The impact of age on blastocyst scoring after single and double embryo transfers

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ABSTRACT

Objective: The aim of the present study was to assess the putative relationship between patient's age and blastocyst scores, in single (SET) or double (DET) transfer cycles, that resulted in single or twin pregnancy or non-pregnancy. Second, we analyzed the effect of maternal age on clinical gestation and implantation rates after single and double blastocyst transfers.

Methods: Retrospective analysis of 164 assisted reproduction cycles with embryo transfers.

Results: Data demonstrated that for both, young (<35 years of age) and older (≥35 years of age) women, trophoctoderm (TE) score is the most important parameter to assess concerning embryo selection. However, inner cell mass (ICM) also plays an important role on blastocyst selection in the group of older patients. In addition, our data shows that for young women the transfer of a single blastocyst results in similar gestational rates as those for DET.

Conclusion: 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". DETs should be considered, particularly after previous cycles with pregnancy failures.

Keywords: Maternal age, blastocyst score, SET, DET.

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 SET cycles 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 in the laboratory. Considering that not all cleavage stage embryos reach the blastocyst stage, they 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 days 5 or 6, have higher likelihoods of implantation and pregnancy after transfer (Gardner *et al.*, 1999). On the other hand, it is not uncommon for patients to have more than one or two blastocysts on days 5 or 6 to transfer or to cryopreserve. Again, a selection must take place to choose the embryo with the highest likelihoods of implantation and gestation. Frequently, embryo selection is based on morphological parameters. Possibly, the most widely used blastocyst scoring system is the one proposed by Gardner & Schoolcraft (2000), which takes into account three

scores for each embryo: its ICM and TE quality and, finally, the blastocyst expansion/hatching (EH) status. Not surprisingly, better 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 ICM, it was reasonable to ponder 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. However, a recent study on single blastocyst transfer, showed that TE quality was the single most important parameter for a successful pregnancy and live birth (Ahlström *et al.*, 2011). Subsequently, the same group of authors reported that similar blastocyst quality criterion should be used in frozen embryo transfers (Ahlström *et al.*, 2013). In this case however, in addition to trophoctoderm quality, the degree of blastocoel re-expansion post-cryopreservation, also played an important role in the prediction of live births.

One point that was not taken into account in those studies was the age of the patients, when blastocyst scores were evaluated (Ahlström *et al.*, 2011; 2013). In a previous study on early embryo development (Bos-Mikich *et al.*, 2001) we observed that the occurrence of the early cleavage (EC) phenomenon depended on maternal age. Early cleavage embryos with 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 (Van Kooij *et al.*, 1996; Lass *et al.*, 1998; Schoolcraft *et al.*, 1999; Schoolcraft *et al.*, 2000; Wittermer *et al.*, 2000). The original study from Gardner *et al.* (1999) did not find any statistical difference in age between the three groups of patients that presented one, two or none top grade blastocysts. However, the overall mean age of the patients was quite young, around 33 years old. The number of patients older than 35 years grows continuously in Assisted Reproduction (AR) programs. Thus, it is important to assess the impact of maternal age on blastocyst quality in this population of women, in order to best advise them on their treatment and pregnancy probabilities with extended embryo culture.

The aim of the present study was to assess the impact of the three blastocyst scores (Gardner & Schoolcraft, 2000) on gestation rates, after single and double embryo transfers of fresh and vitrified blastocysts taking into account maternal age. The effect of maternal age on chemical and clinical gestation and implantation rates after single or double blastocyst transfers was also analyzed.

MATERIALS AND METHODS

In this retrospective study, fresh and cryopreserved cycles of Assisted Reproduction were analyzed, in which patients received a single or two blastocyst(s), between 2012 and 2014. Only cycles in which blastocyst grading scores were recorded clearly were included. Exclusion criteria included patient incomplete records, no gestational information or blastocyst images of poor quality. Clinical gestation and implantation rates were analyzed for SET and DET, the patients were divided into four age groups (<35, 35-37, 38-39, 40-42 years old; Morin *et al.*, 2014). To analyze the impact of ICM, TE and EH scores on clinical gestation rates for SET or DET cycles that resulted in single (SETs) or twin (DETs) pregnancies, or non-gestations, patients were divided into 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 intracytoplasmic sperm injection (ICSI). Embryo transfer was performed on days 5 or 6 if one or two good quality blastocysts were available. One or two embryos with the best score were transferred either on day 5 or day 6 post-insemination. The study outcomes were positive serum βhCG test and clinical pregnancy, which was defined as the presence of intrauterine gestational sac containing one embryo with a functioning heart.

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 number 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 (2000) scoring system. By using this scoring method, embryos received a score from 1 to 6 according to their EH status, with grade "6" related to hatching blastocysts. ICM and TE were given scores A, B or C, with scores A and B corresponding to the best organized ICMs and trophoblast cells forming a continuous epithelium. Grade C related to very small or scattered ICM cells and few and large trophoblastic cells. A single embryologist performed the blastocyst scores. 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 detected by US were considered for analysis.

Cryopreservation

After transfer, surplus embryos that reached the blastocyst stage at days 5 or 6 were vitrified by cryoloop (Mukaida *et al.*, 2001) using the Cryotech Vitrification Kit, and rewarmed according to the "Cryo top" technique.

Outcome measures and Statistical Analysis

Implantation (IR), Chemical (β-hCG) and clinical pregnancy (CPR) rates were tabulated and compared for fresh and cryopreserved transfers for patients in the 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.

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 was registered, so they were excluded from the implantation rate and blastocyst score analysis.

Chemical and clinical gestation and implantation rates

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

No fresh SET was performed for patients aged 35 years or older, because of clinic policy.

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 ($P < 0.001$).

For patients aged 38-39 and 40-42 years old, the 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, for patients aged <35 compared with patients aged 35-37, 38-39 and 40-42 years (Table 2).

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

Table 1: Clinical characteristics of fresh and cryopreserved SETs.

Age Group	SET									
	Fresh					Cryopreserved				
	N TR	N(+) βhCG(%)	N Clin. gest(%)	N Impl. Site(%)	N Twin Gest(%)	N TR	N(+) βhCG(%)	N Clin. gest(%)	N Impl. Site(%)	N Twin 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 patients aged <35 and 35-37 years old ($P < 0.001$)

NTR = number of embryo transfers.

Table 2: Clinical characteristics of fresh and cryopreserved DETs.

Age	DET									
Group	Fresh					Cryopreserved				
	N TR(%)	N(+) β hCG(%)	NClin. gest(%)	NImpl. Site(%)	NTwin Gest(%)	N TR	N(+) β hCG(%)	NClin. gest(%)	NImpl. 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)

*One 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.

NTR = number of embryo transfers.

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

Fresh embryos				
Age	<35		≥35	
Bl. Score	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)	-	-	-
	<i>P</i> >0.99		<i>P</i> >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)	-	-
	<i>P</i> >0.99		<i>P</i> =0.33	
TE				
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)	-
	<i>P</i> =0.44		<i>P</i> >0.99	
Total	9	8	2	1

Twin gestations

No twin gestation was detected for patients in fresh or cryopreserved SETs (Table 1). Fresh and cryopreserved DETs resulted in high twin-pregnancy rates. No significant differences were observed between groups regarding twin-pregnancy 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 TE scores

between pregnant and non-pregnant women for both age groups (Table 3).

Interesting to notice, was the fact that embryos presenting the lower expansion scores (1 or 2) showed a trend to higher frequencies in the group of non-pregnant patients (Tables 3 and 4). Among patients aged <35 years, the 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 group of older patients (≥35yrs), significant differences in the distribution of ICM and trophectoderm scores, but not in expansion score between pregnant and non-pregnant women. Trophectoderm scores were highly significant for both age groups. The majority of blastocysts that resulted in preg-

Table 4: Blastocyst characteristics in SET and DET vitrified/warmed 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	
TE				
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		-<0.001	
Total	23	47	10	58

nancy received TE scores "A" or "B", whereas the majority of the embryos transferred to patients that did not achieve a gestation received a score "C" TE (Table 4).

DISCUSSION

Our results 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, 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 (Koryntová *et al.*, 2005; Yang *et al.*, 2012; Chai *et al.*, 2014). The present study divided patients into four age groups as recent reports have been using this system when describing women's fertility status (Morin *et al.*, 2014). In contrast to the data reported by Wen *et al.* (2013), 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 average pregnancy and implantation rates, in comparison with the group of patients aged >38 years.

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 (Capalbo *et al.*, 2014). 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 the literature. A recent publication using simple logistic analysis (Van den Abbeel *et al.*, 2013) found that, contrary to strong evidences showing that the most important parameter, when choosing a blastocyst for transfer, is its trophectoderm score (Ahlström *et al.*, 2011; Hill *et al.*, 2013; Honnma *et al.*, 2012), blastocyst expansion is the most powerful marker of successful implantation and gestation to term. Our data partially agrees with the last authors. 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 pregnancy. However, as mentioned above, it has been credited that among the three blastocyst scores, trophectoderm 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 births for single-blastocyst transfers (Ahlström *et al.*, 2011; Hill *et al.*, 2013; Honnma *et al.*, 2012). The present analysis also showed a trend towards TE grades A or B, in patients that reached pregnancy. In addition, significant differences in TE scores were observed in cryopreserved cycles, between pregnant and non-pregnant women, for both age groups. However, ICM grading showed a significant trend towards scores "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 patients age. Considering that we did not notice a decline in the implantation rates among older women, our results may suggest that a good quality ICM plays a more important role in establishing a successful gestation in older patients (≥ 35 years of age), because their overall embryo quality is diminished, as oocyte quality declines with aging. Thus, cells from 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 a successful implantation.

Some limitations of the present study should be mentioned. First, embryo scoring is a subjective assessment, which may include inter-observer variations, when the same embryo is analyzed by different embryologists. Also, the number of patients in groups aged above 38 years is considerably smaller than in the younger groups, when pregnancy and implantation rates after SETs and DETs were analyzed. Last, the number of patients was considerably smaller in the older group in fresh cycles, when blastocyst scores were related to pregnancy or non-pregnancy. However, these limitations do not apply to the clear effect of inner cell mass quality in establishing pregnancy in women older than 35 years of age, in cryopreserved cycles.

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 years, fresh and cryopreserved blastocyst transfers. Wen *et al.*, (2013) 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.* (2014). 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 (Prados *et al.*, 2014) is the fact that despite the increased risks of gestational and neonatal problems associated with twin pregnancies, 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. In addition, 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, when assessing the impact of each blastocyst score on their pregnancy and implantation potential.

CONFLICT OF INTERESTS

No conflict of interest have been declared.

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