Metabolic characterization of the bovine blastocyst, inner cell mass, trophoctoderm and blastocoel fluid

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The formation of a viable blastocyst is dependent upon the establishment of a correct inner cell mass (ICM):trophoctoderm cell ratio but little is known about the metabolism of the two cell populations or about the composition of blastocoel fluid. In this study, the metabolism of intact bovine blastocysts, isolated ICM and trophoctoderm was examined in terms of glucose and pyruvate uptake, lactate production, and amino acid consumption or production. The concentration of these nutrients in blastocoel fluid was also determined. The metabolism of glucose, pyruvate and lactate differed significantly between the isolated ICM and trophoctoderm. Isolated trophoctoderm had a higher pyruvate ($P < 0.001$) and lower glucose ($P < 0.05$) consumption, and higher lactate production ($P < 0.05$) than did ICM. The consumption or production of amino acids by ICM and trophoctoderm also differed, with the trophoctoderm displaying a higher turnover (the sum of production and consumption). The ICM and trophoctoderm both depleted arginine, aspartate and leucine,

whereas the production of alanine was consistent. Isolated ICM depleted a further six amino acids, which appeared during trophoctoderm culture; the reverse trend was observed for the remaining amino acids. The concentration of lactate in blastocoel fluid was significantly higher than in synthetic oviductal fluid supplemented with amino acids and BSA (SOFaaBSA; $P < 0.05$). However, glucose ($P < 0.05$) and pyruvate ($P < 0.001$) concentrations were both lower. Aspartate, glutamate, glycine, alanine and tryptophan were present at significantly higher concentrations in blastocoel fluid than in SOFaaBSA, whereas threonine and asparagine concentrations were significantly lower. The metabolism of composite blastocysts, obtained by summing the consumption and production profiles of the ICM and trophoctoderm, and taking into account their respective number of cells, was higher than that of intact blastocysts, indicating that upon isolation of the two cell populations there may be disruption to paracrine interactions or the onset of culture-induced cellular stress or both.

Introduction

Development of mammalian preimplantation embryos involves the formation of the blastocyst in which the outer trophoctoderm cells surround the blastocoel containing the inner cell mass (ICM). The trophoctoderm is the first epithelium to form during mammalian development and gives rise to extraembryonic structures, whereas the ICM develops principally into the embryo proper.

The cells of the trophoctoderm are polarized, with a microvillous apical surface and a relatively smooth basolateral surface that borders the blastocoel fluid (Ducibella and Anderson, 1975), whereas those of the ICM are apolar and devoid of microvilli (Pratt, 1985). The trophoctoderm acts as a selective barrier to the movement of solutes into and out of the blastocoel; in particular, the Na^+/K^+ -ATPase localized to the basolateral and apical membranes of trophoctoderm cells transports Na^+ into the blastocoel cavity, with water following osmotically

(Biggers *et al.*, 1988; Watson and Barcroft, 2001). Regulation of blastocoel fluid formation, and hence the provision of nutrients for the ICM, is determined by the transporting activity of the trophoctoderm (Jaszczak *et al.*, 1972; Robinson and Benos, 1991; Brison and Leese, 1993). Differences between trophoctoderm and ICM have been examined in terms of gene expression (Fleming and Hay, 1991; Slager *et al.*, 1991; Kirchhof *et al.*, 2000), protein synthesis (Handyside and Johnson, 1978) and metabolism (Hewitson and Leese, 1993).

The number of cells in the ICM correlates strongly with normal fetal development (Lane and Gardner, 1997) and the establishment of a correct ICM:trophoctoderm cell ratio is considered essential to ensure subsequent embryo viability (Fleming, 1987).

The early cleavage stages of development of bovine preimplantation embryos rely principally on the oxidation of nutrients, specifically pyruvate for energy provision (Thompson *et al.*, 1996). In common with other species (Leese, 1995), there is an increase in glucose consumption and lactate production at compaction (Thompson *et al.*, 1996). Thus, cattle blastocysts rely

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principally on oxidative phosphorylation (86%) and glycolysis (14%) to generate ATP to support cavitation (Rieger *et al.*, 1992; Donnay and Leese 1999).

Amino acids are added to media for culture of early cattle embryos where they serve a variety of physiological functions including: protein synthesis (Epstein and Smith, 1973), energy provision (Partridge and Leese, 1996; Jung *et al.*, 1998), osmoregulation (Baltz, 2001), pH regulation (Baltz, 1993; Edwards *et al.*, 1998), protection against oxidative stress (Lindenbaum, 1973) and removal of ammonium (Leese, 1991; Partridge and Leese, 1996; Donnay and Leese, 1999).

Robinson and Benos (1991) and Hewitson and Leese (1993) hypothesized that the trophectoderm spares glucose for use by ICM in rabbits and mice, respectively. In mice, the two cell lineages differ in their metabolism of glucose and pyruvate, particularly with respect to their reliance on glycolysis. Murine ICM cells have a glycolytic index (the proportion of glucose converted to lactate) of about 100%, which is twice that of the trophectoderm (Hewitson and Leese, 1993). Furthermore, these lineages differ in their glucose storage, as indicated by the higher glycogen content of the trophectoderm compared with the ICM (Edirisinghe *et al.*, 1984).

To the best of our knowledge, there is no information regarding the metabolic properties of the individual cell populations in cattle blastocysts or on the composition of blastocoel fluid. Therefore, the metabolism of isolated bovine ICM and trophectoderm was examined in the present study in terms of glucose and pyruvate uptake, lactate production and amino acid consumption and production. The same measurements have also been obtained for intact blastocysts. This has enabled determination of the extent to which the metabolism of a bovine blastocyst is the sum of its constituent parts.

Materials and Methods

Collection and maturation of oocytes

All chemicals were purchased from Sigma (Poole) unless otherwise stated. Ovaries were obtained from a local abattoir and transported to the laboratory in phosphate-buffered saline (PBS) supplemented with 25 μ l antibiotic-antimycotic (100 ml⁻¹) (Gibco Life Technologies, Paisley) at room temperature. Ovarian follicles (3–10 mm in diameter) were punctured and the follicular fluid was aspirated into Hepes-buffered TCM199 medium containing 50 μ g kanamycin monosulphate ml⁻¹, 2 μ g heparin ml⁻¹ (H0777) and 1.5 g BSA l⁻¹ (A6003). Cumulus-oocyte complexes (COCs) with several layers of granulosa cells and an evenly shaded cytoplasm were selected, transferred into maturation medium (bicarbonate-buffered TCM199 supplemented with 10% fetal bovine serum, 0.025 iu FSH/LH (Ferring Pharmaceuticals, Langley), 0.47 μ g epidermal growth factor ml⁻¹ (long EGF; E4269) and 10.9 ng fibroblast growth factor

ml⁻¹ (bovine FGF; F0291)) and incubated for 24 h at 39°C under a humidified atmosphere of 5% CO₂ in air. After maturation, the oocytes were washed twice in Hepes-buffered Tyrode's albumin-lactate-pyruvate (Hepes-TALP) and fertilization-TALP (Fert-TALP; Lu *et al.*, 1987) supplemented with 10 μ g heparin ml⁻¹, 600 μ g penicillamine ml⁻¹ and 220 μ g hypotaurine ml⁻¹.

In vitro fertilization and culture

Spermatozoa were prepared from a frozen-thawed semen sample from a single sire of proven fertility. The semen was thawed in a waterbath at 37°C, layered on to a discontinuous Percoll (Pharmacia Biotech, St Albans) gradient (45:90) and centrifuged at 2100 *g* for 25 min. The pellet was resuspended in Hepes-TALP and centrifuged for a further 10 min at 1500 *g*. The resulting motile sperm pellet was resuspended in Fert-TALP. The sperm suspension was adjusted to give a concentration of 1×10^6 spermatozoa ml⁻¹ and added to fertilization wells each containing 45–65 oocytes, which had been washed twice in Hepes-TALP and Fert-TALP. Spermatozoa and oocyte complexes were co-incubated at 39°C under a humidified atmosphere of 5% CO₂ in air. After 18 h, any remaining cumulus cells were removed by vortexing in Hepes-buffered synthetic oviductal fluid (SOF) supplemented with minimum essential and non-essential medium amino acids (Gibco), 1 mmol glutamine l⁻¹ and 4 mg BSA ml⁻¹ (Hepes-SOFaBSA; Tervit *et al.*, 1972). The resultant putative zygotes were washed twice in Hepes-SOFaBSA, placed in groups of 15 in 20 μ l droplets of SOFaBSA under mineral oil and incubated at 39°C in a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂.

Immunosurgery

Immunosurgery was performed on day 8 expanded blastocysts. The zonae pellucidae were removed from unhatched blastocysts by treatment with 0.5% (w/v) pronase at 39°C. The zona pellucida-free embryos were returned to culture medium for 2–3 h to recover before incubation in anti-bovine serum (B8270; 10% (v/v) in Hepes-SOF). The embryos were washed in Hepes-SOF and incubated in guinea-pig complement serum (S1639; 25% (v/v) in Hepes-SOF) supplemented with 0.01 mg propidium iodide ml⁻¹ at 39°C. Treated blastocysts were transferred to individual 2 μ l droplets of Hepes-SOF and the ICM was removed from the lysed trophectoderm cells by aspiration through a narrow bore pipette.

Trophectoderm biopsy

Zona pellucida-free day 8 blastocysts were transferred to individual microdroplets of Hepes-SOF. Holding pipettes and sharp tipped glass needles were made from 1 mm glass capillary tubing with the aid of a microforge

(Research Instruments, Penryn) and siliconized with dimethyldichlorosilane solution to minimize cell adhesion to the glass. A narrow bore holding pipette, mounted on a micromanipulator (Leica, Milton Keynes), was used to maintain the blastocyst in position. Trophectoderm cell biopsies were obtained by free hand slicing through the mural trophectoderm using the sharp tip of a glass needle. The biopsies were returned to culture for 1 h to allow the trophectoderm cells to recover.

Incubation of blastocysts, ICM and trophectoderm vesicles

For the measurement of glucose and pyruvate uptake and lactate production, blastocysts, ICM and trophectoderm cells were transferred to 80 nl droplets of SOFaaBSA, whereas for amino acid analysis, they were transferred to 1 μ l droplets of SOFaaBSA. All droplets were incubated under a mineral oil overlay in a humidified atmosphere of 5% CO₂ in air at 39°C. For the measurement of carbohydrate metabolism, the duration of incubation was 30 min for intact blastocysts and 1 h for isolated ICM and trophectoderm vesicles. The production and consumption of amino acids were measured over 5 h for blastocysts, ICM and trophectoderm vesicles. Control droplets were incubated alongside the embryo-containing drops to account for any non-specific loss of nutrients.

Counting of number of cells

After incubation, blastocysts, isolated ICM and trophectoderm vesicles were transferred to microdroplets of SOFaaBSA supplemented with 1 mg bisbenzimidazole ml⁻¹ and cultured for 1 h. They were removed, fixed in absolute ethanol, mounted individually in droplets of glycerol on a microscope slide and the cells were counted three times using a microscope (Leica).

Micropuncturing technique

The method for blastocyst micropuncturing and aspiration of blastocoel fluid was adapted from Brison *et al.* (1993). In brief, expanded day 8 blastocysts were removed from culture and transferred to a 10 nl droplet of pre-warmed Hepes-SOFaaBSA under a mineral oil underlay. The blastocysts were immobilized by a holding pipette connected to an air-filled syringe and mounted on a micromanipulator. The medium surrounding the embryo was gently removed to ensure that the site of micropuncture was not contaminated by external culture medium. A finely pulled, oil-filled pipette was introduced through the mural trophectoderm to avoid damaging the ICM cells, and blastocoel fluid was aspirated gently until the blastocyst had fully collapsed around the pipette. The retrieved fluids were expelled into a dish under oil and frozen at -80°C alongside 0.5 nl control droplets of

SOFaaBSA. The pipettes for micropuncture and aspirating blastocoel fluid samples were made using a Pul-1 pipette puller (World Precision Instruments, Stevenage).

Ultramicrofluorometric assays

The concentrations of pyruvate, lactate and glucose in the blastocoel fluid and incubation droplets were measured non-invasively using ultramicrofluorometric techniques (Leese and Barton, 1984; Gardner and Leese, 1986). The changes in fluorescence were quantified using a Fluovert microscope (Leica) with photomultiplier and photometer attachments.

Amino acid analysis

Spent and control incubation droplets were diluted 1:40 in high performance liquid chromatography (HPLC) grade water (Fisher Scientific, Loughborough); pooled droplets of blastocoel fluid (2–7 nl) were diluted in 25 μ l water. The amino acid concentrations in the incubation droplets and blastocoel fluid were measured by reverse-phase HPLC as described previously (Lamb and Leese, 1994; Houghton *et al.*, 2002) using a Kontron 500 Series automated HPLC system, with a Jasco F920 fluorescence detector and a 4.5 $\times$ 250 mm Hypersil ODS-16 column (Jones Chromatography, Mid Glamorgan). Amino acid concentrations in experimental samples were evaluated by reference to control samples of known amino acid concentration. This method did not allow the detection of taurine, proline or cysteine.

Statistical analysis

Glucose and pyruvate consumption, and the production of lactate and amino acid consumption and production by blastocysts, ICM and trophectoderm vesicles were expressed as mean pmol per cell per h $\pm$ SEM. Glycolytic index was determined as the proportion of glucose converted to lactate and was expressed as a percentage. The concentrations of glucose, pyruvate, lactate and amino acids in the blastocoel fluid were expressed as mean mmol l⁻¹ $\pm$ SEM. As small numbers of replicates were used in each batch, the data were compared using Kruskal–Wallis test. No significant differences between groups were recorded and so the different cohorts of blastocysts were pooled, before analysis by Student's *t* test and one-way ANOVA with *post hoc* Fisher's least significant difference.

Results

Metabolic assessment of blastocysts, ICM and trophectoderm for glucose, lactate and pyruvate

The uptake of glucose and pyruvate, and production of lactate by whole blastocysts, isolated ICM and

Table 1. Number of cells of intact bovine blastocysts, isolated inner cell mass (ICM) and trophectoderm vesicles

Cell population	Number of replicates	ICM		Trophectoderm	
		Number of cells	Proportion (%)	Number of cells	Proportion (%)
Blastocyst	31	37.72 ± 1.29	31.19 ± 0.37	83.21 ± 2.72	68.81 ± 0.37
Isolated ICM	25	32.82 ± 1.55	–	–	–
Isolated trophectoderm	22	–	–	35.35 ± 1.88	–

Fig. 1. Glucose and pyruvate consumption and lactate production by intact bovine blastocysts (□), isolated inner cell mass (▨) and trophectoderm vesicles (■). Data are expressed on a per cell basis ± SEM. ^{a–f}Different letters indicate significant differences between cell populations (lactate: $P < 0.05$; glucose: $P < 0.05$; pyruvate: $P < 0.001$).

trophectoderm are shown (Fig. 1). The metabolic profiles of the ICM and trophectoderm differed significantly. The trophectoderm showed a significantly higher pyruvate ($P < 0.001$) and lower glucose ($P < 0.05$) consumption, and an increased lactate ($P < 0.05$) production compared with the ICM on a per cell basis. The intact blastocyst profiles tended to reflect those of the isolated ICM rather than the trophectoderm.

The proportion of ICM cells in a day 8 expanded blastocyst was 31.19% compared with 68.81% for trophectoderm (Table 1). The metabolism of a composite blastocyst derived from the values for isolated ICM and trophectoderm metabolism and number of cells (Fig. 1 and Table 1) is shown (Fig. 2) alongside that of control blastocysts. Pyruvate consumption and lactate production were significantly higher in the composite blastocyst, whereas glucose consumption was significantly lower ($P < 0.001$). The glycolytic indices of isolated ICM and intact blastocyst were comparable (Fig. 3), but were markedly lower than those of the composite blastocyst and isolated trophectoderm ($P < 0.001$).

Blastocoel fluid

The concentrations of glucose, lactate and pyruvate in bovine blastocoel cavity and SOFaaBSA controls are

Fig. 2. The metabolism of a composite bovine blastocyst (■) constructed on the basis of the data in Fig. 1 and Table 1, and of an intact blastocyst (□). *Denotes significant difference between intact and composite blastocysts ($P < 0.001$).

Fig. 3. Glycolytic index of intact and composite bovine blastocysts, and isolated inner cell mass (ICM) and trophectoderm. ^{a–c}Different letters indicate significant differences ($P < 0.001$).

shown (Fig. 4). The concentration of lactate in blastocoel fluid was significantly higher, and pyruvate and glucose concentrations were significantly lower, than in SOFaaBSA culture medium.

Assessment of amino acids of blastocysts, ICM and trophectoderm

The production or consumption of amino acids from SOFaaBSA culture medium by intact blastocysts, isolated

ICM and trophoctoderm vesicles was measured using reverse-phase HPLC (Fig. 5). The uptake or production of all amino acids was significantly higher in the isolated cell lineages compared with intact blastocysts. All types of cell depleted aspartate, arginine and leucine, whereas alanine was produced consistently. Isolated ICM depleted asparagine, glycine, threonine, tyrosine, tryptophan and phenylalanine, whereas these were produced during the culture of trophoctoderm vesicles. The reverse trend (that is, production in ICM culture and consumption in trophoctoderm culture) was observed for methionine, valine, isoleucine, glutamate, serine, histidine and glutamine.

The production and consumption of amino acids by intact and composite blastocysts is shown (Fig. 6). Production of most of the amino acids increased in the composite blastocyst compared with the intact blastocyst; however, glutamate, histidine, glycine and threonine showed the opposite profile, that is, increased production in the intact blastocyst.

A summary of total amino acid production and consumption by intact blastocysts, ICM and trophoctoderm vesicles is shown (Fig. 7). The isolated cell lineages have significantly increased total amino acid production and consumption profiles compared with intact blastocysts. This is reflected in total amino acid turnover (total production plus total consumption): blastocyst: 0.142; ICM: 1.21; and trophoctoderm: 1.05 pmol per cell per h. Amino acid consumption values were higher for ICM than trophoctoderm, but production values were similar. There was no difference in turnover between the ICM and trophoctoderm ($P < 0.05$).

Amino acid composition of blastocoel fluid

A comparison of the concentrations of amino acids in bovine blastocoel fluid and SOFaaBSA is shown (Table 2). Most amino acids were present at higher concentrations in blastocoel fluid than in SOFaaBSA; however, only the concentrations of aspartate, glutamate, glycine, alanine and tryptophan were significantly higher. In contrast, concentrations of asparagine and threonine were significantly lower in blastocoel fluid than in SOFaaBSA.

Discussion

The energy-generating pathways available to the ICM and trophoctoderm are the same as for somatic cells and include oxidative metabolism, notably of pyruvate, and the glycolytic conversion of glucose to lactate (Rieger *et al.*, 1992; Thompson *et al.*, 1996; Donnay and Leese, 1999). As only mural trophoctoderm cells were isolated in the present study, the discussion will disregard any possible contribution of polar trophoctoderm cells to intact blastocyst metabolism. The ICM had lower pyruvate consumption per cell than did the isolated

Fig. 4. Composition of blastocoel fluid in day 8 expanded bovine blastocysts (■, $n=8$) and culture medium (□, $n=8$, Hepes-buffered synthetic oviductal fluid (SOF) supplemented with minimum essential and non-essential medium amino acids, 1 mmol glutamine l⁻¹ and 4 mg BSA ml⁻¹). Asterisks denote significant differences between blastocoel fluid and culture medium (* $P < 0.05$ and ** $P < 0.001$).

Table 2. Amino acid concentration in control synthetic oviductal fluid (SOF) supplemented with minimum essential and non-essential medium amino acids, 1 mmol glutamine l⁻¹ and 4 mg BSA ml⁻¹ (control SOFaaBSA; $n=10$) and blastocoel fluid ($n=9$)

Amino acid	Control SOFaaBSA (mmol l ⁻¹)	Blastocoel fluid (mmol l ⁻¹)
Asp	0.15 ± 0.02	5.62 ± 0.26**
Glu	0.15 ± 0.00	8.85 ± 0.54**
Asn	0.07 ± 0.00	0.03 ± 0.02*
Ser	0.15 ± 0.04	0.62 ± 0.07
His	0.13 ± 0.01	0.42 ± 0.02
Gln	0.80 ± 0.02	0.86 ± 0.05
Gly	0.21 ± 0.05	2.16 ± 0.51**
Thr	2.67 ± 0.26	0.90 ± 0.06**
Arg	0.53 ± 0.01	0.93 ± 0.08
Ala	0.29 ± 0.05	2.97 ± 0.49**
Tyr	0.37 ± 0.05	0.55 ± 0.10
Trp	0.08 ± 0.03	0.48 ± 0.20*
Met	0.11 ± 0.03	0.19 ± 0.40
Val	0.48 ± 0.06	0.71 ± 0.15
Phe	0.26 ± 0.04	0.53 ± 0.14
Ile	0.40 ± 0.01	0.64 ± 0.13
Leu	0.42 ± 0.02	0.91 ± 0.06
Lys	0.36 ± 0.01	0.53 ± 0.04

Asterisks indicate significant differences compared with control SOFaaBSA group (* $P < 0.05$ and ** $P < 0.001$).

trophoctoderm and intact blastocysts, which is indicative of relatively low oxidative metabolism. However, the glycolytic index of the ICM (79%) indicated that 21% of the glucose consumed was fully oxidized, thereby possibly reducing the requirement for pyruvate consumption and oxidation. The metabolism of the isolated trophoctoderm differed greatly from that of the ICM, and was characterized by higher pyruvate consumption,

Fig. 5. Amino acid consumption or production by intact bovine blastocysts ($\square$, $n=28$), isolated inner cell mass ($\square$, $n=20$) and trophoblast vesicles ($\blacksquare$, $n=14$). ^{a-c}For each amino acid, bars with different letters indicate significant differences between cell populations ($P < 0.05$).

Fig. 6. Amino acid consumption or production by intact bovine blastocysts ($\square$) and composite blastocysts ($\blacksquare$) constructed on the basis of the data in Table 1 and Fig. 5. The consumption or production of all amino acids differed significantly between composite and intact blastocysts ($P < 0.001$).

lower glucose uptake and higher lactate output. The glycolytic index of the trophoblast (390%) indicated that all the glucose consumed was probably converted to lactate and that further lactate was derived from an additional source or sources. Two possibilities are by the conversion from pyruvate via lactate dehydrogenase (LDH) or from amino acid catabolism, as occurs in the placenta (Bassett, 1991).

The pronounced difference in carbohydrate metabolism between the trophoblast and ICM may reflect their diverging roles in embryological development. Upon hatching, the trophoblast undergoes a period of rapid growth and elongation before attachment to the maternal caruncles. Enhanced glycolysis and, probably, glutaminolysis, best maintain an adequate provision of metabolic intermediates for biosynthetic pathways, as indicated by the metabolism of rapidly dividing cells *in vitro* (Newsholme *et al.*, 1989; Leese, 1993; Wojtczak, 1996).

Amino acids improve the development of preimplantation mouse (Gardner and Lane, 1993), rat (Miyoshi *et al.*, 1995), sheep (Gardner *et al.*, 1994) and cow (Rosenkrans and First, 1994) embryos *in vitro*, and increase the number of cells in mouse blastocyst ICM (Lane and Gardner, 1997). Bovine embryos have a relatively low consumption and production of amino acids until the onset of genome activation, at the 8–16-cell stage (Frei *et al.*, 1989), when there is a rapid increase, which continues throughout development to the blastocyst stage (Partridge and Leese, 1996; Jung *et al.*, 1998).

Total amino acid turnover (that is, the sum of consumption and production) did not differ between isolated ICM and trophoblast, but overall total consumption was significantly higher for the ICM than the trophoblast. This finding indicates that the ICM may have a greater requirement for amino acids, perhaps due to differences in rates of protein synthesis (Handyside and Johnson, 1978) and cell division (Harlow and Quinn, 1982). In turn, this is likely to be a function of expression and localization of amino acid transporters (Miller and Schultz, 1985). Overall, the increased amino acid profile observed in the isolated cell lineages in the present study may reflect metabolic adaptation of trophoblast and ICM to an artificial '*in vitro*' culture environment (Morris *et al.*, 2002) or a loss of metabolic control through the disruption of paracrine interactions upon isolation.

Certain amino acids consistently appeared (alanine and glutamine) or were depleted (aspartate, arginine and leucine) from the medium during incubation of trophoblast and ICM. The production of alanine may result from amino nitrogen fixation, to prevent the accumulation of potentially toxic ammonium ions (Gardner and Lane, 1993; Partridge and Leese, 1996; Donnay and Leese, 1999). This may explain why all cell populations consistently produced alanine, that is, to eliminate ammonium.

Fig. 7. Total amino acid production (□) or consumption (■) by intact bovine blastocysts, inner cell mass (ICM) and trophoblast vesicles. ^{a-c} or ^{x-z} Different letters indicate significant differences between cell populations (^{a-c} $P < 0.05$ and ^{x-z} $P < 0.001$).

Arginine and leucine were consistently depleted, in agreement with reports for *in vivo* and *in vitro* cattle blastocysts (Lee and Fukui, 1996; Partridge and Leese, 1996; Jung *et al.*, 1998) and human embryos (Houghton *et al.*, 2002). Leucine consumption may have resulted from its role as a signalling molecule that is capable of regulating gene expression, protein synthesis and blastocyst growth (Fox *et al.*, 1998; Martin and Sutherland, 2001; Van Winkle, 2001). Similarly, arginine may be required for the production of nitric oxide, which regulates embryonic growth (Gouge *et al.*, 1998). Van Winkle (2001) suggested that arginine and leucine might promote the growth of blastocysts by increasing nutrient supply and protein synthetic capacity via enhanced signalling. Therefore, the consistent consumption of these amino acids may indicate their requirement in blastocyst cells for gene regulation, cell signalling and protein synthesis.

Asparagine is incorporated into proteins, but is only taken up when *de novo* synthesis is insufficient to meet demand, for example in rapidly dividing cells such as malignant cells; this may account for the opposing profiles of asparagines, that is, consumption by the ICM and production by the trophoblast.

The glucose and pyruvate uptake and lactate production values for the intact bovine blastocysts fell within the range reported in other studies (Thompson *et al.*, 1996; Donnay and Leese, 1999), with the exception of glycolytic index; 73% in the present study and 100% in that of Thompson *et al.* (1996). The reasons for this may lie in minor differences in embryo production protocols, for example, the inclusion of amino acids in the fertilization medium used in the present study. Brief exposure to amino acid-free conditions has protracted effects on preimplantation embryos, and results in culture-induced stress (Lane and Gardner, 1998).

There were significant differences between the metabolism of the intact and composite blastocysts, obtained by summing the consumption and production profiles of

the isolated ICM and trophectoderm, and taking into account their representative numbers of cells. The composite blastocyst showed increased pyruvate consumption and lactate production with a decreased glucose uptake compared with that of the intact blastocyst. The intact blastocyst had a lower glycolytic index (73%) than did the composite blastocyst (233%). This lower index indicates that glucose may be metabolized more efficiently by the intact blastocyst in terms of ATP generation, with a greater reliance on oxidative metabolism, as upregulation of glycolysis is a marker of metabolic stress (Lane and Gardner, 1996; Leese *et al.*, 1998). In contrast, Hewitson and Leese (1993) reported that metabolism of mouse composite blastocysts equalled that of intact blastocysts. These differences may be a consequence of differences in embryo glycolytic index between the two species. One possibility is that by virtue of their smaller volume mouse embryos are better able to satisfy their oxygen requirement *in vitro*, and therefore their capacity for aerobic respiration, than bovine embryos (Byatt-Smith *et al.*, 1991).

When ICM and trophectoderm are isolated from the blastocyst, the paracrine signalling mechanisms governing their metabolism *in situ* may be lost, resulting in self-directed regulation. In addition, increased culture-induced stress of both lineages may have occurred due to a loss of homeostatic regulation and protection within the intact blastocyst. The differences in metabolism may also be attributed to alterations in the microenvironment of the trophectoderm and the ICM. The apical and basolateral surfaces of trophectoderm cells are normally exposed to different environments (external and blastocoel fluid), whereas the ICM is normally exposed to a single specialized microenvironment, that is, blastocoel fluid.

Culture of *in vivo*- and *in vitro*-derived bovine embryos results in alanine production and arginine and leucine consumption (Lee and Fukui, 1996; Partridge and Leese, 1996; Jung *et al.*, 1998; Kuran *et al.*, 2002). A similar pattern was observed for both the intact and composite blastocysts. The composite blastocyst was metabolically more active in terms of amino acid turnover than the intact blastocyst, as it was with pyruvate, glucose and lactate, possibly reflecting alterations in trophectoderm and ICM metabolism due to a change in culture environment upon the loss of the blastocoel fluid.

The concentrations of pyruvate, glucose and lactate in blastocoel fluid differed significantly from those of the external medium. Pyruvate and glucose concentrations were lower in blastocoel fluid than in SOFaaBSA, whereas lactate concentration was higher. These results parallel data recorded in mice, rats and rabbits (Quinn and Wales, 1973; Brison *et al.*, 1993), except that in rats, pyruvate concentration was higher in blastocoel fluid than externally (Brison *et al.*, 1993). The lower pyruvate and glucose concentrations in bovine blastocoel fluid

may be due to slow transport into the cavity, via facilitated transport or utilization by the ICM and trophectoderm or both (Brison *et al.*, 1993; Hewitson and Leese, 1993).

The concentrations of substrates in blastocoel fluid are probably maintained within a range optimal for ICM development (Brison *et al.*, 1993). In this respect, it has been demonstrated that high external concentrations of glucose inhibit ICM development within rat blastocysts (De Hertogh *et al.*, 1991). The homeostatic mechanism used by preimplantation embryos to regulate substrate concentrations within the blastocoel cavity is dependent upon the expression and localization of glucose transporters (GLUTs). Pantaleon and Kaye (1998) suggested that the apical distribution of GLUT3 on trophectoderm membranes is responsible for the uptake of external glucose, which in combination with the basolaterally expressed GLUT1 provides glucose to the ICM. The localization of GLUT2 to membranes facing the blastocoel fluid may be indicative of a regulatory role in maintaining glucose concentration in the cavity; however, the precise mechanism remains to be determined.

Aspartate, glutamate, glycine and tryptophan were present at higher concentrations in blastocoel fluid compared with the external environment. However, the reverse was observed for threonine and asparagine concentrations, which were significantly lower in blastocoel fluid. It is possible that the high glutamate concentration within the cavity was due to its formation from α -ketoglutarate as the final reaction in amino acid deamination. Alanine appears in increasing amounts at every stage of development of bovine preimplantation embryos (Partridge and Leese, 1996; Jung *et al.*, 1998; Kuran *et al.*, 2002). Hence, it is not surprising that it appears in such high concentrations within the blastocoel cavity (2.9 mmol l^{-1}). In contrast, the concentrations of threonine, glutamine and asparagine were at lower or similar concentrations in the blastocoel to those of the external medium. A number of studies have revealed the presence of Na^+ -dependent and -independent transporters in the blastocyst. However, their specific localization in the ICM and trophectoderm, and role in determining the amino acid profile of blastocoel fluid, remain to be determined (Miller and Schultz, 1985; Jamshidi and Kaye, 1995; Van Winkle, 2001).

In conclusion, the intact blastocyst and isolated cell lineages differ markedly in their utilization and production of glucose, lactate, pyruvate and amino acids. Our data indicate that, upon isolation, the ICM and trophectoderm lose metabolic control, possibly through the absence of paracrine interactions or culture-induced cellular stress or both. This culminates in increased metabolic activity, as illustrated by the discrepancy between the 'intact' and 'composite' blastocysts.

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