

New Zealand Society of Animal Production online archive

This paper is from the New Zealand Society for Animal Production online archive. NZSAP holds a regular annual conference in June or July each year for the presentation of technical and applied topics in animal production. NZSAP plays an important role as a forum fostering research in all areas of animal production including production systems, nutrition, meat science, animal welfare, wool science, animal breeding and genetics.

An invitation is extended to all those involved in the field of animal production to apply for membership of the New Zealand Society of Animal Production at our website www.nzsap.org.nz

[View All Proceedings](#)

[Next Conference](#)

[Join NZSAP](#)

The New Zealand Society of Animal Production in publishing the conference proceedings is engaged in disseminating information, not rendering professional advice or services. The views expressed herein do not necessarily represent the views of the New Zealand Society of Animal Production and the New Zealand Society of Animal Production expressly disclaims any form of liability with respect to anything done or omitted to be done in reliance upon the contents of these proceedings.

This work is licensed under a [Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International License](http://creativecommons.org/licenses/by-nc-nd/4.0/).



You are free to:

Share— copy and redistribute the material in any medium or format

Under the following terms:

Attribution — You must give [appropriate credit](#), provide a link to the license, and [indicate if changes were made](#). You may do so in any reasonable manner, but not in any way that suggests the licensor endorses you or your use.

NonCommercial — You may not use the material for [commercial purposes](#).

NoDerivatives — If you [remix, transform, or build upon](#) the material, you may not distribute the modified material.

<http://creativecommons.org.nz/licences/licences-explained/>

Expression of milk genes in ruminant mammary tissue, is it subject to local controlling factors?

A.J. MOLENAAR, S.R. DAVIS AND R.J. WILKINS

AgResearch, Ruakura Agricultural Research Centre, Private Bag 3123, Hamilton, New Zealand.

ABSTRACT.

Previous *in situ* hybridisation studies from our laboratory have shown that expression of certain milk protein genes is very high in some areas of the mammary glands of sheep and cattle, while in other areas containing an abundance of fat globules, it is virtually zero. We wished to determine if this heterogeneity was due to local variations in the concentrations of lactogenic hormones and/or their receptors. Artificially increasing the local concentration of lactogenic hormones in the sheep udder was attempted by inserting time release capsules containing prolactin, hydrocortisone and insulin directly into the gland up to one week before sacrifice. The local concentration of prolactin, and the milk gene expression relative to controls, appeared unchanged by the implants. The immunoreactivity of prolactin and its receptor in the mammary epithelial cell cytoplasm and in particular, the nuclei, were demonstrated and appeared to vary with the secretory activity of the cell.

Keywords: Prolactin, Prolactin Receptor, Milk gene, Mammary, Sheep, α -Lactalbumin, Lactoferrin, *in situ* hybridisation, immunohistochemistry, Time release implant.

INTRODUCTION

It is well known that prolactin plays a key role in the growth, development, and differentiation of normal mammary glands (Banerjee and Vonderhaar, 1992). The aim of this experiment was to investigate whether variations in local prolactin concentrations are responsible for the observed heterogeneous pattern of milk gene expression (Molenaar *et al.*, 1991, 1992). Time release capsules containing prolactin were inserted beneath the skin of sheep udders. Later the udders were examined using *in situ* hybridisation (ISH) and immunohistochemistry (IHC) with a variety of milk gene related probes and antibodies to prolactin and prolactin receptor.

METHODS

Time release capsules manufactured by Innovative Research of America, Ohio, USA each containing 1 mg prolactin, 5 mg hydrocortisone and 5 mg insulin were inserted beneath the skin and lateral suspensory ligaments of the same hemisphere of udders from 5 ewes at day 7 and again at day 3 before sacrifice. The hormones insulin and hydrocortisone, although present endogenously in sufficient amounts, were included as 'insurance' to facilitate the action of prolactin. Placebo pellets were similarly inserted in the opposite udder. The matrix of the pellets was modified to permit a maximised local release of the impregnated hormones over 60 days and to minimise the systemic effects. Two ewes were pregnant (86/150 and 141/150 days gestation), 2 were lactating (8 and 9 weeks), and one was involuting (4 days after cessation of a 56 day lactation). After humane killing, the udders were removed, perfused with 10% formalin via the pudic arteries and immersed in formalin for 1 day. A 3 mm slice, bisecting the pellets and

representing the entire gland, was taken through both sides and wax embedded.

Five μ m sections were cut, mounted onto glass slides and probed with 35 S labelled antisense cRNA for α -lactalbumin (Hurley and Schuler, 1987), α -S1-casein (Stewart *et al.*, 1984), lactoferrin (Mead and Tweedie, 1990), and selected slides with antibodies to ovine prolactin (NIDDK-anti-oPRL-IC-1) and its receptor (Okamura *et al.*, 1989). ISH was performed as previously published (Molenaar *et al.*, 1991, 1992). For IHC, a rabbit polyclonal anti-sheep prolactin and a mouse anti-rat liver prolactin receptor antibody was used at a dilution of 1/100 with the BiogenixTM (Biogenix laboratories, San Roman, CA, USA) 'supersensitive' kit. Negative controls, for both ISH and IHC, consisted of serial sections on the same slide which were either probed with 35 S labelled sense cRNA or where the primary antibody was omitted (as appropriate), and treated identically to the test sections. In each case the tissue did not stain.

RESULTS

No consistent histological nor mRNA concentration differences were observed between the test and control udders for any of the genes examined (Figures A & B), nor was lactation maintained in involuting udders (B). Prolactin levels were not found to be elevated above endogenous levels even around the sites of the implant (C). Pituitary and mammary tissue, taken from a lactating, non implanted ewe, was used for positive controls of the immunohistochemistry. Prolactin was found in the cytoplasm of the pituitary lactotrophs (D1) and in the cytoplasm and in particular, the nuclei of actively secreting mammary epithelial cells (D2). Prolactin receptors were also located in the nucleus and cytoplasm of these cells (E1), but

were not apparent in nuclei of alveoli whose lumina were packed with fat globules (ie in stasis) (Holst *et al.*, 1987). Some staining was also evident on membranes of fat globules within the lumen of the alveoli in stasis (E1, F). The intensity of staining for the prolactin receptor was greatest in active alveoli (that is alveoli with a ruffled appearance where the epithelial cells were cuboidal and columnar) (Nolin, 1979), moderate in full (distended) and lowest in alveoli in stasis (E).

FIGURE A: In situ hybridisation with ^{35}S labelled α -lactalbumin cRNA on lactating mammary adjacent to a 60 day time release implant (arrow), placed 3 days before slaughter and containing (1) 1 mg prolactin, 5 mg hydrocortisone and 5 mg insulin and (2) placebo. Magnification x 10.

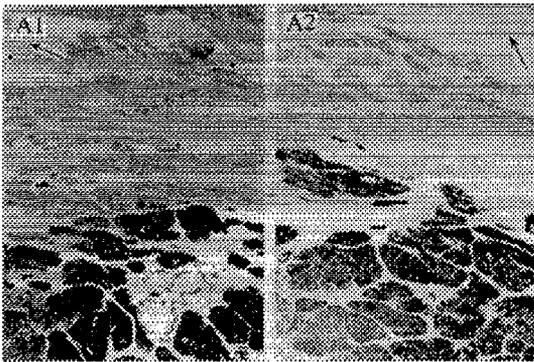


FIGURE B: In situ hybridisation with ^{35}S labelled lactoferrin cRNA on involuting mammary adjacent to an implant (arrow), placed 3 days before slaughter containing. (1) hormones and (2) placebo as described in A. Magnification x 10.

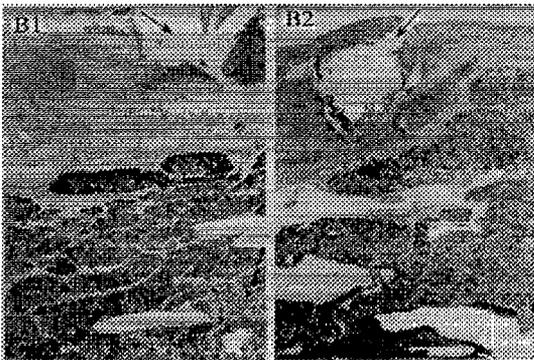


FIGURE C: Immunohistochemistry with prolactin antibody on adjacent tissue sections to those described in B and detected with diaminobenzidine. Not counterstained. Magnification x 10.

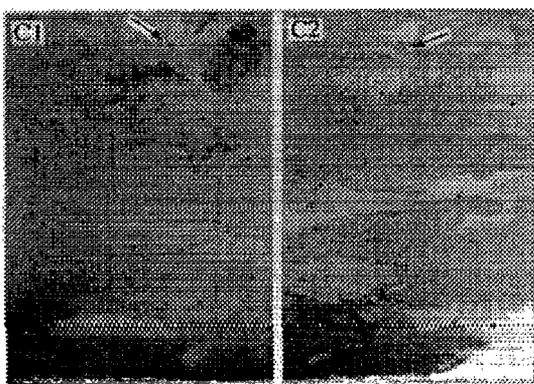


FIGURE D: Immunohistochemistry with prolactin antibody on pituitary (1) (arrow shows cytoplasmic staining) and mammary (2) from a lactating ewe (arrow shows nuclear staining) and detected with diaminobenzidine. Not counterstained. Magnification x 400.

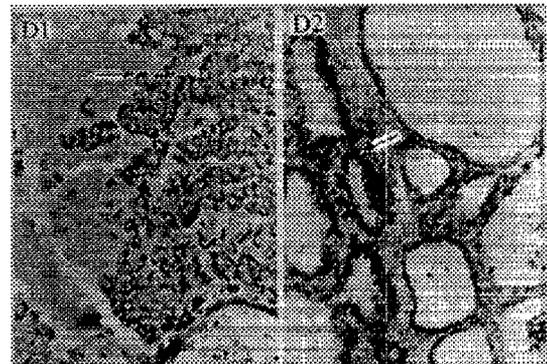


FIGURE E: Immunohistochemistry with prolactin receptor antibody on mammary from a lactating ewe. Alveoli are shown in stasis on the upper part, and active alveoli on the lower (1). The arrow shows labelling of the fat globule membrane. In (2), alveoli are shown that are active (lower) and that are engorged (upper). Detected with diaminobenzidine. Not counterstained. Magnification x 400

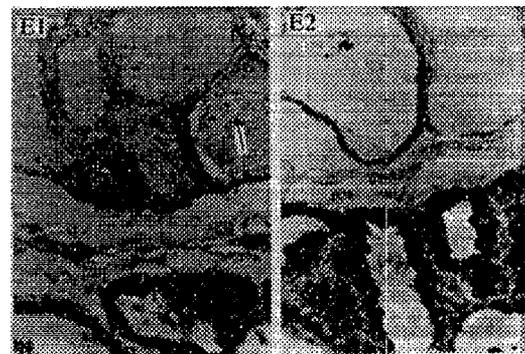
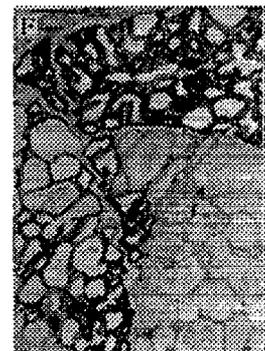


FIGURE F: Immunohistochemistry for prolactin receptor on mammary tissue from the same animal as E but in a separate experiment. Active (upper), engorged (lower left), and stasis (lower right) alveoli are shown. Detected with aminoethylcarbazole. Magnification x 100.



DISCUSSION

The regime of hormones used in this experiment is one that is commonly used to induce milk protein synthesis in cell culture (Doppler *et al* 1989) and in tissue explants (Mao and Bremel 1990, Shamay *et al.*, 1992). Prolactin impregnated pellets from the same source (Innovative Research) have

been used successfully by other investigators for different studies (Ng, et al.; 1987). This is the first known report of a study of this nature so without guidelines we decided that the most appropriate course of action was just to try the experiment using the best information available to us. It was expected that about 16 ug of prolactin would be released per day at a continuous rate but we would not be able to estimate the local concentration that would be attained. We did not know how far or how fast the hormones would travel from the implant but expected to be able to follow this immunohistochemically.

Clearly, the techniques of ISH (as shown by different hybridisation patterns with different probes) and IHC (as shown by different subcellular staining patterns between pituitary and mammary epithelial cells) worked. So it was concerning that a gradient of prolactin could not be demonstrated immunohistochemically with the reagents available at the time. This can be a trying procedure with mammary tissue due to the presence of immunoglobulins and biotin in the gland, both of which are components of the detection system. However more than 200 ng of prolactin was detected by radioimmunoassay in an overnight elution from a fresh pellet. So either the prolactin did not diffuse at all or more likely, it very rapidly entered the systemic circulation. It may be that both the techniques of IHC and ISH are too insensitive to pick up a slight difference in expression levels, or it may be that the appropriate time frame was not examined. In cell culture, prolactin elicits a 5 fold increase in protein kinase C activity (a component of the signaling pathway leading to cell division) within 5-15 minutes of exposure. By 24 hours however, this enzyme's activity diminishes to that of its control (Banerjee and Vonderhaar, 1992). Prolactin stimulates lactose biosynthesis in 4-6 hours in cultured mammary gland explants from pregnant mice due to an increase in galactosyl transferase activity. α -Lactalbumin activity is increased at 24 hours but the study was not extended beyond this time and so it was not reported if these effects were maintained (Jagoda and Rillema, 1991). Prolactin is released from the pituitary during suckling (Ostrom, 1990). It may in fact be, that the secretory activity of mammary epithelial cells is stimulated by these pulses of prolactin, and that the cells become non responsive to a prolonged increase in the basal level of prolactin. If this were true then the use of hormone capsules may not have been appropriate for this type of experiment.

The localisation of prolactin and its receptor in the cell nucleus and cytoplasm, is of interest as the mechanism of prolactin action is still unclear (Banerjee and Vonderhaar, 1992; Seddiki and Ollivier-Bousquet, 1991). It is known that prolactin binds to a receptor located in the cell membrane (Posner and Khan, 1984) and is internalised into the cell where it, and or its receptor, through different routes, affect several cellular processes including gene expression and secretion (Seddiki and Ollivier-Bousquet, 1991). Despite intensive study, a second messenger system has still not been conclusively determined. Our results indicate that one or more of prolactin effects may be mediated by the translocation of the entire prolactin/receptor complex to the cell nucleus.

In addition, it was of interest that the amount of prolactin receptor staining appears to be related to the physiological state of the mammary epithelial cells. This is consistent with

observations in rats (Nolin, 1979) where the secretory activity and histological appearance of the cells was related to the prolactin concentrations and in goats (McKinnon *et al.*, 1991) where the total prolactin receptor number was reduced in response to incomplete milking of the gland.

Biologically active prolactin is transferred to milk by a receptor mediated process (Gala *et al.*, 1980; Seddiki and Ollivier-Bousquet, 1991). The immunoreactivity we observed in the fat globule membranes implies that some prolactin receptors are located on the apical cell surface as this is the source of the fat globule membrane. It has been shown that prolactin can have an inhibitory effect in other systems such as wool growth (Allan Pearson, personal communication). It is tempting then, to speculate that among the myriad roles of prolactin (Wallis, 1988), one may be that its accumulation in the milk could exert a negative influence on milk synthesis by acting through the apical membrane, in contrast to its positive effects exerted via the basal membrane receptors.

ACKNOWLEDGEMENTS.

We are grateful to the National Hormone and Pituitary Program, University of Maryland for the gift of the prolactin antibody, to Dr Paul Kelly for the gift of the prolactin receptor antibody, to Basil Young for cutting the sections and to Warwick Donaldson for care of the animals.

REFERENCES

- Banerjee, R.; Vonderhaar, B.J. 1992. Prolactin-induced protein kinase C activity in a mouse mammary cell line (NOG-8). *Molecular Endocrinology* **90**: 61-67.
- Doppler, W.; Groner, B.; Ball, R.K. 1989. Prolactin and glucocorticoid hormones synergistically induce expression of transfected rat β -casein gene promoter constructs in a mammary epithelial cell line. *Proceedings of the National Academy of Science*. **86**: 104-108.
- Gala, R.R.; Forsyth, I.A.; Turvey, A. 1980. Milk prolactin is biologically active. *Life Science* **26**: 987-993.
- Holst, B.D.; Hurley, W.L.; Nelson, D.R. 1987. Involution of the bovine mammary gland: histological and ultrastructural changes. *Journal of Dairy Science* **70**: 935-944.
- Hurley, W.L.; Schuler, L.A. 1987. Molecular cloning and nucleotide sequence of bovine α -lactalbumin cDNA. *Gene* **61**: 119-112.
- Jagoda, C.A.; Rillema, J.A. 1991. Temporal effect of prolactin on the activities of lactose synthetase, α -lactalbumin and galactosyl transferase in mouse mammary gland explants. *Proceedings of the Society for Experimental Biology and Medicine* **197**: 431-434.
- McKinnon J.; Knight C.H.; Flint, D.J.; Wilde C.J. 1991. Effect of milking frequency and efficiency on goat mammary prolactin receptor number. *Proceedings of the British Endocrine Society April meeting*: Abstract 167.
- Mao, F.C.; Bremel, R.D. 1990. Prolactin control of α -lactalbumin and β -lactoglobulin in mammary explants of dairy heifers. In *Brief Communications of the XXIII International Dairy Congress, Montreal, October 8-12*. **54**: Abstract 338.
- Mead, P.E.; Tweedie, J.W. 1990. cDNA and protein sequence of bovine lactoferrin. *Nucleic Acids Research*. **18**: 7167.
- Molenaar, A.J.; Davis, S.R.; Wilkins, R.J. 1991. Localisation of α -lactalbumin milk gene expression in sheep mammary tissue. *1991 Proceedings of the New Zealand Society of Animal Production* **51**: 97-101.
- Molenaar, A.J.; Davis, S.R.; Wilkins, R.J. 1992. Expression of α -lactalbumin, α -S1-casein and lactoferrin genes is heterogeneous in sheep and cattle mammary tissue. *Journal of Histochemistry and Cytochemistry* **40**: 611-618.
- Ng, M.C.; Tsui, J.Y.; Merola, L.O.; Unakar, N.J. 1987. Effect of prolactin on galactose cataractogenesis. *Ophthalmic Research* **19**: 82-94.

- Nolin, J.M. 1979. The prolactin incorporation cycle of the milk secretory cycle. *Journal of Histochemistry and Cytochemistry* **27**: 1203-1204.
- Okamura, H.; Zachwieja, J.; Raguet, S.; Kelly, P.A. 1989. Characterisation and Applications of Monoclonal Antibodies to the Prolactin Receptor. *Endocrinology* **124**: 2499-2508.
- Ostrom, K.M., 1990. A review of the hormone prolactin during lactation. *Progress in Food and Nutrition Science* **14**: 1-44.
- Posner B.I.; Khan. 1983. In *Prolactin and prolactinomas*. (Eds G. Tolis, C. Stefanis, T. Mountokalakis and F. Labrie). Raven Press New York. pp 9-18.
- Seddiki, T.; Ollivier-Bousquet, M. 1991. Temperature dependence of prolactin endocytosis and casein exocytosis in epithelial mammary cells. *European Journal of Cell Biology*. **55**: 60-71.
- Shamay, A.; Pursel, V.G.; Wall, R.J.; Hennighausen, L. 1992. Induction of Lactogenesis in transgenic virgin pigs: evidence for gene and integration site-specific hormonal regulation. *Molecular Endocrinology* **6**: 191-197.
- Stewart, A.F.; Willis, I.M.; Mackinlay, A.G. 1984. Nucleotide sequences of bovine α -S1-casein and k-casein cDNAs. *Nucleic Acids Research* **12**: 3895-3907.
- Wallis, M. 1988. Mechanism of action of prolactin. In *Hormones and their Actions, Part II*. Eds B.A. Cooke, R.J.B. King and H.J. van der Molen. Elsevier Science Publishers BV. pp 295-319.