apoptotic signals (reviewed by Green & Streuli, 2004). Recent studies have also demonstrated a down-regulation of β1-integrin and TJ protein expression, reflecting a loss of cell-ECM communication and TJ integrity, respectively, during rodent mammary involution (McMahon *et al.*, 2004, Appendix VII; Chapter 4). However, while milk accumulation plays a primary role in the initiation of these processes, it is uncertain whether this is due to chemical inhibition by milk products or to the physical presence of stored milk activating stretch-sensitive pathways.

Evidence supporting the chemical, local regulation of mammary function comes from several experiments in lactating goats, where the increased milk production response to

more frequent milk removal was demonstrated to be due to removal of a chemical inhibitor rather than relief from the physical presence of accumulated milk (Henderson & Peaker, 1984, 1987). Subsequently, a bioactive protein fraction was found in milk which inhibited milk secretion both *in vitro* and *in vivo*, and was, therefore, named the feedback inhibitor of lactation (FIL) (Wilde *et al.*, 1987a, 1988, 1995). In addition, casein-derived phosphopeptides were recently reported to cause local disruption of TJ integrity and inhibit milk secretion in goats and cows (Shamay *et al.*, 2002, 2003). The local synthesis of serotonin by the mammary epithelium has also been implicated in a negative feedback, autocrine-paracrine loop that opposes endocrine stimulation of milk production and secretion in lactating mice (Matsuda *et al.*, 2004). Other potential chemical regulators of mammary function include factors which accumulate in milk during involution, and which have been shown to stimulate apoptosis under certain conditions. These include α-lactalbumin (Hakansson *et al.*, 1995, 1999), IGFBP-5 (Tonner *et al.*, 1997, 2002; Marshman *et al.*, 2003), and TGF-β1 (Letterio *et al.*, 1994; Atwood *et al.*, 1995; Kordon *et al.*, 1995). However, direct evidence for a role of either FIL or casein phosphopeptides in stimulating apoptosis during milk accumulation is yet to be provided. Moreover, their specific mechanisms of action and kinetics of feedback inhibition remain unclear.

Other studies in lactating goats and cows have reported that physical distension of the mammary epithelium rather than chemical inhibition is responsible for initiating reductions in TJ integrity and milk secretion during extended periods of milk accumulation (Fleet & Peaker, 1978; Peaker, 1980; Stelwagen *et al.*, 1998a). While there are always dangers in extrapolating results to other species, the initiation of the first, reversible, stage of mammary apoptosis and involution even in the presence of systemic galactopoietic hormones in rodents (Quarrie *et al.*, 1996; Li *et al.*, 1997a; Marti *et al.*, 1997; Chapter 4), suggests that further investigation of physical distension as the primary trigger of local intra-mammary signalling is warranted.

This mechanism is likely to be initiated when the distension of the alveolar epithelium by accumulated milk products causes the MECs to become stretched or flattened (Richardson, 1947; Chapters 3 and 4). Changes in cell shape have been shown to induce intracellular signalling events (mechanotransduction) in lung, uterine and bladder

tissues (Wirtz & Dobbs, 1990; Daifotis *et al.*, 1992; Yamamoto *et al.*, 1992) and have been proposed in mammary tissue (Stelwagen *et al.*, 1997; Davis *et al.*, 1999; Stelwagen, 2001). Physical distension of the mammary epithelium is likely to result in mechanical tension on both cell-cell and cell-ECM interactions, which may lead to loss of survival signals and initiate pro-apoptotic signals. The down-regulation of β1-integrin (McMahon *et al.*, 2004, Appendix VII) and TJ protein expression (Chapter 4) during rodent mammary engorgement would therefore, be implicated in this mechanotransduction cascade. Furthermore, integrins have been shown to be mechanosensors in other cell types (Geiger & Bershadsky, 2002), while the integral transmembrane TJ protein, occludin, was down-regulated during stretch in lung alveolar epithelial cells (Cavanaugh *et al.*, 2001). This leads to the question of whether it is possible to induce down-regulated β1-integrin and TJ protein expression and initiate mammary apoptosis by flattening the alveolar epithelium.

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 (Hanwell & Linzell, 1972; Li *et al.*, 1997a; Nguyen & Pollard, 2000; McMahon *et al.*, 2004; Chapter 4). Therefore, in this study rat mammary glands were acutely distended to the equivalent of approximately 6 h milk accumulation, using isosmotic sucrose solution, with the aim of accelerating the process of involution. This hypothesis will be supported if early mammary involution events, such as acute changes in the protein expression of β1-integrin and TJ components (occludin, claudin-1 and ZO-1), the up-regulation of the pro-apoptotic marker pSTAT3, and the initiation of apoptosis are observed following induced physical distension.

5.2 MATERIALS AND METHODS

5.2.1 Animals and tissue collection protocols

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 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.) to induce mammary engorgement. 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), equivalent to approximately 6 h worth of milk secretion[1], up-the-teat into the lumen of 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. Dams were killed by carbon dioxide gas and cervical dislocation either immediately or 1, 3, and 6 h after sealing teats. Mammary tissue was collected post-mortem for each treatment and frozen on liquid nitrogen before storage at –80 °C as outlined in section 2.2.1.2. A 5 mm thick slice through a mammary gland was also collected for each treatment and fixed for 24 h in 4% paraformaldehyde for histological analyses as described in section 2.2.1.2.

[1] 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). Raw mammary weight data were kindly provided by the Dairy Science and Technology Group, AgResearch Ruakura (refer to Chapter 4 and McMahon *et al.*, 2004, Appendix VII). 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 (Hanwell & Linzell, 1972; Grigor *et al.*, 1986, 1987; Grigor & Thompson, 1987). 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 (DeNuccio & Grosvenor, 1971).

5.2.2 Intra-mammary infusion procedure

Animals were restrained in a plastic rat cone and then anaesthetised with propofol (15 mg/kg bodyweight i.v. Rapinovet, Schering-Plough Animal Health Ltd.) for the duration of the procedure (10-15 mins). To help visualise the teat orifice and facilitate even distribution of infused sucrose in the mammary gland, 100 mu i.v. oxytocin (Oxytocin V, Vetpharm (NZ) Ltd.) 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 cleaned liberally with 70% ethanol. The teat was gently grasped between thumb and forefinger and a small amount of milk ejected to visualise the opening of the teat canal. The fine tip of a glass capillary tube (1.0 mm OD, Fisher Scientific) that had been drawn out over a flame and fire-polished to ~0.2-0.3 mm was gently inserted into the teat orifice (Fig. 5.1). 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 & Engineering). The teat was then sealed using surgical adhesive to prevent pups from removing the gland's contents.

5.2.3 Histological analysis

Formalin-fixed tissue slices were processed through a range of alcohol washes and then embedded in paraffin wax before mounting onto slides and staining with H&E for histological analyses (see sections 2.2.2.1 and 2.2.2.2). The histological features were noted for each slide and a representative area was photographed under 100 x and 400 x magnifications using a ProgRes C14 digital camera (JENOPTIK Laser) and Paint Shop Pro 7.02 software (Jasc Software Inc.).

5.2.4 ISEL of apoptotic nuclei

ISEL was performed on suckled (control), teat-sealed (engorged) and acutely distended (infused) mammary sections from 3 representative animals as described in section 2.2.2.3. Qualitative and quantitative analysis of the number of positive ISEL nuclei was

carried out for each section as described in section 2.2.2.3. Briefly, ISEL nuclei were identified as either located within the secretory epithelial layer or the lumen of mammary alveoli. Each count of ISEL nuclei per 100 x magnification field was incremented by 1 (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.

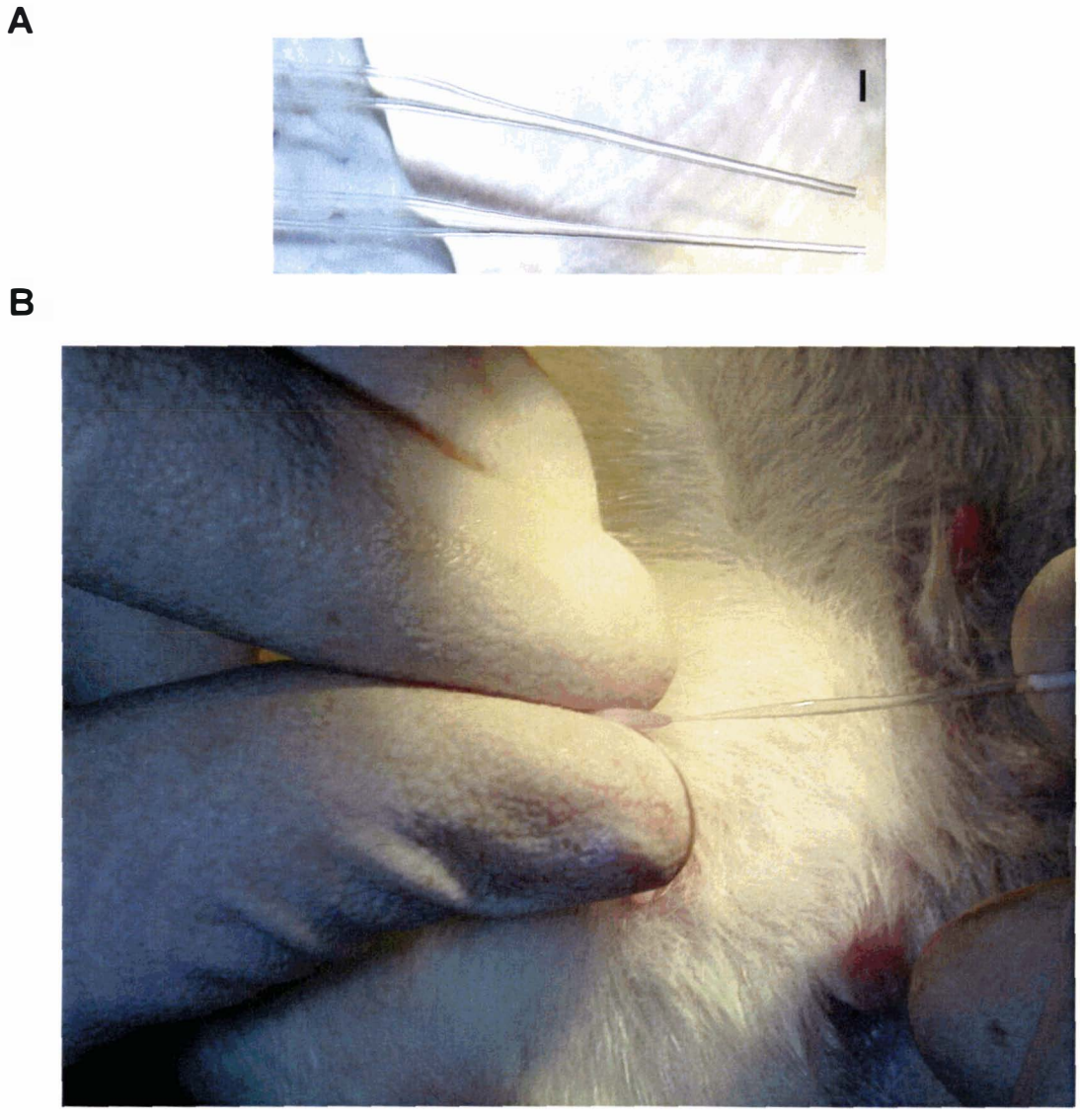


Figure 5.1. The use of fine drawn-out glass pipettes for performing intra-mammary infusions in rats.
(**A**) Two glass capillary tubes (1.0 mm OD) which have been drawn out over a flame and fire-polished to ~0.2-0.3 mm OD. The scale bar equals 1.0 mm. (**B**) A glass pipette gently inserted into the teat orifice of an inguinal mammary gland.

5.2.5 Real-time RT-PCR

Total RNA was extracted from a 150 mg aliquot of ground mammary tissue using TRIzol (Invitrogen) and 1 μg was treated with 1 U DNaseI (Invitrogen) (sections 2.2.3 and 2.2.4). Samples were purified through RNeasy gel columns (QIAGEN Sciences) and converted to cDNA using the SuperScript II Reverse Transcriptase First-Strand Synthesis System kit (Invitrogen) (section 2.2.4). cDNA products were diluted 5-fold in TE (equivalent to approximately 10 ng/μl reversely transcribed total RNA) and then samples (1 μl) were assayed in duplicate, by quantitative real-time RT-PCR with SYBR Green I Chemistry using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) as described in section 2.2.5. cDNA samples were assayed on a 384-well optical plate (Applied Biosystems), which included an assay for an endogenous control gene (ubiquitin) as well as primers for the target gene of interest (i.e., occludin or ZO-1). Ubiquitin was selected as a more suitable endogenous control than β-actin following the acute physical distension of rat mammary glands, as described in section 12.1.2.2, Appendix IV. The sequences of the primer sets and other relevant details are outlined in Table 4.1. Primers were used at a final concentration of 300 nM in each 15 μl real-time reaction. A standard curve of serial dilutions of rat mammary cDNA template, as well as RT-negative and 'no-template' control reactions were included on each plate for each primer pair.

Real-time PCR was performed under the following conditions: 95 °C for 10 min, then 40 cycles of 95 °C for 15 s, 56 °C for 30 s, 72 °C for 30 s and 78 °C for 10 s. The C_T values generated for each real-time PCR reaction were used to quantify the relative abundance of each gene using the relative standard curve method (Applied Biosystems) as described in section 2.2.5.4. The real-time PCR efficiency rates per cycle for investigated genes were: occludin (E = 1.74), ZO-1 (E = 1.86), β-actin (E = 1.78) and ubiquitin (E = 1.89). The specificity of real-time reactions were confirmed by dissociation curve analysis, gel electrophoresis (section 2.2.5.3) and sequencing of amplified products (refer to section 4.2.4, results not shown)

5.2.6 Western immunoblotting

Protein extracts were prepared from aliquots of ground mammary tissue as described in section 2.2.7, and proteins separated by electrophoresis on either 8%, 15% or 7% 15-well SDS-PAGE gels for occludin, claudin-1 or ZO-1 detection, respectively (refer to section 2.2.8.1). For β1-integrin, STAT3 and pSTAT3 detection, samples were electrophoresed on 7.5% 26-well Criterion Precast Tris-HCl gels (133 x 87 x 1.0 mm) (Bio-Rad Laboratories) in the Criterion Cell apparatus (Bio-Rad Laboratories) for approximately 1 h at 200 V in electrode buffer (0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS, pH 8.3). 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 and probed with primary antibodies to rabbit anti-human β1-integrin, and pSTAT3 (Tyr 705), rabbit anti-mouse STAT3 (obtained from Santa Cruz Biotechnology), and rabbit anti-human occludin, claudin-1 and ZO-1 (all from Zymed Laboratories Inc.), as described in section 2.2.8.2. 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).

5.2.7 Immunohistochemistry

Immunohistochemistry with primary antibodies to rabbit anti-human occludin (1: 1000 dilutions; Zymed Laboratories Inc.) was performed on representative control, engorged and infused mammary sections as described in section 2.2.9.

5.2.8 Data and statistical analyses

Data were analysed by ANOVA in GenStat (releases 7.1 and 8.1; Lawes Agricultural Trust, 2003 and 2005), 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 100 x

magnification field. Relative quantification of mRNA expression following real-time PCR was performed using the standard curve method (Applied Biosystems) as described in section 2.2.5.4. The amount of target gene in each sample was normalised to the amount of ubiquitin to control for the initial concentration of cDNA, and the resulting values $\log_{10}$-transformed for statistical analysis. Densitometry results from western blotting were also $\log_{10}$-transformed and adjusted for between gel variations. The levels of mRNA and protein expression were 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 (* $P<0.05$, ** $P<0.01$, *** $P<0.001$). Correlations between the qualitative and quantitative scores of ISEL apoptotic nuclei were performed in Minitab (Minitab Release 14.20, 2005; Minitab Inc., USA).

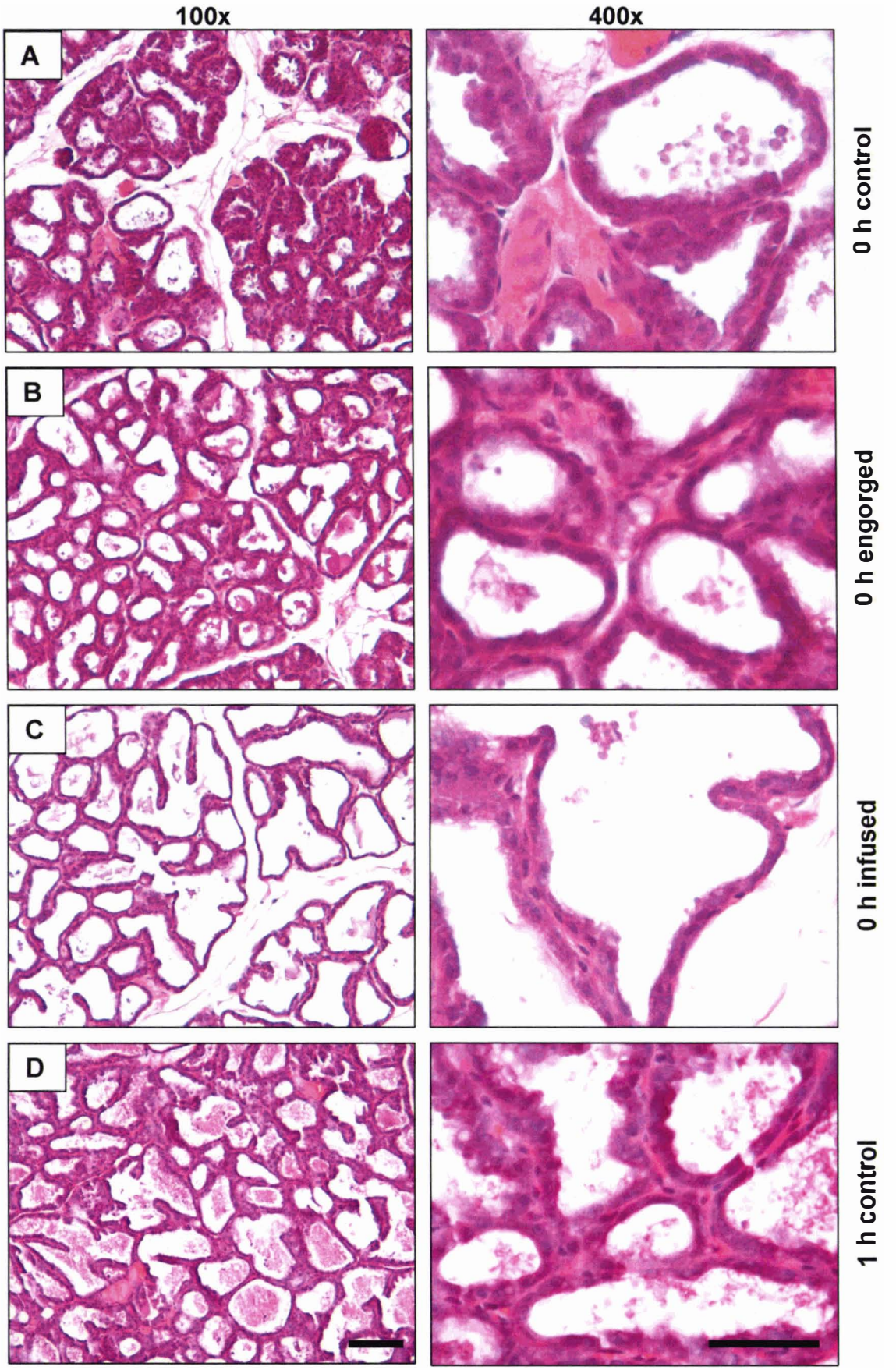
5.3 RESULTS

5.3.1 Histological morphology

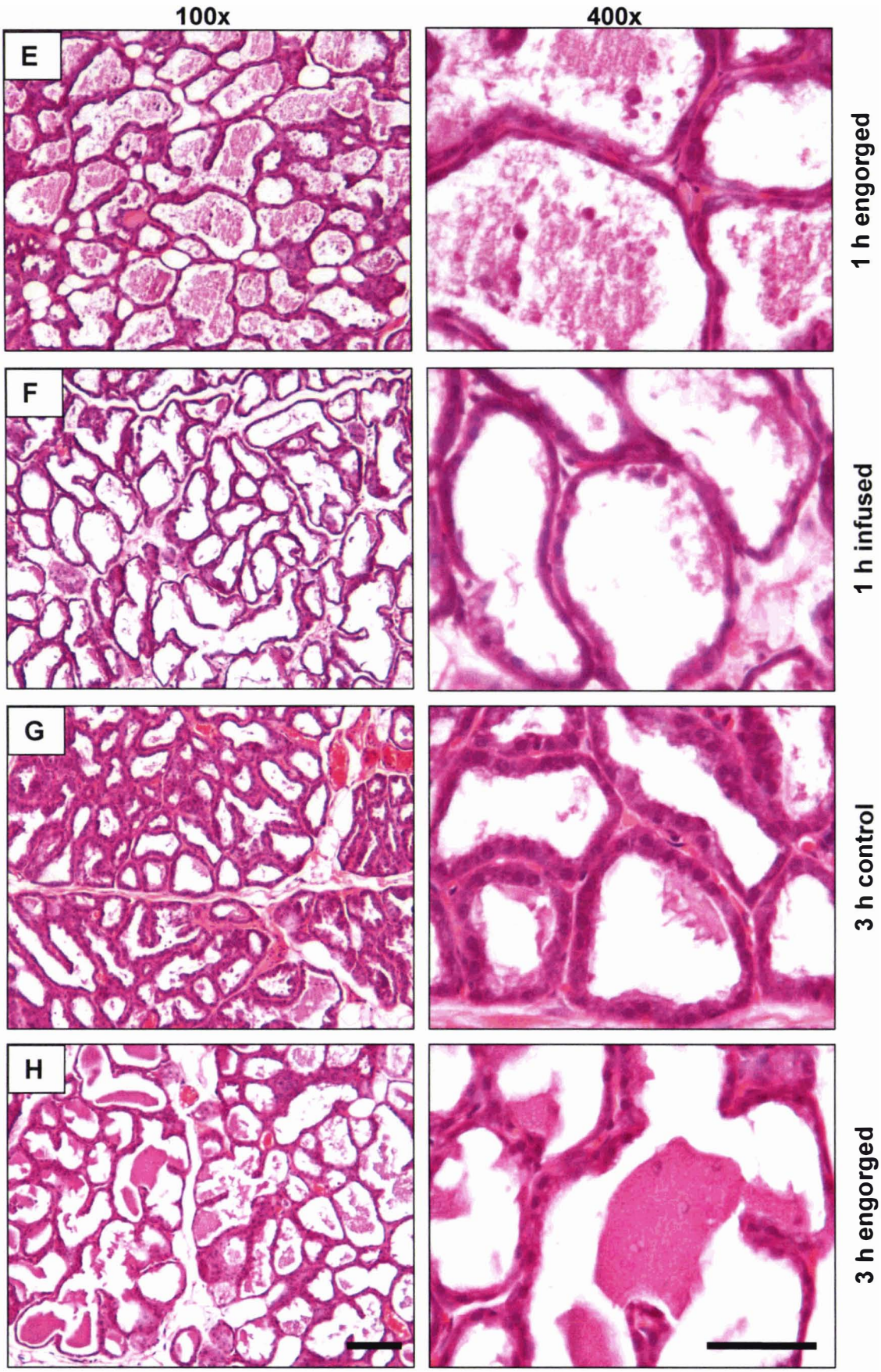
5.3.1.1 Time course of morphological changes following the acute physical distension of rat mammary glands

Changes in alveolar morphology following the acute physical distension of rat mammary glands compared with their respective suckled and engorged glands are shown in Figure 5.2. The histological sections from suckled (control) glands (Fig. 5.2A, D,G and J) were typical of lactating alveoli and consisted of a single layer of cuboidal epithelium surrounding each lumen, which were small-relaxed to moderately-open depending upon the amount of milk secretion contained within them. Within 1 h of teat-sealing (Fig. 5.2E) a small increase in alveolar size due to milk accumulation was apparent between engorged and control glands. However, this difference was much more noticeable at 3 and 6 h, with distended alveolar lumina and flattened epithelium observed in teat-sealed glands (Fig. 5.2H and K).

Infusing the mammary gland with the equivalent of approximately 6 h worth of milk secretion caused an immediate increase in alveolar lumen size and flattening of the secretory epithelium (Fig. 5.2C). Histological examination of tissue sections revealed that the infusate had reached the majority of alveolar lobules, but some peripheral alveoli were not distended. At 1 h the alveoli of infused glands (Fig. 5.2F) appeared distended and did not contain as much milk secretion as their respective engorged glands, but by 3 h (Fig. 5.2I) a few areas contained collapsed alveoli. This was more apparent by 6 h (Fig. 5.2L) with some areas containing large, open, stretched alveoli while others showed signs of vesicle (i.e., coalescing milk fat and proteins) engorgement and/or collapse with rounded epithelial cells protruding into the lumen and discernable gaps between adjacent cells. Most of these cells were still attached to the basement membrane, although some had detached and were present within alveolar lumina along with apoptotic bodies and neutrophils. The latter were also observed in the interalveolar stroma and connective tissue.



(Figure 5.2 is continued overleaf)



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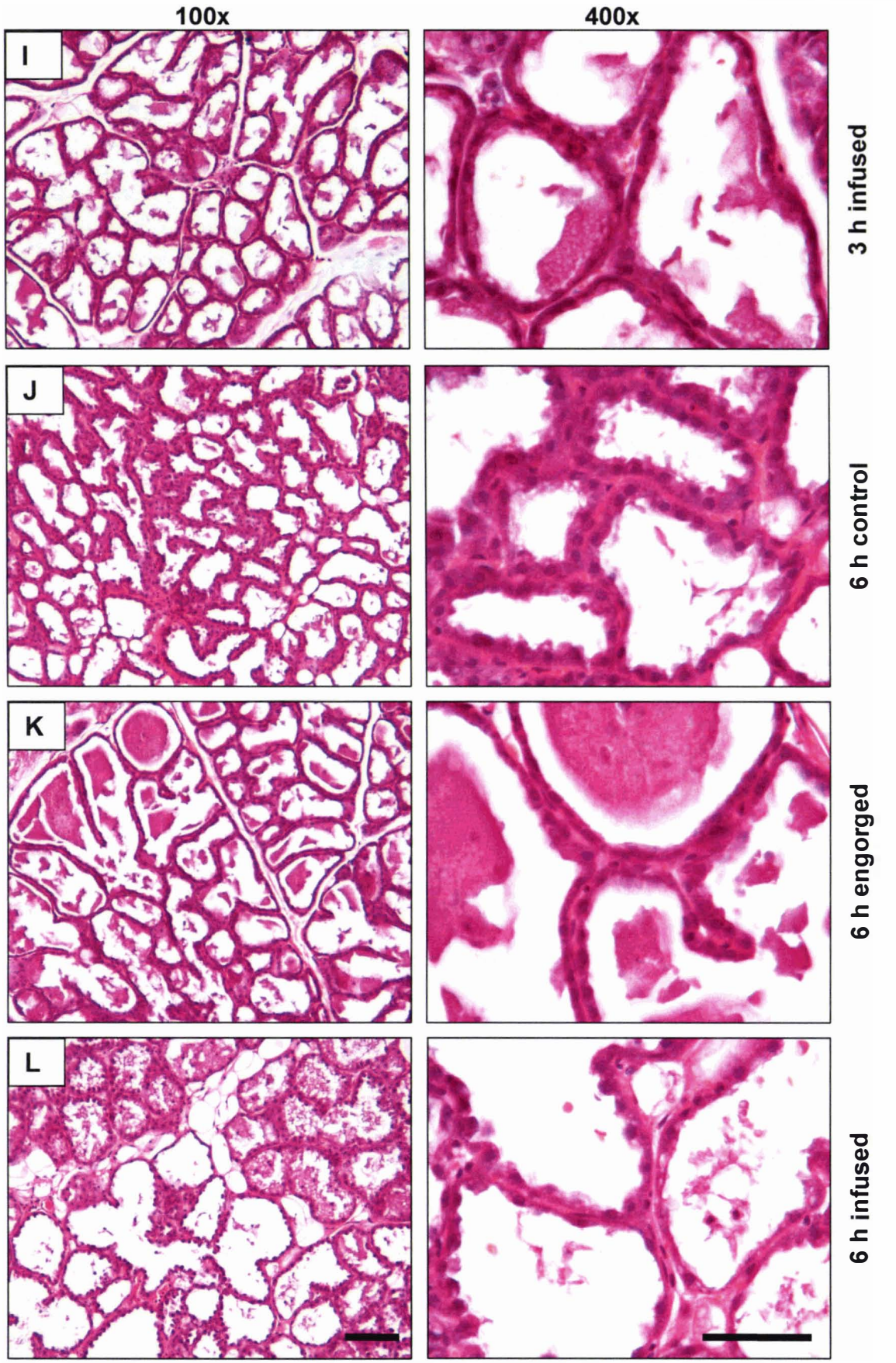


Figure 5.2. Morphological changes in rat mammary glands following acute physical distension. Representative H&E stained sections are shown for suckled (control) glands and corresponding teat-sealed (engorged) and acutely distended (infused) glands at 0 h (**A, B, C**); 1 h (**D, E, F**); 3 h (**G, H, I**); and 6 h (**J, K, L**), respectively. Refer to the text for a detailed description of histological features. Scale bars are at 100 μm and 50 μm for 100 x and 400 x magnifications, respectively.

5.3.2 ISEL of apoptotic nuclei

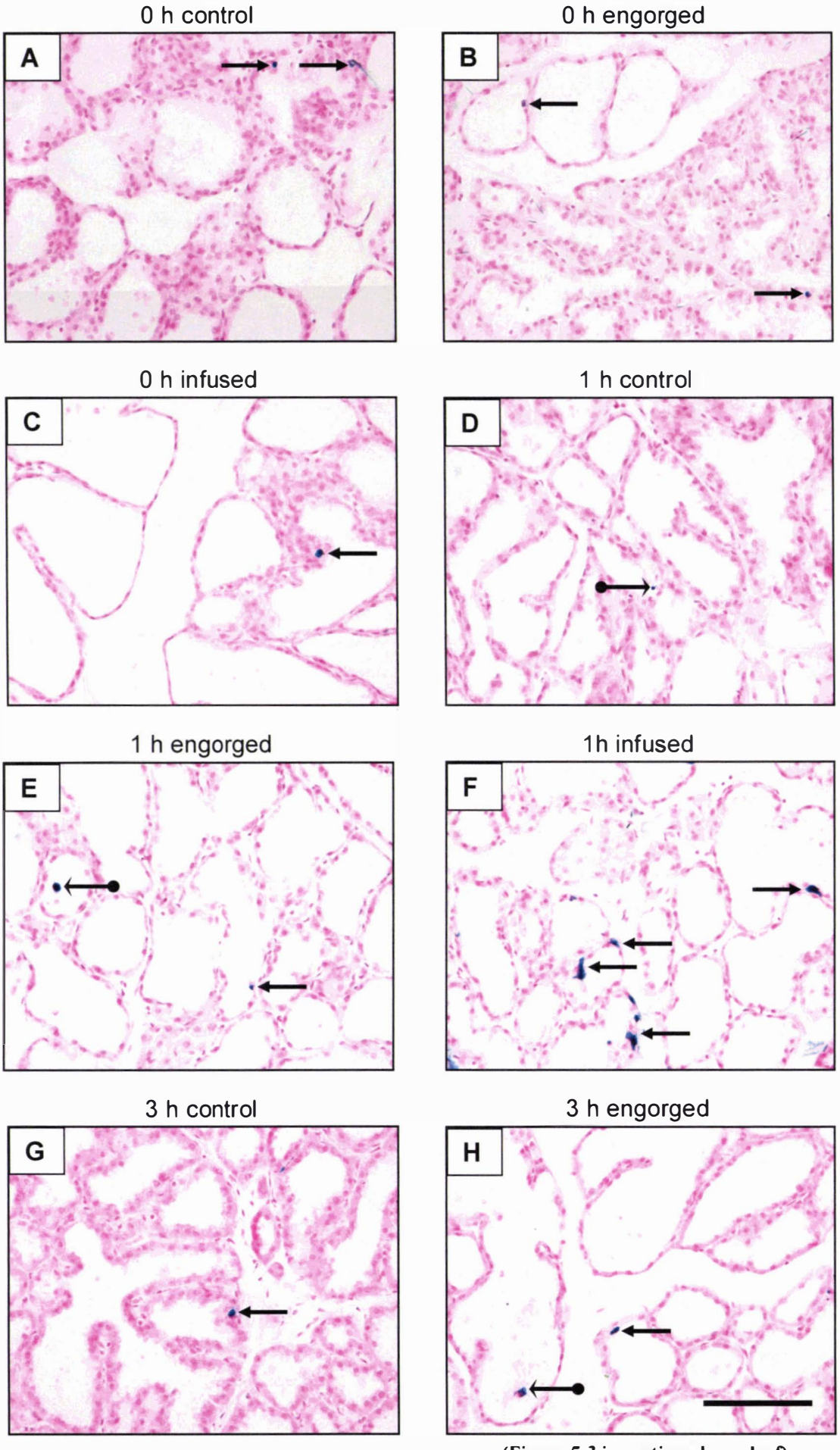
5.3.2.1 Time course of the onset of apoptosis following the acute physical distension of rat mammary glands

ISEL was used to detect apoptosis in rat mammary tissue following acute physical distension, with representative sections shown in Figure 5.3. Apoptotic nuclei were not labelled in negative controls (Fig. 5.3M and N). Sections were given a qualitative score between 1 (lowest) to 5 (highest) of the level of apoptosis with means for the control, engorged and infused glands at each time point presented in Fig. 5.4. Numbers of positive ISEL nuclei were then counted to provide a quantitative measurement of apoptosis (Fig. 5.5 and 5.6). There was a strong positive correlation between the quantitative and qualitative scores of apoptosis ($r = 0.88$, $P<0.001$; Fig. 5.7), and similar results were obtained from both datasets, substantiating the findings discussed below.

A low frequency of apoptotic cells was detected in suckled (control) glands (Fig. 5.3 and 5.4) with no changes ($P>0.05$) in the number of total ISEL nuclei per alveolus during the course of the experiment (Fig. 5.5A). However, by 1 h there was a small increase in the qualitative score of apoptosis ($P<0.05$, Fig. 5.3 and 5.4) and the total number of ISEL nuclei per alveolus ($P<0.001$, Fig. 5.5A) for infused glands compared with control glands. By 3 h and 6 h this difference was dramatic, with higher scores of apoptosis ($P<0.001$; Fig. 5.3 and 5.4) and 17.7-fold and 11.2-fold increases ($P<0.001$) in total numbers of ISEL nuclei detected per alveolus for infused glands compared with control glands (Fig. 5.5A). The qualitative score of apoptosis and the total number of ISEL nuclei per alveolus were also increased for engorged glands compared with controls by 6 h ($P<0.05$, Fig. 5.3 and 5.4, and $P<0.001$, Fig. 5.5A; respectively). Furthermore, by 3 h and 6 h there were significantly higher qualitative scores of apoptosis ($P<0.01$ and $P<0.05$, respectively; Fig. 5.4) and numbers of total ISEL nuclei per alveolus in infused glands compared with engorged glands (12.4-fold and 3.7-fold, $P<0.001$; respectively; Fig. 5.5A), indicating that the acute physical distension procedure had accelerated the induction of apoptosis in rat mammary glands.

Numbers of ISEL nuclei located within epithelia were increased ($P<0.001$) at 1, 3 and 6 h, while luminal ISEL nuclei increased ($P<0.001$) at 3 h and 6 h, in infused glands compared with control glands (Fig. 5.6A). Numbers of epithelial ISEL nuclei per alveolus were also higher in engorged glands compared with control glands at 1 h ($P<0.01$) and 6 h ($P<0.001$), but there were no significant differences for luminal ISEL nuclei ($P>0.05$). Moreover, by 3 h and 6 h there were greater ($P<0.001$) numbers of epithelial and luminal ISEL nuclei per alveolus in infused glands compared with engorged glands (Fig. 5.6A). However, apoptosis was greater ($P<0.001$) in the epithelial layer than within alveolar lumens for all treatments. Luminal ISEL apoptotic nuclei in infused glands at 6 h were associated with small numbers of neutrophils (Fig.5.3M and N) and sloughed epithelial cells, although we were unable to distinguish between the apoptosis of these cell types.

The number of alveoli per 100 x magnification field was used to correct counts of ISEL nuclei per field for changes in alveolar lumen size following induced physical distension and teat sealing. Similar results were observed when the data were analysed as the uncorrected number of ISEL nuclei per field (Fig. 5.5B and 5.6B), and there was a strong positive association between the number of total ISEL nuclei per alveolus and per field ($r = 0.99$, $P<0.001$; Fig. 5.8). There was a significant decrease in the number of alveoli per field in infused glands compared with control glands at 3 h ($P<0.001$) and 6 h ($P<0.05$), and compared with engorged glands at 0 h and 3 h ($P<0.05$ each) (Fig. 5.9). A reduced number of alveoli per field was 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, and agrees with the increased alveolar lumen size in infused glands described in section 5.3.1.1 above.



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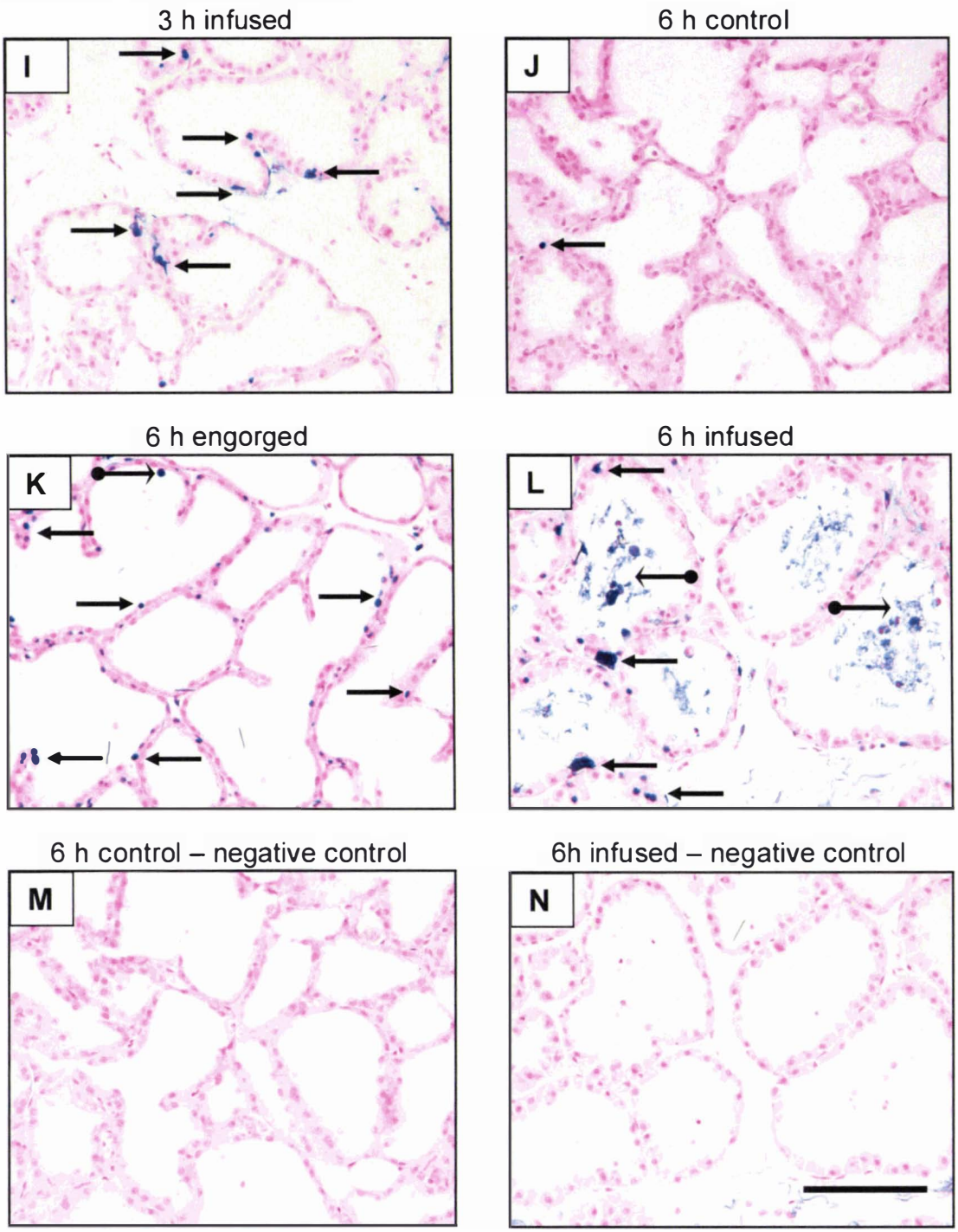


Figure 5.3. *In situ* end-labelling (ISEL) of apoptotic nuclei in rat mammary tissue following acute physical distension.

Representative sections are shown at 200 x magnification for suckled (control) glands and corresponding teat-sealed (engorged) and acutely distended (infused) glands at 0 h (**A, B, C**); 1 h (**D, E, F**); 3 h (**G, H, I**); and 6 h (**J, K, 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 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.

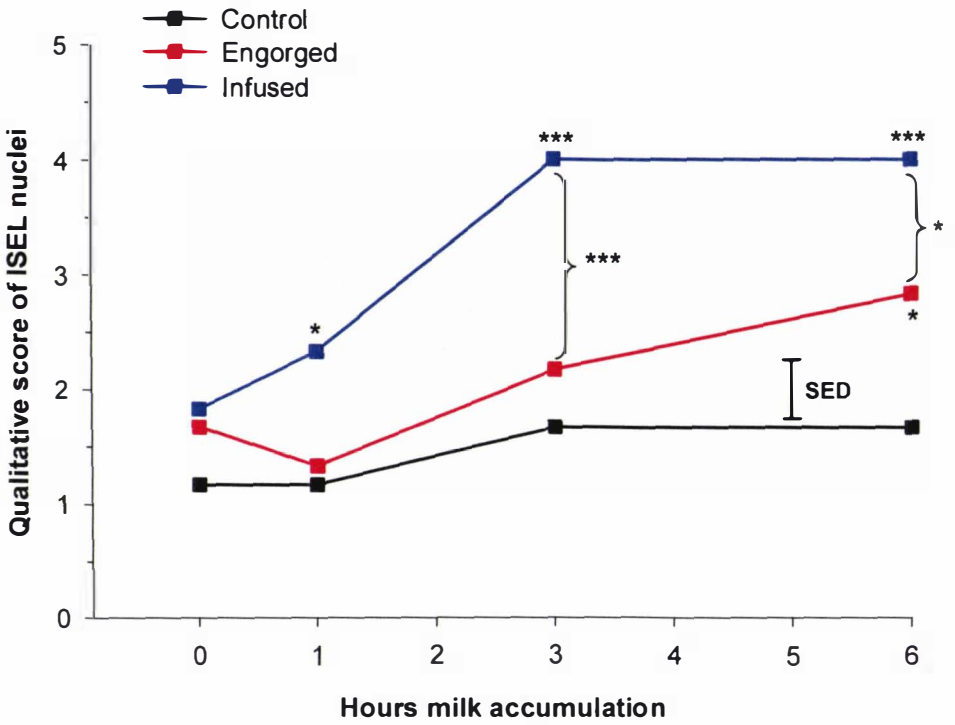


Figure 5.4. Qualitative analysis of *in situ* end-labelled (ISEL) apoptotic nuclei following the acute physical distension of rat mammary glands.

Data are expressed as the mean qualitative score of the level of apoptosis on a scale of 1 to 5 where; 1 = none to low, 2 = low to moderate, 3 = moderate to high, 4 = high and 5 = very high numbers of positive ISEL nuclei 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 control, engorged and infused glands at each time point (* $P<0.05$, *** $P<0.001$ relative to control glands, } = P-value significant for infused glands relative to engorged glands). There were no differences ($P>0.05$) in the qualitative score of apoptosis between time points for control glands.

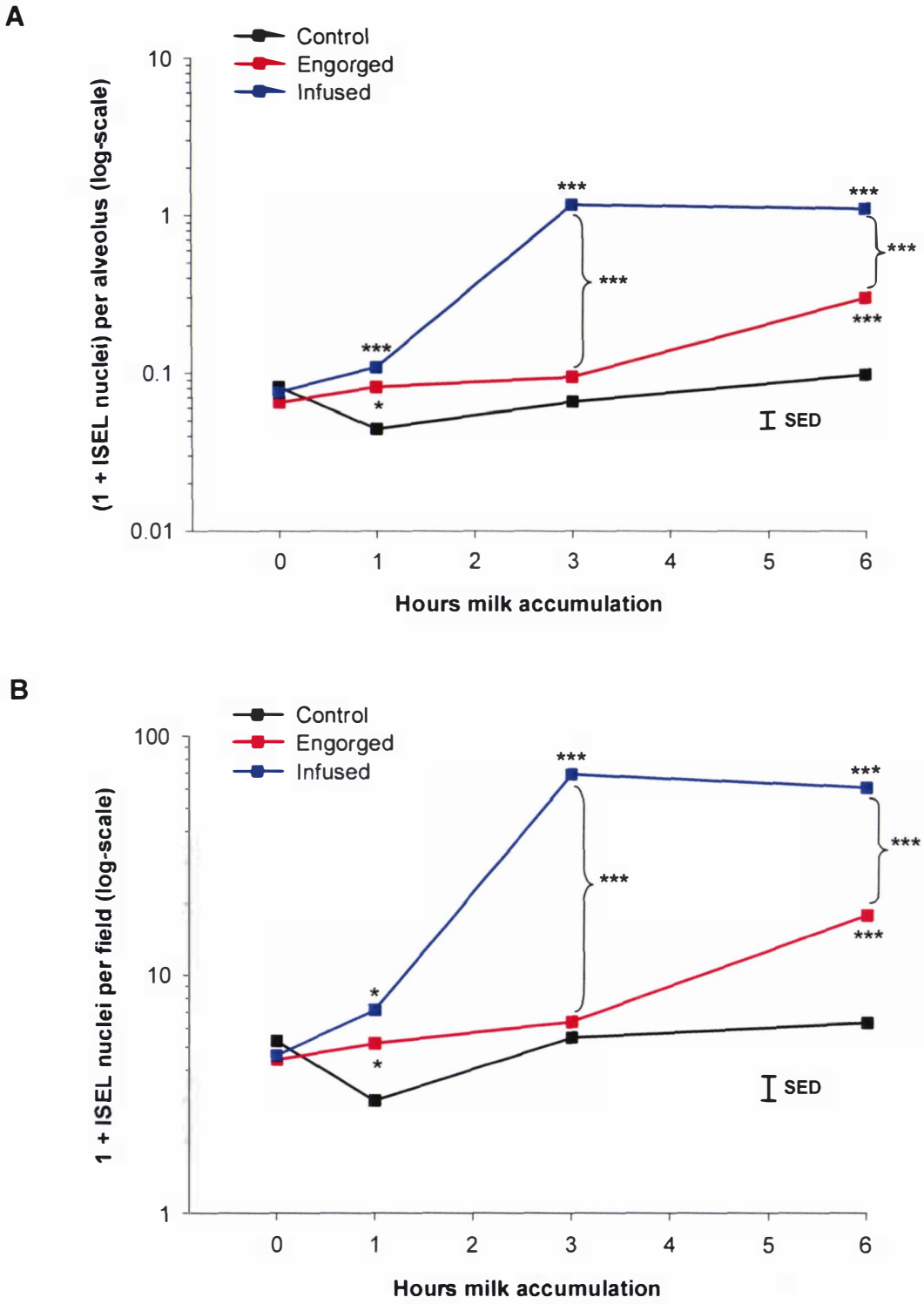


Figure 5.5. Quantitative analysis of the total number of *in situ* end-labelled (ISEL) apoptotic nuclei following the acute physical distension of rat mammary glands.
Data are expressed as the back-transformed mean number of total 1+ ISEL nuclei per alveolus (**A**) and per 100 x magnification field (**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 control, engorged and infused glands at each time point (* $P<0.05$, *** $P<0.001$ relative to control glands, } = P-value significant for infused glands relative to engorged glands). There were no differences ($P>0.05$) in numbers of ISEL apoptotic nuclei per alveolus and per field between time points for control glands.

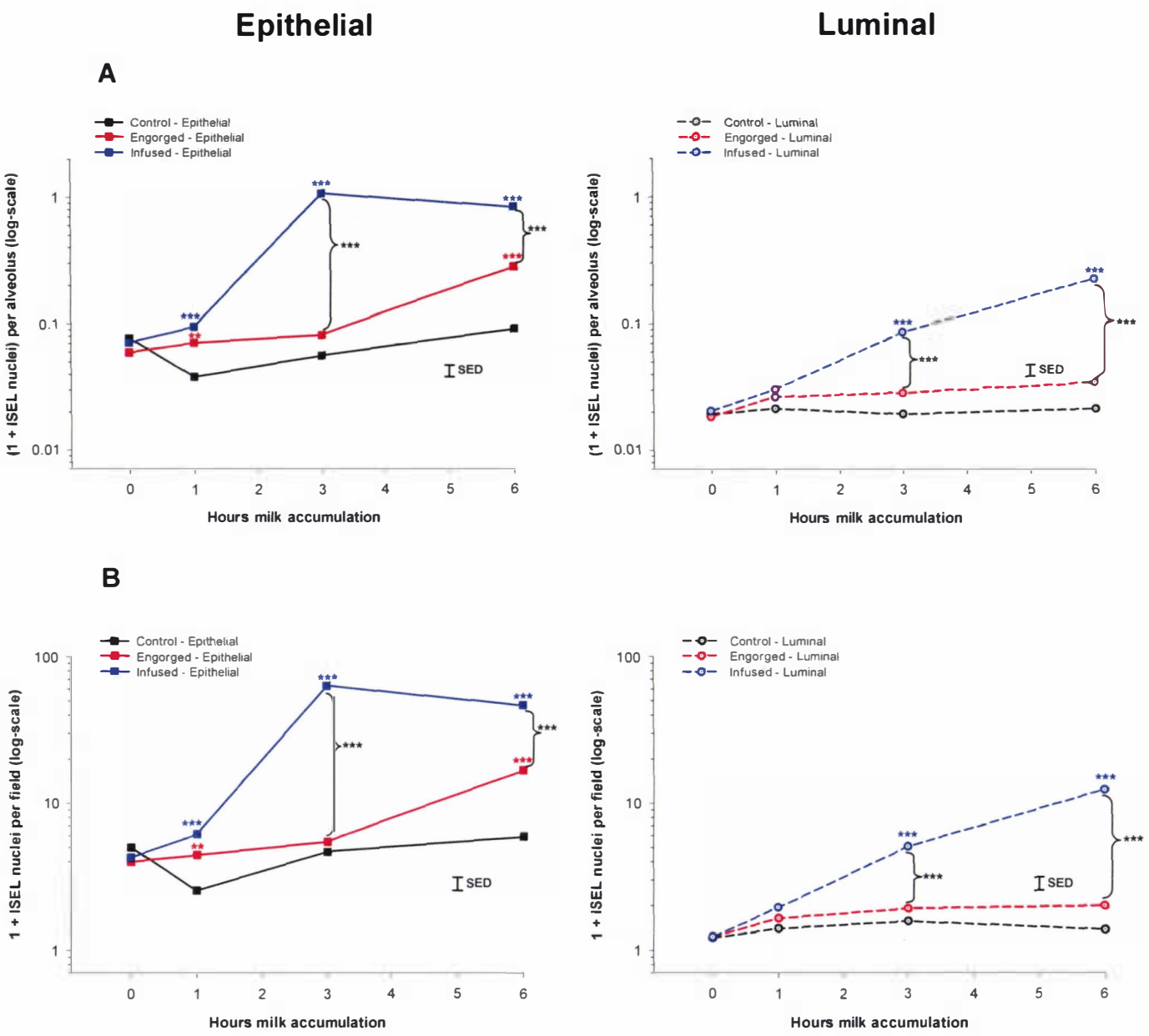


Figure 5.6. Quantitative analysis of the number of *in situ* end-labelled (ISEL) apoptotic nuclei located within alveolar epithelia or lumina following the acute physical distension of rat mammary glands.

Data are expressed as the back-transformed mean number of epithelial and luminal 1+ ISEL nuclei per alveolus (**A**) and per 100 x magnification field (**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. There were no differences (P>0.05) in numbers of epithelial or luminal ISEL nuclei per alveolus and per field between time points for control glands, except for slightly lower (P<0.05) numbers of epithelial ISEL nuclei at 1 h compared with 0 and 6 h.

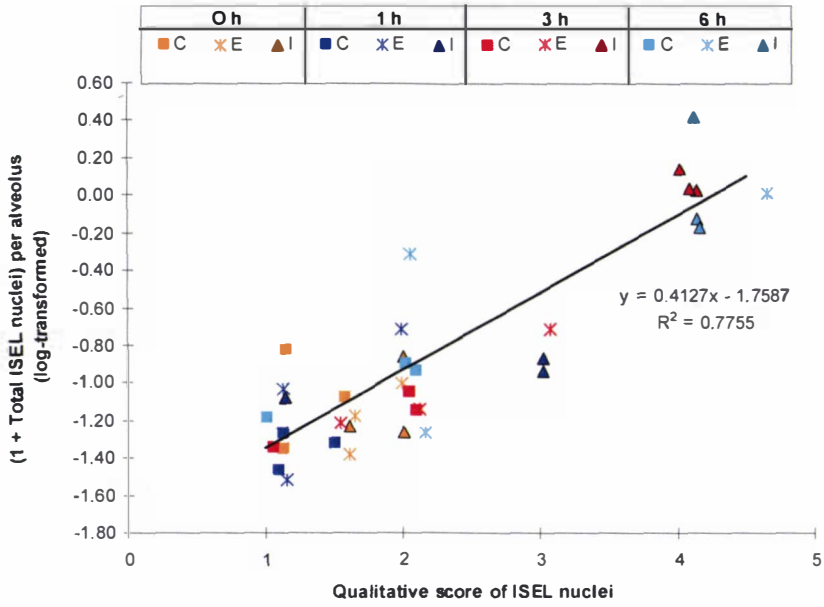


Figure 5.7. The relationship between qualitative and quantitative analyses of *in situ* end-labelled (ISEL) apoptotic nuclei following the acute physical distension of rat mammary glands.
Data are expressed as the qualitative score of the level of apoptosis on a scale of 1 to 5 where; 1 = none to low, 2 = low to moderate, 3 = moderate to high, 4 = high and 5 = very high numbers of positive ISEL nuclei, and as the log-transformed mean number of total 1+ ISEL nuclei per alveolus, 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.

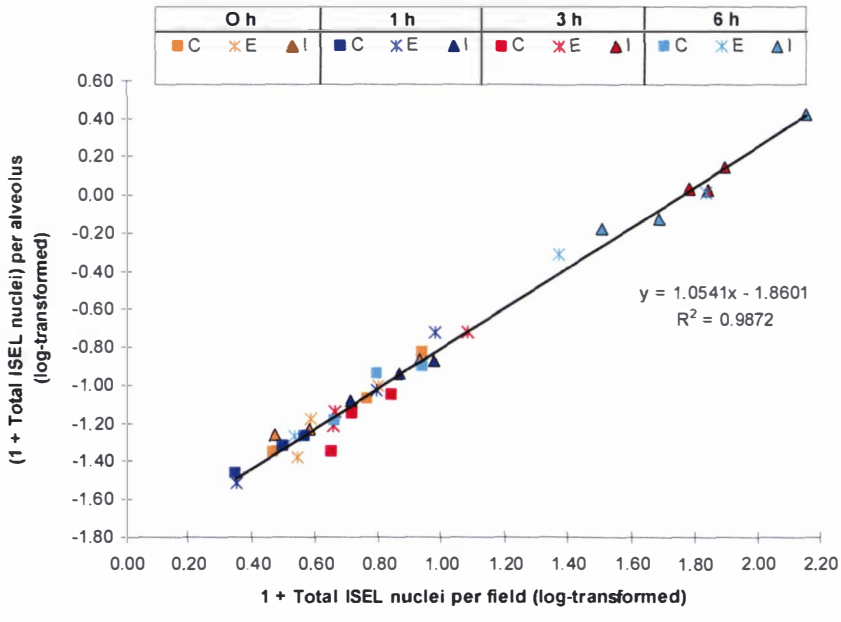


Figure 5.8. The relationship between the number of *in situ* end-labelled (ISEL) apoptotic nuclei per field and per alveolus following the acute physical distension of rat mammary glands.
Data are expressed as the log-transformed mean number of total 1+ ISEL nuclei per 100 x magnification field and per alveolus 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.

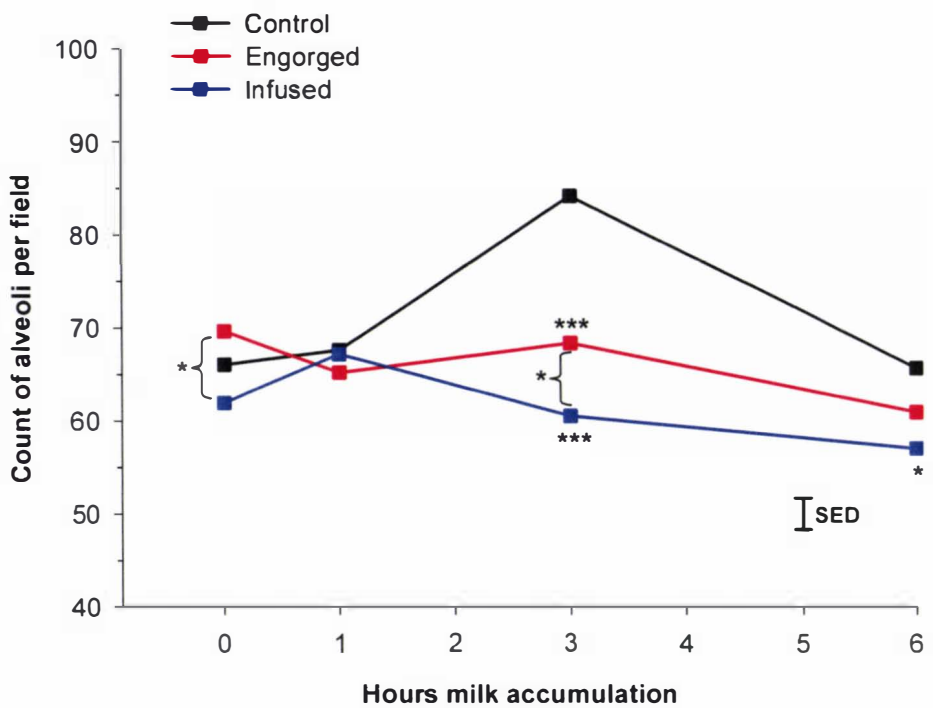


Figure 5.9. The number of alveoli per field following the acute physical distension of rat mammary glands.

Data are expressed as the mean number of alveoli per 100 x magnification field 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 control, engorged and infused glands at each time point (* P<0.05, *** P<0.001 relative to control glands, { = P-value significant for infused glands relative to engorged glands). The numbers of alveoli per field were greater (P<0.01) in control glands at 3 h compared with 0, 1 and 6 h, with no other differences (P>0.05) between time points.

5.3.3 Gene expression of TJ proteins

5.3.3.1 Time course of changes in mRNA expression of TJ proteins following the acute physical distension of rat mammary glands

TJ protein mRNA expression was examined following acute physical distension and mammary engorgement, induced by teat-sealing (Fig. 5.10). Occludin and ZO-1 mRNA expression followed a similar pattern with higher levels in infused glands than in control glands at 0 h (1.7-fold, P<0.01 and 2.6-fold, P<0.001; respectively). However, the levels of occludin and ZO-1 mRNA were also unexpectedly greater in engorged glands compared with controls at 0 h (1.5-fold, P<0.05 and 2.1-fold, P<0.01; respectively), when both glands would have been in a recently suckled state. Moreover, occludin and ZO-1 mRNA expression in control glands were lower at 0 h than at 6 h (P<0.05 and P<0.01, respectively), and there were no differences (P>0.05) between treatments at 1 and 3 h. Therefore, the differences between treatments at 0 h should be interpreted with caution. Nevertheless, occludin and ZO-1 mRNA expression were decreased in infused glands compared with control (1.5-fold, P<0.05 and 1.7-fold, P<0.05; respectively) and engorged (1.7-fold, P<0.01 and 2.0-fold, P<0.01; respectively) glands within 6 h of teat-sealing.

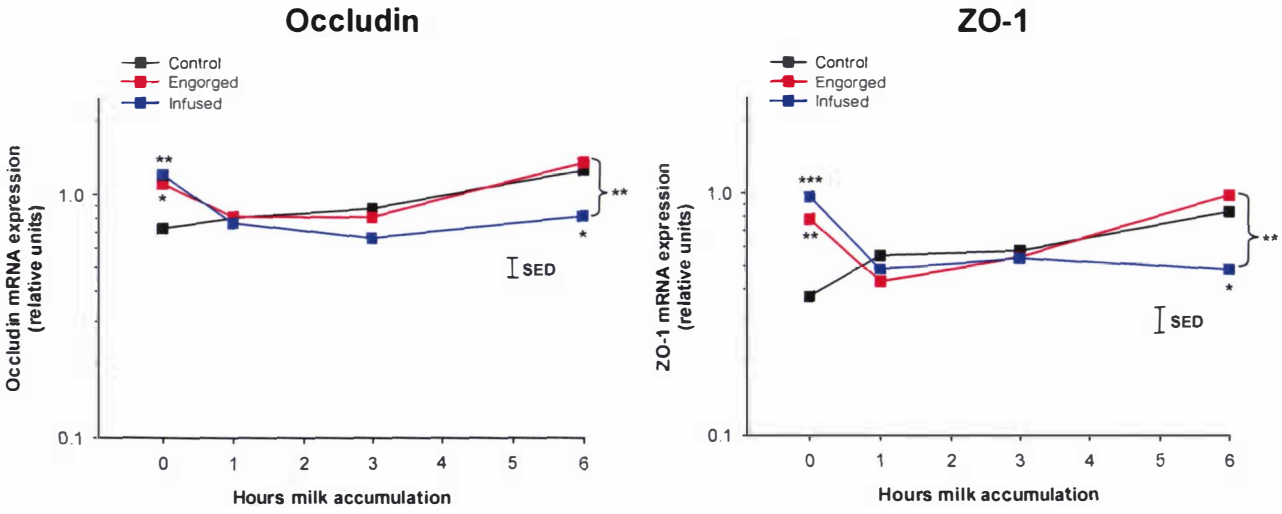


Figure 5.10. The pattern of gene expression of tight junction proteins following the acute physical distension of rat mammary glands.
Changes in mRNA levels of occludin and ZO-1 in the suckled (control), teat-sealed (engorged) and acutely distended (infused) glands of animals at 0, 1, 3, and 6 h (n=6 per time point) following teat sealing. Data are expressed as back-transformed mean relative units with the SED to compare control, engorged and infused glands at each time point (* P<0.05, ** P<0.01, *** P<0.001 relative to control glands, { = P-value significant for infused glands relative to engorged glands). In control glands, occludin and ZO-1 mRNA expression were decreased at 0 h compared with 6 h (P<0.05 and P<0.01, respectively), with no other differences (P>0.05) between time points.

5.3.4 Western blot analysis of β1-integrin, STAT3 and TJ protein expression

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

Western blotting was used to determine the pattern of TJ protein expression in NP-40-soluble and -insoluble fractions following induced physical distension of rat mammary glands. Expression of β1-integrin and pSTAT3 in the soluble fraction were also examined as examples of proteins well known to be down-regulated and up-regulated, respectively, during mammary apoptosis and involution (western analyses provided courtesy of Dr. Kuljeet Singh and Joanne Dobson, Dairy Science and Technology Group, AgResearch Ruakura). A single band for β1-integrin was detected at ~80 kDa, which aligned with positive controls (results not shown). A lower MW band was also detected in close proximity, although only the ~80 kDa band of β1-integrin altered consistently with treatment as reported previously (McMahon *et al.*, 2004; Appendix VII). 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. The expression 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 (Fig. 5.11A and B). In contrast, pSTAT3 was dramatically 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) (Fig. 5.12A and B). However, expression of total STAT3 was down-regulated only 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 (Fig. 5.12A and B).

Immunoreactive bands for occludin (~60 kDa), claudin-1 (~22 kDa and ~28 kDa) and ZO-1 (~225 kDa) were detected as described in section 4.3.4.1. Occludin protein expression was significantly up-regulated in the soluble (mean 1.6-fold, $P<0.05$) and insoluble (mean 6.0-fold, $P<0.001$) fractions of infused glands compared with control

and engorged glands immediately following physical distension at 0 h (Fig. 5.13A and B). However, expression in the soluble fraction 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. Furthermore, expression was decreased (1.6-fold, $P<0.05$) in infused glands compared with engorged glands at 6 h. Occludin protein expression in the insoluble fraction also declined for infused glands to levels similar to those in control and engorged glands by 1 h and 3 h, although expression at 6 h was increased for engorged glands relative to control (2.8-fold, $P<0.05$) and infused (3.0-fold, $P<0.01$) glands. The claudin-1 ~28 kDa band was significantly decreased in engorged glands (4.2-fold, $P<0.05$), but not in infused glands, compared with controls by 6 h (Fig. 5.14A and B). However, there were no changes ($P>0.05$) in expression of the claudin-1 ~22 kDa band between treatments. ZO-1 protein expression was decreased by 1.6-fold in the soluble ($P<0.05$) and insoluble ($P<0.1$) fractions in engorged glands, but not in infused glands, compared with controls by 6 h (Fig. 5.15A and B).

In control glands, there were no significant differences ($P>0.05$) between time points for β1-integrin, occludin and ZO-1 proteins in the soluble fraction. However, claudin-1 ~28 kDa band expression in the soluble fraction was lower ($P<0.01$) at 3 h than 0 and 1 h for control glands, with no other differences ($P>0.05$) between time points. Expression of the claudin-1 ~22 kDa band significantly declined between 0 and 6 h time points for all treatments. STAT3 expression 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 ($P>0.05$) were detected between time points. In the insoluble fraction, occludin and ZO-1 protein expression in control glands were generally greater (at least $P<0.05$) at 0 h than other time points.

Even sample loading of SDS-PAGE gels was verified by Coomassie Blue staining of untransferred gels for soluble and insoluble protein fractions (Fig. 5.16), while the effective transfer of proteins onto a nitrocellulose membrane was confirmed by Ponceau S staining (results not shown).

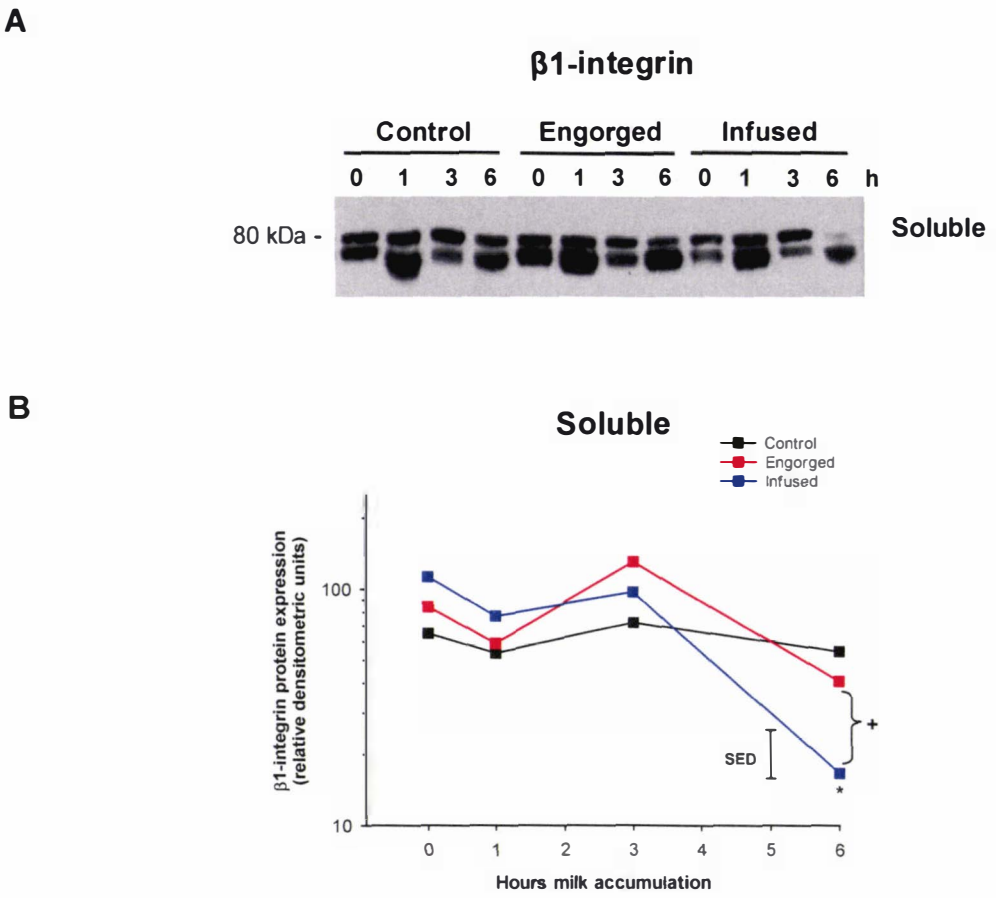


Figure 5.11. The pattern of β1-integrin protein expression following the acute physical distension of rat mammary glands.

Densitometric analyses of western blots are shown for the expression levels 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 where twenty micrograms of protein was loaded into each lane, 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). There were no differences (P>0.05) between time points in β1-integrin expression for control glands. The primary antibody used was rabbit anti-human β1-integrin (1:3000 dilution; obtained from Santa Cruz Biotechnology). Data provided courtesy of Dr. Kuljeet Singh and Joanne Dobson, Dairy Science and Technology Group, AgResearch Ruakura.

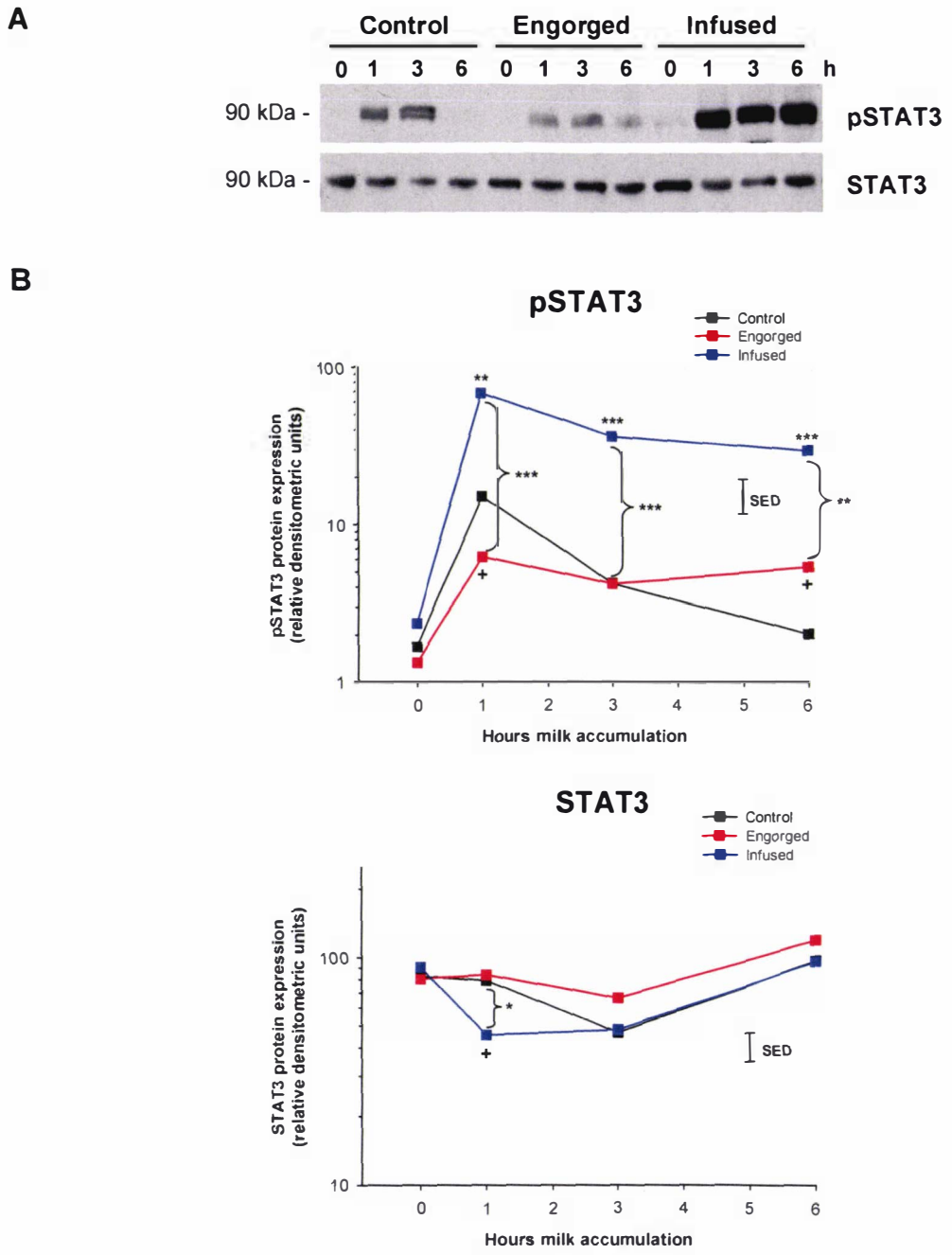


Figure 5.12. The pattern of pSTAT3 and STAT3 protein expression following the acute physical distension of rat mammary glands.

Densitometric analyses of western blots are shown for the expression levels 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 where twenty micrograms of protein was loaded into each lane, 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). In control glands, STAT3 expression 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, but no other differences ($P>0.05$) were detected between time points. The primary antibodies used were rabbit anti-human pSTAT3 (1:2000 dilution) and rabbit anti-mouse STAT3 (1:10,000 dilution) (both obtained from Santa Cruz Biotechnology). Data provided courtesy of Dr. Kuljeet Singh and Joanne Dobson, Dairy Science and Technology Group, AgResearch Ruakura.

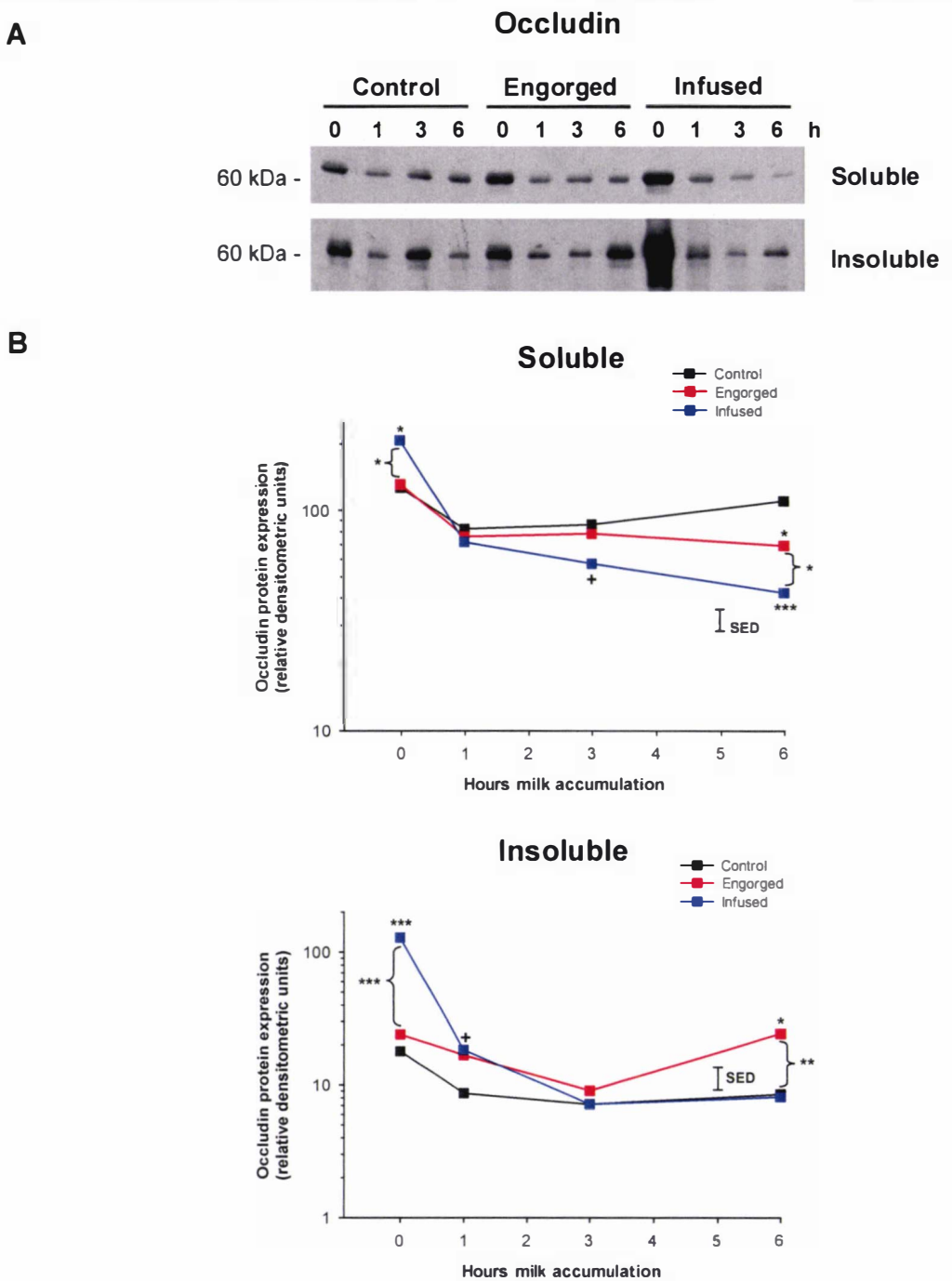


Figure 5.13. The pattern of occludin protein expression following the acute physical distension of rat mammary glands.

Densitometric analyses of western blots are shown for the expression levels of occludin in NP-40-soluble and –insoluble 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 where twenty micrograms of protein was loaded into each lane, 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). There were no differences (P>0.05) between time points in occludin expression in the soluble fraction for control glands. However, expression in the insoluble fraction was higher (P<0.05) at 0 h than 3 and 6 h, with no other differences (P>0.05) between time points. The primary antibody used was rabbit anti-human occludin (1:80,000 and 1:20,000 dilutions for soluble and insoluble fractions, respectively; obtained from Zymed Laboratories Inc.).

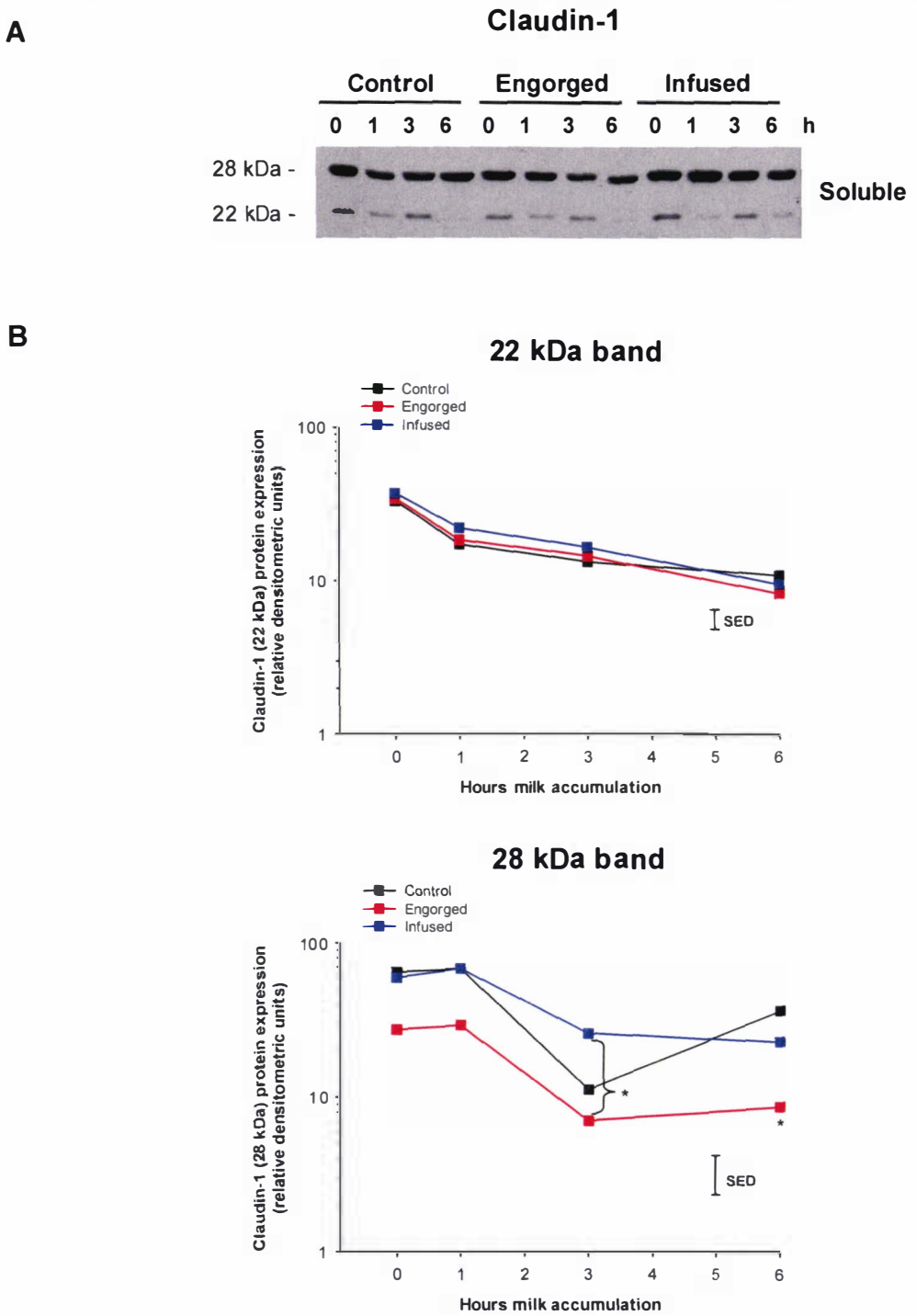


Figure 5.14. The pattern of claudin-1 protein expression following the acute physical distension of rat mammary glands.

Densitometric analyses of western blots are shown for the expression levels of 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 where forty micrograms of protein was loaded into each lane, 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). In control glands, claudin-1 ~28 kDa band expression was lower ($P<0.01$) at 3 h than 0 and 1 h, with no other differences ($P>0.05$) between time points. However, expression of the claudin-1 ~22 kDa band significantly declined between 0 and 6 h time points for all treatments. The primary antibody used was rabbit anti-human claudin-1 (1:1000 dilutions; obtained from Zymed Laboratories Inc.).

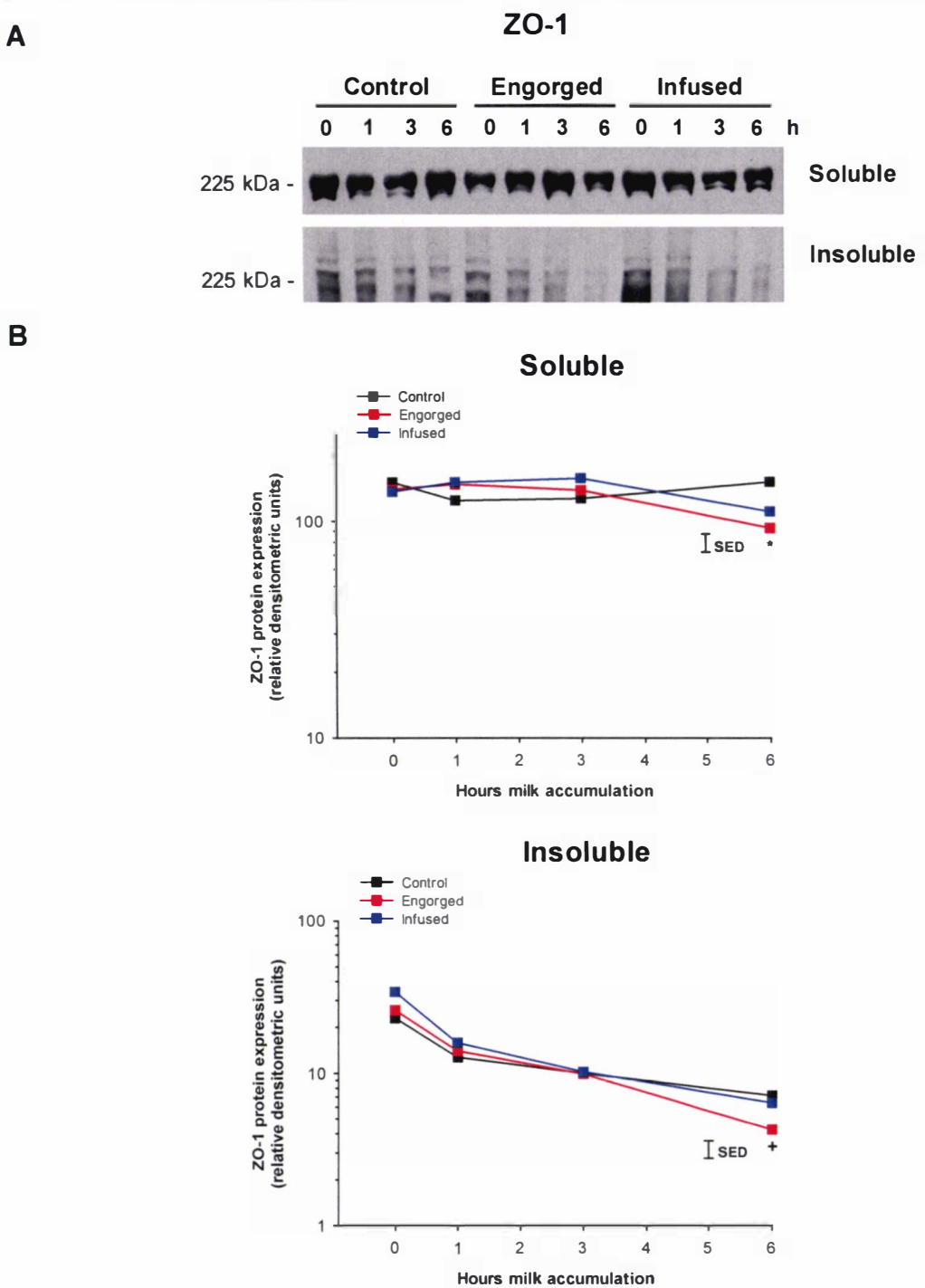


Figure 5.15. The pattern of ZO-1 protein expression following the acute physical distension of rat mammary glands.

Densitometric analyses of western blots are shown for the expression levels of ZO-1 in NP-40-soluble and –insoluble 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 where twenty micrograms of protein was loaded into each lane, 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). There were no differences ($P>0.05$) in ZO-1 expression in the soluble fraction between time points for control glands. However, ZO-1 expression in the insoluble fraction declined ($P<0.001$) between 0 and 6 h time points for all treatments. The primary antibody used was rabbit anti-human ZO-1 (1:10,000 and 1:1000 dilutions for soluble and insoluble fractions, respectively; obtained from Zymed Laboratories Inc.).

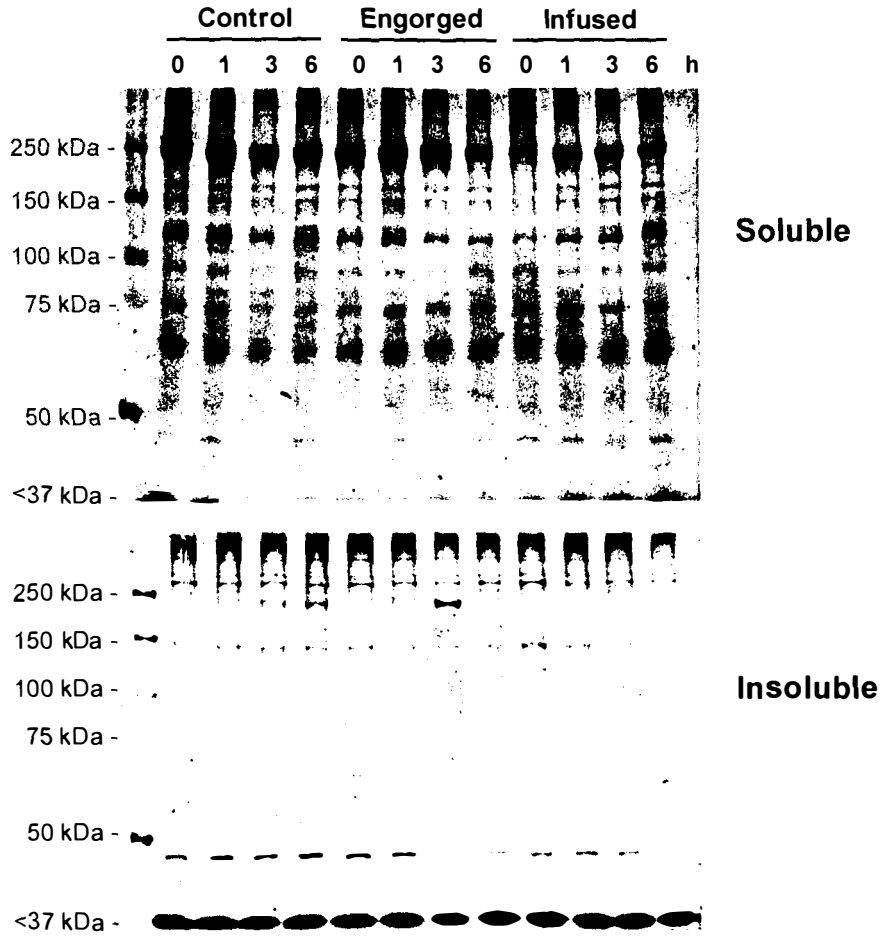


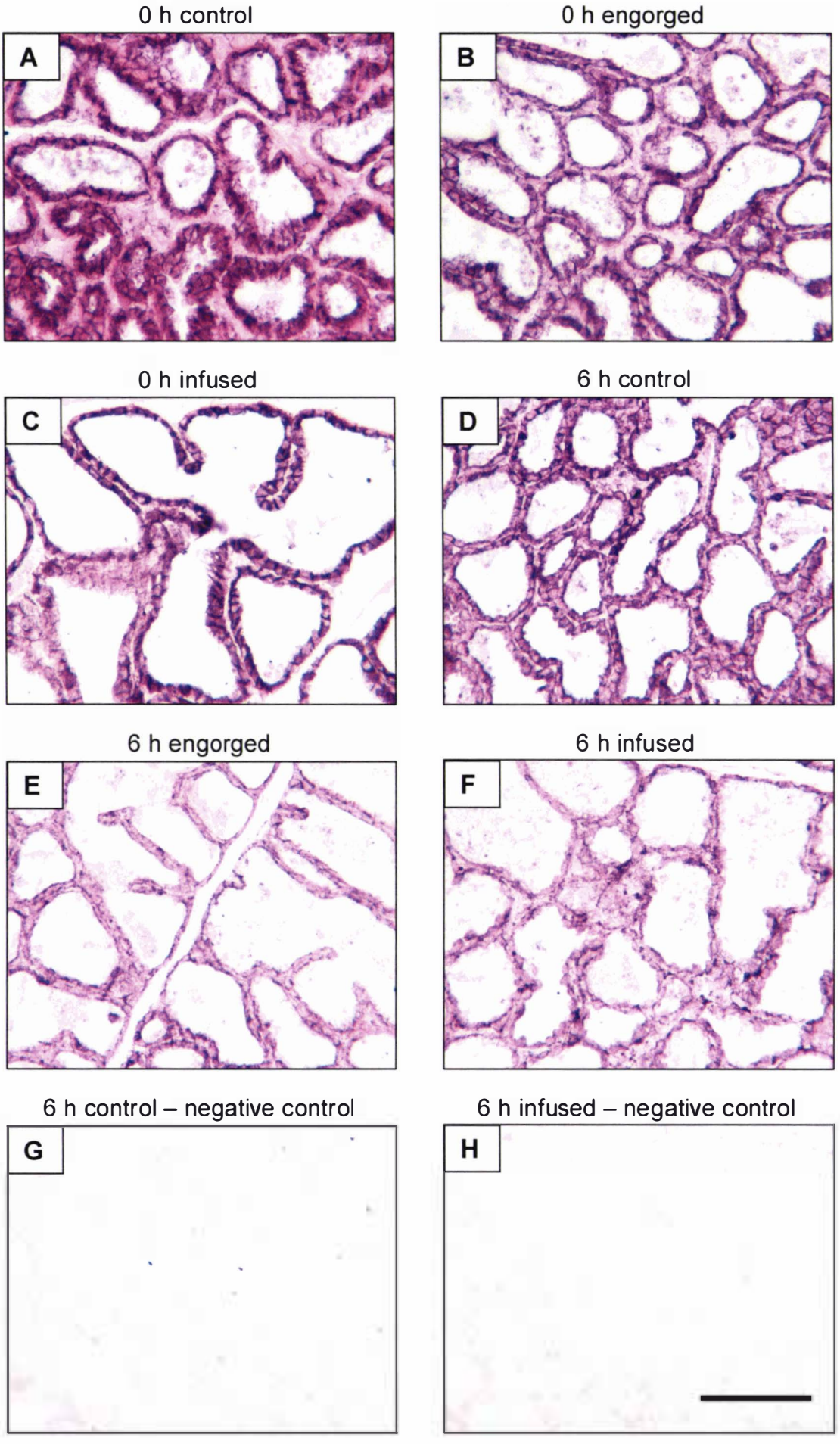
Figure 5.16. Coomassie blue stained gels loaded with protein samples from control, engorged and infused rat mammary glands following teat sealing.
Representative 8% SDS-PAGE gels containing NP-40-soluble and –insoluble protein fractions extracted from suckled (control), teat-sealed (engorged) and acutely distended (infused) mammary glands of animals at 0, 1, 3, and 6 h following teat sealing (n=6 per time point). Twenty micrograms of protein was loaded into each lane, except lane 1 which contains 5 µl of the Precision Plus Protein Standard (BioRad Laboratories). Gels were not transferred and were stained with Coomassie blue for 18 h then de-stained in 25% methanol until optimal colour intensity was obtained.

5.3.5 Immunohistochemistry of occludin protein expression

5.3.5.1 Time course of changes in occludin protein expression and localisation following the acute physical distension of rat mammary glands

Immunohistochemistry was used for localisation of occludin protein expression in suckled, engorged and infused rat mammary tissue. A strong signal for occludin protein was detected on the apical, basal and especially lateral membranes of secretory epithelial cells, while the cytoplasm was diffusely labelled, in suckled alveoli from control glands (Fig. 5.17A and D). By 6 h of teat-sealing, occludin labelling was reduced as alveoli became engorged and distended with milk (Fig. 5.17E). Furthermore, while the occludin signal was strong in infused glands immediately after induced distension at 0 h (Fig. 5.17C), it had decreased within 6 h (Fig. 5.17F) consistent with the down-regulation of occludin protein expression reported in section 5.3.4.1. The membranes of cells located within alveolar lumina were not labelled. There was also no signal on concurrent negative control sections where primary antibodies to occludin were either omitted (Fig. 5.17G and H), or substituted with IgG at the same molar concentration (results not shown).

Figure 5.17. Occludin protein expression and localisation in rat mammary tissue following acute physical distension.
Representative sections labelled with antibodies to occludin are shown at 200 x magnification for suckled (control) glands and corresponding teat-sealed (engorged) and acutely distended (infused) glands at 0 h (**A, B, C**); and 6 h (**D, E, F**), respectively. The omission of primary antibodies to occludin was used to provide concurrent negative controls, with representative sections shown for 6 h control (**G**) and infused (**H**) glands. Sections are lightly counterstained with eosin and the scale bar equals 100 μm.


0 h control
A
0 h engorged
B
0 h infused
C
6 h control
D
6 h engorged
E
6 h infused
F
6 h control – negative control
G
6 h infused – negative control
H

5.4 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 β1-integrin and occludin protein expression, and the increase in pSTAT3 protein expression, 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 primary trigger of mammary apoptosis and involution (Davis *et al.*, 1999; Stelwagen, 2001).

This 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 milkings) stopped milk secretion within 1-2 days (Fleet & Peaker, 1978), and acute distension of the mammary gland, with isosmotic sucrose solution to raise the intra-mammary pressure to levels normally observed following the cessation of milking, reduced the rate of milk secretion within 6 h (Peaker, 1980). Furthermore, in an experiment with cows which were milked once daily, and were also infused with an intra-mammary solution of sucrose and lactose equivalent to "5 hours worth" of milk, TJs became leaky much earlier (after 7 h instead of 17 h) and milk secretion was inhibited more than in ODM control cows (Stelwagen *et al.*, 1998a). In these experiments dilution of potential chemical inhibitor(s) in milk would have occurred. Thus these results indicate that physical distension plays a major role in regulating mammary function during infrequent milking or the cessation of milk removal.

Other researchers have reported that a chemical inhibitory mechanism(s) may also control local mammary function. In an experiment 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 (Henderson & Peaker, 1984). 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 isosmotic sucrose solution, the milk secretion rate increased leading to the suggestion that dilution of a chemical inhibitor occurred (Henderson & Peaker, 1987).

This role of chemical inhibition has been attributed to FIL, a small whey protein which inhibits milk secretion *in vitro* and *in vivo* (Wilde *et al.*, 1995). However, to date there is no published information regarding the complete amino acid sequence of FIL or identification of the gene coding it. Furthermore, while FIL has been demonstrated to inhibit milk protein secretion *in vitro* by blocking the early stages of constitutive secretory pathway at the level of ER-Golgi transport (Rennison *et al.*, 1993), its exact mode of action and kinetics of feedback inhibition *in vivo* are still uncertain.

Several alternative chemical regulatory systems have recently been proposed. In the first mechanism, casein phosphopeptides derived from massive activation of plasmin protease activity disrupt TJs and inhibit milk secretion during extended periods of milk accumulation in goats and cows (Shamay *et al.*, 2002, 2003; Silanikove *et al.*, 2006), 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 MECs (Silanikove *et al.*, 2000).

A separate mechanism 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 (Matsuda *et al.*, 2004). While this homeostatic regulation appears important for suppression of milk secretion in response to milk accumulation within alveolar lumens, the specific mechanisms governing this process are not yet established.

The inconsistencies between studies supporting chemical inhibition and those supporting physical distension, indicate that despite decades of research the mechanisms regulating local control of mammary function still require clarification. While the current study supports physical distension as a regulator of mammary function during

extended periods of milk accumulation, it cannot be ruled out that both chemical and physical mechanisms, acting either independently or in concert, finely control mammary responses to the frequency and completeness of milk removal.

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 (Fleet & Peaker, 1978; Peaker, 1980). It is unlikely that either sucrose or lactose themselves act as chemical inhibitors, as neither crosses the apical membrane into MECs (Fleet & Peaker, 1978; Peaker, 1978). 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 prevent osmotic water movements (Linzell *et al.*, 1976; Peaker, 1977). Furthermore, it is also unlikely that the observed changes in protein expression and apoptosis in the current 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 (DeNuccio & Grosvenor, 1971).

Instead these changes in protein expression and apoptosis were most likely induced by physical stretching of the mammary epithelium - which was confirmed by histological observations that the majority of alveoli were distended and MECs 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 was supported by 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 MECs 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. This was supported by a gradual decrease in occludin protein and mRNA expression in infused glands.

However, expression levels of occludin protein and mRNA, and ZO-1 mRNA, were initially dramatically up-regulated in infused glands immediately following the acute physical distension procedure. Surprisingly, the mRNA, but not protein, expression levels of occludin and ZO-1 were also greater at 0 h in teat-sealed glands compared with controls. It is unclear why this occurred as both glands were in a recently suckled state, although the differences in mRNA expression between glands at 0 h should be interpreted carefully as mRNA expression in control glands was significantly lower at 0 h than at 6 h. Nevertheless, the dramatic up-regulation of occludin protein expression in infused glands immediately after induced physical distension may reflect an attempt to maintain mammary TJ integrity before the system was overwhelmed. Rapid increases in occludin protein expression, presumably to enhance TJ synthesis and repair, have also been reported in response to TJ breakdown by low-calcium conditions in mouse MECs *in vitro* (Stelwagen & Callaghan, 2003).

Furthermore, TJs have previously been reported to respond to mechanical tension as alterations in TJ strand arrangement and orientation occur when MECs are stretched *in vitro* (Pitelka & Taggart, 1983), while increased TJ permeability occurs during cyclic stretch in lung alveolar epithelial cells (Cavanaugh *et al.*, 2001; Cavanaugh & Margulies, 2002; Cavanaugh *et al.*, 2006). The latter was associated with down-regulated occludin protein expression, but no differences were detected in ZO-1 protein expression (Cavanaugh *et al.*, 2001). While the transient increase and then decline in occludin protein and mRNA expression in the current study were accompanied by similar changes in ZO-1 mRNA expression, there were no changes in the protein expression of either ZO-1 or claudin-1 in response to acute mammary physical distension.

Instead, there were decreases in the protein expression of ~28 kDa claudin-1 and ZO-1 by 6 h in engorged teat-sealed glands compared with suckled controls. In the previous Chapter 4, it was shown that a transient rise in the ~22 kDa band relative to the ~28 kDa band of claudin-1 occurs by 6 h after teat-sealing. However, in the current experiment, while the ~28 kDa band decreased, no increase in the ~22 kDa band was observed. The reason for this discrepancy is unclear, and it is possible that it would be observed if the experiment was extended past 6 h following teat-sealing. The small decrease in ZO-1

protein expression in engorged glands by 6 h is similar to the progressive down-regulation observed following teat-sealing in Chapter 4. Therefore, changes in the protein expression of occludin, but not claudin-1 and ZO-1, were accelerated by physical distension compared with milk accumulation alone. This indicates that changes in TJ protein expression during mammary apoptosis and involution are not necessarily regulated in concert, and that occludin protein expression is the most responsive to physical distension of the mammary epithelium.

Occludin is an integral transmembrane TJ protein in numerous epithelia (reviewed by Schneeberger & Lynch, 2004). Overexpression of occludin in MDCK cells decreased TJ permeability (McCarthy *et al.*, 1996), while introduction of COOH-terminally truncated occludin resulted in increased TJ permeability (Chen *et al.*, 1997). In the mammary gland, occludin may also play a vital role in TJ formation and closure during lactogenesis as occludin -/- mice have impaired lactation and fail to suckle their young (Saitou *et al.*, 2000). Furthermore, as occludin protein expression is down-regulated during mammary apoptosis and involution (Chapters 3 and 4), and experimentally-induced truncation of occludin causes MEC apoptosis *in vitro* and *in vivo* (Beeman & Neville, 2001), occludin may act as a cell survival 'signal'.

A rapid loss of cell-ECM survival signalling 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 (McMahon *et al.*, 2004, Appendix VII; Singh *et al.*, 2005, Appendix VI). The present study demonstrated that in the rat mammary gland, the loss of β1-integrin protein expression was 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 (Boudreau *et al.*, 1995; Streuli *et al.*, 1995; Pullan *et al.*, 1996; Faraldo *et al.*, 1998, 2002; Naylor *et al.*, 2005). Moreover, integrins can function as mechanotransducers in response to mechanical stress in other cell/tissue types (reviewed by Shyy & Chien, 1997; Geiger & Bershadsky, 2002); with β1-integrin-signalling pathways mediating mechanical stretch-induced apoptosis in vascular smooth muscle cells (Wernig *et al.*, 2003).

Therefore, the changes in occludin and β1-integrin protein expression 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 polymerised, 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 (Dewey, 1984; Wechezak *et al.*, 1985; Cavanaugh *et al.*, 2001, 2006). In this regard, cyclic stretch in lung alveolar epithelial cells alters F-actin distribution and increases TJ permeability (Margulies *et al.*, 1999; Cavanaugh *et al.*, 2001, 2006). These mechanisms are related as disruption of the F-actin cytoskeleton perturbs TJ structure and function (Madara, 1992; Cavanaugh *et al.*, 2001), and stabilising F-actin during cell stretching reduces the stretch-induced permeability increase (Cavanaugh *et al.*, 2006).

In the mammary gland reorganisation of the cytoskeleton may occur in response to flattening of the secretory epithelium during milk accumulation (Hurley, 1989) and could directly contribute to the decrease in milk secretion, as chemically-induced disruption of the cytoskeleton/microtubule network results in inhibited milk secretion and can be used to accelerate the process of mammary involution (Patton, 1976a&b; Guerin & Loizzi, 1978; Nickerson *et al.*, 1980; Oliver & Sordillo, 1989). Cytoskeletal changes, along with mechanical tension on cell-cell and cell-ECM interactions, would therefore be implicated in a mechanotransduction cascade ultimately leading to mammary apoptosis and involution (Davis *et al.*, 1999; Stelwagen, 2001).

In addition, this study has directly demonstrated that mammary apoptosis can be induced by physical stretching of the mammary epithelium as dramatic increases in the numbers 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 (Migheli *et al.*, 1995), and a similar induction in apoptosis in thymus glands has been documented within 2 h following dexamethasone treatment of adrenalectomised rats (Compton & Cidlowski,

1986). The active, phosphorylated, form of STAT3, but not total STAT3, was dramatically up-regulated within 1 h following the acute physical distension procedure. pSTAT3 is a marker of apoptosis and the acute phase immune response, and is greatly increased during the early stages of both rodent (Philp *et al.*, 1996; Li *et al.*, 1997a; Chapman *et al.*, 1999) and bovine (Dr. K Singh, unpublished observations) mammary involution. Moreover, STAT3 is a key regulator of this process as conditional knockout of mammary STAT3 suppressed MEC apoptosis and delayed involution in mice (Chapman *et al.*, 1999). In this study we show that STAT3 is activated in response to induced physical distension of the mammary epithelium, which implies that STAT3 participates in mechanotransduction pathways during extended periods of milk accumulation.

In conclusion, this study showed that physical distension accelerated the onset of the first stage of mammary apoptosis and involution, compared with milk accumulation alone. Therefore, these data support a role for mechanotransduction during apoptosis and involution of mammary glands. However, to further examine mechano-sensitive processes, an *in vitro* model is required which clearly separates the physical stretching of MECs from the actions of accumulated milk products. The development of this model and the effect of stretching MECs *in vitro* on β1-integrin, pSTAT3 and TJ protein expression are presented in the next chapter.

CHAPTER SIX

Effects of stretching rodent mammary epithelial cells *in vitro* on tight junction protein expression

6.1 INTRODUCTION

Both during extended milking intervals and the early stages of mammary involution, milk accumulates within the alveolar compartment resulting in physical distension of the secretory epithelium. The consequent change in epithelial cell shape, from a cuboidal to a flattened morphology (Richardson, 1947; Chapters 3 and 4), may initiate changes in gene expression through mechanotransduction pathways, leading to the loss of secretory activity and the induction of pro-apoptotic processes (Davis *et al.*, 1999; Stelwagen, 2001; Chapter 5). Down-regulation of β1-integrin and TJ protein expression reflect a loss of cell-ECM communication and TJ integrity, respectively, during the initial stages of mammary apoptosis and involution (McMahon *et al.*, 2004, Appendix VII; Singh *et al.*, 2005, Appendix VI; Chapters 3 and 4).

The results of the previous chapter (Chapter 5) showed that physical distension of rat mammary glands, induced by an infusion of isosmotic sucrose solution, accelerated this process, thus, supporting the hypothesis that physical distension is a primary regulator of mammary function. However, inconsistencies between *in vivo* sucrose infusion studies exist, with some authors showing that physical distension results in increased TJ permeability and reduced milk secretion (Fleet & Peaker, 1978; Peaker, 1980; Stelwagen *et al.*, 1998), while others have concluded that a locally-active chemical inhibitor(s) reduces milk secretion by negative feedback during extended periods of milk accumulation (Henderson & Peaker, 1984, 1987) in goats and cows. An *in vitro* model may provide better control of these variables by isolating the effects of physical stretching of MECs from the actions of accumulated milk products.

It is well documented that MECs can be cultured *in vitro* on plastic where they will form confluent cell monolayers with uplifted regions or "domes" due to cell secretion

and the development of cell polarity. Addition of the lactogenic hormones, insulin, prolactin and glucocorticoids, induces the formation of TJs and the expression of the TJ proteins, occludin, claudin-1 and ZO-1 (Singer *et al.*, 1994; Stelwagen *et al.*, 1999; Stelwagen & Callaghan, 2003). However, on standard tissue culture plastic MECs exhibit a spread, flattened morphology and fail to express milk proteins in the presence of lactogenic hormones. A change in cell shape to a rounded morphology is sufficient to induce lactoferrin expression (Roskelley *et al.*, 1995; Close *et al.*, 1997), but the additional presence of an ECM substratum is required for MECs to secrete milk proteins in response to lactogenic hormones *in vitro* (Medina *et al.*, 1987; Eisenstein & Rosen, 1988; Roskelley *et al.*, 1995). Under these conditions MECs differentiate into 3-D structures called mammospheres, which are reminiscent of alveolar acini *in vivo* (Barcellos-Hoff *et al.*, 1989). While mammospheres provide an *in vitro* model of the lactating mammary gland, their 3-D structures mean that they are challenging to physically stretch with few techniques available to investigate and manipulate cells under 3-D conditions (Alcaraz *et al.*, 2004). Moreover, any mechanical manipulation of mammospheres is potentially confounded by the effects of secreted milk components.

In contrast, there are numerous biomechanical tools and techniques to probe single cells, groups of cells or full monolayers in 2-D cultures (reviewed by Alcaraz *et al.*, 2004). Although the role of the cell adhesion environment has received much attention in MECs, the use of biomechanical tools to expand our knowledge of the physical/mechanical forces experienced by MECs has been limited to very few studies. Pitelka and Taggart (1983) showed that mechanical forces generated in MEC sheets regulate the orientation and arrangement of TJ strands. Furuya *et al.* (1993) also demonstrated that intracellular Ca^{2+} increased in MECs after local mechanical deformation and that this local effect spread rapidly to neighbouring cells, a process that may contribute to the co-ordinated control of casein phosphorylation and secretion in the mammary gland. These results, along with those of studies on the culture of MECs in different adhesive environments (Roskelley *et al.*, 1995; Close *et al.*, 1997; Weaver & Bissell, 1999), support a role for mechanical and structural signals in the regulation of MEC function.

Indeed, secretory epithelial cells in the lactating mammary gland are subjected to a dynamic mechanical environment in response to the secretion, accumulation, and subsequent removal of milk. Milk is secreted apically from MECs into the alveolus where it accumulates due to the tone of the alveolar milk ducts providing resistance to milk flow into the ducts/cisternal system. Valve-like structures have been identified where the milk duct exits the alveolus, and these may have a role in generating resistance to alveolar drainage (Caruolo, 1980). The build up of secreted milk within the alveolus exerts an outward-acting compressive force on the MECs and the surrounding basement membrane (Paszek & Weaver, 2004). This causes the alveolus to physically distend or stretch, and is similar to the tensional stress found in a urine-expanded bladder (Bross *et al.*, 2003). This acute milk compression force is also transmitted to the myoepithelium and will be sensed and resisted by the surrounding stromal tissue (Paszek & Weaver, 2004). Upon suckling/milking the myoepithelium contracts in response to oxytocin stimulation, which exerts an acute inward-acting tensional force on the MECs, squeezing the milk out from the alveolus and into the larger ducts to enable milk removal.

However, infrequent or cessation of suckling/milk removal will result in chronic milk accumulation in the alveolus and a progressive increase in the magnitude of the compression force such that the MEC shape changes from a cuboidal to a flattened morphology as the alveolus distends (Richardson, 1947; Chapters 3 and 4). This process is postulated to be a key trigger initiating the subsequent reduction in secretory activity and ultimately, the onset of mammary apoptosis and involution (Davis *et al.*, 1999; Stelwagen, 2001; Chapter 5), with local chemical inhibitory factors and reduced systemic galactopoietic hormone levels also playing major roles in this process (Wilde *et al.*, 1999).

To model, *in vitro*, the tensional stress encountered during extended periods of milk accumulation, a technique is required which applies uniform equibiaxial stretch to MECs (i.e., increases the cell's surface area by stretching it equally in all directions). There are several techniques available which variously involve culturing cells on a circular flexible/elastic membrane, and subsequently deforming the membrane substratum in two (biaxial strain) directions using either air/fluid displacement, vacuum,

or direct displacement with a convex template or an indenter (Schaffer *et al.*, 1994; Brown, 2000). However, deformation by altering the air pressure, injecting fluid or applying a convex template beneath a circular membrane do not provide a uniform biaxial stretch as the degree of cell stretch varies with the distance from the centre of the membrane (Schaffer *et al.*, 1994; Brown, 2000). The most accurate devices apply uniform biaxial stretch by direct displacement of the circular flexible membrane, which is constrained at its periphery, with a circular indenter of nearly the same diameter as the membrane substratum (Hung & Williams, 1994; Schaffer *et al.*, 1994; Lee *et al.*, 1996; Tschumperlin & Margulies, 1998). The stretch applied to the cells is a function of the strain applied to the flexible membrane, and these devices can be used to apply either static or cyclic stretch.

These techniques have demonstrated that cell-cell and cell-ECM contacts in a number of non-mammary tissue types respond to mechanical stretch, participating in signalling events that lead to changes in gene and protein expression and altered cellular function (Shyy & Chien, 1997; Cavanaugh *et al.*, 2001, 2006; Ko *et al.*, 2001; Geiger & Bershadsky, 2002; Wernig *et al.*, 2003). The loss of cell-ECM survival signalling via β1-integrin and the down-regulation of TJ proteins (occludin, claudin-1 and ZO-1) and increased TJ permeability occurring during extended periods of milk accumulation have, therefore, been implicated in mechanotransduction events in the mammary gland (McMahon *et al.*, 2004, Appendix VII; Singh *et al.*, 2005, Appendix VI; Chapters 3 and 4). Furthermore, acute physical distension of rat mammary glands with an infusion of isosmotic sucrose solution accelerated the onset of apoptosis, the up-regulation of the pro-apoptotic marker pSTAT3, and the loss of β1-integrin and occludin protein expression, compared with milk accumulation alone (Chapter 5). While this *in vivo* study supported a role for mechanotransduction in the regulation of mammary function, an *in vitro* model would enable further investigation of the specific physical mechanism(s) whereby stretching MECs induces changes in protein expression. Therefore, the goal of this study was to design a simple cell stretch device to test the hypothesis that the stretching of MECs *in vitro* induces changes in the protein expression of β1-integrin, pSTAT3, and TJ components (occludin, claudin-1 and ZO-1).

6.2 MATERIALS AND METHODS

6.2.1 The cell stretch device

The simple custom-made device (co-designed and manufactured by Brian Atkins, AgResearch Ruakura; Fig. 6.1 and 6.2) is similar to those presented previously (Hung & Williams, 1994; Schaffer *et al.*, 1994; Lee *et al.*, 1996; Tschumperlin & Margulies, 1998). The device consists of an aluminium alloy metal cassette which holds a 6-well BioFlex plate containing flexible membranes (35 mm diameter, 0.020″ thick; Flexcell International) over 6 plastic circular indenters (27.5 mm diameter) that are fixed directly underneath the centre of each well. The flexible membranes serve as the deformable substratum for cell attachment. The device is designed to stretch the 6 wells of the BioFlex plate simultaneously. This is performed by screwing a clear acrylic plate down over the BioFlex plate until the membranes are displaced over the indenters to the required depth. The indenters are flat with a raised outer circumference, which was designed to minimise the area of contact with the membrane, reduce friction, and give a more uniform membrane deformation.

The magnitude of stretch depends upon the depth of displacement of the membrane by the indenters; with one turn of the device resulting in an indentation depth of 1.5 mm. Using a circular indenter of nearly the same diameter as the membrane substrate results in radial and circumferential strains of equal magnitude (i.e., uniform equibiaxial stretch; Fig. 6.2) (Schaffer *et al.*, 1994). However, while this can be assumed to be correct for the majority of the membrane which is stretched across the indenter, the strain at the edges of the membrane (where it does not stretch across the indenter) will not be uniform (Dr. Paul Shorten, AgResearch Ruakura; personal communication). This is similar to existing cell stretch devices capable of applying uniform equibiaxial strain using either indenter displacement of flexible membranes (Hung & Williams, 1994; Schaffer *et al.*, 1994; Tschumperlin & Margulies, 1998), or vacuum-operated membrane deformation across loading posts, such as the FX-4000 Flexercell Tension Plus System (Flexcell International).

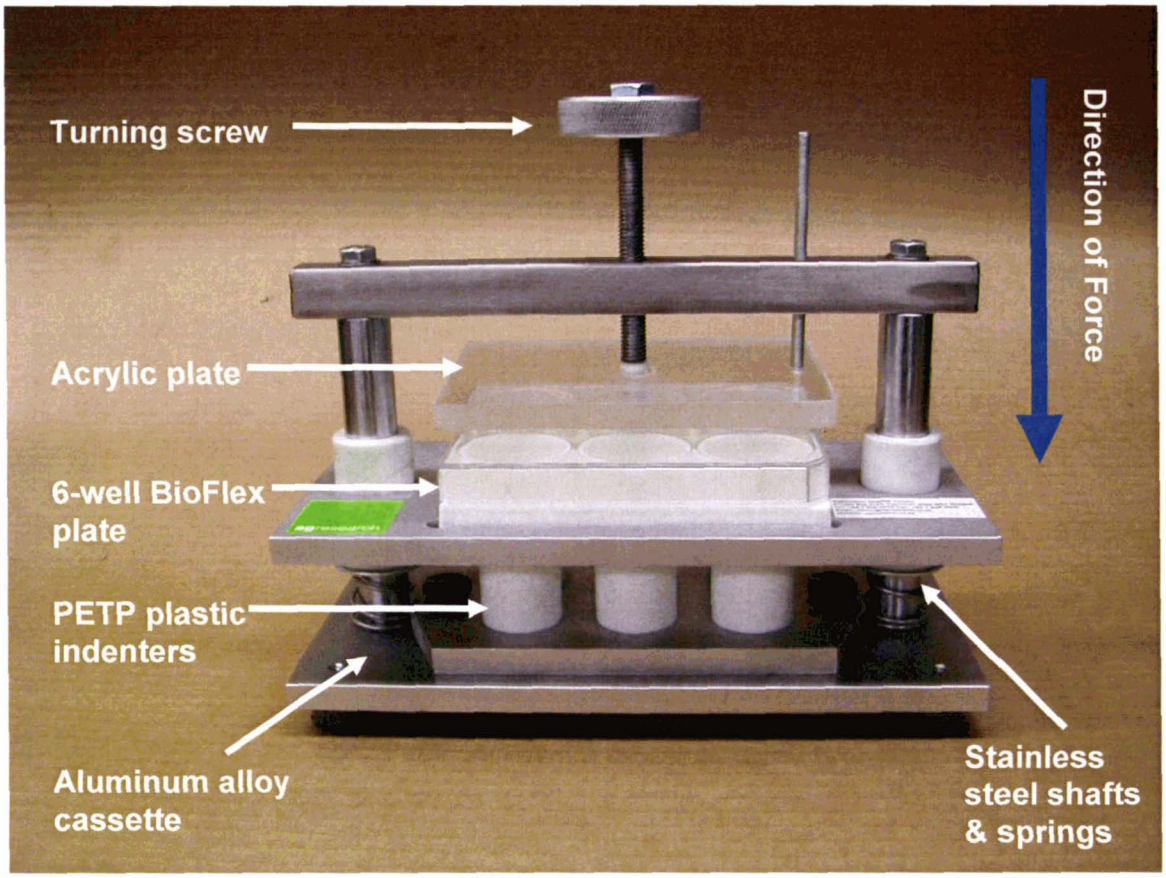


Figure 6.1. A simple custom-made device to stretch MECs *in vitro*.
A photograph depicting the side view of the cell stretch device containing a 6-well BioFlex plate (Flexcell International) with flexible membranes in the undeformed (i.e., rest) position. To perform the stretching, the acrylic plate is screwed down over the BioFlex plate until the membranes are displaced over the indenters to the required depth.

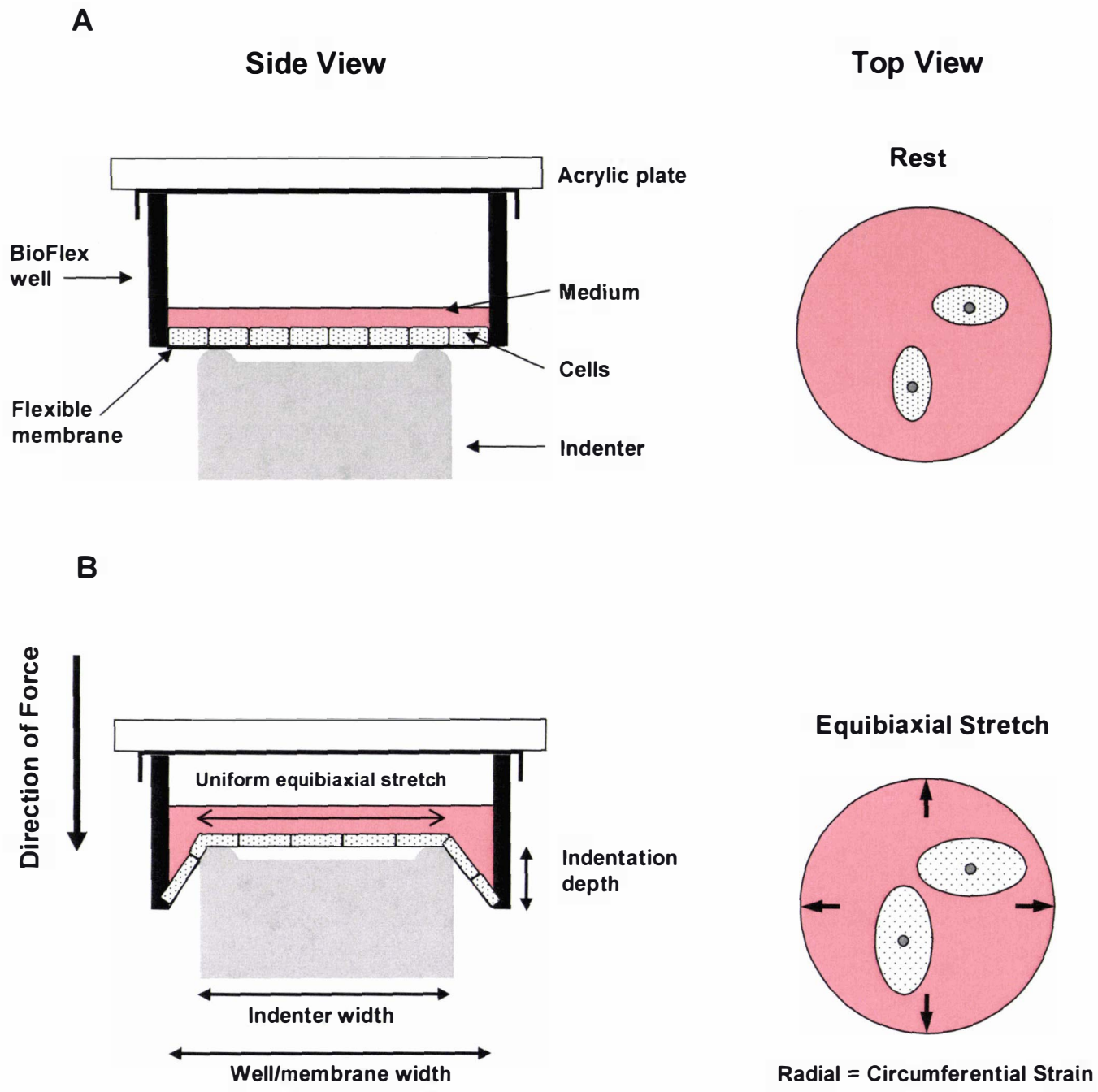


Figure 6.2. Schematic diagram of a simple custom-made device designed to stretch MECs *in vitro*. Depicted are the side view and the top view of the flexible membrane in the (**A**) undeformed (i.e., rest) position and (**B**) deformed over the indenters during uniform equibiaxial stretching. Note the strain at the edges of the membrane (where it does not stretch across the indenter) will not be uniform. The diagram is not to scale.

Stretching causes the membrane surface area (MSA) to increase and the % change in MSA (ΔMSA) for each indentation depth is represented by:

$$\%\Delta MSA = \left[\frac{\left[\frac{\pi r_0^2 + S}{\pi r_1^2} \right] - 1}{1} \right] \times 100$$

Where:

$$S = \pi (r_1 + r_0) \sqrt{d^2 + (r_1 - r_0)^2}$$

r_1 = ½ (27.5) mm = radius of the indenters

r_0 = ½ (35) mm = radius of the wells

d = number of turns x 1.5 mm = indentation depth

(Equations were kindly derived by Dr. Paul Shorten, AgResearch Ruakura)

The relationship between the indentation depth and the % ΔMSA is shown in Figure 6.3. The maximum ΔMSA that can be performed using this cell stretch device is 47%.

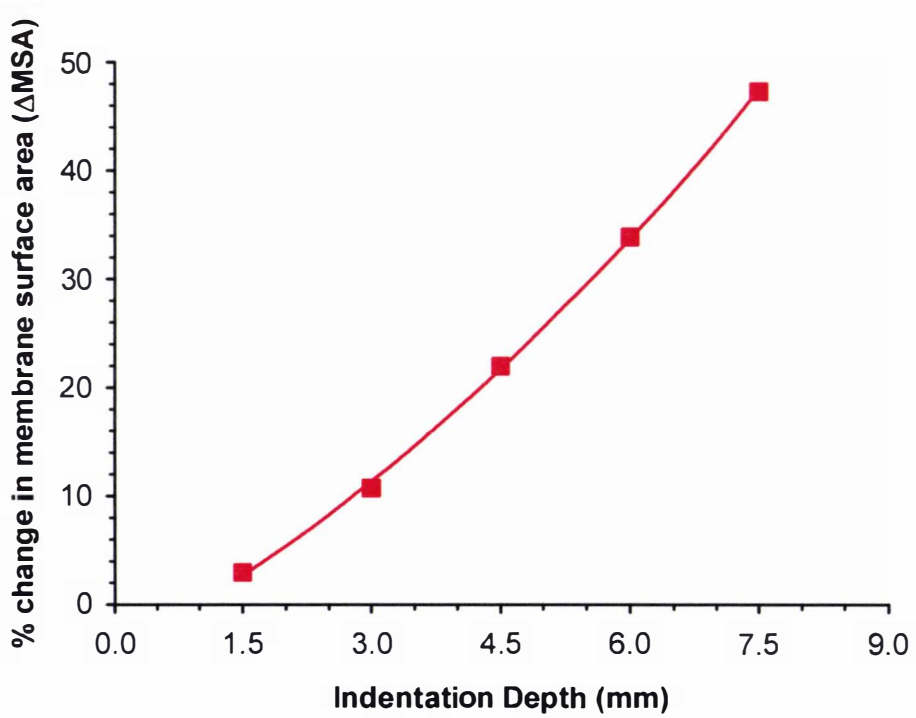


Figure 6.3. The relationship between the indentation depth (mm) of the cell stretch device and the maximum % change in membrane surface area (ΔMSA).
Where 1 turn of the cell stretch device is equal to an indentation depth of 1.5 mm and the maximum number of turns that can be done is 5, corresponding to a maximum ΔMSA of 47%.

6.2.2 Cell culture protocols

The murine mammary cell line COMMA-1D (Danielson *et al.*, 1984), between passages 19 to 23 since initial isolation, was used in all experiments. The COMMA-1D cells were a gift from Dr. Dan Medina (Baylor College of Medicine, Houston, Texas, USA) and were cultured essentially as previously described (Stelwagen *et al.*, 1999). COMMA-1D cells were seeded at a density of 4 x 10^5 cells per well onto flexible membranes in 35 mm BioFlex 6-well plates (Flexcell International) containing 2 ml growth medium (1 x Dulbecco's modified Eagle's medium (DMEM): F12 nutrient mix, pH 7.4; GIBCO Products, Invitrogen) supplemented with 2% foetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 292 μg/ml L-glutamine (all obtained from GIBCO Products, Invitrogen), 300 ng/ml BSA, 5 μg/ml insulin, 5 ng/ml epidermal growth factor (EGF) (all obtained from Sigma Chemical Company). Cells were cultured at 37 °C in an atmosphere of 5% CO_2 and 95% air at all times and the medium was changed every 1-2 days. Once cells had reached confluency they were incubated for 24 h in priming medium (same as growth medium, but without EGF) before the addition of 1 μg/ml prolactin and 1 μM dexamethasone (both purchased from Sigma Chemical Company) to the medium for a further 48 h to induce MEC differentiation. To perform cell stretching, one plate at a time was placed on the cell stretch device (Fig. 6.1) and the membranes stretched continuously at a maximum 47% ΔMSA for the required length of time. An extra 2 ml differentiation medium was added to each well prior to stretching to ensure cells were still covered with medium during membrane deformation. Immediately following stretching, wells were observed for signs of disruption to the cell monolayer.

6.2.3 Optimisation of the cell attachment surface

The cell stretch device was designed to be used with commercially available 6-well BioFlex plates (Flexcell International) which contain flexible membranes in a range of coated surfaces. In a preliminary study, the BioFlex untreated, Collagen Type I-, and laminin-coated membranes were used as flexible growth surfaces for COMMA-1D cells, and were compared with standard 6-well tissue culture plastic plates (NunclonΔ

polystyrene; Nunc). COMMA-1D cells were cultured as described in section 6.2.2 above, except they were seeded at a slightly higher density of 4.8 x 10^5 cells per well. Cells were examined daily to determine a suitable surface for attachment, growth, and differentiation before stretching and then after stretching at a maximum 47% ΔMSA for 3 h.

6.2.4 Western immunoblotting for β1-integrin, STAT3 and TJ proteins

COMMA-1D were grown on Collagen Type I flexible membranes in 35 mm BioFlex 6-well plates (Flexcell International) under the same conditions as described above (section 6.2.2). Cells were then subjected to continuous stretching at a maximum 47% ΔMSA for 1, 3, 8, or 15 h. A non-stretched plate for each time point was used to provide a control and the experiment was repeated on three separate occasions. None of the wells had any cell layers peeling off after stretching. Immediately following treatments the cells were washed twice in ice-cold phosphate buffered saline (PBS; 160 mM sodium chloride, 3 mM potassium chloride, 8 mM di-sodium hydrogen phosphate, 1 mM potassium di-hydrogen phosphate; Dulbecco 'A' solution, Oxoid Ltd). The cells from two wells of each plate were then each scraped from the flexible membrane in 400 µl low salt buffer containing 1% (v/v) NP-40 detergent and protease inhibitors (section 2.2.7) for protein extraction (n=6 per treatment per time point). Cells were lysed with two short bursts using an Omni TH Tissue Homogeniser (Omni International) and then rotated for 30 min at 4 °C. A 150 µl aliquot was saved for analysis of the total cell homogenate fraction. The remaining homogenate was centrifuged at 10,000 x *g* for 30 min at 4 °C and the supernatant collected as the NP-40-soluble protein fraction. To collect the NP-40-insoluble fraction, the remaining pellet was resuspended by sonication in 100 µl of low salt buffer (containing 1% (v/v) NP-40 detergent, 1% (v/v) SDS and protease inhibitors) and rotated for 30 min at 4 °C. Protein samples were mixed with Laemmli loading buffer and boiled for 5 min as described in section 2.2.7, with the protein content of each sample determined using the BCA method.

Protein samples (20 µg) from the non-stretched control and stretched wells of each replicate were then allocated to one of two 26-well Criterion Precast Tris-HCl gels (133

x 87 x 1.0 mm) (Bio-Rad Laboratories) so that there were three replicates for each time on each gel. SDS-PAGE gels contained either 7.5% (STAT3, pSTAT3 and ZO-1 detection), 10% (occludin detection), or 15% (claudin-1 detection) polyacrylamide. Electrophoresis was then carried out in the Criterion Cell apparatus (Bio-Rad Laboratories) for approximately 1 h at 200 V in electrode buffer (0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS, pH 8.3) (refer to section 2.2.8.1). Separated proteins were then transferred onto Hybond-ECL nitrocellulose membranes (Amersham Biosciences) using the Trans-Blot Cell (Bio-Rad Laboratories) wet blotting system and probed with primary antibodies to rabbit anti-human pSTAT3 (Tyr 705) (Santa Cruz Biotechnology), occludin, claudin-1 and ZO-1 (all from Zymed Laboratories Inc.), and rabbit anti-mouse STAT3 (Santa Cruz Biotechnology) as described in section 2.2.8.2. Occludin-probed membranes were stripped (refer section 2.2.8.3) and re-probed with rabbit anti-human β1-integrin (Santa Cruz Biotechnology). 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).

6.2.5 Data and statistical analyses

Densitometry data from western blotting were $\log_{10}$-transformed and analysed by ANOVA in GenStat (releases 7.1 and 8.1; Lawes Agricultural Trust, 2003 and 2005) with blocking on replicate and adjusting for between gel variations to detect differences between control and stretched samples at each time point. Data are presented as back-transformed means (relative units) for the control and stretched samples at each time point with the SED between means. The least significant differences identify the means significantly different from each other (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

6.3 RESULTS

6.3.1 Optimisation of the attachment surface for cell stretching *in vitro*

The BioFlex untreated, Collagen Type I-, and laminin-coated membranes were used as flexible growth surfaces for COMMA-1D cells, and were compared with standard tissue culture plastic wells. The flexible membranes were then stretched at 47% ΔMSA for 3 h, with the results summarised and presented in Table 6.1. COMMA-1D cells were observed to attach and initiate growth/proliferation within 24 h after seeding, regardless of the growth surface. However, with the exception of the Collagen Type I-coated surface, the cells took longer to reach confluency on the BioFlex flexible surfaces compared with plastic. Furthermore, the cell monolayers started to peel away from the untreated BioFlex flexible membrane surfaces after they had reached confluency and during the differentiation stage. Moreover, because peeling had occurred before the cells had been stretched this meant that the cell monolayer came away from the surface during stretching. A small amount of peeling of the cell monolayer was also observed after stretching for laminin-coated membranes. This is obviously undesirable as, unless cells remain attached to the flexible growth surface during membrane deformation, they will not be stretched. Thus, the untreated and laminin-coated surfaces were unsuitable to use in the *in vitro* cell stretch model. By comparison, COMMA-1D cells showed a similar attachment and growth pattern on Collagen Type I-coated surfaces compared with standard tissue culture plastic. COMMA-1D cells also adhered well to Collagen Type I-coated flexible membranes during stretching and, therefore, these were used in all subsequent experiments.

Protein expression of β1-integrin, pSTAT3, total STAT3, occludin, claudin-1 and ZO-1 were confirmed for COMMA-1D cells grown on Collagen Type I membranes (Fig. 6.4), indicating that the phenotype was similar to standard tissue culture plastic (results not shown).

Table 6.1. Optimisation of the attachment surface for cell stretching *in vitro*.
COMMA-1D cells were grown on standard tissue culture plastic (NunclonΔ polystyrene; Nunc), or BioFlex flexible membranes (Flexcell International) which were either untreated, or thinly coated with Collagen Type I or laminin. Cells were examined daily during the growth, priming (i.e., growth arrest) and differentiation stages of culture before stretching and then after stretching at a maximum 47% ΔMSA for 3 h.

Growth surface	Time taken to reach confluency (days)	Observations before stretching			Observations after stretching
		Growth	Priming	Differentiation	
Plastic	4	Excellent cell attachment, spreading and growth.	Cell monolayers were confluent with uplifted regions or "domes".	Cell monolayers were confluent with large domes clearly visible.	N/A
Untreated BioFlex	6	Good cell attachment, but slower spreading and growth compared with plastic. Tendency of cells to remain in clumps.	Cell monolayers were confluent with domes.	Cell monolayers were confluent with large domes clearly visible. However, some slight peeling of cell monolayers was observed around the edges of the wells.	Very poor cell attachment. Cell monolayers were badly peeled away from the edges of the wells. In the centre of the wells peeling also occurred around the edges of domes.
Collagen Type I BioFlex	4	Excellent cell attachment, spreading and growth. Similar to plastic.	Cell monolayers were confluent with domes.	Cell monolayers were confluent with large domes clearly visible.	Excellent cell attachment with no peeling of cell layers observed.
Laminin BioFlex	7	Good cell attachment, but slower spreading and growth compared with plastic. Tendency of cells to remain in clumps.	Cell monolayers were confluent with domes.	Cell monolayers were confluent with large domes clearly visible.	Poor cell attachment. Slight peeling of cell monolayers was observed around the edges of the wells.

6.3.2 Western blot analysis of β1-integrin, STAT3 and TJ protein expression

6.3.2.1 Expression and solubility of β1-integrin, STAT3 and TJ proteins in COMMA-1D cells grown on Collagen Type I BioFlex membranes

The protein expression of β1-integrin, pSTAT3, total STAT3, occludin, claudin-1 and ZO-1 were examined in non-stretched COMMA-1D cell homogenates before and after centrifugation to obtain soluble supernatant and insoluble pellet fractions (Fig. 6.4). Single immunoreactive bands were detected at ~80 kDa for β1-integrin and at ~90 kDa for total STAT3 in COMMA-1D cells. Antibodies against pSTAT3 detected multiple bands at ~90 kDa consistent with phosphorylation, and which agreed with the positive control (24 h weaned, rat mammary gland). Another more intense signal for COMMA-1D cells was detected at ~200 kDa and assumed to be the pSTAT3 homodimer. Occludin protein migrated as multiple bands at ~60 kDa, while antibodies against ZO-1 detected the α^{+}-and α^{-}-isoforms at ~225 kDa. Bands for claudin-1 protein were detected at ~22 kDa, ~28 kDa and ~80 kDa, with the later assumed to be the claudin-1 homodimer.

In general, expression of each protein was enriched in the insoluble fraction compared with total and soluble fractions. Only a faint signal was detected in the soluble fraction for β1-integrin, pSTAT3, STAT3 and ZO-1 proteins. However, expression of the ~22 kDa band of claudin-1 was enriched in the soluble fraction while the ~28 kDa and ~80 kDa bands were not detected, suggesting that the ~22 kDa band was more soluble.

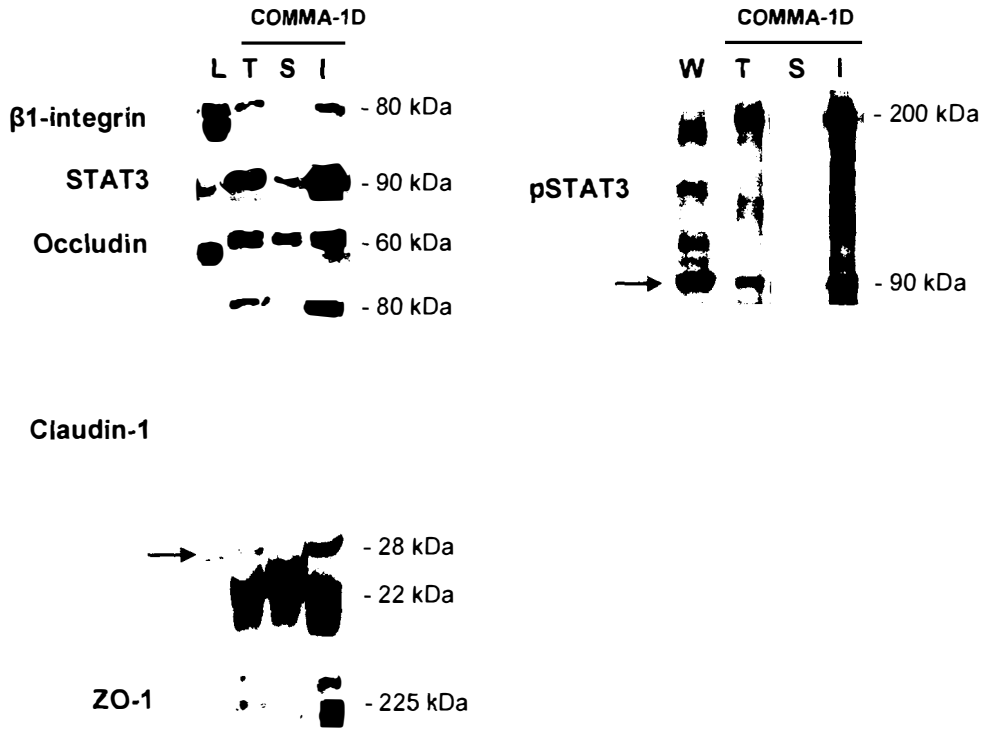


Figure 6.4. Western blots showing the expression of β1-integrin, STAT3 and tight junction proteins in COMMA-1D cell homogenates before and after centrifugation.
Non-stretched COMMA-1D cells grown on Collagen Type I BioFlex flexible membranes (Flexcell International) were homogenised in low salt buffer with 1% NP-40 detergent (total unspun homogenate (**T**)), then centrifuged at 10,000 x *g* for 30 min at 4 °C and the supernatant collected as the detergent-soluble fraction (**S**). The remaining detergent-insoluble pellet (I) was resuspended in low salt buffer with 1% NP-40 detergent and 1% SDS. The soluble fraction from a lactating (**L**) or 24 h weaned (**W**) rat mammary sample was loaded in lane 1 as a positive control. Twenty micrograms of protein was loaded into each lane. The primary antibodies used were rabbit anti-human β1-integrin (1:3000 dilution), and pSTAT3 (Tyr 705) (1:1000 dilution for soluble fractions and 1:30,000 dilution for total and insoluble fractions), rabbit anti-mouse STAT3 (1:30,000 dilution) (obtained from Santa Cruz Biotechnology), and rabbit anti-human occludin (1:100,000 dilution), claudin-1 (1:3000 dilution) and ZO-1 (1:10,000 dilution) (all from Zymed Laboratories Inc.).

6.3.2.2 Time course of changes in β1-integrin, STAT3 and TJ protein expression during cell stretching in vitro

Changes in protein expression during *in vitro* cell stretching were examined for β1-integrin (Fig. 6.5), pSTAT3 (Fig. 6.6), total STAT3 (Fig. 6.7), and ZO-1 (Fig. 6.10) in total and insoluble fractions, and for occludin (Fig. 6.8) and claudin-1 (Fig. 6.9) the soluble fraction was also examined. In the insoluble fraction, expression of β1-integrin was reduced by 3 h (2.1-fold decrease, $P<0.05$) and STAT3 by 8 h (1.2-fold decrease, $P<0.05$) in stretched samples compared with controls. In the total fraction, expression of the claudin-1 ~22 kDa band was also slightly decreased for stretched compared with control samples by 15 h (1.3-fold, $P<0.1$). However, expression was increased for the claudin-1 ~80 kDa homodimer (1.5-fold, $P<0.05$), pSTAT3 (2.1-fold, $P<0.1$) and ZO-1 (2.9-fold, $P<0.1$). No other significant changes in protein expression were detected during cell stretching *in vitro*. Furthermore, protein expression in the total, soluble and insoluble fractions of control samples did not consistently vary between time points and an equivalent loading of samples was confirmed by Coomassie Blue staining of untransferred SDS-PAGE gels (Fig. 6.11) and Ponceau S staining of western blot membranes (results not shown).

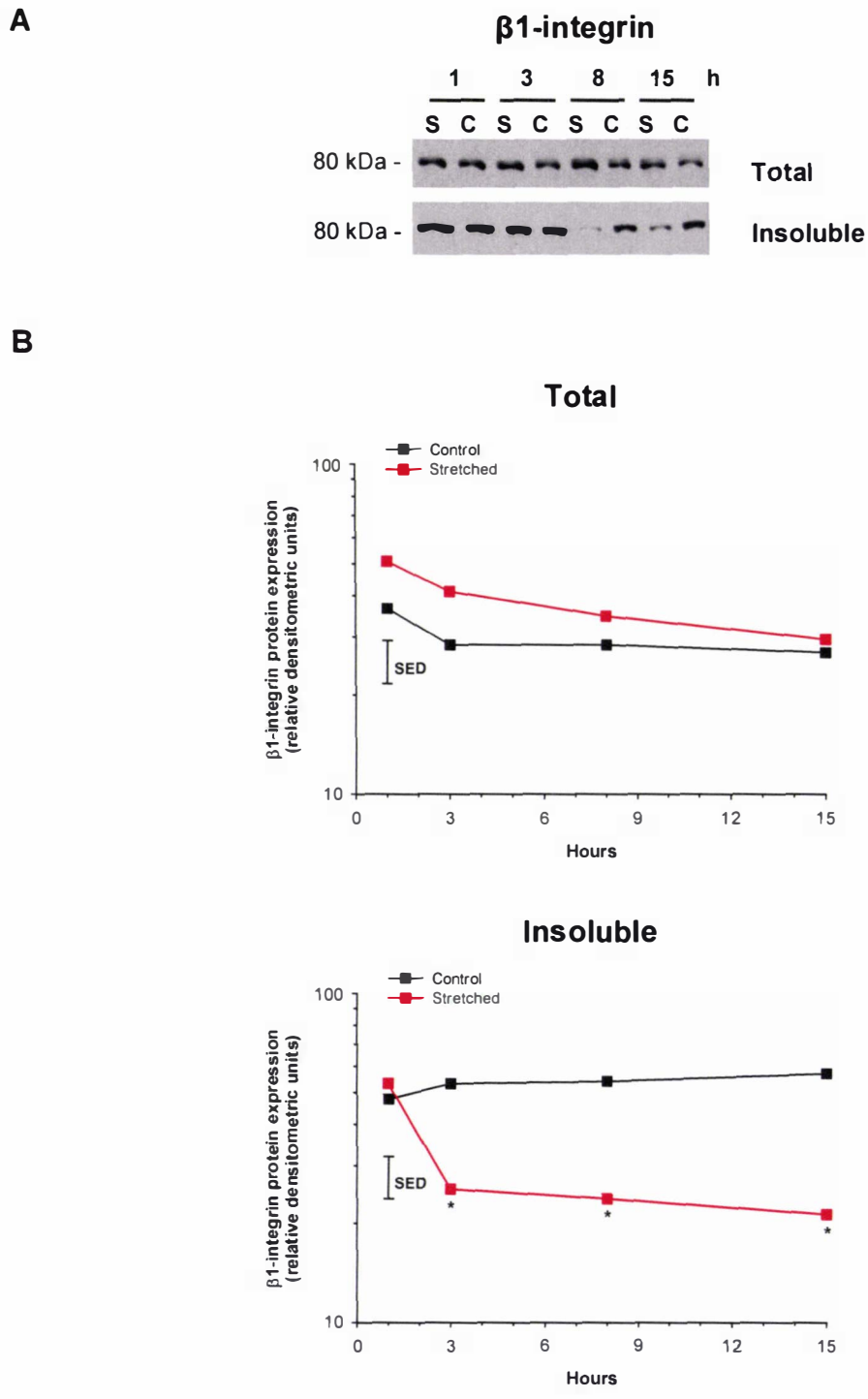


Figure 6.5. The pattern of β1-integrin protein expression during cell stretching *in vitro*.
Densitometric analyses of Western blots are shown for the expression levels of β1-integrin in NP-40-total and –insoluble protein fractions extracted from COMMA-1D cells that were either stretched (**S**) or non-stretched (control (**C**)) for 1, 3, 8, or 15 h (n=6 per time point). Results are presented as: (**A**) representative Western blots where twenty micrograms of protein was loaded into each lane, and (**B**) back-transformed mean relative densitometric units with the SED to compare control and stretched samples at each time point (* $P<0.05$). There were no differences ($P>0.05$) in β1-integrin expression between time points for control samples. The primary antibody used was rabbit anti-human β1-integrin (1:1000 dilutions; obtained from Santa Cruz Biotechnology).

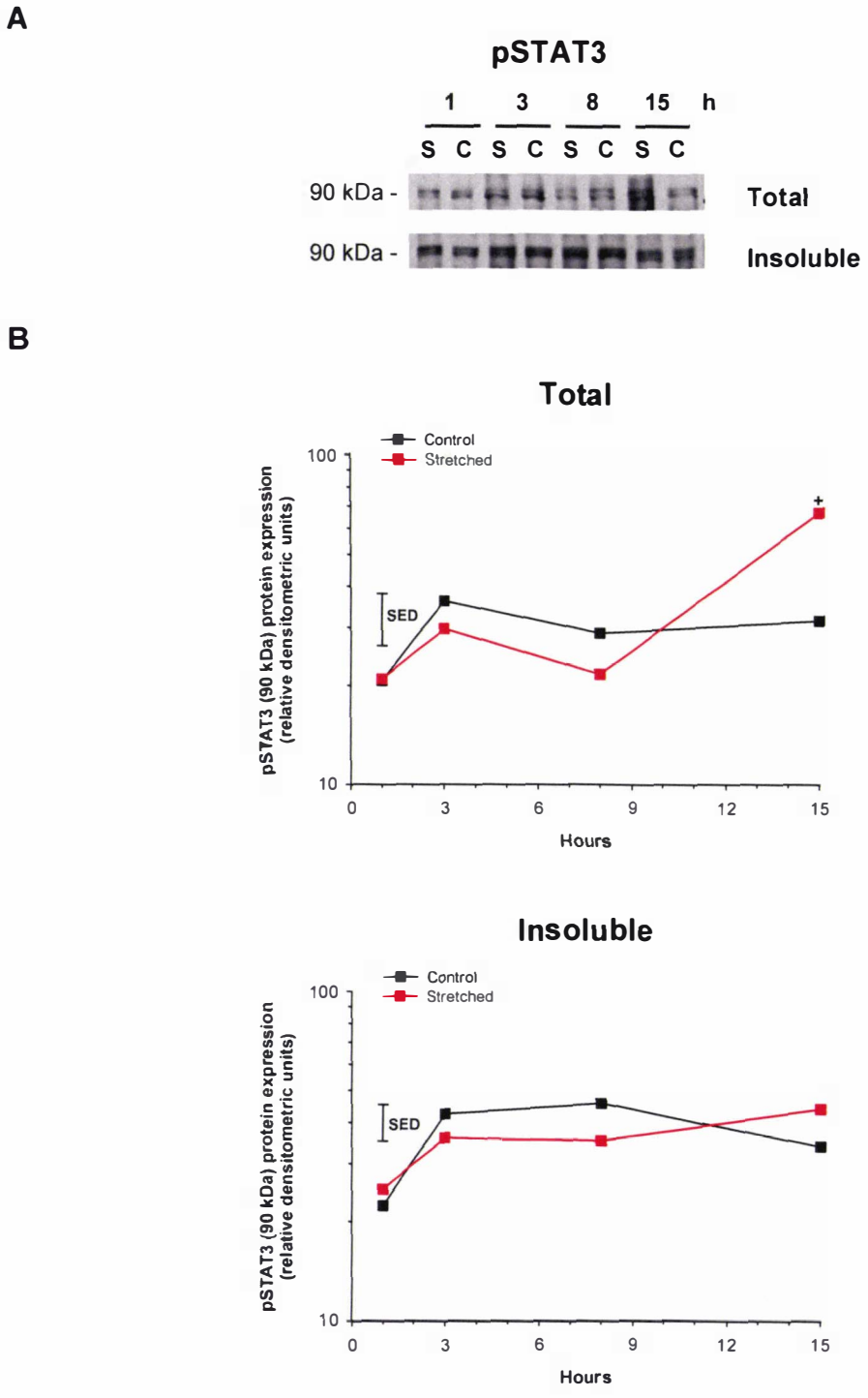


Figure 6.6. The pattern of pSTAT3 protein expression during cell stretching *in vitro*.
Densitometric analyses of Western blots are shown for the expression levels of pSTAT3 in NP-40-total and –insoluble protein fractions extracted from COMMA-1D cells that were either stretched (**S**) or non-stretched (control (**C**)) for 1, 3, 8, or 15 h (n=6 per time point). Results are presented as: (**A**) representative Western blots where twenty micrograms of protein was loaded into each lane, and (**B**) back-transformed mean relative densitometric units with the SED to compare control and stretched samples at each time point ($^{+}$ $P<0.1$). There were no differences ($P>0.05$) in pSTAT3 expression between time points for control samples, except for lower expression in the insoluble fraction at 1 h compared with the other time points ($P<0.05$), which was also observed for stretched samples. The primary antibody used was rabbit anti-human pSTAT3 (1:100,000 dilutions; obtained from Santa Cruz Biotechnology).

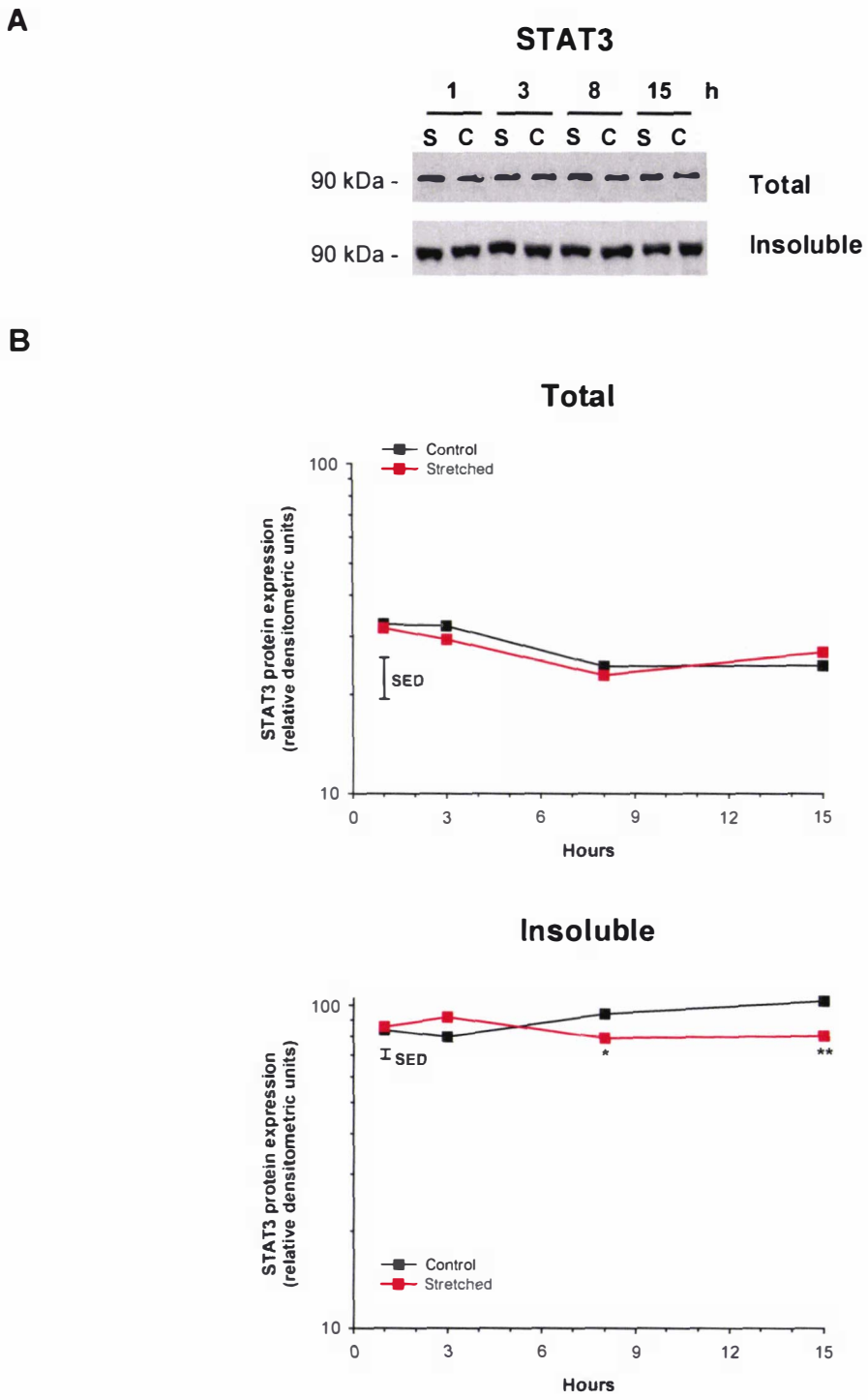


Figure 6.7. The pattern of STAT3 protein expression during cell stretching *in vitro*.
Densitometric analyses of Western blots are shown for the expression levels of STAT3 in NP-40-total and –insoluble protein fractions extracted from COMMA-1D cells that were either stretched (**S**) or non-stretched (control (**C**)) for 1, 3, 8, or 15 h (n=6 per time point). Results are presented as: (**A**) representative Western blots where twenty micrograms of protein was loaded into each lane, and (**B**) back-transformed mean relative densitometric units with the SED to compare control and stretched samples at each time point (* $P<0.05$, ** $P<0.01$). There were no differences ($P>0.05$) in STAT3 expression between time points for control samples, except for lower expression in the insoluble fraction at 3 h compared with 15 h ($P<0.05$). The primary antibody used was rabbit anti-mouse STAT3 (1:100,000 dilutions; obtained from Santa Cruz Biotechnology).

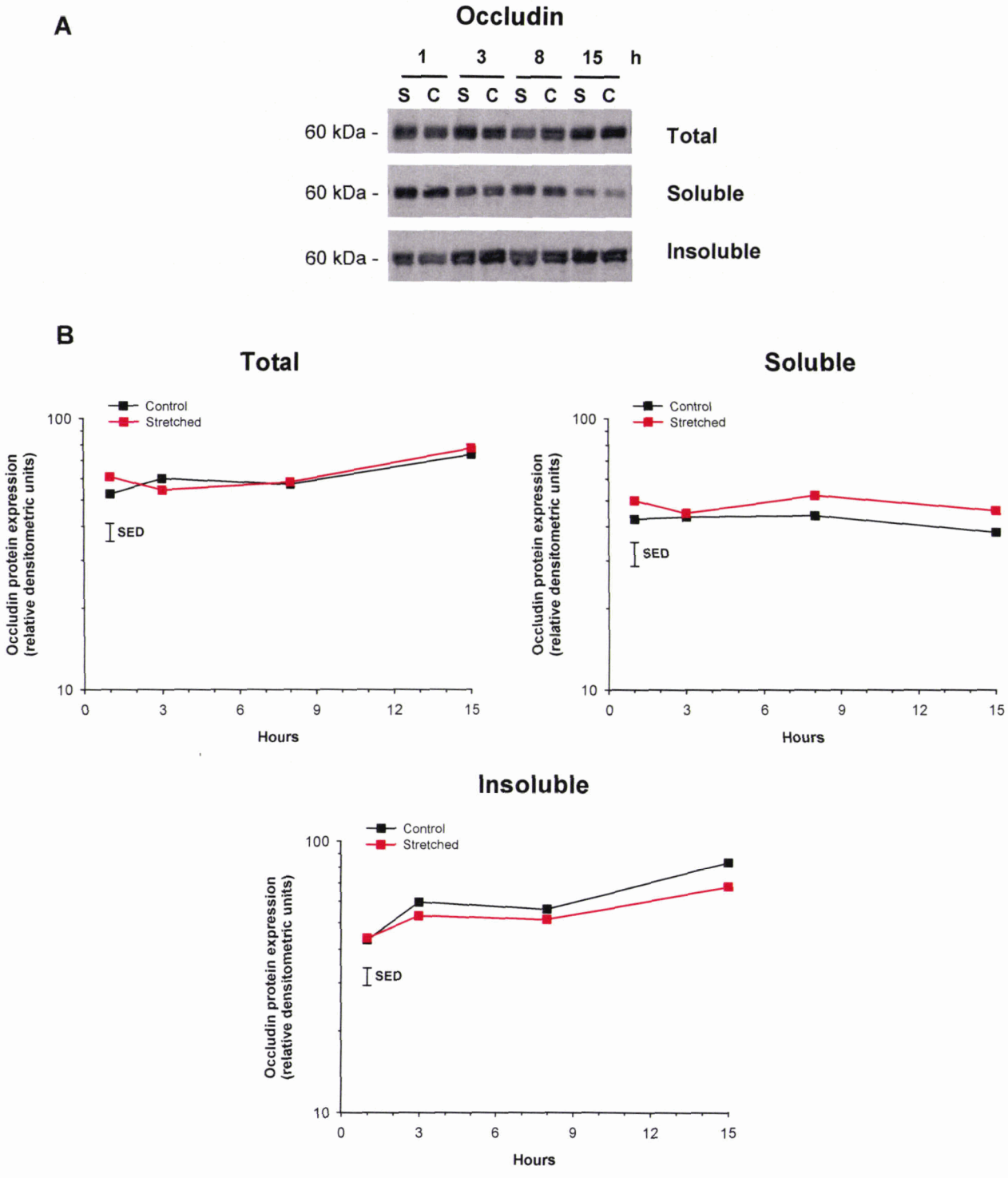
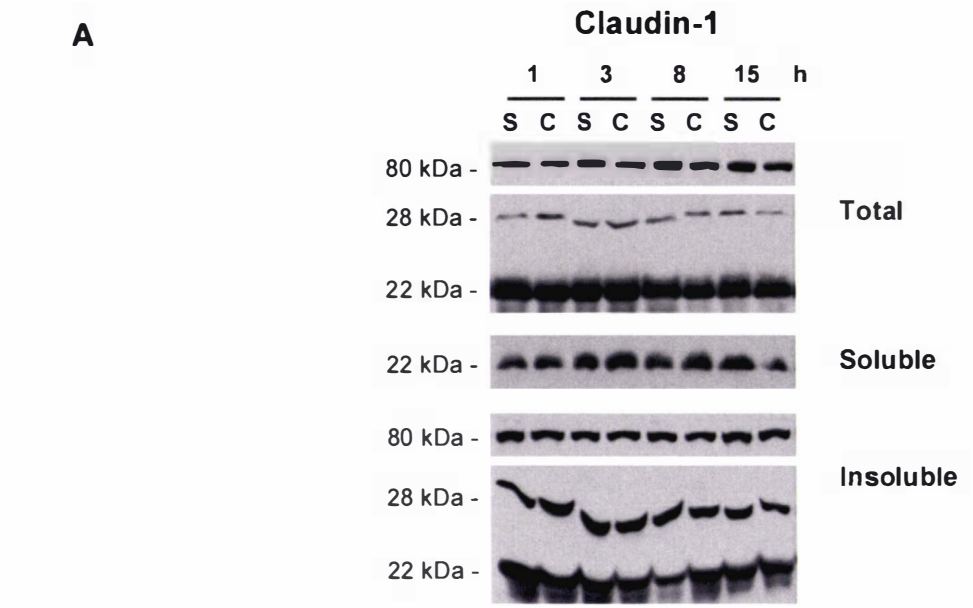
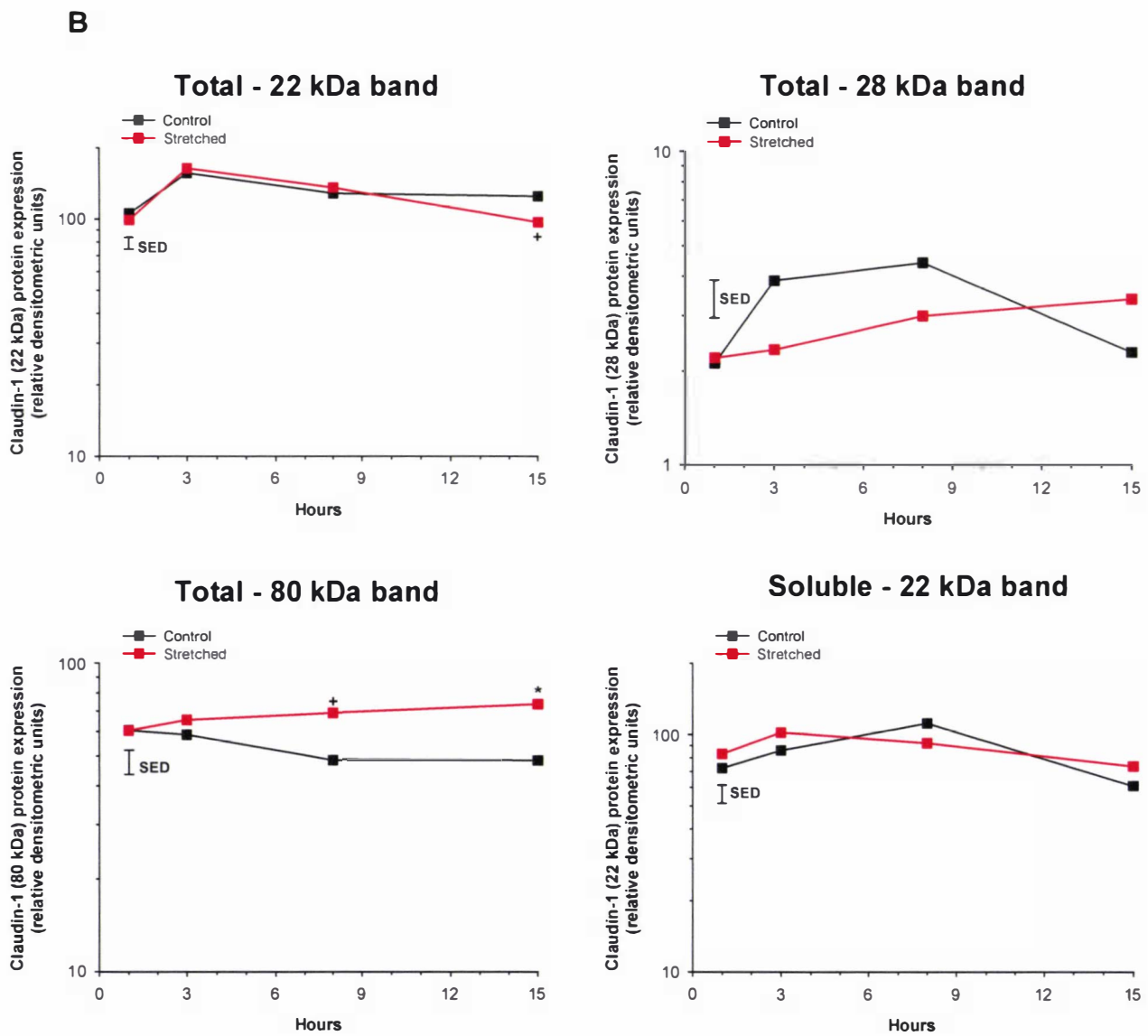


Figure 6.8. The pattern of occludin protein expression during cell stretching *in vitro*.
Densitometric analyses of Western blots are shown for the expression levels of occludin in NP-40-total, –soluble and –insoluble protein fractions extracted from COMMA-1D cells that were either stretched (**S**) or non-stretched (control (**C**)) for 1, 3, 8, or 15 h (n=6 per time point). Results are presented as: (**A**) representative Western blots where twenty micrograms of protein was loaded into each lane, and (**B**) back-transformed mean relative densitometric units with the SED to compare control and stretched samples at each time point. There were no differences ($P>0.05$) in occludin expression in the soluble fraction between time points for control samples. However, higher expression was detected in the total fraction at 15 h compared with 1 h ($P<0.05$) and in the insoluble fraction compared with 1 h ($P<0.01$), 3 and 8 h ($P<0.05$ each), with no other differences between time points for control samples. The primary antibody used was rabbit anti-human occludin (1:100,000 dilutions; obtained from Zymed Laboratories Inc.).





(Figure 6.9 is continued overleaf)

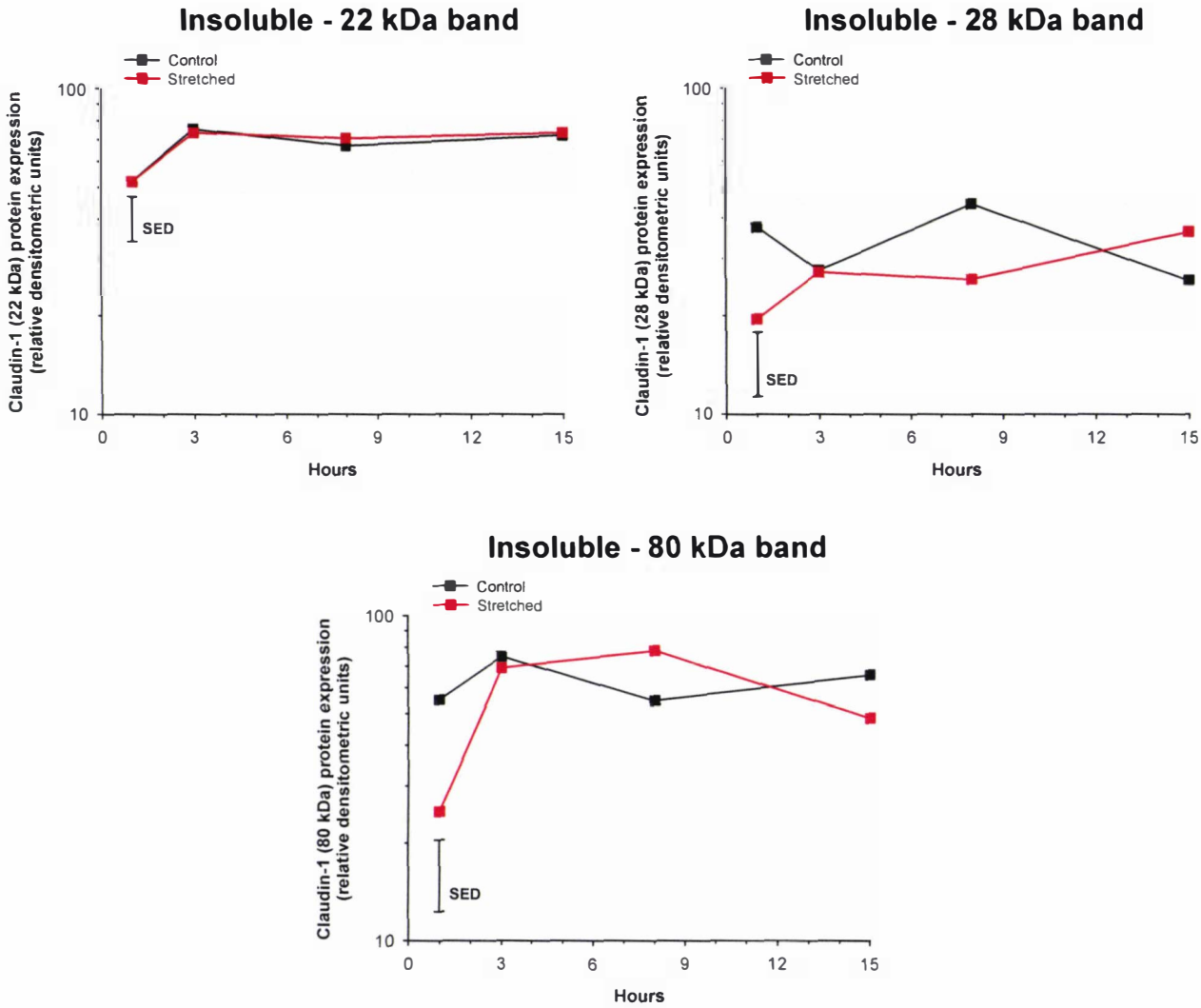


Figure 6.9. The pattern of claudin-1 protein expression during cell stretching *in vitro*.
Densitometric analyses of Western blots are shown for the expression levels of claudin-1 in NP-40-total, –soluble and –insoluble protein fractions extracted from COMMA-1D cells that were either stretched (**S**) or non-stretched (control (**C**)) for 1, 3, 8, or 15 h (n=6 per time point). Results are presented as: (**A**) representative Western blots where twenty micrograms of protein was loaded into each lane, and (**B**) back-transformed mean relative densitometric units with the SED to compare control and stretched samples at each time point ($^{+}$ $P<0.1$, * $P<0.05$). There were no differences ($P>0.05$) in claudin-1 expression between time points for control samples. The primary antibody used was rabbit anti-human claudin-1 (1:1000, 1:10,000 and 1:3000 dilutions for total, soluble and insoluble fractions, respectively; obtained from Zymed Laboratories Inc.).

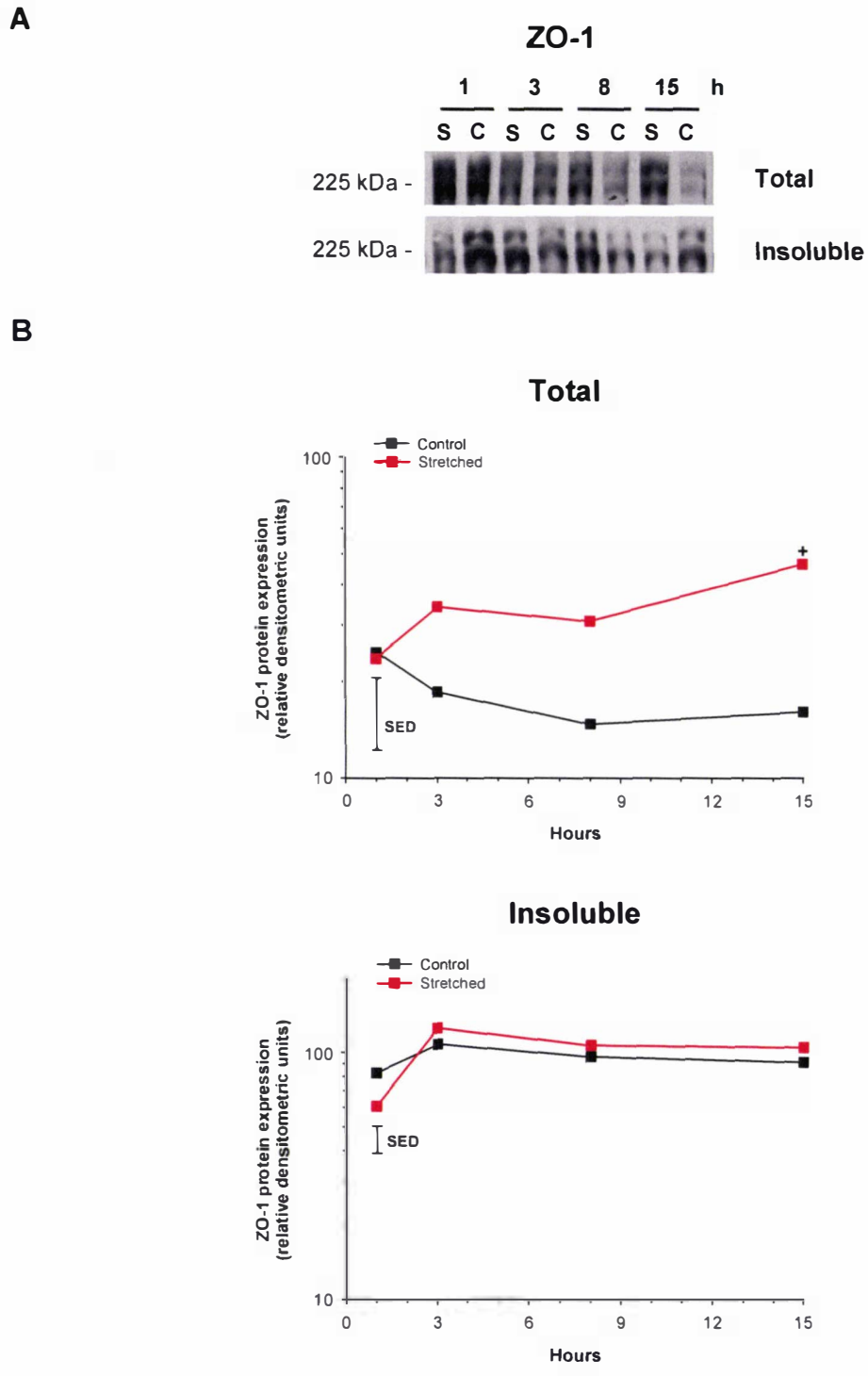


Figure 6.10. The pattern of ZO-1 protein expression during cell stretching *in vitro*.
Densitometric analyses of Western blots are shown for the expression levels of ZO-1 in NP-40-total and –insoluble protein fractions extracted from COMMA-1D cells that were either stretched (**S**) or non-stretched (control (**C**)) for 1, 3, 8, or 15 h (n=6 per time point). Results are presented as: (**A**) representative Western blots where twenty micrograms of protein was loaded into each lane, and (**B**) back-transformed mean relative densitometric units with the SED to compare control and stretched samples at each time point ($^{+}$ $P<0.1$). There were no differences ($P>0.05$) in ZO-1 expression between time points for control samples. The primary antibody used was rabbit anti-human ZO-1 (1:7500 dilutions; obtained from Zymed Laboratories Inc.).

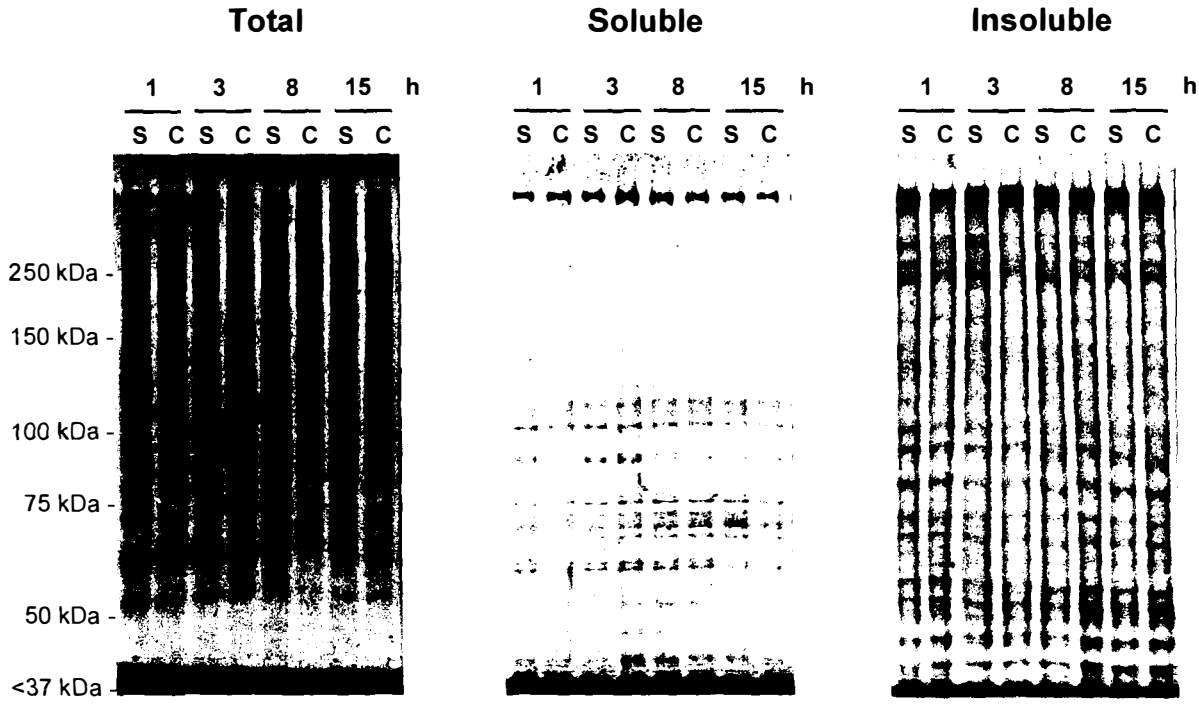


Figure 6.11. Coomassie blue stained gels loaded with protein samples from control and stretched COMMA-1D mammary epithelial cells.
Representative 7.5% SDS-PAGE gels containing NP-40–total, –soluble and –insoluble protein fractions extracted from COMMA-1D cells that were either stretched (**S**) or non-stretched (control (**C**)) for 1, 3, 8, or 15 h. Twenty micrograms of protein was loaded into each lane. Gels were not transferred and were stained with Coomassie blue for 18 h then de-stained in 25% methanol until optimal colour intensity was obtained.

6.4 DISCUSSION

An *in vitro* model was developed to stretch MECs, mimicking the flattening in cell shape postulated to initiate changes in gene and protein expression (mechanotransduction) during mammary engorgement *in vivo*. The effect of stretching MECs *in vitro* on the expression of proteins differentially regulated during bovine and rodent mammary gland apoptosis and involution was then investigated. These included β1-integrin and the TJ proteins, occludin, claudin-1, and ZO-1, which are down-regulated during the initial phase of mammary apoptosis and involution (McMahon *et al.*, 2004, Appendix VII; Singh *et al.*, 2005, Appendix VI; Chapters 3 and 4), and the activated phosphorylated version of STAT3 which is strongly up-regulated (Philp *et al.*, 1996; Li *et al.*, 1997a; Chapman *et al.*, 1999).

A simple custom-made device was used to continuously stretch MECs *in vitro* for up to 15 h, which resulted in small changes in the protein expression of β1-integrin, pSTAT3, total STAT3, claudin-1 and ZO-1. This corroborates previous findings that MECs are mechanically responsive *in vitro* (Pitelka & Taggart, 1983; Furuya *et al.*, 1993) and *in vivo* (Chapter 5). The cell stretch device is similar to others previously reported to impose a uniform equibiaxial stretch to a cell-laden flexible membrane (Hung & Williams, 1994; Schaffer *et al.*, 1994; Lee *et al.*, 1996; Tschumperlin & Margulies, 1998). While the strain at the edges of the membrane (where it does not stretch across the indenter) will not be uniform, this is only a small proportion of the total surface area of the circular membrane, which is essentially deformed into a circle of larger diameter. The cell stretch device is theoretically capable of stretch magnitudes resulting in increases of MSA of up to 47%. This is directly comparable to a similar device frequently used to stretch lung alveolar epithelial cells (Tschumperlin & Margulies, 1998).

The stretch applied to the polarised MEC monolayer is a function of the strain applied to the flexible membrane - the assumption being that cells remain attached and membrane strains are transferred to the attached cells. In a preliminary study, MECs were shown to attach, proliferate and differentiate on flexible membranes treated with Collagen Type I in a similar manner to those cultured on plastic. Furthermore, MECs

remained attached to the Collagen Type I-treated membrane during stretching, with no visible signs of disruption to the cell monolayer even after 15 h of continuous strain. This indicates that the cell monolayer stretched to accommodate the increase in MSA. However, a change in cell surface area would have to be confirmed microscopically using a single-well device modified to stretch a flexible membrane over an annular indenter which is fitted above a microscope lens (Schaffer *et al.*, 1994; Lee *et al.*, 1996; Tschumperlin & Margulies, 1998). Such a device would be useful for real-time visualisation of the cells during stretching. Furthermore, it would open up opportunities for the use of fluorescent immunocytochemistry to track changes over time in the expression and/or distribution of components of mechanosensitive cellular structures, such as the F-actin cytoskeleton (Dewey, 1984; Wechezak *et al.*, 1985), cell-ECM focal adhesions (Shyy & Chien, 1997; Geiger & Bershadsky, 2002), adherens junctions (Ko *et al.*, 2001) and TJ complexes (Cavanaugh *et al.*, 2001).

It was not possible to directly assess TJ formation and permeability in the *in vitro* cell stretch model described here due to the requirement for measurements of transepithelial electrical resistance (TER) or paracellular movement of labelled tracers across cell monolayers to be made using a permeable membrane. The commercial flexible membranes used in the cell stretch model were not available in a permeable state. Therefore, this would require the manufacture of custom-made wells containing flexible, permeable membranes, and the subsequent modification of the cell stretch device. However, a novel *in vitro* method to measure TJ integrity has recently been reported for lung alveolar epithelial cells cultured onto an impermeable substrate (Cavanaugh & Margulies, 2002). This involved adding a fluorescently-tagged ouabain derivative to the apical medium and assessing the extent of binding of the tracer to Na^+-K^+-ATPases located only on the basolateral membrane, with increased binding indicating increased TJ permeability. Validation of this technique in MECs may provide a suitable measure of TJ permeability for future investigations using the *in vitro* cell stretch model described in this chapter.

In the current study, TJ formation in MECs was confirmed by the presence of occludin, claudin-1 and ZO-1 protein expression by western blot analysis (Stelwagen *et al.*, 1999; Stelwagen & Callaghan, 2003). A small decrease in the expression of the claudin-1 ~22

kDa band was detected in total cell homogenates after 15 h of continuous cell stretching, while the claudin-1 ~80 kDa homodimer and ZO-1 were increased. However, no changes were observed in NP-40 detergent-soluble and –insoluble fractions which were enriched for the claudin-1 ~22 kDa band, and the ~28 and ~80 kDa claudin-1 bands plus ZO-1, respectively. The resistance of TJ proteins to solubilisation in detergent-salt extractions indicates their incorporation into the TJ-cytoskeleton complex and is associated with reduced TJ permeability (Sakakibara *et al.*, 1997; Wong, 1997; Chen *et al.*, 2000). Furthermore, occludin protein expression was not changed by up to 15 h of cell stretching *in vitro*. Overall, these results indicate that TJ integrity was probably maintained during the *in vitro* stretching of MECs. However, subtle changes in the distribution and/or phosphorylation states of TJ proteins cannot be ruled out at this stage.

These results were unexpected as TJs have been previously reported to respond to mechanical tension. Cyclic stretching of lung alveolar epithelial cells perturbed the distribution and expression of occludin, and resulted in increased TJ permeability (Cavanaugh *et al.*, 2001, 2006; Cavanaugh & Margulies, 2002). Alterations in the orientation and arrangement of TJ strands also occurred when MECs were stretched uniaxially *in vitro* (Pitelka & Taggart, 1983). Induced physical distension of rat mammary glands *in vivo* transiently up-regulated the expression levels of occludin protein and mRNA, and ZO-1 mRNA, followed by an accelerated decrease in expression (Chapter 5). This was associated with the initiation of mammary apoptosis and involution, the up-regulation of the pro-apoptotic and immune response marker pSTAT3, and the down-regulation of the cell-ECM survival factor β1-integrin. Interestingly, stretching MECs *in vitro* also resulted in a rapid (by 3 h) and sustained decrease in β1-integrin protein expression enriched in the NP-40-insoluble fraction. The expression of pSTAT3 in total cell homogenate was doubled by 15 h, while total STAT3 was slightly decreased in the NP-40-insoluble fraction by 8 h and at 15 h. These results suggest that β1-integrin and pSTAT3 play significant roles in mechanotransduction events in response to stretching of the mammary epithelium both *in vitro* and *in vivo*.

Integrin-mediated cell adhesion is well documented to participate in mechanosensory signalling in various other tissues (Shyy & Chien, 1997; Geiger & Bershadsky, 2002). In particular, the FAK/Src pathway appears to be involved in the transduction of mechanical signals by integrins (Geiger *et al.*, 2001). In the lactating mammary gland, this pathway participates in cell-ECM survival signalling (Farrelly *et al.*, 1999; Gilmore *et al.*, 2000; McMahon *et al.*, 2004, Appendix VI). However, cell-ECM communication is rapidly lost during mammary engorgement, with the down-regulation of β1-integrin, FAK and down-stream pro-survival effectors ($PI3K_{(p85\alpha)}$ and Akt/PKB) thought to contribute to the onset of apoptosis and involution (Schwertfeger *et al.*, 2001; McMahon *et al.*, 2004, Appendix VII; Singh *et al.*, 2005, Appendix VI; Abell *et al.*, 2005). Furthermore, the activation of pSTAT3 during involution has recently been shown to initiate mammary apoptosis by inducing expression of $PI3K_{(p55\alpha)}$ and $PI3K_{(p50\alpha)}$ regulatory subunits to down-regulate $PI3K_{(p85\alpha)}$-Akt-mediated survival signalling (Abell *et al.*, 2005). Therefore, the down-regulation of β1-integrin and the up-regulation of pSTAT3 in response to stretching *in vitro* (current study) and *in vivo* (Chapter 5) indicate that this process is initiated by the physical distension of the mammary epithelium during extended periods of milk accumulation. Further work is required to directly show that the loss of β1-integrin and increase in pSTAT3 results in apoptosis in response to the physical stretching of MECs.

In this regard, the *in vitro* cell stretch model has several limitations in imitating the physical distension of the mammary gland *in vivo*. The COMMA-1D cell monolayer contains only epithelial cells; it does not contain the underlying myoepithelial cells, fibroblasts, and nor was a basement membrane/ECM used. It also lacks neutrophils, macrophages and other immunocytes, so is unable to mimic the immune response of mammary glands to chronic milk accumulation (Chapters 3 and 4) or induced, acute, physical distension (Chapter 5). Importantly, it does not have apical milk accumulation and the associated changes in milk composition and chemical inhibitory milk components. While COMMA-1D cells will form mammospheres and secrete milk components in response to the appropriate lactogenic hormone and ECM substratum (Medina *et al.*, 1987; Eisenstein & Rosen, 1988), their 3-D structures mean that they are difficult to mechanically stretch. Developing techniques which will allow uniform distension of lactating MECs such as found in mammospheres will provide a more

representative *in vitro* model of the processes occurring during extended periods of milk accumulation.

The current *in vitro* cell stretch model is effective in that it clearly isolates the physical stretching of MECs from the confounding effects of chemical inhibitors in milk and the effects of vesicle engorgement, which is challenging to achieve with mammospheres and *in vivo*. Conversely, as local chemical and physical mechanisms, along with systemic hormone withdrawal, may act in concert to finely regulate rodent mammary function, this may explain why the changes in protein expression described in this study tended to be small and the effects on TJs were uncertain.

This model also provides the opportunity to apply a uniform equibiaxial strain to cells and to directly control the degree and duration of that stretch. While the strains incurred by MECs *in vivo* have been described (Alcaraz *et al.*, 2004; Paszek & Weaver, 2004), they have yet to be quantified. However, Kim *et al.* (1997) reported that the alveolar lumen size was maximally increased by 3-fold at 12 h compared with 0 h of weaning in mice. In the rodent mammary gland, processes initiating the progressive reduction in milk secretion, loss of survival signals and increase in pro-apoptotic signals occur within the first 6 -12 h of milk accumulation (Hanwell & Linzell, 1972; Li *et al.*, 1997a; Nguyen & Pollard, 2000; McMahon *et al.*, 2004; Chapter 4). Therefore, subjecting MECs to an equibiaxial stretch at 47% ΔMSA for up to 15 h should be sufficient to mimic early mechanotransduction events. Similar strains have been previously reported to down-regulate occludin protein expression and increase TJ permeability in lung alveolar epithelial cells *in vitro* (Cavanaugh *et al.*, 2001, 2006; Cavanaugh & Margulies, 2002) and rapidly up-regulate *c-jun* mRNA expression in fibroblasts (Levinson *et al.*, 2002).

In conclusion, this study described the development of a simple cell stretch device and showed that stretching MECs *in vitro* induced small changes in protein expression, demonstrating that MECs are responsive to changes in cell shape. While the results are inconclusive regarding the role of *in vitro* stretching on the regulation of TJ proteins, the data supports further investigation into mechanotransduction events in the mammary gland. In particular, the results point to crosstalk between cell-ECM survival signalling

and STAT3 death signalling through the PI3K-Akt pathway as a candidate for regulation by physical distension of the mammary epithelium in response to extended periods of milk accumulation.

CHAPTER SEVEN

General Discussion and Conclusions

This study investigated the regulation of TJ proteins during the early stages of mammary apoptosis and involution, with particular emphasis on the role of physical distension of the mammary epithelium in the regulatory process. A sudden decrease in the frequency of milk removal (e.g., ODM) from the mammary gland, or complete cessation of milk removal, results in extended periods of milk accumulation within the alveolar compartment. Local intra-mammary signals are rapidly initiated which progressively lead to reduced milk secretion, increased apoptosis and eventually, mammary involution if the gland remains unmilked (Quarrie *et al.*, 1994, 1996; Li *et al.*, 1997a; Marti *et al.*, 1997). This process is associated with an increase in TJ permeability, the timing of which coincides, approximately, with the decline in milk secretion rate in both ruminants (Stelwagen *et al.*, 1994b, 1997; Stelwagen, 2001) and rodents (Hanwell & Linzell, 1972; Yamamuro & Sensui, 1994; Kim *et al.*, 1997).

The present study showed that the integral transmembrane TJ proteins, occludin and claudin-1, and the cytoplasmic TJ protein, ZO-1, were decreased in both bovine and rat mammary glands during apoptosis and involution following the abrupt cessation of milk removal (Chapters 3 and 4). In the rat, the rapid down-regulation of TJ protein expression and the initiation of MEC apoptosis occurred only in glands which had been teat-sealed to induce mammary engorgement, with no changes detected in suckled glands from the same animal. Therefore, these responses were consistent with the local regulation of mammary function.

Reduced levels of TJ protein expression occur during TJ disruption in other epithelial cell types (Li & Mrsny, 2000; Tian & Phillips, 2002; Ma *et al.*, 2004). Conversely, increased occludin and ZO-1 protein expression were demonstrated during decreased TJ permeability in response to glucocorticoids and/or prolactin in mouse MECs *in vitro* (Singer *et al.*, 1994; Woo *et al.*, 1996; Stelwagen *et al.*, 1999). In the present study the

down-regulation of TJ protein and mRNA expression in bovine mammary glands by 36 h post-milking occurred shortly after the previously reported rises in plasma lactose concentrations at 18 h that are proposed to signal an initial increase in TJ permeability (Stelwagen *et al.*, 1997). However, the decreases in TJ protein expression preceded the large drop in intramammary pressure and volume between 3 to 7 d of involution (Mackenzie, 1968; Fleet & Peaker, 1978; Hurley, 1989) that is associated with a massive loss of TJ integrity.

In rats, changes in protein expression occurred by 6 h, 12 h and 18 h following teat-sealing for claudin-1, occludin, and ZO-1, respectively. A rise in mammary TJ permeability, as indicated by increased plasma lactose concentrations, was previously reported to begin about 7 h following weaning (Yamamuro & Sensui, 1994). In the present study, mammary glands reached a maximum mass at 12 h following teat-sealing (McMahon *et al.*, 2004; Appendix VII), after which time the mass of glands declined indicating reabsorption of secreted milk. This suggestion was supported by the presence of very large numbers of milk vesicles, consisting of coalescing fat droplets and proteins within engorged alveolar lumens, which would have been too large to pass through leaky TJs (Molenaar *et al.*, 1996a), by 18 h.

These results imply that the down-regulation of occludin, claudin-1 and ZO-1 contribute to the progressive loss of TJ integrity during extended periods of milk accumulation. Other members of the claudin family (Tsukita *et al.*, 2001), or scaffolding and signalling proteins linked to the TJ (reviewed by Matter & Balda, 2003; Schneeberger & Lynch, 2004; Aijaz *et al.*, 2006), may be down-regulated earlier than the proteins that were studied here. Alternatively, changes in phosphorylation states and/or the cellular distribution of TJ components may provide important signals initiating losses of TJ integrity. It would be valuable to examine these possibilities in future studies to explore more fully the intracellular mechanisms by which TJ proteins are regulated in response to mammary engorgement.

Nevertheless, the changes in TJ protein expression reported here preceded the onset of MEC apoptosis, supporting TJ disruption as a relatively early event in mammary apoptosis and involution. While the breakdown of specialised cell-cell junctions is the

first visible stage of MEC apoptosis during mammary involution prior to cytoplasmic and nuclear condensation (Walker *et al.*, 1989; Strange *et al.*, 1992; Tenniswood *et al.*, 1992), it is still unclear whether changes in TJ proteins are causal to the onset of apoptosis. However, experimentally-induced truncation of occludin results in MEC apoptosis both *in vivo* and *in vitro* (Beeman & Neville, 2001), indicating that intact occludin protein is necessary as a cell survival 'signal'.

The present study also provided a detailed account of the time course of the initiation of apoptosis relative to changes in alveolar histological morphology following the abrupt cessation of milk removal (Chapters 3 and 4). In rats, large numbers of apoptotic nuclei were detected at 18 h, 24 h and 36 h following teat-sealing, coincident with heavy vesicle engorgement of alveoli. These results show that the onset of apoptosis in rats occurs 12-18 h after teat-sealing, which is earlier than previously indicated in reports in mice where apoptosis was not measured until 24-48 h after teat-sealing (Quarrie *et al.*, 1995, 1996; Marti *et al.*, 1997). In bovine alveolar tissue, an increase in the numbers of apoptotic nuclei occurred by 72 h and 8 d following the abrupt cessation of milk removal in mid-lactation, and was associated with vesicle engorgement and signs of alveolar collapse and regression. Previously, increased apoptosis has been demonstrated during gradual involution occurring during the latter part of lactation in goats and cows (Knight & Peaker, 1984; Wilde *et al.*, 1997; Li *et al.*, 1999; Capuco *et al.*, 2001).

Mammary apoptosis was generally greater in cells from the epithelial layer surrounding the alveolar lumen than in cells located within the alveolar lumen, except at 36 h in rats and at 8 d in bovine when there were large numbers of apoptotic nuclei in alveolar lumens. This was associated with increased numbers of leukocytes (e.g., neutrophils and macrophages) in alveolar lumens. However, it was not possible to distinguish if the apoptotic nuclei were from sloughed MECs or from the invading leukocytes. While an investigation of the role of leukocytes in mammary apoptosis and involution was outside the scope of this project, it should be considered for future studies.

The data also showed that individual cows varied in their rates of involution, with differences between cows apparent by 36 h post-milking. Animals with higher numbers of apoptotic nuclei showed clear signs of engorgement and involution of the secretory

alveoli with decreased α-lactalbumin and α-S1-casein mRNA expression and increased expression of the immune-associated genes, lactoferrin and MSAA. In addition, there were differences within cows in the stage of involution of individual lobules and alveoli, indicating that alveoli enter involution asynchronously. These results may help explain the variation between cows in the extent of the initial, acute milk production loss in response to ODM.

The loss of TJ protein expression and the onset of apoptosis and involution in bovine mammary glands were not as rapid, nor as extensive, as those that occurred in rats. Ruminants appear to tolerate the effects of extended periods of milk accumulation better than rats and, by delaying commitment to apoptosis, retain the ability to re-initiate lactation if circumstances alter. In dairy cows lactation can be almost fully restored after 7 d (Dalley & Davis, 2006) and partially reinstated after 11 d (Noble & Hurley, 1999) of involution. In contrast, the critical time for lactation reversal lies between 48 h and 72 h in rodents (Jaggi *et al.*, 1996; Li *et al.*, 1997a; Sorensen & Knight, 1997). Lactation can be restored to mice up to 48 h following litter removal, but after 72 h recovery is very limited as the mammary glands progress into the second, irreversible, stage of involution, which involves extensive proteolytic degradation of the ECM and remodelling of the lobular-alveolar structures (Jaggi *et al.*, 1996; Lund *et al.*, 1996; Li *et al.*, 1997a). Systemic galactopoietic hormone signalling also appears to play an important role in rodents as, while local signals (i.e., milk accumulation) are sufficient to initiate apoptosis and involution, a fall in the levels of prolactin and glucocorticoids is required for progression into the irreversible phase of mammary involution (Li *et al.*, 1997a).

The identity of the primary signal triggering the onset of mammary apoptosis and involution in response to local milk accumulation is unknown, but is related to either the chemical presence of inhibitory factors in milk, or to the physical presence of stored milk activating stretch-sensitive pathways. FIL (Wilde *et al.*, 1995), casein-derived phosphopeptides (Shamay *et al.*, 2002; 2003; Silanikove *et al.*, 2006), and serotonin (Matsuda *et al.*, 2004) are among the chemical factors thought to regulate local mammary function. However, the exact mechanisms by which these factors inhibit milk secretion and initiate mammary apoptosis and involution remain to be determined.

Other studies, in lactating goats and cows, have reported that physical distension of the mammary epithelium rather than chemical inhibition were responsible for initiating reductions in TJ integrity and milk secretion during extended periods of milk accumulation (Fleet & Peaker, 1978; Peaker, 1980; Stelwagen *et al.*, 1998a). The change in epithelial cell shape from a cuboidal to a flattened morphology as the alveoli distends to accommodate secreted milk (Richardson, 1947; Chapters 3 and 4) may initiate mechanotransduction signalling events that culminate in the loss of secretory activity and the induction of apoptosis (Davis *et al.*, 1999; Stelwagen, 2001).

Physical distension of the mammary epithelium is likely to result in mechanical tension on both cell-cell and cell-ECM interactions, which may lead to loss of survival signals and initiate pro-apoptotic signals. The rapid down-regulation of TJ proteins (occludin, claudin-1 and ZO-1; Chapters 3 and 4) and increased TJ permeability (Hanwell & Linzell, 1972; Fleet & Peaker, 1978; Yamamuro & Sensui, 1994; Kim *et al.*, 1997; Stelwagen *et al.*, 1997) occurring during extended periods of milk accumulation may therefore, participate in these mechanotransduction events. Concurrent studies in our lab have also recently demonstrated a loss of cell-ECM survival signalling through β1-integrin and FAK during the first, reversible, stage of mammary apoptosis and involution (McMahon *et al.*, 2004, Appendix VII; Singh *et al.*, 2005, Appendix VI). Therefore, the role of physical distension of MECs in initiating apoptosis and down-regulating β1-integrin and TJ protein expression was examined using *in vivo* (Chapter 5) and *in vitro* (Chapter 6) approaches.

In the *in vivo* study, rat mammary glands were acutely distended to the equivalent of approximately 6 h worth of milk accumulation, using an isosmotic sucrose solution, with the aim of initiating the process of involution. The results showed that induced physical distension of the mammary epithelium accelerated the onset of apoptosis, the up-regulation of the pro-apoptotic and immune response marker pSTAT3, and the loss of β1-integrin and occludin protein expression, compared with the effects of milk accumulation alone. This procedure would have diluted any potential chemical inhibitor(s) in milk. Thus, this work supports the hypothesis that physical distension of the mammary epithelium during extended periods of milk accumulation is a primary trigger of mammary apoptosis and involution (Davis *et al.*, 1999; Stelwagen, 2001).

These results are also consistent with earlier studies in goats and in cows, which demonstrated that induced physical distension of mammary glands, by an isosmotic sucrose/lactose solution, increased TJ permeability and inhibited milk secretion (Fleet & Peaker, 1978; Peaker, 1980; Stelwagen *et al.*, 1998a). However, other researchers have reported that milk secretion rate was increased when milk removed at an extra daily milking was replaced immediately by an equal volume of isosmotic sucrose solution to maintain the gland's distension (Henderson & Peaker, 1984), and when milk stored in the mammary gland was diluted with isosmotic sucrose solution (Henderson & Peaker, 1987). These results lead to the suggestion that a chemical mechanism(s) may also control local mammary function by feedback inhibition. Therefore, it cannot be ruled out that both chemical and physical mechanisms, acting either independently or in concert, finely control mammary responses to the frequency and completeness of milk removal.

Mammary TJs were previously reported to respond to mechanical tension as alterations in the orientation and arrangement of TJ strands occurred when MECs were stretched i*n vitro* (Pitelka & Taggart, 1983). In the current *in vivo* study, occludin protein and mRNA, and ZO-1 mRNA expression were initially dramatically up-regulated immediately following the acute physical distension procedure, but then gradually declined to be down-regulated after 6 h. The rapid increase in expression may reflect an attempt to maintain mammary TJ integrity before the system was overwhelmed, with the subsequent down-regulation of TJ protein expression providing evidence of a loss of TJ integrity. However, in contrast to milk accumulation alone, there were no changes in the protein expression of claudin-1 and ZO-1 within 6 h of induced physical distension of rat mammary glands. This indicates that changes in TJ protein expression during mammary apoptosis and involution are not necessarily regulated in concert, and that expression of the integral transmembrane TJ protein, occludin, is the most responsive to physical distension of the mammary epithelium.

Furthermore, this study demonstrated that the onset of mammary apoptosis in response to induced physical distension was accompanied by the dramatic up-regulation of the pro-apoptotic factor pSTAT3, and the down-regulation of the cell-ECM survival factor β1-integrin. Integrin-mediated cell adhesion has been reported to participate in

mechanosensory signalling in various other cell types (Shyy & Chien, 1997; Geiger & Bershadsky, 2002). These results suggest that loss of β1-integrin-mediated cell-ECM communication (McMahon *et al.*, 2004, Appendix VII; Singh *et al.*, 2005, Appendix VI) and an increase in pSTAT3 (Philp *et al.*, 1996; Li *et al.*, 1997a; Chapman *et al.*, 1999; Dr. K. Singh, unpublished observations) play significant roles in mechanotransduction events during apoptosis and involution of rat and bovine mammary glands. STAT3 is a key regulator of this process as conditional knockout of mammary STAT3 suppressed MEC apoptosis and delayed involution in mice (Chapman *et al.*, 1999). Conversely, mice expressing a dominant-negative form of β1-integrin in their mammary glands have increased apoptosis during lactation (Faraldo *et al.*, 1998) and accelerated loss of secretory function during involution (Faraldo *et al.*, 2002). STAT3 death signalling has recently been linked to β1-integrin-FAK survival signalling through the downstream PI3K-Akt pathway. The activation of STAT3 during involution initiates mammary apoptosis by inducing expression of $PI3K_{(p55\alpha)}$ and $PI3K_{(p50\alpha)}$ regulatory subunits to down-regulate $PI3K_{(p85\alpha)}$-Akt-mediated survival signalling (Abell *et al.*, 2005). The results of this thesis indicate that this process is triggered by physical distension of the mammary epithelium, and should be subjected to further investigation.

While the present *in vivo* study supported a role for mechanotransduction in the regulation of mammary function, an *in vitro* model would enable further investigation of the specific physical mechanism(s) whereby stretching MECs induces changes in protein expression. In the final study, an *in vitro* model was developed which clearly separated the physical stretching of MECs from the actions of accumulated milk products (Chapter 6). A simple custom-made device was used to apply uniform equibiaxial stretch to COMMA-1D cells, a well-characterised murine MEC line, that were attached to a Collagen Type 1-coated flexible membrane. The device was theoretically capable of stretch magnitudes resulting in increases in the surface area of the flexible membrane substratum of up to 47%, and was similar to others previously reported in the literature (Hung & Williams, 1994; Schaffer *et al.*, 1994; Lee *et al.*, 1996; Tschumperlin & Margulies, 1998). Continuous stretching of MECs *in vitro* at 47% ΔMSA for up to 15 h resulted in small changes in the protein expression of β1-integrin, pSTAT3, total STAT3, claudin-1 and ZO-1. These results confirmed previous

findings that MECs are mechanically responsive *in vitro* (Pitelka & Taggart, 1983; Furuya *et al.*, 1993) and *in vivo* (Chapter 5).

However, the results were inconclusive regarding the role of stretching MECs *in vitro* on TJs. After 15 h of continuous cell stretching a small decrease in the expression of the claudin-1 ~22 kDa band was detected in total cell homogenates, while the claudin-1 ~80 kDa homodimer and ZO-1 were increased. No changes were observed in NP-40 detergent-soluble and –insoluble fractions which were enriched for the claudin-1 ~22 kDa band, and the ~28 and ~80 kDa claudin-1 bands plus ZO-1, respectively. Occludin protein expression in MECs also remained unchanged during 15 h of stretching *in vitro*. Overall, these results indicate that TJ integrity was probably maintained during the *in vitro* stretching of MECs. This was unexpected in light of the changes in occludin protein expression in response to *in vivo* mammary physical distension discussed above (Chapter 5), and of previous reports that cyclic stretching of lung alveolar epithelial cells perturbed the distribution and expression of occludin, and resulted in increased TJ permeability (Cavanaugh *et al.*, 2001, 2006; Cavanaugh & Margulies, 2002). However, changes in the distribution and/or phosphorylation states of TJ proteins and their signalling partners cannot be ruled out during *in vitro* stretching of MECs and should be investigated further.

Stretching MECs *in vitro* resulted in a rapid (by 3 h) and sustained decrease in β1-integrin protein expression enriched in the NP-40-insoluble fraction, whereas the expression of pSTAT3 in total cell homogenate was doubled at 15 h. These results support the findings of the *in vivo* study which showed that a decrease in β1-integrin protein expression and a dramatic increase in pSTAT3 protein expression occurred during apoptosis initiated by acute physical distension of rat mammary glands (Chapter 5). Further work is now required to determine whether the loss of β1-integrin and increase in pSTAT3 directly results in apoptosis in response to the physical stretching of MECs.

Subsequent experiments leading on from this project could also include the development of 3-D techniques which would allow uniform distension of lactating MECs, such as found in mammospheres, to provide a more representative *in vitro*

model of the processes occurring during extended periods of milk accumulation. The use of bovine MECs rather than rodent MECs in the *in vitro* cell stretch model would also enable a more direct comparison to mimic the physical distension of the alveolar epithelium in response to ODM or complete cessation of milk removal in dairy cows.

In summary, the results of this thesis contribute to the understanding of local regulation of mammary function, and in particular the role of physical distension and the TJ complex, during extended periods of milk accumulation. Expression of the major TJ proteins, occludin, claudin-1, and ZO-1, was down-regulated in both bovine and rat mammary glands during apoptosis and involution in response to the abrupt cessation of milk removal (Chapters 3 and 4). Induced physical distension of rat mammary glands *in vivo* transiently up-regulated the expression levels of occludin protein and mRNA, and ZO-1 mRNA, followed by an accelerated decrease in expression compared with milk accumulation alone. This was associated with the initiation of apoptosis, the up-regulation of the pro-apoptotic factor pSTAT3, and the down-regulation of the cell-ECM survival factor β1-integrin. These results support the hypothesis that physical distension of the mammary epithelium induces mechanotransduction events which trigger the onset of mammary apoptosis and involution in response to extended periods of milk accumulation. While stretching MECs *in vitro* did not conclusively alter TJ protein expression, the overall results of this project support further investigation into the role of the TJ complex in mechanotransduction pathways. In addition, the crosstalk between cell-ECM survival signalling and STAT3 death signalling through the PI3K-Akt pathway is another candidate for regulation by physical distension of the mammary epithelium. The results presented in this thesis provide a foundation from which to examine the role of mechanotransduction events in the mammary gland in more detail.

CHAPTER EIGHT

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APPENDIX I

List of Company Addresses

- Accelrys Inc., San Diego, California, USA.
- AgResearch Ltd., Ruakura Agricultural Research Centre, Private Bag 3123, Hamilton, NZ.
- Ambion, Inc., 2130 Woodward St., Austin, Texas 78744-1832, USA.
- Amersham Biosciences UK Ltd., Amersham Pl., Little Chalfont, Buckinghamshire HP7 9NA, England.
- Applichem GmbH, Ottoweg 10 b, D-64291, Darmstadt, Germany.
- Applied Biosystems, 850 Lincoln Centre Dr., Foster City, California 94404, USA.
- BDH Laboratory Supplies, Poole, England.
- Biogenex, 4600 Norris Canyon Rd., San Ramon, California 94583, USA.
- Biolab Scientific, Bush Rd., Albany, Auckland, New Zealand.
- Bio-Rad Laboratories, 2000 Alfred Nobel Dr., Hercules, California 94547, USA.
- CHEMICON International, Inc., 28820 Single Oak Dr., Temecula, California 92590, USA.
- Dako Cytomation, 6392 Via Real, Carpinteria, California 93013, USA.
- Dural Plastics & Engineering, Auburn, New South Wales, Australia.
- Eastman Kodak Company, Rochester, New York, USA.
- Eppendorf AG, Barkhausenweg 1, 22339 Hamburg, Germany.
- Fisher Scientific, Pittsburgh, Pennsylvania, USA
- Flexcell International Corporation, Hillsborough Business Center, 437 Dimmocks Mill Rd., Hillsborough, North Carolina 27278, USA.
- Gelman Laboratory, Pall Corporation, 600 South Wagner Rd., Ann Arbor, Michigan, USA.
- GIBCO Products, Invitrogen, 3175 Staley Rd., P.O. Box 68, Grand Island, New York 14072, USA.
- Informax Inc., USA.
- Invitrogen, 1600 Faraday Ave., Carlsbad, California 92008, USA.
- Invitrogen (NZ), Auckland 1135, New Zealand.

- Janke & Kunkel, Staufen, Germany.
- Jasc Software Inc., 7905 Fuller Rd., Eden Prairie, Minnesota 55344, USA.
- JENOPTIK Laser, Optik, Systeme GmbH, Division Digital Cameras, Oskar-Von-Miller-Straβe 1a, D-85386 Eching, Germany.
- Leica Microsystems, Heidelberger Straβe 1779, Nussloch 69226, Germany.
- Loctite Australia Pty. Ltd., Caringbah, New South Wales, Australia.
- LKB-Produkter AB, Box 305, S-161 26, Bromma, Sweden.
- Millipore Corp., 290 Concord Rd., Billerica, Massachusetts 01821, USA.
- Minitab Inc., Quality Plaza, 1829 Pine Hall Rd, State College, Pennsylvania 16801-3008, USA.
- Molecular BioProducts, Inc., San Diego, California, USA.
- New England Biolabs, 240 County Rd., Ipswich, Massachusetts 01938-2723, USA.
- Nunc A/S, Kamstrupvej 90, PO Box 280, DK-400, Roskilde, Denmark.
- Omni International, Inc., Suite 100, Warrenton, Virginia 20187, USA.
- Olympus Optical Co., Inc., Tokyo, Japan.
- Oxoid Ltd., Basingstoke, Hampshire, England.
- Promega Corporation, 2800 Woods Hollow Rd., Madison, Wisconsin 53711-5399, USA.
- QIAGEN Sciences, 19300 Germantown Rd., Germantown, Maryland 20874, USA.
- Roche Applied Science, Mannheim, Germany.
- Sakura-Finetek Europe BV, Hoge Rijndijk 48 a, 23282 AT Zoeterwoude, The Netherlands.
- Santa Cruz Biotechnology, Inc., 2145 Delaware Ave., Santa Cruz, California 95060, USA.
- Schering-Plough Animal Health Limited, Upper Hutt, New Zealand.
- Serva Electrophoresis GmbH, Carl-Denz-Str. 7, Heidelberg, Germany.
- Sharps Grain & Seed, Carterton, New Zealand.
- Sigma Chemical Company, PO Box 14508, St. Louis, Missouri 63178, USA.
- Vetpharm (NZ) Ltd., Glenfield, Auckland, New Zealand.
- Waikato DNA Sequencing Facility, University of Waikato, Hamilton, New Zealand.
- Whatman International Ltd., Maidstone, England.
- Zymed Laboratories Inc., 561 Eccles Ave., South San Francisco, California 94080, USA.

APPENDIX II

pGem-T Easy Vector System

10.1 pGEM-T EASY VECTOR MAP AND SEQUENCE

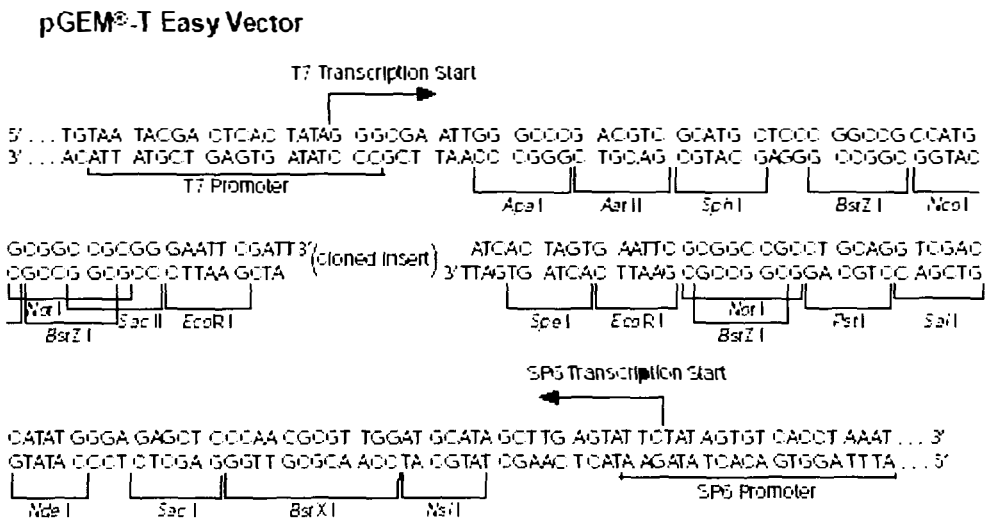
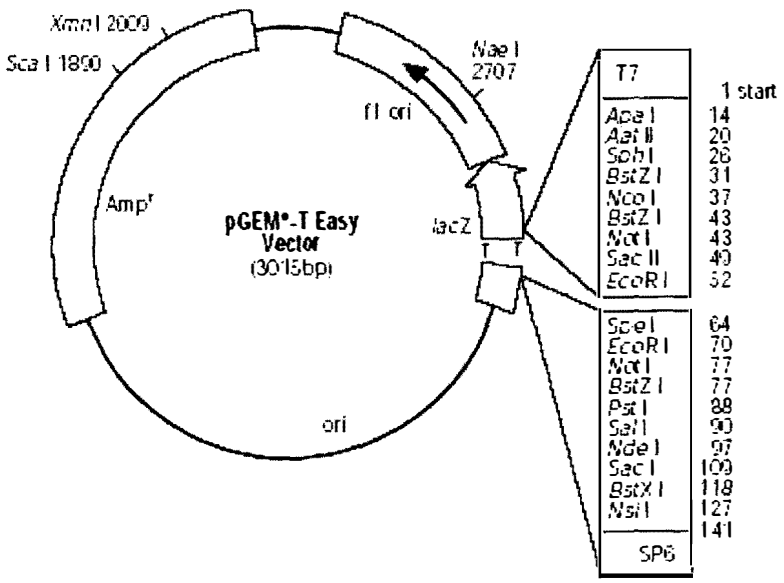


Figure 10.1. The promoter and multiple cloning sequence of the pGEM-T Easy Vector.
The top strand of the sequence shown corresponds to the RNA synthesised by T7 RNA Polymerase. The bottom strand corresponds to the RNA synthesised by SP6 RNA Polymerase. The pGEM-T Easy Vector contains multiple restriction sites within the multiple cloning region. (Adapted from "pGEM®-T and pGEM®-T Easy Vector Systems, Technical Manual No. 042"; Promega Corporation, 2003).



pGEM®-T Easy Vector Sequence reference points:

Base pairs (bp)	3015
T7 RNA polymerase transcription initiation site	1
multiple cloning region	10-128
SP6 RNA polymerase promoter (-17 to +3)	139-158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176-197
lacZ start codon	180
lac operator	200-216
β-lactamase coding region	1337-2197
phage f1 region	2380-2835
lac operon sequences	2836-2996, 166-395
pUC/M13 Forward Sequencing Primer binding site	2949-2972
T7 RNA polymerase promoter (-17 to +3)	2999-3

Figure 10.2. pGEM-T Easy Vector circle map and sequence reference points.
The pGEM-T Easy Vector contains multiple restriction sites within the multiple cloning region. (Adapted from "pGEM®-T and pGEM®-T Easy Vector Systems, Technical Manual No. 042"; Promega Corporation, 2003).

APPENDIX III

Contiguous Nucleic Acid Sequences for Bovine Tight Junction Proteins

```
1    GCGGAGACGGCGGGACTCCGGGGAGAGCTGGCCGGAACGCCGAGTANCCC
51   GGGCGGGGAGCCGCAGATTGGCTGATCTCAGGAGGCTCTGGCAGTGCTCA
101  TCCTGAAGATCAAGTGACCACTGACACCAGCCATGTCATCCAGGCCTTTT
151  GAAAGTCCACCTCCTTATAGACCTGATGAATTCAAACCTAATCACTACAC
201  ACCAAGCAATGACATATATAGTGGAGAGATGCACGTTCGACCAATGCTCT
251  CTCAGCCAGCATATTCCTTCTACCCGGAAGATGAAATTCTCCACTTCTAC
301  AAATGGACCTCTCCTCCCGGAGTAATTCGGATCCTGTCTATGCTTGGTAT
351  CGTGATGTGCATCGNCATTTTCG
```

Figure 11.1. Bovine occludin ortholog, partial nucleic acid sequence.
This contiguous sequence (contig) (373 bp) was built from expressed sequence tags (ESTs) obtained from the AgResearch EST database, and which have been submitted to Genbank under the accession numbers: DY070480 and DY156224. It is a partial sequence similar to published Genbank occludin sequences for bovine (predicted XM 589919 similar to occludin; 77.4% sequence identity), human (U53823; 83.6% sequence identity), canine (U49221; 84.6% sequence identity) and rat (AB016425; 67.5% sequence identity). Sequence similarities were calculated using the Smith-Waterman local alignment program in EMBOSS-GUI v1.14 (EMBNET, http://www.uk.embnet.org). The positions of PCR primers are bold/underlined with the PCR product (183 bp) highlighted in grey (refer to Table 3.2, Chapter 3).

```
1    CCACGCGTCCGGGAGCAGCCTCAGCTTCTCGCCTCCGAACCGCAGCGCCG
51   ACCAAGACCCGGACCCGGACCCGGACCCGGTCCCAGAGCTTCTCCGACGT
101  CAGCGCAGGGAGCAGGAGTCCCTGAAATAACTTCTTCCGCTAGGCTCCGT
151  CACGCCCGGGAGTCCCGGGTGCGCACCTGCAAACTCCGCCTTGTGCACCT
201  GCTGCCCCTGAGCCAGCGCGGGCGCCCGAGCGAGTCATGGCCAACGCGGG
251  GTTGCAGCTGCTGGGCTTCATCCTGGCGTTTCTGGGCTGGATCGGCTCCA
301  TCGTCAGCACGGCGCTGCCCCAGTGGAAAGTTTACTCCTATGCTAGTGAC
351  AACATCGTGACGGCCCAGGCCATCTATGAGGGGCTGTGGATGTCCTGCGT
401  GTCGCAGAGCACCGGGCAGATCCAGTGCAAAGTCTTCGACTCCTTGCTGA
451  ATCTGAACAGCACTTTGCAAGCAACCCGTGCCTTGATGGTGATTGGCATC
501  CTGCTGGGACTAATAGCCATCTTTGTGGCCACCGTTGGCATGAAGTGTAT
551  GAAGTGCATGGAAGACGACGAGGCACAGAAGATGCGGATGGCTGTCTTTG
601  GGGGCGTGATCTTTCTTATTTCAGGTCTGGCTATTTTAGTTGCCACAGCA
651  TGGTATGGCAATAGAATTGTTCAAGAATTCTATGACCCCATGACCCCGGT
701  CAATGCCAGGTATGAATTTGGTCAGGCTCTCTTCATTGGCTG
```

Figure 11.2. Bovine claudin-1 ortholog, partial nucleic acid sequence.
This contiguous sequence (contig) (742 bp) was built from expressed sequence tags (ESTs) obtained from the AgResearch EST database, and which have been submitted to Genbank under the accession numbers: DY073072, DY100198, DY183610, and DY168138. It is a partial sequence similar to published Genbank claudin-1 sequences for bovine (NM 001001854; 100% sequence identity), human (NM 021101; 87.3% sequence identity) and rat (NM 031699; 79.9% sequence identity). Sequence similarities were calculated using the Smith-Waterman local alignment program in EMBOSS-GUI v1.14 (EMBNET, http://www.uk.embnet.org). The positions of PCR primers are bold/underlined with the PCR product (395 bp) highlighted in grey (refer to Table 3.2, Chapter 3).

```
1    ACCGTCACGCCAGCATACAATCGATTCACACCAAAACCATACACAAGCTC
51   TGCACGGCCATTTGAACGCAAGTTTGAAAGCCCTAAATTCAACCACAATC
101  TCTTGCCGAATGAAACCGCACACAAACCTGACCTGTCCTCCAAAGCCCCC
151  GCTTCTCCGAAAACCCTCCTGAAGTCTGCACAGCCTCCTGAGTTTGACAG
201  TGGCGTGGAGACCCTCTCTGTCCACACTGACAACAAGCTGAAATACCAAG
251  TAAACAGTGTTAGCATGGGGCCGAGAGCAGTGCCCGTGAGTCCCTCAGCC
301  GTGGAGGAAGATGAAGACGAAGACGGTCACACAGTGGTGGCTACTGCCCG
351  CGGCGTGTTTAACAGCAATGGTGGGGTGCTGAGCTCCATTGAGACCGGTG
401  TCAGCATCATCATCCCCCAGGGAGCCATTCCCGAGGGGGTGGAACAGGAG
451  ATCTACTTCAAGGTCTGCCGCGACAACAGCATCCTGCCGCCTTTGGACAA
501  AGAGAAGGGTGAGACCCTGCTGAGCCCCCTAGTGATGTGTGGGCCCCATG
551  GCCTCAAGTTCCTGAAGCCCGTGGAGCTGCGCCTACCACACTGTGCGTCC
601  ATGACTCCTGACGGTTGGTCTTTTGCTCTAAAATCATCCGACTCCTCGTC
651  GGGTGATCCTAAAACCTGGCAAAACAAGTGTCTTCCTGGAGATCCAAATT
701  ATCTTGTTGGAGCAAACTGTGTTTCTGTCCTGATTGACCACTTTTAATCC
751  TTAAAATATATGAACTTGATTAAATAATGTGAAACTGGGTTAAAGTTACT
801  AAATCTAAAATGGAACCACTCTATCAAGTAGTACCTTTTCCTGGAGTTGA
851  TCCTGCAGTGTGTTAGTTTTACGCGCTGTGTTTGGGTGGGGGAGGCTTGA
901  ATGTGCAACCCCTGGGAAACATGCTTGCCCACTGCCCATTTGGGGGCTTG
951  GTGTGGGCTGGAGGCTTTAAA
```

Figure 11.3. Bovine ZO-1 ortholog, partial nucleic acid sequence.
This contiguous sequence (contig) (971 bp) was built from expressed sequence tags (ESTs) obtained from the AgResearch EST database, and which have been submitted to Genbank under the accession numbers: DY061280, and DY086244. It is a partial sequence similar to published Genbank ZO-1 sequences for bovine (predicted XM 582218; 100% sequence identity), human (NM 003257; 74.3% sequence identity) and rat (predicted XM 218747; 70.5% sequence identity). Sequence similarities were calculated using the Smith-Waterman local alignment program in EMBOSS-GUI v1.14 (EMBNET, http://www.uk.embnet.org). The positions of PCR primers are bold/underlined with the PCR product (303 bp) highlighted in grey (refer to Table 3.2, Chapter 3).

APPENDIX IV

Identification and Selection of Endogenous Controls for Relative Quantification of Gene Expression by Real-Time PCR

12.1 ANALYSIS OF ENDOGENOUS CONTROL GENE EXPRESSION DURING MAMMARY ENGORGEMENT

Selection of an appropriate endogenous control is vital to obtaining accurate results from relative quantification of gene expression by real-time PCR. Normalisation of target gene expression to an endogenous control, often a housekeeping or maintenance gene, corrects results for differences in the initial amount of cDNA present in each sample. Therefore, the ideal endogenous control gene should be expressed at a constant level in all samples regardless of experimental treatments, developmental stage and tissue type. It is also desirable for the endogenous control gene to have either the same or slightly higher expression than target genes. Hence, an endogenous control gene should be validated for the particular experimental system under study (Dheda *et al*, 2004; 2005), which in this case are bovine (Chapter 3) and rat (Chapters 4 & 5) models of mammary engorgement.

The housekeeping genes 18s rRNA, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin and ubiquitin are commonly used as endogenous controls during real-time PCR. However, GAPDH was not considered to be a suitable endogenous control in the present studies due to numerous reports suggesting that its use is inappropriate, as mRNA expression can vary widely between samples and with differing physiological, developmental and disease states (reviewed by Bustin, 2000; 2002; Dheda *et al*., 2004; 2005). 18s rRNA, which makes up a large percentage of total cellular RNA, has been suggested as a more reliable internal standard (Bustin, 2000; 2002). However, it was unsuitable to use in the present studies as first strand cDNA synthesis was performed with Oligo(dT) primers which hybridise to 3' poly(A) tails

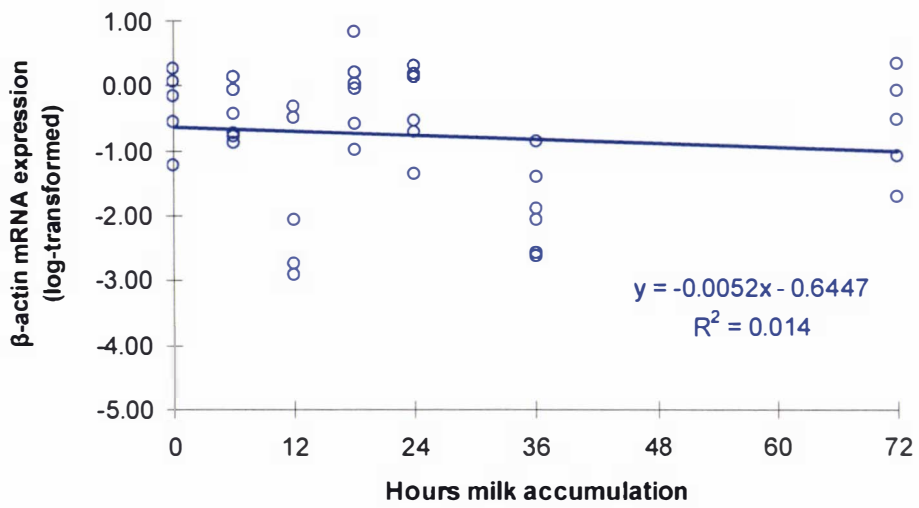
enriching the cDNA for mRNA rather than rRNA transcripts. While β-actin is widely used as an endogenous control gene, it should be evaluated in the system under study as its levels of transcription can vary under different experimental conditions (Bustin, 2000; Dheda *et al.*, 2004; 2005). Therefore, the mRNA expression of β-actin was compared with that of ubiquitin, which was previously reported to be constantly expressed in bovine mammary tissue (Pfaffl *et al.*, 2003; Schmitz *et al.*, 2004), in order to select the most suitable endogenous control for normalisation of target gene expression across bovine and rat models of mammary engorgement.

12.1.1 Bovine models of mammary engorgement

12.1.1.1 Time course of changes in mRNA expression of β-actin and ubiquitin during bovine mammary engorgement

In a preliminary experiment, the mRNA expression of β-actin and ubiquitin were compared in bovine alveolar mammary tissue taken between 0 and 72 h post-milking (Fig. 12.1A and B); refer to Chapter 3 for experimental design). Expression of β-actin and ubiquitin mRNA was variable between individual cows within each time point post-milking. However, β-actin mRNA expression was more variable within and between time points post-milking than ubiquitin expression. This result is similar to northern blotting data which showed that variation in mammary β-actin mRNA expression between animals at either ~5 h (lactating) or ~72 h (involuting) post-milking was as great as variation between individual cows within each group, and that expression was equal to or slightly higher in involuting glands (Wiens *et al.*, 1992). Ubiquitin was previously reported to be a high performing internal standard both within and across bovine tissue types (Tichopad *et al.*, 2004), and was used to normalise the mRNA expression of major milk proteins and immune-associated genes in bovine mammary tissue following real-time PCR (Pfaffl *et al.*, 2003; Schmitz *et al.*, 2004). Therefore, ubiquitin was selected to normalise the expression of TJ genes in bovine mammary glands for experiments 1 and 2, as described in section 3.2.4.

A



B

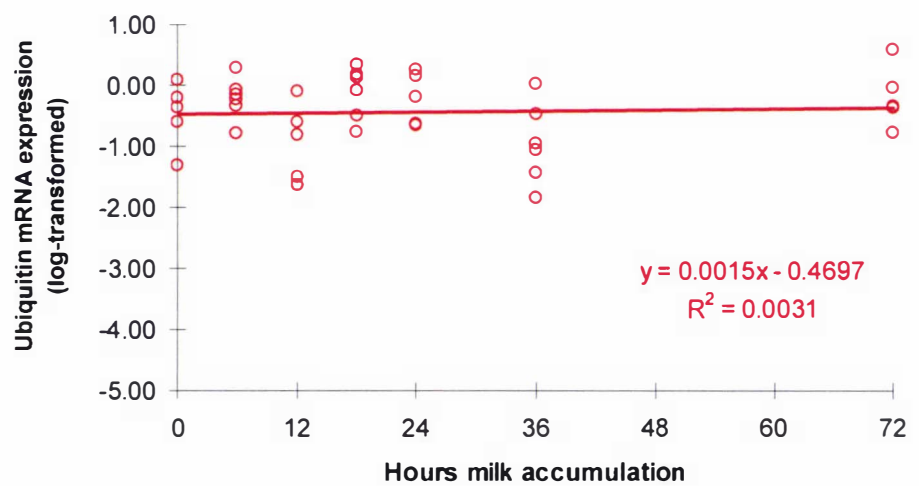


Figure 12.1. Evaluation of β-actin and ubiquitin mRNA expression for use as endogenous controls during the engorgement of bovine mammary glands.
Changes in mRNA levels of the housekeeping genes, β-actin and ubiquitin, were compared in alveolar mammary tissue of lactating cows at 0, 6, 12, 18, 24, 36 and 72 h following the last milking (n=≥ 5 per time point). Data are presented as the relative amounts of log-transformed β-actin (**A**) and ubiquitin (**B**) mRNA expression for individual cows and the corresponding linear regression equations across time.

12.1.2 Rat models of mammary engorgement

12.1.2.1 Time course of changes in mRNA expression of β-actin and ubiquitin during rat mammary engorgement

The mRNA expression of β-actin and ubiquitin were compared during the engorgement of rat mammary glands (refer to Chapter 4 for experimental design). Both β-actin and ubiquitin had strong mRNA expression in lactating rat mammary tissue, although levels were slightly higher for ubiquitin (Fig. 12.2A). Furthermore, ubiquitin was more evenly expressed across the time course of mammary engorgement than β-actin (Fig. 12.2A and B). Levels of β-actin mRNA were increased ($P<0.01$) in engorged glands compared with their suckled controls by ~3.0-fold at 18 h and 36 h following teat sealing (Fig. 12.2B). Fitting a linear regression line to the data showed that the slope for engorged glands increased ($P<0.001$) from 0 h to 36 h, whereas the slope for control glands remained flat with no differences ($P>0.05$) between time points (Fig. 12.2A). In contrast, ubiquitin mRNA expression was not significantly different ($P>0.05$) between control and engorged glands, with slopes of approximately zero for both treatments (Fig 12.2A and B). Therefore, ubiquitin was used to normalise β-actin mRNA expression, which increased ($P<0.001$) 1.9-fold by 18 h, 2.0-fold by 24 h and 2.6-fold by 36 h following teat sealing in engorged glands compared with controls (Fig. 12.2C). This result agrees with previous reports that mouse mammary β-actin mRNA was increased within 12 to 24 h of involution following pup removal (Wiens *et al.*, 1992). Consequently, ubiquitin was chosen to normalise the expression of TJ genes of interest following real-time PCR as described in section 4.2.4.

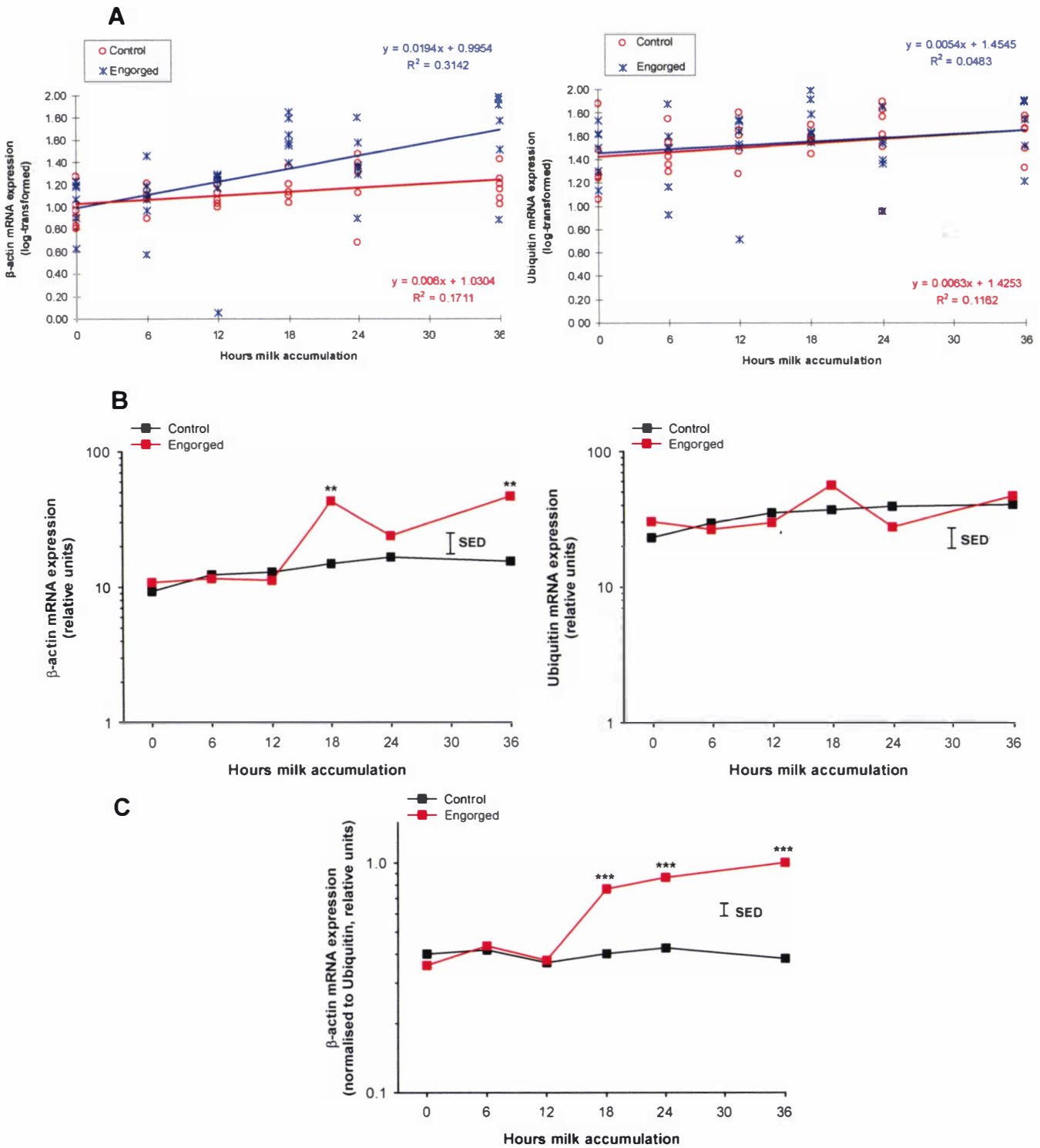


Figure 12.2. The pattern of β-actin and ubiquitin gene expression during the engorgement of rat mammary glands.

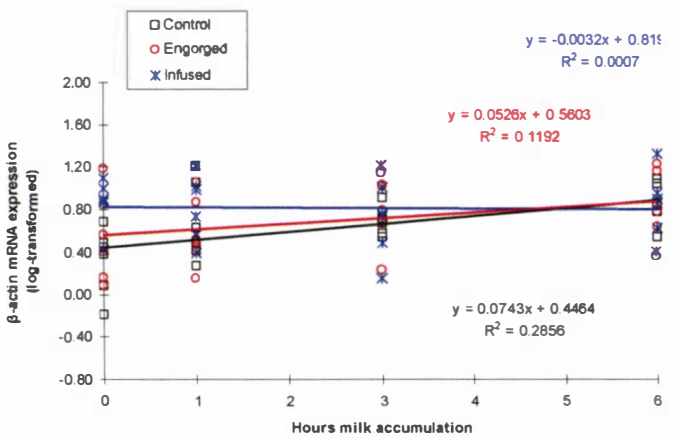
Changes in mRNA levels of the housekeeping genes, β-actin and ubiquitin, were compared in suckled (control) and engorged glands of animals at 0, 6, 12, 18, 24, and 36 h following teat sealing (n=6 per time point). Data are presented as: (**A**) the relative amounts of log-transformed β-actin and ubiquitin mRNA expression for the control and engorged glands of each animal and the corresponding linear regression equations for each treatment across time, (**B**) back-transformed means of β-actin and ubiquitin mRNA expression (relative units) and (**C**) back-transformed means of β-actin normalised to ubiquitin mRNA expression (relative units) with the SED to compare control and engorged glands at each time point (** $P<0.01$, *** $P<0.001$). There were no differences ($P>0.05$) in β-actin and ubiquitin mRNA expression between time points for control glands.

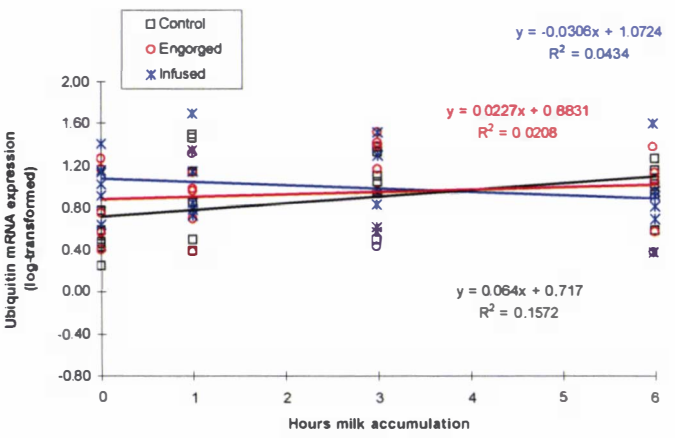
12.1.2.2 Time course of changes in mRNA expression of β-actin and ubiquitin following acute physical distension of rat mammary glands

The patterns of β-actin and ubiquitin mRNA expression following induced physical distension of rat mammary glands (refer to Chapter 5 for experimental design) were compared to select a suitable endogenous control during real-time RT-PCR. Levels of β-actin mRNA were higher at 0 h in infused glands compared with control ($P<0.01$) and engorged ($P<0.05$) glands (Fig. 12.3B). Ubiquitin mRNA expression followed a similar pattern, with lower amounts in control glands than engorged ($P<0.1$) and infused glands ($P<0.01$) at 0 h (Fig. 12.3 B). However, there were no differences ($P>0.05$) between treatments at 1, 3 and 6 h for β-actin and ubiquitin mRNA expression. Furthermore, in control glands both β-actin and ubiquitin mRNA levels were lower at 0 h than 1 h ($P<0.1$ and $P<0.05$, respectively), 3 h ($P<0.05$ and $P<0.01$, respectively) and 6 h ($P<0.01$ and $P<0.05$, respectively), but no other differences ($P>0.05$) were detected between time points for engorged and infused glands (Fig. 12.3B). These results suggest that the decreases at 0 h in control glands were due to a lower amount of starting cDNA rather than an actual treatment effect. This was confirmed when levels of β-actin mRNA were normalised to ubiquitin mRNA, as no differences ($P>0.05$) were detected between or within treatments during the course of the experiment (Fig. 12.3C). Therefore, although regression trends across treatments were not dramatically different between β-actin and ubiquitin mRNA levels (Fig. 12.3A); ubiquitin was chosen as a more suitable endogenous control due to prior reports of β-actin mRNA expression increasing during rodent mammary involution (Wiens *et al.*, 1992; section 12.1.2.1).

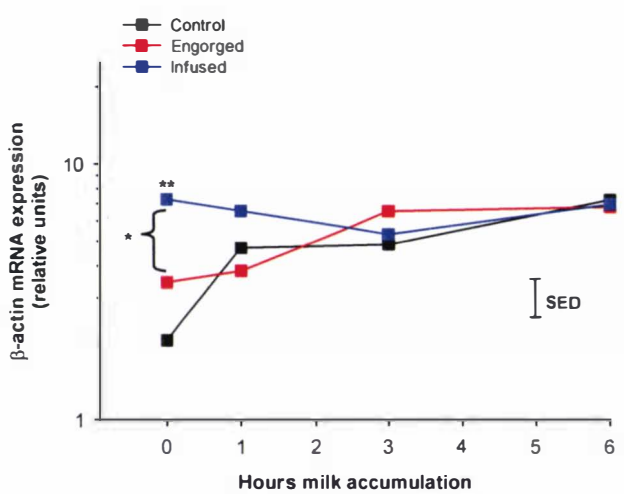
Figure 12.3. The pattern of β-actin and ubiquitin gene expression following the acute physical distension of rat mammary glands.
Changes in mRNA levels of the housekeeping genes, β-actin and ubiquitin, were compared in suckled (control), teat-sealed (engorged) and acutely distended (infused) glands of animals at 0, 1, 3, and 6 h (n=6 per time point) following teat sealing. Data are presented as: (**A**) log-transformed β-actin and ubiquitin mRNA expression (relative units) for the control, engorged and infused glands of each animal and their corresponding linear regression equations, (**B**) back-transformed means of β-actin and ubiquitin mRNA expression (relative units) and (**C**) back-transformed means of β-actin normalised to ubiquitin mRNA expression (relative units) with the SED to compare control, engorged and infused glands at each time point (⁺ $P<0.1$, * $P<0.05$, ** $P<0.01$ relative to control glands, { = P-value significant for infused glands relative to engorged glands). There were no differences ($P>0.05$) in β-actin normalised to ubiquitin mRNA expression between time points for control glands. However, both β-actin and ubiquitin uncorrected mRNA levels were lower at 0 h than 1 h ($P<0.1$ and $P<0.05$, respectively), 3 h ($P<0.05$ and $P<0.01$, respectively) and 6 h ($P<0.01$ and $P<0.05$, respectively), with no other differences ($P>0.05$) between time points for control glands.

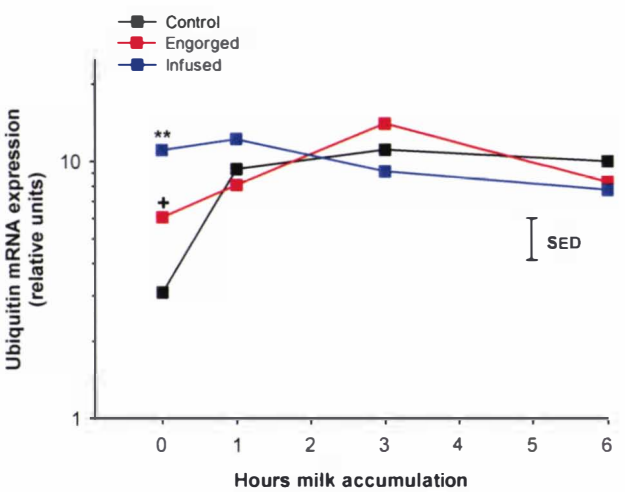
A


Control
Engorged
Infused
y = -0.0032x + 0.819
R² = 0.0007
y = 0.0526x + 0.5603
R² = 0.1192
y = 0.0743x + 0.4464
R² = 0.2856
β-actin mRNA expression (log-transformed)
Hours milk accumulation

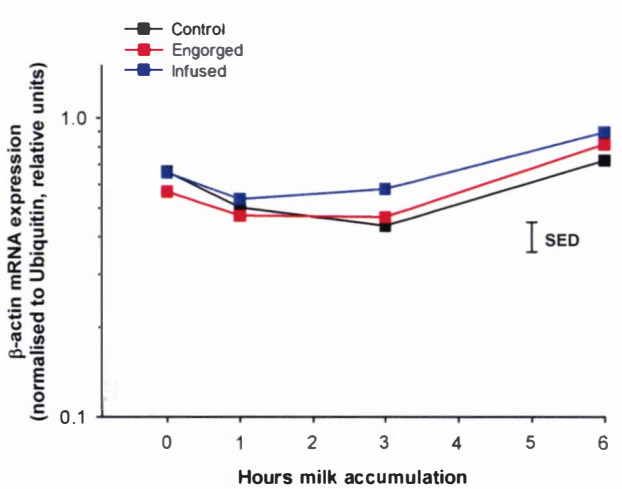

Control
Engorged
Infused
y = -0.0306x + 1.0724
R² = 0.0434
y = 0.0227x + 0.8831
R² = 0.0208
y = 0.064x + 0.717
R² = 0.1572
Ubiquitin mRNA expression (log-transformed)
Hours milk accumulation

B


Control
Engorged
Infused
β-actin mRNA expression (relative units)
SED
Hours milk accumulation


Control
Engorged
Infused
Ubiquitin mRNA expression (relative units)
SED
Hours milk accumulation

C


Control
Engorged
Infused
β-actin mRNA expression (normalised to Ubiquitin, relative units)
SED
Hours milk accumulation

APPENDIX V

The Relationships between Apoptosis and Milk Protein Gene Expression during Bovine Mammary Engorgement

The relationships between the number of apoptotic cells and the mRNA expression of major milk proteins and immune-associated genes, as analysed by northern blotting, during bovine mammary engorgement are presented in Figures 13.1 and 13.2, respectively (refer to Chapter 3 for further details). The mRNA expression levels of α-lactalbumin and α-S1-casein were inversely associated with the number of ISEL nuclei per alveolus ($r = -0.64$, $P<0.001$; $r = -0.71$, $P<0.001$, respectively) and the qualitative score of apoptosis ($r = -0.48$, $P<0.05$; $r = -0.53$, $P<0.01$, respectively) (Fig. 13.1A and B). MSAA mRNA expression was positively correlated to the number of ISEL nuclei per alveolus ($r = 0.76$, $P<0.001$) and the qualitative score of apoptosis ($r = 0.46$, $P<0.05$) (Fig. 13.2A). Lactoferrin mRNA expression was also positively associated with the number of ISEL nuclei per alveolus ($r = 0.46$, $P<0.05$), but was not significantly correlated to the qualitative score of apoptosis ($r = 0.24$, $P>0.05$) (Fig. 13.2B). Correlations using the real-time RT-PCR generated mRNA expression of these genes gave similar results (refer to Fig. 3.9 and Fig 3.10, Chapter 3).

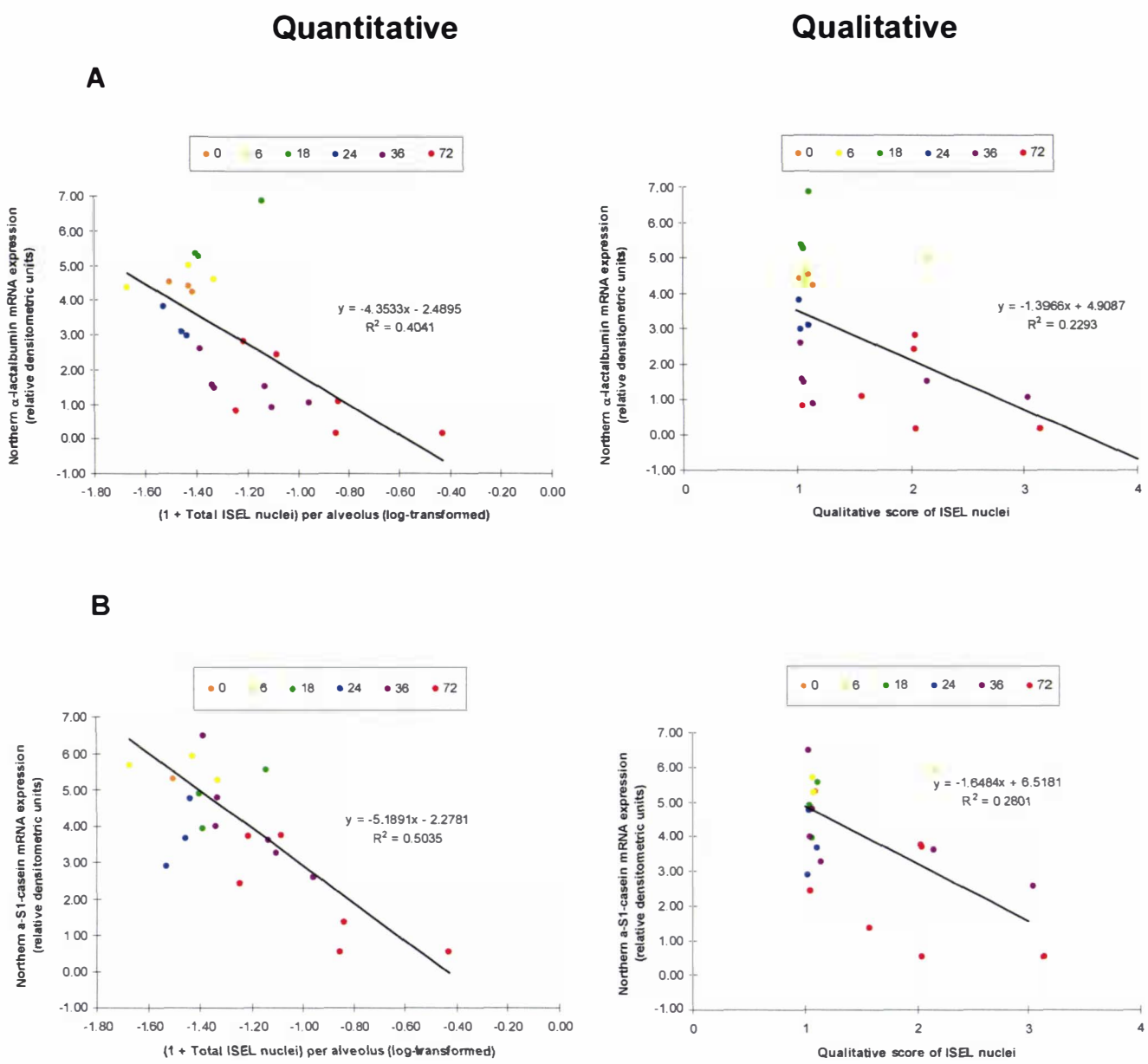


Figure 13.1. The relationship between the level of apoptosis and northern milk protein mRNA expression during the engorgement of bovine mammary glands.

(**A**) α-lactalbumin and (**B**) α-S1-casein mRNA expression levels are negatively correlated with quantitative and qualitative analyses of apoptosis. Milk protein mRNA expression data were analysed by northerns and are expressed as relative densitometric units for each animal at 0, 6, 18, 24 h (n=3 per time point) and at 36 and 72 h (n=6 per time point) following the last milking. For analysis of *in situ* end-labelling (ISEL), data for each animal are expressed as either: the qualitative score of the level of apoptosis on a scale of 1 to 5 where; 1 = none to low, 2 = low to moderate, 3 = moderate to high, 4 = high and 5 = very high numbers of positive ISEL nuclei, or the $\log_{10}$-transformed mean number of total 1+ ISEL nuclei per alveolus. The qualitative score of ISEL nuclei are graphed with jitter to distinguish between data points.

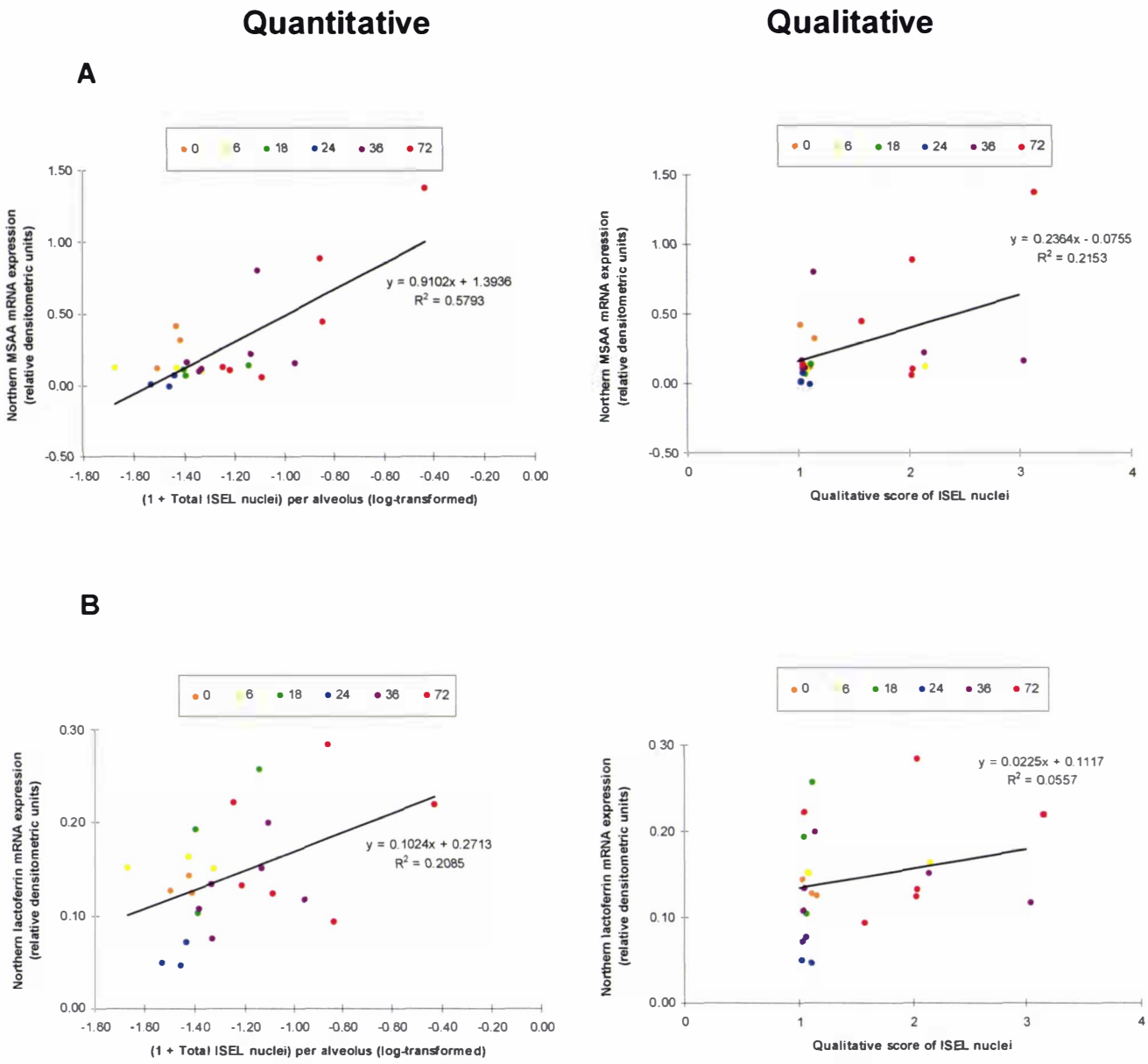


Figure 13.2. The relationship between the level of apoptosis and northern immune-associated gene expression during the engorgement of bovine mammary glands.
(**A**) mammary serum amyloid A3 (MSAA) and (**B**) lactoferrin mRNA expression levels are positively correlated with quantitative and qualitative analyses of apoptosis. Immune-associated gene expression data were analysed by northerns and are expressed as relative densitometric units for each animal at 0, 6, 18, 24 h (n=3 per time point) and at 36 and 72 h (n=6 per time point) following the last milking. For analysis of *in situ* end-labelling (ISEL), data for each animal are expressed as either: the qualitative score of the level of apoptosis on a scale of 1 to 5 where; 1 = none to low, 2 = low to moderate, 3 = moderate to high, 4 = high and 5 = very high numbers of positive ISEL nuclei, or the $\log_{10}$-transformed mean number of total 1+ ISEL nuclei per alveolus. The qualitative score of ISEL nuclei are graphed with jitter to distinguish between data points.

APPENDIX VI

Publications Arising from this Work

14.1 LIST OF PUBLICATIONS

14.1.1 Refereed papers

Cooper, C. V., Stelwagen, K., Singh, K., Farr, V. C., Prosser, C. G., and Davis, S. R. (2004): Expression of the tight junction protein zonula occludens-1 during mammary engorgement. *Proceedings of the New Zealand Society of Animal Production* **64**, 43-47.

Singh, K., Dobson, J., Phyn, C. V. C., Davis, S. R., Farr, V. C., Molenaar, A. J., and Stelwagen, K. (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. *Livestock Production Science* **98**, 67-78.

14.1.2 Conference abstracts

14.1.2.1 Oral Presentations

Cooper, C. V., Stelwagen, K., McMahon, C. D., Singh, K., Farr, V. C., and Davis, S. R. (2003): Tight junction (TJ) protein expression during engorgement of rat and bovine mammary glands. *Journal of Dairy Science* **86 (Supplement 1)**, 33 (Abstract # 131).

Cooper, C. V., Stelwagen, K., Singh, K., Farr, V. C., Prosser, C. G., and Davis, S. R. (2004): Expression of tight junction proteins during bovine mammary engorgement. *The 14th Annual Queenstown Molecular Biology Meeting, 28 Nov – 1 Dec 2004, Queenstown, New Zealand*. Abstract # 22.

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14.1.2.2 Poster Presentations

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