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The Integration of Morphokinetics and Amino Acids Assessments for Day 5 Embryos

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Abstract

Background: The intracytoplasmic sperm injection (ICSI) technique has shown great success in the assisted reproduction field. Amino acids, abundant in female reproductive fluids, are crucial for mammalian embryo culture media.

Objective: This study explores the relationship between the amino acid profile in a 5-day-old embryo culture medium and embryo implantation potential in in vitro fertilization (IVF). It combines two non-invasive methods: amino acid quantification in the culture medium and morphokinetic analysis using the time-lapse technique, to link embryological processes with biochemical dynamics.

Materials and Methods: Ovarian stimulation and oocyte collection, semen preparation and assessment, embryo culturing, scoring, and selection with a time-lapse monitoring system were assessed in the Reproductive Centre of Agial Hospital, Alexandria, Egypt. On day 5, culture media in which the embryos were developed, were collected and analyzed for 20 amino acids using liquid chromatography–mass spectrometry (LC–MS). Statistical methods estimated amino acid consumption, release, and their correlations.

Results: The study highlights the importance of specific amino acids, such as valine, threonine, serine, cysteine, and alanine which were prominently produced in the culture media of developing embryos. While other amino acids exhibited decreased levels in both developing and arrested embryos. Notably, histidine, lysine, cystine, aspartate, glutamate, glutamine, and glycine, were significantly absorbed from the media.

Conclusion: Our findings suggest that the combined use of amino acid profiling and morphokinetic analysis can help select the most competent embryo, potentially increasing IVF success rates, reducing the incidence of multiple births, and predicting implantation potential in morphologically excellent embryos.

Keywords: Amino acids; embryo development potential; Intracytoplasmic Sperm Injection; Noninvasive assessment; Time-lapse

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Original article

Introduction

Reproduction is the biological process that maintains the continuity of life. During the last decades, the world has shown a high prevalence of infertility. Infertility is described as a disease of the reproductive system defined by the failure to achieve a clinical pregnancy after 12 months or more of regular, unprotected sexual intercourse, according to the World Health Organization (WHO) (1). Reasons for infertility are shown either in men or women, or both of them (2). In vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) appear to be the most effective micro-assisted reproductive techniques for overcoming infertility (3). Methods of IVF have progressed drastically since the first IVF baby was born in 1978 (4). The technique of conventional IVF involves introducing insemination of oocyte with a certain concentration of prepared sperm , and it can be a solution for female infertility rather than male infertility. ICSI, as an assisted fertilization procedure, was introduced in 1992 (4,5), and it is currently accounting for 70-80% of the performed cycles (6). In ICSI, a single sperm cell is injected directly into the cytoplasm of an oocyte. This technique is used to prepare the gametes for the production of embryos that would be transferred to a maternal uterus (7). After the injection of the oocytes, embryos are cultured in media for 3 or 5 days. The media that are used to culture human preimplantation embryos are considered to be one of the most crucial factors affecting the success rates of ICSI (8).

The quality of embryos depends largely on the culture media, which provide the necessary elements and energy for their development (9). To support the in vitro growth of gametes and embryos, an appropriate culture media is required (10). Moreover, amino acids are essential components of the media used to culture human zygotes until they reach the blastocyst stage in vitro (11, 12).

Several studies have indicated that a subset of embryos exhibiting normal development display heightened consumption of leucine from the culture medium. Furthermore, significant correlations have been observed between developmental potential and the profiles of arginine, glutamine, methionine, and asparagine (13). The concentration of amino acids in the medium of human zygotes developed into the 2-cell stage exhibited notable changes, as indicated by research findings. Specifically, the turnover of three amino acids—asparagine, glycine, and leucine—was significantly associated with clinical pregnancy and live birth outcomes (1).

During culturing the embryos in the media for 3 or 5 days, the embryos undergo assessments in different ways to evaluate their development. The most common method for evaluations is non-invasive assessments, which depend on morphology, analysis of the culture media, or morphokinetics by using a time-lapse system (TLS), which is the most recently used technique. During the last decade, these new technologies were found to monitor the development of embryos (14). TLS is programmed to capture the development of the embryos at frequent time intervals of 5 to 20 minutes for 5 days. A built-in camera in the incubator captures snapshots of a dynamic process (15). In a time-lapse incubator, the embryos can be kept in optimal conditions throughout their culture, such as pH, temperature, humidity, light exposure, and respiration (16). The importance of this technique is to observe the development of the embryo over the incubation period, starting with the appearance of the pronuclei (2 PN) and two polar bodies that assure fertilization, without exposing the embryos or removing them from their optimal conditions. TLS makes it possible to precisely identify the beginning, length, and spacing between cell divisions (17). It provides exact information about developmental kinetics as well as embryo morphology and allows accurate observation of cellular uncommon events, such as direct cleavage into three cells, blastomeric fusion, multinucleation, and fragment reabsorption (14, 15). Therefore, TLS shows accurate assessment and reduces errors, which depends on the embryologist's expertise and capabilities (16).

The objective of the present study was to evaluate the metabolomic profile, especially the levels of 20 amino acids, in embryo culture media and explore their correlation with the morphokinetic parameters using a time-lapse incubator for the grading of day 5 embryos produced by the ICSI process. By integrating biochemistry and embryology through two non-invasive assessments, our aim is to enhance the predictive capability of pregnancy outcomes.

Materials and Methods

Samples Collection

The culture media of Ninety- three embryos derived from 35 women undergoing ICSI treatment for pregnancy at the Reproductive Centre of Agial Hospital, Alexandria, Egypt, were collected on day 5 of development. Women were selected in the study within the age range of 25 to 35 years. The exclusion criteria for recipients in this study were females with ovarian pathologies such as polycystic ovaries (PCO) or endometriosis. Ethical approval for this study was obtained from the Ethics Committee of the Faculty of Medicine, Alexandria University, with a serial number (0108104).

Ovarian stimulation and Oocyte collection

Figure. 1 illustrates the general steps, including oocyte and sperm assessment and injection. Oocyte aspiration was performed 36 hours after the injection of human chorionic gonadotropin (r-HCG). Ovarian stimulation followed the previously known guidelines for stimulation (18). Follicular aspiration was done when the follicles' diameter reached around 18 µm. Follicles were aspirated with an aspiration needle using transvaginal ultrasound, collected in tubes, and put in a portable incubator at 37 °C. Oocytes were identified using a stereomicroscope by observing the oocyte-cumulus complex (19). Oocytes were collected in a petri dish containing wash media (Ham's F10, Irvine, USA) to remove the granulosa cells and the follicular fluid. Then they were kept in a central well dish that contained IVF media (Vitrolife, Sweden) for 30 minutes in a humidified incubator (Labotect, Germany) at 37 ºC, 6.5% CO2, and 5% O2 (20).

Oocyte denudation, which is the removal of the cumulus and corona cells to be ready for injection, was done after 30-60 minutes of oocyte retrieval. First, denudation was done enzymatically by placing the matured oocyte in a small dish containing 80 µl of the HYASE or hyaluronidase enzyme (SAGE, Origio, Denmark) for 30 seconds, which dissolves huge parts of the cumulus to make the denudation easier (19, 21). The second step was mechanically where the oocytes were placed in a buffer media called gamete and by using strippers of different sizes (170 and 130 µm) to remove the corona cells and ensure that the oocytes were completely neat and ready for injection (22). Denuded oocytes are cultured in small dishes containing IVF media, covered with paraffin oil (Nidacon, Sweden), and kept in an incubator at 6.5 % CO2 and 5% O2 to be ready for injection (19). Oocytes were classified according to their maturity and only metaphase II oocytes (diagnosed by the presence of the first polar body) are injected (23).

Semen Assessment

After the collection of seminal fluid from the patient, the semen sample was left at room temperature for a maximum period of 1 hour to be liquefied. Then semen volume, concentration, motility, and morphology were assessed. Frequent variability among ejaculates in individuals is a common issue (24). Before the selection and injection of sperm, semen was prepared and concentrated to get high-quality sperm samples with progressive motility.

Semen Preparation

During semen preparation, it is important to remove unwanted cells, including epithelial cells, erythrocytes, leukocytes, and immature, immotile, and non-viable spermatozoa, that inhibit the capacitation and the ability for fertilization (19). Density gradient and swim-up preparation techniques were used. PureSperm®100 (Nidacon, Sweden) is a standard sterile colloidal silica suspension used for density gradient centrifugation of sperm. (24).

Briefly, a conical tube was prepared with 3 layers: a layer of 80 % sperm grad (pure sperm) with a high density to remove all the weak grade C unwanted cells and immotile sperms, and a layer of 40% sperm grad with a lower density to avoid the low-quality sperms being transferred before the semen is loaded to form the third layer. The sample undergoes centrifugation at 1200 rpm for 15 minutes. Then it undergoes a double wash by using Hams F10 (Irvine, USA) to remove the toxicity of PureSperm®100. Immediately before injection, 1µl of the sperm suspension was diluted with 4µl of polyvinylpyrrolidone (PVP) (SAGE,Origio, Denmark) in a gamete medium (Vitrolife, Sweden) placed in the middle of the plastic petri dish. PVP was necessary to increase the medium's viscosity, which should facilitate sperm handling by slowing down their motion and reducing fluid movement in micro-pipettes (25). About 3 µl of concentrated sperm suspension was put straight into the injection dish to load the search dishes. Finally, selection plates were prepared, and sperms were selected under an inverted microscope at 40x magnification and were ready for oocyte injection (26).

Embryo culture

After insemination and injection of oocytes with selected sperms, they were placed in a time-lapse petri dish containing the culture media, covered with paraffin oil, and incubated in the time-lapse incubator (ESCO, Senghafora) at 37 ºC, pH 7.3, under a gas phase of 5% O2 and 6.5% CO2 for 5 days. Embryos are photographed in time-lapse monitoring devices at intervals of five to twenty minutes (27).
Embryos are kept in culture media containing all the sources of energy, minerals, and metabolic requirements and maintained in conditions that represent the female uterus (19). The used media was SAGE media (Origio, Denmark).

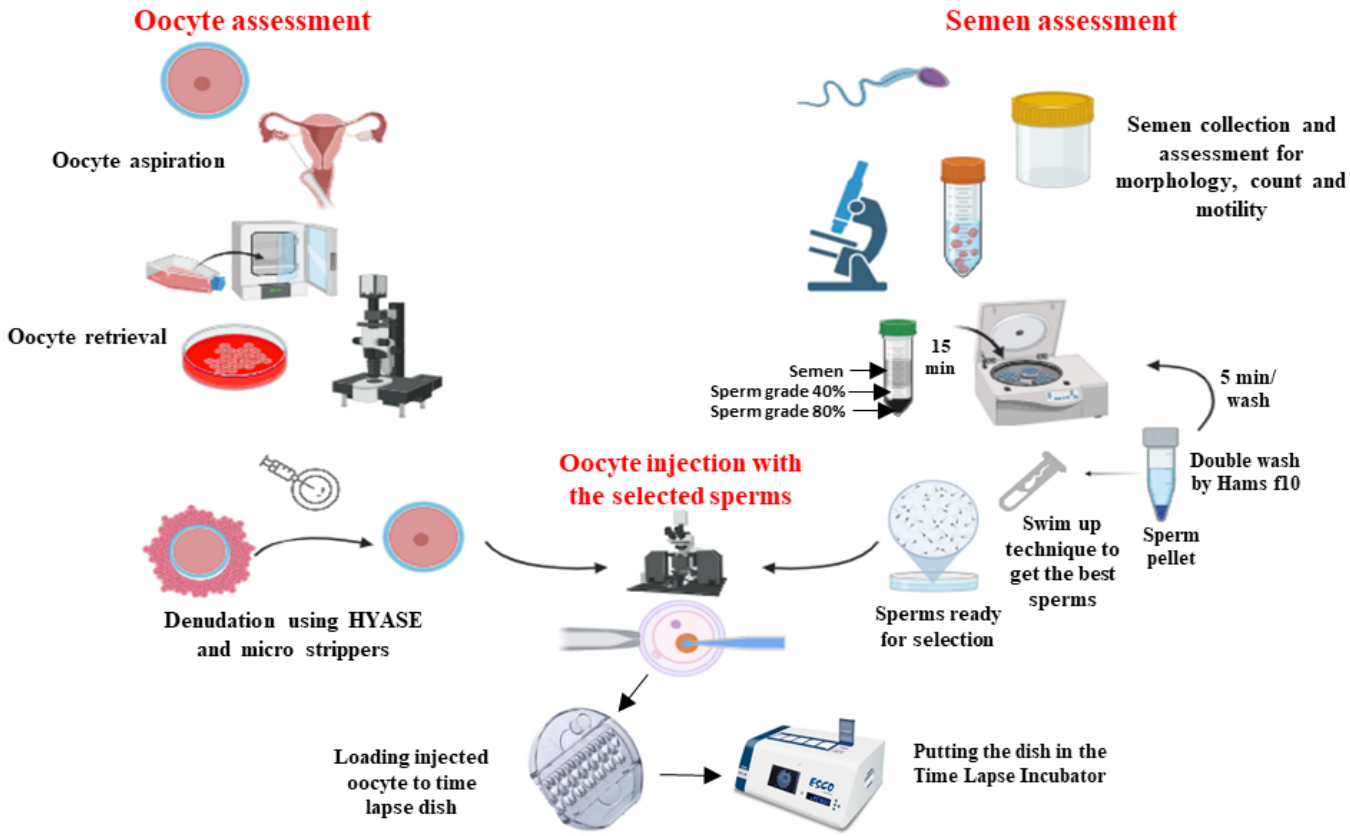


Figure 1. **Diagrammatic illustration of oocyte and sperm assessment and injection.**

The media contains amino acids such as arginine, asparagine, aspartic acid, cysteine, glutamate, glycine, histidine, leucine, lysine, methionine, phenylalanine, proline, serine, alanine, cystine, tryptophan, tyrosine, and valine. Also, it contains human albumin solution, gentamicin, glucose, lactate, and pyruvate, and some minerals in the form of potassium chloride (KCl), monopotassium phosphate (KH_2PO_4), magnesium sulfate ($MgSO_4$), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), and monosodium phosphate (NaH_2PO_4).

Assessment of embryos

Developmental events and embryo morphology were noted, along with the exact timing of the cell divisions that were observed in the hours following ICSI. There are many algorithms developed to evaluate embryo quality. Time-lapse grading is one of the advanced technologies for selection and grading. Time-lapse grading depends on estimating division time and rate, in this way, it is possible to predict embryonic development and the potential of implantation (28). Embryos spend either 3 or 5 days in incubators. After this period, embryos undergo assessment. Currently, the primary method used to determine embryo viability during the embryo culture phase is morphological examination (29), Morphological grading is applied according to Gardner grading system by quantifying three characteristics of embryo morphology which are expansion; trophectoderm quality; and inner cell mass (ICM) quality. The description of expansion status is given on a six-point rating system. Grades A, B, and C are used to describe the trophectoderm and ICM's qualities (30).

The final assessment was obtained from the time-lapse according to a flow- chart that identified the grades of the embryos, and this was done by annotation of specific times (Figure. 2). The embryos were divided throughout the monitoring period, and those reached the blastocyst on day 5, were designated as developing (63 embryos). While the embryos that failed to develop at any point during incubation were designated as arresting (30 embryos). The main timings used in time-lapse are: T0 which is the time of the sperm injection per oocyte, but practically, it is the mid-time point from when injection begins and ends for that patient's cohort of oocytes. This time point is used as a start time for the variables below which are measured in hours post injection, T2, which is the time of the first cell cleavage, or mitosis. T2 is the first frame at which the two blastomeres are completely

separated by individual cell membranes., T3 which is the time of the first observation of three discrete cells and this marks initiation of the second round of cleavage,and T5 at which the division reaches 5 cells. The embryo is described a morula when it starts the 4th round of division. The embryo is usually in day 4 and is composed of 16 to 32 blastomeres (31).

First, the final morphology on day 5 will be observed. If the embryo is a developed blastocyst, we will start to evaluate the embryo grade according to its morphokinetics. The first parameter that will be observed is T3. If its range is between (34 and 40 hours), the grade of the embryo will be A or B. But if it is out of that range, the embryo grade will be C or D (Figure. 2). Then we continue to observe the second parameter, which is the duration of the second cell cycle (CC2, the difference between T3 and T2). If the range is between (9 and 12 hours), the grade of the embryo will be A+ or A in the case of T3, was in the normal range. But if the CC2 is out of range, the embryo grade will be B+ or B in the case of T3, was in the normal range. If the CC2 is in the normal range but the T3 is out of range, the embryo grade will be C+ or C. While if the CC2 is out of the normal range and the T3 is out of the normal range, the embryo grade will be D+ or D. The final time parameter to be observed to subgrade the embryo, is the T5 (normal range between 45 and 55 hrs). If the T5 is in its normal range then the subgrade of the embryo will be A+ or B+ or C+ or D+ but if it is out of the normal range then the subgrade of the embryo will be A or B or C or D (Figure. 2). If the embryo is arrested (non-viable) on day 5, the grade will be F as its time parameters will be out of all normal ranges (Figure. 2).

Depending on this criterion of time lapse grading, we have chosen grade A and grade B, (t3=34-40 hrs and t5= 45-55 hours) to be under the category of developing embryos. Where after analysis, grade A showed no huge differences in their features or their amino acids concentrations from grade B.

Amino acids analysis of the culture media by Liquid chromatography–mass spectrometry (LC–MS)

Ninety-three samples of human culture media in which the embryos were developed were collected on day 5. The media were immediately frozen at -80 ºC until amino acid analysis. Samples of collected culture media underwent thawing using the Eksigent ekspertTM ultra-LC 100 system (Dublin – California, USA) as a chromatographic system. Culture media was prepared by methanol precipitation (33). A 3 µL aliquot of supernatant was diluted to 100 µL in 0.1% formic acid in HPLC-grade water. Amino acids were separated using an ACE 3 AQ column (150 × 0.5 mm, HiChrom, UK) maintained at ambient temperature. The injection volume was 2 µL.

Separation was accomplished using a gradient of 2 to 20% formic acid (0.1%) in acetonitrile over 5 min, at a flow rate of 20 µL/min. Amino acids' standard solutions containing 2.5 µmol/mL each of histidine (His), cysteine (Cys), leucine (Leu), isoleucine (Ile), lysine (Lys), methionine (Met), phenylalanine (Phe), threonine (Thr), tryptophan (Trp), valine (Val), alanine (Ala), arginine (Arg), aspartic acid (Asp), asparagine (Asn), glutamic acid (Glu), glutamine (Gln), glycine (Gly), proline (Pro), serine (Ser), tyrosine (Tyr), and cystine were prepared. Embryo-free control media incubated alongside the embryo media were treated in the same manner to illustrate any non-specific amino acid degradation or appearance. Mass spectrometric detection was performed on the AB SCIEX Triple Quad 5500 (AB SCIEX instruments, Foster City, CA) in MRM mode. A Turbo VTM Ion Source (ESI) interface in positive ionization mode was used. Both ultra-high-performance liquid chromatography (UHPLC) and mass spectrometer were controlled remotely using Analyst® software v. 1.6. (AB SCIEX, Foster City, Canada).

Statistical analysis of the data

Data were analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp.). The data were confirmed as being normally distributed using the Shapiro-Wilk test. Statistical analyses were conducted using a One-way ANOVA test for normally distributed quantitative variables to compare between more than two groups. The Post Hoc test (Tukey) was used for pairwise comparisons. Quantitative data were described using the mean and standard deviation (33). A P-value of 0.05 was considered to be significant. For the correlation between embryo morphokinetic parameters, the Pearson coefficient was used to correlate between two normally distributed quantitative variables.

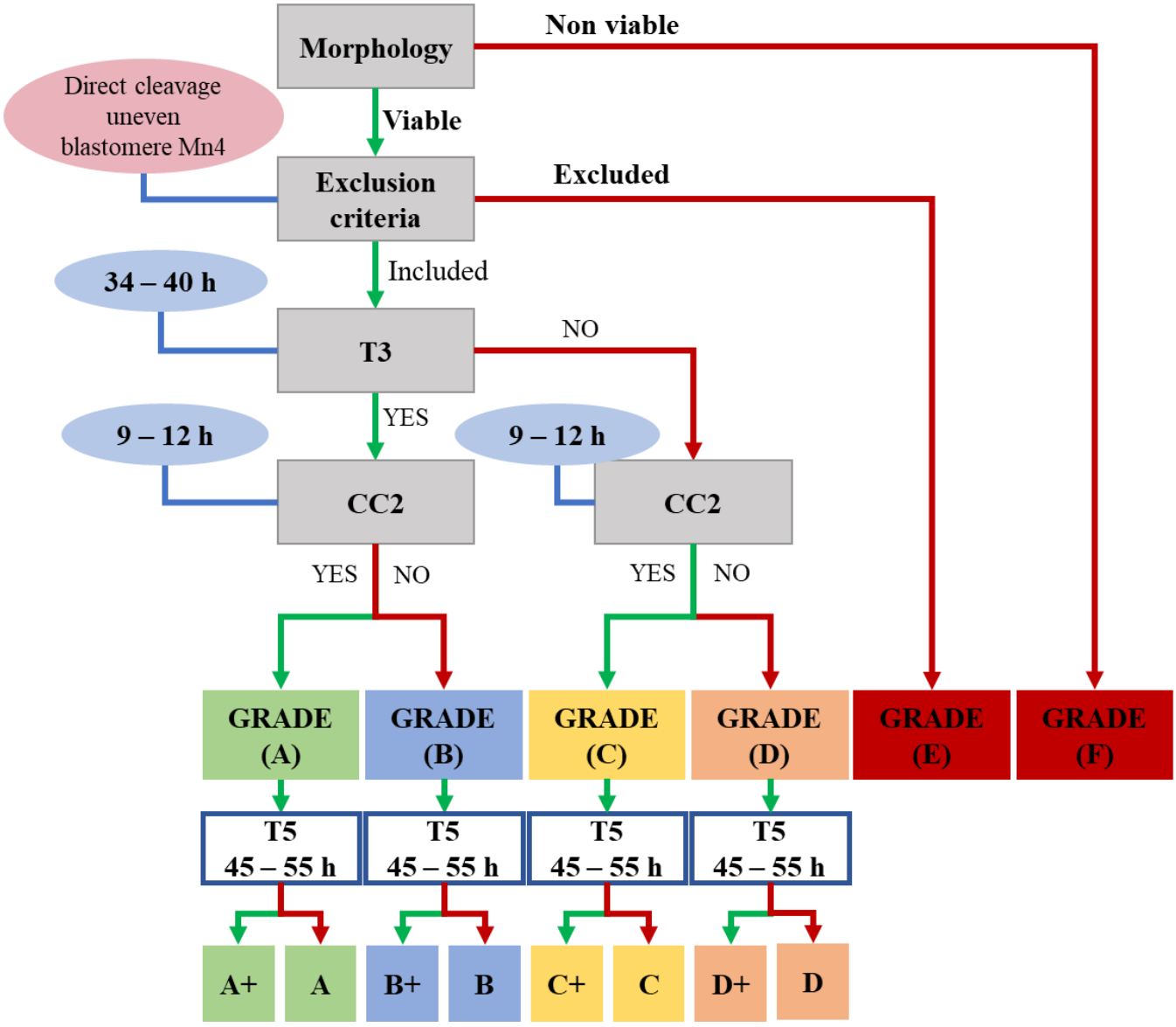


Figure 2. Embryo scoring and selection with time lapse monitoring system. Scoring was performed by analysis of time-lapse images of each embryo on an external computer with software developed for time-lapse image analysis (32). The precise timing of cell division and developmental parameters, such as blastomere symmetry and multinucleation, were determined. These deriving the following morphokinetic parameters: time of cleavage to two-, three-, four- or five blastomere embryo (T2, T3, T4 or T5). Additionally, the duration of the second cell cycle (CC2) which is the duration of the two-blastomere embryo phase (CC2=T3 −T2) was calculated (32). The grading was started for day 5 embryos that are viable and have acceptable morphology, but the embryos that show no acceptable morphology or viability, they considered as arrested or grade F.

Results

Morphokinetic parameters of developing and arrested day 5 embryos

Ninety-three human culture medium samples were collected after their embryos were examined. The embryos, that divided throughout the monitoring period and reached the blastocyst stage on day 5, were designated as developing (grades A and B, 63 samples). The other embryos that did not develop and had stopped cleaving at any stage throughout the incubation period were considered arrested embryos (30 samples), Table 1.

Table 1: Embryo characteristics: number and maternal age

Embryo quality	Number of embryos	Maternal age (Mean ± SD)
All	93	30 ± 2
Developing	63	31 ± 3
Arrested	30	32 ± 3

Tables 2 and 3 describe the morphokinetic data and embryos image determined by the time-lapse incubator for both developing and arrested embryos. The data represents a significant delay in the most important three times (T2, T3, and T5) in the arrested embryos compared to the developing embryos. The range of T2 is 25.2 ± 1.0 h for developing embryos and 33.08 ± 4.7 h for arrested embryos. The T3 range is 38.03 ± 1.5 h for developing embryos and 47.08 ± 3.7 h for arrested embryos, while the T5 range is 50.3 ± 1.6 h for developing embryos and 66.3 ± 6.7 h for arrested embryos. The CC2 value, which is T3-T2, was 12.7 ± 1.9 h for developing embryos and 14.3 ± 5.5 h for arrested embryos (Table 2).

Table 2: Morphokinetic parameters of developing and arrested embryos to the blastocyst stage

Parameters/ h	Developing embryos	Arrested embryos
T2	25.45± 1.05 (25.29)	33.33± 5.41* (32.1)
T3	36.43± 1.17 (36.12)	48.66± 4.26* (48.6)
T5	50.25± 1.67 (50.29)	66.46± 7.85* (65.4)
CC2	10.98± 1.40 (11.36)	15.34± 2.50 (15.05)

Table 3 represents the time-lapse imaging of embryos during different times of division, which are considered important parameters of grading. In this table, the images of developing and arrested embryos at different times were captured by the Esco time-lapse incubator at Agial Hospital. For developing, there are two images at each time, one for grade A and the other for grade B, which both are considered to be developing and were captured at the optimum time. The morphology of the embryos could be near each other for developing and arrested but at developing, they have reached this stage in the normal range, while at the arrested they have delayed time, and sometimes they cannot complete division and development.

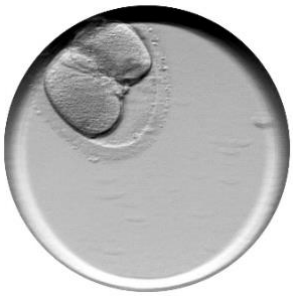
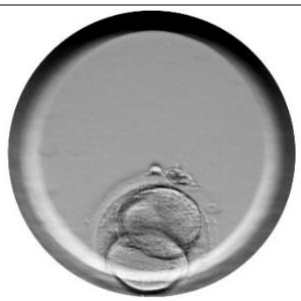
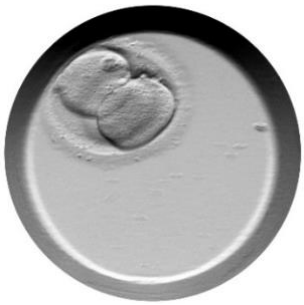
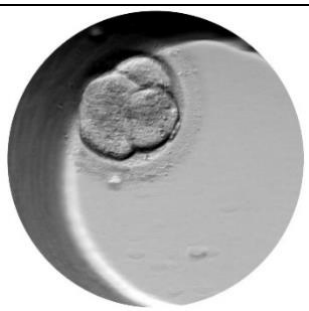
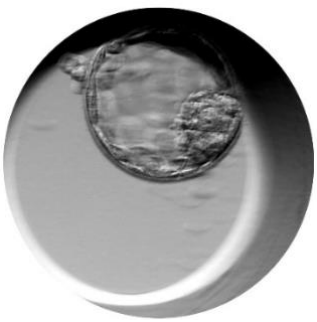
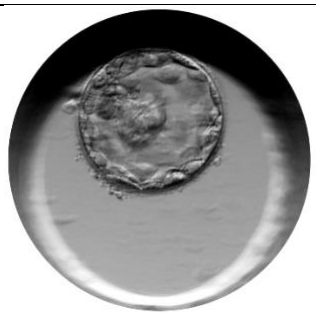
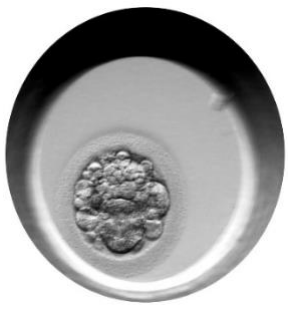
Amino acids concentrations in the culture media of developing and arrested day 5 embryos

The concentration of amino acids in the culture media of developing embryos (A and B) and arrested embryos are illustrated in Table 4 and Figure. 3. A significant ($p \leq 0.05$) increase in the concentration of five amino acids (Thr, Val, Ala, Ser, and Cys) was observed in the media of developing embryos compared to their corresponding levels in the control media (embryo-free media). On the other hand, the concentrations of His, Lys, cystine, Asp, Glu, Gln, and Gly were significantly decreased in the media of developing embryos compared to that of the control media. In the culture media of arrested embryos, concentrations of Ala, Gln, Ile, and Pro were significantly increased, while Ser and Gly concentrations were significantly decreased compared to control samples. The concentrations of other amino acids such as Phe, Met, TrP, Leu, Arg, and Tyr showed a non-significant change between the media of developing and arrested embryos and control media (Table 4 and Figure. 3).

Amino acids release and consumption in the culture media of developing and arrested day 5 embryos

The changes in amino acid concentrations in the media of developing and arrested embryos are clearly illustrated in Figure. 4 and Table 5. Total amino acids release and consumption are determined by positive or negative values after subtraction from the control values. The positive values mean that the amino acids are released in the media, while the negative values mean that the amino acids are consumed by the embryo. Table 5 illustrates that the number of amino acids that are consumed and released in the media of developing embryos are greater than that in the arrested one. Interestingly, the essential amino acids are neither be consumed nor released in the culture media of the arrested embryos compared to the embryo-free control media. While in the media of the developing embryos, Thr and Val were highly released, and His and Lys were consumed. Data also revealed that, Ser is released in the culture media of developing embryos and consumed from the media of arrested embryos. Moreover, arrested embryos are marked by the release of Gln, Ile, and Pro in their culture media, and these amino acids were either consumed (Gln) or there was no change (Ile and Pro) in the culture media of developing embryos. These data do not show only the amino acids that

Table 3: Time-lapse imaging of embryos during different times

Parameters/ h/ definition	Developing embryos		Arrested
T2/ The time at which the cells begin to divide into two cells. It begins after forming the pronuclear. It ranges between 21.4-34.8 hrs for the embryo to be classified as developing embryo.	 **24 h**	 **21 h**	 **40 h**
T3/ The time at which the embryo divides to three blastomeres. It ranges between 34- 40 hrs for the embryo to be classified as developing embryo.	 **35 h**	 **31 h**	 **48 h**
T5/ The time at which the embryo divides into five blastomeres It ranges between 44- 55 hrs for the embryo to be classified as developing embryo.	 **45 h**	 **44 h**	 **63 h**
End of incubation at day 5	  Developing embryos are formed as a blastocyst with very good morphology of trophectoderm and inner cell mass.		 Arrested embryos which could not complete its development and forms cellular fragments.
The images of developing and arrested embryos at different times were captured by Esco MIRI time lapse incubator MTL-0038, Denmark, at Agial Hospital, Alexandria, Egypt.			

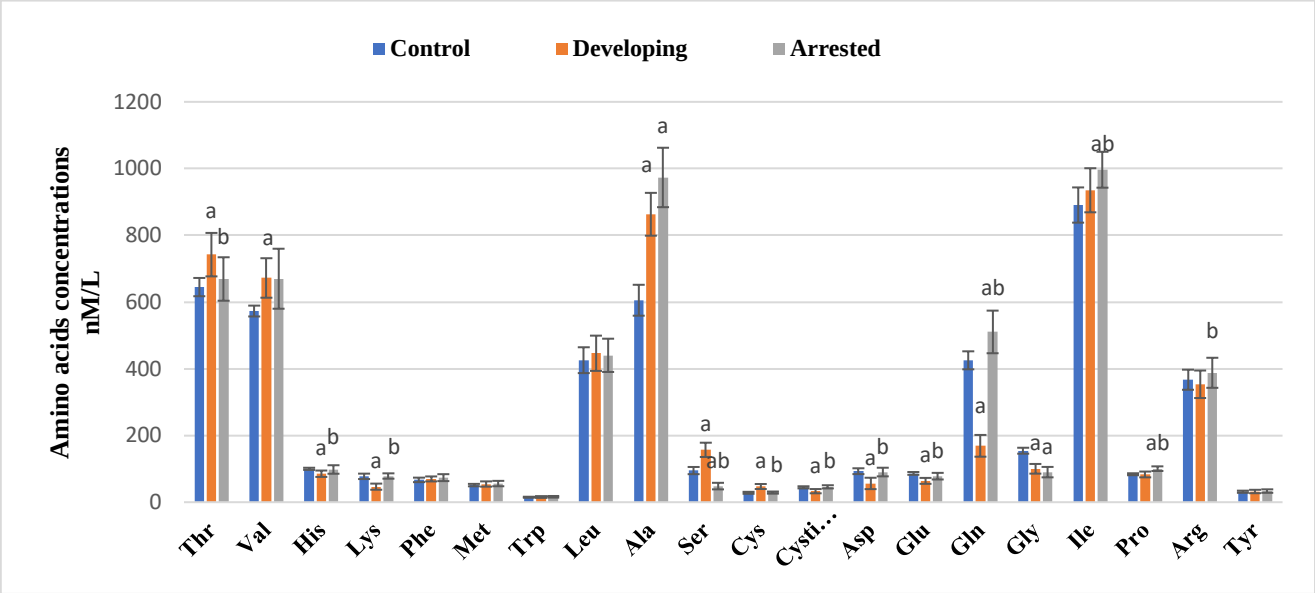


Figure 3. Amino acid concentrations in the culture media of developing and arrested embryos compared to embryo-free control media. The data was expressed using mean± SD. A One-way ANOVA test was used to compare the studied groups.

a: Significant with Control. b: Significant with media of developing embryos

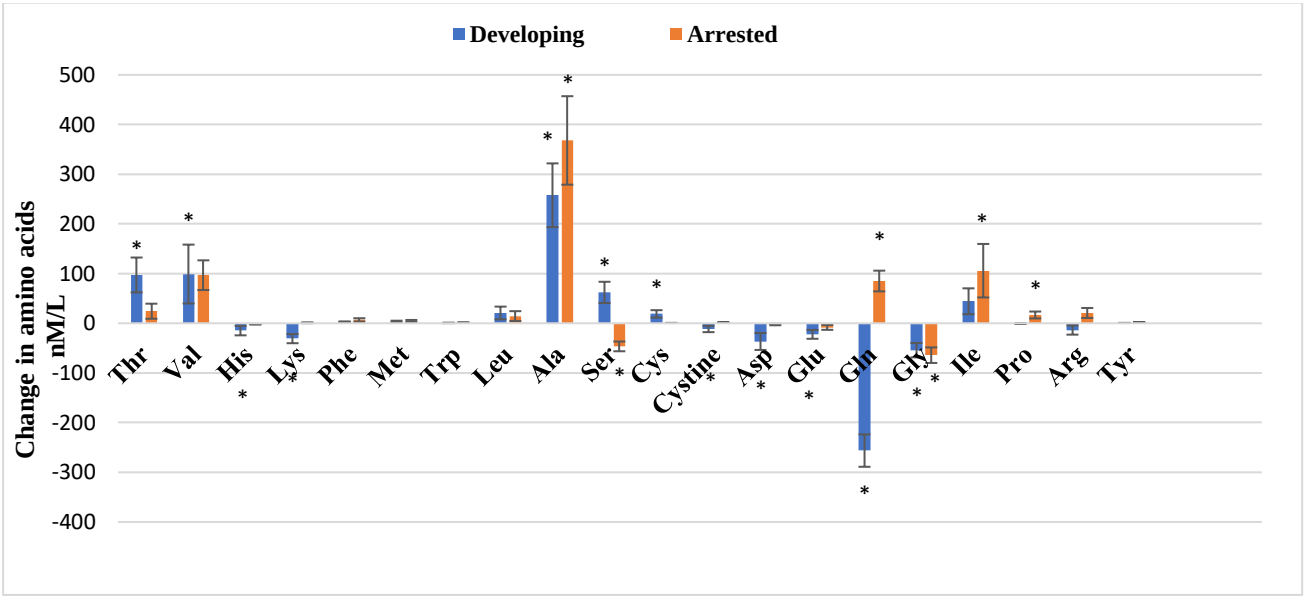


Figure 4. The changes in the amino acids concentrations in the culture media of developing (grad A and B) and arrested embryos compared to embryo- free control media. Data was expressed as Mean± SD.

(*) means that amino acid is significant either released or consumed and that illustrated in the graph where positive values means released and negative values are consumed also that's described at figure 5.

Table 4: **The concentrations of amino acids (nmol/l) in the culture media of developing and arrested embryos compared to the control group (embryo-free media) on day 5**

Groups / Amino acids	Control media	Media of developing embryos	Media of arrested embryos
Essential amino acids			
Thr	644.75±27.4	742±65.12[a]	669±65.16[b]
Val	573±16.26	672.12±59.2[a]	669.8±89.9
His	100.22±3.25	85.3±9.57[a]	98.01±12.72[b]
Lys	77.47±8.1	46.5±9.18[a]	78.47±8.0[b]
Phe	66.87±6.75	69.69 ± 7.47	73.61±10.41
Met	51.26±3.62	54.49±7.94	55.87±8.09
Trp	14.83±1.51	15.55±2.62	16.17±2.40
Leu	425.67±38.74	446.4±52.8	440.2±49.84
Non-essential amino acids			
Ala	605.3±46.32	863.0 ±44[a]	973.37±89.04[a]
Ser	94.80±10.78	156.94±21.4[a]	48.27±9.82[ab]
Cys	28.12±2.93	46.98±7.51[a]	28.77±3.16[b]
Cystine	44.53±3.14	33.0±6.35[a]	46.07±4.73[b]
Asp	92.88±8.77	56.12±17.06[a]	90.37±12.95[b]
Glu	86.29±4.21	63.92±8.85[a]	78.00±9.88[b]
Gln	425.2±27	168.9±32.6[a]	510.0±63.86[ab]
Gly	154.3±8.91	100.10±14.49[a]	90.09±15.65[a]
Ile	890.68±52.8	935±66.04	996.51±53.82[ab]
Pro	83.74±2.93	83.14±8.7	100.43±6.96[ab]
Arg	367.02±30.15	353.43±41.27	387.85±45.02[b]
Tyr	31.36 ±3.21	32.03±5.15	33.3±5.21

Data was expressed using Mean± SD. One-way ANOVA test was used to compare between the studied groups.

Statistically significant values are at $p \leq 0.05$.

a: Significant with Control.

b: Significant with media of developing embryos

Table 5: Consumed and released amino acids in the culture media of developing (grad A and B) and arrested embryos compared to embryo-free media at day 5

Developmental stage Day 5	Consumed amino acids		Released amino acids	
	Developed embryos	Arrested embryos	Developed embryos	Arrested embryos
	His^{E}	Ser^{NE}	Thr^{E}	Ala^{NE}
	Lys^{E}	Gly^{NE}	Val^{E}	Gln^{NE}
	$Cystine^{NE}$		Ala^{NE}	Ile^{NE}
	Asp^{NE}		Ser^{NE}	Pro^{NE}
	Glu^{NE}		Cys^{NE}	
	Gln^{NE}			
	Gly^{NE}			

Superscripts depict the amino acid category. E=essential amino acids, NE=non-essential amino acid

were released or consumed but also show the differences in the production and consumption of amino acids between the developing and arrested embryos. Therefore, these findings can be used as a good marker for differentiation and can help in the prediction of the embryos' quality.

Amino acid turnover in the culture media of developing and arrested day 5 embryos

Rather than being the amino acids taken up from the culture medium, the majority of amino acids were liberated into it (Figure 5). Consequently, positive values were attained by the balance. Figure. 5 shows that the total amount of amino acids consumed by developing embryos is significantly higher than that consumed by arrested embryos, and vice versa for the released amino acids.

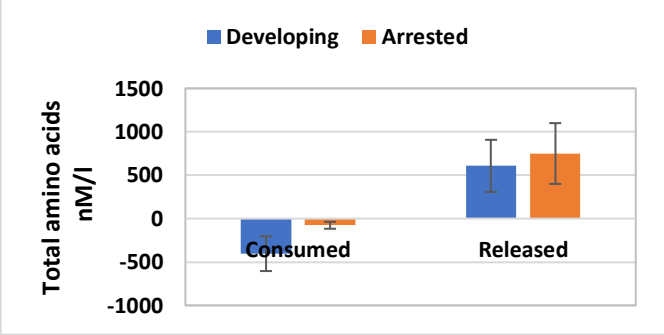


Figure 5. Amino acid turnover in the culture media of developing and arrested day 5 embryos.

Values are the sum of the amino acids concentrations that are significantly increased or decreased in the medium.

The correlation between morphokinetic parameters and the amino acid concentrations (consumed or released) in the media of developing and arrested embryos

Table 6 revealed that most of the significant positive correlations were obtained between the essential (Phe, Met, Trp, and Leu) and non-essential (Ile, Pro, and Tyr) amino acids whose concentrations were non-significantly changed in the media of developing embryos compared to the embryo-free control media. The essential amino acid leucine showed a significant positive correlation with each of Phe, Met, Trp, Ile, Pro, and Tyr. Moreover, Ile, Pro, and Tyr are positively correlated with Phe, Met, Trp, and Leu. Additionally, Pro and Tyr are positively correlated with Glu. On the other hand, Asp, which is highly consumed from the media of developing embryos, is positively correlated with Glu and negatively correlated with CC2. Also, Table 6 confirms that CC2 is positively correlated with T3 and negatively correlated with T2. Furthermore, His is positively correlated with cystine, Glu, Pro, T2, and T5.

The correlation between the amino acids in the media of arrested embryos showed a different pattern than that of developing embryos. Interestingly, there is a significant positive correlation between all of the essential amino acids except Lys and Ile (Table 7). Moreover, Glu, Arg, and Tyr (non-changed non-essential amino acids) have a significant positive correlation with all the essential amino acids except Lys and Ile (Thr, Val, His, Phe, Met, and Trp). Ile, a released amino acid, is negatively correlated with Ser and Gly, which are consumed amino acids. Unlike the developing embryos, the arrested embryos showed a significant positive correlation between T3 and T2.

Aspartate is one of the most important amino acids for embryos' growth. In the media of developing embryos, Asp was negatively correlated with most of the essential amino acids and also negatively correlated with T3 and CC2. In the media of arrested embryos, Asp was positively correlated with all of the essential amino acids and negatively correlated with T2, T3, and T5.

Discussion

Recently, ICSI has been the most significant breakthrough in the treatment of infertility. It was originally designed to overcome male infertility barriers (34). In our study, we used to integrate the embryo grade and the amino acid profile of the embryos' culture media at day 5 in a time-lapse incubator. We employed two non-invasive methods to assess human embryonic quality. One method involved the determination of the amino acids profile in embryo culture media, showing promise in enhancing individual embryo selection for clinical IVF transfers (35). The other method utilizes a time-lapse incubator, which combines conventional morphological observation and grading of embryos with the analysis of their morphokinetics. Most IVF labs utilize time-lapse monitoring systems, which enable them to control for anomalies that traditional microscopy could overlook (36-38). It is significant to note that incubation conditions, including oxygen levels and amino acids composition, have an impact on embryonic metabolism (39). Amino acids, especially essentials, play vital roles in fetal nutrition and early embryonic signaling pathways. Studies found that blastocysts cultured in a medium with essential amino acids produced larger fetuses (40,41).

Interestingly, amino acid turnover shows significance differences in most of the papers that worked in this area. This variation depends mainly on the use of various analytical methods, sample preparation, and the selection of the culture medium (1, 42, 43). In our study, we used Origio SAGE culture media, a one-step medium used for developing the blastocysts. This medium contains all the essential and non-essential amino acids, and this gives the embryos the ability to choose their needs for amino acids (39, 43). In this study, the levels of twenty amino acids in the culture media were analyzed using LC– MS. Following 5 days of incubation, the amino acid levels exhibited variability among different developing and arrested embryos. While some amino acids increased in developing embryos, others decreased, and certain amino acids showed no significant changes. Furthermore, there is a high correlation observed between T3 and some amino acids such as Thr, Val, His, Phe, Trp, Arg, Leu, Tyr, and Pro. T3 is considered a predictor of blastocyst formation, which is the surge of division where cells start dividing into four cells (44). At this stage, the cell needs the most important precursors of the amino acids to help the embryo develop.

In our study, the amino acids' profiles in the media of developing embryos after 5 days of incubation exhibited a significant increase in Ala, Thr, Ser, Cys, and Val levels. This observation aligns with the findings of Huo, Zhu (45), who detected Ser, Thr, and Ala in the culture medium and noted significant differences between pregnant and non-pregnant groups. Conversely, Phe, Met, Trp, Leu, Arg, Proline, Ile, and Tyr showed no significant changes in the media of developing embryos, while His, Lys, Gln, Glu, Asp, Gly, and cystine exhibited significant depletion. These findings differ from other researches due to many factors, including the use of different culture media, time of sample collection, and method for determination of amino acids concentration.

Alanine was found to be released in both developing and arrested embryos, being the most abundant amino acid released in the media of developing embryos. During amino acid metabolism, excess nitrogen needs to be excreted, often in the form of ammonia, which can be toxic to cells (46). Orsi and Leese (47) noted the release of nitrogen as free ammonium ions, with alanine being produced following the transamination of pyruvate [48]. Additionally, the accumulation of alanine in the medium is believed to serve as a route for ammonia disposal by the early embryo via glutamate dehydrogenase and alanine transaminase, explaining the elevated concentrations observed (48). Drabkova, Andrlova (39) supported this, suggesting that ammonia accumulation may contribute to the elevation of alanine in the cultural media. Moreover, the amount of alanine produced by the arrested embryos is greater than that produced by developing embryos, and that is also confirmed by Houghton, Hawkhead (13), who explains this phenomenon indirectly supports the proposition that alanine production provides a mean of disposing of toxic ammonium.

Interestingly, our findings revealed a significant depletion of glutamine levels in developing embryos, whereas an increase was observed only in arrested embryos' media. Elevated concentrations of glutamine in the culture media of arrested embryos may be related to an accumulation of ammonia (39).

Table 6: Correlation between amino acids and the time-lapse parameters of developing embryos.

	Thr	Val	His	Lys	Phe	Met	Trp	Leu	Ala	Ser	Cys	Cystine	Asp	Glu	Gln	Gly	Ile	Pro	Arg	Tyr	T2	T3	T5	CC2
Thr		(+)	(+)	(-)	(+)	(-)	(-)	(-)	(-)	(-)	(-)	(+)	(-)	(-)	(+)	(-)	(-)	(+)	(-)	(-)	(-)	(-)	(+)	(+)
Val			(-)	(-)	(+)	(+)	(+)	(+)	(-)**	(+)	(+)	(-)	(-)	(-)	(-)	(+)	(+)*	(+)	(-)	(+)	(-)	(+)	(-)	(+)
His				(-)	(+)	(+)	(+)	(+)	(+)	(-)	(-)	(+)*	(+)	(+)*	(-)	(+)	(+)	(+)**	(+)	(+)	(+)*	(+)	(+)*	(-)
Lys					(+)	(-)	(-)	(+)	(+)	(+)	(-)	(-)	(-)	(-)	(+)	(-)	(+)	(-)	(+)	(-)	(-)	(+)	(+)	(+)
Phe						(+)	(+)	(+)*	(-)	(-)	(-)	(+)*	(-)	(+)	(+)	(+)	(+)*	(+)*	(+)	(+)	(+)	(+)	(-)	(-)
Met							(+)**	(+)**	(+)	(-)	(-)	(+)	(+)	(+)**	(+)	(+)	(+)*	(+)*	(+)	(+)**	(+)	(+)	(-)	(-)
Trp								(+)**	(-)	(-)	(+)	(-)	(+)	(+)**	(-)	(+)	(+)*	(+)*	(-)	(+)**	(+)	(+)	(-)	(+)
Leu									(-)	(+)	(+)	(+)	(-)	(+)	(+)	(+)	(+)*	(+)*	(-)	(+)**	(-)	(+)	(-)	(+)
Ala										(-)	(-)**	(+)	(+)	(+)	(-)	(+)	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(-)
Ser											(+)	(-)	(-)	(-)	(+)	(-)	(+)	(-)	(-)	(-)	(+)	(+)	(+)	(+)
Cys												(-)	(-)	(-)	(-)	(+)	(+)	(-)	(-)	(+)	(-)	(+)	(-)	(+)
Cystine													(+)	(+)	(+)	(+)	(+)	(+)*	(+)	(-)	(+)	(-)	(+)	(-)
Asp														(+)**	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(-)	(+)	(-)*
Glu															(-)	(+)	(+)	(+)**	(+)	(+)**	(+)	(-)	(+)	(-)
Gln																(-)	(-)	(-)	(+)	(-)	(-)	(+)	(-)	(+)
Gly																	(+)	(+)	(+)	(+)*	(+)	(-)	(-)	(-)
Ile																		(+)*	(+)	(+)*	(+)	(+)	(-)	(+)
Pro																			(+)	(+)*	(+)	(+)	(+)	(-)
Arg																				(-)	(+)	(-)	(-)	(-)
Tyr																					(+)	(+)	(-)	(+)
T2																						(+)	(+)	(-)**
T3																							(+)	(+)**
T5																								(-)
CC2																								

Pearson coefficient was used to correlate between two normally distributed quantitative variables. The positive correlation is indicated with the symbol (+), a negative correlation with (-). a. Cannot be computed because at least one of the variables is constant. *. Correlation is significant at the 0.05 level (2-tailed)., **. Correlation is significant at the 0.01 level (2-tailed).

Table 7: **Correlation between amino acids and the time-lapse parameters of arrested embryos.**

	Thr	Val	His	Lys	Phe	Met	Trp	Leu	Ala	Ser	Cys	Cystine	Asp	Glu	Gln	Gly	Ile	Pro	Arg	Tyr	T2	T3	T5	CC2
Thr		(+)*	(+)*	(+)	(+)*	(+)*	(+)*	(+)	(-)	(+)	(+)	(+)*	(+)	(+)*	(+)	(+)*	(+)	(+)	(+)*	(+)*	(+)	(+)	(-)	(-)
Val			(+)*	(-)	(+)*	(+)	(+)*	(+)	(-)	(-)	(-)	(+)	(+)	(+)**	(+)**	(+)**	(+)**	(+)	(+)**	(+)**	(+)	(+)	(-)	(+)
His				(+)	(+)*	(+)*	(+)*	(+)*	(+)	(+)	(-)	(+)	(+)	(+)**	(+)	(+)	(+)	(+)	(+)	(+)**	(+)	(+)	(-)	(-)
Lys					(+)	(-)	0	(+)	(+)*	(-)	(-)	(-)	(+)	(+)	(-)	(-)	(+)	(-)	(-)	(-)	(+)	(+)	(+)	(+)
Phe						(+)*	(+)*	(+)	(-)	(+)	(-)	(+)	(+)	(+)**	(+)	(+)**	(+)	(-)	(+)*	(+)*	(+)	(+)	(-)	(+)
Met							(+)*	(+)	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)*	(+)*	(+)	(-)	(-)	(-)
Trp								(+)	(-)	(+)	(+)	(+)	(+)	(+)**	(+)	(+)**	(+)	(+)	(+)*	(+)*	(+)	(+)	(-)	(-)
Leu									(+)	(-)	(+)	(-)	(+)	(+)**	(+)	(+)	(+)*	(-)	(+)	(+)	(+)	(+)	(-)*	(-)
Ala										(+)	(-)	(-)	(+)	(+)	(+)	(-)	(+)	(-)	(-)	(-)	(+)	(+)	(+)	(+)
Ser											(-)	(+)	(-)	(+)	(+)	(+)	(-)	(+)	(-)	(+)	(+)	(+)	(+)	(+)
Cys												(+)	(-)	(-)	(-)*	(-)	(-)	(+)	(+)	(+)	(+)	(+)	(-)	(-)
Cystine													(+)	(+)	(-)	(+)	(-)	(+)	(+)**	(+)**	(+)	(+)	(+)	(-)
Asp														(+)	(+)	(-)	(+)	(-)	(+)	(+)	(-)	(-)	(-)	(+)
Glu															(+)**	(+)**	(+)	(+)	(+)*	(+)*	(+)	(+)	(-)	(-)
Gln																(+)**	(+)	(-)	(+)	(+)	(-)	(-)	(-)	(+)
Gly																	(-)	(+)	(+)	(+)**	(+)	(+)	(-)	(-)
Ile																		(-)	(+)	(+)	(-)	(-)	(+)	(+)
Pro																			(+)	(+)	(+)*	(+)*	(-)	(-)
Arg																				(+)**	(+)	(+)	(-)	(-)
Tyr																					(+)	(+)	(-)	(-)
T2																						(+)**	(-)	(-)**
T3																							(-)	(-)
T5																								(+)
CC2																								

Pearson coefficient was used to correlate between two normally distributed quantitative variables. The positive correlation is indicated with the symbol (+), a negative correlation with (-). a. Cannot be computed because at least one of the variables is constant. *. Correlation is significant at the 0.05 level (2-tailed)., **. Correlation is significant at the 0.01 level (2-tailed).

Additionally, increased glutamine consumption in developing embryos' media was concluded to be a compensatory mechanism aimed at mitigating oxidative stress in embryos (49).

Glutamine serves as a conditionally essential amino acid, playing pivotal roles beyond protein synthesis. These include maintaining cellular energy through the tricarboxylic acid cycle, regulating acid-base balance, and contributing to the synthesis of nonessential amino acids, lipids, and nucleotides (50). These may explain the depleted glutamine levels in developing embryos due to its consumption. Additionally, Rieger (51) found that glutamine uptake is elevated during blastocyst expansion in two and four-cell bovine embryos due to increased protein synthesis. Conversely, Miao, Feng (49) stated that poor-quality embryos consumed considerably more glutamine than good-quality embryos.

A study by Alexiou and Leese (52) highlighted the significance of glycine, glutamine, and aspartate in proliferating somatic cells, as they provide nitrogen atoms essential for de novo synthesis of purine and pyrimidine precursors of nucleic acids. Thus, these amino acids are crucial for early embryo development, promoting the progression of day 2 human embryos to blastocysts in culture. Despite their roles in nucleic acid synthesis, our study revealed a positive correlation between aspartate and glycine. Serine and threonine levels exhibited significant elevation in the media of developed embryos, as confirmed by Huo, Zhu (45), who found that serine and threonine concentrations were elevated, in the culture media of day 3 embryos, particularly in the pregnant group. While in the media of arrested embryos, serine was elevated and there was no significant change observed for threonine. The increase in serine levels in the developing embryos' media may be attributed to its biosynthetic pathways, which originate from aspartate and other amino acids of the aspartate family, such as Lys, Met, and Ile. In blastocysts during development, threonine tends to accumulate, unlike most other amino acids (11, 12). The trophectoderm possibly produces threonine by hydrolyzing protein in vitro (53). Trophoblast cells are capable of this process through pinocytosis, followed by protein digestion (54). These mechanisms likely contribute to the observed elevation of threonine levels in the media of developing embryos.

Cysteine is also one of the elevated amino acids in the media of developing embryos while remaining unchanged in arrested embryos' media. Suggesting a potential mechanism for embryos to regulate cysteine levels within the media as a defense against oxidative stress. Furthermore, there is a negative correlation between cysteine and cystine, which increased in the media of developing embryos. This may be attributed to redox activity occurring within the embryos, which typically operates in equilibrium (55). Leese, McKeegan (46) elucidated that cysteine, in the form of selenocysteine, serves as a constituent of certain glutathione peroxidase, an important component of the glutathione cycle responsible for the regeneration of this key intracellular antioxidant. This cycle operates in oocytes and early embryos, emphasizing the significance of cysteine, particularly in its selenocysteine form, in antioxidant defense mechanisms.

In the present study, a significant depletion in aspartate levels was observed in the culture media of developing embryos, while no change was observed in arrested embryos. This depletion can be attributed to the essential role of aspartate in metabolism. The reduced levels of aspartate in developing embryos align with findings by Drabkova, Andrlova (39), but contradict those of Huo, Zhu (45), who determined high levels of aspartate in day 3 embryos in the pregnant group. Additionally, Houghton, Hawkhead (13) reported the appearance of aspartate at embryos developing from morula to blastocyst. Aspartate is considered one of the most metabolically essential amino acids during the preimplantation phase due to its role in regulating the activity of the malate-aspartate shuttle, which facilitates the regeneration of NAD+ in the cytoplasm (8, 56). This process provides the co-factor required for glucose metabolism (57). On the other hand, inhibition of the malate-aspartate shuttle in the pre- and post-compaction stages significantly reduces blastocyst development and quality and impairs subsequent fetal and placental growth (58, 59). This may explain the high consumption of aspartate in the media of developing embryos. Additionally, the positive correlation observed between aspartate and tryptophan in our study may be attributed to their roles in NAD+ synthesis. Moreover, the study shows a positive correlation between alanine and aspartate, and this is due to their roles, which depend on each other for Krebs cycle and ATP production. Part of aspartate is converted to oxaloacetate by aspartate aminotransferase, and alanine is converted into pyruvate, which enters the Krebs cycle for energy production (60).

Glutamate, for instance, is synthesized and subsequently decomposes to produce α-ketoglutaric acid, which generates ATP through the citric acid cycle. Additionally, glutamate serves as a secondary source of carbon and nitrogen for the re-synthesis of pyrimidines and hydrazines, while acting as a reducing agent to safeguard cells from oxidative stress [45]. These processes likely contribute to the heightened consumption and diminished levels of glutamate observed in the media of developing embryos. Essential amino acids like lysine, which decreased in the media of developing embryos remained unchanged in the media of arrested embryos, play a vital role in stimulating blastocyst growth by enhancing embryo cleavage rate. Additionally, research by Van Winkle, Galat (61) suggested that lysine is probably converted to glutamate within these cells. Moreover, human embryonic stem cells in culture exhibit a higher consumption of lysine compared to their production levels (61).

Glycine is predominantly consumed by both developing and arrested embryos due to its role in conjunction with taurine and hypotaurine, which help maintain osmotic homeostasis and regulate cell volume (62). The utilization of these metabolites as organic osmolytes may be unique to the early embryo, avoiding the need for high quantities of ions, which could otherwise alter cell biochemistry and electrophysiology (63). Our research shows a negative correlation between glycine and threonine, this is due to threonine dehydrogenase, which catalyzes the conversion of threonine to glycine and acetyl CoA (64). Glycine in embryonic stem cells is crucial to sustaining the specific epigenetic modifications needed to preserve their pluripotency (65, 66). Moreover, a study by Brison, Houghton (1) demonstrated a significant association of asparagine, glycine, and leucine with clinical pregnancy and live birth.

Several amino acids, Phe, Met, Trp, Arg, Leu, and Tyr, exhibited no significant change in their levels in the media of both developing and arrested embryos compared to the control media. This observation raises questions regarding whether these amino acids were not consumed by the embryos from the media, or if they were released and utilized in an equilibrium manner. Notably, tryptophan plays a pivotal role in niacin synthesis, which is essential for the production of NAD, a coenzyme crucial for energy production (67).

Proline exhibited a significant increase in the media of arrested embryos compared to the control while remaining unchanged in developing embryos. Additionally, a positive correlation was observed between glutamate and proline. Its elevation may be due to excess production of proline to reduce the oxidative reaction, which results in the arrested embryos helping the embryo to develop. Studies have noted that chronic exposure to proline can decrease cellular reactive oxygen species (ROS) levels through various context-dependent mechanisms. One such mechanism involves proline serving as a ROS scavenger due to its secondary amine group (68-70), and by contributing to the production of the cell's major antioxidant, GSH (69). Therefore, the increase in proline levels in the media of arrested embryos may signify an adaptive response by the embryo to enhance its chances of survival. Alternatively, this elevation could stem from cellular decomposition within the arrested embryos, leading to a higher release of amino acids responsible for oxidative reactions such as proline.

Time-lapse monitoring is a valuable tool in laboratory practice. Research suggests that early embryo cleavage is a reliable indicator of developmental capacity (71). Our utilization of time-lapse monitoring systems enables the detection of anomalies, such as multinucleation or asynchronous division, which may otherwise go unnoticed under traditional microscopy, helping us to detect the arrested embryos at an earlier time (38). Our research focuses on investigating the correlation between human embryonic kinetic events and metabolism. We have identified a relationship between times of division (t2, t3, and t5) and embryo metabolism for both developing and arrested embryos. In developing embryos, the consumption of histidine is positively correlated to the division times t2 and t5. While in arrested embryos, the release of proline into the media is positively correlated to the division times t2 and t3. This correlation can serve as a distinguishing marker between the two grades of embryos.

Generally, based on the current results, we can conclude that developing embryos consume a higher number of amino acids compared to arrested embryos. That is contradicted with Houghton, Hawkhead (13), who found that arrested embryos had a greater amino acid uptake when developing from morula to blastocyst stage. However, our study has several limitations that merit consideration. Firstly, we only collected culture medium on day 5 of embryonic culture. Further investigation is needed to determine if similar results would be obtained with collection at alternate time points. Secondly, the number of clinical samples was limited. Future research should aim to increase the sample size based on power calculations to validate our current findings. Thirdly, we did not explore whether the metabolic profiles associated with

embryo quality could provide further insight into the predictive capacity of amino acid profiling for IVF pregnancy outcomes.

Conclusion

Among the suggested markers for developing embryos are elevated alanine, valine, cystiene, threonine, and serine. However, alanine cannot be used as an accurate marker as it significantly increases in developed and arrested embryos. Moreover, the significant depletion in serine levels can be used as a marker for arrested embryos, in addition to the elevated levels of proline, isoleucine, and glutamine. Therefore, applying two non-invasive techniques, can be an excellent way to ensure the selection of the best embryo to increase the success rate and avoid multiple births. Although it may require a longer processing time, the amino acid profile by LC-MS can be used for difficult cases with a high abortion rate as a confirmatory assessment but can't be used as a primary assessment but can be used to predict the implantation potential of morphologically excellent embryo.

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References

1. Brison, D.R., et al., Identification of viable embryos in IVF by non-invasive measurement of amino acid turnover. Hum Reprod, 2004. 19(10): p. 2319-24.
2. Kumar, N. and A.K. Singh, Trends of male factor infertility, an important cause of infertility: A review of literature. Journal of human reproductive sciences, 2015. 8(4): p. 191-196.
3. Alukal, J.P. and D.J. Lamb, Intracytoplasmic sperm injection (ICSI)--what are the risks? The Urologic clinics of North America, 2008. 35(2): p. 277-x.
4. Eskew, A.M. and E.S. Jungheim, A History of Developments to Improve in vitro Fertilization. Missouri medicine, 2017. 114(3): p. 156-159.
5. Gozlan, I., et al., Comparing intracytoplasmic sperm injection and in vitro fertilization in patients with single oocyte retrieval. Fertil Steril, 2007. 87(3): p. 515-8.
6. Sánchez-Calabuig, M.J., et al., Potential Health Risks Associated to ICSI: Insights from Animal Models and Strategies for a Safe Procedure. Frontiers in public health, 2014. 2: p. 241-241.
7. Merchant, R., G. Gandhi, and G.N. Allahbadia, In vitro fertilization/intracytoplasmic sperm injection for male infertility. Indian journal of urology : IJU : journal of the Urological Society of India, 2011. 27(1): p. 121-132.
8. Mantikou, E., et al., Embryo culture media and IVF/ICSI success rates: a systematic review. Hum Reprod Update, 2013. 19(3): p. 210-20.
9. Zhao, Q., et al., Noninvasive metabolomic profiling of human embryo culture media using a simple spectroscopy adjunct to morphology for embryo assessment in in vitro fertilization (IVF). Int J Mol Sci, 2013. 14(4): p. 6556-70.
10. Geber, S., L. Sales, and M.A. Sampaio, Laboratory techniques for human embryos. Reprod Biomed Online, 2002. 5(2): p. 211-8.
11. Martin, P.M., A.E. Sutherland, and L.J. Van Winkle, Amino Acid Transport Regulates Blastocyst Implantation1. Biology of Reproduction, 2003. 69(4): p. 1101-1108.
12. Van Winkle, L.J. and H.R. Dickinson, Differences in Amino Acid Content of Preimplantation Mouse Embryos that Develop in Vitro Versus in Vivo: In Vitro Effects of Five Amino Acids that are Abundant in Oviductal Secretions1. Biology of Reproduction, 1995. 52(1): p. 96-104.
13. Houghton, F.D., et al., Non-invasive amino acid turnover predicts human embryo developmental capacity. Human Reproduction, 2002. 17(4): p. 999-1005.
14. Dominguez, F., et al., New strategy for diagnosing embryo implantation potential by combining proteomics and time-lapse technologies. Fertil Steril, 2015. 104(4): p. 908-914.
15. Milewski, R. and A. Ajduk, Time-lapse imaging of cleavage divisions in embryo quality assessment. Reproduction, 2017. 154(2): p. R37-R53.
16. Tabibnejad, N., Embryo Morphokinetics Based on Time-Lapse Observation. 2017.
17. Meseguer, M., et al., Embryo incubation and selection in a time-lapse monitoring system improves pregnancy outcome compared with a standard incubator: a retrospective cohort study. Fertil Steril, 2012. 98(6): p. 1481-9 e10.
18. Shrestha, D., X. La, and H.L. Feng, Comparison of different stimulation protocols used in in vitro fertilization: a review. Ann Transl Med, 2015. 3(10): p. 137.
19. Geber, S., L. Sales, and M.A.C. Sampaio, Laboratory techniques for human embryos. Reproductive BioMedicine Online, 2002. 5(2): p. 211-218.
20. Patrat, C., et al., Optimal timing for oocyte denudation and intracytoplasmic sperm injection. Obstet Gynecol Int, 2012. 2012: p. 403531.
21. Moura, B.R., et al., Low concentration of hyaluronidase for oocyte denudation can improve fertilization rates and embryo quality. JBRA Assist Reprod, 2017. 21(1): p. 27-30.
22. Van de Velde, H., et al., Effect of timing of oocyte denudation and micro-injection on survival, fertilization and embryo quality after intracytoplasmic sperm injection. Human reproduction (Oxford, England), 1998. 13: p. 3160-4.

23. Álvarez, C., et al., In vitro maturation, fertilization, embryo development & clinical outcome of human metaphase-I oocytes retrieved from stimulated intracytoplasmic sperm injection cycles. The Indian journal of medical research, 2013. 137(2): p. 331-338.
24. Schwartz, D., et al., Within-subject variability of human semen in regard to sperm count, volume, total number of spermatozoa and length of abstinence. J Reprod Fertil, 1979. 57(2): p. 391-5.
25. Balaban, B., et al., An alternative to PVP for slowing sperm prior to ICSI. Human reproduction (Oxford, England), 2003. 18: p. 1887-9.
26. Palermo, G.D., et al., Intracytoplasmic sperm injection (ICSI) in extreme cases of male infertility. PLoS One, 2014. 9(12): p. e113671.
27. Lundin, K. and H. Park, Time-lapse technology for embryo culture and selection. Ups J Med Sci, 2020. 125(2): p. 77-84.
28. Armstrong, S., et al., Time-lapse systems for embryo incubation and assessment in assisted reproduction. Cochrane Database Syst Rev, 2018. 5(5): p. CD011320.
29. Uyar, A. and E. Seli, Metabolomic assessment of embryo viability. Semin Reprod Med, 2014. 32(2): p. 141-52.
30. Gardner, D.K., et al., Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. Fertil Steril, 2000. 73(6): p. 1155-8.
31. Basile, N., et al., The use of morphokinetics as a predictor of implantation: a multicentric study to define and validate an algorithm for embryo selection. Hum Reprod, 2015. 30(2): p. 276-83.
32. Basile, N., et al., The use of morphokinetics as a predictor of implantation: a multicentric study to define and validate an algorithm for embryo selection. Hum Reprod, 2015. 30(2): p. 276-83.
33. Armstrong, M., K. Jonscher, and N.A. Reisdorph, Analysis of 25 underivatized amino acids in human plasma using ion-pairing reversed-phase liquid chromatography/time-of-flight mass spectrometry. Rapid Commun Mass Spectrom, 2007. 21(16): p. 2717-26.
34. Glenn, T., A. Kotlyar, and D. Seifer, The Impact of Intracytoplasmic Sperm Injection in Non-Male Factor Infertility—A Critical Review. Journal of Clinical Medicine, 2021. 10: p. 2616.
35. Sturmey, R.G., D.R. Brison, and H.J. Leese, Assessing embryo viability by measurement of amino acid turnover. Reproductive BioMedicine Online, 2008. 17(4): p. 486-496.
36. Drabkova, P., L. Andrlova, and R. Kandar, An HPLC method for the determination of selected amino acids in human embryo culture medium. Biomed Chromatogr, 2017. 31(2).
37. Chamayou, S., et al., The use of morphokinetic parameters to select all embryos with full capacity to implant. J Assist Reprod Genet, 2013. 30(5): p. 703-10.
38. Kalatova, B., et al., Tripolar mitosis in human cells and embryos: occurrence, pathophysiology and medical implications. Acta Histochem, 2015. 117(1): p. 111-25.
39. Drabkova, P., et al., Amino acid metabolism in human embryos. Physiol Res, 2016. 65(5): p. 823-832.
40. Van Winkle, L.J., Amino Acid Transport Regulation and Early Embryo Development. Biology of Reproduction, 2001. 64(1): p. 1-12.
41. Lane, M. and D.K. Gardner, Differential regulation of mouse embryo development and viability by amino acids. J Reprod Fertil, 1997. 109(1): p. 153-64.
42. Consensus Group, C., 'There is only one thing that is truly important in an IVF laboratory: everything' Cairo Consensus Guidelines on IVF Culture Conditions. Reproductive BioMedicine Online, 2020. 40(1): p. 33-60.
43. Houghton, F.D., et al., Non-invasive amino acid turnover predicts human embryo developmental capacity. Hum Reprod, 2002. 17(4): p. 999-1005.
44. Castelló, D., et al., How much have we learned from time-lapse in clinical IVF? Molecular Human Reproduction, 2016. 22(10): p. 719-727.
45. Huo, P., et al., Non-invasive Amino Acid Profiling of Embryo Culture Medium Using HPLC Correlates With Embryo Implantation Potential in Women Undergoing in vitro Fertilization. Front Physiol, 2020. 11: p. 405.
46. Leese, H., P. McKeegan, and R. Sturmey, Amino Acids and the Early Mammalian Embryo: Origin, Fate, Function and Life-Long Legacy. International Journal of Environmental Research and Public Health, 2021. 18: p. 9874.
47. Orsi, N.M. and H.J. Leese, Ammonium exposure and pyruvate affect the amino acid metabolism of bovine blastocysts in vitro. Reproduction, 2004. 127(1): p. 131-40.
48. Donnay, I. and H.J. Leese, Embryo metabolism during the expansion of the bovine blastocyst. Mol Reprod Dev, 1999. 53(2): p. 171-8.
49. Miao, S.B., et al., Glutamine as a Potential Noninvasive Biomarker for Human Embryo Selection. Reprod Sci, 2022. 29(6): p. 1721-1729.
50. Chen, P.R., et al., Glutamine supplementation enhances development of in vitro-produced porcine embryos and increases leucine consumption from the medium. Biol Reprod, 2018. 99(5): p. 938-948.
51. Rieger, D., Relationships between energy metabolism and development of early mammalian embryos. Theriogenology, 1992. 37(1): p. 75-93.
52. Alexiou, M. and H.J. Leese, Purine utilisation, de novo synthesis and degradation in mouse preimplantation embryos. Development, 1992. 114(1): p. 185-92.
53. Van Winkle, L. and R. Ryznar, Amino Acid Transporters: Roles for Nutrition, Signalling and Epigenetic Modifications in Embryonic Stem Cells and Their Progenitors. 2019. p. 1-13.
54. Sun, C., et al., Mouse early extra-embryonic lineages activate compensatory endocytosis in

response to poor maternal nutrition. Development (Cambridge, England), 2014. 141.
55. Zhou, Z., et al., Using cysteine/cystine to overcome oxidative stress in goat oocytes and embryos cultured in vitro. Mol Med Rep, 2016. 14(2): p. 1219-1226.
56. Wale, P.L. and D.K. Gardner, Oxygen Regulates Amino Acid Turnover and Carbohydrate Uptake During the Preimplantation Period of Mouse Embryo Development1. Biology of Reproduction, 2012. 87(1): p. 24, 1-8.
57. Gardner, D. and A. Harvey, Blastocyst metabolism. Reproduction, fertility, and development, 2015. 27.
58. Mitchell, M., et al., Disruption of Mitochondrial Malate-Aspartate Shuttle Activity in Mouse Blastocysts Impairs Viability and Fetal Growth1. Biology of Reproduction, 2009. 80(2): p. 295-301.
59. Wakefield, S.L., M. Lane, and M. Mitchell, Impaired Mitochondrial Function in the Preimplantation Embryo Perturbs Fetal and Placental Development in the Mouse1. Biology of Reproduction, 2011. 84(3): p. 572-580.
60. Marchese, L., et al., The Uptake and Metabolism of Amino Acids, and Their Unique Role in the Biology of Pathogenic Trypanosomatids. Pathogens, 2018. 7(2).
61. Van Winkle, L.J., V. Galat, and P.M. Iannaccone Lysine Deprivation during Maternal Consumption of Low-Protein Diets Could Adversely Affect Early Embryo Development and Health in Adulthood. International Journal of Environmental Research and Public Health, 2020. 17, DOI: 10.3390/ijerph17155462.
62. Baltz, J.M. and C. Zhou, Cell volume regulation in mammalian oocytes and preimplantation embryos. Mol Reprod Dev, 2012. 79(12): p. 821-31.
63. Leese, H.J., Quiet please, do not disturb: a hypothesis of embryo metabolism and viability. Bioessays, 2002. 24(9): p. 845-9.
64. Han, C., et al., Regulation of L-Threonine Dehydrogenase in Somatic Cell Reprogramming. Stem Cells, 2013. 31(5): p. 953-965.
65. Shyh-Chang, N., et al., Influence of Threonine Metabolism on S-Adenosylmethionine and Histone Methylation. Science, 2013. 339(6116): p. 222-226.
66. Shengya, T., et al., Glycine cleavage system determines the fate of pluripotent stem cells via the regulation of senescence and epigenetic modifications. Life Science Alliance, 2019. 2(5): p. e201900413.
67. Fukuwatari, T. and K. Shibata, Nutritional aspect of tryptophan metabolism. Int J Tryptophan Res, 2013. 6(Suppl 1): p. 3-8.
68. Liang, X., et al., Proline mechanisms of stress survival. Antioxid Redox Signal, 2013. 19(9): p. 998-1011.
69. Krishnan, N., M.B. Dickman, and D.F. Becker, Proline modulates the intracellular redox environment and protects mammalian cells against oxidative stress. Free Radic Biol Med, 2008. 44(4): p. 671-81.
70. Matysik, J., et al., Molecular mechanism of quenching of reactive oxygen species by proline under stress in plants. Current Science, 2002. 82.
71. Wong, C., et al., Time-lapse microscopy and image analysis in basic and clinical embryo development research. Reprod Biomed Online, 2013. 26(2): p. 120-9.