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Research

# Development, amino acid utilization and cell allocation in bovine embryos after *in vitro* production in contrasting culture systems

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**The effects of protein-supplemented and protein-free media on amino acid uptake, protein synthesis and cell differentiation in bovine blastocysts were investigated. Four formulations of synthetic oviduct fluid were used. Each formulation was identified by the principal supplement: bovine serum albumin (0.4%, w/v); polyvinyl alcohol (0.3%, w/v); or either of two steer sera (10%, v/v). After zygote culture, blastocyst yields (day 7.5) were lowest in protein-free medium and highest in albumin-supplemented medium. Subsequent 12 h incubation in the presence of both essential and non-essential amino acids was used for the measurement of amino acid flux. All blastocysts released alanine but consumed aspartate ($P < 0.001$) and the extent was influenced by prior culture conditions. Aspartate uptake was lower in blastocysts produced in protein-free conditions ($P < 0.05$) than in blastocysts produced in albumin-supplemented conditions. Consumption indices for 16 other amino acids were not influenced by blastocyst source. Cell counts and hatching incidences were highest for albumin-supplemented blastocysts, but were similar among blastocysts from the protein-free and serum-dependent treatments. Crucially, the use of protein-free medium for zygote culture did not compromise resultant blastocysts in terms of either *de novo* protein synthesis ([$^3$H]phenylalanine incorporation) or trophectoderm function (phenotype based on interferon-tau detection). Thus, although blastocyst yields were compromised after zygote culture in a protein-free (*vis-à-vis* albumin-supplemented) medium, amino acid flux was qualitatively conserved, and only quantitatively modified in the case of alanine and aspartate. Moreover, vital properties of blastocysts that were produced, including *de novo* protein synthesis and trophectodermal cell function, apparently were not adversely affected by protein deprivation.**

## Introduction

Two major considerations governing choice of culture system for *in vitro* production of bovine and other mammalian embryos are the efficiency of blastocyst production and the developmental competence of those blastocyts produced. The efficiency of blastocyst production is readily quantified, but it is more difficult to determine the developmental capabilities of individual blastocysts, either in terms of current functional competence or generation of viable offspring. Although production of healthy offspring is the ultimate indicator of developmental capacity – and failure in this regard is one reason for seeking alternatives to serum-dependent cultures (Leese *et al.*, 1998; Gardner, 1999; McEvoy *et al.*, 2000a) – integrity of embryos at the blastocyst stage is crucial too. Studies of bovine and ovine embryos produced *in vitro* have demonstrated that they differ from embryos produced *in vivo* in morphology, metabolism and gene expression (Wrenzycki *et al.*, 1996; Thompson, 1997; Holm and Callesen, 1998; Abe *et al.*, 1999a; Niemann and Wrenzycki, 2000). In addition, there is evidence that choice of culture conditions and constituents can affect embryo morphology (Abe *et al.*, 1999b), carbohydrate metabolism (Rieger *et al.*, 1995; Eckert *et al.*, 1998; Krisher *et al.*, 1999), apoptosis (Byrne *et al.*, 1999) and, in both the short and longer term, expression of developmentally important genes (Natale *et al.*, 1998; Wrenzycki *et al.*, 1999, 2001; Young *et al.*, 2001).

Significant advances have been made towards a comprehensive understanding of bovine embryo needs and preferences in terms of energy substrate use. In contrast, progress towards quantifying the extent to which amino acids are either needed or metabolized in various culture environments has been limited (Thompson, 2000). Until recently, this was of little consequence for commercial embryo production because, whenever amino acids were added to media, most formulations also included proteins, which, via endocytosis, could augment amino acid supplies to the embryo (Pemble and Kaye, 1976; Thompson *et al.*, 1998). However, the need for more precision has arisen because of interest in protein-free media (McEvoy *et al.*, 2000a), which would avoid the biohazards associated with serum and albumin (Guerin *et al.*, 1997), particularly in the UK and other countries where these products are not pathogen-free.

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Opinion is divided on the question of whether protein provision is an obligatory requirement for optimal bovine embryo production *in vitro*. Protein-free media can support zygote development to the blastocyst stage, but have yet to emulate conventional formulations in terms of blastocyst production. Nevertheless, zygotes cultured in chemically defined media in the absence of protein have generated blastocysts, which have subsequently developed *in vivo* to produce healthy calves (Keskintepe *et al.*, 1995; Holm *et al.*, 1999; Hernandez-Fonseca *et al.*, 2002). Most success with defined media has derived from an appreciation that, when protein is excluded, there is a need for formulations that take account of the fact that in addition to a macromolecular substitute, such as polyvinyl alcohol or hyaluronic acid, contaminants normally present in albumin or serum must be provided (Kuran *et al.*, 2001).

It has been shown that protein content (Thompson *et al.*, 1998), but not protein synthesis (Thompson *et al.*, 1998; Kuran *et al.*, 2001), is compromised in embryos produced in protein-free media; however, whether this compromise is detrimental remains uncertain. McEvoy and Sreenan (1990) demonstrated that demi-embryos can generate healthy calves, indicating that protein mass *per se* is less crucial for development and survival of blastocysts after culture than the capacity of the blastocyst to synthesize protein *de novo*. Consequently, amino acid availability, flux and utilization will be key determinants of the capacity of the embryo to develop and survive after culture. Partridge and Leese (1996) and Jung *et al.* (1998) have reported amino acid consumption at various preimplantation stages after production in albumin-supplemented synthetic oviduct fluid (SOF) medium. Using a similar strategy, Lee and Fukui (1996) reported amino acid uptake by morulae and blastocysts produced via two-stage culture, using a protein-free formulation up to 30 h after fertilization, with the addition of bovine serum albumin thereafter. Analogous investigations would help to determine the impact of contrasting *in vitro* production environments on amino acid uptake by bovine embryos. However, such work to our knowledge has not yet been reported.

A prerequisite for survival of bovine blastocysts after culture *in vitro* is the ability to generate a trophectoderm-derived protein signal (interferon tau, IFN-τ), which is crucial for establishment of pregnancy. Each blastocyst must be capable of efficient *de novo* protein synthesis and have an adequate complement of differentiated trophectoderm cells. Consequently, any *in vitro* production system that compromises these features cannot be considered suitable, irrespective of whether the culture medium includes proteins, such as serum and albumin.

The present study investigated amino acid flux (uptake or release), *de novo* protein synthesis and allocation of cells to inner cell mass (ICM) and trophectoderm (TE) in blastocysts produced in a protein-free medium and compared the results with those for embryos produced in either albumin- or serum-supplemented systems, to determine the impact of contrasting media formulations on bovine embryo production and viability. Two sera were used, which were each processed in standard conditions but with different concentrations of some key amino acids, notably glycine and serine, to resolve whether exogenous protein composition is likely to influence amino acid consumption profiles. Preliminary results for some of this work have been reported (Kuran *et al.*, 1999; McEvoy *et al.*, 2000b).

## Materials and Methods

All reagents and media were from Sigma Chemical Co. (Dorset, UK) unless otherwise stated.

### In vitro *maturation and fertilization*

Ovaries were collected from beef heifers at slaughter and transported to the laboratory at approximately 35°C in PBS (Oxoid, Basingstoke) containing 0.05 g kanamycin sulphate $l^{-1}$. Cumulus–oocyte complexes were recovered from follicles 2–8 mm in diameter by aspiration, using an 18 g needle and 5 ml syringe, and were collected in 2–3 ml of Hepes-buffered Medium 199 containing Earle's salts and supplemented with 10% (v/v) steer serum (Globepharm, Surrey) and antibiotics (50 000 iu penicillin $l^{-1}$ and 50 mg streptomycin sulphate $l^{-1}$). The morphology of the cumulus–oocyte complexes was assessed before maturation and only oocytes with compact, non-atretic cumulus investment and evenly granulated cytoplasm were selected for maturation. The maturation medium used was bicarbonate-buffered Medium 199 with Earle's salts (Life Technologies, Paisley) supplemented with 10% (v/v) steer serum and antibiotics (50 000 iu penicillin $l^{-1}$ and 50 mg streptomycin sulphate $l^{-1}$). After washing twice in this medium, selected cumulus–oocyte complexes were matured in 30 mm diameter culture dishes (3 ml medium per dish) for 24 h at 38.5°C in a humidified atmosphere of 5% $CO_2$ in air.

Frozen–thawed motile spermatozoa from a single bull were used for *in vitro* fertilization at $1 \times 10^6$ motile spermatozoa $ml^{-1}$ of fertilization medium following a standard swim-up procedure (Parrish *et al.*, 1986) in 1 ml of a modified calcium-free Tyrode's albumin lactate pyruvate (TALP) capacitation medium containing 0.6% (w/v) BSA (fraction V), 50 000 iu penicillin $l^{-1}$ and 50 mg streptomycin sulphate $l^{-1}$ (pH 7.4). Fertilization medium was modified TALP (Lu *et al.*, 1987) supplemented with 0.2 µmol penicillamine $l^{-1}$, 0.1 µmol hypotaurine $l^{-1}$, 0.02 µmol adrenaline $l^{-1}$, 0.6% (w/v) fatty acid-free BSA, 30 mg heparin $l^{-1}$, 50 000 iu penicillin $l^{-1}$ and 50 mg streptomycin sulphate $l^{-1}$ (pH 7.8). After maturation, most of the cumulus cells were removed by gentle pipetting before the oocytes were washed twice in Hepes-buffered TALP supplemented with 0.3% (w/v) BSA (fraction V), 50 000 iu penicillin $l^{-1}$ and 50 mg streptomycin sulphate $l^{-1}$. Matured oocytes (15–20 ova per drop) were placed in the fertilization medium droplets (50 µl final volume) in which oocytes and spermatozoa were co-incubated under mineral oil for 22 h at 38.5°C in a humidified atmosphere of 5% $CO_2$ in air.

### In vitro *culture*

After insemination, putative zygotes were removed and washed twice in a Hepes-buffered modification of SOF medium (Tervit *et al.*, 1972). Randomly selected groups of ten zygotes were placed in 20 µl microdrops of one of four media formulations and cultured in 5% $CO_2$, 5% $O_2$ and 90% $N_2$ at 38.5°C in ten replicates. All droplets were renewed every 48 h. All four media formulations were derivatives of a basal bicarbonate-buffered SOF medium (Kuran *et al.*, 2001). The supplements added to complete the different formulations were: (i) 10.0% (v/v) steer serum A (SSA); (ii) 10.0% (v/v) steer serum B (SSB); (iii) 0.4% (w/v) fatty acid-free bovine serum albumin (BSA) plus 2.0% (v/v) basal medium eagle (BME) essential amino acids (EAA; × 50) and 1.0% (v/v) minimum essential medium (MEM) non-essential amino acids (NEAA; × 100) (SBSA); and (iv) 0.3% (w/v) polyvinyl alcohol (PVA) plus 0.5 mmol sodium citrate $l^{-1}$ plus 5 µg epidermal growth factor (EGF; tissue culture grade) $l^{-1}$ plus 1% (v/v) NEAA (SPVA). L-glutamine (1 mmol $l^{-1}$) was present in all four formulations. The sera used (Table 1) were from steers fed for 10 days on a diet with either a high (190 g dry matter $kg^{-1}$; serum A donors) or low (120 g dry matter $kg^{-1}$; serum B donors) protein content. When used as culture supplements *in vitro*, these differed (A > B) in their capacity to increase ammonia concentrations (Carolan *et al.*, 1997). Moreover, ovine embryos produced in the presence of one of these sera have shown features characteristic of the large offspring syndrome (Sinclair *et al.*, 1999). Zygotes assigned to SPVA were washed in gas-equilibrated SPVA medium ten times before introduction to the culture drops. On day 3 of development (day 0 = *in vitro* fertilization), cleavage rate was recorded and cleaved zygotes were cultured in groups of four per 20 µl drop until day 7. At day 7, blastocyst yields, quality grades (Lindner and Wright, 1983) and precise developmental stages were recorded.

**Table 1.** Amino acid, insulin-like growth factor I (IGF-I), urea, ammonia and lipid profiles of the steer sera used

| | Serum A | Serum B |
|---|---|---|
| Amino acid (nmol $g^{-1}$) | | |
| Alanine | 312.5 | 353.3 |
| Anserine + tryptophan | 30.0 | 33.9 |
| Arginine | 160.9 | 138.1 |
| Asparagine | 65.2 | 63.5 |
| Aspartate | 5.2 | 7.4 |
| α-amino *n*-butyric acid | 9.1 | 11.1 |
| Carnosine | 12.5 | 26.1 |
| Citrulline | 89.1 | 84.3 |
| Glutamate | 145.4 | 129.7 |
| Glutamine | 217.7 | 150.2 |
| Glycine | 300.0 | 361.5 |
| Histidine | 66.3 | 70.8 |
| 1-methyl histidine | 7.5 | 13.9 |
| 3-methyl histidine | 4.0 | 4.6 |
| Isoleucine | 88.8 | 109.4 |
| Leucine | 143.1 | 156.7 |
| Lysine | 121.6 | 146.7 |
| Methionine | 19.4 | 21.7 |
| Ornithine | 109.5 | 128.6 |
| Phenylalanine | 55.4 | 64.7 |
| Phosphoserine | 9.3 | 20.6 |
| Proline | 86.6 | 101.4 |
| Serine | 123.8 | 166.5 |
| Taurine | 77.8 | 61.8 |
| Threonine | 75.1 | 97.5 |
| Tyrosine | 52.9 | 55.0 |
| Valine | 198.6 | 231.4 |
| IGF-I (µg $l^{-1}$) | 553.8 | 398.6 |
| Urea (nmol $g^{-1}$) | 6693.5 | 3472.1 |
| Ammonia (µmol $l^{-1}$) | 509.5 | 296.8 |
| Lipid (µmol $l^{-1}$) | | |
| HDL-Cholesterol | 0.71 | 0.57 |
| LDL-Cholesterol | 0.61 | 0.51 |
| Total cholesterol | 1.09 | 0.34 |
| Triglycerides | 2.94 | 2.23 |

HDL: high density lipids; LDL: low density lipids.

### *Assay of amino acid flux in blastocysts from different culture environments*

Blastocysts produced in SSA, SSB, SBSA and SPVA systems were washed in SOF supplemented with 0.1% (w/v) PVA plus 2% (v/v) BME EAA (× 50) and 1% (v/v) MEM NEAA (× 100). Between 6 and 16 blastocysts per 20 µl droplet were incubated in the same medium for 1 h in a humidified atmosphere of 5% $CO_2$, 5% $O_2$ and 90% $N_2$ to allow them to equilibrate. Blastocysts were then transferred to 20 µl of fresh 'test' droplets of this same medium and incubated for 12 h (day 7.5 to day 8.0) in the same environment. Droplets without embryos were used as 'control' references. At the end of incubation, 10 µl of medium was removed from each 'test' and 'control' droplet, and stored at –20°C for amino acid analysis. Blastocysts were then used for determinations of *de novo* protein synthesis or to quantify the numbers of cells present. Individual amino acid concentrations in frozen–thawed incubation medium droplets were determined by reverse phase HPLC (Waters Pico Tag Amino Acid Analyser) after derivatization of the samples using phenylisothiocyanate (Heinrikson and Meredith, 1984). For 18 amino acids, indices of depletion from, or release into, the incubation medium by blastocysts were measured as the difference in amino acid concentrations between 'test' and 'control' samples. Cysteine and tryptophan concentrations were not assayed.

### *Differential staining of ICM and TE cells*

After the amino acid metabolism studies, expanded blastocysts (replicates one to four, inclusive) were subjected to zona pellucida removal by the method of Van Soom *et al.* (1996). Briefly, embryos were washed in PBS and then, in

groups of three to five, were exposed to prewarmed (37°C) pronase solution (0.5% protease) for approximately 5 min for SPVA embryos and for 1 min for all other embryos. The embryos were then transferred to prewarmed acid Tyrode solution (pH 2.1) for either 2 min (SPVA embryos) or 1 min (other treatments), before being placed promptly in Hepes-buffered SOF. The TE and ICM cells of hatched and zona pellucida-free expanded blastocysts were subjected to immunosurgery using the method of Stojkovic *et al.* (1998). In this procedure, embryos were washed four times in prewarmed PBS and incubated in a 1:2 dilution of rabbit antiserum in PBS for 45 min at 39°C in a humidified atmosphere of 5% $CO_2$ in air. The rabbit antiserum was raised against a mixture of recombinant bovine IFN-τ and serum-containing medium conditioned by trophoblastic vesicles produced *in vitro* (Stojkovic *et al.*, 1998). The embryos were then washed four times in prewarmed PBS and incubated in PBS supplemented with 5% (v/v) guinea-pig complement and 50 mg propidium iodide $l^{-1}$ for 45 min at 39°C in a humidified atmosphere of 5% $CO_2$ in air. This procedure resulted in selective antibody-mediated complement lysis of trophectoderm cells and staining of trophectoderm cell nuclei. Subsequently, embryos were washed in PBS and placed in cold absolute ethanol containing 25 mg bisbenzimide (Hoechst 33342) $l^{-1}$ for 30 min at 4°C. This procedure resulted in the staining of all cell nuclei (inner cell mass and lysed trophectoderm) and the fixation of the embryo. Embryos were subsequently washed in absolute ethanol and mounted in a glycerol drop on a glass slide and covered with a coverslip. ICM and TE cells were counted using a fluorescence microscope.

### *Total cell counts and blastocyst diameters*

Blastocysts (replicates five, nine and ten) retrieved from the amino acid flux assay droplets were washed twice in Hepes-buffered SOF and fixed in a solution of ethanol and acetic acid (volume ratio = 3:1) for a minimum of 24 h. Subsequently, each blastocyst assigned to this process was placed on a glass slide, dried, stained with 15 µl of 10 mg bisbenzimide stain (Hoechst 33342) $l^{-1}$ prepared in 2.3% (w/v) sodium citrate and gently overlaid with a coverslip, which was sealed along its edges with varnish. The total number of cells in each blastocyst was determined by counting stained nuclei with the aid of a fluorescence microscope.

### De novo *protein synthesis*

Blastocysts (replicates six to eight) were washed four times in phosphate-buffered ovum culture medium (OCM; Imperial Laboratories, Andover) and incorporation of [$^3$H]-phenylalanine was determined, using the method described by McEvoy *et al.* (1997). Briefly, labelling of *de novo* synthesized protein was carried out in separate wells of 16-well dishes, with each well containing 15 µl [$^3$H]-phenylalanine solution; this comprised, in a 1:9 volume ratio, 10 × Dulbecco's modified Eagle's medium (DMEM) and aqueous L-[2,6-$^3$H]phenylalanine (37 MBq $ml^{-1}$, specific activity 2.07 TBq $mmol^{-1}$; Amersham International, Little Chalfont). The addition of individual embryos in 100 µl OCM resulted in a total volume of 115 µl per well, which was then incubated for 2 h at 38.5°C. At the end of the incubation period, embryos were washed four times in ice-cold OCM, and each embryo was stored at –80°C in 50 µl OCM until processing. The processing protocol (McEvoy *et al.*, 1995) involved dissolution in 50 µl of 0.6 mol NaOH $l^{-1}$, protein precipitation in 30 µl of 2.0 mol perchloric acid (PCA) $l^{-1}$ and repeated washing with 100 µl of 0.2 mol PCA $l^{-1}$. The protein was redissolved in 100 µl of 0.3 mol NaOH $l^{-1}$, and was added, together with 100 µl of 0.5 mol HCl $l^{-1}$ to prevent chemiluminescence, to a vial containing 5 ml liquid scintillation counter cocktail (Emulsifier Safe; Canberra Packard, Pangbourne). The radioactivity of the samples, together with the appropriate controls, was determined in a β-counter and results, after correction for background values, were expressed as d.p.m.

### *Statistical analysis*

Zygote cleavage, blastocyst production, hatching and 'ICM/total' cell data were analysed using Generalized Linear Model procedures for binomial data within the GENSTAT statistical package (Genstat 5, Version 4.1, 1998; Lawes Agricultural Trust, Rothamstead). The model, which facilitated pairwise comparisons, included 'replicate' and 'treatment'. Indices of *de novo* protein synthesis ([$^3$H]-phenylalanine incorporation; $\log_{10}$ d.p.m. counts) were tested using an REML procedure (Genstat 5) with 'replicate' as the random factor and 'treatment', 'stage' and 'treatment × stage' as fixed factors. The same procedure was used for analysis of blastocyst diameter data, with 'treatment' or 'presence versus absence of protein' as fixed factors, and for analysis of cell counts ($\log_{10}$-transformed data), with 'treatment' or 'presence versus absence of albumin' as fixed factors. When REML indicated significance, pairwise comparisons were carried out using Tukey's test based on SED values. Individual net depletion data for amino acids were tested for significance from zero by the Student's *t* test, and treatment effects were compared using one-way ANOVA and Tukey's pairwise test. Results are presented as untransformed mean (± SEM) values, except for d.p.m. values which are $\log_{10}$-transformed.

## Results

### *Embryo development* in vitro

The presence of serum compromised cleavage of zygotes in comparison with cleavage achieved in either SBSA or SPVA ($P < 0.001$; Table 2), and there was no difference between SSA and SSB or between SBSA and SPVA. However, when expressed as a percentage of cleaved zygotes, the protein-free medium yielded fewer blastocysts on day 7 ($P < 0.001$) than any of the protein-supplemented formula-

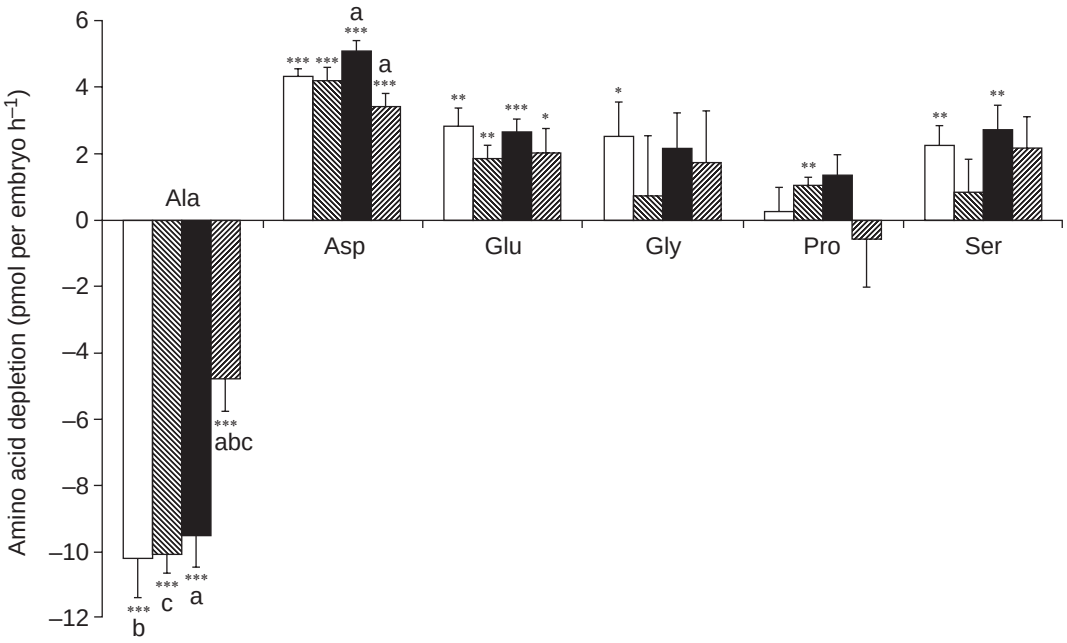


**Fig. 1.** Depletion of non-essential amino acids after 12 h of incubation (day 7.5 to day 8.0) of bovine blastocysts produced *in vitro* in the presence of serum (SSA, □; SSB, ▨) or albumin (SBSA, ■) or in the absence of protein (SPVA, ▨) in protein-free 'test' medium (synthetic oviduct fluid, polyvinyl alcohol, essential amino acids and non-essential amino acids). Values are mean ± SEM net depletion data ($n$ = 8–10 replicates) expressed in pmol per embryo $h^{-1}$. Negative values denote net amino acid release from embryos. Differences from 'control' (embryo-free) droplet values are denoted by asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$) whereas, for each amino acid, treatments with superscripts in common differ significantly ($^{a}P < 0.05$, $^{b}P < 0.01$, $^{c}P < 0.01$).

tions, and SBSA yielded fewer blastocysts than each of the serum-supplemented cultures ($P < 0.001$). By day 8, blastocyst production efficiency in the defined production system, in terms of both total and good quality blastocysts (grade 1: excellent; grade 2: very good), was no more than half that achieved in all other formulations ($P < 0.001$). In contrast, by day 8, the efficiency of generating good quality blastocysts in SBSA cultures was not significantly different from that recorded in the presence of serum.

Of the total numbers of blastocysts produced in each treatment, the percentages classified as good quality (grades 1 and 2) were not significantly different. However, after culture in the absence of exogenous protein or in the presence of either serum, the incidence of blastocyst hatching was significantly lower ($P < 0.01$) than that achieved in the formulation that relied on albumin. The hatching outcomes in each of the serum-supplemented systems were contrary to intuitive expectations based on timing of initial blastocoel formation. Embryos cultured in the presence of serum tended to reach blastocyst stages earlier, with more than 70% of those forming a blastocyst having done so by early day 7, compared with just 50 and 46% for SBSA and SPVA, respectively.

### *Amino acid depletion by embryos from culture media*

Individual amino acid depletion indices (mean ± SEM net values; pmol per embryo $h^{-1}$) after 12 h incubation of blastocysts produced in the four distinct culture systems are presented (Figs 1 and 2; Table 3). Of the 18 amino acids that were studied, only alanine concentrations consistently increased in the culture media (that is, values differed significantly from 'control' readings), reflecting significant net release, denoted as negative depletion values, by blastocysts from all four culture systems ($P < 0.001$; Fig. 1). In contrast, nine amino acids were taken up in significant amounts by blastocysts produced in SBSA compared with five, five and three amino acids by those produced in SSA, SSB and SPVA, respectively. Embryos from all four culture systems used took up significant amounts of aspartate ($P < 0.001$). Glutamate was also taken up by embryos from all treatments (SSA, $P < 0.001$; SSB, $P < 0.01$; SBSA, $P < 0.001$; SPVA, $P < 0.05$), whereas arginine was depleted from 'test' medium by SBSA ($P < 0.001$), SSA ($P < 0.001$) and SSB ($P < 0.01$) embryos, but not by SPVA embryos. Embryos produced in SSA depleted both serine ($P < 0.01$) and glycine ($P < 0.05$), whereas those produced in SSB took

up proline ($P < 0.01$) and isoleucine ($P < 0.05$). In addition to aspartate and glutamate, SBSA embryos took up serine ($P < 0.01$), histidine ($P < 0.05$), tyrosine ($P < 0.05$), methionine ($P < 0.05$), isoleucine ($P < 0.05$) and lysine ($P < 0.05$). Histidine ($P < 0.01$) was also taken up by embryos produced in SPVA.

SPVA embryos released less alanine than did blastocysts generated in the presence of either albumin ($P < 0.05$) or serum ($P < 0.01$) (Fig. 1). Blastocysts produced in protein-free conditions also depleted less aspartate than blastocysts generated in the presence of albumin ($P < 0.05$). Collectively, blastocysts from serum-free cultures (SBSA and SPVA) depleted more histidine than did blastocysts from serum-supplemented (SSA and SSB) cultures ($P < 0.05$). Analysis of mean (± SEM) indices for net flux (depletion or release) of all other essential and non-essential amino acids indicated no other significant treatment effects. Mean (± SEM) concentrations of six amino acids were not significantly altered by any treatment (Table 3).

### *Blastocyst cell proliferation and differentiation*

ICM, TE and the total number of cells recorded for blastocysts from the different culture systems (replicates one to four, inclusive) are presented (Fig. 3). Blastocysts produced in the presence of albumin had more ICM cells ($P < 0.05$) than did those from serum-supplemented formulations. The same outcome applied for total number of cells. However, for blastocysts generated in the absence of protein, neither ICM nor total number of cells differed from any protein-inclusive treatment. The number of trophectoderm cells did not differ significantly among treatments. The proportions of cells allocated to ICM were similar in blastocysts from albumin-supplemented (0.37 ± 0.01) and protein-free media (0.36 ± 0.02) and, in each case, exceeded the corresponding proportions for those from either SSA (0.29 ± 0.02; $P < 0.001$) or SSB (0.31 ± 0.02; $P < 0.01$ versus SBSA; $P < 0.05$ versus SPVA).

### *Total number of cells and blastocyst diameters*

Among blastocysts produced in replicates five, nine and ten, cell proliferation was again greater after culture in albumin-supplemented medium (mean ± SEM = 107 ± 11) than in all of the other formulations ($P < 0.001$), and none of these differed from the others (SSA = 73 ± 9; SSB = 69 ± 6; SPVA = 79 ± 7). Thus, although the number of cells tended to be lower than those reported for replicates one to four inclusive, the relative impact of the different media was unchanged.

The most obvious difference between blastocysts from the defined and protein-supplemented cultures was that the former were less expanded (mean ± SEM = 168 ± 5 μm; $n = 19$) than blastocysts produced in the presence of protein ($P < 0.01$). Corresponding zona pellucida-inclusive diameters of blastocysts produced in SSA, SSB and SBSA were 181 ± 3 μm ($n = 27$), 188 ± 5 μm ($n = 28$) and 186 ± 5 μm ($n = 18$), consistent with the overall evidence (Table 2) that fewer were hatching by day 8 (1%) than for serum-derived (SSA, 4%; SSB, 5%) and, more emphatically, albumin-derived (18%) blastocysts.

### De novo *protein synthesis*

Incorporation of [$^3$H]phenylalanine into PCA-precipitable protein (replicates six to ten) was similar, within-stage, for blastocysts generated in protein-supplemented and protein-free culture systems (Table 4). Only expanding blastocysts generated in the presence of serum B had inferior indices of protein synthesis compared with those produced in the presence of albumin. Mean (± SEM) cumulative indices ($\log_{10}$ d.p.m.) of [$^3$H]phenylalanine incorporation were 3.10 ± 0.05, 2.98 ± 0.05, 3.29 ± 0.04 and 3.02 ± 0.05 for blastocysts produced in SSA, SSB, SBSA and SPVA, respectively.

## Discussion

Amino acids, by virtue of their roles in protein synthesis, osmoregulation, nucleotide substrate provision and induction or control of gene expression, are among the factors most likely to influence preimplantation development, signalling and subsequent viability of mammalian embryos. Therefore, it is encouraging that, in the present study, amino acid utilization indices of blastocysts from a defined, protein-free system generally did not differ from those of blastocysts produced, albeit more efficiently, in albumin- or serum-supplemented systems.

Previous investigations have shown that bovine embryos produced *in vivo* or *in vitro* use most amino acids that are available to them. The significant and consistent exception is alanine, which invariably is released into the environment of the embryo (Lee and Fukui, 1996; Partridge and Leese, 1996; Jung *et al.*, 1998; Donnay and Leese, 1999; Donnay *et al.*, 1999). The same overall pattern with respect to amino acid flux was maintained in the present study, although the greatest legacy of blastocyst origin was that the magnitude of alanine release was almost halved among those generated in protein-free conditions. This finding may reflect a reluctance to relinquish any amino acid when, as demonstrated by Thompson *et al.* (1998), the protein complement of such embryos is already diminished. However, it is unlikely that the diminished release of alanine reflects increased retention as free amino acid in an unaltered form; rather, it is more likely to be used for protein synthesis or, perhaps, as a glucogenic precursor of pyruvate. The extent of alanine release from embryos produced in albumin-supplemented medium in the present study (mean = 9.5 pmol per embryo h$^{-1}$) is similar to that reported by Lee and Fukui (1996) and by Jung *et al.* (1998) for IVF-derived bovine blastocysts produced *in vitro* in SOF medium supplemented with albumin and subsequently incubated for 10 h in conditions equivalent to those used in the present study. Partridge and Leese (1996) also recorded highly significant, albeit less marked, alanine release from

**Table 2.** *In vitro* development of bovine embyos in serum supplemented (SSA, SSB), albumin supplemented (SBSA) and protein-free (SPVA) synthetic oviductal fluid media formulations

| Characteristic | SSA | SSB | SBSA | SPVA |
|---|---|---|---|---|
| Number of zygotes | 87 ± 6 | 85 ± 6 | 89 ± 6 | 123 ± 9 |
| Zygotes cleaved / total (%) | 62 ± 3$^{e}$ | 63 ± 2$^{e}$ | 76 ± 2$^{f}$ | 73 ± 2$^{f}$ |
| Day 7 blastocysts (% of cleaved) | 26 ± 4$^{e}$ | 24 ± 3$^{e}$ | 15 ± 2$^{fg}$ | 8 ± 2$^{fh}$ |
| Days 7 and day 8 blastocysts (% of cleaved) | 36 ± 3$^{ae}$ | 34 ± 4$^{e}$ | 30 ± 2$^{be}$ | 15 ± 2$^{f}$ |
| Grade 1 and 2 blastocysts (% of cleaved) | 22 ± 3$^{e}$ | 23 ± 3$^{e}$ | 20 ± 2$^{e}$ | 10 ± 2$^{f}$ |
| Grade 1 and 2 blastocysts (% of total) | 61 ± 3 | 66 ± 5 | 69 ± 3 | 60 ± 4 |
| Hatching blastocysts (% of grades 1 and 2) | 4 ± 2$^{c}$ | 5 ± 2$^{c}$ | 18 ± 4$^{d}$ | 1 ± 1$^{c}$ |

Values are presented as mean ± SEM from ten replicates.
Within rows, values with different superscripts differ as follows: a versus b, $P < 0.05$; c versus d, $P < 0.01$; e versus f, $P < 0.001$; g versus h, $P < 0.001$.

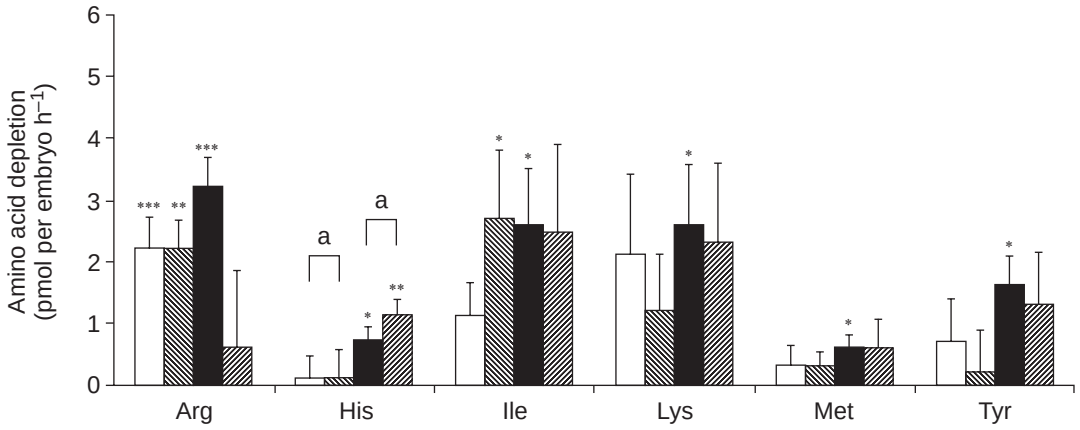


**Fig. 2.** Depletion of essential amino acids after 12 h of incubation (day 7.5 to day 8.0) of bovine blastocysts produced *in vitro* in the presence of serum (SSA, □; SSB, ▨) or albumin (SBSA, ■) or in the absence of protein (SPVA, ▨) in protein-free 'test' medium (synthetic oviduct fluid, polyvinyl alcohol, essential amino acids and non-essential amino acids). Values are mean ± SEM net depletion data ($n$ = 8–10 replicates) expressed in pmol per embryo h$^{-1}$. Differences from 'control' (embryo-free) droplet values are denoted by asterisks (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$). Note that, in the case of histidine, presence or absence of serum significantly affected depletion rates ($^{a}P < 0.05$).

both *in vivo*-derived blastocysts (approximately 3 pmol per embryo h$^{-1}$) and blastocysts produced *in vitro* in albumin-supplemented SOF medium (approximately 4 pmol per embryo h$^{-1}$). None of those studies reported amino acid flux in blastocysts produced in defined or serum-supplemented conditions.

Profiles for glycine, the single most abundant amino acid in the bovine oviduct and uterus (Elhassan *et al.*, 2001), although consistently indicating net utilization rather than release, were significantly different from control droplet values only for blastocysts produced in the presence of serum A. Serine, which can be converted to provide glycine and the active carbon atoms for purine synthesis (Jozwik *et al.*, 1999), was consumed in significant amounts by blastocysts produced in the presence of the same serum. In contrast, neither glycine nor serine was sought by blastocysts produced in the presence of serum B, arguably because the latter was a richer source of each. However, such a deduction was not always valid: for instance, despite being more abundant in serum B, both proline and isoleucine were avidly used by blastocysts grown in the presence of serum B but not serum A. Consequently, the present study indicates that amino acid profiles of ill-defined exogenous proteins are poor predictors of the extent to which amino acid requirements of embryos are fulfilled by such supplements.

The amino acid most avidly used by all blastocysts in the present study, regardless of origin, was aspartate, and this is in agreement with earlier reports (Lee and Fukui, 1996; Partridge and Leese, 1996). However, uptake was lower among blastocysts produced in defined medium than among those from albumin-supplemented conditions. Blastocysts produced in the presence of albumin were more advanced, so it is plausible that this finding merely reflects increasing demand for aspartate as embryos develop (Partridge and Leese, 1996). Alternatively, the possibility that blastocysts produced in defined conditions have more limited uptake capabilities cannot be exluded. How-

**Table 3.** Depletion indices for amino acids that were not influenced by prior embryo culture treatment and the concentrations of which, after 12 h incubation in the presence of embryos, did not differ significantly from those for 'control' (embryo-free) droplets

| | SSA | SSB | SBSA | SPVA |
|---|---|---|---|---|
| | Number of replicates | | | |
| Amino acid | 10 | 8 | 8 | 9 |
| Asparagine | 0.8 ± 0.6 | 1.1 ± 0.5 | 0.4 ± 0.5 | 2.4 ± 1.5 |
| Glutamine | –2.0 ± 2.6 | –0.6 ± 3.1 | 1.2 ± 2.9 | 0.3 ± 6.9 |
| Leucine | 1.9 ± 1.2 | 1.3 ± 0.9 | 1.1 ± 0.8 | 2.3 ± 1.6 |
| Phenylalanine | 2.0 ± 1.9 | 3.5 ± 1.8 | 1.3 ± 0.6 | 1.4 ± 0.9 |
| Threonine | 0.3 ± 1.3 | 0.6 ± 0.5 | 1.4 ± 0.9 | 1.9 ± 1.2 |
| Valine | 3.9 ± 1.2 | 1.1 ± 0.8 | 1.0 ± 0.6 | 2.6 ± 1.3 |

Values are mean ± SEM net depletion data ($n$ = 8–10 replicates) expressed in pmoles per embryo $h^{-1}$.
*In vitro* development of bovine embyos in serum supplemented (SSA, SSB), albumin supplemented (SBSA) and protein-free (SPVA) synthetic oviductal fluid media formulations

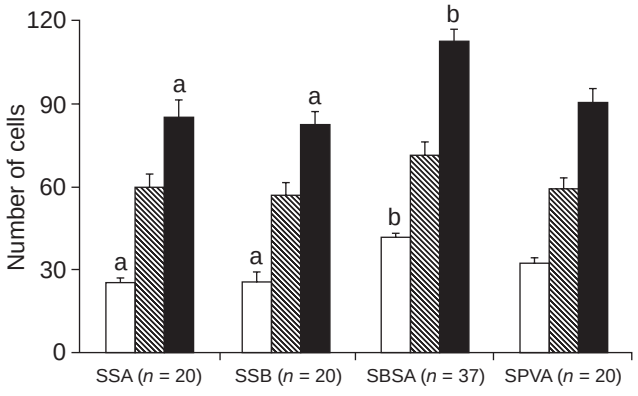


**Fig. 3.** Mean (± SEM) inner cell mass (ICM, □), trophectoderm (TE, ▨) and total number of cells (■) for bovine blastocysts (replicates one to four) produced in the presence of serum (SSA; SSB) or albumin (SBSA) or in the absence of protein (SPVA). Cells were identified on the basis of phenotype, using a differential staining procedure, which discriminated between cells that produced interferon τ (IFN-τ) (TE cells) and those which did not (ICM cells). Within each cell category, all significant differences are indicated as follows: a versus b ($P < 0.05$).

ever, a comparison of SBSA- and SPVA-derived blastocysts, showed that no other amino acid index, except that of alanine, was found to differ significantly. This finding does not support any major disruption of amino acid profiles in the latter population although, whether expressed in absolute or comparative terms, amino acid flux data must be interpreted with caution. For example, the question as to whether the consistently similar relative demand for amino acids is indicative of analogous intracellular amino acid precursor pools, and hence comparable capacities for *de novo* protein synthesis, is a matter for further investigation. However, as demonstrated in a study by Kuran *et al.* (2001), *de novo* synthesis of acid-precipitable protein in the present study was comparable, within-stage, for blastocysts produced in either albumin-supplemented or protein-free conditions. Thompson *et al.* (1998) reported similar findings even though, as in both the present and previous studies, blastocyst diameters were greater among their albumin-derived embryos.

In contrast to the non-significant net uptake of histidine by serum-derived embryos, this essential glucogenic $C_5$ amino acid was avidly used by blastocysts produced in either albumin-dependent ($P < 0.05$) or protein-free ($P < 0.01$) conditions. Although blastocysts generated in the presence of either serum would have had access to various forms of this amino acid, demand for histidine among blastocysts produced in the defined system, and to a lesser extent among those provided with albumin, may reflect a tendency to compensate for an earlier lack of this particular amino acid.

Demand for another glucogenic $C_5$ amino acid, arginine, an effector of improved nutrient supply and increased protein synthesis in mammalian embryos (Van Winkle, 2001), was significant among blastocysts from the three protein-supplemented systems. Lee and Fukui (1996) also reported significant arginine uptake by blastocysts produced in the presence of albumin, but Partridge and Leese (1996) did not. Therefore, access to albumin does not necessarily equate with adequate or optimal provision of required amino acids, a fact frequently underlined by the beneficial effects of amino acid addition to media containing supplementary albumin (Takahashi and First, 1992; Gardner *et al.*, 1994; Steeves and Gardner, 1999). In the present study, variable but low arginine uptake was a feature of blastocysts from protein-free medium but reasons for this are not clear. One possibility, applicable to other amino acids too, is that endogenous protein degradation contributed to arginine requirements. Although not sustainable, such a process may be more prevalent among blastocysts from defined conditions (Thompson *et al.*, 1998) and ought to provide a near-ideal amino acid supply in the short term.

Although our PVA-supplemented formulation yielded

**Table 4.** Indices of de *novo* synthesis of protein by bovine blastocysts produced *in vitro* in the presence of serum (SSA; SSB) or albumin (SBSA) or in the absence of protein (SPVA)

| Blastocyst categories | SSA | SSB | SBSA | SPVA |
|---|---|---|---|---|
| Early to mid-blastocyst (*n*) | 2.6 ± 0.21 (6) | 2.8 ± 0.03 (3) | 3.2 ± 0.06 (3) | 3.0 ± 0.06 (8) |
| Expanding blastocyst (*n*) | 3.1 ± 0.06 (21) | 2.9 ± 0.09[a] (12) | 3.3 ± 0.08[b] (8) | 3.0 ± 0.10 (9) |
| Expanded to hatched blastocyst (*n*) | 3.2 ± 0.07 (23) | 3.1 ± 0.07 (17) | 3.3 ± 0.05 (23) | 3.2 ± 0.06 (5) |

Results are expressed as mean (± SEM) values for [$^3$H]phenylalanine incorporation ($\log_{10}$ d.p.m.) into perchloric acid-precipitable protein.
Within rows, values with different superscripts are significantly different ($P < 0.01$).

fewer blastocysts, those that were produced showed analogous differentiation, based on trophectoderm-specific IFN-τ gene expression, compared with that of blastocysts generated in the presence of protein. Use of this 'phenotypic' assay avoided the limitations and errors inherent in alternative 'inside–outside' immunoassay procedures which presuppose and depend on 'robust' cell–cell cohesion. The assay data from the present study are consistent with the findings reported by Wrenzycki *et al*. (1999) that production of bovine embryos in chemically defined culture environments does not compromise IFN-τ gene expression. In view of the critical role played by this trophectoderm-specific protein in ensuring maternal recognition and establishment of pregnancy, this may be very important.

Exclusion of essential amino acids from the defined medium formulation in the present study was guided by the outcome of earlier work in our laboratory, which indicated that blastocyst production was virtually nullified by their inclusion at a standard rate (McEvoy *et al*., 2000c). However, rather than resorting to their complete exclusion, alternative 'non-standard' inclusion rates (see, for example, Liu and Foote, 1995) may help to safeguard development by virtue of the influence of essential amino acids on expression of stress-responsive and other genes in preimplantation embryos, in particular those that contain amino acid response elements. Notable among these are CCAAT/enhancer binding protein homologous protein (*CHOP*) genes, the asparagine synthetase (*AS*) gene and the insulin-like growth factor binding protein-I (*IGFBP-I*) gene (Fafournoux *et al*., 2000). *CHOP* (also known as *gadd153*) is ubiquitous and, in collaborative studies led by W. D. Rees, we have demonstrated that its expression in bovine embryos is altered *in vitro* in response to stress (Fontanier-Razzaq *et al*., 2001). *CHOP* gene expression precedes induction of apoptosis in embryonic cells (Fontanier-Razzaq *et al*., 1999) and is thought to be involved in ensuring the elimination of aberrant or damaged cells. Consequently, serum, which has been shown to restrain *CHOP* gene expression (Kashima *et al*., 1995), may serve only to perpetuate developmental errors.

When embryos are produced *in vitro* in the absence of exogenous proteins, they are denied the facility to endocytose polypeptides in a manner known to occur *in vitro* (Pemble and Kaye, 1976; Dunglison and Kaye, 1995; Thompson *et al*., 1998) and likely to be availed of *in vivo*. Whether such deprivation is undesirable is a matter of ongoing debate, and opinions are divided on the necessity to include protein *in vitro*. Normal offspring have been generated after *in vitro* culture of rodent, ruminant and pig embryos in protein-free conditions (Bavister, 1995; Keskintepe *et al*., 1995; Holm *et al*., 1999; McEvoy *et al*., 2000c; Hernandez-Fonseca *et al*., 2002; Yoshioka *et al*., 2002), but there is ample evidence, including that from the present study, to indicate that embryos produced in such conditions are, in the short term, functionally modified. A crucial question, therefore, is whether such functional modification is ultimately benign or inevitably harmful; thus far, the former remains a reasonable possibility. Moreover, in countries where the alternative option (that is, protein provision) is neither ideal nor hazard-free, decision-making ought to be governed by the relative rather than absolute merits of protein-free *in vitro* embryo production strategies.

In conclusion, the present study contributes to the ongoing investigations and debates concerning the options available to embryologists, and consequences of their decisions, in relation to *in vitro* production of mammalian embryos, especially wherever biohazard-free protein supplements are not readily available. The results from the present study demonstrate that depriving preimplantation bovine embryos of exogenous protein throughout the first week after fertilization resulted in halving of blastocyst yields in the absence of essential amino acids. However, there was encouraging evidence that blastocysts generated from protein-deprived embryos were developmentally competent in terms of amino acid utilization, cell differentiation and protein synthesis.

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