Ammonium exposure and pyruvate affect the amino acid metabolism of bovine blastocysts *in vitro*

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Abstract

The accumulation of ammonium is a major artefact of *in vitro* embryo culture. This study has examined ammonium production and potential mechanisms of disposal in preimplantation bovine blastocysts. Embryos were produced by *in vitro* maturation and fertilisation of oocytes, and cultured in synthetic oviduct fluid containing amino acids and BSA (SOFaaBSA). Ammonium/urea concentrations were determined enzymatically. Amino acid appearance/disappearance 'profiles' of single blastocysts were determined at 0, 1.25 and 2.5 mM NH₄Cl (with or without 0.33 mM pyruvate), and with or without 10 mM dipicolinic acid (DPCA; a glutamate dehydrogenase (GLDH) inhibitor) or 2 mM amino-oxyacetate (AOA; a transaminase inhibitor). Free ammonium was produced at a rate of 4.281 ($\pm$ 0.362) pmol/embryo/h, while urea production was undetectable. The presence/absence of pyruvate affected amino acid profiles, especially alanine appearance ($P < 0.001$), glutamate disappearance ($P < 0.05$) and overall turnover (the sum of appearance and disappearance) ($P < 0.001$). GLDH inhibition with DPCA had no effect on amino acid overall disappearance, but glutamate disappearance increased, while that of arginine decreased ($P < 0.05$). The transaminase inhibitor, AOA, depressed turnover ($P < 0.05$), aspartate and glutamate disappearance, and alanine appearance. Thus, bovine blastocysts release ammonium as free ions or fix them, not as urea, but as alanine, possibly glutamine and, less likely, arginine. An active role for GLDH and transaminases in regulating blastocyst amino acid metabolism was demonstrated.

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Introduction

Although preimplantation embryos can be cultured in the absence of amino acids (Caro & Trounson 1986, Fissore *et al.* 1989), their inclusion in both fertilisation and culture media enhances development and blastocyst cell numbers in the mouse (Gwatkin 1966, Spindle & Pedersen 1973, Ali *et al.* 1993, Gardner & Lane 1993, Ho *et al.* 1995, Lane & Gardner 1997*a,b*, Nakazawa *et al.* 1997, Biggers *et al.* 2000, Summers *et al.* 2000), cattle (Lee & Fukui 1996), human (Devreker *et al.* 2001) and hamster (Kane & Bavister 1988, McKiernan *et al.* 1995). Amino acids serve a variety of physiological functions, including: the synthesis of proteins and nucleotides (Epstein & Smith 1973, Alexiou & Leese 1992, Katchadourian *et al.* 1994), nutrition and energy provision (Lane & Gardner 1997*a*, 1998, Gardner 1998, Houghton *et al.* 2002), osmoregulation (Van Winkle & Campione 1996, Dumoulin *et al.* 1997, Dawson *et al.* 1998), protection against oxidative stress (Lindenbaum 1973, Nasr-Esfahani *et al.* 1992), pH regulation (Bavister & McKiernan 1993, Edwards *et al.* 1998),

signalling molecule biosynthesis (Wu & Morris 1998), trophoblast differentiation (Martin & Sutherland 2001) and basement membrane formation between primitive endoderm and ectoderm (Biggers *et al.* 2000). It is not surprising, therefore, that supplementing embryo culture medium with a mixture of amino acids reduces culture-induced metabolic perturbations and associated loss of viability in murine blastocysts (Lane & Gardner 1998) and overcomes developmental blocks in their leporine counterparts (Liu *et al.* 1996). It has also been suggested that *in vitro* produced embryos should be exposed to amino acids as early as the oocyte stage, as this increases oocyte maternal mRNA levels and promotes preimplantation development (Watson *et al.* 2000). In support of this opinion, it is notable that a brief exposure of zygotes to amino acid-free conditions depresses their developmental capacity and blastocyst cell numbers (Gardner & Lane 1996).

Exogenous amino acid consumption and incorporation by preimplantation murine and bovine embryos increases

throughout development, particularly after the 8-cell and compaction stages (Tasca & Hillman 1970, Brinster 1971, Epstein & Smith 1973, Kaye *et al.* 1982, Partridge & Leese 1996). Although beneficial effects on preimplantation development may be obtained through the addition of amino acids *in vitro*, their spontaneous breakdown above that resulting from metabolic deamination can compromise development through increases in ammonium concentration (Gardner & Lane 1993, Katchadourian *et al.* 1996). Exposure of ruminant and rodent preimplantation embryos to ammonium results in reduced intracellular pH, depressed oxidative phosphorylation (Lane *et al.* 2002), decreased blastocyst cell number (Gardner & Lane 1993), altered developmental kinetics, fetal retardation (McEvoy *et al.* 1997, Sinclair *et al.* 1999) and exencephaly (Lane & Gardner 1994). Intriguingly, increased ammonium production also appears to be a product of protein-free culture conditions (Berg *et al.* 2002). In the bovine embryo, ammonium toxicity depends on both the concentration and the stage of development during which exposure occurs. Thus, exposure to ammonium during *in vitro* fertilisation at modest concentrations in the range 29–88 μM improves subsequent development, whilst exposure during preimplantation culture at any concentration is detrimental (Hammon *et al.* 2000a).

The mechanism(s) by which ammonia is detrimental – or beneficial – to gametes and embryos is unclear. Gardner and Lane (1993) have proposed that ammonia may mediate its adverse effects through a decrease in the concentration of α -ketoglutarate, by promoting its conversion to glutamate. This would result in a reduction in flux through the tricarboxylic acid (TCA) cycle and reduce ATP production. Hammon *et al.* (2000a) suggest that this could limit ATP production *post* compaction consistent with the observed decrease in blastocyst (production) rate. These authors have also suggested that the vulnerability of the embryo after compaction may be due to the accumulation of ammonium in the blastocoel fluid through Na^+/K^+ ATPase and $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter activity. To overcome these problems *in vitro*, Lane and Gardner (1995) devised a technique for the *in situ* enzymatic removal of ammonium in culture, which increased murine blastocyst cell number, implantation rate, fetal development and weight after transfer.

Stress due to exposure of preimplantation embryos to excess ammonium may also occur *in vivo*, particularly in domestic ruminants. This occurs when animals consume excess rapidly degradable nitrogen in the absence of readily fermentable carbohydrate (e.g. as found in spring pasture), or when feed is supplemented with excess urea (McDonald *et al.* 1995, McEvoy *et al.* 1997, Sinclair *et al.* 1999). Urea is hydrolysed by the urease activity of rumen microorganisms, resulting in the production of ammonium (McDonald *et al.* 1995). In this case, the rumen microflora cannot maximise microbial protein synthesis from dietary nitrogen, urea or ammonium. Furthermore, dietary protein may also be deaminated and used as a microbial

energy source, thereby releasing even larger amounts of ammonium into the circulation and increasing the risk of toxicity before it is converted to urea and removed by the kidneys (McDonald *et al.* 1995, Papadopoulos *et al.* 2001). Effects on fertility are particularly evident when such dietary changes are implemented around the time of mating or insemination (Papadopoulos *et al.* 2001, Dawuda *et al.* 2002). Whether elevated systemic concentrations of ammonia/ammonium ($\text{pK}_a = 9.24$) or urea in ruminants reduce embryo survival by disrupting the follicular, oviductal and/or uterine environments remains a topic of discussion (Fahey *et al.* 2001, Papadopoulos *et al.* 2001, Kenny *et al.* 2002). Nonetheless, it has been demonstrated that cattle with increased circulatory levels of urea had altered uterine fluid composition, decreased uterine pH and reduced conception rates (Papadopoulos *et al.* 2001). Despite having no effect on ovulation rate (Fahey *et al.* 2001), elevated systemic urea adversely affects oocytes and/or the follicular environment, and leads to reduced embryo development and quality, in terms of disrupted blastocyst metabolism, possibly through alterations in reproductive tract pH (McEvoy *et al.* 1997, Hammon *et al.* 2000b, Fahey *et al.* 2001, Papadopoulos *et al.* 2001, Dawuda *et al.* 2002). This may affect embryos in the long-term through ‘reprogramming’ during the earliest stages of embryo development (McEvoy *et al.* 1997, Kwong *et al.* 2000).

The mechanism(s) by which early embryos dispose of ammonium has been little investigated. Partridge and Leese (1996) and Donnay *et al.* (1999) suggested that pyruvate, after transamination to alanine, may potentially be used as an ammonium sink, thereby preventing the build-up of ammonium ions in the culture medium. The possibility that embryos (as undifferentiated cells) possess the urea cycle, localised in the adult to the liver, has never been examined.

In order to study the effects of ammonium in bovine blastocysts, we have: (i) measured ammonium production during embryo culture; (ii) tested whether embryos produce urea; (iii) investigated the effects of ammonium loading on blastocyst amino acid metabolism in the presence and absence of pyruvate, over a range of ammonium concentrations; and (iv) examined the metabolic effect(s) of inhibiting glutamate dehydrogenase (GLDH) and transaminase enzymes in general.

Materials and Methods

Culture of bovine embryos

All chemicals were purchased from Sigma, Poole, Dorset, UK, unless otherwise specified. Cattle embryos were generated by *in vitro* maturation and fertilisation of oocytes aspirated from abattoir-derived ovaries, as previously described (Thompson *et al.* 1996). Briefly, oocyte–cumulus complexes were matured for 24 h in tissue culture medium 199 supplemented with 10% fetal calf serum, 1 $\mu\text{g}/\text{ml}$

oestradiol, 10.9 ng/ml fibroblast growth factor, 0.47 µg/ml epidermal growth factor, 0.025 IU luteinising hormone (Ferring Pharmaceuticals, Slough, Berks, UK), 0.025 IU follicle-stimulating hormone (Ferring Pharmaceuticals), 24 µg/ml apo-transferrin and 1 mM pyruvate. Fertilisation for 18 h was performed in Fertilisation Tyrode's Albumin Lactate Pyruvate (Fert TALP) medium with 1×10^6 sperm/ml freeze-thawed semen from a sire of proven fertility.

Presumptive zygotes were cultured in groups of 14 in 20 µl drops of synthetic oviduct fluid supplemented with amino acids and bovine serum albumin (SOFaaBSA; Tervit *et al.* 1972) under a mineral oil overlay at 39°C and a humidified 5% O₂/5% CO₂/90% N₂ atmosphere, until allocated to their experimental groups after 168 h of culture in this medium.

Experimental media and amino acid profiling

We have previously determined that chronic exposure to 0.5 and 1.5 mM ammonium chloride affects murine embryo cell number and blastocyst rate respectively, with 3.0 mM being the highest concentration permissive to development (N M Orsi & H J Leese, unpublished data). We therefore tested the effects of acute ammonium loading at concentrations of 0, 1.25 and 2.5 mM on bovine embryo amino acid profiles (see below) in the presence or absence of 0.33 mM pyruvate. Single expanding blastocysts of comparable, good morphology were selected at 168 h of culture and were placed in 1 µl incubation drops under mineral oil at 39°C under a humidified 5% CO₂ atmosphere for 12 h. In order of increasing ammonium concentration (0 (control), 1.25 and 2.5 mM), and in the presence of pyruvate, 29, 27 and 19 single blastocysts respectively, had their amino acid profiles quantified. In the absence of pyruvate, 41, 20 and 18 embryos respectively, were used.

In order to examine the potential ammonium fixation and amino acid catabolic pathways in the preimplantation bovine embryo, amino acid profiles were also determined for blastocysts cultured in ammonium-free SOFaaBSA in the presence of 10 mM dipicolinic acid (DPCA) ($n = 15$ replicates), an inhibitor of GLDH, and 2 mM amino-oxyacetate (AOA), a generic transaminase inhibitor ($n = 36$ replicates) (Broeder *et al.* 1994, Hewitson *et al.* 1996).

Amino acid analyses

Spent incubation drops were diluted 1:40 with high-performance liquid chromatography (HPLC)-grade water (Fisher Scientific, Loughborough, Leics, UK) and analysed by reverse-phase HPLC on a Kontron 500 Series automated HPLC system fitted with a Jasco F920 fluorescence detector and a 4.5 × 250 mm Hypersil ODS-16 column (Jones Chromatography, Hengoed, Mid Glamorgan, UK), as previously described (Houghton *et al.* 2002). As an internal, non-metabolisable standard to all SOFaaBSA formulations, 0.5 mM D-α-amino butyric acid (Sigma) was

added. Using this method, it was not possible to measure proline or cysteine.

Quantification of ammonium and urea production

Early day 7 cattle blastocysts (day 0 = fertilisation) were placed in groups of nine in 15 µl SOFaaBSA for 40 h, and allowed to undergo expansion and hatching ($n = 7$ replicates). Spent culture medium samples (10 µl) were diluted 1:2.5 with sterile water and assayed for ammonium with a COBAS MIRA autoanalyser (Roche Instruments, UK) using a commercial kit (Sigma) for the quantitative enzymatic determination of ammonia. The method was based on the reductive amination of 2-oxoglutarate, using GLDH and NADPH. The decrease in absorbance at 340 nm, due to the oxidation of NADPH, was proportional to the ammonium concentration. Unknown concentrations were determined by reference to a standard curve.

Urea measurements were initially performed with nine early cattle blastocysts incubated in 15 µl of SOFaaBSA for 40 h ($n = 9$ replicates). However, urea production could not be detected, so the number of embryos, their density and incubation time were therefore increased to 30 blastocysts in 15 µl for 30 h ($n = 6$ replicates). Spent culture drops that had contained 20 embryos/20 µl, displaying a blastocyst rate of at least 20% after 192 h of culture, were also assayed ($n = 13$ replicates). Analyses were performed on a COBAS MIRA autoanalyser using a commercial kit (Sigma) for the quantitative enzymatic determination of urea after its conversion to ammonium by urease. The decrease in absorbance at 340 nm, due to oxidation of NADH, was proportional to urea concentration. Unknown concentrations were determined by reference to a standard curve.

Data presentation and statistical analysis

Ammonium and amino acid appearance/depletion were expressed in pmol/embryo/h. Amino acid profiles with increasing concentrations of ammonium in standard or pyruvate-free SOFaaBSA were compared by a mixed model nested (hierarchical) two-way analysis of variance using a general linear model to account for imbalances in the statistical design, with a *post hoc* Bonferroni test to determine statistical differences between individual groups. Non-parametric sets were compared by Scheirer-Ray-Hare tests (by ranking and cumulative χ^2 -square). The same approach was applied to comparisons with the inhibitors AOA and DPCA. Relative turnover amino acid profiles were expressed as % turnover (see below). All percentages were arcsine log transformed for statistical analysis, and all data were $\pm$ S.E.M.

The following terminology was adopted to describe amino acid profiles: (i) quantitative: refers to the appearance/disappearance of individual amino acids; (ii) overall appearance/disappearance refers to the sum of all amino acids appearing/disappearing; (iii) turnover refers to the sum of 'absolute' values (i.e. ignoring sign) for amino

acid appearance and disappearance; and (iv) relative turnover is applied to appearance/disappearance profiles, expressed as a percentage of turnover on a per embryo basis, thus accounting for differences in metabolic activity between individual embryos.

Results

Ammonium and urea production by bovine blastocysts

Ammonium was produced by cattle blastocysts at a rate of $4.281 (\pm 0.362)$ pmol/embryo/h during the blastocyst expansion and hatching period. The production of urea by cattle embryos was undetectable in all samples assayed.

Effects of pyruvate and ammonium on bovine blastocyst amino acid profile

Amino acid 'appearance' data were assigned positive values, while 'disappearance' data were given negative values. The presence or absence of pyruvate affected the quantitative profiles of histidine, glycine, tryptophan ($P < 0.05$) and alanine ($P < 0.001$), as well as overall amino acid appearance and turnover ($P < 0.001$). By contrast, ammonium addition affected the profile of asparagine ($P < 0.05$) and valine ($P < 0.001$), whereas that of aspartate and glutamate ($P < 0.05$) was affected by both ammonium and pyruvate. Overall quantitative amino acid disappearance, and the profiles of serine, glutamine, threonine, arginine, tyrosine, methionine, phenylalanine, isoleucine, leucine and lysine were all unaffected by either ammonium or pyruvate. These data are illustrated in Table 1.

While overall quantitative amino acid disappearance was not affected by the presence of pyruvate or ammonium (-12.165 ± 1.081 to -19.151 ± 2.317 pmol/embryo/h range), overall quantitative appearance was significantly decreased in the absence of pyruvate in the medium ($P < 0.01$). In particular, the 1.25 mM ammonium pyruvate group appearance was significantly higher than that of the pyruvate-free control (14.927 ± 1.249 and 9.500 ± 0.967 pmol/embryo/h respectively; $P < 0.05$), while none of the other values differed significantly. Amino acid turnover was significantly reduced by the absence of pyruvate ($P < 0.05$) (Fig. 1).

The presence/absence of pyruvate affected the relative turnover profiles of aspartate, serine, alanine ($P < 0.001$), glycine, tryptophan ($P < 0.01$) and isoleucine ($P < 0.05$). Ammonium loading affected histidine profile ($P < 0.05$). The methionine profile ($P < 0.01$) was affected by both parameters. Relative turnover profiles of glutamate, asparagine, glutamine, threonine, arginine, tyrosine, valine, phenylalanine, leucine and lysine were all unaffected by either ammonium or pyruvate (Table 2). Relative turnover, overall appearance and disappearance were also unaffected by these compounds (ranges of 40.665 ± 6.469 to 55.432 ± 2.968 and 44.568 ± 2.968 to 59.335 ± 6.469 respectively).

Table 1 Quantitative amino acid profiles of bovine blastocysts incubated in the presence or absence of pyruvate at various ammonium concentrations.

Amino acid	0.33 mM Pyruvate control	0.33 mM Pyruvate NH ₄ Cl	0.33 mM Pyruvate 1.25 mM NH ₄ Cl	0.33 mM Pyruvate 2.5 mM NH ₄ Cl	Pyruvate-free control	Pyruvate-free 1.25 mM NH ₄ Cl	Pyruvate-free 2.5 mM NH ₄ Cl
ASP	-4.119 ± 0.316^a	-3.841 ± 0.314^{ac}	-3.841 ± 0.314^{ac}	-4.090 ± 0.331^a	-1.028 ± 0.225^b	-1.710 ± 0.325^{bc}	-2.485 ± 0.414^c
GLU	-1.844 ± 0.351^a	-1.210 ± 0.368^{ac}	-1.210 ± 0.368^{ac}	-1.495 ± 0.456^{ad}	0.215 ± 0.231^b	-0.350 ± 0.298^{bcd}	-1.181 ± 0.356^{ac}
ASN	-0.140 ± 0.073^{ab}	0.146 ± 0.060^a	0.146 ± 0.060^a	-0.045 ± 0.097^{ab}	-0.989 ± 0.431^b	0.077 ± 0.117^{ab}	-0.061 ± 0.100^{ab}
SER	-0.230 ± 0.096	-0.278 ± 0.071	-0.278 ± 0.071	-0.493 ± 0.105	-0.174 ± 0.195	-0.397 ± 0.257	0.032 ± 0.246
HIS	-0.450 ± 0.507	0.006 ± 0.329	0.006 ± 0.329	-0.740 ± 0.375	0.445 ± 0.250	0.156 ± 0.245	0.032 ± 0.181
GLN	0.433 ± 0.637	1.638 ± 0.663	1.638 ± 0.663	0.156 ± 0.843	-1.389 ± 0.745	0.735 ± 0.778	0.287 ± 1.090
GLY	0.501 ± 0.102	0.299 ± 0.069	0.299 ± 0.069	0.261 ± 0.126	0.201 ± 0.160	-0.089 ± 0.207	0.029 ± 0.164
THR	-0.359 ± 0.341	0.532 ± 0.257	0.532 ± 0.257	-0.703 ± 0.543	-0.345 ± 0.194	-0.277 ± 0.304	-0.558 ± 0.362
ARG	-4.864 ± 0.640	-3.115 ± 0.361	-3.115 ± 0.361	-4.528 ± 0.678	-3.857 ± 0.412	-3.463 ± 0.655	-3.700 ± 0.654
ALA	7.523 ± 0.593^a	7.289 ± 0.619^a	7.289 ± 0.619^a	7.668 ± 1.016^a	2.508 ± 0.294^b	3.674 ± 0.481^b	4.168 ± 0.515^b
TYR	-0.056 ± 0.101	0.184 ± 0.056	0.184 ± 0.056	-0.045 ± 0.060	-0.121 ± 0.067	0.038 ± 0.067	-0.201 ± 0.145
TRP	-0.0253 ± 0.064	0.115 ± 0.046	0.115 ± 0.046	0.253 ± 0.104	-0.099 ± 0.107	-0.052 ± 0.153	-0.0581 ± 0.100
MET	-0.098 ± 0.123	-0.110 ± 0.097	-0.110 ± 0.097	-0.033 ± 0.143	0.006 ± 0.223	0.617 ± 0.328	-0.125 ± 0.117
VAL	-1.021 ± 0.273^{ac}	1.195 ± 0.536^b	1.195 ± 0.536^b	-0.542 ± 0.408^{ac}	-0.381 ± 0.292^{ac}	-0.345 ± 0.301^{ab}	-0.174 ± 0.371^{ab}
PHE	-0.167 ± 0.116	0.204 ± 0.069	0.204 ± 0.069	-0.104 ± 0.090	-0.0394 ± 0.121	-0.027 ± 0.097	-0.070 ± 0.174
ILE	-1.291 ± 0.208	-0.383 ± 0.183	-0.383 ± 0.183	-0.932 ± 0.291	-0.919 ± 0.256	-0.469 ± 0.299	-0.896 ± 0.422
LEU	-0.584 ± 0.216	-0.340 ± 0.125	-0.340 ± 0.125	-0.672 ± 0.103	-0.488 ± 0.122	-0.115 ± 0.124	-1.495 ± 1.189
LYS	0.805 ± 0.428	0.431 ± 0.287	0.431 ± 0.287	0.934 ± 0.429	0.964 ± 0.481	-0.118 ± 0.544	-0.484 ± 0.510

Within each amino acid, values with no superscripts in common differ significantly ($P < 0.05$).

Figure 1 Amino acid turnover of bovine blastocysts incubated in the presence or absence of pyruvate at various ammonium concentrations.

Effects of DPCA and AOA on bovine blastocyst amino acid profile

Quantitatively, DPCA affected the appearance/disappearance of glutamate, glutamine, threonine, arginine, valine and isoleucine, while AOA affected the appearance/disappearance of aspartate, glutamate, serine, glycine, alanine, valine and isoleucine ($P < 0.05$) (Table 3). Overall appearance with 10 mM DPCA was significantly higher than with 2 mM AOA ($P < 0.05$) while control values were intermediate (16.620 ± 1.722 , 10.300 ± 1.035 and 13.452 ± 1.249 respectively). By contrast, overall disappearance in the control group was significantly higher than with 2 mM AOA ($P < 0.05$), while 10 mM DPCA values were intermediate (-19.150 ± 2.317 , -13.773 ± 1.035 and -14.446 ± 1.291 respectively). AOA also significantly reduced overall amino acid turnover, compared with control and DPCA values (24.072 ± 1.345 compared with 32.603 ± 2.176 and 31.066 ± 2.014 respectively) ($P < 0.05$). DPCA differed from AOA by displaying higher aspartate and glutamate disappearance, lower arginine and serine disappearance, higher glycine appearance, lower threonine appearance, and higher overall appearance ($P < 0.05$).

DPCA affected the relative turnover profiles of glutamate, histidine, arginine ($P < 0.001$), tyrosine, tryptophan, phenylalanine and isoleucine ($P < 0.05$). AOA affected the metabolism of threonine ($P < 0.001$), tryptophan ($P < 0.01$), phenylalanine and isoleucine ($P < 0.05$) (Table 4). Neither inhibitor had significant effects upon relative turnover overall appearance (control 43.327 ± 3.565 ; 10 mM DPCA 51.497 ± 4.580 and 2 mM AOA 43.028 ± 3.112) or disappearance (control 56.673 ± 3.565 , 10 mM DPCA 48.503 ± 4.580 and 2 mM AOA 56.972 ± 3.112).

Discussion

We were unable to detect the formation of urea by early bovine embryos. Although such embryos are unlikely to be exposed to excessive ammonium concentrations in the

maternal tract under proper herd management or in the wild, they nonetheless release free NH_4^+ . The average production of $4.281 \text{ pmol/embryo/h}$ over the cavitation to hatching period is comparable to the $1.0 \text{ pmol/embryo/h}$ determined for the mouse blastocyst (Gardner & Lane 1993), as cattle embryos are roughly four times metabolically more active than their murine counterparts (Leese & Barton 1984, Thompson *et al.* 1996). Since the embryos tested were all blastocysts of comparable morphology, our observations do not preclude that ammonium production may be related not only to developmental stage, but also to embryo quality.

The overall amino acid appearance/disappearance profiles observed were similar to those reported by Partridge and Leese (1996) and Jung *et al.* (1998). Some amino acids were depleted from the medium while others, notably alanine, appeared. The present data indicated a significant decrease in alanine production in the absence of pyruvate, in agreement with the findings of Donnay *et al.* (1999). These data suggest that pyruvate can be transaminated to alanine in cattle blastocysts, thereby providing a route for the embryo to dispose of ammonia, in addition to that lost directly as NH_4^+ (Fig. 2). However, there was no significant dose–response increase in alanine appearance with increasing concentrations of ammonium. This could be interpreted as meaning that alanine production is not an adaptive response geared towards detoxification of increasing concentrations of ammonium in the embryo (since the alanine transaminase reaction is not regulated (Zubay 1993)), and/or that the transamination pathway is saturated at low concentrations of ammonium. Decreases in bovine blastocyst alanine output have also been documented in instances of protein deprivation in culture (Kuran *et al.* 2002, Orsi & Leese 2003). However, it is speculated that the relatively brief pyruvate starvation period will have been insufficient to alter embryo protein content/protein synthesis significantly. Glutamate may also be involved in the fixation of ammonium via its conversion to glutamine, catalysed by glutamine synthetase (Fig. 2) (Salway 1996), as observed during systemic hyperammonaemia (Stryer 1988). Quantitatively, glutamate

Table 2 Relative turnover amino acid profiles of bovine blastocysts incubated in the presence or absence of pyruvate at various ammonium concentrations.

Amino acid	0.33 mM Pyruvate control	0.33 mM Pyruvate NH ₄ Cl	0.33 mM Pyruvate NH ₄ Cl	Pyruvate-free control	Pyruvate-free 1.25 mM NH ₄ Cl	Pyruvate-free 2.5 mM NH ₄ Cl
ASP	16.908 ± 4.545 ^a	14.247 ± 0.962 ^a	14.025 ± 0.936 ^a	5.814 ± 0.615 ^b	7.861 ± 1.001 ^{ab}	9.708 ± 1.307 ^{ab}
GLU	6.220 ± 0.760	5.343 ± 0.882	5.020 ± 0.914	5.235 ± 0.686	4.721 ± 0.781	5.269 ± 1.001
ASN	0.943 ± 0.125	0.899 ± 0.144	0.925 ± 0.176	4.027 ± 1.427	1.686 ± 0.360	1.780 ± 0.462
SER	1.836 ± 0.603 ^a	1.332 ± 0.222 ^a	1.933 ± 0.205 ^a	3.319 ± 0.683 ^{ab}	2.865 ± 0.552 ^{ab}	5.335 ± 1.180 ^b
HIS	5.839 ± 1.042 ^a	3.104 ± 0.782 ^{ab}	3.238 ± 0.651 ^{ab}	4.640 ± 0.670 ^{ab}	3.755 ± 1.321 ^{ab}	1.474 ± 0.254 ^b
GLN	9.372 ± 1.413	9.749 ± 1.325	10.589 ± 1.635	12.983 ± 1.109	13.336 ± 1.990	10.171 ± 2.350
GLY	2.085 ± 0.238 ^{ab}	1.493 ± 0.242 ^a	1.187 ± 0.189 ^a	3.215 ± 0.501 ^b	2.134 ± 0.755 ^{ab}	2.941 ± 0.554 ^{ab}
THR	4.568 ± 0.490	3.607 ± 0.597	5.731 ± 0.818	4.239 ± 0.396	4.888 ± 1.067	4.109 ± 0.532
ARG	17.929 ± 3.840	12.088 ± 1.320	13.584 ± 1.509	15.966 ± 1.180	16.249 ± 1.573	16.534 ± 1.885
ALA	30.269 ± 7.370 ^a	26.917 ± 1.802 ^a	24.927 ± 1.588 ^{ab}	11.026 ± 1.268 ^b	15.082 ± 1.577 ^{ab}	16.539 ± 1.999 ^{ab}
TYR	1.120 ± 0.161	0.964 ± 0.140	0.706 ± 0.131	1.141 ± 0.159	1.166 ± 0.167	1.211 ± 0.214
TRP	0.781 ± 0.158	0.580 ± 0.173	0.901 ± 0.336	1.760 ± 0.256	1.853 ± 0.844	1.371 ± 0.419
MET	2.406 ± 0.978 ^{abcd}	1.238 ± 0.256 ^{ad}	1.594 ± 0.299 ^{abcd}	4.162 ± 0.588 ^{bc}	5.063 ± 1.183 ^c	0.939 ± 0.308 ^d
VAL	5.243 ± 1.555	7.003 ± 1.918	4.509 ± 0.592	5.179 ± 0.501	4.600 ± 0.809	5.398 ± 0.822
PHE	1.501 ± 0.244	1.249 ± 0.156	1.002 ± 0.147	2.001 ± 0.556	1.591 ± 0.228	1.367 ± 0.315
ILE	4.735 ± 0.832	2.677 ± 0.406	3.316 ± 0.494	4.742 ± 0.433	3.931 ± 0.735	5.367 ± 0.592
LEU	4.392 ± 1.654	2.397 ± 0.285	2.620 ± 0.382	2.325 ± 0.407	1.925 ± 0.429	5.459 ± 2.537
LYS	6.049 ± 1.097	5.112 ± 0.809	5.103 ± 0.654	8.227 ± 1.066	7.294 ± 1.545	5.028 ± 0.950

Within each amino acid, values with no superscripts in common differ significantly ($P < 0.05$).**Table 3** Quantitative amino acid appearance and disappearance of blastocysts incubated with 10 mM DPCA or 2 mM AOA.

Amino acid	Control	10 mM DPCA	2 mM AOA
ASP	-4.119 ± 0.316 ^a	-4.545 ± 0.410 ^a	-2.482 ± 0.191 ^b
GLU	-1.844 ± 0.351 ^a	-3.426 ± 0.404 ^b	-0.619 ± 0.272 ^c
ASN	-0.140 ± 0.073	0.189 ± 0.059	0.108 ± 0.128
SER	-0.230 ± 0.096 ^a	0.068 ± 0.027 ^a	-0.394 ± 0.071 ^b
HIS	-0.450 ± 0.507	0.064 ± 1.223	0.078 ± 0.399
GLN	0.433 ± 0.637 ^a	3.003 ± 0.989 ^{bc}	1.023 ± 0.623 ^{ac}
GLY	0.501 ± 0.102 ^a	0.427 ± 0.190 ^a	-0.027 ± 0.129 ^b
THR	-0.358 ± 0.341 ^a	1.512 ± 0.430 ^b	-0.154 ± 0.231 ^a
ARG	-4.864 ± 0.640 ^a	-1.869 ± 0.634 ^b	-4.407 ± 0.370 ^a
ALA	7.523 ± 0.593 ^a	6.124 ± 0.624 ^{ab}	4.958 ± 0.370 ^b
TYR	-0.056 ± 0.101	0.098 ± 0.047	0.010 ± 0.108
TRP	-0.025 ± 0.064	0.058 ± 0.021	-0.012 ± 0.023
MET	-0.098 ± 0.123	-0.080 ± 0.149	-0.048 ± 0.113
VAL	-1.021 ± 0.273 ^a	0.399 ± 0.190 ^b	0.072 ± 0.484 ^b
PHE	-0.167 ± 0.116	0.063 ± 0.063	0.091 ± 0.147
ILE	-1.291 ± 0.208 ^a	-0.260 ± 0.169 ^b	-0.263 ± 0.311 ^b
LEU	-0.584 ± 0.216	-0.641 ± 0.169	-0.316 ± 0.311
LYS	0.805 ± 0.428	0.993 ± 0.309	0.366 ± 0.268

Values with no superscripts in common differ significantly ($P < 0.05$).**Table 4** Relative turnover amino acid appearance and disappearance of bovine blastocysts incubated with 10 mM DPCA or 2 mM AOA.

Amino acid	Control	10 mM DPCA	2 mM AOA
ASP	16.908 ± 4.545	14.114 ± 1.197	11.005 ± 0.631
GLU	6.220 ± 0.760 ^a	11.010 ± 1.213 ^b	5.613 ± 0.634 ^z
ASN	0.943 ± 0.125	0.915 ± 0.173	0.989 ± 0.149
SER	1.836 ± 0.603	2.467 ± 0.461	1.495 ± 0.238
HIS	5.839 ± 1.042 ^a	12.245 ± 2.247 ^b	4.317 ± 0.984 ^a
GLN	9.372 ± 1.413	10.855 ± 2.081	8.701 ± 1.169
GLY	2.085 ± 0.238	1.685 ± 0.476	1.679 ± 0.193
THR	4.568 ± 0.490 ^a	6.148 ± 0.974 ^a	3.050 ± 0.441 ^b
ARG	17.929 ± 3.840 ^{ab}	8.092 ± 1.785 ^a	19.613 ± 1.654 ^b
ALA	30.269 ± 7.370	19.235 ± 2.035	21.689 ± 1.112
TYR	1.120 ± 0.161 ^a	0.453 ± 0.080 ^b	0.958 ± 0.182 ^{ab}
TRP	0.781 ± 0.159 ^a	0.283 ± 0.059 ^b	0.411 ± 0.055 ^b
MET	2.406 ± 0.978	0.812 ± 0.393	1.989 ± 0.326
VAL	5.242 ± 1.555	2.207 ± 0.370	5.388 ± 0.725
PHE	1.501 ± 0.244 ^a	0.728 ± 0.151 ^b	1.306 ± 0.187 ^b
ILE	4.735 ± 0.832 ^a	1.867 ± 0.365 ^b	3.899 ± 0.388 ^b
LEU	4.392 ± 1.654	2.206 ± 0.301	2.345 ± 0.327
LYS	6.049 ± 1.097	4.678 ± 0.793	5.553 ± 0.612

Values with no superscripts in common differ significantly ($P < 0.05$).

disappearance was affected by both pyruvate and ammonium. More glutamate disappeared with increasing ammonium concentration, but only in the absence of pyruvate available for transamination to alanine. Quantitative and relative turnover profiles of serine, glycine and tryptophan in the absence of pyruvate may be accounted for by the potential of these amino acids to be converted to pyruvate (Stryer 1988, Salway 1996), providing an alternative source of this α -ketocarboxylic acid to that supplied exogenously.

Amino acid turnover was depressed in the absence of pyruvate, most likely due to the decrease in alanine

Figure 2 Schematic representation of amino acid metabolism in the bovine blastocyst including the site of action of AOA and DPCA, and the potential pathways of ammonium generation and fate (inner cell mass and trophectoderm contributions are not distinguished). *Energy metabolism, protein synthesis, potential nitrogen fixation; ¹nitric oxide production, *not* urea production.

output, as this was the single most important amino acid that contributed to turnover. Intriguingly, there was no evidence that embryos compensated for the absence of pyruvate by a general increase in amino acid consumption. This inability to substitute alternative oxidative substrates for pyruvate has already been noted for the early cleavage stages in the mouse (Trimarchi *et al.* 2000).

The amino acid profiles obtained with 10 mM DPCA were unexpected, since GLDH catalyses the oxidative deamination of glutamate to α -ketoglutarate, and ultimately regulates oxidative consumption of amino acids (Stryer 1988). Not only was there no reduction in the disappearance or turnover of amino acids, but glutamate disappearance itself was increased. Assuming that DPCA is a specific inhibitor for GLDH, the concentration of this compound used may have been insufficient for complete inhibition, or there could have been increased glutamate conversion to glutamine (see above). Isoleucine disappearance was lower with DPCA, both quantitatively and relative to turnover. Isoleucine catabolism, like that of other branched-chain amino acids, involves conversion of α -ketoglutarate to glutamate through aminotransferase activity. It is unlikely that GLDH inhibition would have reduced the availability of α -ketoglutarate, as this can be generated through TCA cycle activity. One may speculate that the reduction in isoleucine disappearance may partly have accounted for the increase in glutamate disappearance with DPCA, as the one was roughly equivalent to the other on a mole:mole basis. Less arginine disappeared in the presence of DPCA. This may have reflected the increase in glutamate disappearance under these conditions, as arginine can be derived from glutamate (via carbamoyl phosphate, citrulline and argininosuccinate), thereby acting as an ammonium sink, without the involvement of the urea-producing arginase reaction (Fig. 2). Measurement of the activities of carbamoyl phosphate synthetase, ornithine transcarbamoylase, argininosuccinate synthetase and argininosuccinase might help to clarify this possibility.

AOA is a generic transaminase inhibitor that prevents the transfer of α -amino groups from donor amino acids to α -ketoacids. Quantitatively, the fall in aspartate and glutamate disappearance, and alanine appearance could be explained by the inhibition of their respective aminotransferases by AOA. However, the reasons for the alterations in glycine, valine, threonine, tryptophan and phenylalanine profiles were unclear. The fall in overall amino acid appearance and turnover could have been anticipated, in view of the fall in alanine appearance, which accounted for about 25% of turnover and around 80% of appearance.

In conclusion, bovine blastocysts produce free ammonium, but can also fix amino nitrogen/ammonium, not through the production of urea, but by the pyruvate-dependent synthesis of alanine and, potentially, of glutamine and arginine. Amino acid profiles of preimplantation blastocysts are affected by pyruvate availability, and, to a

lesser extent, by ammonium loading. The use of aminotransferase and GLDH inhibitors also affected amino acid profiles, indicating that these enzymes play an active role in embryo amino acid catabolism.

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