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Original Article

Disposition of embryos from women who only produced morphologically poor embryos on day three

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ARTICLE INFO

Article history:
Received 25 July 2019
Accepted 11 January 2021
Available online 15 January 2021

Keywords:
In vitro fertilization
Morphologically poor embryo
Cryopreservation
Frozen embryo transfer
Blastocyst transfer

ABSTRACT

Background: The presence of only morphologically poor embryos (MPEs) on day3 is common in autologous *in vitro* fertilization (IVF), particularly among p Tel: 886-7-7317123 Ext. 8916. Fax: 886-7-7322915.atients who have advanced maternal age or are poor responders. However, there are limited data regarding the disposition of embryos from patients who only produced MPEs on day3. The present study was designed to investigate the possible benefits of extended culturing MPEs. Try to detect whether the extended culture (day4 or day5 culture) can improve the live birth rate per cycle?

Methods: This retrospective, observational, single-center, cohort study examined 224 IVF/intracytoplasmic sperm injection (ICSI) cycles between January 2010 and June 2015, in which women only produced MPEs on day3. A total of 544 MPEs were analyzed. The defines a day3 embryo as an MPE if it fails to develop to eight cells, blastomeres of equal size, and less than 20% cytoplasmic fragments. Of the 224 cycles, 89 (39.7%) underwent fresh embryo transfer on day3, and 135 (60.3%) underwent extended culture. Of the 135 extended cultures, 54 cycles (40.0%) experienced day4, or day5 embryo transfer, 16 cycles (11.9%) had all embryos frozen, and 65 cycles (48.1%) had total embryo arrest.

Results: Analysis of patient baseline demographic data, cycle characteristics, and cycle outcomes for day3 transfer group and extended culture group indicated that a higher body mass index in the day3 transfer group was the only significant difference ($p = 0.006$). Both fresh transfer groups had low live birth rates (LBRs) (4.5% *vs.* 7.4% $p = 0.46$). After extended culture, 65 cycles (48.1%) were cancelled because the embryos exhibited developmental arrest and 70 cycles (51.9%) grew to day4 or day5. Thirteen frozen embryo transfer (FET) cycles and 22 frozen blastocysts derived from MPEs were thawed. There were more high-quality embryos ($p < 0.001$), higher implantation rates (IRs) ($p = 0.038$), and higher LBRs

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Peer review under responsibility of Chang Gung University.
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https://doi.org/10.1016/j.bj.2021.01.002
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($p = 0.042$) for embryos that underwent FET cycles. MPES in extended culture transfer have favorable survival than MPES in day3 transfer.
Conclusion: The extended culture of MPEs in fresh transfer cycles did not increase the LBR. However, younger females with the extended culture of MPEs followed by FET resulted in significantly higher LBRs and may be a feasible strategy to improve outcomes for patients with poor embryo quality. However, day3 embryo transfer may be a better choice if a fresh transfer is unrestricted and avoid the cycle cancellation. Extended culture may decrease to the transfer of developmental potential arrest embryos to patients.

At a glance commentary

Scientific background on the subject

The presence of only morphologically poor embryos (MPEs) on day three is common in autologous in vitro fertilization cycles of patients with advanced maternal age and in low-responding patients. However, there are limited data regarding the disposition of embryos from patients who only produced MPEs on day3.

What this study adds to the field

Day3 embryo transfer may be a better choice if a fresh transfer is unrestricted and avoid the cycle cancellation. Extended culture may decrease to the transfer of developmental potential arrest embryos to patients.

Morphological evaluation is the main method used to select the most competent embryos for transfer, and previous studies have demonstrated a strong association between embryo morphology and clinical pregnancy rate. Embryos with high-quality morphology are also more likely to be euploid [1–3], and the morphologic grade of euploid blastocysts influences implantation and subsequent pregnancy rate [4]. Conversely, transfer of morphologically poor embryos (MPEs) results in a low live birth rate (LBR) and increases the risk of poor health in offspring [5–9].

The presence of only MPEs on day three (day3) is common in autologous *in vitro* fertilization (IVF) cycles of patients with advanced maternal age (AMA) and in low-responding patients. Sermondade et al. reported that the incidence rate of only MPEs is approximately 10% at the first IVF attempt, and the recurrence rate is 3% [10]. Patients with only MPEs on day3 be responsible for remarkably decreased pregnancy rates and live birth rates in IVF programs [10], and most of MPEs will arrest in in-vitro culture conditions [11,12].Embryologists and clinicians are uncomfortable when faced with decisions involving the ongoing culture of MPEs, as the true potential of an MPE to yield a viable fetus is uncertain [13]. When the patient has only MPEs available, the patient chooses to transfer, but with expected live birth rate is low, or another choice are abandoned embryos without transfer, both of which will bring a relatively economical and psychological burden. To avoid generally destroyed poor-quality embryos, Sallem et al. report that day5 blastocyst extended culture of embryos after vitrification improves ART outcomes [14]. Regardless of the morphology and number of embryos, a cryopreservation and delayed embryo transfer program is requisite are the presence of risk factors for OHSS, the need for pre-implantation genetic diagnosis or screening (PGD/PGS) or the presence of embryo/endometrial asynchrony (e.g., ovarian stimulation with premature progesterone elevation on HCG day) [15–17]. But in addition to the conditions mentioned above, limited data are available on the disposition of embryos from women who only produce MPEs. Moreover, there is no unified guideline available for the fate decision of such embryos [18]. The actual probability of the successful IVF cycles of poor quality embryos has not been thoroughly studied [18]. The present study was, therefore, designed to investigate the possible benefits of extended culturing MPEs. Try to detect whether the extended culture (day4 or day5 culture) can improve the live birth rate per cycle? We conduct a retrospective study of the clinical outcomes of only MPE cycles on day3, without restricting our analysis to poor responders and older patients. We also compared the results with fresh day3 transfer with day4 or day5 transfer, and the clinical outcomes of fresh transfer with frozen embryo transfer (FET) of MPEs.

Materials and methods

Patients

This retrospective observational, single-center cohort study examined 224 IVF/intracytoplasmic sperm injection (ICSI) cycles in which only MPEs were produced on day3 after oocyte retrieval at the Kaohsiung Chang Gung Memorial Hospital between January 1, 2010 and June 30, 2015. The rationality of the two treatment strategies regarding only MPEs on day3 was developed in our institute, share extended culture decision of making between different physician and patient couples:

Rationale 1: Extended culture can serve as a strong diagnostic tool [19], waiting morula stage embryos have undergone genome activation [20], yielding useful information regarding the implantation potential of the human embryo. MPEs on day3 can reach the blastocyst stage, to embryo implant, and to give healthy babies [14]. It may decrease to transfer developmental arrest with chromosomal abnormalities embryos to patients [11,12].

Rationale 2: Cycle cancellation may cause economic burdens and psychological distress [21] for patients with MPE cycles. Transfer MPEs embryos early in these cycles to avoid

embryo waste. Although more euploid embryos can develop to blastocysts than aneuploid embryos, morphological analysis is not enough to select against chromosome abnormalities. Some euploid embryos may also arrest in the extend culture [22]. Extended culture should only be offered primarily to good prognosis patients to facilitating a natural selection of good quality embryos while lowering the multiple gestation rates following IVF [23,24], and this group should be specially defined in each clinical set-up. A total of 544 MPEs were analyzed. Veeck's morphological grading system was used [25]. This system defines a day3 embryo as a MPE if it fails to develop to Grade 1 (eight cells, blastomeres of equal size, and no cytoplasmic fragments) or Grade 2 (eight cells, blastomeres of equal size, and <20% cytoplasmic fragments). All MPE cycles were classified as fresh transfer on day3 (Group A) or extended culture (Group B). The extended culture group (Group B) included three subgroups: Group B1 had fresh transfer on day4 or day5; Group B2 had all embryos frozen on day4 or day5, followed by thawing and transfer; and Group B3 had embryo arrest [Fig. 1].

Ethics, consent and permissions

This study was approved by the Institutional Review Board and the Ethics Committee of Chang-Gung Medical Foundation, Taiwan. All patients provided informed consent.

Ovarian stimulation protocols

Controlled ovarian stimulation (COS), oocyte retrieval, embryo culture, and embryo transfer were performed as previously described [11,26]. The agonist protocol for COS were individualized according to the ovarian reserve, based on the antral follicle count (AFC) and level of anti-Müllerian hormone (AMH), age, baseline level of serum follicle stimulating hormone (FSH), and previous response to COS. Briefly, the protocol consisted of pituitary downregulation using leuprolide acetate (Lupron®; Takeda, Tokyo, Japan) with an initial dose of gonadotropin (human menopausal gonadotropin [hMG] or FSH [purified or recombinant]). The gonadotropin dose was individualized for each patient (range: 150–300 IU per day). Further dose adjustments were made based on the ovarian response, serum level of estradiol (E2), and ultrasonographic monitoring of follicular growth. When the leading follicle had a diameter of 18–20 mm, leuprolide acetate and FSH were discontinued, and recombinant human chorionic gonadotropin (hCG; Ovidrel®; Serono, Modugno, Italy) was administered. Oocyte retrieval was performed 36–38 h later by transvaginal aspiration under ultrasound guidance. Standard IVF/ICSI procedures were used for oocyte fertilization as previously described [11,26].

Assessment of fertilization, embryo culture, and embryo grading

The embryos were cultured in G1™ medium (Scandinavian IVF Science) on day1 to day3, and in G2™ medium (Scandinavian IVF Science) on day3 to day5. Veeck's morphological grading system [25] was modified and adopted for day3 embryo scoring, and the following definitions were used: Grade 1, eight cells, blastomeres of equal size and no cytoplasmic fragments (embryo score = 4); Grade 2, eight cells with blastomeres of equal size and less than 20% cytoplasmic fragments (embryo score = 3); Grade 3, eight cells with uneven blastomere sizes and no cytoplasmic fragments (embryo score = 2); Grade 4, four or eight cells with more than 20% fragmentation (embryo score = 1); and Grade 5, few blastomeres of any size and with major or complete fragmentation (embryo score = 1). Embryos that failed to meet the criteria of Grade 1 or Grade 2 were classified as MPEs. Blastocysts were defined as high-quality embryos if they were full, had development of an inner cell mass containing numerous tightly packed cells, and had a trophectoderm with many cells forming a cohesive epithelium. The embryos were transferred on day3, day4, or day5 after oocyte retrieval. The benefits of blastocyst transfer (BT) are that it facilitates the selection of high-quality embryos, it leads to a high implantation rate, and it lowers the risk of multiple pregnancies. Since 1999, our program has routinely offered elective BT to patients with three or more top quality eight-cell embryos on day3 [11]. Luteal phase support continued until the day pregnancy was confirmed by detection of hCG in the urine, and was provided for an additional four weeks if conception occurred.

The protocol for vitrification and warming was adapted from publications by Mukaida et al. and other researchers

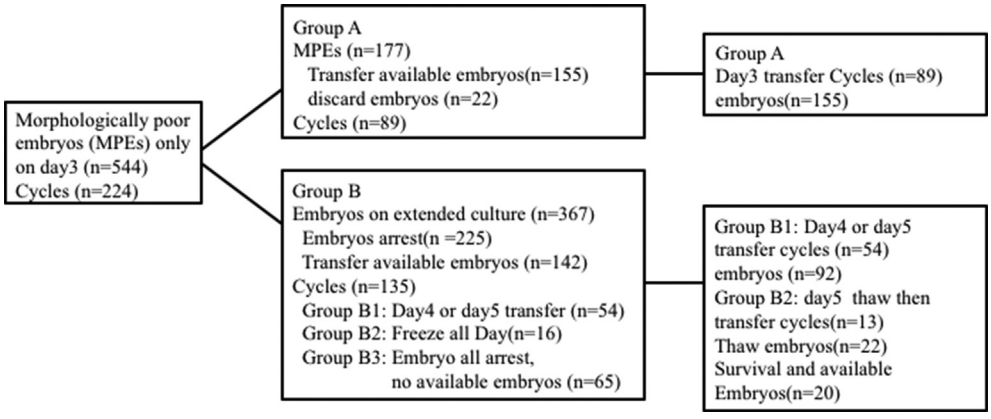

Fig. 1 Flow chart of study population.

[27,28]. The blastocysts were assessed based on their morphologic appearance and the presence of blastocoel expansion under a dissecting microscope at about 2 h after warming. Blastocysts with a morphologically intact inner cell mass, a trophectoderm, and a re-expanding blastocoel were determined to have survived. A biochemical pregnancy was defined by a positive urinary hCG test or a serum hCG concentration above 10 IU/L. The clinical implantation rate (IR) was defined as the number of gestational sacs per number of embryos transferred. Clinical pregnancy was defined as the presence of an intrauterine gestational sac with positive cardiac movement on ultrasound [29]. The pregnancy outcome was recorded for all pregnant women using a postal questionnaire or by telephone. The LBR per transfer was defined as the proportion of IVF cycles reaching embryo transfer that resulted in the birth of at least one live-born child. The cumulative pregnancy rate was followed up until December 2016.

Statistical analysis

The primary outcome measure was the LBR of fresh and FET cycles. Non-normally distributed continuous variables are expressed as medians and interquartile ranges unless otherwise stated. Continuous and categorical variables were compared using the Mann–Whitney test and Fisher's exact test, respectively. The potential clustering effect between groups had been evaluated [30]. Since the dataset contains multiple embryos for the same patient, modeling cycle as a random effect to account for data clustering for 224 cycles with 544 MPEs by linear mixed model.

The statistical analysis was conducted using the Statistical Program for Social Sciences (SPSS Inc., Version 15.0, Chicago, U.S.A.) and MedCalc Statistical Software version 16.8 (MedCalc Software, Ostend, Belgium; http://www.medcalc.org; 2016). Multivariable logistic regression analysis was used to assess the relationship of liver birth rate with age of female partners, Fresh ET or FET, BMI, day3 or extended culture, primary or secondary infertility, duration of infertility, number of oocytes retrieved, number of embryos transferred, number of two pronuclear (PN), totally embryos score and available. A full model, with the inclusion of all variables to adequately control for potential confounding, was implemented and the results are presented as adjusted odds ratios (aORs) and 95% confidence interval (95% CIs). All *p*-values were two-sided, and values less than 0.05 were considered statistically significant.

Results

This retrospective analysis evaluated 544 MPEs that were collected from 224 IVF/ICSI cycles that contained only MPEs on day3. Of these 224 cycles, 89 (39.7%) underwent fresh embryo transfer on day3 (Group A), and 135 (60.3%) underwent extended culture (Group B). In Group B, 54 cycles (40.0%) underwent day4 or day5 embryo transfer (Group B1), 16 cycles (11.9%) had all embryos frozen (Group B2), and 65 cycles (48.1%) exhibited total embryo arrest (Group B3) [Fig. 1]. A total of 155 day3 MPEs were transferred, and 22 were discarded due to developmental delay beyond 2 days. Additionally, 367 MPEs underwent extended culture; 92 (25.1%) were freshly transferred and 50 (13.6%) were frozen at day5, with evidence of development to the morula or blastocyst stage. The other 225 (61.3%) MPEs were developmentally arrested. The blastocyst formation rate of the MPEs was 9.5% (35/367), and high-quality blastocysts were recovered in 8.5% (3/35) of all blastocysts derived from MPEs.

Analysis of patient baseline demographic data and cycle characteristics for Group A and Group B [Table 1] indicated that a greater body mass index in Group A was the only significant difference ($p = 0.006$). Analysis of the pregnancy rate and live birth rate in the two groups [Table 1] indicated no significant differences.

Further analysis of Group B indicated that a high percentage of the 65 cycles (48.1%) were unusable because the embryos exhibited developmental arrest. However, 70 cycles (51.9%) continued growing to day4 or day5. In addition, 54 of these 70 cycles (77.1%) underwent fresh transfer, and the other 16 cycles were all frozen due to prevention of ovarian hyperstimulation syndrome (N = 4), pre-implantation diagnosis/screening (N = 2), premature progesterone elevation (N = 4), abnormal endometrial preparation after mild stimulation (N = 2), and unexpected delayed development of embryos (N = 4).

Notably, both fresh transfer groups (Table 2. Group A vs. Group B1) had very low LBRs (4.5% *vs.* 7.4% $p = 0.46$) and identical abortion rates (20%). There was no evidence of aneuploidy or other major congenital anomalies in the offspring. Table 2 also shows the pregnancy outcomes of fresh day4 or day5 transfer with MPEs (Group B1) and FET with MPEs (Group B2). There were 13 FET cycles, 22 frozen embryos were thawed, and 20 embryos were recovered for transfer. The post-thaw survival rate was 90.1% (20/22). Patients in Group B2 had more post-thaw embryos with good quality ($p < 0.001$), a higher IR ($p = 0.038$), and a higher LBR ($p = 0.042$). Patients in Group B2 also had a tendency for a higher clinical pregnancy rate, but this was not significant. There was no evidence of aneuploidy or major congenital anomalies in the offspring. Surplus embryos were scarce in two groups. Fig. 2 compares the pregnancy rates of the three groups. Since the dataset contains multiple MPEs for the same patient, modeling cycle as a random effect to account for data clustering for 224 cycles with 544 MPEs by linear mixed model. We found day4 or day5 transfer (GroupB1) has significant survival for embryos than day3 transfer embryos (Group A) [Table 3]. However, MPEs with extended culture then thaw embryos transfer (Group B2) with significant implantation outcome (Table 3: Group A vs. Group B2: Coefficient = −0.233, $p < 0.001$; Group B1 vs. Group B2: Coefficient = −0.203, $p = 0.002$).

Multiple Logistic regression analysis of the relationship of patient and treatment characteristics with live birth rate per cycle or per MPE. After adjustment for confounding, the consistent factors that remained significant were age of female partners (aOR = 0.783, 95% CI = 0.659 to 0.930; aOR = 0.565, 95% CI = 0.455 to 0.702), and fresh or FET (aOR: 7.396, 95% CI: 1.704 to 32.104; aOR: 21.673, 95% CI: 4.250 to 110.513) [Table 4 and Table 5].

Table 1 Characteristics of patients in Group A and Group B.

	Group A Day 3 Embryo transfer	Group B Extended culture	*p* value
No. of cycles	89	135	
Age of female partners (y)	38.4 (35.2–40.5)	37.7 (34.8–40.4)	NS
Age of male partner (y)	39.1 (36.0–43.0)	39.0 (35.7–42.3)	NS
Body mass index (kg/m^2)	22.3 (20.7–25.2)	21.1 (20.1–23.1)	0.006[a]
Duration of infertility (y)	4 (2–6)	3 (2–5)	NS
Baseline FSH (mIU/ml)	7.40 (6.0–9.5)	6.45 (5.1–10.1)	NS
Serum AMH, ng/mL	1.41 (0.8–2.2)	1.67 (0.7–2.93)	NS
Infertility, % (n) Primary/secondary	53.4%/46.5% (46/40)	57.2%/42.8% (79/59)	NS
Days of FSH treatment	8 (7–9)	9 (8–10)	NS
IVF/ICSI %(n)	59.3%/40.7% (51/35)	65.2%/34.8% (90/48)	NS
Endometrial thickness on hCG day	1.3 (1.2–1.6)	1.3 (1.1–1.5)	NS
Total FSH dose (IU)	2100 (1335–2700))	2100 (1560-2625	NS
E2 on hCG day (pg/mL)	1034 (596–1706)	1135 (524–1988)	NS
P4 (ng/mL) on hCG day	0.68 (0.41–1.17)	0.71 (0.38–1.37)	NS
No. of oocytes retrieved	3 (2–3)	3 (2–6)	NS
No. of mature oocytes retrieved	1 (0–1)	1 (1–2)	NS
No of 2 PN	1 (1–2)	1 (1–3)	NS
Pregnancy rate per transfer	5.6% (5/89)	13.4% (9/67)	NS
Pregnancy rate per cycle	5.6% (5/89)	6.6% (9/135)	NS
Live birth rate per transfer	4.5% (4/89)	11.9% (8/67)	NS
live birth rate per cycle	4.5% (4/89)	5.9% (8/135)	NS

Basic demographic and clinical parameters of Day 3 transfer with morphologic poor embryos (group A) and extended culture with morphologic poor embryos (group B).
Note: Values are shown %(*n*) or median (interquartile range) [a] $p < 0.05$.
NS: not significant.
AMH: anti-Müllerian hormone;2 PN: two pronuclear; hCG: human chorionic gonadotropin; FSH: follicle stimulating hormone; IVF: *in vitro* fertilization; ICSI: intracytoplasmic sperm injection.

Table 2 Comparison of clinical outcomes of morphologically poor embryos (MPEs) only on day 3 cycles: Fresh Day 3 transfer (group A), fresh Day 4 or day5 transfer after extended culture (group B-1) and frozen embryo transfer (group B-2).

	Group A Day 3 transfer	Group B-1 Day4 or day5 transfer after Extended culture	Group B-2 Frozen embryo transfer
No. of cycles	89	54	13
Mean no. of embryos transferred	2 (1–2)	2 (1–2)	1.5 (1–2)
Good-quality embryo in transferred	0% (0/155)	0% (0/92)	20% (4/20)*
Biochemical pregnancy	5.6% (5/89)	14.8% (8/54)	38.4% (5/13)
Clinical pregnancy rate, % (n)	5.6% (5/89)	9.3% (5/54)	30.1% (4/13)
Implantation rate, (%) (n)	2.6% (4/155)	7.6% (7/92)	25% (5/20)*
Abortion rate (%)	20% (1/5)	20% (1/5)	0% (0/4)
Live birth rate (%)	4.5% (4/89)	7.4% (4/54)	30.1% (4/13)*
Multiple gestation (%)	0% (0/4)	25% (1/4)	25% (1/4)

Note: Values are % (*n*) or median (interquartile range) GroupB1vs. GroupB2 *$p < 0.05$.

Discussion

A limited number of treatment strategies are available for these patients who only produce MPEs on day3 that simultaneously reduce embryo waste and lead to a high LBR. Besides, little is known about the consequences of extended culture or freezing/thawing transfer of MPEs on pregnancy outcomes.

Extended embryo culture allows improved embryo selection for transfer, and potentially increases the pregnancy rate per embryo transferred [31]. Our study reported that extended culture before fresh transfer did not increase the LBR compared to day3 embryo transfer. More specifically, both approaches led to very low LBRs (day3 *vs.* day4 or day5: 4.5% *vs.* 7.4%; $p = 0.46$) and identical abortion rates (20%). Another finding of our study is that extended culture with vitrification followed by FET led to a significantly increased LBR compared to day4 or day5 fresh transfer [Tables 2 and 4]. Besides, it is still consistent that our previous finding that age is a major prognosticator in females with poor ovarian response cycles [32][Table 4].

We also found that extended culture led to the loss of most MBEs (62.4% embryo arrest); Therefore, day3 cleavage-embryo transfer may be a better choice if a fresh transfer is

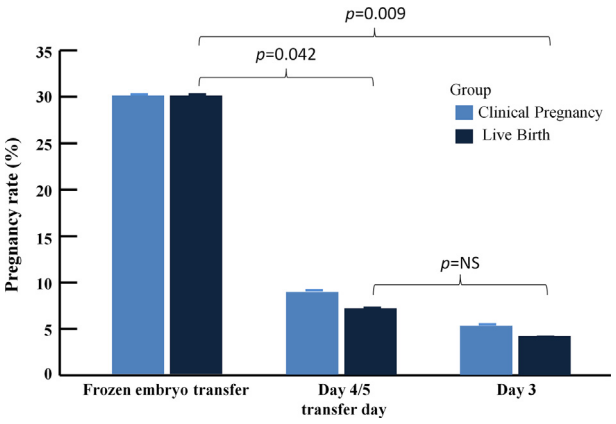

Fig. 2 Comparison of pregnancy outcomes in Day 3, Day 4/5 and frozen embryo transfer.

unrestricted and avoid the cycle cancellation. Day4 or day5 fresh transfer after extended culturing does not increase the LBR but may decrease to transfer developmental potential arrest embryos to patients.

Indeed, individualize MPEs after extended culture and transfer had more significant survival than day3 transfer MPEs [Table 3]. Previous studies reported that MPEs could develop to the blastocyst stage and were capable of implantation [13,33,34]. Previous research indicated that blastocyst-stage transfer of poor-quality cleavage-stage embryos results in a higher IR [8]. However, no other evidence supports the use of extended culturing to improve the pregnancy outcomes for MPEs.

Extended culture of MPEs followed by FET resulted in significantly higher LBRs. These findings are consistent with previous studies. For example, Kaartinen et al. reported that repeated IVF/ICSI cycles could be avoided by using frozen blastocysts that develop from poor-quality cleavage-stage embryos [35]. Nakagawa et al. found comparable perinatal outcomes from using frozen cleavage-stage poor-quality embryos and using good embryos [5]. Shaw-Jackson et al. also reported that vitrification of blastocysts derived from poor-quality cleaved embryos can produce high pregnancy rates after warming [21]. Additionally, Xu et al. found that day 5 blastocysts derived from non-top-quality day 3 embryos after cryopreservation had better clinical outcomes than those from day 3 cleavage-stage embryos and day 6 blastocysts [36]. Thus, FET significantly improves the LBR for poor quality embryos. This may be because of the dual selection of embryos by extended culture and then by freezing/thawing, which helps to identify MPEs most suitable for implantation.

Blastomere morphology, cleavage kinetics, and the extent of cytoplasmic fragmentation are parameters used to assess cleavage-embryo quality [25]. Fragmentation is a common observation in 75% of human *in vitro*-produced embryos, but can be variable. The presence of fragmentation inversely correlates with embryo viability [37–39]. The mechanisms of embryo fragmentation remain unclear, but may correlate with maternal factors, progression of meiotic and mitotic cell cycles, or apoptosis/necrosis [40–44]. Substantial fragmentation can obscure the identification of other blastomeres and make assessment difficult. Thus, extending the culture to the blastocyst stage allows identification of viable embryos with the greatest potential for implantation [45]. The *in vitro* culture conditions artificially mimic the natural environment, and expose embryos to developmental stress. The extended culture of MPEs is similar to a "functional assay", and allows selection of the most viable embryos [19]. Culturing MPEs to cell differentiation and the activation of the embryonic genome at the blastocyst stage may also improve embryo selection [24,46].

The freezing/thawing process is another type of embryo selection. A previous study indicated that the euploidy rate in excellent blastocysts was 56.4%, and that morphological parameters were not well correlated with successful implantation [2]. The freezing/thawing process may be considered a stress to embryos. Blastocysts from MPEs have the advantages of having more cells and a higher membrane/cytoplasmic ratio, factors that can compensate for partial cryoinjury [47]. As mentioned above, significant fragmentation of MPEs may make morphologic grading more difficult during fresh cycles. However, the freezing/thawing process may reduce the impact of these nonviable fragmentations, and thereby facilitate morphological grading. Besides, the decline of fragmentation may benefit the growth of other blastomeres. The phenomenon is consistent with our recent finding [48].

Table 3 Morphologically poor embryos (MPEs) after extended culture and transfer or day3 transfer by linear mixed model.

MPEs arrest or not			
Parameter	Coefficient	Standard error	*p* value
intercept	0.722	0.084	<0.001
Group A vs. Group B2	−0.689	0.092	<0.001*
Group B1 vs. Group B2	−0.045	0.095	0.636
Group A vs. Group B1: $p < 0.001$*			
MPEs implant or not			
Parameter	Coefficient	Standard error	*p* value
intercept	0.265	0.057	<0.001
Group A vs. Group B2	−0.233	0.061	<0.001*
Group B1 vs. Group B2	−0.203	0.064	0.002*
Group A vs. Group B1: $p = 0.412$.			
Group A: fresh transfer on day3.			
Group B1: fresh transfer on day4 or day5.			
Group B2: all embryos frozen on day4 or day5, followed by thawing and transfer.			

Table 4 Multivariate analysis of factors affecting live birth per cycle.

	Adjusted OR	95% Confidence interval for adjusted OR Lower bound	Upper bound	*p value*
Age of female partners (y)	0.783	0.659	0.930	**0.005**
Fresh ET or FET	7.396	1.704	32.104	**0.008**
Day3 or extended culture	3.314	0.629	17.468	NS
Body mass index (kg/m^2)	1.070	0.850	1.346	NS
Duration of infertility (y)	0.798	0.577	1.102	NS
Primary/secondary	4.290	0.901	20.426	NS
No. of oocytes retrieved	0.676	0.396	1.155	NS
No of 2 PN	0.983	0.515	1.875	NS
Totally embryos score	0.885	0.538	1.456	NS
Totally embryos available	1.321	0.540	3.229	NS
Transfer number	1.581	0.377	6.631	NS

NS: not significant.
2 PN: two pronuclear.
ET: embryo transfer.
FET: frozen embryo transfer.

Table 5 Multivariate analysis of factors affecting live birth per MPE.

	Adjusted OR	95% Confidence interval for adjusted OR Lower bound	Upper bound	*p value*
Cycle	1.024	1.102	1.036	**<0.001**
Age of female partners (y)	0.565	0.455	0.702	**<0.001**
Fresh ET or FET	21.673	4.250	110.513	**<0.001**
Day3 or extended culture	12.219	2.512	59.433	**0.002**
Body mass index (kg/m^2)	1.044	0.896	1.216	NS
Duration of infertility (y)	0.894	0.714	1.120	NS
Primary/secondary	16.185	3.740	70.038	**<0.001**
No. of oocytes retrieved	1.329	1.128	1.565	**<0.001**
No of 2 PN	0.337	0.202	0.563	**<0.001**
Totally embryos score	0.447	0.313	0.638.	**<0.001**
Totally embryos available	0.488	0.139	1.708	NS
Transfer number	31.915	6.279	162.212	**<0.001**

NS: not significant.
2 PN: two pronuclear.
ET: embryo transfer.
FET: frozen embryo transfer.

Therefore, the dual selection of MPEs may lead to the transfer of the most competent embryos.

The endometrial environment of a fresh cycle is more hostile to MPEs. A top-quality embryo transferred by FET may have the same potential as an embryo from a fresh cycle [49]. However, little information exists regarding the implantation potential of MPEs by fresh transfer and the FET of poor-quality embryos. Our data showed that the IR significantly increased only after FET, and higher embryo grades were present after thawing of MPEs. Wirleitner et al. also reported the expansion of blastocysts on day 5, and that fresh transfer and FET groups had similar IRs and LBRs. In this case, non-top-quality day 5 blastocysts transferred by FET led to higher IRs and LBRs than fresh embryo transfer [50]. Although the reasons for the apparently better outcome in FET cycles is not entirely clear, several authors speculated that the endometrial and hormonal environment might be less hostile to embryos from frozen cycles [51,52].

Many studies provided strong evidence of decreased endometrial receptivity in stimulated cycles, which significantly limits the success of fresh embryo transfer [16,53–56]. FET cycles may provide the MPEs with more physiologically favorable endometrial conditions, and therefore increase the IR. However, it is unclear whether cryopreservation has a direct or indirect impact on embryo quality [57]. Despite the routine and widespread use of embryo cryopreservation, little is known about the effect of cryopreservation on the embryo's genome and its structural/functional conformation. The extent of fragmentation during fresh culture is inversely proportional to pregnancy rate, and embryo viability is severely compromised when fragmentation exceeds 50% [39,58]. However, Fernandez-Gallardo et al. reported that the IR of vitrified day 3 embryos was only determined by the number of cells lost, the occurrence of mitosis resumption, and the specific number of blastomeres, and that IR was unaffected by fragmentation, blastomere symmetry, or volume change [59]. Our previous study also found

post-thaw survival rate and blastocyst formation rate were satisfactory, with about half of all heavily fragmented morulas developing into blastocysts [48]. Additional studies are needed to determine the impact of the freezing/thawing process on fragmentation and embryo quality.

The present study had several limitations. First, because our sample size was relatively small, a study of a larger sample is needed for confirmation. Second, the retrospective design of our study could have led to selection bias, because we did not establish the groups by randomization. A prospective randomized controlled trial should be performed to confirm our results. Third, the relationship between pregnancy outcome and single MPE transfer requires further investigation due to a mean of approximately two MPEs were transferred to most patients in this study. Finally, we did not apply a pre-implantation genetic screen (PGS) for these MPEs due to cost concerns. Therefore, use of a PGS may be another method to help select the most suitable MPEs.

Conclusion

This study characterized a particular subgroup of patients who only produced MPEs on day3. We found that the extended culture of MPEs followed by fresh transfer led to a similar LBR as day3 fresh transfer. Both approaches resulted in very low LBRs, and had identical abortion rates. Younger females with the extended culture of MPEs followed by FET led in significantly higher LBRs and may be a feasible strategy to improve outcomes for patients with poor embryo quality. However, day3 embryo transfer may be a better choice if a fresh transfer is unrestricted and avoid the cycle cancellation. Day4 or day5 extended culture may decrease to the transfer of developmental potential arrest embryos to patients.

Funding

This study was supported by grants CMRPG8G0071-73 and CMRPG8H0981, CMRPG8K0061 from the Chang Gung Memorial Hospital.

Consent to publish

We have obtained consent to publish patient data from the study participants.

Conflicts of interest

The authors declare that they have no competing interests.

Acknowledgements

We thank the embryologists at Kaohsiung Chang Gung Memorial Hospital for their assistance and Registered Nurse Yun-Fang Chiang for assistance in patient registration and data collection.

We sincerely appreciate the Biostatistics Center, Kaohsiung Chang Gung Memorial Hospital, for performing the statistical analysis.

REFERENCES

[1] Minasi MG, Colasante A, Riccio T, Ruberti A, Casciani V, Scarselli F, et al. Correlation between aneuploidy, standard morphology evaluation and morphokinetic development in 1730 biopsied blastocysts: a consecutive case series study. Hum Reprod 2016;31:2245–54.

[2] Capalbo A, Rienzi L, Cimadomo D, Maggiulli R, Elliott T, Wright G, et al. Correlation between standard blastocyst morphology, euploidy and implantation: an observational study in two centers involving 956 screened blastocysts. Hum Reprod 2014;29:1173–81.

[3] Alfarawati S, Fragouli E, Colls P, Stevens J, Gutierrez-Mateo C, Schoolcraft WB, et al. The relationship between blastocyst morphology, chromosomal abnormality, and embryo gender. Fertil Steril 2011;95:520–4.

[4] Irani M, Reichman D, Robles A, Melnick A, Davis O, Zaninovic N, et al. Morphologic grading of euploid blastocysts influences implantation and ongoing pregnancy rates. Fertil Steril 2017;107:664–70.

[5] Nakagawa K, Ojiro Y, Nishi Y, Sugiyama R, Motoyama H, Sugiyama R. Perinatal outcomes of patients who achieved pregnancy with a morphologically poor embryo via assisted reproductive technology. Arch Gynecol Obstet 2016;293:183–8.

[6] Oron G, Son WY, Buckett W, Tulandi T, Holzer H. The association between embryo quality and perinatal outcome of singletons born after single embryo transfers: a pilot study. Hum Reprod 2014;29:1444–51.

[7] Mendoza R, Perez S, de Los Santos MJ, Larreategui Z, Ayerdi F, Exposito A, et al. Congenital malformations, chromosomal abnormalities and perinatal results in IVF/ICSI newborns resulting from very poor quality embryos: a case-control study. Gynecol Obstet Invest 2015;79:83–9.

[8] Ebner T, Yaman C, Moser M, Sommergruber M, Polz W, Tews G. Embryo fragmentation in vitro and its impact on treatment and pregnancy outcome. Fertil Steril 2001;76:281–5.

[9] Hardarson T, Caisander G, Sjogren A, Hanson C, Hamberger L, Lundin K. A morphological and chromosomal study of blastocysts developing from morphologically suboptimal human pre-embryos compared with control blastocysts. Hum Reprod 2003;18:399–407.

[10] Sermondade N, Delarouziere V, Ravel C, Berthaut I, Verstraete L, Mathieu E, et al. Characterization of a recurrent poor-quality embryo morphology phenotype and zygote transfer as a rescue strategy. Reprod Biomed Online 2012;24:403–9.

[11] Lan KC, Huang FJ, Lin YC, Kung FT, Hsieh CH, Huang HW, et al. The predictive value of using a combined Z-score and day 3 embryo morphology score in the assessment of embryo survival on day 5. Hum Reprod 2003;18:1299–306.

[12] Hardy K, Spanos S, Becker D, Iannelli P, Winston RM, Stark J. From cell death to embryo arrest: mathematical models of human preimplantation embryo development. Proc Natl Acad Sci U S A 2001;98:1655–60.

[13] Hershko Klement A, Ovadia M, Wiser A, Berkovitz A, Shavit T, Nemerovsky L, et al. What we learned from extended culture of 'rejected' day-3 cleavage stage embryos: a prospective cohort study. J Ovarian Res 2017;10:35.

[14] Sallem A, Santulli P, Barraud-Lange V, Le Foll N, Ferreux L, Maignien C, et al. Extended culture of poor-quality

supernumerary embryos improves ART outcomes. J Assist Reprod Genet 2018;35:311–9.
[15] Blockeel C, Drakopoulos P, Santos-Ribeiro S, Polyzos NP, Tournaye H. A fresh look at the freeze-all protocol: a SWOT analysis. Hum Reprod 2016;31:491–7.
[16] Evans J, Hannan NJ, Edgell TA, Vollenhoven BJ, Lutjen PJ, Osianlis T, et al. Fresh versus frozen embryo transfer: backing clinical decisions with scientific and clinical evidence. Hum Reprod Update 2014;20:808–21.
[17] Tsai YR, Huang FJ, Lin PY, Kung FT, Lin YJ, Lin YC, et al. Progesterone elevation on the day of human chorionic gonadotropin administration is not the only factor determining outcomes of in vitro fertilization. Fertil Steril 2015;103:106–11.
[18] Kirillova A, Lysenkov S, Farmakovskaya M, Kiseleva Y, Martazanova B, Mishieva N, et al. Should we transfer poor quality embryos? Fertil Res Pract 2020;6:2.
[19] Balaban B, Urman B. Embryo culture as a diagnostic tool. Reprod Biomed Online 2003;7:671–82.
[20] Fabozzi G, Alteri A, Rega E, Starita MF, Piscitelli C, Giannini P, et al. Morphological assessment on day 4 and its prognostic power in selecting viable embryos for transfer. Zygote 2016;24:477–84.
[21] Milazzo A, Mnatzaganian G, Elshaug AG, Hemphill SA, Hiller JE. Depression and anxiety outcomes associated with failed assisted reproductive technologies: a systematic Review and meta-analysis. PloS One 2016;11:e0165805.
[22] Qi ST, Liang LF, Xian YX, Liu JQ, Wang W. Arrested human embryos are more likely to have abnormal chromosomes than developing embryos from women of advanced maternal age. J Ovarian Res 2014;7:65.
[23] Garcia-Velasco JA, Simon C. Blastocyst transfer: does it really affect the outcome? Curr Opin Obstet Gynecol 2001;13:299–304.
[24] Gardner DK, Schoolcraft WB, Wagley L, Schlenker T, Stevens J, Hesla J. A prospective randomized trial of blastocyst culture and transfer in in-vitro fertilization. Hum Reprod 1998;13:3434–40.
[25] Veeck LL. An atlas of human gametes and conceptuses: an illustrated reference for assisted reproductive technology. New York, USA: CRC Press; 1999.
[26] Lin PY, Huang FJ, Kung FT, Lin YC, Chiang HJ, Lin YJ, et al. Reassessing the feasibility of the zygote score for predicting embryo viability in IVF/ICSI using the GnRH antagonist protocol compared to the long protocol. PloS One 2017;12:e0171465.
[27] Mukaida T, Takahashi K, Kasai M. Blastocyst cryopreservation: ultrarapid vitrification using cryoloop technique. Reprod Biomed Online 2003;6:221–5.
[28] Lin PY, Huang FJ, Kung FT, Wang LJ, Chang SY, Lan KC. Comparison of the offspring sex ratio between fresh and vitrification-thawed blastocyst transfer. Fertil Steril 2009;92:1764–6.
[29] Zegers-Hochschild F, Adamson GD, de Mouzon J, Ishihara O, Mansour R, Nygren K, et al. The international committee for monitoring assisted reproductive technology (ICMART) and the world health organization (WHO) revised glossary on ART terminology, 2009. Hum Reprod 2009;24:2683–7.
[30] Luke DA. Multilevel modeling. Thousand Oaks, CA: Sage; 2004.
[31] Maheshwari A, Hamilton M, Bhattacharya S. Should we be promoting embryo transfer at blastocyst stage? Reprod Biomed Online 2016;32:142–6.
[32] Su YT, Lin PY, Huang FJ, Kung FT, Lin YJ, Tsai YR, et al. Age is a major prognosticator in extremely low oocyte retrieval cycles. Taiwan J Obstet Gynecol 2017;56:175–80.
[33] Stone BA, Greene J, Vargyas JM, Ringler GE, Marrs RP. Embryo fragmentation as a determinant of blastocyst development in vitro and pregnancy outcomes following embryo transfer. Am J Obstet Gynecol 2005;192:2014–9. discussion 2019-20.
[34] Shaw-Jackson C, Bertrand E, Becker B, Colin J, Beaudoin-Chabot C, Rozenberg S, et al. Vitrification of blastocysts derived from fair to poor quality cleavage stage embryos can produce high pregnancy rates after warming. J Assist Reprod Genet 2013;30:1035–42.
[35] Kaartinen N, Das P, Kananen K, Huhtala H, Tinkanen H. Can repeated IVF-ICSI-cycles be avoided by using blastocysts developing from poor-quality cleavage stage embryos? Reprod Biomed Online 2015;30:241–7.
[36] Xu L, Chen X, Tian X, Liu Y, Wang N, Ye D, et al. Comparison of clinical outcomes of blastocysts derived from non-top quality embryos and cleavage-stage high-quality embryos in frozen-thawed embryo transfer cycles. Nan fang yi ke da xue xue bao= Journal of Southern Medical University 2015;35:481–5.
[37] Hardy K. Apoptosis in the human embryo. Rev Reprod 1999;4:125–34.
[38] Staessen C, Camus M, Bollen N, Devroey P, Van Steirteghem AC. The relationship between embryo quality and the occurrence of multiple pregnancies. Fertil Steril 1992;57:626–30.
[39] Ziebe S, Petersen K, Lindenberg S, Andersen AG, Gabrielsen A, Andersen AN. Embryo morphology or cleavage stage: how to select the best embryos for transfer after in-vitro fertilization. Hum Reprod 1997;12:1545–9.
[40] Tilly JL. Commuting the death sentence: how oocytes strive to survive. Nat Rev Mol Cell Biol 2001;2:838–48.
[41] Pirkkala L, Nykanen P, Sistonen L. Roles of the heat shock transcription factors in regulation of the heat shock response and beyond. Faseb J 2001;15:1118–31.
[42] Van Blerkom J. Mitochondria in human oogenesis and preimplantation embryogenesis: engines of metabolism, ionic regulation and developmental competence. Reproduction 2004;128:269–80.
[43] Stensen MH, Tanbo TG, Storeng R, Abyholm T, Fedorcsak P. Fragmentation of human cleavage-stage embryos is related to the progression through meiotic and mitotic cell cycles. Fertil Steril 2015;103:374–81. e4.
[44] Chi HJ, Koo JJ, Choi SY, Jeong HJ, Roh SI. Fragmentation of embryos is associated with both necrosis and apoptosis. Fertil Steril 2011;96:187–92.
[45] Stecher A, Vanderzwalmen P, Zintz M, Wirleitner B, Schuff M, Spitzer D, et al. Transfer of blastocysts with deviant morphological and morphokinetic parameters at early stages of in-vitro development: a case series. Reprod Biomed Online 2014;28:424–35.
[46] Shapiro BS, Harris DC, Richter KS. Predictive value of 72-hour blastomere cell number on blastocyst development and success of subsequent transfer based on the degree of blastocyst development. Fertil Steril 2000;73:582–6.
[47] Mesut N, Ciray HN, Mesut A, Aksoy T, Bahceci M. Cryopreservation of blastocysts is the most feasible strategy in good responder patients. Fertil Steril 2011;96:1121–5.e1.
[48] Tsai NC, Su YT, Lin YJ, Chiang HJ, Huang FJ, Kung FT, et al. Developmental potential of surplus morulas with delayed and/or incomplete compaction after freezing-thawing procedures. Reprod Biol Endocrinol 2019;17:87.
[49] Veleva Z, Orava M, Nuojua-Huttunen S, Tapanainen JS, Martikainen H. Factors affecting the outcome of frozen-thawed embryo transfer. Hum Reprod 2013;28:2425–31.
[50] Wirleitner B, Schuff M, Stecher A, Murtinger M, Vanderzwalmen P. Pregnancy and birth outcomes following fresh or vitrified embryo transfer according to blastocyst morphology and expansion stage, and culturing strategy for delayed development. Hum Reprod 2016;31:1685–95.
[51] Shih W, Rushford DD, Bourne H, Garrett C, McBain JC, Healy DL, et al. Factors affecting low birthweight after assisted reproduction technology: difference between transfer of fresh

and cryopreserved embryos suggests an adverse effect of oocyte collection. Hum Reprod 2008;23:1644–53.
[52] Halliday JL, Ukoumunne OC, Baker HW, Breheny S, Jaques AM, Garrett C, et al. Increased risk of blastogenesis birth defects, arising in the first 4 weeks of pregnancy, after assisted reproductive technologies. Hum Reprod 2010;25:59–65.
[53] Van Vaerenbergh I, Van Lommel L, Ghislain V, In't Veld P, Schuit F, Fatemi HM, et al. In GnRH antagonist/rec-FSH stimulated cycles, advanced endometrial maturation on the day of oocyte retrieval correlates with altered gene expression. Hum Reprod 2009;24:1085–91.
[54] Evans J, Hannan NJ, Hincks C, Rombauts LJ, Salamonsen LA. Defective soil for a fertile seed? Altered endometrial development is detrimental to pregnancy success. PloS One 2012;7:e53098.
[55] Haouzi D, Assou S, Dechanet C, Anahory T, Dechaud H, De Vos J, et al. Controlled ovarian hyperstimulation for in vitro fertilization alters endometrial receptivity in humans: protocol effects. Biol Reprod 2010;82:679–86.
[56] Boomsma CM, Kavelaars A, Eijkemans MJ, Fauser BC, Heijnen CJ, Macklon NS. Ovarian stimulation for in vitro fertilization alters the intrauterine cytokine, chemokine, and growth factor milieu encountered by the embryo. Fertil Steril 2010;94:1764–8.
[57] Kopeika J, Thornhill A, Khalaf Y. The effect of cryopreservation on the genome of gametes and embryos: principles of cryobiology and critical appraisal of the evidence. Hum Reprod Update 2015;21:209–27.
[58] Stylianou C, Critchlow D, Brison DR, Roberts SA. Embryo morphology as a predictor of IVF success: an evaluation of the proposed UK ACE grading scheme for cleavage stage embryos. Hum Fertil 2012;15:11–7.
[59] Fernandez Gallardo E, Spiessens C, D'Hooghe T, Debrock S. Effect of embryo morphology and morphometrics on implantation of vitrified day 3 embryos after warming: a retrospective cohort study. Reprod Biol Endocrinol 2016;14:40.