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ASSISTED REPRODUCTION TECHNOLOGIES

Patient selection criteria for blastocyst transfers in extended embryo culture programs

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Abstract
Purpose To identify the correlation between different cycles, patient factors and blastocyst characteristics.
Methods The study included 420 patients undergoing ICSI cycles and 2781 graded blastocysts, which took into account the blastocyst quality. The correlations between the blastocyst parameters and the patient and cycle characteristics were assessed.
Results The blastocyst development was negatively correlated with the maternal age, BMI and dose of FSH. The ICM was negatively correlated with the FSH dose, whereas the TE quality was influenced by the FSH dose, the maternal age and the number of retrieved oocytes. The embryo morphology on days two and three may predict the blastocyst developmental competence.
Conclusions Older patients and patients with high BMI should not be included in extended embryo culture programmes. The extended culture may not favour embryos with poor morphology on days two and three of development. Additionally, a lower ovarian stimulation and decreased oocyte yields may lead to the development of high-quality blastocysts.

Keywords Blastocyst · Embryo · Morphology · Maternal age · FSH

Capsule Older patients and patients with high BMI should not be included in extended embryo culture programmes. Moreover, lower ovarian stimulation and decreased oocyte yields lead to the development to high quality blastocysts.

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Introduction

An extended embryo culture and the transfer of blastocyst-stage embryos are associated with increased implantation rates compared with the transfer of cleavage-stage embryos [5, 36]. A prolonged culture period allows for a better selection of more advanced embryos that are not arrested because the laboratory assessment is undertaken after the embryonic genome has begun to be expressed, which starts between the four- and eight-cell stages of human embryo development [44]. At this stage, the sperm-derived genes that influence the embryo viability have also been activated, which allow for the selection of a genetically normal embryo. In addition, it has been reported that a better synchronisation between the endometrium function and the embryo development is possible with blastocyst-stage embryo transfers [24, 48].

Moreover, because of their high implantation success rates, single blastocyst transfers may increase pregnancy rates and reduce multiple gestations [13, 38]. In fact, it is necessary to decrease the assisted-reproduction-induced iatrogenic multiple pregnancies due to health, economic and legal issues in several countries [1].

Even though the improved culture media systems have increased the proportion of embryos that reach the blastocyst stage in vitro [41, 49]. the proportion of embryos that reach this stage is still unpredictable [29, 40, 47], and some assisted reproduction centres are reluctant to adopt extended

embryo cultures to avoid any embryo transfer cancellation due to an unpredictable rate of blastocyst development.

Various factors, such as the culture medium characteristics [2, 4, 41], the laboratory conditions [23, 27, 46], the oocyte quality [6, 18], and the sperm origin [32], may be responsible for this variation in the rate of blastocyst formation. However, the influence of the patient and cycle characteristics on blastocyst formation is not understood. Moreover, although early embryo morphology scoring systems have been developed [7, 33], it has been suggested that their use is limited by significant intraobserver and interobserver variability [3]. Furthermore, there is no consensus regarding the value of the currently accepted morphological criteria that is used with early embryos to predict subsequent in vitro blastocyst development.

A morphological grading system, which was first described by Gardner and Schoolcraft [12], has been widely used for the selection of the blastocysts for embryo transfer. According to this system, three parameters are graded: the degree of blastocoel expansion and hatching status, the size and compactness of the inner cell mass (ICM), and the cohesiveness and number of trophectoderm (TE) cells. The identification of the different factors that contribute to the blastocyst quality is of paramount importance for the selection of patients who could benefit from extended embryo culture programmes. Therefore, the goal of the present study was to identify the correlation between the different cycles and patient factors and the blastocyst characteristics.

Materials and methods

Study design

This study included 2,781 embryos, which were obtained from 420 patients undergoing intracytoplasmic sperm injection (ICSI) cycles between January and December 2011. All of the embryos were evaluated 16–18 h post-ICSI and on days two, three and five of development. All the cases with severe spermatogenic alteration, including frozen and surgically retrieved sperm, were excluded from the study.

The blastocysts were graded according to a modified version of the Gardner and Schoolcraft system [12]. The morphological characteristics of the blastocysts, namely, the degree of blastocoel expansion and hatching status, the size and compactness of the ICM, and the cohesiveness and number of TE cells, were correlated to the following parameters: maternal age, paternal age, FSH dose (for controlled ovarian stimulation), 17β-estradiol levels on the hCG trigger day, number of aspirated follicles, number of retrieved oocytes, maternal body mass index (BMI), sperm concentration, sperm motility and sperm morphology.

The influences of the cause of infertility and the embryo quality on days two and three of development on the blastocyst morphological parameters were also evaluated.

A written informed consent, in which patients agreed to share the outcomes of their cycles for research purposes, was obtained; the study was approved by the local institutional review board.

Controlled ovarian stimulation & laboratory procedures

A controlled ovarian stimulation was achieved by pituitary blockage using a GnRH antagonist (Cetrotide; Serono, Geneva, Switzerland), and the ovarian stimulation was performed using recombinant FSH (Gonal-F; Serono, Geneva, Switzerland).

The follicular growth was monitored using transvaginal ultrasound examination starting on day four of gonadotropin administration. When adequate follicular growth and serum E2 levels were observed, recombinant hCG (Ovidrel; Serono, Geneva, Switzerland) was administered to trigger the final follicular maturation. The oocytes were collected 35 h after hCG administration through transvaginal ultrasound ovum pick-up.

The recovered oocytes were assessed to determine their nuclear status, and those in metaphase II were submitted to ICSI following routine procedures [35].

Embryo morphology evaluation

The embryo morphology was assessed 16–18 h post-ICSI and on the mornings of days two, three and five of embryo development using an inverted Nikon Diaphot microscope (Eclipse TE 300; Nikon, Tokyo, Japan) with a Hoffmann modulation contrast system under 400X magnification.

To evaluate the cleavage-stage morphology, the following parameters were recorded: the number of blastomeres, the percentage of fragmentation, the variation in blastomere symmetry, the presence of multinucleation and the defects in the zona pellucida and cytoplasm. The high-quality cleavage-stage embryos were defined as those with all of the following characteristics: 4 cells on day two or 8–10 cells on day three, <15 % fragmentation, symmetric blastomeres, the absence of multinucleation, colourless cytoplasm with moderate granulation and no inclusions, the absence of perivitelline space granularity and the absence of zona pellucida dysmorphism. Embryos lacking any of these characteristics were considered to be of low quality.

To evaluate the blastocyst-stage morphology, the size and compactness of the ICM and the cohesiveness and number of TE cells were recorded. The embryos were given a numerical score from one to six based on their degree of expansion and hatching status as follows: 1, an early blastocyst with a blastocoel that is less than half of the volume

of the embryo; 2, a blastocyst with a blastocoel that is greater than half of the volume of the embryo; 3, a full blastocyst with a blastocoel that completely fills the embryo; 4, an expanded blastocyst; 5, a hatching blastocyst; and 6, a hatched blastocyst. The ICM of full, expanded, hatching, and hatched blastocysts was classified as either high quality (tightly packed with many cells) or low quality (loosely grouped with several or few cells). Similarly, the TE was also classified as either high quality (many cells forming a cohesive epithelium) or low quality (few cells forming a loose epithelium or very few cells).

Statistical analyses

Pearson correlation analyses were performed to study the relationship between the blastocyst morphological parameters and the cycle and patient characteristics. These results were expressed in terms of the correlation coefficient (CC) and p-value. If a significant correlation was found, the data were also compared using Student's *t*-test or variance analysis, and the results were expressed as the average±standard deviation with the corresponding p-values.

In addition, binary regression analyses were used to evaluate the influence of the causes of infertility and embryo quality on the blastocyst morphological parameters on days two and three of development. The results were expressed as odds ratios (ORs), 95 % confidence intervals (CI), and p-values.

The results were considered to be significant at the 5 % critical level ($p<0.05$). The data analysis was performed using the Minitab (version 14) Statistical Program.

Results

A significant negative correlation was found between the maternal age (CC: -0.095, p<0.001), BMI (CC: -0.038, p< 0.001) and total FSH dose (CC: -0.038, p=0.046) and the degree of blastocyst expansion and hatching status (Table 1). These results were confirmed by variance analysis, which demonstrated a significant difference in the degree of blastocyst expansion and hatching status among the groups in relation to the maternal age (**1**: 34.2±5.03; **2**: 33.7±5.45; **3**: 33.5±4.62; **4**: 32.3±4.59; **5**: 31.3±6.18, p<0.001), the maternal body mass index (**1**: 23.1±6.3; **2**: 22.7±7.0; **3**: 22.3±7.5; **4**: 21.8±7.6; **5**: 21.2±8.8, p=0.030) and the FSH dose (**1**: 2194.4±675.1; **2**: 2165.0±706.8;**3**: 2082.3±712.9; **4**: 2071.5±673.5; **5**: 2033.7±615.4, p=0.017).

The FSH dose was also negatively correlated with both the ICM (CC: -0.031, p=0.044) and the TE quality (CC: -0.073, p<0.001) (Table 1). These findings were also confirmed by Student's *t*-test, which demonstrated a significant difference in the ICM (high quality: 2115.0±661.1; low quality: 2200.5±691.2, p<0.001) and TE (high quality: 1938.0±721.1; low quality: 2164.5±721.2, p<0.001) groups in relation to the FSH dose.

Table 1 Pearson correlation between the blastocyst quality and the following variables: maternal age, paternal age, FSH dose, 17β-estradiol level, aspirated follicles, retrieved oocytes, maternal body mass index, sperm concentration, sperm motility and sperm morphology

Response variable	Predictor variable	Correlation coefficient	*p*
Degree of expansion and hatching status	Maternal age	−0.095	<0.001
	Paternal age	0.005	0.851
	FSH dose	−0.068	<0.001
	17β-estradiol level	0.028	0.248
	Aspirated follicles	0.017	0.400
	Retrieved oocytes	0.003	0.894
	Maternal BMI	−0.038	0.046
	Sperm concentration	−0.006	0.836
	Sperm motility	−0.032	0.260
	Sperm morphology	−0.014	0.632
ICM quality	Maternal age	−0.005	0.755
	Paternal age	−0.010	0.731
	FSH dose	−0.031	0.044
	17β-estradiol level	−0.004	0.832
	Aspirated follicles	−0.020	0.215
	Retrieved oocytes	−0.015	0.356
	Maternal BMI	0.011	0.520
	Sperm concentration	−0.004	0.887
	Sperm motility	−0.007	0.808
	Sperm morphology	−0.009	0.753
TE quality	Maternal age	−0.067	<0.001
	Paternal age	0.035	0.226
	FSH dose	−0.073	<0.001
	17β-estradiol level	0.036	0.465
	Aspirated follicles	−0.006	0.736
	Retrieved oocytes	−0.030	0.050
	Maternal BMI	−0.014	0.401
	Sperm concentration	−0.007	0.816
	Sperm motility	0.026	0.373
	Sperm morphology	0.022	0.450

ICM Inner cell mass, *TE* trophectoderm cells, *BMI* body mass index

The maternal age (CC: -0.067, p<0.001) and the number of retrieved oocytes (CC: -0.030, p=0.050) were negatively correlated with the TE quality (Table 1). These results were also confirmed by Student's *t*-test, which showed a significant difference between the TE groups in relation to the maternal age (high quality: 32.74±4.38; low quality: 33.8± 5.63, p=0.007) and the number of retrieved oocytes (high quality: 11.5±8.50; low quality: 14.3±7.63, p<0.001).

In the analysis of the causes of infertility, the logistic regression showed that all the blastocyst morphological

parameters were negatively influenced by the presence of ovarian factor infertility. The presence of polycystic ovarian syndrome negatively influenced the degree of blastocyst expansion and hatching status. Moreover, the presence of endometriosis negatively influenced both the ICM and TE morphologies (Table 2).

However, the presence of male factor infertility did not have any impact on blastocyst quality (Table 2). Furthermore, it was noted that a positive correlation existed between the day two and day three embryo quality and the degree of blastocyst expansion, the hatching status and the ICM quality; however, no correlation was found with the TE quality (Table 3).

Table 3 Binary regression analysis of the embryo quality variables on days two and three that may affect the blastocyst quality

Response variable	Predictor variable	*p*	OR	CI: Lower	CI: Upper
Embryo quality on day two	Degree of expansion and hatching status	<0.001	1.86	1.82	1.90
	ICM quality	0.017	1.68	1.50	1.92
	TE quality	0.118	1.27	1.12	1.49
Embryo quality on day three	Degree of expansion and hatching status	<0.001	1.78	1.74	1.83
	ICM quality	0.010	1.58	1.39	1.86
	TE quality	0.041	3.31	1.32	5.99

ICM Inner cell mass, *TE* trophectoderm cells, *OR* odds ratio, *CI* confidence interval

Discussion

The transfer of human embryos at the blastocyst stage is becoming more common in the practice of assisted reproduction [28]. The advantages of blastocyst-stage embryo transfer include better synchronisation between the endometrium and the embryo and the possible selection of embryos with a higher implantation potential [34]. The disadvantages of the extended embryo culture are the unpredictable rate of blastocyst development and the risk of embryo transfer cancellation.

In the present study, we evaluated the possible factors that may influence blastocyst formation and morphology. The maternal age and total FSH dose appeared to play a role in the blastocyst developmental capacity. The FSH dose correlated with the quality of both the ICM and TE, and the maternal age correlated with the TE status.

Similar to our findings, several other studies have also shown a decrease in the blastocyst development rate with increasing female age [14, 20, 42]. Other than advanced maternal age, no clearly established factor has been described for the development of embryos with poor morphology. It is typical that more than half of the retrieved oocytes from women older than 40 years of age that underwent IVF treatment have abnormal chromosomes [11]. This result is in agreement with our findings, which demonstrated that the maternal age influences the blastocyst developmental competence. However, the blastocyst morphology was unaffected by the paternal age or other sperm parameters.

Previous reports have suggested a negative paternal influence on the blastocyst developmental capacity [8, 43]. It has been demonstrated that oocytes injected with spermatozoa with high motility have a significantly higher chance of reaching the blastocyst stage [30, 43]. It has also been reported that paternal cigarette smoke exposure affects the embryonic implantation ability [21]. However, the results of the above-mentioned studies should be interpreted with caution because some of these results were based on the continued culture of embryos that were considered unsuitable for transfer or cryopreservation on day three of development. In addition, these studies predate recent improvements in the culture media and may represent development under sub-optimal culture conditions.

Our findings also suggest a significant correlation between the FSH dose and the blastocyst quality. The relationship between the elevated FSH dose used for ovarian stimulation and the embryo quality remains a topic of heated discussion among practitioners of assisted reproduction. There is growing evidence in animal studies to support the hypothesis that elevated FSH is an underlying cause of embryo defects [9, 10, 39]. However, whereas animal studies seem to support an association between FSH exposure and

Table 2 Binary regression analysis of the causes of infertility that may affect blastocyst quality

Response variable	Predictor variable	*p*	OR	CI: Lower	CI: Upper
Male factor	Degree of expansion and hatching status	0.961	1.00	0.95	1.05
	ICM quality	0.885	0.94	0.41	2.15
	TE quality	0.935	0.98	0.65	1.49
Ovarian factor	Degree of expansion and hatching status	0.025	1.92	1.86	0199
	ICM quality	0.003	1.44	1.26	0.76
	TE quality	0.003	2.31	1.34	3.99
PCOS	Degree of expansion and hatching status	0.029	1.10	1.01	1.20
	ICM quality	0.809	1.09	0.54	2.20
	TE quality	0.589	1.53	0.33	7.19
Endometriosis	Degree of expansion and hatching status	0.775	1.01	0.94	1.08
	ICM quality	0.029	1.53	1.30	1.94
	TE quality	0.016	2.04	1.14	3.63

PCOS Polycystic ovarian syndrome, *ICM* Inner cell mass, *TE* trophectoderm cells, *OR* odds ratio, *CI* confidence interval

embryonic defects, human studies have been more inconsistent [25, 26, 31, 37, 45].

In our study, the FSH dose and the number of retrieved oocytes had a negative impact on the TE quality. Our results are in agreement with recent studies that have shown that milder ovarian stimulation reduces the likelihood of segregation errors during early embryo cleavages [16, 22] and results in high-quality embryos for transfer, as indicated by good embryo morphology [17]. The observation that higher FSH doses and oocyte yields are associated with impaired blastocyst development may be due to an supraphysiologic ovarian stimulation that interferes with the cell machinery during oocyte formation. These findings suggest that lower oocyte yields may represent a better response to ovarian stimulation because it allows the development of only the most competent follicles and oocytes.

The influence of the cause of infertility on the blastocyst developmental competence was also evaluated in this study. It was noted that the presence of ovarian factor infertility had a negative impact on all aspects of the blastocyst quality. Moreover, the presence of PCOS and endometrioses had a negative effect on the degree of blastocyst development and the ICM and TE quality, respectively. A recent study concluded that the blastocyst development rate is not related to the cause of infertility; however, the study may have been underpowered to detect differences [19].

The decline in the blastocyst developmental competence in the presence of female factor infertility is in agreement with our findings regarding the relationship between the maternal age and the blastocyst quality. It is generally accepted that the activation of the full embryonic genome is complete after the 8-cell stage [44]. At this stage, the paternally activated genes are expressed and may influence the embryo viability. However, it has been reported that the oocyte quality has a more drastic effect on the blastocyst developmental capacity than the sperm quality; therefore, the oocyte is mainly responsible for the blastocyst developmental capacity.

It was clearly demonstrated that the embryo morphology on both days two and three may predict the blastocyst developmental competence; therefore, the quality of the cleavage-state embryo may be predictive of the quality of the blastocyst formation. In fact, our data showed that a good morphology on day three increases the likelihood that a blastocyst will be of good quality by up to threefold. Conversely, previous studies have suggested that the early embryo morphology is a poor predictor of the blastocyst quality in extended culture [14, 15]. A recent study from Guerif et al. [15] suggested that the relationship between early morphological parameters and the blastocyst morphology is weak. However, it was demonstrated that the degree of fragmentation on day three should be taken into account in the selection of the best blastocyst for transfer.

Several studies have shown improved outcomes with blastocyst transfer; however, not all patients may benefit from postponing the embryo transfer until day five of development. The results presented here suggest that older patients, patients with high BMI, and those couples with female factor infertility should not be included in the extended embryo culture programmes. An extended culture may also not favour embryos with poor morphology on days two and three of development. In addition, our evidence suggests that lower ovarian stimulation and decreased oocyte yields may represent a more appropriate response to ovarian stimulation and thus may lead to the development of high-quality blastocysts.

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