

The importance of the cleavage stage morphology evaluation for blastocyst transfer in patients with good prognosis

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Abstract

Purpose To evaluate: (i) the influence of morphology at cleavage stage on blastocyst formation and implantation, and (ii) whether the transfer of low-quality embryos on day-three would be a better approach than the transfer at blastocyst stage.

Methods This study included 8,444 embryos obtained from 1,125 patients undergoing ICSI cycles between January/2011 and September/2013. The influence of the quality of the embryo on days-two and -three on blastocyst formation and implantation success was evaluated. Moreover, the implantation potential of low-quality embryos, at cleavage stage, transferred on day-three was compared with the implantation potential of low-quality embryos, at cleavage stage, transferred on day-five.

Results Low-quality embryos on day-two had an approximate 20 % decreased chance of achieving the blastocyst stage, and blastocysts derived from low-quality embryos on day-two had a nearly 40 % decrease in the implantation chance. Low-quality embryos on day-three had a 30 % decreased chance of achieving the blastocyst stage, and blastocysts derived from low-quality embryos on day-three had an almost 40 % decreased implantation chance. The implantation rate didn't differ when low-quality embryos on the cleavage stage were

transferred on day-three or left in culture and transferred on day-five.

Conclusions The transfer of low-quality embryos on day-three is a better approach than transfer at the blastocyst stage. In addition, the embryo morphology evaluation at the cleavage stage is still needed for the selection of the embryo with the best implantation potential in extended embryo culture programmes.

Keywords Assisted reproduction · Blastocyst · Morphology · Extended embryo culture · Day two morphology · Day three morphology

Introduction

The success of in vitro fertilisation (IVF) depends on the production of viable embryos with high implantation potential. Although high-quality embryos may be available for transfer, choosing the best embryo for transfer has become the main challenge in IVF.

Performing serial observations of embryo morphology is a common technique to evaluate embryos and has been considered a key predictor of implantation and pregnancy [1–10]. However, morphological assessments have some limitations. This is a highly subjective method [11, 12], and the correlation between morphological parameters and embryo implantation potential is unclear [13]. To perform the assessment, embryos are removed briefly from the incubator and placed under a microscope, but due to concerns for the safety and stability of culture conditions, the observation of embryos outside the incubator should be avoided as much as possible.

Time-lapse imaging is a non-invasive, emerging technology that allows 24-h monitoring of embryo development, offering the possibility of increased quantity and quality of morphological information without disturbing the culture

Capsule Embryo morphology evaluation at the cleavage stage is still needed for the selection of the embryo with the best implantation potential in extended embryo culture programmes.

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conditions [9, 14–17]. However, the acquisition of time-lapse images is complicated because it requires an incubation chamber equipped with cameras, and the majority of the currently available technologies are extremely expensive and unsuitable for routine use in an IVF laboratory [18]. Moreover, although the safety of this technique has been previously studied [19], acquisition of time-lapse images requires periodic illumination of embryos during development, which could potentially harm developing embryos [20]. Additionally, it was reported that the use of time-lapse observation had no effect on IVF pregnancy rates [21].

Prolonging the embryo culture period allows for a better selection of embryos for transfer because laboratory assessment is undertaken after the embryonic genome has begun to be expressed [22]. Recently, extended embryo culture and blastocyst-stage embryo transfers have been correlated with increased implantation rates and reduced rates of multiple pregnancies [23, 24].

In fact, by culturing embryos to the blastocyst stage, after which point the embryonic genome has been activated, it is possible to identify the embryos that have undergone a developmental block on days two or three. Therefore, the most promising embryos reaching the blastocyst stage may be selected for transfer. However, because of our current inability to predict which cleavage-stage embryos will develop into viable blastocysts [25, 26], assisted reproduction centres are reluctant to adopt extended embryo culture to avoid embryo transfer cancellation [27].

Although it has been reported that the transfer of blastocysts drastically increases the implantation rate [28–31], we have previously reported that extended culture may not favour embryos with poor morphology on days two and three of development [32]. However, it is unclear whether transfer of low quality embryos at the cleavage stage is a better approach than culturing these embryos until day five. Additionally, the importance of the cleavage stage embryo morphology evaluation for day five embryo transfer programmes in patients with good prognosis is unknown.

The goals of the present study were to: (i) evaluate the influence of embryonic morphology at days two and three on blastocyst formation and implantation capacity, and (ii) investigate whether the transfer of low quality embryos at the cleavage stage would be a more successful approach than extended embryo culture and transfer in the blastocyst stage.

Material and methods

Study design

This study included 8,444 embryos obtained from 1,125 patients undergoing intracytoplasmic sperm injection (ICSI) cycles between January 2011 and September 2013 in a private

assisted reproduction centre. All cases included good patients, as described elsewhere [33]: (i) undergoing their first IVF cycles, (ii) <38 years old, (iii) no diagnosis of endometriosis, and (iv) ≥ eight oocytes retrieved.

From those embryos, 6,164 were cultured until day five and 2,280 embryos were transferred on day three. The day of the embryo transfer was not chosen randomly, instead of that it depended on the clinician choice, and it did not take into consideration the cycles characteristics.

All of the embryos were evaluated at 16–18 h post-ICSI and subsequently on days two, three and, for extended culture, also on day five of development. All cases of severe spermatogenic alteration, including frozen and surgically retrieved sperm, and embryos transferred on days one, four or six were excluded from the study.

The influence of the quality of the embryo on days two and three on successful blastocyst formation and implantation was evaluated. When the implantation rate was either 100 % or 0 %, the influence of the quality of the embryo on days two and three on the blastocyst implantation capacity was also investigated.

In addition, a comparison was made between the implantation potential of low quality embryos at the cleavage stage that were transferred on day three versus day five.

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

Controlled ovarian stimulation & laboratory procedures

Controlled ovarian stimulation was achieved by pituitary blockage using a gonadotropin-releasing hormone (GnRH) antagonist (Cetrotide, Serono, Geneva, Switzerland), and ovarian stimulation was performed using recombinant FSH (Gonal-F; Serono, Geneva, Switzerland).

Follicular growth was followed by a transvaginal ultrasound examination starting on day four of gonadotropin administration. When adequate follicular growth and serum oestradiol levels were observed, recombinant human chorionic gonadotropin (hCG) (Ovidrel; Serono, Geneva, Switzerland) was administered to trigger the final follicular maturation. Oocytes were collected 35 h after hCG administration by transvaginal ultrasound ovum pick-up.

The nuclear status of the recovered oocytes was assessed, and oocytes in metaphase II were submitted to ICSI following routine procedures [34].

Embryo morphology evaluation

Embryo morphology was assessed by two well-trained embryologists at 16–18 h post-ICSI and on the mornings of days two, three and five of embryo development using an inverted

microscope (Eclipse TE 300; Nikon, Tokyo, Japan) with a Hoffmann modulation contrast system under 400× magnification.

All embryos were photographed and whenever there was a disagreement a third embryologist was involved.

For the cleavage stage morphology assessment, the following parameters were recorded: (i) the number of blastomeres, (ii) the percentage of fragmentation, (iii) the variation in blastomere symmetry, (iv) the presence of multinucleation and (v) defects in the zona pellucida and cytoplasm. High-quality cleavage stage embryos were defined as those having all of the following characteristics: (i) 4 cells on day two or 8–10 cells on day three, (ii) <15 % fragmentation, (iii) symmetric blastomeres, (iv) absence of multinucleation, (v) colourless cytoplasm with moderate granulation and no inclusions, (vi) absence of perivitelline space granularity and (vii) absence of zona pellucida dysmorphism. Embryos lacking any of the above characteristics were considered to be of low quality.

To evaluate the blastocyst-stage morphology, the size and compactness of the inner cell mass (ICM) and the cohesiveness and number of trophectoderm 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

Binary Logistic Regressions were performed to study the influence of the quality of the embryo on days two and three on successful blastocyst formation and implantation chance. The results were expressed as odds ratios (OR) with 95 % confidence intervals (CI), and *p* values were calculated.

The regression models were adjusted for male and female age, body mass index (BMI), number of retrieved oocytes, endometrial thickness, sperm concentration and sperm motility, as these variables would affect the results.

To compare the pregnancy rate of low quality embryos at cleavage stage transferred either on days three or five of development, Chi-squared analyses was performed.

For patient and cycle characteristics, dichotomic variables were evaluated by Chi-squared or Fisher exact test and data were expressed as percentages. Continuous variables were

evaluated by ANOVA and data were expressed as average± standard deviation.

Results were considered to be significant at the 5 % critical level ($p < 0.05$). Data analysis was carried out using the Minitab (version 14) Statistical Program.

Results

Blastocyst formation and implantation were negatively influenced by the quality of the embryo on days two and three. In extended embryo cultures, embryos of low quality on day two had an almost 20 % decreased chance of achieving the blastocyst stage, and blastocysts derived from low quality embryos on day two had an approximate 40 % decrease in the odds of implantation. Embryos classified as low quality on day three had a 30 % decreased chance of achieving the blastocyst stage, and blastocysts derived from low quality embryos on day three demonstrated an almost 40 % decreased implantation chance (Table 1).

The implantation rate did not differ when low quality embryos on day two or three were left in culture and transferred at the blastocyst stage or at the cleavage stage (25.06 % vs. 20.39 %, $p = 0.320$, for day two; 26.25 % vs. 23.08 %, $p = 0.432$, for day three). Conversely, high quality embryos on days two or three demonstrated an increased implantation rate if left in culture and transferred at the blastocyst stage rather than at the cleavage stage (22.54 % vs. 38.16 %, $p = 0.036$, for day two; 22.97 % vs. 41.18 %, $p = 0.035$, for day three).

The characteristics of cycles in which embryo transfer was performed at the cleavage stage or at the blastocyst stage were equally distributed among the groups, except for the number of transferred embryos (Table 2).

Discussion

Extending the duration of embryo culture to the blastocyst stage offers many advantages over the transfer of cleavage stage embryos, including a higher implantation rate, the selection of the most viable embryo for transfer, a decrease in the number of embryos transferred, and a better synchronisation between the embryo and the endometrium at the time of embryo transfer [36–38].

According to The Practice Committee of the American Society for Reproductive Medicine and the Practice Committee of the Society for Assisted Reproductive Technology, the ability to [39] produce blastocysts varies widely among individual patients, ranging from 0 % to almost 100 %. Consequently, the incidence of cancelled transfers may be higher in unselected patients. It has been previously described that poor embryo morphology on days two and three of development do

Table 1 Binary regression analysis of the influence of embryo quality on days two or three on blastocyst formation and implantation chance

Predictor variable	Response variable	P	OR	CI: Lower	CI: Upper
Embryo quality on day two	Blastocyst formation	0.001	0.82	0.73	0.92
	Blastocyst implantation	<0.001	0.58	0.43	0.77
Embryo quality on day three	Blastocyst formation	<0.001	0.69	0.61	0.78
	Blastocyst implantation	0.001	0.63	0.48	0.83

OR: odds ratio, and CI: confidence interval

not favour blastocyst formation [32]. There is a general consensus that low quality embryos may have an increased implantation chance when incubated in vivo compared to in vitro conditions. In a recent report, it was demonstrated that in a specific subgroup of patients who repeatedly exhibited a poor quality embryo morphology phenotype, the zygote transfer may provide a valid alternative solution [40]. In fact, in a previously published meta-analysis, it was described that the embryo transfer cancellation rate is more than two fold higher in extended embryo culture programs than in patients in whom a cleavage-stage embryo transfer was performed [35]. However, embryos that reach the blastocyst stage have a significantly increased chance of implantation [41].

In the present study, the transfer of low quality embryos on day three was compared with extended embryo culture and transfer in the blastocyst stage. Although the blastocyst formation rate may be compromised by the poor morphology on the early stages of development, low quality embryos at the cleavage stage have the same implantation potential whether transferred on day three or day five.

The number of embryos that should be cultured to guarantee that a blastocyst will be available for transfer on day five is still not clear. It has been predicted that 35 % of zygotes would reach the blastocyst stage on day five [42]. Based on this study, we used a threshold of eight oocytes to guarantee that the results would not be biased by the number of zygotes obtained. Our evidence suggests that even in cycles where eight or more oocytes were retrieved, low quality embryos at

the early stages of development could benefit from transfer at the cleavage stage because these embryos have a 30 % decreased chance of achieving the blastocyst stage.

While it has been shown that blastocyst transfer does not improve the likelihood of implantation of poor morphology embryos, our results demonstrated that high quality embryos transferred on day five have a significantly increased chance of implantation compared to embryos transferred at the cleavage stage. This increased likelihood is most likely due to more accurate embryo selection that occurs at day five compared to day three.

These findings are in agreement with the ASRM committee opinion, which supports the idea that blastocyst transfer yields a significantly higher live-birth rate following fresh transfer in patient populations with good prognosis [43]. However, it remains to be elucidated whether the morphology evaluation at the early stages of development is still needed in this group of patients.

Time-lapse studies suggest that scoring of early embryo development is limited if based on static observations because embryo morphology can change within short time intervals and thus may mislead an assessment performed at a static time point [44]. Using traditional incubators, the need to obtain a detailed image of embryo development must be balanced with the risk of compromising stable culture conditions because frequent evaluation outside the incubator exposes embryos to undesirable changes in temperature, humidity and gas composition [45].

Table 2 Comparison of patient and cycle characteristics when embryo transfer was performed at the cleavage stage or at the blastocyst stage

Variable	Cleavage stage embryo transfer (n=203)	Blastocyst stage embryo transfer (n=922)	p
Female Age (years)	32.01±4.54	31.08±4.23	0.182
Male age (years)	36.55±7.08	36.82±6.73	0.459
FSH dose (IU)	2,221±577	2,282±995	0.486
No. of follicles	18.03±8.02	21.5±11.03	0.326
No. of oocytes retrieved	15.4±3.11	18.9±6.03	0.541
No. of MII oocytes	10.05±4.21	13.31±4.05	0.3221
Fertilisation Rate	79.3±13.8	80.3±13.	0.593
Endometrial thickness (mm)	10.64±2.38	10.77±1.96	0.755
Transferred embryos	2.4±0.53	2.1±0.59	<0.001

MI: Metaphase II

To date, substantial effort has been directed at enhancing the viability of in vitro-cultured embryos, such as minimising the stress to which embryos are exposed, to improve the blastocyst formation chance. It has been suggested that blastocyst development and quality is increased at physiological oxygen tension [46]. It was also reported that temperature recovery after a five-door opening/closing procedure is approximately five minutes for mini-incubators and 30 min for conventional incubators [47]. Therefore, the ability of incubators to maintain a stable microenvironment significantly influences the formation of good early-stage embryos and the subsequent blastocyst formation rate [45].

In the present study, we challenged the predictive value of the cleavage stage embryo morphology on blastocyst formation and implantation success. It was hypothesised that embryo morphology evaluations at the cleavage stages would be dismissed in extended embryo culture programs, especially for good prognosis patients. This would minimise the stress to which embryos are exposed. Previous studies have demonstrated that the type of culture media does not affect embryo development and implantation, regardless of whether single-step or sequential culture media is used. It has also been shown that, if laboratory conditions are closely monitored and precautions are taken against atmospheric fluctuations, renewing culture media is unnecessary for optimal embryo development [48–50].

Our results, however, demonstrated that the embryo morphology on days two and three is an important indicator to predict blastocyst development and implantation. When the morphology is compromised at the cleavage stage, the probability of blastocyst formation may be impaired, and the odds of implantation may decrease by almost 40 %.

In conclusion, our results suggest that the transfer of low quality embryos on days two or three is a better approach than the transfer at the blastocyst stage. In addition, the embryo morphology evaluation at the cleavage stage is still needed for the selection of the embryo with the best implantation potential in extended embryo culture programmes.

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