Article

Amino acid and fatty acid composition of follicular fluid as predictors of in-vitro embryo development

Kevin Sinclair is an Associate Professor and Reader in Developmental Biology at the University of Nottingham, UK. His work focuses on metabolic programming during early mammalian development, where epigenetic outcomes are determined in embryonic cells and tissues, and long-term developmental consequences assessed in offspring. Early studies investigated the effects of culture media composition on fetal development in ruminants. Recent studies have focused on the effects of specific dietary nutrients around the time of conception on epigenetic programming of adult health and disease in offspring. His long-term objective is to identify the features of those eggs and/or embryos that give rise to viable and healthy offspring.

Dr Kevin Sinclair

KD Sinclair[1,3], LA Lunn[1,2], WY Kwong[1], K Wonnacott[1], RST Linforth[1], J Craigon[1]
[1]School of Biosciences, University of Nottingham, Sutton Bonington, Leicestershire LE12 5RD, UK; [2]School of Human Development, University of Nottingham, Queens Medical Centre, Nottingham NG7 2UH, UK
[3]Correspondence: Tel: +44 115 9516053; Fax: +44 115 9516060; e-mail: Kevin.Sinclair@nottingham.ac.uk

Abstract

The value of using the amino acid and fatty acid composition of follicular fluid as predictors of embryo development was assessed in a bovine model of in-vitro maturation (IVM), IVF and blastocyst culture (IVC). A total of 445 cumulus–oocyte complexes (COC) aspirated from visually healthy follicles underwent IVM and IVF singly (n = 138) or in groups (n = 307). Of these COC, 349 cleaved (78%) following IVF and 112 went on to form blastocysts (32% of cleaved) following IVC. Culture method (singly or in groups) had no effect on development. In contrast to fatty acids, which had no predictive value, the amino acid composition of follicular fluid was associated with morphological assessments of COC quality and with post-fertilization development to the blastocyst stage. Principal component analysis identified two amino acids (i.e. alanine and glycine) that had the highest value for predicting early post-fertilization development. The predictive value of these two amino acids, in terms of the percentage of oocytes that cleaved following IVF, was greatest for COC with the poorest morphological grades but, with respect to blastocyst yields, was independent of morphological grade, and so may serve as a useful additional non-invasive measure of COC quality.

Keywords: amino acids, blastocysts, fatty acids, follicular fluid, IVF, oocyte

Introduction

The quest for non-invasive methodologies to identify the most viable embryos for transfer following IVF continues. Morphological assessments based on cell number and appearance have been refined in recent times to include characteristics of the pronucleate embryo and rates of cleavage, leading to the development of computer algorithms based on sequential assessments of embryo development (Gardner and Sakkas, 2003). Whilst such methodologies introduce an element of objectivity, and may outperform conventional morphological assessments in the hands of some, their predictive value remains modest (Neuber *et al*., 2006). Alternative or additional approaches for the non-invasive assessment of embryo viability include quantitative assessments of embryo metabolism (Brison *et al*., 2007). The focus to date has been on profiling selective classes of metabolites where amino acids have featured heavily. On the basis of the 'quiet embryo hypothesis' (Leese, 2002), the depletion and appearance (termed turnover) of amino acids in media following short-term culture has been determined and related to human blastocyst development *in vitro* (Houghton *et al*., 2002) and, more recently, to pregnancy outcome in a retrospective clinical study involving 53 cycles of IVF treatment using intracytoplasmic sperm injection (Brison *et al*., 2004). In that study, non-invasive assessments of amino acid turnover were found to be largely independent of other known determinants of pregnancy, such as morphological grade, so that a combination of assessments (e.g. amino acid metabolism and morphological grade) could potentially increase the chances of selecting developmentally competent embryos. Whilst less attention has been paid to other classes of metabolite, a recent

study highlighted the importance of fatty acid metabolism in human preimplantation embryos by assessing the accumulation of ^{13}C-labelled fatty acids in relation to blastocyst development *in vitro* (Haggarty *et al.*, 2006). The uptake of specific fatty acids was dependent on stage of development; embryos that went beyond the 4-cell stage had higher concentrations of unsaturates and lower concentrations of saturates.

Whilst considerable efforts have been directed towards metabolic profiling of media during embryo culture, less attention has been given to the metabolite composition of follicular fluid or granulosa cells in relation to post-fertilization development (Singh and Sinclair, 2007). Intrafollicular concentrations of meiosis-activating sterols, cholesterol and progesterone were not related to measures of post-fertilization development in women with polycystic ovary syndrome undergoing IVF (Bokal *et al.*, 2006). In contrast, Bedaiwy *et al.* (2007) reported both positive and negative associations between specific interleukins and pregnancy outcome following IVF in women, confirming the value of follicular-fluid markers in the selection of developmentally competent eggs for IVF, or embryos for transfer.

In the present study a well-characterized bovine model was used to determine the value of follicular fluid composition as a predictor of post-fertilization development to the blastocyst stage *in vitro*. In keeping with the metabolite turnover studies conducted with embryos described above, both the amino acid and fatty acid composition of follicular fluid were measured. Specific aspects of this study that are of clinical interest include: (i) immature oocytes recovered from non-stimulated ovaries; (ii) in-vitro maturation; and (iii) blastocyst culture.

Materials and methods

All reagents and media were from Sigma-Aldrich (Poole, Dorset, UK) unless stated.

In order to retain individual oocyte identity, whilst allowing embryos to benefit from group culture (Gopichandran and Leese, 2006), cumulus–oocyte complexes (COC) were matured *in vitro* (IVM), fertilized (IVF) and the resulting zygotes cultured (IVC) individually using the Well-of-the-Well (WOW) system (Vajta *et al.*, 2000). Individual wells within wells were prepared to give a final diameter of approximately 0.8 mm, thus allowing for cumulus expansion during IVM.

Bovine ovaries obtained from local abattoirs were transported to the laboratory within 1 h in warm (37°C) phosphate-buffered saline. COC, intended for individual culture in WOWs, were aspirated from visually healthy 6–8 mm follicles using a 19 gauge needle and 1 ml syringe, both of which were renewed for each follicle. It was decided to mature COC from these medium-sized follicles, as the post-fertilization developmental potential of oocytes from such follicles is greater than for oocytes recovered from smaller (<5 mm) antral follicles (e.g. Sinclair *et al.*, 2000). Individual identity was retained throughout. Once COC from each 6–8 mm follicle were recovered, the remaining aspirates were centrifuged at 18,600 *g* for 1 min, and supernatants transferred to 1.5 ml Eppendorf tubes, snap frozen in liquid nitrogen and stored at –80°C until analysis. In addition, for the purposes of quality control, within each replicate, COC aspirated from 3–10 mm follicles were matured in groups of 50–60, which is standard practice in our laboratory. Zygotes derived from these oocytes were subsequently cultured in groups of 25–30 in 400 µl medium contained in four-well dishes.

In-vitro embryo production

Apart from those features listed above, in all other respects methods of embryo production were as described previously by Sinclair *et al.* (2000) and Adamiak *et al.* (2005). COC were graded on a four-point scale by the number of compact cumulus cell layers and granulation of the oocyte cytoplasm. The scale was based on that of Goodhand *et al.* (1999). Briefly, grade 1 COC had more than five layers of compact cumulus cells, with a clear, even ooplasm; grade 2 COC had less than five layers of compact cumulus cells, with a clear, even ooplasm; grade 3 COC had less than five layers of cumulus cells which were slightly expanded, and the ooplasm was slightly uneven; grade 4 COC ranged from those with less than five layers of expanding cumulus and uneven ooplasm to denuded oocytes, and COC that were fully expanded. Denuded oocytes and fully expanded COC were discarded and so did not feature in any subsequent analysis. COC were matured in bicarbonate-buffered TCM199 with Earle's salts and 10% fetal calf serum, 10 µg/ml FSH, 10 µg/ml LH and antibiotics (penicillin, 50 U/ml; and streptomycin, 50 µg/ml). They were matured for 20–24 h at 38.8°C in a humidified atmosphere of 5% CO_2 in air.

Frozen semen from a single bull was thawed at 37°C and layered (125 µl) under 3 ml of a modified calcium-free tyrode albumin lactate pyruvate medium (TALP) in conical tubes for a swim-up procedure as described previously (Adamiak *et al.*, 2005). Motile spermatozoa were counted and added in a maximum of 40 µl capacitation medium to 460 µl fertilization medium containing matured oocytes (up to 30 per well), to give a final concentration of 1×10^6 motile spermatozoa per ml of fertilization medium. Fertilization medium was a modified TALP supplemented with 4.58 nmol/ml hypotaurine, 2.72 nmol/ml epinephrine, with 0.6% w/v fatty-acid-free bovine serum albumin (BSA), 10 µg/ml heparin, 0.556 nmol/ml caffeine and antibiotics. The oocytes and spermatozoa were incubated in 500 µl for 22 h at 38.8°C in a humidified atmosphere of 5% CO_2 in air. Putative zygotes were cultured in groups of 25–30 (or 10 in the WOW system) in 400 µl synthetic oviductal fluid medium (Holm *et al.*, 1999), containing (µmol/ml): 25 $NaHCO_3$, 108 NaCl, 7.2 KCl, 1.8 $CaCl_2$, 1.2 KH_2PO_4, 1.5 $MgSO_4$, 7.27 sodium pyruvate, 5.35 sodium lactate, 0.2 L-glutamine, 0.34 sodium citrate, supplemented with 0.3% w/v fatty acid-free BSA, 0.5% v/v non-essential amino acids, and 4.5% v/v essential amino acids and antibiotics. Putative zygotes were incubated in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2 at 38.8°C. On day 2 of development (day 0 = IVF), the percentage of inseminated oocytes that cleaved and cell numbers were recorded, and cleaved zygotes were then cultured until day 8. On day 8, embryo stage and grade was recorded according to the International Embryo Transfer Society classification system (Stringfellow and Seidel, 1998).

Amino acid and fatty acid analyses

Follicular fluid samples were thawed on ice prior to analysis. Amino acids were isolated from samples and derivatized using the EZ:Faast™ amino acid kit (Phenomenex, Macclesfield, UK). Briefly, 25 µl of each sample was combined with 20 nmol

(100 μl) norvaline, which acted as an internal standard. This solution was passed through the EZ:Faast solid phase extraction absorbent, which was washed with 200 μl propanol. A solution of propanol and sodium hydroxide (200 μl) was then used to remove the absorbent (and the amino acids retained on it) from the pipette tip. Chloroform (50 μl) and iso-octane (100 μl) were then sequentially added to the solution to derivatize the amino acids. The amino acids were recovered in the upper organic layer, dried down under a stream of nitrogen and the sample re-dissolved in 100 μl iso-octane:chloroform (80:20 v/v) prior to analysis using gas chromatography-mass spectrometry (GC-MS). Where necessary, samples were stored at 4°C for a maximum of 24 h prior to analysis.

For GC-MS analysis of amino acids, 1 μl of the sample was injected in splitless mode (split closed for 10 s) using an AS800 autosampler (PerkinElmer, Beaconsfield, UK). The injector of the GC8000 gas chromatograph (Fisons, Manchester, UK) was maintained at 250°C, with an initial oven temperature of 90°C which was increased to 320°C at 20°C/min (transferline from the oven to mass spectrometer, 300°C). Helium (12 psi (82.8 kPa)) was used as the carrier gas to elute the amino acids from the ZB-AAA column (10 m × 0.25 mm internal diameter). The MD800 mass spectrometer (Fisons) was operated in selected ion mode recording ions 101, 114, 116, 130, 144, 146, 155, 156, 158, 172, 180, 184, 243 and 244 with a dwell time of 0.03 s. Calibration was achieved by comparison of peak areas for the amino acids in standard and sample runs after adjustment for variation in the peak area of the internal standard. The method permitted the analysis of 18 amino acids. Cysteine and arginine were not detected by this method.

For the analysis of fatty acids in follicular fluid, between 40 and 100 μl (depending on the volume that remained following amino acid analysis) of each sample was combined with 100 μl (200 μg/ml) of internal standard (pentadecanoic acid; C15:0). Fatty acids were extracted using a 1:2 mixture of chloroform:methanol based on the method of Bligh and Dyer (1959), and methylated using methanolic HCl (5% v/v). Fatty acid methyl esters were re-suspended in hexane, and 2 μl was injected in splitless mode using an Agilent GC6890 gas chromatographer equipped with flame ionised detection (Agilent Technologies, Cheadle, UK) using a 100 m Varian CP7489 capillary column (internal diameter 0.2 μm) (Varian Scientific, Oxford, UK). Oven temperature increased from 59°C to a final temperature of 240°C over 55 min. Separation was based on column retention time. Hexane was injected between samples to prevent carry-over contamination. Calibration was achieved by comparison of peak areas for fatty acids with reference to a known standard (37 component mix; Supelco, Poole, UK) using Agilent HPCORE chemstation software (version 1.1). Twenty quantitatively significant and/or biologically important fatty acids were identified for subsequent statistical analysis.

Statistical analyses

Method of embryo culture (Group versus WOW) was formally compared by assessing the percentage of oocytes that cleaved and blastocysts that formed following insemination. Data were analysed using a generalized linear model within Genstat release 9.1 (Genstat, 2007) assuming binomial errors and with logit link functions. Terms fitted to the model were replicate and method of culture (group versus WOW). Spearman's rank correlation coefficients were generated to relate amino acid and fatty acid composition of follicular fluid to oocyte grade (assessed visually on a four-point scale, with grades ascribed to the nearest half point).

Analysis of the association between amino acid and fatty acid composition of follicular fluid and post-fertilization development began by comparing metabolite composition of follicular fluid for oocytes that failed to cleave following insemination, oocytes that cleaved following insemination but arrested soon thereafter, and oocytes that cleaved and went on to form blastocysts by day 8 following insemination. This was achieved by ANOVA. Residual plots were checked for normality and data were $\log_{10}$ transformed where necessary to normalize the residuals. Such data are presented as geometric means, with log means presented in parentheses. Standard errors of differences (SED) in such cases are presented on the log scale.

As there were correlations among the levels of different amino acids from the same follicle, it was inappropriate to consider amino acids to be independent explanatory variables when fitting linear models to explain the proportion of inseminated oocytes that cleaved, or the proportion of cleaved oocytes that went on to form blastocysts. It was necessary to have explanatory variables that encapsulated the variation among the amino acids but were also independent of each other. Principal component (PC) analysis of amino acid levels defines such variables as a linear combination of the measured amino acid levels for each follicle. Most of the variation in amino acid concentrations was explained by relatively few principal components (many fewer than the original number of explanatory variables in the data). Therefore, it was possible to include most of the variation in the amino acid data by fitting only a few independent explanatory variables defined by these principal components. Consequently, further assessments of the association between post-fertilization development and follicular fluid composition employed principal components analysis (Genstat, 2007). This was conducted for amino acids and fatty acids separately. In each case scores for the first five PC, which explained 98% of the variation in metabolite concentrations, were incorporated into generalized linear models, which assumed binomial errors and with logit links as before, so that the final list of terms fitted to each model was replicate, COC grade, and PC 1–5. The latent vector loadings (which indicate the relative importance of the individual acids to a particular PC) for any PC that contributed significantly to a linear model were examined to determine which acids were most influential in defining the PC. Principal component analyses were re-run using only the data from a reduced subset of acids excluding those that were among the least influential. The generalized linear models were fitted again using these new PC. When the new PC contributed significantly to the linear models then these models were used to predict the percentage of oocytes that cleaved or went on to form blastocysts following insemination. These predictions were based on fewer amino acid measurements than would be required for models based on the original PC.

Results

Method of in-vitro embryo production

A total 445 COC were matured and inseminated singly in WOWs ($n = 138$) or as groups ($n = 307$). Of these COC, 349 cleaved and 112 went on to form blastocysts. Cleavage was assessed 48 h

after IVF commenced. Zygotes that cleaved were classified at the point of assessment as follows: 2–3 cells (20%), 4–7 cells (47%), 8–11 cells (31%) and ≥12 cells (2%). Overall, the percentage of inseminated oocytes that cleaved averaged 78% for both WOW and group culture methods, and this value did not differ between the two systems of culture (**Figure 1**). Similarly, percentage of blastocysts from cleaved COC averaged 32%; this was within the range expected (Adamiak *et al.*, 2005, 2006), and did not differ between the two methods of culture. Although some zygotes that failed to reach the blastocyst stage by day 8 were at or around the mid- to late-morula stage, the vast majority had arrested between the 4- and 16-cell stage, coincident with the initiation of transcriptional activity in the bovine embryo (Memili *et al.*, 1998). The percentage of oocytes that cleaved following insemination differed ($P = 0.025$) according to COC morphological grade (grade: 1, 91.7 ± 4.3%; 2, 71.0 ± 6.9%; 3, 70.8 ± 6.1%; 4, 92.1 ± 8.3%). The percentage of blastocysts from cleaved also differed ($P = 0.01$) according to COC grade (grade: 1, 33.9 ± 5.7%; 2, 20.6 ± 5.8%; 3, 8.8 ± 3.8%; 4, 13.5 ± 9.8%). However, neither stage of blastocyst development (early blastocyst, 12%; blastocyst, 77%; expanded blastocyst, 4%; and hatched blastocyst, 7%) nor blastocyst grade (median = grade 1) differed by COC grade, and these parameters were also not affected by system of culture.

Follicular fluid composition and COC grade

A number of amino acids were found to be either positively or negatively correlated with COC grade (**Table 1**). For example, as the proportion (percentage of total amino acids) of glutamate in follicular fluid increased, so COC grade improved ($P < 0.001$). In contrast, as the proportion of leucine in follicular fluid increased, so COC grade worsened ($P < 0.001$). Fewer fatty acids were correlated to COC grade (**Table 2**). However, increasing levels of palmitic acid were associated with morphologically poor COC ($P = 0.028$). In contrast, increasing levels of stearic acid were associated with morphologically good COC ($P = 0.022$).

The amino acid concentration (µmol/ml) of follicular fluid was greater for those follicles that gave rise to oocytes that cleaved, and/or developed to the blastocyst stage following insemination, relative to those follicles that gave rise to oocytes that either failed to fertilize or to cleave following fertilization (**Table 3**). Expressed as a percentage of total amino acids, follicular fluid values for glutamate, glycine, isoleucine, leucine, lysine, methionine, phenylalanine, proline and tyrosine differed for follicles that gave rise to oocytes that developed to blastocysts following insemination relative to those that failed to cleave following insemination. Values for isoleucine, phenylalanine and proline also differed for follicles that gave rise to oocytes that cleaved, but subsequently arrested, relative to those that failed to cleave (**Table 3**). In contrast, neither the fatty acid concentration (µg/ml) nor composition (percentage total fatty acids) of follicular fluid differed between the three developmental categories (**Table 4**).

PC analyses

PC analysis was conducted for amino acid and fatty acid concentrations (i.e. µmol/ml, amino acids; and µg/ml, fatty acids) in follicular fluid in turn. In each case, the first five PC accounted for approximately 98% of the variation for each group of metabolites within follicular fluid. When the terms replicate and COC grade, followed by PC 1–5, were incorporated into generalized linear models of percentage cleavage following insemination and blastocyst yields, COC grade was related to the percentage that cleaved ($P = 0.02$) and blastocyst yield (blastocysts of inseminated, $P = 0.008$; blastocysts of cleaved, $P = 0.04$). None of the PC based on the fatty acid concentration of follicular fluid was related to either the percentage that cleaved or blastocyst yield. However, two of the PC for amino acid concentration of follicular fluid were related to oocytes cleaved of inseminated (i.e. PC1, $P < 0.005$; PC5, $P < 0.004$) and blastocysts of inseminated (i.e. PC1, $P = 0.09$; PC5, $P = 0.028$).

From this PC analysis the following amino acids were identified as being most influential (i.e. had the greatest positive and/or negative latent vector loadings) in defining these two PC: alanine, glycine, proline and valine (for PC1), and glutamate, glycine and valine (for PC5). Therefore, to simplify future predictions a second PC analysis was performed using just these five amino acids. The linear models based on the new PC identified two new PC (i.e. PC1a ($P = 0.006$) and PC4a ($P = 0.008$)) that explained oocytes cleaved of inseminated. PC4a also predicted blastocysts of inseminated ($P = 0.04$), but not blastocysts of cleaved ($P = 0.09$). PC1a was also not significant for blastocysts of either inseminated or cleaved in this regard ($P = 0.098$).

Next, predicted relationship between PC1a, PC4a and oocytes cleaved of inseminated for each of the COC grades (i.e. grades 1–4) was considered. **Figure 2** shows that amino acid composition is more important when predicting the percentage of oocytes that cleave following insemination for the lower grade oocytes (i.e. grades 3 and 4). For all COC grades, however, PC4a was less influential than PC1a. **Table 5** presents the latent vector loadings for PC1a and PC4a. These loadings show the relative importance of the different amino acids. With the possible exception of proline, all five amino acids were influential in defining both PC1a and PC4a. As a predictor of the percentage of oocytes that cleaved following insemination, however, glycine and alanine (with their high negative loadings for PC1a and high positive loadings for PC4a) would be predicted to have the greatest effect. That is, an increase in both glycine and alanine concentrations within follicular fluid would decrease PC1a and increase PC4a, resulting in an increased predicted percentage of oocytes that cleave following insemination.

Finally, the relationship between PC4a and blastocyst yield (blastocysts of inseminated), again for each of the four COC grades (**Figure 3**), was considered. As stated earlier, blastocyst yields increased with COC grade and with PC4a. Consequently, based on their latent vector loadings (**Table 5**), the relative contributions of alanine, glutamate, glycine and valine are similar; although it is possible to infer that whilst alanine and glycine are positively associated with blastocyst yield, glutamate and valine are negatively associated with blastocyst yield.

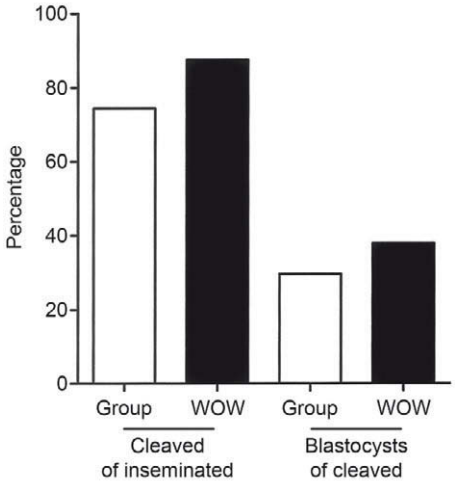

Figure 1. Effect of culture method (Well-of-the-Well, WOW) versus group culture) on the percentage of oocytes that cleaved following insemination and blastocysts of cleaved. Group culture based on 11 replicates, WOW culture based on 14 replicates.

Table 1. Spearman's rank correlation coefficients for the composition of amino acids in follicular fluid (percentage, total amino acids) and cumulus–oocyte grade[a].

Amino acid	r_s	P-*value*
Alanine	–0.108	NS
Asparagine	0.111	NS
Aspartate	–0.180	0.045
Glutamate	–0.389	<0.001
Glutamine	0.023	NS
Glycine	–0.201	0.026
Histidine	0.228	0.011
Isoleucine	0.285	0.001
Leucine	0.402	<0.001
Lysine	0.296	0.001
Methionine	0.297	0.001
Phenylalanine	0.268	0.003
Proline	0.269	0.003
Serine	0.023	NS
Threonine	0.007	NS
Tryptophan	0.137	NS
Tyrosine	0.317	<0.001
Valine	0.218	0.015

[a]Cumulus–oocyte (COC) grade (1 = good; 4 = poor): i.e. a negative correlation indicates that the amino acid is positively associated with COC quality.

Table 2. Spearman's rank correlation coefficients for the composition of selected fatty acids in follicular fluid (percentage total fatty acids) and cumulus–oocyte grade[a].

Fatty acid	r_s	P-*value*
Palmitic (16:0)	0.205	0.028
Stearic (18:0)	–0.213	0.022
Palmitoleic (16:1)	–0.075	NS
Oleic (18:1, n-9)	0.140	NS
Linoleic (18:2, n-6)	0.005	NS
Arachidonic (20:4, n-6)	0.151	NS
γ-Linolenic (18:3, n-6)	–0.089	NS
α-Linolenic (18:3, n-3)	–0.057	NS
Eicosapentaenoic (20:5, n-3)	0.079	NS
Docosahexaenoic (22:6, n-3)	0.118	NS

[a]Cumulus–oocyte (COC) grade (1 = good; 4 = poor): i.e. a negative correlation indicates that the fatty acid is positively associated with COC quality. NS = not significant.

Table 3. Amino acid composition (percentage total amino acids) of follicular fluid.

	Failed to cleave	*Cleaved but arrested*	*Developed to blastocysts*	*SED*	P-*value*
Follicular fluid samples	26	77	23		
Total amino acids (µmol/ml)	4.29[a] (0.632)	5.28[b] (0.723)	5.42[b] (0.734)	(0.034)	0.014
Alanine	11.19	11.65	11.98	0.622	
Asparagine	0.87	0.91	0.86	0.046	
Aspartate	0.49	0.59	0.59	0.071	
Glutamate	3.81[a] (0.581)	5.27[ab] (0.722)	6.53[b] (0.815)	(0.717)	0.057
Glutamine	13.28	13.73	13.55	1.126	
Glycine	13.15[a]	14.60[a,b]	16.14[b]	0.817	0.009
Histidine	1.99 (0.299)	1.81 (0.257)	1.80 (0.255)	(0.047)	
Isoleucine	3.72[a]	3.45[b]	3.08[c]	0.163	0.006
Leucine	4.45[a]	4.07[a]	3.67[b]	0.191	0.004
Lysine	5.89[a]	5.18[a,b]	4.71[b]	0.360	0.023
Methionine	0.47[a]	0.43[a]	0.36[b]	0.028	0.003
Phenylalanine	2.94[a]	2.64[b]	2.45[c]	0.103	<0.001
Proline	18.45[a]	16.26[b]	16.33[b]	0.729	0.007
Serine	3.05	3.27	3.22	0.204	
Threonine	2.97	3.08	2.91	0.157	
Tryptophan	1.28	1.32	1.25	0.072	
Tyrosine	3.05[a] (0.484)	2.81[a] (0.449)	2.51[b] (0.399)	(0.023)	0.013
Valine	6.90 (0.839)	6.42 (0.808)	5.87 (0.769)	(0.038)	

Values are expressed as means.
Values for individual amino acids are expressed as percentages of the total amino acids for failed-to-cleave, cleaved-but-arrested, and developed-to-blastocysts categories.
Data in parentheses are $\log_{10}$ transformed mean and standard error of difference (SED) values. Corresponding geometric means are presented before these values.
[a,b,c]Means within a row with different superscripts are different at the $P < 0.05$ level of significance.

Table 4. Fatty acid composition (percentage total fatty acids) of follicular fluid.

	Failed to cleave	*Cleaved but arrested*	*Developed to blastocysts*	*SED*
Follicular fluid samples	23	72	20	
Total fatty acids (µg/ml)	590.0	636.0	620.0	55.3
Saturated[a]	28.5	27.6	27.9	0.84
Unsaturated[a]	71.5	72.0	71.7	1.56
Monounsaturated[a]	25.1	23.0	24.0	1.24
Polyunsaturated[a]	46.4	49.0	47.7	1.63
n-6 series[a]	34.7	37.3	36.9	2.24
n-3 series[a]	11.6	11.7	10.8	1.28
Selected fatty acids				
Palmitic (16:0)[a]	15.1	14.2	14.5	0.62
Stearic acid (18:0)[a]	10.8	11.1	11.2	0.46
Palmitoleic (16:1)[a]	4.2	4.3	5.0	0.60
Oleic acid (18:1, n-9)[a]	20.5	18.1	18.6	1.09
Linoleic (18:2, n-6)[a]	29.5	31.7	31.3	2.13
Arachidonic (20:4, n-6)[a]	3.1	2.9	3.0	0.27
γ-Linolenic (18:3, n-6)[a]	0.2	0.6	0.4	0.19
α-Linolenic (18:3, n-3)[a]	10.5	10.5	9.7	1.22
Eicosapentaenoic (20:5, n-3)[a]	0.01	0.03	0.00	0.02
Docosahexaenoic (22:6, n-3)[a]	1.1	1.2	1.1	0.16

[a]Values are expressed as percentages of the total fatty acids for failed-to-cleave, cleaved-but-arrested, and developed-to-blastocysts categories.
SED = standard error of difference.

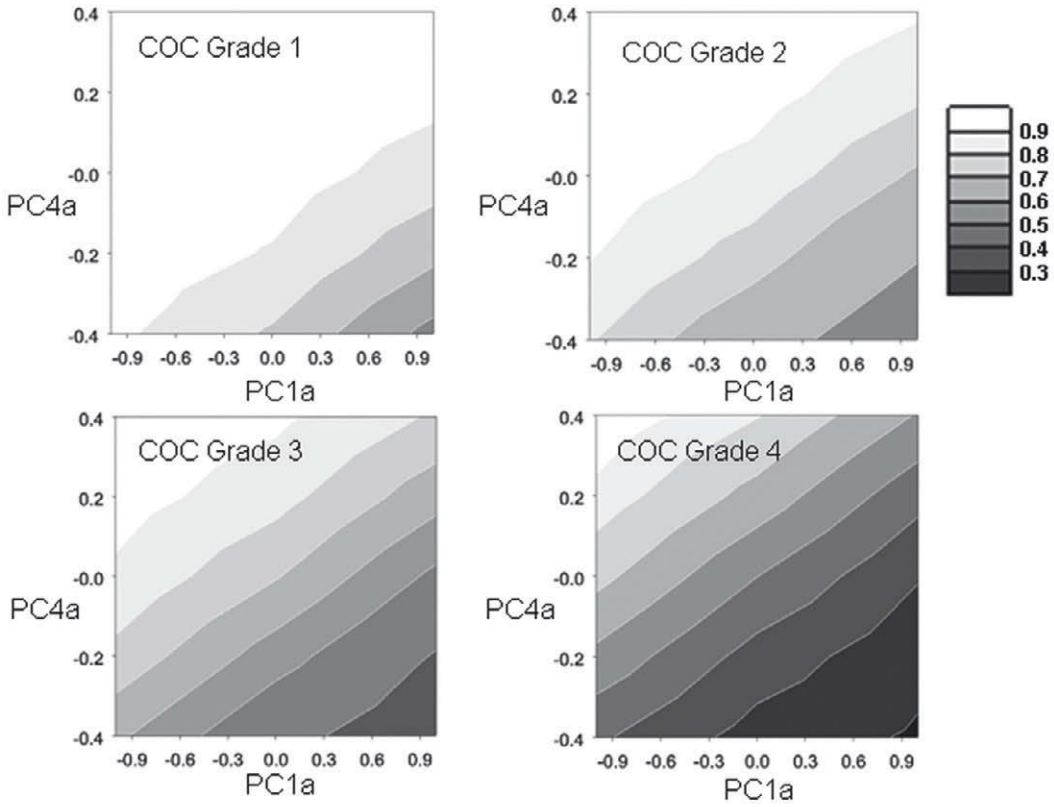

Figure 2. Cumulus–oocyte complex (COC) grade, and principal components (PC) 4a and 1a as predictors of the proportion of oocytes that cleaved following IVM and IVF. Contour lines depict different proportions of cleaved oocytes (from 0.2 to 0.9) following IVF; they illustrate that the proportion of oocytes that cleave improves as PC1a decreases and as PC4a increases.

Table 5. Latent vector loadings for principal components (PC) 1a and 4a.

Amino acid	*PC1a*	*PC4a*
Alanine	–0.481	0.190
Glutamate	–0.120	–0.518
Glycine	–0.450	0.517
Proline	–0.591	0.054
Valine	–0.450	–0.652

Discussion

The main findings from the current study were that, in contrast to fatty acids which appeared to have no predictive value, the amino acid composition of follicular fluid was associated with morphological assessments of COC quality and with post-fertilization development *in vitro* up to the blastocyst stage. PC analysis identified five amino acids (i.e. alanine, glutamate, glycine, proline and valine), which were particularly influential in this regard. Of these five amino acids, glycine and alanine were most influential in determining post-fertilization development. It would appear, however, that when it comes to the proportion of oocytes that cleave following insemination, the predictive value of these amino acids is greatest for COC with the poorest morphological grades (**Figure 2**). In contrast, the predictive value of these amino acids, with respect to blastocyst yields, is independent of COC grade (**Figure 3**) where, in addition to alanine and glycine, glutamate and valine are likely to be influential.

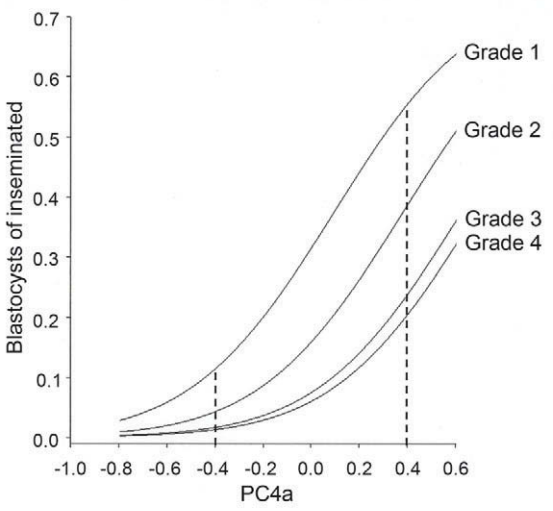

Figure 3. Cumulus–oocyte complex (COC) grade and principal component (PC) 4a as predictors of blastocyst yields (proportion of inseminated) following IVM, IVF and IVC. Dashed lines mark the data range for the current study. Blastocyst yields increase with COC grade and PC4a.

Follicle size and oocyte quality

To ascertain the significance of these observations it is necessary to place them in context of the procedures employed, and to contrast these with those used in clinical practice. In the current study, it was elected to mature oocytes from medium-sized (6–8 mm) follicles that exhibited no overt signs of atresia. As stated earlier in this article, the post-fertilization developmental potential of oocytes from such follicles is greater than for oocytes recovered from smaller (<5 mm) antral follicles (e.g. Sinclair *et al.*, 2000; Machatkova *et al.*, 2004; Lequarre *et al.*, 2005). Transcript levels for a number of developmentally important genes involved in cell signalling, cell cycle progression, chromatin modelling and chromosome segregation have been shown to increase in bovine germinal vesicle oocytes with follicle size (from <3 to >8 mm); and the expression of at least two of these genes (i.e. *Homo sapiens* pituitary tumour-transforming 1 and *Bos taurus* cyclin B2) were also greater in early cleaving zygotes (Mourot *et al.*, 2006).

The percentages of oocytes that cleaved following insemination declined as COC grades increased in the current study up to grade 3, but were higher for grade 4 COC. This phenomenon, where COC showing signs of early expansion and a slightly granulated ooplasm develop better *in vitro* following IVF, has been observed previously (e.g. Blondin and Sirard, 1995); although in the current study it was not associated with improved blastocyst yields which, in fact, declined with advancing grade. Blondin and Sirard (1995) postulated that the oocyte of an early atretic follicle may undergo changes similar to oocytes from pre-ovulatory follicles. Indeed, working with stimulated cycles in the cow, Blondin *et al.* (2002) later demonstrated that a 48 h period of FSH withdrawal (which they termed 'coasting'), followed by a single bolus of LH given 6 h prior to egg recovery, led to improved blastocyst yields following IVM, IVF and IVC of up to 80%. The authors found that, whilst working with more defined culture conditions, and culturing embryos in small groups from separate oocyte donors, this protocol led to improved blastocyst yields of up to 54% (Adamiak *et al.*, 2006). Although morphological assessments of human COC, and their value as a predictor of subsequent development, have not been fully explored, one recent report with COC subjected to IVM following a truncated course of FSH prior to aspiration indicated that the 'B' grade COC (four to five layers of slightly expanded cumulus) were developmentally more competent than the 'A' grade COC (more than five layers of compact cumulus) (Sato *et al.*, 2007).

Amino acid composition of follicular fluid

The mean concentration of amino acids within follicular fluid (**Table 3**), although in agreement with values for bovine follicular fluids from the study of Orsi *et al.* (2005), was greater than those (2.1 µmol/l) for the equivalent set of amino acids reported for human follicular fluid from the stimulated cycles of Józwik *et al.* (2006), and for porcine follicular fluid (1.9 µmol/l; in 3–8 mm follicles) from the study of Hong and Lee (2007). Nevertheless, closer inspection of these data reveals that the relative proportions of the different amino acids are strikingly similar across species. The exceptions are glutamate in the pig, glutamine in the human and proline in the cow. However, the mean concentration of glutamine in the current study of 714 µmol/l was similar to the value of 441 µmol/l for human follicular fluid reported by Józwik *et al.* (2006), suggesting that the relative proportions of amino acids in follicular fluid may be more similar between the cow and human, than the pig. Further reassurances about the general applicability of our findings stems from the fact that Orsi *et al.* (2005) observed the amino acid composition of bovine follicular fluid to be independent of oestrous cycle stage and follicle size, although again this does not appear to be the case in the pig (Hong and Lee, 2007). Nevertheless, considered together, these observations suggest that the relationships reported here may be more widely applicable, even for cycles undergoing conventional (ovarian stimulated) IVF (Nargund *et al.*, 2007).

PC analysis revealed that two amino acids (i.e. alanine and glycine) were more influential than others in predicting the

developmental potential of oocytes. Their value in predicting the percentage of oocytes that cleave following insemination was greatest for COC with the poorest morphological grades (**Figure 2**). In contrast, their value in predicting blastocyst yields was independent of COC grade (**Figure 3**), where glutamate and valine also featured. Alanine and glycine are two of the more abundant amino acids found in follicular fluid (**Table 3**), and in oviductal and uterine fluids (Hugentobler *et al*., 2007), where they are believed to have a number of cellular functions in addition to protein synthesis including nucleotide biosynthesis and osmoregulation (glycine: Steeves *et al*., 2003; Salway, 2004), and ammonium detoxification (alanine: Humpherson *et al*., 2005). Both alanine and glycine added to chemically defined bovine embryo culture media improved blastocyst development (Lee and Fukui, 1996). In the current study PC1a would be increased and PC4a would be decreased by a decrease in either of these two amino acids. From **Figure 2**, therefore, it appears that the percentage of oocytes that cleaved following insemination for the 'robust' grade 1 COC were least affected by these changes, whereas the percentage of oocytes that cleaved following insemination for the more 'delicate' grade 4 COC diminished sharply as glycine and alanine concentrations declined. Similarly, follicular fluid concentrations of alanine and glycine are predicted to increase and concentrations of glutamate and valine predicted to decrease as PC4a increases. These changes were associated with an increase in blastocyst yields (**Figure 3**).

Conclusions

The amino acid but not fatty acid composition of follicular fluid is related to post-fertilization development *in vitro* and so may, in addition to morphological grade, serve as a useful predictor of oocyte quality. Glycine and alanine would appear to be most influential in this regard. However, whilst the amino acid composition of follicular fluid may be broadly similar across species, and is largely independent of follicle size, its relationship to oocyte quality may not be universally consistent. The relationship may differ depending on the inherent developmental potential of the oocyte, which, in turn, may be dependent on follicle size and pharmacological interventions prior to egg recovery. Considered in a clinical context, therefore, the findings from the current study may be most applicable to 'natural cycle IVF' (Nargund *et al*., 2007).

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