Bioluminescent Determination of Lactose Secretion: A Measure of the *In Vitro* Performance of Mammary Acini from Lactating Rats

B. H. Choi, K. W. Stewart[1], S. R. Davis[2] and K. H. Myung*

Department of Animal Science, Chonnam National University, Kwangju 500-757, Korea

ABSTRACT : A culture system for lactating rat mammary acini was evaluated, where the primary indicator of performance was lactose secretion, measured by a sensitive bioluminescence assay. Lactose secretion was reduced by half ($p<0.01$) over the first 6 h of culture by overnight feed withdrawal (FW) from tissue donors but was sensitive to increased glucose concentration in the culture media ($p<0.001$) up to 30 mM. Lactose production of cells from fed donors over the first 6 h in culture in 30 mM glucose was 8.9 fmol/cell/h – a rate calculated to be about half that *in vivo*. No significant difference was shown in lactose secretion by cells from fed or FW rats over 6-24 h. Lactose secretion was 3.6 fmol/cell/h by cells from fed animals in 40 mM glucose concentration media over the 6-24 h culture period. Addition of insulin to the culture media had no effect on rates of lactose secretion while addition of prolactin and hydrocortisone, with or without insulin, significantly ($p<0.001$) decreased lactose production over both 0-6 h and 6-24 h culture periods. Lactose synthesis in vitro was significantly enhanced by aeration of the media during collagenase digestion of mammary tissue ($p<0.05$). No improvement in lactose secretion was effected by shaking of cells during culture, Matrigel coating of culture dishes or change in cell density over a range up to 2.5 million cells per ml. ***(Asian-Aust. J. Anim. Sci. 2002. Vol 15, No. 2 : 274-278)***

Key Words : Lactose, Lactation, Mammary, Cell Culture, Hormones

INTRODUCTION

Throughout lactation, mammary secretory epithelial cells either synthesize or procure from the serum an impressive variety of amino acids, sugars, fats, salts, vitamins and other organic molecules which are assimilated into mammary specific proteins, sugars and fats and secreted via the apical cell surface as milk. Among the components of milk, lactose is unusual in that the mammary cell is the only animal cell that produces this disaccharide, making lactose a distinctive marker of mammary cell function. Further, lactose is a major osmotic component of milk and, hence, is a major driver of water secretion into milk.

The rate of lactose synthesis in the intact lactating rat undergoes marked variation in relation to nutritional and physiological factors (Carrick and Kuhn, 1978; Wilde and Kuhn, 1979; Bussman et al.; Ward and Kuhn, 1984). Furthermore, lactose synthesis by mammary epithelial cells from lactating animals has been shown to be difficult to maintain *in vitro* (Cline et al., 1982). Nevertheless, Foster (1978) showed rates of lactose synthesis approaching *in vivo* rates in mammary cells from rats and mice using dispersed mammary epithelial cells *in vitro*, at least over short periods of culture.

Of the methods available to provide quantifiable levels of performance *in vitro*, the use of dispersed cell aggregates obtained by collagenase digestion is potentially the most practical (Wilde and Kuhn, 1979). However, assessment of *in vitro* relative to *in vivo* performance requires the direct measurement of milk components in culture media. Measurement of lactose secretion in past studies has usually required measurement of radioactive glucose incorporation into lactose (Wilde and Kuhn, 1979). Development of bioluminescent methods for lactose measurement (Arthur et al., 1989) has allowed the adaptation of this assay for measurement of lactose in small aliquots of culture media (Davis et al., 1993). In the present paper we used a bioluminescence assay for lactose to evaluate the *in vitro* performance of mammary acini from lactating rats relative to *in vivo* function. In particular, effects of nutritional state of tissue donors, glucose availability, hormonal treatments, aeration during tissue digestion, Matrigel coating for cell attachment and cell density at seeding were examined over 24 h of culture.

MATERIALS AND METHODS

Animals

Primiparous Sprague-Dawley rats rearing litters of 10-12 pups, were used on days 12-16 of lactation. Rats normally had free access to food and water. Tissues from starved rats were taken after overnight withdrawal of food (18-20 h).

Preparation and incubation of acini

Rats were anesthetized by intraperitoneal injection of 0.35 ml (60 mg/ml) sodium pentobarbitone. Mammary tissue (approximately 2 g) from the inguinal mammary

* Corresponding Author: K. H. Myung. Tel.: +82-62-530-2122, Fax: +82-62-530-2129, E-mail: khmyung@chonnam.ac.kr

[1] The Waikato Polytechnic, Private Bag 3036, Hamilton, New Zealand.

[2] AgResearch, Ruakura Research Centre, Private Bag 3122, Hamilton, New Zealand.

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glands was removed, placed in sterile digestion medium and transported to the laboratory. Tissue was sliced into smaller pieces and added to 10 ml digestion medium (medium 199 with Earle's salts plus L-glutamine and 25 mM Hepes (pH 7.4) containing 26 mM $NaHCO_3$, penicillin (100 IU/ml), streptomycin (0.1 mg/ml), amphotericin (0.25 μg/ml), collagenase (18.5 mg) and DNAse (5 mg). 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 medium was incubated at 37°C for 70 min on a rotary shaker at 150 oscillations/min. with aeration by O_2/CO_2 (20:1) gas during digestion. The resultant suspension was then filtered through 500 μm mesh Nitex cloth. The filtrate was centrifuged (500 rpm for 3 min.) and cells collected and washed in warm culture medium (composition as digestion medium except for collagenase and DNAse)

Following gentle disruption of cell clumps by hand shaking, total cell numbers were determined in a haemocytometer following crystal violet staining and total live cells were assessed by trypan blue exclusion. The whole preparation generally took 90 min.

The digestion method described gave a good yield of acini (approximately 3×10^7 cells per gram of tissue) of high viability by trypan blue exclusion (data not shown).

Incubation of acini

Acini were cultured with or without shaking at 37°C for up to 24 h in 12-well cultures dishes with or without Matrigel coating in 0.7 ml culture medium with or without hormones (insulin, 10 μg/ml; hydrocortisone, 1 μg/ml; prolactin, 3 μg/ml; all from Sigma Chemical Co., St Louis, Mo.). The glucose concentrations of the medium were adjusted from 5 to 40 mM. Seeding density was normally 0.5×10^6.

Lactose determination

Lactose content of the culture medium was determined by a bioluminescence assay by the method of Arthur et al., (1989), following deproteinization of samples with $ZnSO_4$.

Bioluminescence was measured with a Turner Designs (Sunnydale, CA) TD-20/20 luminometer. Assay sensitivity for lactose was 1 μM.

Statistical analysis

Treatment effects were assessed by one-way analysis of variance using SAS (1997; Cary, North Carolina, SAS Institute).

RESULTS

Effects of glucose concentration in vitro and nutritional status of tissue donor

Acini showed a linear increase in lactose secretion with increasing glucose concentration (up to a maximum at 30 mM) for cells from both fed and starved rats ($p<0.001$) over 0-6 h of culture (figure 1). The cells from fed rats produced significantly ($p<0.01$) more lactose than cells from starved rats over 0-6 h of culture. The least square means for 'fed' and 'starved' cells were 7.3 and 3.8 fmol/cell/h, respectively and the standard error of the difference between them was 1.2 (figure 1). Lactose secretion at 5 mM glucose concentration was 5.0 fmol/cell/h while at 30 mM glucose, lactose secretion was 8.9 fmol/cell/h (figure 1).

The same trends were apparent in lactose secretion over 6-24 h of culture except that differences in lactose production between cells from fed and starved rats were not statistically significant ($p>0.05$; figure 2). Lactose secretion rates were significantly ($p<0.001$) lower in the 6-24 h period. Lactose secretion rates of cells from fed rats for 0-6 h and 6-24 h culture periods were 7.4 and 2.6 fmol/cell/h, respectively.

Hormonal and matrigel effects

Prolactin, hydrocortisone and the combination of all three hormones significantly ($p<0.001$) inhibited lactose secretion in 30 mM glucose concentration media over the 0-6 h culture period (figure 3). Insulin alone (10 μg/ml) had no effect on lactose secretion (figure 3). Once again there was a significant difference ($p<0.001$) between 0-6 h and 6-24 h culture periods in lactose production, irrespective of

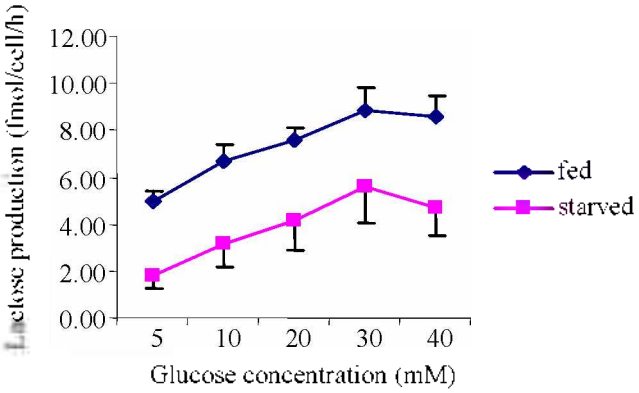

Figure 1. Effects of glucose concentration and nutritional state of tissue donors on lactose production of lactating rat mammary acini over 0-6 h of culture. Cells were taken from fed and starved (18 h feed withdrawal) rats (n=7 for each group) and cultured in medium 199 with shaking, as described in Methods section. Seeding density was 0.5×10^6 cells per 0.7 ml medium. There was a statistically significant increase in lactose secretion with increasing glucose concentration ($p<0.001$) and a significant difference of in vitro lactose output between fed and feed withdrawal rats (18 h) ($p<0.01$). Error bars are standard errors of mean.

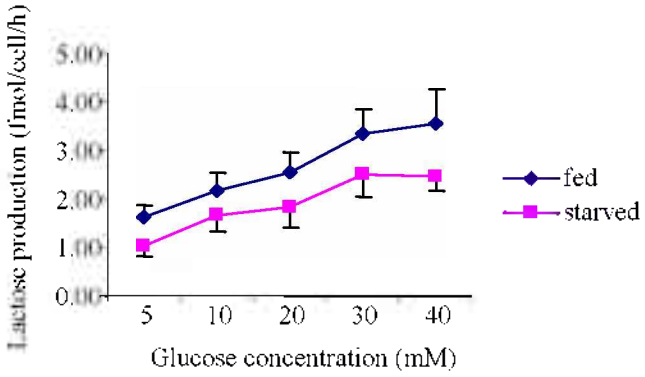

Figure 2. Effects of glucose concentration and nutritional state of tissue donors on lactose production of lactating rat mammary acini over 6-24 h of culture. Cells were taken from fed and starved (18 h feed withdrawal) rats (n=7 for each group) and cultured in medium 199 with shaking, as described in Methods section. Seeding density was 0.5×10^6 cells per 0.7 ml medium. There was a statistically significant increase in lactose secretion with increasing glucose concentration ($p<0.001$) but differences between fed and feed withdrawal rats (18 h) were not significant ($p>0.05$). Error bars are standard errors of mean.

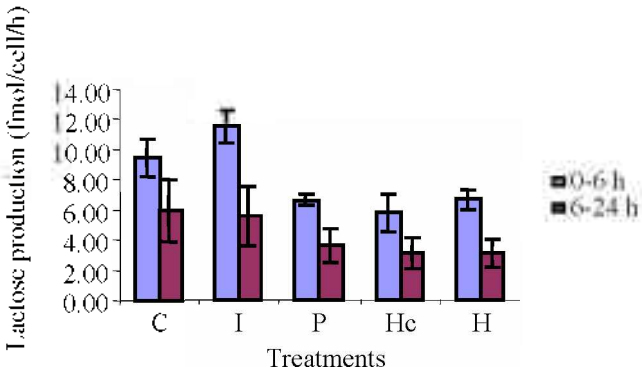

Figure 3. Effects of hormone treatment and time course on lactose production of lactating rat mammary acini cultured in 4 mM $NaHCO_3$ 199 medium with shaking as described in Methods section. Seeding density was 0.5×10^6 cells per 0.7 ml medium. There were statistically significant reductions in lactose output associated with prolactin (P), hydrocortisone (Hc) alone and combined with insulin (I) hormone treatments ($p<0.002$) over the 0-6 h time period. Error bars are standard errors of mean.

any hormonal treatments. The least square means for lactose secretion during the 0-6 h and 6-24 h culture periods were 8.0 and 4.3 fmol/cell/h, respectively (sed 0.6: figure 3).

There was no effect of Matrigel coating during incubation on lactose secretion of cells from fed rats in 30 mM glucose concentration media during both 0-6 and 6-24 h culture periods (data not shown).

Aeration and cell density effects

Aeration with O_2/CO_2 (20:1) gas during tissue digestion significantly ($p<0.05$) increased lactose secretion by cells from fed rats in 30 mM glucose concentration media over 0-6 h culture. The least square means of oxygenated and non-oxygenated cells were 9.3 and 4.0 fmol/cell/h respectively (sed 1.2; figure 4). Shaking during in vitro culture was without effect on lactose secretion over 24 h (data not shown).

There was no effect of cell density at seeding (up to 2×10^6 per well) on lactose secretion in 30 mM glucose concentration media over a 6 h culture period (figure 5).

DISCUSSION

Development and use of a bioluminescence assay for lactose allowed assessment of the *in vitro* performance of lactating mammary cells in small (<50 µl), serially-sampled, volumes of culture media. Rates of lactose secretion in vitro were close to 9 fmol/cell/h over 0-6 h of culture and in the presence of 30 mM glucose. *In vivo*, it can be calculated that the rate of lactose synthesis is 16-20 fmol/cell/h based on milk yield of 2.4 ml/g tissue/d at 2.6% lactose (Linzell, 1972) and tissue DNA content of 3.5 mg /g tissue (Knight et al., 1984). While our measured *in vitro* rates were lower than those *in vivo*, it is likely that the observed decline in lactose secretion seen over 24 h had, in fact, begun at 0 h. Thus, over the first hour of culture, cell performance may have approached *in vivo* rates. Foster (1978) determined a rate of lactose synthesis in rat mammary acini of 18 fmol/cell/h over the initial 4 h of culture using a radioisotope method.

In vivo, there is a close dependence of lactose synthesis on food intake (Wild and Kuhn, 1979). However, Wilde and

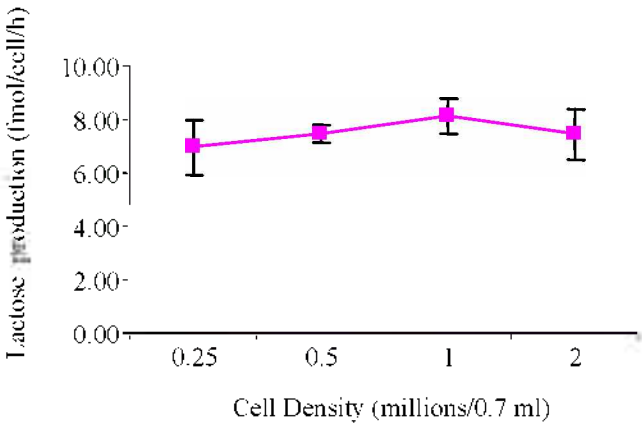

Figure 4. Effect of cell density on lactose production of lactating rat mammary acini over 0-6 h of culture. Cells were taken from fed rats (n=3 for each group) and cultured in medium 199 with shaking as described. Error bars are standard errors of mean.

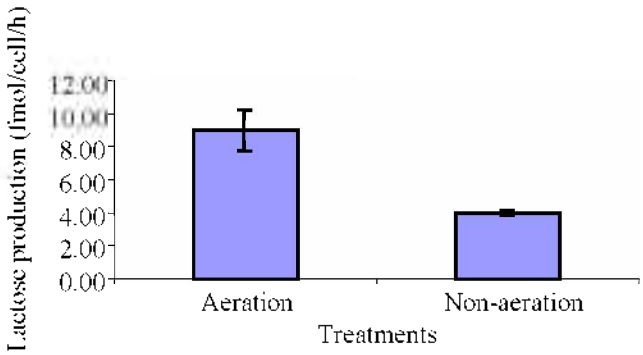

Figure 5. Effect of aeration during mammary tissue digestion on lactose production of lactating rat mammary acini in a culture. The aeration was achieved by bubbling O_2/CO_2 (20:1) gas. Cells were taken from fed rats (n=3 for each group) and cultured in as described. Seeding density was 0.5×10^6 cells per 0.7 ml medium. There was a statistically significant difference by analysis of variance between aeration and non-aeration ($p<0.05$) Error bars are standard errors of mean.

Kuhn (1979) could not establish any difference in rates of lactose synthesis *in vitro* in cells from fed and starved rats using ^{14}C-glucose incorporation to assess lactose synthesis. Our data using the bioluminescence assay show that there is, in fact, a substantial effect of overnight feed withdrawal on lactose output *in vitro* that was not remedied by the presence of insulin in the culture medium. These results suggest that the feed withdrawal treatment resulted in some impairment of the synthetic machinery of the mammary gland.

The *in vitro* data are in contrast to observations in vivo where feed withdrawal in rats caused lactose synthesis to decline to almost zero (Bussman et al., 1986). It is possible that the differences between *in vivo* and *in vitro* results stem from a metabolic inhibition of the mammary gland during feed withdrawal, which is relieved, in part, by removal of the cells to culture media. A period of 48 h of feed withdrawal results in a marked reduction in α-lactalbumin mRNA concentrations and in protein secretion rates in vitro (Geursen et al., 1987; Geursen and Grigor, 1987). Further, Grimble et al., (1987) have shown that feeding low protein diets will restrict milk volume through a mechanism that involves a reduction in α-lactalbumin expression. Thus effects of feed withdrawal on milk volume are probably mediated by impairment of lactose synthesis through restriction of α-lactalbumin availability at the Golgi. This raises a major question as to whether the effects of refeeding on lactose synthesis are mediated by insulin or in combination with another hormone. While the *in vitro* data suggest the latter the declining function of the secretory cells *in vitro* may mean that theses cells became insensitive to hormonal stimulation during preparation.

In contrast to the rat, ruminant cells in culture do not demonstrate any effect of feed withdrawal from the tissue donor on *in vitro* performance as assessed by lactose secretion (Davis et al., 1993).

Peak lactose production was achieved at a glucose concentration of 30 mM in the culture medium, suggesting that there was some impairment of glucose transport for the cells in culture. Similar improvements in performance of rodent cells in culture with increased glucose, were obtained by Cline et al. (1982) and Foster (1978). However, increased provision of glucose did not prevent a continual decline in lactose synthesis *in vitro*.

Although the lack of effect of insulin on lactose synthesis agrees with some previous observations (Carrick and Kuhn, 1978; Wild and Kuhn, 1979), the inhibition of lactose synthesis by other lactogenic hormones in this experiment was unexpected and is currently unexplained.

However, again there are conflicting data in the literature. Insulin stimulated production of lactose by rat mammary slices *in vitro* by 2.5 fold associated with an apparent shift in the NAD/NADH ratio toward a more oxidised state (Martin and Baldwin, 1971). Furthermore, it was demonstrated that in lactating mice, mammary alveoli require continued presence of either hydrocortisone or prolactin with insulin for maximal accumulation of α-lactalbumin (Cline et al., 1982). It is possible that in our experiment prolactin and hydrocortisone receptors were saturated in excised tissue because pups were not removed until the dams were anesthetised. However, the reason why the lactogenic hormones reduced lactose synthesis is still not clear. The time course of the decline in lactose production (figure 3) can be compared with an apparent sharp decrease of lactose synthesis after 24 h cultivation (Cline et al., 1982) and decreased protein synthesis after 2 h in culture (Martin and Baldwin, 1971).

Lactose production was enhanced 2.3 fold by aeration during the collagenase digestion period indicating that the mammary cells were sensitive to oxygen availability during digestion. To what extent performance is impaired in the 'aerated cells' remains unknown, suffice to say that periods of anoxia during cell washing are not easy to avoid.

The decline in lactose synthesis seen with increasing cell density at seeding with ruminant cells (Rao et al., 1975; Davis et al., 1993) was not observed in the present experiment. Davis et al. (1993) confirmed that the inhibition was not arising through inadequate supply of substrate. So, it appears that cell densities of up to 2.5 million cells per ml medium (2 cm^2 well) do not impair performance of rat mammary cells, in contrast to the sheep data.

No effect of Matrigel (a laminin-rich, mixture of extracellular matrix components) on lactose production was

observed. While attachment to a matrix such as matrigel can improve cell development and survival *in vitro* it is unlikely the culture system allowed sufficient time for cells to attach to the matrigel and enjoy the benefits of matrix attachment which have been demonstrated previously.

Ours and other studies have demonstrated the difficulties in maintaining function of lactating cells *in vitro*. While our results give pointers to some areas of performance sensitivity there still remains an underlying trend of mammary cells from lactating animals to stop lactation *in vitro*, as exemplified by the ever-declining rate of lactose synthesis. Wheeler et al. (1995) showed that with sheep mammary cells there was no evidence of RNA synthesis occurring *in vitro*, indicative of a fundamental change in the regulation of the metabolism of these cells.

In conclusion, methods have been developed to prepare, culture and monitor the productivity of lactating rat mammary acini *in vitro*. A new observation was that lactose secretion of mammary cells prepared from rats unfed for 18 h was substantially impaired. While some optimisation of culture conditions was achieved, procedures that might improve the long-term maintenance of lactating acini in culture will require methodical dissection of the hormonal and, possibly, morphological requirements of lactating cells to synthesise and secrete milk constituents. However, the bioluminescence assay for lactose provides a useful and sensitive tool with which to rapidly assess lactation performance *in vitro*.

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