Prolonged culture of blastocysts after thawing as a tool for improving prediction of success

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Abstract

Purpose A few years ago, we started to use a new freeze–thaw protocol for the frozen embryo transfer cycles. Instead of thawing the embryos 2–4 h prior to the transfer, we started thawing the embryos 20–22 h prior to the transfer. The aim of this study was to compare the pregnancy rate in cases of embryos that continued to develop in the post-thawing culture to that of embryos that did not.

Methods A retrospective cohort study of blastocyst freeze/thaw cycles vitrified on day 5, thawed and transferred after 20–22 h in the culture, between January 2012 and December 2016.

Results A total of 375 patients were included in the analysis. Two hundred twenty-eight embryos graded as good, 87 graded as fair, and 60 graded as poor embryos were transferred. The clinical pregnancy rate (50% vs. 19.5% vs 3.3% $p < 0.01$) and the ongoing pregnancy rate (38.5% vs. 13.6% vs 1.7% $p < 0.01$) were higher in cases of good embryo quality compared with fair and poor-quality embryos, respectively. For good embryos, progressing to a better grade during the culture did not change the clinical pregnancy rate (51.3% vs. 46.2% $p = \text{NS}$) or the ongoing pregnancy rate (38.5% vs. 37.5% $p = \text{NS}$). For fair embryos, progressing to a better grade during the culture resulted in a higher clinical pregnancy rate (25.4% vs 9% $p = 0.05$).

Conclusions The development of the fair embryos in the culture has a highly positive impact on the pregnancy rate and this factor should be taken into consideration before deciding how many embryos to transfer.

Keywords ART · Freeze–thaw cycles · Frozen embryo transfers · Vitrification

Introduction

Recently, several studies have demonstrated a significantly increased clinical pregnancy rate per transfer in the FET (frozen embryo transfer) cycles versus the fresh cycles [1–4], and there has been a trend toward FET cycles in our clinic during the last 3 years. There are many factors that may have an impact on the pregnancy rate. These factors include the age of the patient, number of failed IVF cycles in the past and the blastocyst quality [5–8].

Five years ago we started to use a new freeze–thaw protocol for frozen embryo transfer cycles. Instead of thawing the

blastocysts 2–4 h prior to the transfer, we started thawing the blastocysts 20–22 h prior to the transfer. In the majority of the cases, we observed that the blastocysts continued to develop in the culture and improved their grading. In other cases, although the blastocysts were viable after warming, they did not develop further in culture. The aim of this study was to compare the pregnancy rate in cases of blastocysts that continued to develop after warming to that of blastocysts that did not.

Materials and methods

This was a single center, retrospective cohort study of 375 blastocysts freeze/thaw cycles vitrified on day 5, warmed and transferred after 20–22 h in the culture, between January 2012 and December 2016. The study was approved by the Research Ethics Board at Mount Sinai Hospital in Toronto. Only blastocysts vitrified on day 5 were included in the study, and only cycles with single blastocyst transfer were included in the study. Embryo post PGS were not included in the study.

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Frozen cycle endometrial preparation

The frozen blastocysts were thawed and transferred during natural cycles or hormonally substituted frozen embryo transfer (FET) cycles.

Artificial hormone replacement Patients started on days 2–3 of the cycle with oral administration of 2 mg of estradiol (Estrace) twice daily for endometrial preparation, which was increased by a step-up protocol to 8 mg/d. An ultrasound endometrial assessment performed about 10 days later assessed the lining as ready for the ET procedure when the endometrial thickness was ≥ 6.5 mm. If not adequate, endometrial estrogen priming continued and ultrasound assessment was undertaken to confirm further endometrial thickening. Participants commenced luteal support via vaginal administration of progesterone suppositories 200 mg three times daily according to the proposed day of blastocyst thawing and transfer. The blastocysts were thawed on day 5 of progesterone and transferred on day 6 of progesterone.

Natural cycles Following spontaneous menstruation, patients were monitored by serial ultrasound for endometrial thickness, follicular development, LH, and progesterone levels, until a rise in LH level was observed (LH level exceeds 180% of the baseline value), corresponding to a day prior OPU/ovulation. On the following day, progesterone suppositories 200 mg three times daily were started. The blastocysts were thawed on day 5 of progesterone and transferred on day 6 of progesterone.

Thawing process

The blastocyst was thawed using the embryo warming kit—Irvine scientific Vit kit®-Thaw (catalog no. 90137-SO, Irvine sci. Santa Ana, CA, USA). The warming solutions consisted of Thaw solution (TS), Dilution solution (DS), and Wash solution (WS). The patient specimen to be thawed was removed from dewar and placed into a LN2 thermos by the. The straw was left immersed in liquid nitrogen until solutions and tools were ready. High-security straws (HSV) were used for vitrification. The HSV was clipped and the inner trough with blastocyst could be removed and quickly immersed into 37° TS. The blastocyst was found and left in 37° TS for 1 min. The blastocyst was then transferred to a 50 μ l drop of DS for 4 min and two subsequent drops (50 μ l) of WS for 4 min each. The blastocyst was then transferred to equilibrated global media (supplemented with 20% LGPS) and was cultured for 20–22 h in the medium. The blastocyst was graded directly after it was thawed and again directly before the transfer.

Blastocyst grading

All blastocyst were evaluated by an experienced embryologist using the grading system proposed by Gardner [9]. Each blastocyst was also given a grade according to the simplified SART (Society for Assisted Reproductive Technology) grading system which was proposed by Heitmann et al. [10]. SART grade good was assigned for inner cell mass (ICM) grade A and trophectoderm (TE) grade A or B (AA or AB blastocysts). SART grade fair was assigned for ICM grade B and TE grade A, B, or C (BB, BC, BA, and AC blastocysts). SART grade poor was assigned for any ICM grade C (CC or CB blastocysts).

Outcomes

Clinical pregnancy was defined as visualization of a gestational sac, while ongoing pregnancy necessitated the visualization of fetal cardiac activity on transvaginal ultrasound.

Statistics

Comparison of continuous variables between the two groups was conducted using student *t* test and Mann–Whitney test. Chi-square test and ANOVA were used for comparison of categorical variables. Logistic regression analysis was employed for multivariate analysis. Variables used in the regression model included maternal age, number of eggs retrieved, number of zygotes, and number of blastocysts during the fresh ovum retrieval cycle. Significance was accepted at $p \leq 0.05$. Statistical analyses were conducted using the IBM Statistical Package for the Social Sciences (IBM SPSS v.20; IBM Corporation Inc., Armonk, NY, USA).

Results

A total of 375 frozen, autologous cycles were included in the analysis. Two hundred twenty-seven blastocysts graded as good, 88 graded as fair, and 60 graded as poor blastocysts were transferred (Table 1). Only the first FET cycle for every patients was included in the study. The age, number of eggs retrieved, number of MII and zygotes, and total number of blastocysts developed during the fresh cycle were comparable between the three groups. The clinical pregnancy rate (50.2% vs. 19.3%, $p < 0.01$) and the ongoing pregnancy rate (38.8% vs. 13.6% vs. $p < 0.01$) were higher in cases of good blastocyst quality compared with fair-quality blastocysts, and higher in the fair-quality blastocyst group compared with the poor-quality blastocysts (19.3% vs. 3.3% $p < 0.01$) (Table 1).

Table 1 Comparison between cycles with progression to good-quality, fair-quality, and poor-quality embryos

	Good prognosis	Fair prognosis	Poor prognosis	<i>p</i>
<i>N</i>	227	88	60	
Age (years) (mean ± sd)	35 ± 3.8	35.7 ± 3.7	35.4 ± 3.5	
Number of eggs retrieved (mean ± sd)	15.9 ± 7.7	14.8 ± 7.9	16.4 ± 8.5	
2PN (mean ± sd)	10.4 ± 5.7	9.8 ± 5.9	10.8 ± 5.5	
Total number of blastocysts developed during the fresh cycle (mean ± sd)	5.5 ± 3.5	5.1 ± 3.1	5.1 ± 3.2	
Clinical pregnancy rate	114/227 (50.2%)	17/88 (19.3%)	2/60 (3.3%)	< 0.01
Ongoing pregnancy rate	88/227 (38.8%)	12/88 (13.6%)	1/60 (1.7%)	< 0.01

NS not significant

We performed a multivariate regression analysis adjusting for number of eggs, MII, zygotes, total number of blastocysts during the fresh cycle, and the quality of the blastocyst during the transfer, and found the quality of the blastocyst (OR 3.7, CI 2.1–6.6 $p < 0.001$) to be associated with increased ongoing pregnancy rate.

There were 227 blastocysts graded as good quality prior to the transfer. In this group, 48/227 (21%) were graded as fair-quality blastocysts on day 5 and developed to good-quality blastocysts prior to the transfer, and 179/227 (79%) were good-quality blastocysts both on day 5 and day 6. In the group of good-quality blastocysts, 147/227 (65%) continued to develop to a better grade during the 20–22 h (for example 3AB–4AA, 3AA–4AA). When comparing the pregnancy rate in the group of good-quality blastocysts that developed further during the culture with the group of good-quality blastocysts that did not change in the culture, both the clinical pregnancy rate (51.7% vs. 46.2% $p = \text{NS}$) and the ongoing pregnancy rate (38.7% vs. 37.5% $p = \text{NS}$) were comparable (Table 2).

We performed a multivariate regression analysis adjusting for number of eggs, MII, zygotes, total number of blastocysts during the fresh cycle and the development of the blastocyst during the 20–22 h. We did not find the development of the blastocyst to have any statistically significant impact on the clinical or ongoing pregnancy rate.

There were 88 blastocysts graded as fair quality prior to the transfer. 54/88 (61.3%) were either poor or fair at the thaw before the culture. We found that any blastocyst that developed to a better grade during the culture resulted in a higher pregnancy rate (25.4% vs 9% $p = 0.05$). The ongoing pregnancy rate was not significantly different (18.1% vs. 6% $p = \text{NS}$) (Table 3).

We performed a multivariate regression analysis adjusting for number of eggs, MII, zygotes, total number of blastocysts during the fresh cycle, and the development of the blastocyst during the 20–22 h. We found, both the clinical pregnancy rate (OR = 6.6, CI 1.4–3.3, $p = 0.02$) and the ongoing pregnancy rate (OR = 7.5, CI 1.3–5.0, $p = 0.04$), to be associated with significantly higher in the group in which the blastocysts developed to a better grade compared to non-developing blastocysts.

There were 60 blastocysts graded as poor quality prior to the transfer. Thirty one of them were good quality at the thaw and became poor over the course of 20–22 h of culture. Twenty-three blastocysts were thawed as fair quality and became poor quality after the 20–22 h. Six of them were thawed as poor quality, and still were of poor quality after 20–22 h. In this group, there were only two clinical pregnancies and one ongoing pregnancy. Fourteen blastocysts were vitrified as poor-quality blastocysts. Eight blastocysts developed to fair-quality blastocysts over the course of 20–22 h of culture, and six blastocysts improved their grading but remained poor-quality blastocysts. The clinical pregnancy rate in this group was 14.3% (2/14) but the ongoing pregnancy rate was 0/14.

Discussion

To our knowledge, this is the first report in the literature of this freeze-thaw-culture protocol. Because the blastocyst has 20–22 h to develop in the culture, we have more information regarding the blastocyst prior the transfer and this information can assist us in predicting the pregnancy rate. We have an elective single blastocyst transfer policy in our clinic but in

Table 2 Comparing the pregnancy outcome of good-quality embryos that developed in the culture with good-quality embryos that did not develop in the culture

	Good-quality embryos that developed in the culture	Good-quality embryos that did not develop in the culture	<i>p</i>
<i>N</i>	147	80	–
Clinical pregnancy rate	76/147 (51.7%)	37/80 (46.2%)	NS
Ongoing pregnancy rate	57/147 (38.7%)	30/80 (37.5%)	NS

NS not significant

Table 3 Comparing the pregnancy outcome of fair-quality embryos that developed in the culture with embryos that did not develop in the culture

	Fair-quality embryo that developed in the culture	Fair-quality embryo that did not develop in the culture	<i>p</i>
<i>N</i>	55	33	—
Clinical pregnancy rate	14/55 (25.4%)	3/33 (9%)	0.05
Ongoing pregnancy rate	10/55 (18.1%)	2/33 (6%)	NS

NS not significant

cases of patients with poor prognosis (advanced age or recurrent implantation failure), we do transfer two blastocysts in order to increase the pregnancy rate per cycle. The results of this study could have an influence on the decision regarding the number of blastocysts that should be transferred.

As shown by Elgindy et al. [11] in fresh cycles, the pregnancy outcome is the same whether a day 5 expanded blastocyst is transferred on day 5 or day 6 and therefore we reasoned that the pregnancy rate would not change whether we transferred the blastocyst 2–4 h after the thawing, or if we let the blastocyst grow in the culture for another day after warming. We believe that the additional information gained from this protocol is very important.

We found that transferring a good-quality blastocyst resulted in higher clinical and ongoing pregnancy rates compared to cycles in which fair and poor-quality blastocysts were transferred. These results are similar to data published previously by Heitmann et al. [10] and by Irani et al. [12]. In our cohort, 21% of the transferred good-quality blastocysts were vitrified as fair-quality blastocysts and developed to become a good-quality blastocyst after the 20–22 h in the culture. These 20–22 h in culture distinguished between vitrified fair-quality blastocyst that remained the same grade after culture, and fair-quality blastocysts that developed to good-quality blastocysts. This difference was reflected in the ongoing pregnancy rate. Thus, the decision regarding the number of blastocysts to transfer can be made with more data and may assist in improving the IVF success rate without increasing the risk of twin pregnancies.

In the transferred fair-quality blastocyst group, we found a significant increase in the ongoing pregnancy rate if the blastocyst developed during the post-thawing culture (OR = 7.5, CI 1.3–5.0, $p = 0.04$) compared to the cycles without blastocyst development. The ongoing pregnancy rate was extremely low in the non-developing fair-quality group (6%), and therefore if after 20–22 h there is an arrested fair-quality blastocyst, it may be beneficial to thaw another blastocyst (if available) and transfer both immediately.

There were some blastocysts in the studied cohort that arrested, or degenerated. It has been suggested that blastocysts that fail to develop in vitro would have better outcome in vivo as the uterine cavity might be a better “incubator”. If that hypothesis were true, the cumulative pregnancy rate would have been better in cleavage stage transfers compared to

transfers at the blastocyst stage, and as previously published, there is no evidence of a difference in cumulative pregnancy rates between day 3 vs. day 5 blastocyst transfers [13].

There are a few limitations in our study. The main limitation is the retrospective study design and although the results of this study are promising, further prospective studies will be needed to confirm the findings. There are many other factors that may influence the pregnancy rate including difficulty performing the transfer, endometrial thickness and pattern, and subendometrial contractions to name a few. Those factors were not controlled for in the study.

In conclusion, the new warming protocol with culture for 20–22 h appears to have value in predicting the pregnancy rate. The development of fair blastocysts in culture has a high positive impact on pregnancy rate and this factor should be taken into consideration before deciding how many blastocysts to transfer.

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