Amino acid turnover by elongating cattle blastocysts recovered on days 14–16 after insemination

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Blastocyst elongation from day 14 to day 16 after insemination coincides with a major phase of embryo loss in cattle. Protein synthesis, reflected in protein content, increases markedly over this period but little is known about the amino acid requirement of elongating blastocysts at this time. Cattle blastocysts produced *in vivo* were recovered on days 14–16 after insemination and cultured individually for up to 8 h in synthetic oviduct fluid containing a physiological mixture of amino acids plus 1 mmol glutamine l⁻¹ and 0.1% (w/v) polyvinyl alcohol (SOFaaPVA). After 1, 4 and 8 h in culture, an aliquot of culture medium was removed and the rate of amino acid depletion or production was calculated per unit of protein and per hour of culture. Amino acids were depleted or

produced at different rates. Arginine was depleted from the medium at a significant rate ($P < 0.05$) during all culture periods. Alanine and glutamate were produced at a significant rate ($P < 0.05$) during all culture periods. The rate of alanine production was significantly greater ($P < 0.05$) in blastocysts recovered on day 14 compared with days 15 or 16 after insemination. Alanine production and arginine depletion tended to be greater in smaller embryos recovered on day 14 compared with larger and later stage embryos, indicating that earlier stage embryos may have higher metabolic activity than later stage embryos. Qualitatively, the pattern of amino acid consumption and production during elongation was similar to that shown from the zygote to early blastocyst stage.

Introduction

Protein synthesis by cattle embryos remains relatively constant between the zygote and the eight-cell stage embryo (Frei *et al.*, 1989; Thompson *et al.*, 1998). From the 8–16-cell stage, and with the onset of the maternal to zygote genome transition, protein synthesis increases markedly (Frei *et al.*, 1989; Grealy *et al.*, 1996; Thompson *et al.*, 1998) with concomitant increases in glucose (Rieger *et al.*, 1992; Thompson *et al.*, 1996) and amino acid (Guader-Joly *et al.*, 1997) turnover (that is, consumption and production). This period of increased protein synthesis and metabolic activity coincides with the process of embryo compaction at about day 6. This process is followed, in turn, by blastocyst formation, hatching and elongation, which are accompanied by an exponential increase in embryo size and protein content (Grealy *et al.*, 1996; Thompson *et al.*, 1998; Morris *et al.*, 2000). In the embryo, amino acids are required for protein synthesis and have further roles in metabolism (Gardner, 1998), as energy sources (Rieger and Guay, 1988), as nucleic acid precursors (Leese, 1991), as osmolytes (Steeves and Gardner, 1999), as regulators of pH_i (Bavister and McKiernan, 1993; Edwards *et al.*, 1998) and as signalling molecules (Van Winkle, 2001). Amino acids

are present in the oviduct and uterine fluids of cattle; alanine, glutamate and glycine are present in the highest concentrations (Moore and Bondioli, 1993; Guerin *et al.*, 1995; Elhassan *et al.*, 2001). Amino acid supplementation of culture media has been used to improve the developmental rate of matured, fertilized and cultured cattle embryos *in vitro* (Moore and Bondioli, 1993; Pinyopummintr and Bavister, 1996). Eckert *et al.* (1998) showed that *in vitro* culture, in the absence of protein or amino acids, results in a significant reduction in the proportion of cattle embryos developing to blastocysts. Partridge and Leese (1996) hypothesized a changing requirement for amino acids during early preimplantation cattle embryo development, whereas Steeves and Gardner (1999) demonstrated temporal and differential effects of amino acids on cattle embryo development during *in vitro* culture. Embryos depend on their uterine environment to sustain the exponential growth rates recorded between blastocyst formation at day 7 and day 8 and the elongated stage at day 16 after insemination (Grealy *et al.*, 1996; Morris *et al.*, 2000); this period coincides with the phase of greatest embryo loss in cattle (Diskin and Sreenan, 1980). During this period, because of increased metabolism and protein synthesis, embryos may be more susceptible to environmental stress or other conditions that alter their uterine environment. Indeed, amino acid concentrations in the bovine uterus are affected by heat stress and by the

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hormonal environment (Roussel and Loe, 1973). Although turnover of amino acids by cattle oocytes, zygotes and early stage embryos up to the blastocyst stage has been documented (Partridge and Leese, 1996; Jung *et al.*, 1998), there are no reports on amino acid turnover at the time of blastocyst elongation, a period of rapid growth (Grealley *et al.*, 1996; Morris *et al.*, 2000) and a further critical period in cattle embryo development, after which time most embryos form fetuses that are carried to term (Diskin and Sreenan, 1980). An understanding of amino acid turnover by elongating cattle blastocysts is necessary to understand why this period of development is so susceptible to embryo loss. Therefore, the objective of the present study was to measure amino acid turnover by cattle blastocysts over the period of maximum elongation, from day 14 to day 16 after insemination.

Materials and Methods

Embryo recovery

Recovery of embryos produced *in vivo* was carried out as described by Morris *et al.* (2000). In brief, Hereford-cross heifers were superovulated by the i.m. administration of 1500 iu equine chorionic gonadotrophin (eCG) (Folligon, Intervet UK Ltd, Cambridge) during the mid-luteal phase of the oestrous cycle (days 10–14) and 500 µg cloprostenol (Estrumate, Coopers Animal Health Ltd, Berkhamsted) 48 h later to induce luteolysis. After administration of cloprostenol, the heifers were examined continuously for overt signs of oestrus and those heifers observed in standing oestrus were artificially inseminated by an operator using semen from one sire. Embryo recovery was carried out during mid-ventral laparotomy performed under licence in accordance with the European Community Directive, 86-609-EC. Anaesthesia was induced with thiopentone sodium (5 g, i.v.; Rhone Merieux, Harlow) and maintained by inhalation of halothane (May and Baker Ltd, Dagenham) in oxygen in a closed-circuit apparatus. On days 14–16 after insemination, embryos were flushed from the uterus using a two-way Foley French 14-gauge catheter with a 30 ml balloon (Rusch Inc, NY). The composition of the flushing medium was 139.0 mmol NaCl l⁻¹, 2.7 mmol KCl l⁻¹, 0.89 mmol CaCl₂·2H₂O l⁻¹, 1.47 mmol KH₂PO₄ l⁻¹, 0.49 mmol MgCl₂·6H₂O l⁻¹, 7.46 mmol Na₂HPO₄·2H₂O l⁻¹, 1.0 mmol glucose l⁻¹, 0.5 mmol sodium pyruvate l⁻¹ and 0.1% (w/v) polyvinyl alcohol, pH 7.3.

Embryo culture

The culture medium used was synthetic oviduct fluid (Tervit *et al.*, 1972) with 20 essential and non-essential amino acids at concentrations identical to those present in minimum essential medium (MEM) plus 1 mmol glutamine l⁻¹ (Table 1) and 0.1% polyvinyl alcohol (SOFaaPVA). After recovery, embryos were washed individually three times in 3 ml flushing medium followed by three washes in 3 ml Hepes buffered SOFaaPVA, followed by three washes in bicar-

Table 1. Concentrations of essential and non-essential amino acids present in the culture medium SOFaaPVA

Amino acid	Concentration (mmol l ⁻¹)	Amino acid	Concentration (mmol l ⁻¹)
Alanine	0.10	Leucine	0.40
Arginine	0.73	Lysine	0.50
Asparagine	0.10	Methionine	0.10
Aspartic acid	0.10	Phenylalanine	0.20
Cysteine	0.20	Proline	0.10
Glutamic acid	0.10	Serine	0.10
Glutamine	1.00	Threonine	0.40
Glycine	0.10	Tryptophan	0.05
Histidine	0.27	Tyrosine	0.20
Isoleucine	0.40	Valine	0.40

SOFaaPVA: synthetic oviduct fluid with 20 essential and non-essential amino acids plus 1 mmol glutamine l⁻¹ and 0.1% polyvinyl alcohol.

bonate buffered SOFaaPVA. Embryos recovered on day 14 ($n = 11$), day 15 ($n = 12$) and day 16 ($n = 13$) after insemination were cultured individually in 500 µl of the third wash (embryo medium) in Falcon tubes for up to 8 h at $38.5 \pm 0.5^\circ\text{C}$ under a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂. After embryo removal, a 500 µl aliquot of the third wash (embryo wash) was also cultured as a control for each embryo. Measurements were made at three time points to validate the *in vitro* system as a means of assessing amino acid metabolism. After 1, 4 and 8 h in culture, a 50 µl aliquot was removed from each tube, snap-frozen in liquid nitrogen and stored at -80°C . At each of these time points, the culture medium was replenished with a 50 µl aliquot of fresh medium. At the end of the 8 h culture period, embryos recovered on days 14–15 after insemination were washed four times in 3 ml cold (4°C) 10 mmol PBS l⁻¹ in 35 mm dishes. As embryos recovered on day 16 after insemination are more prone to disintegration, they were washed by gentle agitation in an Eppendorf tube containing 2 ml of cold PBS, followed by centrifugation at 15 000 *g* for 10 min; these steps were repeated three more times. After washing, all the embryos were frozen in Eppendorf tubes at -80°C in 5–10 µl PBS until used for assay. After thawing, the protein content of the embryos was measured (Grealley *et al.*, 1996) using the bicinchoninic acid reagent (Pierce microBCA reagent, Perbio Science).

Amino acid analysis

Amino acid analysis was carried out using the procedure described by Lamb and Leese (1984) and Partridge and Leese (1996). Amino acids are reacted with α -phthalaldehyde to yield highly fluorescent products which are analysed by reverse-phase high pressure liquid chromatography (HPLC). Cysteine and proline could not be detected by this method. For each embryo, a series of amino acid analyses was carried out on an aliquot of the embryo wash followed

by an aliquot of the embryo medium at 1, 4 and 8 h, respectively. For each embryo, a standard amino acid mixture containing 18 amino acids was also analysed to determine the absolute concentrations of amino acids in the spent culture media. The amino acid concentrations found in the medium collected at 4 and 8 h were corrected for the removal of 50 μ l spent medium at the end of the 1 and 4 h time points and the addition of 50 μ l fresh medium at the beginning of the 4 and 8 h time points, respectively. The HPLC assay coefficient of variation was 15.3% ($n = 36$), determined from an aliquot of culture medium analysed with each embryo. The protein content of each embryo was used to calculate amino acid utilization in pmol per μ g protein per hour.

Statistical analyses

Amino acid depletion or production per μ g embryo protein per hour was calculated to normalize for differences in embryo protein content and duration of culture. Amino acid depletion or production during each of the three culture periods was tested for significance from zero by the Student's *t* test. Differences between days or culture periods were analysed by Proc GLM (version 8.01, SAS 2001) to fit a model that allowed for the repeated measures made on the same heifer and included the effect of heifer within days. The main effects of day and culture period and day $\times$ culture period interaction were tested using the heifer within-day mean square as the error term. Significant differences were compared using the Tukey–Kramer option within SAS. Data were log transformed to approximate normal variates before analysis. A probability value of $P < 0.05$ was considered significant. Values are presented as arithmetic means $\pm$ SEM.

Results

Amino acid depletion and production rates pooled across days for elongated cattle blastocysts recovered on days 14–16 after insemination, during 1, 4 and 8 h in culture are shown (Figs 1–3, respectively). There was no day $\times$ culture period interaction on amino acid depletion or production. The depletion or production rates of individual amino acids differed among culture periods. Amino acid depletion was more variable among embryos during the 1 h culture period than during the 4 and 8 h culture periods; however, the pattern of depletion or production was generally independent of culture period. Glutamate and alanine were produced during all culture periods at significant ($P < 0.05$) to highly significant ($P < 0.001$) rates (Figs 1–3). Glycine was produced at a significant ($P < 0.01$) to highly significant ($P < 0.001$) rate during the 4 and 8 h culture periods, respectively, whereas methionine and valine were produced at a significant rate ($P < 0.05$) during the 8 h culture period only. Arginine was depleted from the medium at a significant ($P < 0.05$) to highly significant ($P < 0.001$) rate during all culture periods. Aspartate was

Fig. 1. Amino acid production and depletion rates (mean $\pm$ SEM) by cattle blastocysts at days 14, 15 and 16 after insemination, during 0–1 h of culture (1 h period) in SOFaaPVA containing a physiological mixture of amino acids. Results for all days are combined and are expressed as pmol per μ g protein per hour. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with zero depletion.

Fig. 2. Amino acid production and depletion rates (mean $\pm$ SEM) by cattle blastocysts at days 14, 15 and 16 after insemination, during 1–4 h of culture (4 h period) in SOFaaPVA containing a physiological mixture of amino acids. Results for all days are combined and rates are expressed as pmol per μ g protein per hour. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with zero depletion.

depleted in a significant manner during the 1 and 4 h culture periods ($P < 0.01$), whereas asparagine, phenylalanine, isoleucine, leucine and lysine were depleted at a significant rate ($P < 0.05$) during the 1 h culture period only. The rate of isoleucine depletion was significantly higher ($P < 0.05$) during the 1 h period compared with during the 8 h culture period. There was no effect of time on the rate of depletion or production of any of the other amino acids.

The rate of alanine production pooled across culture periods was higher in embryos recovered on day 14 ($P < 0.05$) compared with embryos recovered on day 15 or

Fig. 3. Amino acid production and depletion rates (mean $\pm$ SEM) by cattle blastocysts at days 14, 15 and 16 after insemination, during 4–8 h in culture (8 h period) in SOFaaPVA containing a physiological mixture of amino acids. Results for all days are combined and rates are expressed as pmol per μ g protein per hour. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with zero depletion.

day 16 (Fig. 4) and tended to be higher for the earlier stage and smaller embryos compared with the later and larger embryos recovered on day 14 after insemination (Fig. 5). Glutamate production pooled across culture periods also tended to be higher in embryos recovered on day 14 compared with embryos recovered on day 15 or day 16 after insemination and followed a similar pattern to alanine production (Fig. 4); however, this difference was not significant. Arginine depletion pooled across culture periods also tended to be higher in embryos recovered on day 14 compared with embryos recovered on day 15 or day 16 after insemination (Fig. 4); however, this difference was not significant. In a similar manner to alanine production, arginine depletion also tended to be higher for the earlier stage and smaller embryos recovered on day 14 compared with the later and larger embryos recovered on day 14 after insemination (Fig. 6). Irrespective of embryo age, alanine and glutamate appearance and arginine depletion in the medium tended to be greatest in smaller embryos. Depletion and production rates were not different between days for any of the other amino acids.

Discussion

This is thought to be the first report to describe amino acid turnover by elongating cattle blastocysts recovered on days 14–16 after insemination. The individual amino acid requirements of cattle embryos recovered on days 14–16 after insemination varied widely, and many amino acids were depleted at different rates whereas others were produced by embryos. Differences in the individual rate of amino acid turnover may be the result of differences in the concentrations of amino acids present in the culture medium and, in turn, competition between amino acids for

amino acid transporters. However, the relationship between amino acid concentrations in the medium and turnover by embryos was not addressed in the present study. The results show that the rate of amino acid depletion was generally higher during the 1 h period of culture compared with the 4 and 8 h culture periods; however, the rate of amino acid production was similar across culture periods. The rate of depletion during 1 h of culture probably reflects the way in which the embryo adapts to an *in vitro* culture environment, whereas the depletion or production rates during 4 and 8 h of culture are more likely to reflect a steady state of amino acid turnover. Glutamine and arginine were depleted numerically at the greatest rate, especially during the 1 h culture period. Glutamine is present at relatively low concentrations in the bovine oviduct and uterine fluids, whereas glycine is the most abundant amino acid (Moore and Bondioli, 1993; Solymosi and Horn, 1994; Guerin *et al.*, 1995; Elhassan *et al.*, 2001). However, glutamine is the preferred amino acid of bovine zygotes produced *in vitro* and of four-cell embryos (Partridge and Leese, 1996) and has been shown to improve embryonic development during *in vitro* culture. In addition to pyruvate and glycine, oxidative metabolism of glutamine is the major energy-producing pathway in cattle oocytes (Rieger *et al.*, 1992) at a time when high concentrations of glucose are detrimental to embryo development. However, the rate of glutamine consumption is relatively low by the time the cattle embryo reaches the blastocyst stage at day 7 after insemination (Rieger *et al.*, 1992; Partridge and Leese, 1996; Jung *et al.*, 1998). However, glutamine consumption increases again during blastocyst expansion (Rieger *et al.*, 1992; Tiffin *et al.*, 1991), at which stage glucose consumption too has increased markedly. In the present study, by the time the blastocyst begins to elongate, glutamine is one of the preferred amino acids. Glutamine is thought to have a dual role during bovine embryo development, including that of an osmolyte and an energy source (Steeves and Gardner, 1999). Arginine was the only amino acid depleted at a significant rate during all culture periods. Arginine depletion has also been reported for cattle blastocysts produced *in vivo* at day 7 after insemination (Partridge and Leese, 1996) and by morulae and blastocysts produced *in vitro* (Lee and Fukui, 1996). In the present study, arginine depletion also tended to be greatest in early stage embryos recovered on day 14 compared with later stage embryos recovered on days 14–16 after insemination. Arginine acts as a substrate for nitric oxide synthesis, which is essential for mouse embryo growth and development (Gouge *et al.*, 1998; Chen-Huei-Wen, 2001). Arginine is thought to promote the growth of blastocysts by increasing the supply of nutrients and also by signalling that results in an increase in the capacity for, and rate of, protein synthesis (Van Winkle, 2001). The tendency for the rate of arginine depletion to decrease from the early stage blastocyst at day 14 to blastocysts at days 15–16 after insemination observed in the present study is in agreement with a decrease in the rate of protein synthesis reported by Morris *et al.* (2000) for

Fig. 4. Comparison of amino acid production and depletion rates (mean $\pm$ SEM) among cattle blastocysts at day 14 (■), day 15 (□) and day 16 after insemination (▨) after culture in SOFaaPVA containing a physiological mixture of amino acids. Results for all culture periods are combined and rates are expressed as pmol per μ g protein per hour. Asterisk denotes significant difference: $P < 0.05$.

Fig. 5. Alanine production as a function of embryo protein content by cattle blastocysts at day 14 (▲), day 15 (■) and day 16 after insemination (●) after culture in SOFaaPVA containing a physiological mixture of amino acids. Results for all culture periods are combined and rates are expressed as pmol per μ g protein per hour.

Fig. 6. Arginine depletion as a function of embryo protein content by cattle blastocysts at day 14 (▲), day 15 (■) and day 16 after insemination (●) after culture in SOFaaPVA containing a physiological mixture of amino acids. Results for all culture periods are combined and rates are expressed as pmol per μ g protein per hour.

blastocysts at day 15 compared with blastocysts at day 14 after insemination. Nitric oxide can regulate cGMP concentrations, and inhibition of nitric oxide synthesis decreases cGMP concentrations in rat endometrium (Durán-Reyes *et al.*, 1999). A decrease in the cGMP content per unit of protein of cattle blastocysts at days 15–16 compared with blastocysts at day 14 after insemination was reported by Grealy *et al.* (1997) and Grealy and Sreenan (1999) and is consistent with the tendency for arginine

depletion in the present study to be lower in cattle blastocysts at days 15–16 compared with blastocysts at day 14 after insemination.

An unexpected result, although consistent with reports by Partridge and Leese (1996), Jung *et al.* (1998) and McEvoy *et al.* (2000) for cattle blastocysts at day 7 after insemination, was the highly significant production of alanine by embryos at all culture periods. Alanine production is also a feature of embryos produced *in vitro*

from the putative zygote to the day 7 blastocyst stage (Partridge and Leese, 1996). Alanine production or output may be a mechanism by which embryos export intracellular ammonia, a by-product of metabolism, a build up of which would be toxic (Gardner and Lane, 1993; Gardner *et al.*, 1994; Lane and Gardner, 1994). The production of glutamate is not a feature of early embryonic stages (Partridge and Leese, 1996; Jung *et al.*, 1998), but in the present study may be an additional mechanism for ammonia disposal in the elongating blastocyst via glutamate dehydrogenase. Ammonia is an inevitable product of the metabolism of amino acids, and as such, alanine or glutamate output may be considered useful markers of protein turnover. Indeed, when alanine production was related to embryo protein content, smaller embryos tended to have a higher rate of alanine production and, by inference, a higher metabolic rate than larger and later stage embryos. This finding is in agreement with a study by Morris *et al.* (2000) in which the rate of protein synthesis by blastocysts at day 13 was found to be higher than that by blastocysts at days 14–15 after insemination. The consistent production of these amino acids in the face of a changing pattern of consumption indicates that this export mechanism operates to full capacity in these embryos. However, although the consumption of individual amino acids tended to be greater during the early culture periods, glutamate and alanine were produced by the embryos at a consistent rate regardless of the culture period. In the present study, amino acid depletion or accumulation is reported on a per embryo mass basis. No attempt was made to differentiate between amino acid depletion and production by the blastocoelic fluid and by the embryo *per se*, an impossible task, practically, in elongating cattle blastocysts. Large differences in the rates of depletion and production for other amino acids were also found among blastocysts at days 14–16 after insemination; however, these differences were not significant. This finding may, in part, be due to the numbers of embryos used in this study, although it is more likely to be the result of the naturally wide variation in developmental stage and size of embryos recovered on each day.

Overall, the pattern of amino acid consumption and production by expanding cattle blastocysts in the present study was similar to the pattern for cattle embryos at other developmental stages (Partridge and Leese, 1996; Jung *et al.*, 1998). These data support previous reports of individual amino acid preferences by cattle embryos and the way in which amino acid preferences change throughout development. In future studies, it will be important to investigate how the requirements of the preimplantation cattle embryo are met by conditions in the uterine environment and how these requirements may, in turn, be affected by the maternal nutritional environment.

This work was supported by the EC Biotech 4th Framework Programme (Contract No. CT-95-0190). The authors wish to thank A. Glynn, P. Joyce, G. Morris, W. Connolly and J. Nally for

technical support, and G. Burke, P. Creavan and P. Reilly for care of the animals.

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Received 15 April 2002.

First decision 15 May 2002.

Revised manuscript received 12 June 2002.

Accepted 16 July 2002.