

Amino Acid Metabolism in Human Embryos

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Summary

The aim of this study was to find some relationship between amino acid metabolism and the embryo morphokinetic parameters studied via time-lapse analysis. Study included 48 human embryo samples and their culture media. Two groups of embryos were identified: embryos reached the 8-cell stage on day 3 (n=34) and embryos failed to develop at any point during the incubation (n=14). Amino acids levels were measured on day 3 of embryo development; using time-lapse analysis, the precise timing of embryo cleavage, synchrony of division, grade of fragmentation etc. were established. No statistically significant differences between dividing and arresting embryos were observed in terms of amino acids production/consumption and turnover. Amino acids which were part of the culture medium did not exhibit any statistically significant correlation with kinetic parameters with the exception of the grade of fragmentation on day 3; there were negative correlation with glutamate, and positive with glutamine, glycine and taurine. In some dividing and in some arresting embryos appeared new amino acids which strongly correlated with each other, with methionine, but not with any other amino acid that is a regular part of the culture medium.

Introduction

In vitro fertilization (IVF) and embryo transfer were first successfully used over 30 years ago. It is reported that IVF is inefficient, only about 30 % of all embryos replaced in the uterus implant and result in a live baby (Kupka *et al.* 2014). The transfer of several embryos increases the probability of success; however the risk of a multiple pregnancy is enhanced (Allen *et al.* 2006, Murray and Norman 2014). Currently a number of European countries are moving towards placing a single embryo *per cycle* (Kupka *et al.* 2014). The main criteria for the selection of suitable embryos for transfer are cell number and morphological appearance. These criteria alone are poor predictors of implantation and propose finding another more objective approach for embryo selection. Embryo metabolism and time-lapse monitoring are new methods in this field (Houghton *et al.* 2002, Brison *et al.* 2004, Borini *et al.* 2005, Machtinger and Racowsky 2013).

Time-lapse monitoring enables continual monitoring of developing embryos and it is an ideal tool to study the dynamic biological processes of early embryo development (Wong *et al.* 2013). It provides information about the exact timing of embryo cleavages, synchrony division, the resorption and the grade of fragmentation, number and size of blastomeres (Lundin *et al.* 2001, Lemmen *et al.* 2008, Pribenszky *et al.* 2010).

Embryo metabolism could be another option in embryo selection. There have been many studies concerned with embryo metabolism, but few of them were conducted on human embryos, because of the practical difficulties involved. Often sugar and amino acid metabolism are discussed. Exogenously supplied amino acids influence human pre-implantation embryo development *in vitro*. Amino acids may be consumed by the embryo or secreted either intact or metabolized. Therefore amino acid turnover can be a suitable marker for the selection of embryos for transfer (Houghton *et al.* 2002, Brison *et al.* 2004, Stokes *et al.* 2007, Seli *et al.* 2008, Picton *et al.* 2010, Pudakalakatti *et al.* 2013, Zhao *et al.* 2013).

There are many methods for the determination of amino acids in biological samples. The choice of method often depends on the nature of analyzed sample and the available laboratory equipment. To date, many separation methods were developed, such as high-performance liquid chromatography (HPLC), gas chromatography and capillary electrophoresis (CE). HPLC methods use various types of detectors, such as ultraviolet/visible, fluorescence, electrochemical and mass spectrometry. Most of amino acids do not contain any strong chromophore or fluorophore in their molecule. To increase of sensitivity, a suitable derivatization is necessary. CE with contactless conductivity detection, characterized with short time of analysis, high sensitivity and minimal sample consumption can be used (Coufal *et al.* 2003, Tůma *et al.* 2010). The main advantage of this technique is direct analysis without derivatization step.

Our work mainly focused on (i) comparing human embryo amino acid metabolism between embryos which failed to develop and those which divided and on day 3 (approximately 72 hour after insemination) achieved the 8-cell stage and (ii) the relationship of human embryo metabolism and embryo morphokinetic parameters using time-lapse monitoring.

Materials and Methods

Samples of Human Embryo Culture Medium

All patients were treated according to standard protocols and gave written informed consent to participate in this research study, which was approved by the Sanus Committee on Human Research (Sanus In Vitro Fertilization Clinic, Pardubice, Czech Republic). An ovarian stimulation with recombinant FSH (150-250 IU/day) was used from the second to third day of the cycle (Puregon, MSD, Oss, Netherlands; Gonal-F, Merck-Serono, London, Great Britain) and in combination with an antagonist of GnRH (0.25 mg/day) from the fifth to sixth day of

the cycle (Orgalutran, MSD, Hoddesdon, Great Britain; Cetrotide, Merck-Serono, London, Great Britain). When at least three follicles reached a mean diameter of 17 mm and an adequate serum E₂ levels appeared, 10,000 IU of hCG (Pregnyl, Organon, Oss, Netherlands) was given to induce ovulation. Oocyte retrieval was carried out transvaginally under ultrasound guidance, 36 h after hCG administration. Immediately after ICSI, embryo culture was carried out in 4-well multidishes (Nunc, Roskilde, Denmark), each containing 500 µl of G-1TMv5 PLUS culture medium (Vitrolife) at 37° C in an atmosphere of 6% CO₂. Twenty hours after ICSI, one randomly selected zygote from each patient was transferred to the G-1TMv5 PLUS culture medium (100 µl) under mineral oil (OvoilTM, Gothenburg, Sweden) in a Primo Vision dish (Cryo Management Ltd., Szeged, Hungary) and placed under the time-lapse microscope (Primo Vision, Gothenburg, Sweden) at 37° C in an atmosphere of 6% CO₂. Images were acquired every 12 min over 48 h of culture. On the third day (48 h after the Primo Vision dish cultivation), the embryo was transferred to new 4-well multidishes in G-2TMv5 PLUS culture medium (Vitrolife). Retrospective analysis of the acquired images of each embryo was done with external computer running specialized software, Primo Vision Analyzer Software (version 4.4.1.01.010, Vitrolife Kfr., Hungary). Using Primo Vision Analyzer Software, we determined the precise timing of cell divisions and other development parameters (Table 1).

After incubation, culture medium in which the embryo was incubated and culture medium, incubated under the same conditions, but without the embryo (blank sample) were transferred into tube and immediately stored at -80 °C.

Sample Preparation and Chromatography Method

For the analysis, 20 µl of culture medium and 20 µl of the internal standard (norvaline) were pipetted into an amber vial and the content mixed. Standards were subjected to the same

procedure as described above for culture medium. The derivatization procedure with o-phthaldehyde and 2-mercaptoethanol was performed using the autosampler. Chromatography of amino acid derivatives was accomplished using a gradient elution on a LiChroCART® 125 x 4 mm i.d., Purospher® STAR RP-18e, 5 µm analytical column (Merck, Darmstadt, Germany) at 37 °C. The amount of amino acids was quantified from a peak area ratio of individual amino acid/internal standard using LCsolution chromatography software (Shimadzu, Kyoto, Japan).

Analytical parameters of presented method were as follows: intra-assay with CV from 2.5 to 6.2% and average recovery from 95.5 to 104.4%. The calibration curves were linear in the whole range tested. The lowest concentrations that could be quantified with acceptable accuracy and precision were from 0.3 µmol/l (130 fmol/inject) to 20.4 µmol/l (8497 fmol/inject). The limits of detection were from 0.1 µmol/l (43 fmol/inject) to 6.7 µmol/l (2804 fmol/inject). Limit of quantification (LOQ) and limit of detection (LOD) were calculated using the following equations: $LOQ = 10 Sa/b$ and $LOD = 3.3 Sa/b$, where Sa is a standard deviation of the intercept and b is a slope of the calibration curve.

Statistical Analysis

Data were analyzed using the Sigmastat version 3.5 (Systat Software Inc., Point Richmond, CA, USA) and the STATISTICA version 12 (StatSoft CR s.r.o., Prague, Czech Republic). The data are presented as mean and the standard error of the mean (SEM), and median and IQR (interquartile range). Differences between developing and arresting embryos were analyzed using the unpaired t-test. The correlation between individual amino acids, the maternal age and the embryo morphokinetic parameters was calculated using Pearson product moment correlation test.

Results

If the embryo divided throughout the monitoring period and reached the 8-cell stage on day 3 (approximately 72 hour after ICSI), it was designated as developing; if the embryo failed to develop at any point during the incubation it was designated as arresting (prior to the 8-cell stage). Forty-eight samples of human culture media in which 14 embryos stopped cleaving and 34 embryos divided were analyzed (Table 1). Embryo morphokinetic parameters and morphological events are only given for developing embryos.

Statistically significant differences in amino acid concentrations in the culture medium before and after incubation were observed in both groups (Table 2). In developing embryos there were only 2 amino acids (aspartate and asparagine) whose concentrations altered slightly (statistically insignificant), whereas there were more amino acids (aspartate, asparagine, serine, glycine and taurine) whose levels remained unchanged in embryos that arrested before 8-cell stage.

On day 3 after ICSI, amino acids metabolism did not differ in developing and arresting embryos (Fig. 1a and 1b) and in some developing and in some arresting embryos new amino acids appeared which were not included in the culture medium. Arginine had the highest levels at the end of incubation (8.9 $\mu\text{mol/l}$ and 8.1 $\mu\text{mol/l}$), followed by leucine (6.1 $\mu\text{mol/l}$ and 5.2 $\mu\text{mol/l}$) then threonine (5.5 $\mu\text{mol/l}$ and 5.2 $\mu\text{mol/l}$), valine (5.4 $\mu\text{mol/l}$ and 5.5 $\mu\text{mol/l}$) and isoleucine (5.3 $\mu\text{mol/l}$ and 4.7 $\mu\text{mol/l}$), histidine (3.8 $\mu\text{mol/l}$ and 2.9 $\mu\text{mol/l}$), tyrosine (2.9 $\mu\text{mol/l}$ and 2.7 $\mu\text{mol/l}$) and phenylalanine (3.4 $\mu\text{mol/l}$ and 2.6 $\mu\text{mol/l}$); tryptophan had the lowest concentration (1.5 $\mu\text{mol/l}$ for developing and 1.4 $\mu\text{mol/l}$ for arresting embryos). The overwhelming majority of amino acids were more released into the culture medium than taken up from it; therefore the balance reached positive values (Fig. 1c). The turnover (sum of the production and consumption of amino acids) was higher in developing (84.5 pmol/embryo/hour) than in arresting embryos (59.1 pmol/embryo/hour), but these results were

statistically insignificant due to the large deviation (for developing embryos the SD was 86.9 pmol/embryo/hour and SEM 14.9 pmol/embryo/hour; for arresting embryos the SD was 47.7 pmol/embryo/hour and SEM 12.7 pmol/embryo/hour).

The correlation between embryo morphokinetic parameters and amino acid metabolism in human embryos are expressed in a simplified figure (Fig. 2; (a) for developing embryos, (b) for arresting embryos). The degree of correlation is represented by the number of asterisks (*), a positive correlation is indicated with the symbol (+), a negative correlation with (-).

Discussion

Currently, the most important criteria for the selection of human embryos for transfer are morphological appearance and cell division. This is quite a subjective approach. For this reason, other alternatives are being searched for, and embryo metabolism could be one of them.

The amino acids turnover of embryos differs significantly between authors. This is caused by the use of various analytical methods, sample preparation and selection of culture medium (Houghton *et al.* 2002, Brison *et al.* 2004, Stokes *et al.* 2007, Seli *et al.* 2008, Picton *et al.* 2010, Pudakalakatti *et al.* 2013, Zhao *et al.* 2013, Wale and Gardner 2012, Hemmings *et al.* 2013, Lamb and Leese 1994, Marhuenda-Egea *et al.* 2011). Therefore it is important to correctly quantify the levels of amino acids in culture medium. It depends on a suitable sample preparation method and on the availability of reliable methods for their quantification. In our opinion, the most important is the composition of culture medium. We used G-1TMv5 PLUS culture medium, which is designed to support the development of the cleavage stage. Eagle's nonessential amino acids, furthermore methionine, taurine and instead of glutamine dipeptide alanyl-glutamine are the main components. Lane and Gardner (1997) observed that nonessential amino acids are necessary and promote cell division, while the addition of

essential amino acids can inhibit stimulatory effect of nonessential amino acids (and may be a possible origin of an increasing concentration of ammonium during incubation). This medium is completely different e.g. from that used in the studies of the working group of Leese (Houghton *et al.* 2002, Brison *et al.* 2004, Stokes *et al.* 2007, Picton *et al.* 2010, Hemmings *et al.* 2013). Their culture medium contains all amino acids and they allow the embryo to choose which amino acids to utilize for its development.

We observed differences in the amino acid levels in the culture medium before and after incubation (Table 2) and were able to detect new amino acids (essential) which appeared in some samples of human embryo culture medium after incubation. Some amino acids were released into the culture medium and their concentrations were higher after incubation in both groups (glutamate, glutamine, glycine, alanine, taurine, methionine). We did not observe any statistically significant changes in amino acid consumption or production between developing and arresting embryos (Fig. 1a and 1b) which was described in many studies (Houghton *et al.* 2002, Brison *et al.* 2004, Picton *et al.* 2010) and the turnover was very similar in both groups (Fig. 1c). It was hypothesized that embryo with a low metabolic activity (so-called quiet metabolism) reflects a less stressed physiology, and such an embryo is classified as viable, consequently optimal for transfer (Baumann *et al.* 2007, Leese *et al.* 2008). This study did not confirm the earlier hypothesis and even contradicts because the turnover was higher in developing embryos, but due to the large standard deviation this observation is statistically insignificant. It is important to note that embryo metabolism is influenced by the incubation conditions, mainly the oxygen level. Wale and Gardner (2012) observed that in 20% oxygen the metabolism of amino acids in mouse embryos is different from that in 5% oxygen and that in atmospheric oxygen embryos displayed greater turnover, which may be due to the fact that the embryos are more stressed.

The levels of alanine and glutamine increased after incubation in all embryos, irrespective of whether they divided or not. Higher levels of these amino acids in the culture medium after embryo incubation may be associated with an accumulation of ammonia. Embryos are able to use pyruvate for conversion to alanine and thus prevent the accumulation of ammonia. In a study by Pudakalakatti *et al.* (2013), successfully implanted embryos exhibited higher alanine levels and a lower pyruvate/alanine ratio in the culture medium, whereas Houghton *et al.* (2002) found statistically significant higher alanine levels on day 2-3 in embryos which arrested before blastocyst formation. Glutamate was also released in many samples into the culture medium. This may be caused by its regeneration from α -ketoglutarate and ammonium in a transamination reaction or *via* the conversion of glutamine to glutamate (Chatot *et al.* 1990). Seli *et al.* (2008) found higher glutamate concentrations in the culture media of embryos that resulted in pregnancy, and postulated that the elevated levels of glutamate may be a reflection of the embryo's ability to reduce the levels of ammonium in the media.

Methionine has an indispensable role in reproduction and embryo development. It initiates protein synthesis; it is a precursor of the essential methyl donor for methylation reactions, and a source of redox regulators. It has a high affinity for the transporters, and may prevent the uptake of other amino acids, thus creating an imbalance in the endogenous pool (Ménézo *et al.* 2013). We observed that this amino acid was more released into the culture medium than taken up and if the increase was higher than 50%, new amino acids (essential) were detected in the spent culture medium. Essential amino acids only appeared in some samples regardless of whether the embryo divided or not; they were not detected in the blank samples and therefore are products of embryo metabolism. In developing embryos, all essential amino acids with the exception of tryptophan strongly correlated with each other and with methionine but not with any other amino acid that is a regular part of the culture

medium. Oocytes and embryos have an endogenous reserve of all amino acids (Hemmings *et al.* 2013, Gardner and Lane 1993) and so they can be released into the culture medium, but we cannot explain why this only happened in some dividing and in some arresting embryos. It is interesting that methionine correlated with all essential amino acids, but, with the exception of alanine, there was no relationship to any of the nonessential amino acids. Nonessential amino acids correlated with each other, but not as much as essential ones.

Time-lapse monitoring becomes a very useful tool in laboratory practice. There are many reports that the early-cleavage of embryos is a good predictor of developmental competence (Wong *et al.* 2013, Lemmen *et al.* 2008, Wong *et al.* 2010, Dal Canto *et al.* 2012). Some authors did not find differences in embryos development and implantation rates between time-lapse imaging and standard incubation (Lundin *et al.* 2001, Cruz *et al.* 2011). Our work attempts to discover the relationship between human embryo kinetic events and its metabolism. One of the earliest works in this field studied this connection in mouse embryos (Lee *et al.* 2015) and to our knowledge there has not been single paper describing this relationship in human embryos. As shown in Fig. 2, we found a relationship between the time of division (tPN, t2, t3, t4 and t8) and human embryo metabolism for developing embryos to the 8-cell stage. In this instance there were higher levels of branched-chain amino acids (BCAA) and also of tryptophan. All of these amino acids exhibited a positive correlation to the division times; in embryos with prolonged cleaving, there were higher levels of BCAA (tPN, t2, t3 and t4) and tryptophan (t8). Changes in nonessential amino acids levels did not exhibit any dependence on the cleaving time. Embryos with higher fragmentation on day 3 had elevated levels of glutamine, glycine and taurine, and decreased levels of glutamate. These embryos had a higher sum of the production of amino acids (releasing amino acids into the medium) and therefore the amino acid turnover was also higher.

The use of time-lapse monitoring system in most of IVF laboratories allows us to control abnormalities that could be easily overlooked with classical microscopy, like multinucleation or asynchronous division (Kalátová *et al.* 2015, Chamayou *et al.* 2013, Kovačik *et al.* 2014). The direct division of one cell into three cells is phenomenon that was observed in 12.2% of ISCI procedures (Chamayou *et al.* 2013). This abnormality can be caused by lack of some proteins in cytokinesis so it would be interesting to monitor amino acids turnover (Wong *et al.*, 2010). Kalátová *et al.* (2015) describe strong correlation between presence of tripolar mitosis and poor early embryo development. Chamayou *et al.* (2013) suggest that these embryos can be implanted successfully and give healthy babies even though the implantation rate is lower.

Of the important factors, that may affect the metabolic activity of embryos, are genetic abnormalities, for example aneuploidy. More than half of human embryos generated during IVF contain aneuploid cells. Aneuploidy rates increase with maternal age. Aneuploid embryos can have normal early development (Fragouli and Wells 2011). Campbell *et al.* (2013) found by time-lapse monitoring differences in timing of initiation of compaction, time to reach full blastocyst stage and initiation of blastulation between aneuploid and euploid embryos. Picton *et al.* (2010) found that in the early stage of division asparagine, glycine and valine turnover was significantly different between genetically normal and abnormal embryos on day 2-3 of culture.

In conclusion, currently there are only a few publications on amino acids turnover of human embryos that use different culture media and procedures. Therefore, in meantime, amino acids turnover cannot be the evaluation criterion for the selection of suitable embryo for transfer.

Conflict of Interest

There is no conflict of interest

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Legend to figures

Fig. 1. Amino acid metabolism in human embryos

(a) Amino acids, which are part of the cultivation media, depletion and appearance by human embryos on day 3 of development. (b) Amino acids, which are not part of the cultivation media, appearance by human embryos on day 3 of development. (c) Total amino acids production, depletion, turnover and balance by human embryos on day 3 of development. The results are expressed as the mean and the standard error of the mean (SEM).

Fig. 2. The correlations between individual amino acids, the maternal age and the embryo morphokinetic parameters

The correlations were calculated using Pearson product moment correlation test in human embryos developing to the 8-cell stage (a) and human embryos arresting prior to the 8-cell stage (b).

Table 1 Embryo characteristic

Embryo quality	n	Maternal age				
		Mean	SEM	Median	IQR	Range
All	48	32	1	32	5	22-40
Arresting	14	31	1	31	4	26-40
Developing	34	33	1	33	6	22-40
Morphokinetic parameters of developing embryos to the 8-cell stage						
		Mean	SEM	Median	IQR	Range
	tPN (min)	1467	25	1422	216	1272-1837
	t2 (min)	1598	29	1562	256	1347-2017
	t3 (min)	2283	37	2286	301	1743-2907
	t4 (min)	2353	34	2374	234	1990-2907
	t5 (min)	3175	47	3152	324	2801-4073
	t8 (min)	3452	59	3391	413	2921-4215
	cc2 (min)	686	23	684	72	84-991
	cc3 (min)	891	24	836	96	732-1464
	s2 (min)	70	17	48	48	0-624
	s3 (min)	277	40	186	158	60-1130
	fragmentation (%)	5	1	5	10	0-15

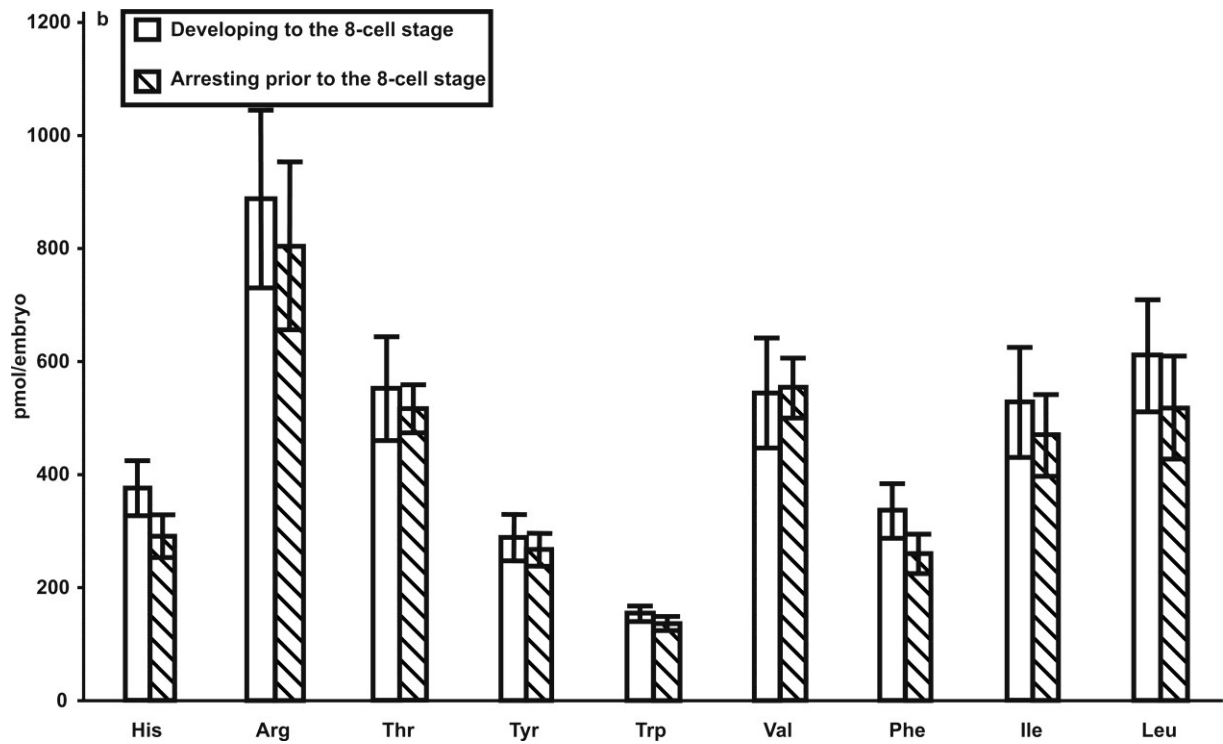
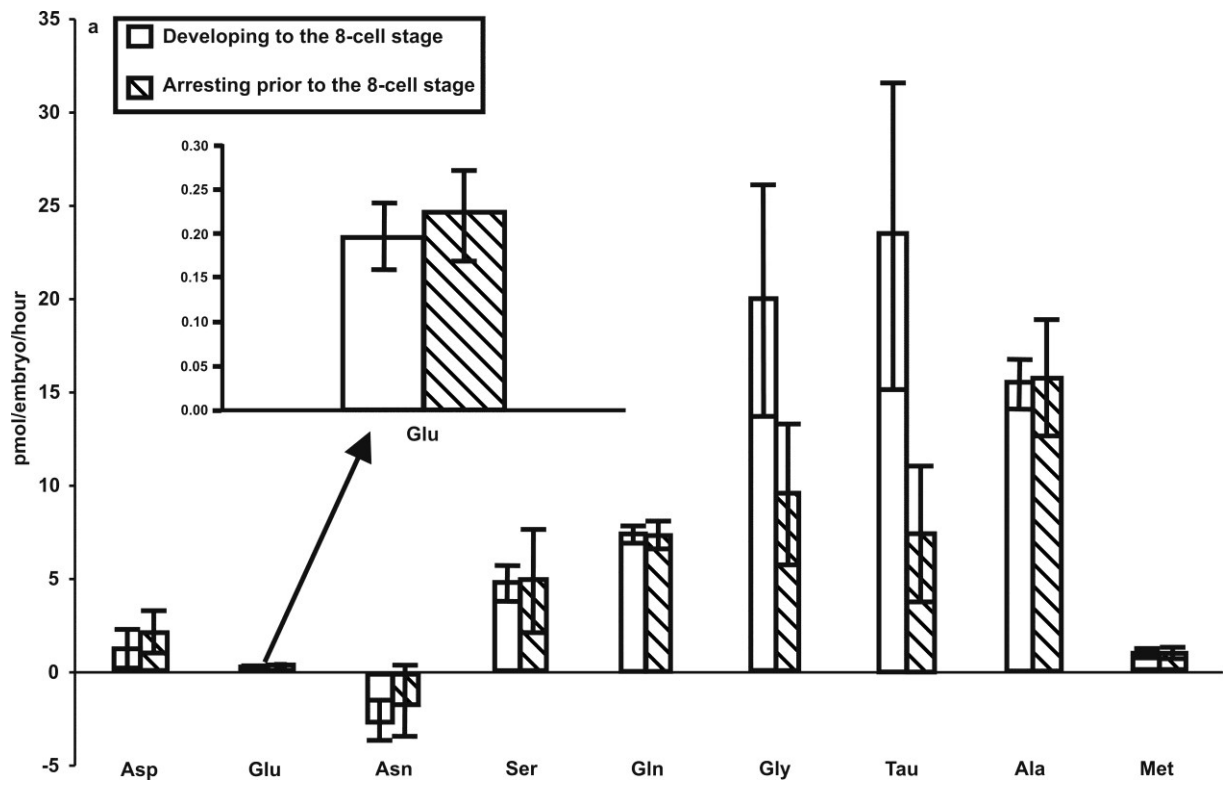
tPN, time when both pronuclei had faded; t2, time of cleavage to two-blastomere embryo; t3, time of cleavage to three-blastomere embryo; t4, time of cleavage to four-blastomere embryo; t5, time of cleavage to five-blastomere embryo; t8, time of cleavage to eight-blastomere embryo; cc2, the duration of the second cell cycle (t3 – t2); cc3, the duration of the third cell

cycle ($t_5 - t_3$); s_2 , the time of synchrony of the second cell cycle ($t_4 - t_3$), from 2 to 4 cells; s_3 , time of synchrony of the third cell cycle ($t_8 - t_5$), from 4 to 8 cells. The grade of fragmentation of embryos was determined on the third day of cultivation.

Table 2 Summary table for the paired t-test investigating the changes in levels of amino acids in the culture medium before and after incubation

Amino acid	Developing embryos				Arresting embryos			
	t	p	Power (1- β)	Conclusion	t	p	Power (1- β)	Conclusion
Asp	-0.833	0.411	0.050	-	-1.781	0.098	0.246	-
Glu	-5.512	<0.001	1.000	<i>Significant</i>	-4.415	<0.001	0.985	<i>Significant</i>
Asn	2.540	0.016	0.621	-	0.847	0.412	0.050	-
Ser	-5.502	<0.001	1.000	<i>Significant</i>	-1.792	0.096	0.268	-
Gln	-14.510	<0.001	1.000	<i>Significant</i>	-10.052	<0.001	1.000	<i>Significant</i>
Gly	-3.669	<0.001	0.946	<i>Significant</i>	-2.519	0.026	0.568	-
Tau	-3.297	0.002	0.880	<i>Significant</i>	-2.048	0.061	0.369	-
Ala	-12.315	<0.001	1.000	<i>Significant</i>	-5.057	<0.001	0.998	<i>Significant</i>
Met	-4.346	<0.001	0.992	<i>Significant</i>	-3.330	0.005	0.848	<i>Significant</i>

The t-test statistic is computed by subtracting the values before the intervention from the value observed after the intervention in each experimental subject. The p value is the probability of being wrong in concluding that there is a true effect. There are significant differences if $p < 0.05$. The power, or sensitivity, of a paired t-test is the probability that the test will detect a difference between treatments if there really is a difference. The closer the power is to 1, the more sensitive the test. Traditionally, the power of the performed test should be > 0.8 . Less than desired power indicates you are less likely to detect a difference when one actually exists. Negative results should be interpreted cautiously.



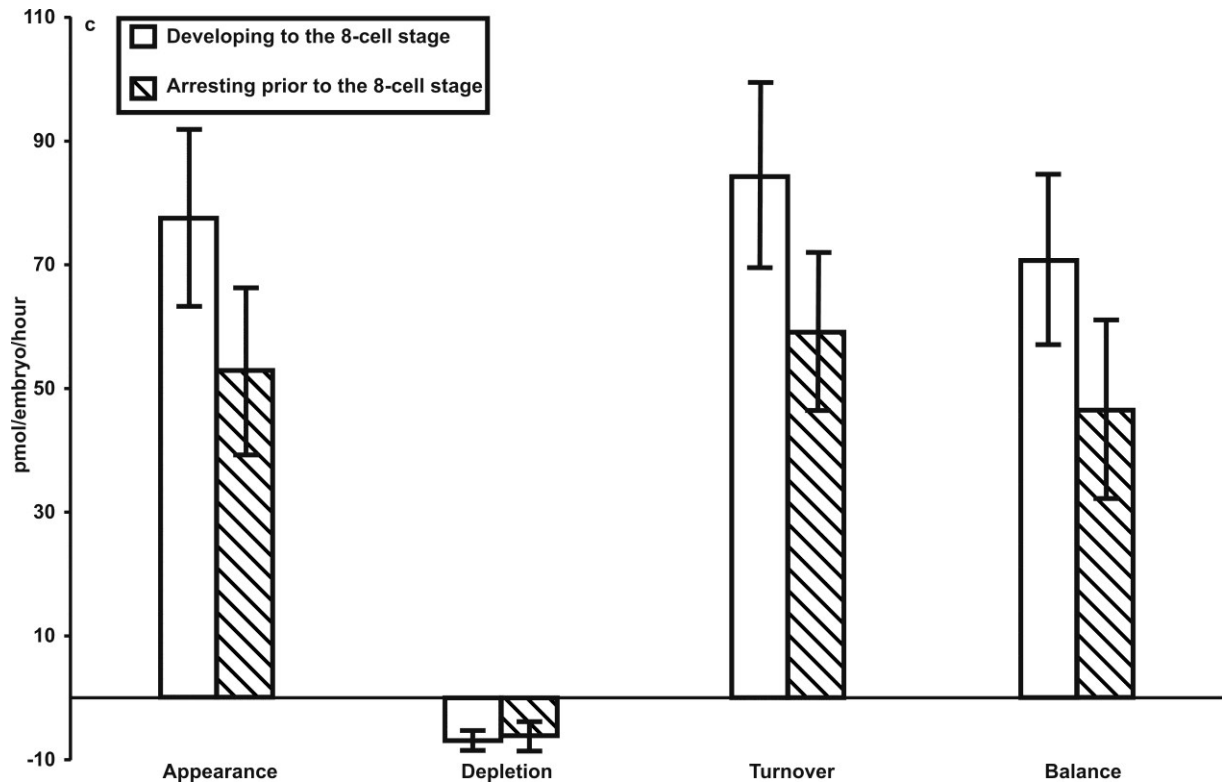


Fig. 1. Amino acid metabolism in human embryos. (a) Amino acids, which are part of the cultivation media, depletion and appearance by human embryos on day 3 of development. (b) Amino acids, which are not part of the cultivation media, appearance by human embryos on day 3 of development. (c) Total amino acids production, depletion, turnover and balance by human embryos on day 3 of development. The results are expressed as the mean and the standard error of the mean (SEM).

