Quantitative and qualitative trophectoderm grading allows for prediction of live birth and gender

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Abstract

Purpose Prolonged in vitro culture is thought to affect pre- and postnatal development of the embryo. This prospective study was set up to determine whether quality/size of inner cell mass (ICM) (from which the fetus ultimately develops) and trophectoderm (TE) (from which the placenta ultimately develops) is reflected in birth and placental weight, healthy live-birth rate, and gender after fresh and frozen single blastocyst transfer.

Methods In 225 patients, qualitative scoring of blastocysts was done according to the criteria expansion, ICM, and TE appearance. In parallel, all three parameters were quantified semi-automatically.

Results TE quality and cell number were the only parameters that predicted treatment outcome. In detail, pregnancies that continued on to a live birth could be distinguished from those

pregnancies that aborted on the basis of TE grade and cell number. Male blastocysts had a 2.53 higher chance of showing TE of quality A compared to female ones. There was no correlation between the appearance of both cell lineages and birth or placental weight, respectively.

Conclusions The presented correlation of TE with outcome indicates that TE scoring could replace ICM scoring in terms of priority. This would automatically require a rethinking process in terms of blastocyst selection and cryopreservation strategy.

Keywords Blastocyst · Inner cell mass · Live-birth · Neonatal outcome · Trophectoderm

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Capsule Quantitative and qualitative trophectoderm grading was found to be associated with gender and allows for prediction of live birth in fresh and vitrified/warmed single blastocyst transfers.

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Introduction

With the development of optimized culture media [1, 2] and culture conditions [3, 4], the practice of culturing embryos for transfer until the blastocyst stage, rather than transferring them at the cleavage stage, has become more common in the field of assisted reproductive technology (ART). Known advantages of blastocyst culture include a better synchronization between developmental stage of the embryo and the (less hostile) intrauterine milieu [5, 6], as well as an accumulation of viable embryos in culture, due to the elimination of extensively mosaic embryos [7, 8].

Indeed, the Practice Committees of the American Society for Reproductive Medicine and the Society for Assisted Reproductive Technology [9] highlighted that “in good prognosis patients, blastocyst transfer results in increased live-birth rates compared to transfer of equal numbers of cleavage-stage embryos.”

Prolonged in vitro culture up to day 5 or 6, however, requires detailed blastocyst scoring systems in order to identify the blastocysts with the highest implantation potential. The most popular scoring system [10] takes three parameters into

consideration: grade of blastocyst expansion, as well as morphology of the inner cell mass (ICM) and the trophectoderm (TE), based on cell number and cohesion. This more qualitative approach has been refined by Santos Filho et al. [11] who implemented a semi-automatic quantitative grading system.

However, it should also be noted that several drawbacks have been reported for blastocyst culture, such as monozygotic twinning [12], preterm delivery [13], congenital malformations [14], or increased birth weight [15].

Particularly, the latter parameter has been investigated in great detail, and it was shown that in ART pregnancies derived from different blastocyst culture media, pre- [16] and postnatal growth [17, 18] is affected. Information as to whether this culture-dependent effect is already noticeable at the blastocyst stage is scarce. This led us to prospectively analyze whether quality/size of ICM and TE, as precursor cells of the proper embryo and placenta, is reflected in birth and placental weight, healthy live-birth rate, and gender after fresh and frozen single blastocyst transfer.

Material and methods

This prospective study comprises all fresh and vitrified/warmed single blastocyst transfers in an 18-month period provided that they fulfilled the following criteria:

- i. Expansion of the blastocyst should at least have reached full stage [10], thus guaranteeing the presence of an extensive blastocoel and distinct cell lineages.
- ii. Photo documentation should have been done by the principal investigator (TE) in order to avoid inter-observer variance [19].
- iii. Only photos that showed ICM and TE in one focal plane were included in order to allow for proper analysis.
- iv. Female age <40 years.

Applying these inclusion criteria, a total of 254 single blastocysts transfers could be evaluated. Fresh cycles were only included once; however, 29 patients (11.4 %) had their subsequent vitrified/warmed cycle within the study period. This resulted in an overall patient number of 225. Vitrified/warmed and fresh cycles did not differ in parental age, BMI, birth order, or smoking behavior ($P>0.05$).

Patients were either stimulated with a GnRH-antagonist protocol (58 %) or an agonist one (42 %). In the first regimen, recombinant products (Elonva® or Puregon®, MSD, Vienna, Austria) were administered in approximately half of the cases ($n=68$), while urinary products (Menopur®, Ferring, Vienna, Austria) were used for the rest ($n=62$). The antagonist injected was Orgalutran® (MSD). In the long protocol, however, down regulation was performed with Buserelin (Suprecur®, Sanofi-Aventis, Vienna, Austria) and the gonadotrophins used were

Puregon® ($n=52$) or Menopur® ($n=43$). Ovulation induction was done with 5000–10,000 IU hCG (Pregnyl®, MSD). Oocyte retrieval was carried out transvaginally under ultrasound guidance 36 h after ovulation was induced. On average, stimulation took 10.8 ± 2.5 days ($\pm$ SD), and the estradiol level on the day of ovulation induction was found to be 1452 ± 1290 pg/ml.

Intracytoplasmic sperm injection (ICSI) ($n=200$) or IVF ($n=25$) was performed no later than 40 h post hCG. A swim-up procedure was used to accumulate spermatozoa for ICSI, while for IVF a Zech-selector (AssTIC Medizintechnik GmbH, Leutsch, Austria) was used, which accumulates sperms without additional centrifugation steps [20]. Fertilization check was carried out 16–20 h post insemination. On day 1, only 2-pronuclear zygotes were kept in culture. This was done in small groups using 30 μ l of EmbryoAssist medium (Origio, Berlin, Germany), as previously published [4]. On day 3 of in vitro culture, embryos were transferred to fresh 30 μ l droplets of BlastAssist medium (Origio). Embryos at cleavage were scored with regard to number and size of blastomeres, as well as presence of fragmentation and multinucleation, according to Alpha and ESHRE guidelines [21]. All cultures resulted in a day 5 (single) blastocyst transfer, of which some 70 % were elective.

Vitrification was done with a commercially available vitrification/warming kit (GM501 VitriStore, Gynemed, Lensahn, Germany), as previously published [22]. In short, ethylene glycol and DMSO were the cryoprotectants in the three-step vitrification procedure (timing: 1 min, 2 min, and 30 s). For the warming process, special care was taken to ensure very high warming rates. Warming occurred in four media based on PBS and HSA with descending (0.5, 0.25, 0.125, and 0 M) concentrations of sucrose (timing: 3, 2, 2, and 1 min). This was followed by direct transfer into pre-warmed medium (BlastAssist; Origio), which was then used for blastocyst transfer.

Since the philosophy and routine performance of our laboratory was to transfer blastocysts with the largest ICM from the very beginning of the implementation of blastocyst culture, no ethical approval was sought. In principle, the blastocyst with the largest ICM was chosen for single transfer out of a pool of given blastocysts. When two blastocysts showed identical ICMs, trophectoderm quality (priority 2) and expansion (priority 3) were taken into consideration. Like in other studies [23], this strategy limited the number of study blastocysts ($n=2$) that showed ICMs of class C [10], e.g., consisting of very few cells.

As a general rule, for vitrification, the quality of the ICM and TE should not be worse than B in order to optimize cryosurvival. The only exception was if a blastocyst was the only one available and the associated ICM was of quality A ($n=3$).

As a result of these implemented standards, we found that the analysis according to Gardner et al. [10] using grades A–C was less promising. We, therefore, aimed to identify additional quantifications of both ICM and TE. In detail, maximum blastocyst diameter (from outer zona to outer zona) was recorded using an imaging and archival software (OctaxEyeware®, MTG, Bruckberg, Germany). It should be highlighted that measuring the zona (instead of the proper blastocyst) was chosen in order to avoid ending up with distorted embryos. This was frequently observed in the presence of fragments and/or excluded blastomeres and in hatching blastocysts. In the rare case of blastocysts with ovoid zonae [24], the mean value between maximal and minimal extension was used ($n=2$).

Since the software used requires images and does not work in situ, the attempt was made to create photos with both ICM and TE in focus. Particular emphasis was placed on capturing an image, where the maximum dimension of the ICM was represented in cases of ovoid shapes. ICM size was quantified with two different techniques; according to Richter et al. [25], and semi-automatically. For the latter mode, ICMs were outlined in great detail (CorelDraw Graphics Suite X4) and the resulting shape (Fig. 1) was then cut out and transferred to a computer-aided design software (Solid Works, Dassault Systèmes Solid Works Corp; Munich, Germany) where the size of the ICM was calculated semi-automatically.

TE was measured quantitatively by counting the number of cells found in the representative cross-section that was associated with the ICM measuring [25]. It is important to note that the term “TE cell number” used in this manuscript, and by others [25], is based on the examination of one representative plane of the TE and does not reflect the overall TE cell number of the blastocyst. Since blastocysts beyond full stage (Gardner scores 4 and 5) were not completely recordable, due to a

Fig. 1 Full IVF blastocyst 3AA according to Gardner et al. [10]. Inner cell mass is highlighted and semi-automatic calculation showed a size of $3286 \mu\text{m}^2$. Trophectoderm cell count was 11. Live-birth of a healthy girl was achieved

discrepancy between the dimension of the blastocyst and the frame used by the Octax Eyeware, cells were counted under the microscope. Thus, the same mode of TE calculation was applied to all blastocysts, but in blastocysts grade 4 and 5, images could not be used as a control. As for ICMs, classical categorization according to the Gardner et al. [10] criteria was also done.

Quantification of warmed blastocysts was performed within 2 h after complete re-expansion had been documented [22]. This was necessary in order to optimize and standardize ICM measuring and TE cell counting.

Statistics

The data were analyzed using SPSS version 22 (SPSS, Inc., Chicago IL). Descriptive statistics gives mean $\pm$ SD. Birth and placental weights were corrected for week of gestation by non-linear regression. It should be noted that the response rate of the maternity hospitals was 100 % in terms of birth weight and 75 % in terms of placental weight. Consequently, enlargement of the placenta relative to the offspring (placental to birth weight ratio) could only be checked in three quarters of the study population.

Our primary outcome data was birth weight. Since the current strategy in in vitro fertilization is to favor the transfer of blastocysts with quality grade A (ICM and TE), we anticipated a strong asymmetry within the quality grades of blastocysts toward grade A. Therefore, we defined the effect of the quality of trophoctoderm cells on the birth weight as the primary outcome. Since the quality of trophoctoderm cells, as well as the number of the cells is so far not used as a selection parameter, we minimize the bias in our analysis. The corresponding power analysis revealed that by anticipating an allocation ratio of 2 between grade A and grade B/C TE cells, we would detect a medium effect size (0.55) with a sample size of at least 63 and 31 samples of grade A and grade B/C, respectively (power 80 %, level of significance 5 %).

Differences among the means of three groups (live birth, pregnancy loss, and no pregnancy) based on all normally distributed parameters were elucidated using a multivariate ANOVA. The overall level of significance was fixed at 5 %. The overall effect of treatment was investigated using Wilks' lambda. Since no positive overall group effects could be detected, follow-up separate univariate ANOVA and post hoc tests were not performed. The same applied to the subpopulation of live birth with respect to the groups “female” and “male.”

Median differences of non-normally distributed parameters across the three groups were analyzed using independent sample median tests. In the case of significant mean differences, we used binary logistic regression analysis to further explore the association between the parameter and live birth. Linear regression models were used to study the correlation between

the (corrected) birth and placental weights and ICM. The effect of vitrification on birth and placental weight were analyzed using a multivariate ANOVA. The overall effect of treatment was investigated using Wilks' lambda. Significant overall treatment effects were analyzed by separate one-way ANOVA. For univariate analysis of categorical variables, we used chi-square tests. Finally, we performed a one-factorial ANOVA to assess mean differences of ICM between the groups hatching, full, and expanded blastocysts.

Results

In 139 out of 254 single blastocyst transfers (54.7 %), a pregnancy (positive hCG) was achieved. No significant difference between fresh (90/162; 55.6 %) and vitrified/warmed transfer (49/92; 53.3 %) was seen. The same held true with respect to live-birth rate since fresh (66/162; 40.7 %) and vitrified/warmed cycles (33/92; 35.9 %) showed similar rates. Pooled live-birth rates of single blastocyst transfers, scored according to Gardner et al. [10], are highlighted in Table 1.

In total, 99 live births (39.0 %) were achieved, of which 3 were monozygotic twins (2 fresh and 1 vitrified/warmed transfer). Mean birth weight of singletons was 3314 ± 703 g, and they were delivered, on average, in gestation week 39 ± 2.5 . Detailed birth weights are presented in Fig. 2. The associated placental weight was 589 ± 136 g. The multivariate ANOVA used to study the effect of vitrification on birth and placental weight revealed an overall positive treatment effect ($P=0.013$). There was no difference in birth weight regarding the method of fertilization.

Malformations were observed in 4.6 % (3/66) of fresh (large foramen ovale, duodenum aplasia, cystic fibrosis) and 3 % (1/33) and 3 % of live births resulting from vitrified/warmed transfers (trisomy 18). It should be stressed that the couple with the baby suffering from cystic fibrosis was not aware of the fact that they both were carriers. In the case of trisomy 18, no prenatal testing was performed.

Table 1 Live-birth rates of single blastocyst transfers subgrouped according to Gardner et al. [10]

Expansion	ICM (A)		ICM (B)	
	TE (A)	TE (B)	TE (A)	TE (B)
3	8/16 (50.0)	5/26 (19.2)	1/2 (50.0)	1/3 (33.3)
4	25/57 (43.8)	19/43 (44.2)	2/3 (66.6)	1/9 (11.1)
5	30/65 (46.2)	7/22 (31.8)	na	0/1
Σ	63/138 (45.7)	31/91 (34.1)	3/5 (60.0)	2/13 (15.4)

Values in parentheses are percentages. Seven blastocysts with quality C ICM or TE were not included. ICM inner cell mass, TE trophoblast, na not applicable

Fig. 2 Presentation of birth weights and gestational week after fresh (circle) or vitrified/warmed (triangle) single blastocyst transfers

Trophoblast

Contrary to the present hypothesis, neither TE quality nor cell number showed an association with placental weight or placental to birth weight ratio.

However, trophoblast quality ($P=0.015$) and cell number ($P=0.026$) were the only parameters that were significantly related to rates of implantation and pregnancy. Mean of TE cell number related to pregnancies that went to live birth were significantly higher than the mean cell number related to the pregnancies that aborted (biochemical pregnancies, missed abortion with and without heart activity) ($P<0.001$). On the other hand, the mean difference in TE cell number between the groups “pregnancy that aborted” and “no clinical pregnancy” was not significantly different (Figs. 3 and 4).

Distribution of live birth differed significantly ($P=0.039$) for the two different trophoblast quality groups (A vs. B/C). Using a binary logistic regression model, the odds ratio for live birth based on the number of trophoblast cells was 1.175 ($P<0.001$, 95 % of confidence interval [1.08, 1.278]). However, more detailed analysis revealed that while the same odds ratio limited to the subpopulation with quality grade A is slightly higher (OR 1.183, $P=0.004$, 95 % of confidence interval [1.055, 1.326]), the result is at the edge of significance for the subpopulation with poorer quality grade (B and C, OR 1.128, $P=0.07$). The odds ratio estimate thus indicates that while a higher number of trophoblast cells is favorable for the prediction of live birth whenever the trophoblast quality grade is A, it might be of less importance in cases where the TE quality is grade B or C (Table 2).

Moreover, trophoblast quality according to Gardner et al. [10] and sex of the offspring (single blastocyst transfers) were found to be associated ($P=0.04$). Odds ratio analysis indicated that male blastocysts have 2.53 times the odds of showing a TE of grade A compared to female ones. Finally, the binary logistic regression model predicts that if the number of trophoblast cells exceeds ten, the probability of life birth is higher than 50 %.

Fig. 3 Comparison of semi-automatic ICM quantification (identity) and area calculation according to Richter et al. [25]

Inner cell mass

Similar to TE, ICM appearance had no predictive power on birth weight and placental to birth weight ratio, respectively. In addition, two-dimensional ICM size did not correlate with the events of implantation and pregnancy, regardless of whether the semi-automatic or the measuring according to Richter et al. [25] was used. In this respect, it could be

Fig. 4 Correlation between TE cell number counted in a representative cross-section and outcome of the single blastocyst transfers ($P<0.001$). In a Box-Whisker Plot, 50 % of values are within the box; 25 % are greater than and 25 % are less than the values within the box. The horizontal line inside the box represents the median value

demonstrated that both methods of ICM calculation correlated significantly with each other ($P<0.001$); however, the latter approach overestimated the size of the ICM (Fig. 3).

The average semi-automatically calculated ICM size was $3702 \mu\text{m}^2$ ($\pm 1216 \mu\text{m}^2$). No significant mean differences between fresh ($3655 \pm 1120 \mu\text{m}^2$) and vitrified/warmed ICMs ($3621 \pm 1286 \mu\text{m}^2$) were seen. The more surprising it was when post hoc univariate analysis showed that vitrified blastocysts led to a significantly higher birth ($P=0.012$) but not placental weight ($P=0.106$), when compared to the fresh counterparts (birth weights 3568 ± 744 vs. 3187 ± 691 g).

Last but not least, hatching blastocysts ($3380 \pm 1032 \mu\text{m}^2$) showed significantly smaller ICMs compared to expanded blastocysts ($3812 \pm 1309 \mu\text{m}^2$) ($P<0.05$) and full blastocysts ($4036 \pm 1180 \mu\text{m}^2$) ($P<0.01$).

Discussion

Human intrauterine growth in ART can be impaired as early as the second trimester of pregnancy [13]. In order to identify an even earlier correlation (day 5) of both cell lineages, ICM and TE, with birth and placental weight respectively, the attempt was made to determine a method for accurate quantification of these morphological blastocyst criteria.

Morphological assessment conducted in IVF labs is often prone to a certain inter- and intra-observer variance, as has been shown for day 3 embryos [19]. This unwanted scenario

Table 2 Quantitative grading of both cell lineages in correlation with classical morphology according to Gardner et al. [10]

	ICM (A)		ICM (B)	
	TE (A)	TE (B)	TE (A)	TE (B)
Expansion grade 3				
<i>n</i>	16	25	2	2
Diameter (μm)	176 $\pm$ 10	175 $\pm$ 7	174 $\pm$ 5	181 $\pm$ 5
ICM size (μm^2)	3643 $\pm$ 1142	4144 $\pm$ 1229	3416 $\pm$ 110	5105 $\pm$ 190
# TE cells	9 $\pm$ 1	8 $\pm$ 2	10 $\pm$ 3	7 $\pm$ 1
Expansion grade 4				
<i>n</i>	59	43	3	8
Diameter (μm)	193 $\pm$ 13	188 $\pm$ 14	210 $\pm$ 17	179 $\pm$ 16
ICM size (μm^2)	3955 $\pm$ 1316	3765 $\pm$ 1200	4233 $\pm$ 2283	3439 $\pm$ 1213
# TE cells	11 $\pm$ 3	10 $\pm$ 3	10 $\pm$ 4	9 $\pm$ 3
Expansion grade 5				
<i>n</i>	66	22	0	1
Diameter (μm)	198 $\pm$ 15	195 $\pm$ 13	na	180
ICM size (μm^2)	3400 $\pm$ 1111	3302 $\pm$ 790	na	3811
# TE cells	12 $\pm$ 3	12 $\pm$ 4	na	13

All values are mean $\pm$ SD. Seven blastocysts with quality C ICM or TE were not included. *ICM* inner cell mass, *TE* trophectoderm, *na* not applicable

also holds true for blastocysts; in particular, the gradual transition from full to expanded blastocyst causes some confusion. Similarly, both cell lineages are classified based on visual information from the embryologist, which could cause subjective interpretation of cell number and cohesion [11].

Unlike ICM, the TE is less characterized by its texture. Admittedly, the perfect method to quantify it would be using both TE thickness and regularity as parameters [11]. Unfortunately, however, such data is not available in routine blastocyst grading schemes. Luckily, a significant correlation between cell number in the TE and its regularity has been demonstrated [11]. Thus, the present approach, to use the number of cells in a cross-sectional circumference of the blastocyst [25] in order to quantify TE, is a feasible option.

The placenta is known to be essential for the maintenance of pregnancy and fetal growth. It has been found that placental weight is closely correlated to birth weight of spontaneously conceived infants [26]. In addition, first trimester placental three-dimensional volume measurements in non-ART pregnancies revealed that the presence of infants “large for gestational age” and “macrosome infants” is in part determined by early placental growth and development [27]. Disproportions between placental weight and birth weight may be more common in ART pregnancies [28].

In the present IVF and ICSI pregnancies, however, quality of trophectoderm, as the source of the placenta, did not allow for such a prediction of birth weight. Furthermore, trophectoderm cell number and classification according to Gardner et al. [10] showed no correlation to placental weight either. This is in contrast to recent findings from a Japanese

group, who reported a correlation between trophectoderm appearance and placental weight in their retrospective analysis of fresh and frozen single blastocyst transfers [29].

On the other hand, both parameters, trophectoderm quality [10] and estimated trophectoderm cell number, did allow for predicting live birth. With respect to this, trophectoderm cell number performed even better, particularly in grade A TEs cell number showed the highest predictive efficiency. One possible explanation why this correlation was not observed in grade B TEs is that in these cases, the TE cross-section that was associated with ICM measuring (and on which cell number calculation was based) was not representative for the whole blastocyst.

Interestingly, we also demonstrated for the first time that implantation and, more interestingly, the fate of an implanted embryo may be predicted on the basis of trophectoderm quality and cell number at blastocysts stage, since live births and early pregnancy losses (biochemical pregnancy, missed abortion) were distinguishable in this respect. So far, only inner cell mass grade was found to be associated with early pregnancy loss, in the time between the positive β -hCG test and confirmation of fetal heart activity [30].

Although this is the first evidence that trophectoderm has a distinct impact on the delivery rate once a patient achieves pregnancy, it is in full accordance with recent publications suggesting the superiority of trophectoderm over inner cell mass scoring in terms of live birth. In detail, Ahlström et al. [23, 31] emphasized the predictive strength of TE grade over ICM for selecting the best blastocyst for both fresh and frozen-thawed cycles. Others took the same stance when they

confirmed the above-mentioned Swedish results, regardless of whether the authors dealt with fresh [32, 33] or cryopreserved blastocysts [34].

Another first-time observation although based on a rather small sample size is the association between trophectoderm quality and the sex of the corresponding offspring. With respect to this, male blastocysts performed significantly better than female ones. This is likely to be the result of a faster cleavage rate in male embryos [35]. Indeed, it has been published that human female embryos exhibited a significantly higher glucose consumption than male ones [36], which could reflect the need for more energy in order to inactivate the second X-chromosome [37] at the expense of a slower mitotic rate. This is in line with observations from time-lapse video recording of sex-determined bovine embryos which indicate that in the presence of glucose (like in the present set up) male embryos cleave faster [38]. In human, gender was also found to be associated with blastocyst grading, male embryos developing at a significantly higher rate than female ones [39]. Such sex-related differences in physiology and kinetics are consistent with documented differences in gene expression [40, 41].

Alternatively, it is hypothesized that female blastocysts suffer from precocious X-chromosome inactivation together with an ICSI-induced decrease in number of trophectoderm cells [42]. This would mean that in cases in which blastocysts are selected according to trophectoderm grade or expansion, it could theoretically result in a shift of sex ratio toward more male babies. Those, and this will be the majority, who rely on inner cell mass size and morphology would not experience such a shift. Such a scenario would explain the divergent statements concerning the sex ratio after blastocyst transfer in literature [35, 43–45].

Richter et al. [25] were the first to move away from the rather simple ICM classification according to Gardner et al. [10] when they introduced a more quantitative grading system for ICMs. The authors calculated its area (in μm^2) by multiplying its longest length and the widest perpendicular width.

Present data shows that estimating the size of the ICM on the basis of a rectangle [25] slightly overestimates its extension. A step toward optimal ICM size calculation was seen in the publication from Santos Filho et al. [11]. These authors introduced a semi-automatic grading technique, which has been adapted by our group and guarantees a well-defined depiction of the ICM outline, allowing for proper calculation of the ICM area.

However, neither this ideal quantification of the ICM size [11] nor the calculation according to Richter et al. [25] allowed for predicting birth weight. This held true for both fresh and vitrified single blastocyst transfers. Interestingly, birth weights from fresh and vitrified blastocysts were found to be different, although their ICM and TE were comparable. The observed difference in birth weight has been postulated in literature before. In detail, a significantly improved neonatal

outcome was observed in vitrified/warmed blastocyst transfers with babies born on average 85 to 145 g heavier as compared to fresh blastocyst transfers [46, 47]. In addition, singletons born after transfer of vitrified blastocysts were at 340 14 % less risk of being born preterm compared with their “fresh” counterparts [48].

Two scenarios may explain the lack of correlation between inner cell mass size and birth weight. One explanation would be that not even the most accurate two-dimensional measurement reflects three-dimensional cell mass and its theoretical impact on neonatal outcome properly. A collateral flaw could be that the timing of ICM size evaluation was inappropriate since differences in the growth rate of both cell lineages have been reported. In detail, ICM cells double between days 5 and 6 (which would be after our ICM evaluation), while the number of TE cells remains virtually unchanged [49]. Thus, theoretically, day 6 ICM scoring would better reflect a possible correlation with fetal development.

With increasing expansion (and cell number), the ICM of a blastocyst gets smaller due to an increasing cohesion of inner mass cells as their number grows, as has been previously suggested by our group [50]. Indeed, present data confirms that hatching blastocysts have a significantly smaller ICM compared to full or expanded blastocysts. However, correction of the data set for the degree of blastocyst expansion did not reach the level of significance either, which could be due to the excessively low sample numbers in the three subgroups full, expanded, and hatching blastocysts (Gardner scores 3–5).

Interestingly, other authors [51] published results showing that embryos of higher ICM grade [10] exhibit a higher birth weight than lower grades (A>B but not A>C). This discrepancy may be either due to the retrospective nature of the above-mentioned paper, or the inter-observer classification differences.

To summarize, to the best of our knowledge, this is the first manuscript dealing with quantitative grading of day 5 blastocysts and its potential association with neonatal outcome. However, the prospective approach of the present study did not allow for patient numbers comparable to those from a retrospective pivotal study published recently [31], but patient cohort was within accepted ranges [51].

On the basis of present data, it can be said that neither qualitative nor quantitative day 5 grading allows for prediction of placental or birth weight (corrected for gestation week). However, the observed correlations of TE performance with sex, as well as the rates of implantation, pregnancy, and pregnancy loss indicate that sooner or later trophectoderm scoring will replace inner cell mass scoring in terms of priority. This would automatically demand a rethinking process in terms of blastocyst selection and cryopreservation strategy.

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References

1. Van Langendonck A, Demylle D, Wyns C, Nisolle M, Donnez J. Comparison of G1.2/G2.2 and Sydney IVF cleavage/blastocyst sequential media for the culture of human embryos: a prospective, randomized, comparative study. *Fertil Steril*. 2001;76:1023–31.
2. Perin PM, Maluf M, Nicolosi Foltran Januário DA, Nascimento Saldiva PH. Comparison of the efficacy of two commercially available media for culturing one-cell embryos in the in vitro fertilization mouse model. *Fertil Steril*. 2008;90:1503–10.
3. Meintjes M, Chantilis SJ, Douglas JD, Rodriguez AJ, Guerami AR, Bookout DM, et al. A controlled randomized trial evaluating the effect of lowered incubator oxygen tension on live births in a predominantly blastocyst transfer program. *Hum Reprod*. 2009;24:300–7.
4. Ebner T, Shebl O, Moser M, Mayer RB, Arzt W, Tews G. Groupculture of human zygotes is superior to individual culture in terms of blastulation, implantation and life birth. *Reprod Biomed Online*. 2010;21:762–8.
5. Gardner DK, Lane M, Calderon I, Leeton J. Environment of the preimplantation human embryo in vivo: metabolite analysis of oviduct and uterine fluids and metabolism of cumulus cells. *Fertil Steril*. 1996;65:349–53.
6. Fanchin R, Ayoubi JM, Righini C, Olivennes F, Schönauer LM, Frydman R. Uterine contractility decreases at the time of blastocyst transfers. *Hum Reprod*. 2001;16:1115–9.
7. Evsikov S, Verlinsky Y. Mosaicism in the inner cell mass of human blastocysts. *Hum Reprod*. 1998;13:151–5.
8. Magli MC, Jones GM, Gras L, Gianaroli L, Korman I, Trounson AO. Chromosome mosaicism in day 3 aneuploid embryos that develop to morphologically normal blastocysts in vitro. *Hum Reprod*. 2000;15:1781–6.
9. American Society for Reproductive Medicine. Blastocyst culture and transfer in clinical-assisted reproduction: a committee opinion. *Fertil Steril*. 2013;99:667–72.
10. Gardner DK, Lane M, Stevens J, Schlenker T, Schoolcraft WB. Blastocyst score affects implantation and pregnancy outcome: towards a single blastocyst transfer. *Fertil Steril*. 2000;73:1155–8.
11. Santos Filho E, Noble JA, Poli M, Griffiths T, Emerson G, Wells D. A method for semi-automatic grading of human blastocyst microscope images. *Hum Reprod*. 2012;27:2641–8.
12. Nakasuji T, Saito H, Araki R, Nakaza A, Nakashima A, Kuwahara A, et al. The incidence of monozygotic twinning in assisted reproductive technology: analysis based on results from the 2010 Japanese ART national registry. *J Assist Reprod Genet*. 2014;31:803–7.
13. Dar S, Librach CL, Gunby J, Bissonnette F, Cowan L, IVF Directors Group of Canadian Fertility and Andrology Society, et al. Increased risk of pretermbirth in singleton pregnancies after blastocyst versus Day 3 embryo transfer: Canadian ART Register (CARTR) analysis. *Hum Reprod*. 2013;28:924–8.
14. Källén B, Finnström O, Lindam A, Nilsson E, Nygren KG, Olausson PO. Blastocyst versus cleavage stage transfer in in vitro fertilization: differences in neonatal outcome? *Fertil Steril*. 2010;94:1680–3.
15. Zhu J, Lin S, Li M, Chen L, Lian Y, Liu P, et al. Effect of in vitro culture period on birthweight of singleton newborns. *Hum Reprod*. 2014;29:448–54.
16. Nelissen EC, Van Montfoort AP, Smits LJ, Menheere PP, Evers JL, Coonen E, et al. IVF culture medium affects human intrauterine growth as early as the second trimester of pregnancy. *Hum Reprod*. 2013;28:2067–74.
17. Dumoulin JC, Land JA, Van Montfoort AP, Nelissen EC, Coonen E, Derhaag JG, et al. Effect of in vitro culture of human embryos on birthweight of newborns. *Hum Reprod*. 2010;25:605–12.
18. Kleijkers SH, van Montfoort AP, Smits LJ, Viechtbauer W, Roseboom TJ, Nelissen EC, et al. IVF culture medium affects post-natal weight in humans during the first 2 years of life. *Hum Reprod*. 2014;29:661–9.
19. Bendus AEB, Mayer JF, Shipley SK, Catherino WH. Interobserver and intraobserver variation in day 3 embryo grading. *Fertil Steril*. 2006;86:1608–15.
20. Ebner T, Shebl O, Moser M, Mayer RB, Arzt W, Tews G. Easy sperm processing technique allowing exclusive accumulation and later usage of DNA-strandbreak-free spermatozoa. *Reprod Biomed Online*. 2011;22:37–43.
21. Alpha Scientists in Reproductive Medicine and ESHRE Special Interest Group of Embryology. The Istanbul consensus workshop on embryo assessment: proceedings of an expert meeting. *Reprod Biomed Online*. 2011;22:632–46.
22. Ebner T, Vanderzwalmen P, Shebl O, Urdl W, Moser M, Zech NH, et al. Morphology of vitrified/warmed day-5 embryos predicts rates of implantation, pregnancy and live birth. *Reprod Biomed Online*. 2009;19:72–8.
23. Ahlström A, Westin C, Reisner E, Wikland M, Hardarson T. Trophoctoderm morphology: an important parameter for predicting live birth after single blastocyst transfer. *Hum Reprod*. 2011;26:3289–96.
24. Ebner T, Shebl O, Moser M, Sommergruber M, Tews G. Developmental fate of ovoid oocytes. *Hum Reprod*. 2008;23:62–6.
25. Richter KS, Harris DC, Daneshmand ST, Shapiro BS. Quantitative grading of a human blastocyst: optimal inner cell mass size and shape. *Fertil Steril*. 2001;76:1157–67.
26. Salafia CM, Zhang J, Charles AK, Bresnahan M, Shrout P, Sun W, et al. Placental characteristics and birthweight. *Paediatr Perinat Epidemiol*. 2008;22:229–39.
27. Schwartz N, Quant HS, Sammel MD, Parry S. Macrosomia has its roots in early placental development. *Placenta*. 2014;35:684–90.
28. Haavaldsen C, Tanbo T, Eskild A. Placental weight in singleton pregnancies with and without assisted reproductive technology: a population study of 536,567 pregnancies. *Hum Reprod*. 2012;27:576–82.
29. Iwasa Y, Kikuchi H, Suzuki A, Yamada K, Kanno H, Murakawa H, et al. Comparison of blastocyst grade and the new-born baby's body weight. *Fertil Steril*. 2014;102(3):e333.
30. Van den Abbeel E, Balaban B, Ziebe S, Lundin K, Cuesta MJ, Klein BM, et al. Association between blastocyst morphology and outcome of single-blastocyst transfer. *Reprod Biomed Online*. 2013;27:353–61.
31. Ahlström A, Westin C, Wikland M, Hardarson T. Prediction of live birth in frozen-thawed single blastocyst transfer cycles by pre-freeze and post-thaw morphology. *Hum Reprod*. 2013;28:1199–209.
32. Hill MJ, Richter KS, Heitmann RJ, Graham JR, Tucker MJ, DeCherney AH, et al. Trophoctoderm grade predicts outcomes of single-blastocyst transfers. *Fertil Steril*. 2013;99:1283–9.
33. Thompson SM, Onwubalili N, Brown K, Jindal SK, McGovern PG. Blastocyst expansion score and trophoctoderm morphology strongly predict successful clinical pregnancy and live birth following elective single embryo blastocyst transfer (eSET): a national study. *J Assist Reprod Genet*. 2013;30:1577–81.
34. Chen X, Zhang J, Wu X, Cao S, Zhou L, Wang Y, et al. Trophoctoderm morphology predicts outcomes of pregnancy in vitrified-warmed single-blastocyst transfer cycle in a Chinese population. *J Assist Reprod Genet*. 2014;31:1475–81.

35. Ménézo YJ, Chouteau J, Torelló J, Girard A, Veiga A. Birth weight and sex ratio after transfer at the blastocyst stage in humans. *Fertil Steril*. 1999;72:221–4.
36. Gardner DK, Wale PL. Analysis of metabolism to select viable human embryos for transfer. *Fertil Steril*. 2013;99:1062–72.
37. Epstein CJ, Smith S, Travis B, Tucker G. Both X chromosomes function before visible X-chromosome inactivation in female mouse embryos. *Nature*. 1978;274:500–3.
38. Peippo J, Kurkilahti M, Bredbacka P. Developmental kinetics of in vitro produced bovine embryos: the effect of sex, glucose and exposure to time-lapse environment. *Zygote*. 2001;9:105–13.
39. Alfarawati S, Fragouli E, Colls P, Stevens J, Gutiérrez-Mateo C, Schoolcraft WB, et al. The relationship between blastocyst morphology, chromosomal abnormality, and embryo gender. *Fertil Steril*. 2011;95:520–4.
40. Kobayashi S, Isotani A, Mise N, Yamamoto M, Fujihara Y, Kaseda K, et al. Comparison of gene expression in male and female mouse blastocysts revealed imprinting of the X-linked gene, *Rhox5/Pem*, at preimplantation stages. *Curr Biol*. 2006;16:166–72.
41. Bermejo-Alvarez P, Rizos D, Rath D, Lonergan P, Gutierrez-Adan A. Sex determines the expression level of one third of the actively expressed genes in bovine blastocysts. *Proc Natl Acad Sci USA*. 2010;23:3394–9.
42. Tarín JJ, García-Pérez MA, Hermenegildo C, Cano A. Changes in sex ratio from fertilization to birth in assisted-reproductive-treatment cycles. *Reprod Biol Endocrinol*. 2014;12:56.
43. Milki AA, Jun SH, Hinckley MD, Westphal LW, Giudice LC, Behr B. Comparison of the sex ratio with blastocyst transfer and cleavage stage transfer. *J Assist Reprod Genet*. 2003;20:323–6.
44. Csokmay JM, Hill MJ, Cioppettini FV, Miller KA, Scott Jr RT, Frattarelli JL. Live birth sex ratios are not influenced by blastocyst-stage embryo transfer. *Fertil Steril*. 2009;92:913–7.
45. Bu Z, Chen ZJ, Huang G, Zhang H, Wu Q, Ma Y, et al. Live birth sex ratio after in vitro fertilization and embryo transfer in China—an analysis of 121,247 babies from 18 centers. *PLoS One*. 2014;9:e113522.
46. Kato O, Kawasaki N, Bodri D, Kuroda T, Kawachiya S, Kato K, et al. Neonatal outcome and birth defects in 6623 singletons born following minimal ovarian stimulation and vitrified versus fresh single embryo transfer. *Eur J Obstet Gynecol Reprod Biol*. 2012;161:46–50.
47. Roy TK, Bradley CK, Bowman MC, McArthur SJ. Single-embryo transfer of vitrified-warmed blastocysts yields equivalent live-birth rates and improved neonatal outcomes compared with fresh transfers. *Fertil Steril*. 2014;101:1294–301.
48. Li Z, Wang YA, Ledger W, Edgar DH, Sullivan EA. Clinical outcomes following cryopreservation of blastocysts by vitrification or slow freezing: a population-based cohort study. *Hum Reprod*. 2014;29:2794–801.
49. Hardy K, Handyside AH, Winston RM. The human blastocyst: cell number, death, and allocation during late preimplantation development in vitro. *Development*. 1989;107:597–604.
50. Ebner T, Vanderzwalmen P, Shebl O, Mayer RB, Moser M, Tews G. Morphology aspects of human blastocysts and the impact of vitrification. *J Reprod Med Endocrinol*. 2011;8:13–20.
51. Licciardi F, McCaffrey C, Oh C, Schmidt-Sarosi C, McCulloh DH, Licciardi F, et al. Birth weight is associated with inner cell mass grade of blastocysts. *Fertil Steril*. 2014;103:382–7.e2.