

Research Article

The Impact of Age on Gestation and Implantation Rates and on Blastocyst Scoring, after Single and Double Embryo Transfers

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Abstract

Successful single embryo transfer (SET) cycles depend primarily on the choice of the best embryo to be transferred. Recent study on single blastocyst transfers has shown that trophectoderm (TE) quality is the most important parameter for successful pregnancy and live birth. Patient's age however, is a key component of a woman's reproductive potential. The present retrospective study aimed to analyze first the effect of maternal age on clinical gestation and implantation rates after single and double blastocyst transfers. Second, patients were divided into two age groups (<35 and >35 years old) and their blastocyst scores were recorded after single or double (DET) transfers resulting in single or twin pregnancy or non-pregnancy.

Our data clearly shows that for young women (<35 years of age) the transfer of a single blastocyst results in similar gestational rates as DETs, without the risk of twin pregnancies. In addition, our data show that for both young and older women TE score is the most important parameter to be assessed for embryo selection. In addition, inner cell mass (ICM) plays an important role in blastocyst selection in older (>35 years of age) patients. We suggest that blastocyst grading for patients aged 35 years or above shall be performed using a strict grading policy, possibly not of a single parameter, but TE, ICM and expansion grades together to choose the "best combined-score blastocyst" and DETs should be considered, particularly after previous cycles with pregnancy failures.

Keywords: Blastocyst score; SET; DET; Maternal age

Introduction

Despite the risks of multiple implantation and gestation, most IVF clinics around the world do not perform SET for fear of dropping their implantation and pregnancy rates. Successful cycles of SET represent the gold standard of an ART Institution. Success on a SET cycle depends primarily on the choice of the best embryo to be transferred. Since the advent of embryo culture to the blastocyst stage, "natural" *in vitro* selection takes place, as not all cleavage stage embryos reach the blastocyst stage and are naturally eliminated from the cohort of putative candidates for transfer. Although there is the possibility that a patient may have no embryo for transfer, embryos that reach the blastocyst stage on day-5 or -6, have higher chances of implantation and pregnancy after transfer [1]. On the other hand, it is not uncommon that patients have more than one or two blastocysts on day-5 or -6 for transfer or cryostorage. Again, a selection must take place to choose the embryo with the highest chances of implantation and gestation, most of the times based on morphological parameters. Possibly, the most widely used blastocyst scoring system is the that one proposed by Gardner & Schoolcraft [2], which takes into account three scores for each embryo: its inner cell mass quality, its trophectoderm quality and finally the blastocyst expansion / hatching (EH) status. Not surprisingly, high implantation and live birth rates were obtained when transferred blastocysts presented top grading for all three scores. Considering that the developing embryo,

gastrula and eventually fetus will develop from the inner cell mass, it was reasonable to consider that the inner cell mass score was the major criterion to be taken into account, when choosing one embryo from a group of blastocysts with similar grading for the three scores. A retrospective study on single blastocyst transfers, however have shown that trophectoderm quality is the single most important parameter for a successful pregnancy and live birth [3]. Subsequently, the same group of authors reported that similar blastocyst quality criterion should be used in frozen embryo transfers [4], where, in addition to the trophectoderm quality, the degree of blastocoel re-expansion post-cryopreservation also played an important role in the prediction of live birth.

One point that was not taken into account in those previous studies was the age of the patients, when blastocyst scores were evaluated [3,4]. In a previous work on early embryo development [5] we observed that the early cleavage (EC) phenomenon is dependent on maternal age. Early cleavage embryos, which presented the highest implantation and gestation rates, occurred more frequently in younger women. Other studies have already shown the effects of maternal age on hormonal treatment to induce follicular growth, ovulation induction, zygote and embryo quality, implantation and gestation rates [6-10]. The original work from Gardner et al. [1] did not find any statistical difference in age between the three groups of patients that presented one, two or none top grading blastocysts. However, the

Table 1: Clinical characteristics of fresh and cryopreserved SETs.

Age Group	SET									
	Fresh					Cryopreserved				
	N TE	N(+) βhCG(%)	NClin. gest(%)	Mmpl. Site(%)	NTwin Gest(%)	N TE	N(+) βhCG(%)	NClin gest(%)	Mmpl. Site(%)	NTwin Gest(%)
<35	7	3(43) ^a	3(43) ^a	3(43) ^a	-	14	9(64) ^a	7(50) ^a	7(50) ^a	-
35-37	-	-	-	-	-	14	3(21) ^b	2(14) ^b	2(14) ^b	-
38-39	-	-	-	-	-	4	-	-	-	-
40-42	-	-	-	-	-	2	-	-	-	-
Total	7	3(43)	3(43)	3(43)	-	34	12(35)	9(26)	9(26)	-

^aNo significant differences in outcomes between fresh or cryopreserved SETs

^bSignificant difference in outcomes between patient ages <35 and 35-37 years old (P<0.001)

overall mean age of the patients was quite low, around 33 years old. The number of patients older than 35 years of age grows continuously in Assisted Reproduction (AR) programs. Thus, it is important to assess the impact of age on blastocyst quality in this population of women, in order to best counsel them about the treatment and their pregnancy probabilities with extended embryo culture.

The aims of the present retrospective study were first to evaluate the effect of maternal age on chemical and clinical gestation and implantation rates after single or double blastocyst transfers. Second, the impact of the three blastocyst scores on gestation [2,8] was assessed on single and double embryo transfers of fresh and vitrified/rewarmed blastocysts taking into account maternal age.

Material and Methods

In this retrospective study, fresh and cryopreserved cycles of Assisted Reproduction using intracytoplasmic sperm injection (ICSI) were analyzed, in which patients received a single or two fresh or vitrified/rewarmed blastocyst(s), between 2012 and 2014. Only cycles in which blastocyst grading scores were clearly registered were included. Clinical gestation and implantation rates were analyzed for SET and DET, where patients were divided into four age groups (<35, 35-37, 38-39, 40-42 years old; [11]). To analyze the impact of ICM, TE and EH scores on clinical gestation rates from SET or DET cycles that resulted in single (SETs) or twin (DETs) pregnancies, or non-gestations, patients were divided in two groups: < 35 and >35 years of age.

Stimulation protocol and embryo transfer

Pituitary suppression was achieved using GnRh antagonist and ovarian stimulation was achieved using recombinant FSH or hMG. When at least one follicle reached 18mm in diameter, patients received a single dose of hCG. Oocyte collection was performed 36 hours after hCG administration and insemination was performed by ICSI. Embryo transfer was performed on day-5 or -6 if one or two good quality blastocysts were available. One or two embryos with the best score were transferred either day-5 or day-6 post-insemination. The study outcomes were positive serum βhCG test and the presence of gestational sac(s) by ultrasound (US), two to three weeks after a positive βhCG test.

Embryo culture and blastocyst scoring

Embryos were cultured from the pronuclear to the blastocyst stage in Global® medium supplemented with 20% SSS. On the morning of day-5 or -6 of culture, the percentage of blastocysts was

recorded. Blastocysts from each patient were photographed prior to transfer. For the vitrified/rewarmed embryos, pictures were taken on the day of rewarming and transfer. For grading, blastocysts were classified using Gardner & Schoolcraft [2,8] scoring system. By using this scoring method, embryos received a score from 1 to 6 according to their EH status, being that grade “6” related to hatching blastocysts. ICM and TE were given scores A, B or C, being that scores A and B corresponded to the best organized ICMs and trophoctoderm cells forming a continuous epithelium. Grade C related to very small or scattered ICM cells and few and large trophoctodermal cells. In order to make a correlation between maternal age, clinical gestation and blastocyst scoring, only SETs and DETs in which 2 or 0 gestational sacs were detected by US were considered for analysis.

Cryopreservation

After transfer, surplus embryos that reached the blastocyst stage at day-5 or -6 were vitrified with a cryoloop [12] using Cryotech Vitrification Kit, and rewarmed according to the “Cryo top” technique.

Outcome measures and Statistical Analysis

Chemical (β-hCG) and clinical pregnancy (CPR) and implantation (IR) rates were tabulated and compared for fresh and cryopreserved transfers for patients in SET and DET groups. Blastocyst scores were assessed on SET and DET in pregnant and non-pregnant women, divided in 2 age groups: <35 and >35 years old. Clinical pregnancy was defined as the presence of one (or two) gestational sac in the uterus.

Differences between groups were assessed by two-tailed Fisher exact-test. A difference of p<0.05 was considered statistically significant.

Results

A total of 35 fresh and 129 cryopreserved single and double blastocyst transfers were retrospectively analyzed.

For two cycles of cryopreserved embryo transfers, no gestational sac US data were registered, so they were excluded from the implantation rate and blastocyst score analysis.

Chemical and clinical gestation and implantation rates

Results show that patients aged <35 years old presented similar chemical, clinical and implantation rates for fresh or cryopreserved, single or double blastocyst transfers (Tables 1 and 2).

Table 2: Clinical characteristics of fresh and cryopreserved DETs.

Age Group	DET									
	Fresh					Cryopreserved				
	N TE(%)	N(+) βhCG(%)	NClin. gest(%)	Mmpl. Site(%)	NTwin Gest(%)	N TE	N(+) βhCG(%)	NClin. gest(%)	Mmpl. Site(%)	NTwin Gest(%)
<35	22	14(64) ^a	14(64) ^a	17(39) ^a	3(21) ^a	49	29(59) ^b	28(58) ^{*b}	37(39) ^b	7(25) ^b
35-37	5	4(80) ^a	3(60) ^a	4(40) ^a	1(33) ^a	25	17(68) ^b	16(64) ^b	17(34) ^b	2(13) ^b
38-39	1	1(100)	-	-	-	11	8(73) ^b	5(50) ^{*b}	7(35) ^b	2(40) ^b
40-42	-	-	-	-	-	10	5(50) ^b	4(40) ^b	4(20) ^b	-
Total	28	19(68)	17(61)	21(38)	4(24)	95	59(62)	53(57)	65(35)	11(21)

^cOne clinical gestation record not found, thus data not included.

^aNo significant differences in outcomes between age groups <35 and 35-37 Fresh DETs

^bNo significant differences in outcomes between different age groups of Cryopreserved DETs

No fresh SET was performed for patients older than 35 years of age.

For patients aged 35-37 years, the transfer of a single cryopreserved embryo resulted in significantly lower chemical, clinical and implantation rates compared with the transfer of two cryopreserved embryos ($P < 0.001$).

Patients aged 38-39 and 40-42 years old, cryopreserved SETs did not result in any gestation.

Double blastocyst transfers resulted in no significant differences in chemical, clinical and implantation rates for fresh and cryopreserved cycles, between patients aged <35 compared with 35-37, 38-39 and 40-42 years old (Table 2).

For patients aged 38-39 and 40-42 years old, cryopreserved DETs resulted in chemical (73% and 50%, respectively), clinical (50% and 40%, respectively) gestations with fairly good implantation (35% and 20%, respectively) rates.

Twin gestations

No twin gestation was detected for patients in fresh or cryopreserved SETs (Table 1).

Fresh and cryopreserved DETs resulted in high twin rates. No significant differences were observed between groups regarding twin rates (Table 2).

Blastocyst scoring

For the analysis of the putative effect of maternal age on blastocyst scores and pregnancy rates, blastocysts from pregnant and non-pregnant patients that had SET or DET (with 2 or 0 gestational sacs) were analyzed and grouped into two groups: <35 or >35 years of age (Tables 3 and 4).

In fresh cycles, blastocyst grading did not show a significant difference in distribution of EH, ICM and TR scores between pregnant and non-pregnant women for both age groups (Table 3).

Interesting to notice, is the fact that embryos presenting the lower expansion scores (1 or 2) showed a trend to higher frequencies in non-pregnant patients (Tables 3 and 4). Among patients aged <35 years old comparison between pregnant and non-pregnant women in cryopreserved cycles showed a significant difference in the distribution of expansion and trophectoderm scores, but not of ICM (Table 4). In contrast, in the older group of patients, significant differences

Table 3: Blastocyst characteristics in SET and DET fresh cycles, considering two age groups of patients.

Fresh embryos				
Age	<35		>35	
	Preg(%)	Non-preg(%)	Preg(%)	Non-preg(%)
EH				
1	-	1/8(12,5)	-	-
2	2/9 (22)	3/8(37,5)	-	-
3	5/9(56)	4/8(50)	1/2(50)	1/1(100)
4	1/9(11)	-	1/2(50)	-
5	-	-	-	-
6	1/9(11)	-	-	-
		P>0.99	P>0.99	
ICM				
A	1/9(11)	-	-	1/1(100)
B	5/9(56)	4/8(50)	2/2(100)	-
C	3/9(33)	4/8(50)	-	-
		P>0.99	P=0.33	
TR				
A	-	2/8(25)	1/2(50)	1/1(100)
B	8/9(89)	5/8(62,5)	-	-
C	1/9(11)	1/8(12,5)	1/2(50)	-
		P=0.44	P>0.99	
Total	9	8	2	1

in the distribution of ICM and trophectoderm scores, but not of expansion score were observed between pregnant and non-pregnant women. Trophectoderm scores presented a high significance for both age groups. The majority of blastocysts that resulted in pregnancy received TE score "A" or "B", whereas the majority of the embryos transferred to patients that did not achieve a gestation received TE score "C" (Table 4).

Discussion

Our results clearly show that for women aged <35 years the transfer of a single fresh or cryopreserved blastocyst results in pregnancy and implantation rates similar to the transfer of two. This is a very reassuring result that should be taken into consideration,

Table 4: Blastocyst characteristics in SET and DET vitrified/rewarmed cycles, considering two age groups of patients.

Cryopreserved cycles				
Age	<35		>35	
Bl.Score	Preg (%)	Non-preg (%)	Preg (%)	Non-preg (%)
EH				
1	-	10/47(21)	-	5/58(9)
2	2/23 (9)	6/47(13)	1/10(10)	12/58(21)
3	12/23(52)	27/47(57)	4/10(40)	32/58(55)
4	6/23(26)	1/47(2)	3/10(30)	6/58(10)
5	2/23(9)	2/47(5)	1/10(10)	2/58(3)
6	1/23(4)	1/47(2)	1/10(10)	1/58(2)
	P=0.0037		P=0.143	
ICM				
A	2/23(8,8)	1/47(2)	2/10(20)	1/58(2)
B	13/23(57)	21/47(45)	8/10(80)	33/58(57)
C	8/23(35)	25/47(53)	-	24/58(41)
	P=0.164		P=0.041	
TR				
A	4/23(17,5)	3/47(6)	6/10(60)	4/48(7)
B	15/23(65)	14/47(30)	3/10(30)	22/58(38)
C	4/23(17,5)	30/47(64)	1/10(10)	32/58(55)
	P=0.007		P<0.001	
Total	23	47	10	58

when performing AR Technologies in this group of patients, to avoid multiple gestations and their undesired consequences. Similar results have already been published for this age group [13-15]. The present study divided patients into four age groups as recent reports have been using this system when describing women's fertility status [11]. In contrast to the data reported by Wen et al. [16], SET of cryopreserved blastocysts for patients aged 35-37 years old resulted in a significantly lower chemical and clinical gestation and implantation rates compared with the younger group of patients (<35 years of age). The reason for this discrepancy may be the fact that the authors grouped young women (<35 years) together with patients aged 37-38 years old enhancing the average pregnancy and implantation rates, in comparison with the group of patients aged >38years.

Another study, on a selected group of patients younger than 35 years showed that SET together with aCGH analysis results in a clinical implantation rate of 70.9% compared with 45.8%, when blastocysts were selected based on morphology only [14]. In our transfers, the best embryos were selected based only on morphology scores as stated by Gardner & Schoolcraft [2,8] and our results were very close (43%) to those reported by Yang et al., [14] for the same age group that did not have blastocyst aCGH analysis. It is possible that, nearly half of the blastocysts transferred to our patients carried undetectable aneuploidies, an inherent imprecision of embryo selection based on morphological parameters only.

In addition, data from the present study demonstrate that for the older age groups, 35-37, 38-39 or 40-42 years of age, no significant decrease in chemical and clinical gestation and implantation rates

were observed after cryopreserved DETs compared with the younger group (<35 years old). One reason for this may be the fact that embryo quality seems to be directly related to aneuploidy rates, as shown in the study of Capalbo et al. [17]. In their study, the authors demonstrated that implantation potential of euploid embryos is similar in patients aged 38-39 and 35 to 37 years old, despite a significant higher rate of aneuploidy in the older group of patients. We did not perform genetic screening of the transferred blastocysts and the selection for transfer was based on morphology scores only. Thus, from our analysis it is possible to suggest that blastocysts selected for transfer based on morphology parameters in the group of patients aged 40-42 years, were euploid embryos with a similar implantation potential to blastocysts from younger patients. Based on these observations, it is important to emphasize the fact that older patients that produce good quality blastocysts, these embryos may have the same implantation and clinical gestation potential, as good quality blastocysts from younger women.

Ideally, AR cycles should perform a genetic screening before SET, regardless whether fresh or cryopreserved embryos are being transferred, for all patients to further increase their implantation and clinical gestation chances. However, genome amplification techniques are expensive and not available on site for the majority of the IVF Institutions. Thus the use of strong morphology parameters for embryo grading still has its place on embryo selection prior to transfer.

The relevance of each of the three blastocyst scores for embryo selection has been a matter of discussion in literature. A recent publication using simple logistic analysis [18] found that, contrary to strong evidences [3,19,20] showing that the most important parameter when choosing a blastocyst for transfer is its trophoctoderm score, blastocyst expansion state is the most powerful marker of successful implantation and gestation to term. Our data partially agree with the last authors in the sense that, a significant difference was observed in the distribution of expansion scores between pregnant and non-pregnant patients in the <35 age group that received cryopreserved embryos. In this particular group of patients, a higher proportion of blastocysts presented expansion/hatching scores 4, 5 and 6 (more expanded or hatching blastocysts), when compared to blastocysts transferred to patients that did not achieve a pregnancy. However, as mentioned above, it has been credited that among the three blastocyst scores, trophoctoderm is the most important parameter to be taken into account when performing blastocyst transfers. TE grading, but not ICM or EH grading, significantly correlated with implantation and live birth for single-blastocyst transfers [3,19,20]. The present analysis also showed a trend towards TE grade A or B in patients that reached a pregnancy and significant differences in TE scores were observed in cryopreserved cycles, between pregnant and non-pregnant women for both groups of age. ICM grading show a significant trend towards score "A" and "B" (more compacted and organized ICM cells) in the group of pregnant women aged >35 years, that received two cryopreserved blastocysts. It is an interesting observation, because the same effect was not observed among the blastocysts transferred in the group of younger patients, as described in previous studies most of which did not take into account patient's age. Considering that we did not notice a decline in the implantation rates among older women when compared with patients <35 years of

age, together these observations may suggest that a good quality ICM plays a more important role in establishing a successful gestation in this group of patients (>35 years of age) because their overall embryo quality is diminished, as oocyte quality declines with aging. Thus, cells that form the ICM are intrinsically less viable in these blastocysts and their number, morphology and aggregation are important factors to promote subsequent gastrulation and fetal development, once implantation has taken place. On the contrary, ICM cells derived from a fertilized oocyte retrieved from a young patient (<35 years of age) may intrinsically be more robust and capable of further development into successful implantation and fetal life, even if they are present in a scattered pattern and small numbers (lower ICM scores). However, as is the case for older patients, blastocysts generated from young patients presenting low or high ICM scores have to show a good quality TE, to ensure first of all a successful implantation.

No twin gestation in fresh or cryopreserved SETs was detected, emphasizing the safety of single transfers to avoid multiple gestations. On the other hand, DETs yielded twin gestation rates between 13% and 40% for all age groups below 40, fresh and cryopreserved blastocyst transfers. Wen et al. [16] reported no multiple pregnancies in SETs and a rate between 19.5 and 13.4% for fresh and frozen DETs, respectively. These numbers emphasize the need to avoid DETs in young patients and our data goes further to show that in patients aged 38-39 years, there is still a very high chance (40%) of twinning after the transfer of two cryopreserved blastocysts. In agreement with our data, no multiple births were observed after elective SET, compared with 35% after elective DET in the study by Prados et al. [21]. Thus, blastocyst DETs should be performed only in particular instances, as in cases of previous gestation failures following single good quality blastocyst transfer and for women aged above 40 years. One interesting aspect to point out from the last report [21] is the fact that despite the increased risks of gestational and neonatal problems, nearly half the patients refused elective SET even after having been well informed about its benefits.

In conclusion, regarding the effect of maternal age on blastocyst scores and transfer outcomes, we may say that for young (<35 years) or older (>35 years) patients the trophectoderm score is the most relevant parameter to be taken into account when performing blastocyst selection. Inner cell mass seems to be an important marker of blastocyst quality and pregnancy potential in older women (>35 years). This is a new finding not reported previously, as former authors did not segregate embryos according to patient's age group, including more advanced maternal age, when assessing the impact of each blastocyst score on their pregnancy and implantation potential.

As previously reported [5] early cleavage (EC) embryos, which result in a higher implantation and gestation rates, are significantly more frequent in younger patients, possibly because of the better quality of their oocytes. Thus, one suggestion for blastocyst transfer in SET programs involving young patients (<35 years old) would be to combine EC and TE/EH morphology parameters to enhance to a maximum implantation and gestation chances, in the absence of a genetic screening. For the older group of patients, in case no embryo shows EC, blastocyst selection shall be performed using a grading policy that combines all three parameters EH, ICM and TE to choose the "best combined-score blastocyst" and DETs should be considered

for this patient population, particularly after previous cycles with gestation failures.

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