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Factors affecting the production of lactose by ovine mammary cells *in vitro*

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ABSTRACT

A method was developed to isolate mammary cell clumps (acini), from lactating sheep and maintain these in culture for more than 48 h. Performance of these cells was assessed by the measurement of lactose secreted into media using a sensitive bioluminescence assay. Lactose production was dependent upon cell density at seeding, lactose output per cell being 5-6 fold higher (6-7 fmol/cell/h) at 0.25×10^6 cells per ml medium compared to 2.5×10^6 cells per ml medium over 24 h of culture. Lactose output declined with time in culture, irrespective of cell density, falling to <1 fmol/cell/h over the period 24-48 h. 2-deoxy glucose uptake by mammary cells tended to increase with time in culture. 2-deoxy glucose uptake was significantly higher in cells from starved sheep ($p < 0.05$) and in cells cultured in medium containing foetal calf serum, insulin, hydrocortisone, prolactin and prostaglandin E_2 ($p < 0.001$).

Lactose output of acini was not affected by starvation of the ewe for 18 h before removing mammary tissue, and increased slightly (11%) at low cell density in response to the additions of hormones and/or foetal calf serum to culture medium ($p < 0.05$).

Keywords: Lactose, lactation, sheep, cell culture, hormones

INTRODUCTION

The development of a technique which allows a 'normal' rate of milk synthesis and secretion from mammary epithelial cells *in vitro* would expedite many investigations of the genetic, endocrine and biochemical control of milk synthesis and secretion.

Currently there are no techniques for mammary cell culture which maintain milk synthesis at rates approximating those *in vivo*. Of the methods available, the use of dispersed cell aggregates (acini) obtained by collagenase digestion is potentially the most productive approach. This method was assessed using acini isolated following collagenase digestion of mammary tissue from lactating goats (Hansen and Knudsen, 1991) demonstrating evidence of secretory function for several days of culture.

We have adapted this procedure to study the productivity of acini from lactating ovine mammary tissue. The objective of the work was to optimise *in vitro* performance with a view to using primary cultures for the investigation of the regulation of lactose and milk protein synthesis and secretion.

Performance of acini in the study reported below was assessed by measurement of lactose secretion by a sensitive bioluminescence assay, allowing, for the first time, the use of small samples of media (<100 μ l) for lactose determination.

MATERIALS AND METHODS

Lactating ewes, 5-8 weeks *post partum*, were used. Mammary tissue (20-30 g) was sampled immediately after slaughter of the ewe by captive-bolt gun. The sample was placed in sterile, base medium (see below) containing 5% foetal calf serum and transported to the laboratory.

Base medium composition was medium 199 with Earle's salts plus L-glutamine and 25 mM HEPES (pH 7.2) containing

5 mM sodium acetate, 26 mM NaHCO_3 , penicillin (100 iu/ml), streptomycin (0.1 mg/ml) and amphotericin (0.25 μ g/ml).

Mammary acini were prepared by the method of Hansen and Knudsen (1991). Tissue (8 g) was sliced into 1-2 g pieces and added to 15 ml base medium containing 10% foetal calf serum (Life Technologies Inc., Auckland) and 1000 u/ml collagenase (Sigma Chemical Co., St Louis, Mo., USA).

The digestion medium was injected repeatedly into the tissue pieces until the tissue was swollen to approximately double its original size. The distended tissue and remaining digest medium were incubated at 37°C for 90 min. on a rotary shaker in 25 ml of base medium with 10% foetal calf serum.

Single cells and acini were separated from larger tissue pieces by filtration through coarse nylon mesh. The filtrate was centrifuged (200 g for 1 min.) and cells collected and washed in base medium. The mixture of acini and single cells were enriched for acini by filtration of the mixture through 50 μ m nylon mesh. Following gentle disruption of cell clumps by vortex mixing, total cell numbers were determined in a haemocytometer (crystal violet stain) and total live cells by trypan blue exclusion.

Cells were cultured for up to 48 h in 24-well culture dishes coated with 'Matrigel' (Integrated Sciences Pty Ltd, Crows Nest, NSW, Australia) in 1 ml base medium with or without the addition of hormones (insulin, 1 μ g/ml; hydrocortisone, 1 μ g/ml; prolactin, 1 μ g/ml; prostaglandin E_2 , 500 ng/ml and 10% foetal calf serum). Medium was changed after 24 h of culture. Seeding density was varied from 0.25 to 5×10^6 cells per well and treatments applied to wells in triplicate.

Lactose Determination

Lactose content of the culture medium was determined by a bioluminescence assay based on the method of Arthur *et*

al., (1989) following deproteinisation of samples with $\text{Ba}(\text{OH})_2/\text{Zn}(\text{OH})_2$. Bioluminescence was measured in a Perkin-Elmer LS-50 spectrometer. Assay sensitivity was 5 μM for lactose.

Glucose Transport

The ability of mammary cells to take up 2-deoxy glucose during culture was assessed using acini cultured on 'Matrigel' at 0, 2, 24 and 48 h. The cultures were carried out in medium 199 (in triplicate) with and without hormones as described above and at a concentration of 2.5×10^6 cells per well. 2-deoxy glucose uptake was determined after incubation of acini for 20 minutes in base medium containing 4 μCi [^3H] - 2-deoxy glucose (Amersham) 1% bovine serum albumin (Sigma Chemical Co.) and 0.5 mM 2-deoxy glucose. Transport was stopped by removing the media and washing the cells with 2.0 ml ice-cold phosphate buffered saline four times at 2 minute intervals. Cells were solubilised in 1.0 ml of 0.1 M NaOH, and transferred to a vial containing 10 ml scintillation fluid ('Ready Safe', Beckman Instruments, Inc., Fullerton, CA., USA) and counted in a liquid scintillation counter.

Statistical Analysis

Treatment effects were assessed by Analysis of Variance using GENSTAT.

RESULTS

The digestion method described gave an adequate yield of acini (approximately 2×10^7 cells per gramme tissue) of high viability (ca. 90% by trypan blue exclusion). Time from tissue collection to culture was 3.5-4 h including a 90 min. period of collagenase digestion.

With acini cultured at 2.5×10^6 cells per ml, lactose secretion into the medium declined from around 3 fmol/h during the first 6 h of culture to about 0.5 fmol/h during the period of 30-48 h in culture (Fig. 1).

Lactose production was slightly, but not significantly ($p > 0.05$) greater in cells harvested from ewes starved for the previous 24 h (Fig. 1). There was no effect of the presence of foetal calf serum and hormones on the level of lactose output to 48 h of culture (Fig. 1).

There was a strong effect of cell density on lactose output, lactose production per cell being 5-6 fold lower over the first 24 h of culture in cells plated at 2.5×10^6 per ml when compared to a plating density of 2.5×10^5 cells per ml (Fig. 2). There was a consistent 9-11% enhancement of lactose output in media containing hormones and foetal calf serum up to a cell density of $1.5 \times 10^6/\text{ml}$ ($p < 0.05$; Fig. 2). There was no effect of previous nutritional status of the ewe on lactose output of mammary cells at any cell density and data from fed and starved animals were therefore combined for Fig. 2.

Lactose output over 48h of culture at 2.5×10^5 and 2.5×10^6 cells per ml is shown in Fig. 3. Lactose output at low cell density was greater in the medium containing hormones but this effect did not achieve statistical significance ($p > 0.05$; $n=3$). Lactose output in the period 24-48 h was < 1 fmol/h at both cell densities. Lactose output over 24-48 h at low cell

FIGURE 1: Time course of lactose production (fmol/cell/h) of ovine mammary acini in a culture. Cells were taken from fed (solid bars) or starved (shaded bars) sheep (18 h; $n=4$ for each group) and cultured in medium 199 as described. Seeding density was 2.5×10^6 cells per ml medium. Differences in production of cells from fed and starved sheep were not statistically significant. There was no effect of hormonal supplement of media on lactose production and data for control and hormonal treatments were therefore combined. Error bars are standard errors of mean.

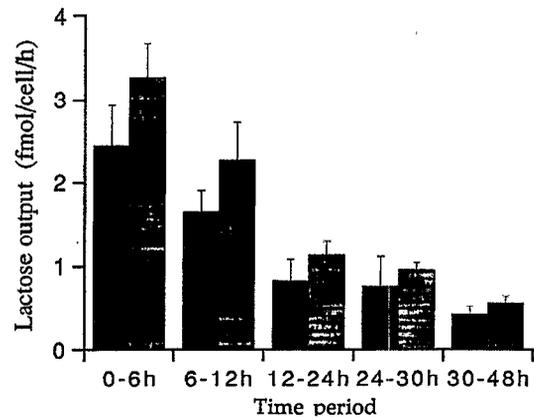
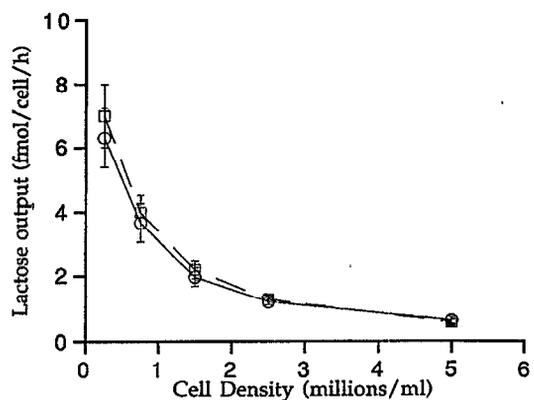


FIGURE 2: Effect of cell density on lactose production (fmol/cell/h) of lactating ovine mammary acini over 0-24 h of culture. As there was no effect of previous nutritional treatment of the ewe on lactose output at any cell density, data from fed and starved sheep were combined. Difference in lactose output of cells seeded up to $1.5 \times 10^6/\text{ml}$ in medium containing (see text; open squares) or with no additions (open circles) was statistically significant by analysis of variance ($p < 0.05$). Error bars are standard error of mean ($n=8$).



density showed more than 6-fold reduction relative to the first measurement period, but was still at a level similar to that shown by the cells at high cell density over the first 24 h.

Glucose Transport

Glucose transport measured by 2-deoxy-glucose uptake into mammary cells was measured at a cell density of 2.5×10^6 cells per ml. Cells from fed sheep showed significantly ($p < 0.05$) lower rates of glucose transport than cells from starved sheep and both cell sources showed increased glucose transport ($p < 0.001$) following incubation with medium containing hormones and foetal calf serum (Fig. 4).

Glucose transport increased in all treatments up to 24 h of culture and was maintained to 48 h (Fig. 4).

FIGURE 3: Time course of lactose production over 48 h of culture by ovine mammary acini seeded at 0.25×10^6 cells per ml in medium with (dark stipple bar) or without (solid bar) hormones or at 2.5×10^6 cells per ml with (open bar) or without (light stipple) hormones. Effects of hormonal treatment over the first 24 h of culture in this case were not statistically significant ($p > 0.05$; $n = 3$). Error bars are standard error of mean.

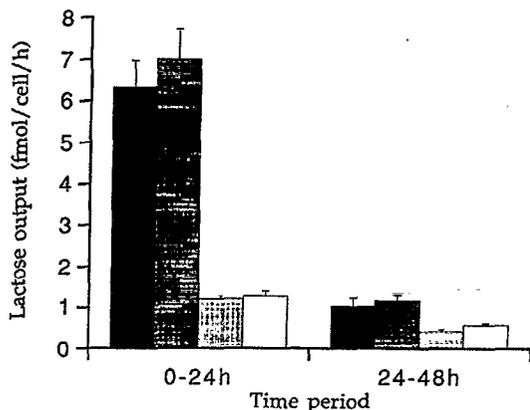
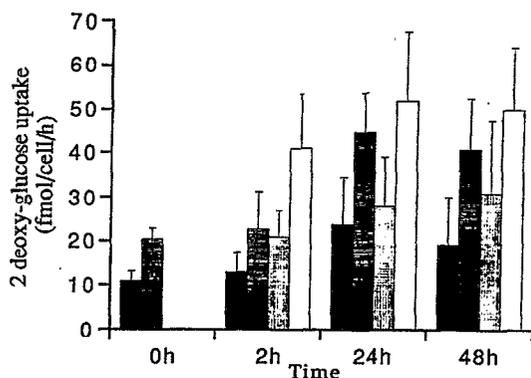


FIGURE 4: Uptake of 2-deoxy glucose by lactating ovine mammary cells from fed or starved sheep following culture up to 48 h in media with and without hormones. Uptake of [^3H] 2-deoxy glucose was measured after 20 min. incubation in control media. Legend is; solid bar, fed control media; dark stipple, starved control; light stipple fed plus hormones; open bar, starved plus hormones. Differences according to nutritional treatment of ewe ($p < 0.05$) and hormonal content of media ($p < 0.05$) were statistically significant. Error bars are standard error of mean.



DISCUSSION

A method has been developed which allows rapid preparation of acini from ovine mammary tissue with adequate yield and >90% viability. The cells, when plated at low density, produce lactose over the first 24 h of culture at a rate (5-10 fmol/cell/h) lower than estimates of *in vivo* productivity (10-40 fmol/cell/h) in ruminants (Larson, 1976). Productivity of cells over the first 6 h of culture at low cell density was higher at 10-20 fmol/cell/h (data not shown). The cells also showed responsiveness to media containing hormones plus foetal calf serum especially at low cell density. Responses were observed for both glucose uptake and lactose output.

The time course of the decline in lactose output was similar to that reported by Rao *et al.*, (1972) who also showed a similar relationship between cell density and cellular production of lactose in bovine mammary cells in disperse culture. These authors also noted that the effect of cell density

on lactose output and the loss of lactose output with time were independent processes, an observation that is supported by the present data (Fig. 3).

The decline in lactose output with cell density is currently unexplained. Rao *et al.*, (1972) could not find any evidence that the temporal decline in lactose output was due to any limitation in substrate supply. We have examined the effect of media changes every 3 h and shown no effect of this treatment on the lactose output of cells cultured at 2.5×10^6 cells per ml, (data not presented) supporting the concept that the inhibition is not arising through inadequate supply of substrate.

Milk yield of ruminants declines by over 50% during short-term starvation (eg. Linzell, 1967) yet the lactose output of mammary acini in culture was unaffected by such pre-treatment of the ewe. A similar observation has been made with acini from the mammary glands of starved, lactating rats (Wilde and Kuhn, 1979). These data support the notion that the impairment of milk production during short-term starvation is through an active inhibition of milk production rather than through any reduction in mammary synthetic capacity.

Mammary glucose transport was enhanced in starved sheep (relative to fed sheep) and was also enhanced by the presence of the foetal calf serum/hormone combination. The rate of glucose uptake was more than adequate to account for lactose output by the mammary cells at all stages of culture. The maintenance of mammary glucose uptake by the cells over 48 h of culture was indicative of the maintenance of cell viability although the decline in lactose output and a concomitant increase in lactate production (data not shown) indicated a change in mammary glucose metabolism as the cells lost their lactational ability.

In conclusion, methods have been developed to prepare, culture and monitor the productivity of lactating, ovine mammary acini *in vitro*. Culture conditions need to be optimised, particularly the nature of the effect of cell density on cell productivity. Procedures which might improve the long-term maintenance of lactating acini in culture will require a methodical dissection of the hormonal and morphological requirements of lactating cells to synthesise and secrete milk constituents.

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