Zona Pellucida Thickness Variation and Occurrence of Visible Mononucleated Blastomeres in Preembryos Are Associated with a High Pregnancy Rate in IVF Treatment

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Purpose: *The ability of six morphological criteria (embryo development rate, fragmentation, regularity of blastomere shape, equality of blastomere size, zona pellucida thickness variation [ZPTV], and visible mononucleated blastomeres [VMBs]) to predict pregnancy in IVF treatment cycles was evaluated.*
Methods: *In order to select a homogeneous study group, 85 consecutive nulliparous couples with single tubal infertility undergoing their first IVF treatment and receiving three preembryos at embryo replacement 2 days after ovum pickup were included.*
Results: *A total of 255 preembryos was replaced two days after ovum pickup and resulted in 34 clinical pregnancies (40%). By logistic regression analysis, ZPTV and VMBs showed highly significant and strong predictive values, whereas none of the other parameters was a significant predictor of pregnancy. In the treatments in which all replaced preembryos had a ZPTV of less than 15%, the pregnancy rate was extremely low (1/22). If the maximum ZPTV of any of the replaced preembryos was in the interval between 15 and 20%, the pregnancy rate was 24.1% (7/29). In the treatments in which at least one preembryo had a ZPTV of more than 20%, the pregnancy rate was 76.5% (26/34). When VMBs were added to the results of the ZPTV analysis, the pregnancy rate was as high as 92.3% (24/26).*
Conclusions: *ZPTV and VMBs seem to be strong predictors of pregnancy in IVF treatment and thus important indicators of good embryo quality.*

KEY WORDS: in vitro fertilization; preembryo; zona pellucida; blastomere; pregnancy.

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INTRODUCTION

Clinical in vitro fertilization (IVF) has been dependent on controlled ovarian hyperstimulation and multiple preembryo replacement to obtain acceptable delivery rates per treatment cycle. However, it has been necessary to counteract an increasing multiple pregnancy rate by reducing the number of preembryos replaced (1). Consequently, today, at most Swedish IVF clinics a maximum of two preembryos is being replaced. Although this practice has reduced the triplet rate to less than 1%, the twin rate is still at the 20 to 30% level. Replacement of only one preembryo in most treatments will most likely be necessary to decrease the multiple birth rate further. From this perspective, choosing the right preembryo is an important challenge in the near future as a means of increasing the proportion of singleton pregnancies in IVF treatment (2). However, selection of the single appropriate preembryo for replacement will require accurate and reliable criteria for embryo quality determination.

Morphological preembryo quality scoring systems are based on criteria registered at light microscopy (3–7). Embryo development rate (EDR) and fragmentation (presence of anucleate fragments in the preembryo) are commonly used criteria (4). In addition, regularity of blastomere shape and equality of blastomere size are included in other scoring systems (5, 6). Morphological scoring systems have shown a low predictive ability, alone or in combination, to select pre-embryos of high developmental potential in IVF practice (4,8,9). Nonmorphological scoring has been disappointing until now, as most methods are complicated and time-consuming (4,8).

In this study we evaluated the ability of six morphological criteria to predict pregnancy in IVF treatment cycles. In addition to EDR, fragmentation, regularity of blastomere shape, and equality of blastomere size, zona pellucida thickness variation (ZPTV) and visible mononucleated blastomeres (VMBs) were studied. Cohen *et al.* (10) found that ZPTV, not the thickness per se, was a strong predictor of IVF treatment outcome. However, the impact of this parameter has so far not been confirmed. The occurrence of VMBs in preembryos has not, to our knowledge, been studied as a predictor of treatment outcome.

MATERIALS AND METHODS

Study Group

During a 21-month period a total of 249 IVF treatment cycles resulted in replacement of preembryos in our clinic. In order to select a homogeneous study group, those couples with the following characteristics treated during this period were included ($n = 85$): first IVF treatment ever, single tubal infertility, nulliparous, and receiving three preembryos at embryo replacement (ER) 2 days after ovum pickup (OPU), as presented in Table I. The women ranged in age from 25 to 39 years, with a mean age of 33.6 years.

Clinical IVF Protocol

All women involved in this study underwent controlled ovarian hyperstimulation after pituitary suppression with a gonadotropin-releasing hormone agonist (Suprefact; Svenska Hoechst AB, Stockholm, Sweden) as described previously (11). After downregulation was verified; ovarian stimulation was performed with human menopausal gonadotropin (hMG; Pergonal; Serono Nordic AB, Sollentuna, Sweden). Follicular development was monitored by vaginal ultrasound measurements of follicles combined with blood samples for estradiol analysis. When the day for OPU was decided, 5000 IU human chorionic gonadotropin (hCG; Profasi; Serono Nordic AB) was given i.m. Approximately 35 hr later OPU was performed by transvaginal ultrasound-guided follicle aspiration.

Table I. Treatment Results in Nulliparous Women with Single Tubal Infertility Who Were Undergoing Their First IVF Treatment Ever in Relation to the Number of Preembryos Replaced During the Study Period[a]

	No. of preembryos			
	1	2	3	4
Patients	10	12	85	17
Pregnancies	2 (20%)	1 (8%)	34 (40%)	7 (43%)

[a] The 85 women who received three preembryos at ER were included in the study.

IVF and Preembryo Culture

For insemination and sperm swim-up, Earl's balanced salt solution (E-7883; Sigma, Stockholm, Sweden) was used with the addition of 0.1 m*M* pyruvate (Sigma), 10,000 IU/ml penicillin (Sigma), and 10% heat-inactivated patient serum. For cleavage culture, the same medium with 15% of the serum was used. Approximately 3 to 5 hr after OPU, 50,000–100,000 motile spermatozoa were added to each oocyte–cumulus kept in test tubes (Falcon 2003) with 1 ml of culture medium. Insemination and cleavage were carried out in airtight CCC boxes (AOK AB, Uppsala, Sweden) gassed with a humidified mixture of 5% CO_2 and 95% air at 37°C. The ova were thoroughly denuded from granulosa cells 16–18 hr after insemination, and fertilization was then assessed by the presence of pronuclei. Cleavage was observed 2 days after OPU and those preembryos with the highest scores according to Puissant *et al.* (5) were selected for replacement. Preembryos with multinucleated blastomeres were not replaced. During the present study period usually three, and never more than four, preembryos were replaced (Table I).

ER and Pregnancy Follow-up

The transcervical replacement procedure was carried out 2 days after OPU using a TDT catheter (Prodimed, Neully-en Thelle, France). The luteal phase was supported with 2500 IU hCG (Profasi) i.m. at 3, 6, and 9 days after ER. Serum hCG was measured 14 days after ER and, when greater than 10 IU/L, repeated 2 days later. Ultrasound examination was performed five weeks from ER for localization of pregnancy and repeated at 9 to 12 weeks of gestational age to confirm fetal viability. Pregnancy implied the observation of a gestational sac at the first ultrasound examination.

Videocinematography and Morphologic Assessment

All replaced pre-embryos were recorded on Super-VHS tape with a color videocamera (Ikegami, Tokyo) mounted on an inverted phase-contrast microscope (Diaphot; Nikon, Solna, Sweden). The settings concerning magnification (× 400) and light (bright field) were constant. The recordings were performed just prior to ER and were later analyzed by an observer who was unaware of the outcome of the treatment cycles. Six morphological parameters were assessed and scored (Table II). A selection based on EDR and fragmentation (5) was already performed when choosing the preembryos for ER. ZPTV was determined by direct measurement of the zona pellucida thickness on the video screen by a ruler at three locations and then calculated as indicated in Table II (10).

Statistics

To test the power of the six morphological parameters to predict pregnancy, the logistic regression method was used, where the outcome pregnancy/not pregnancy was the dependent variable and the six morphological parameters EDR, Frag, Even, Size, VMBs, and ZPTV (Table II) were the independent variables (predictors). The analyses were performed on all six morphological parameters, with the end point being pregnancy or not. The analyses were performed on three sets of calculations for each patient. Either the scores of the three preembryos for each morphological parameter were either added together (cumulative), or an average score was calculated (average), or the maximum score of any of the preembryos was used

Table II. Morphological Parameters

	Subgroup	Score
Embryo developmental rate (EDR), No. of blastomeres	0–1	0
	2–3	1
	4	2
	>4	3
Rate of fragmentation (Frag) (%)	>50	0
	20–50	1
	0–20	2
Regularity in blastomere shape (Even)	≥1 irregular	0
	All regular	1
Identical blastomere size (Size)	>20% deviating	0
	All equal	1
No. of visible mononucleated blastomeres (VMBs)	0	0
	1	1
	2	2
	3	3
	≥4	4
Zona pellucida thickness variation (ZPTV)	$[(z_{max} - z_{mean})/z_{mean}] \times 100$	

(max). In Table III the regression coefficients (B) are presented. The significance level of the coefficients was tested with Wald's statistics. In Table IV, the hit rates, expressed as percentages of the correct predictions of the analyses, are presented. For an introduction to this method of analysis, see Hosmer and Lemeshow (12).

RESULTS

From the 85 women in the study group a total of 569 oocytes was collected and, after insemination, 427 (75%) became fertilized and cleaved. A total of 255 preembryos was replaced and resulted in 34 clinical pregnancies (40% per ER). One pregnancy was ectopic and nine miscarried. Twenty-four pregnancies (18 singletons, 5 twins, and 1 triplet) resulted in 31 live-born children. Thus, the live-born implantation rate was 12%.

The statistical analysis of the morphological parameters (Table III) showed that there were very small differences between the results regardless of whether the maximum, cumulative, or average value of the morphological score of the three preembryos was used. ZPTV and VMBs showed highly significant and strong predictive values. None of the other parameters had a significant predictive value, although it should be remembered that the preembryos were already selected for ER on the basis of EDR and fragmentation.

ZPTV together with the other morphological parameters gave a strong negative prediction (90.2%) as well as a good positive prediction (73.5%) of which ER would result in pregnancy (Table IV).

Pregnancy rate related to ZPTV and/or VMBs is presented in Table V. In the treatments in which all preembryos had a ZPTV of less than 15%, the pregnancy rate was extremely low (1/22). If the maximum ZPTV of any of the replaced preembryos was in the interval between 15 and 20%, the pregnancy rate was 24.1% (7/29). In the treatments in which at least one preembryo had a ZPTV of more than 20%, the pregnancy rate was 76.5% (26/34). When VMB was added to the results of the ZPTV analysis, the pregnancy rate was as high as 92.3% (24/26) in the group in which ZPTV was greater than 20% and at least one blastomere had a visible single nucleus.

DISCUSSION

In this study the two morphological parameters ZPTV and VMBs exhibited high predictive values for

Table III. Significance (*P* Values) and Regression Coefficients (*B*) for the Prediction of Treatment Outcome[a]

Morphological parameter	Maximum		Cumulative		Average	
	P	*B*	*P*	*B*	*P*	*B*
EDR	0.0988	−0.956	0.6978	0.079	0.5014	0.474
Frag	0.8727	−0.087	0.3167	−0.215	0.3247	−0.636
Shape	0.7483	−0.303	0.7781	−0.127	0.9126	−0.159
Size	0.7618	−0.240	0.5940	−0.187	0.6533	−0.470
VMBs	**0.008**	0.906	**0.001**	0.859	**0.001**	2,524
ZPTV	**0.000**	0.192	**0.000**	0.136	**0.000**	0.411

[a] Six morphological parameters were analyzed for the three preembryos (maximum, cumulative, and average).

Table IV. Observed and Predicted Treatment Outcome Using All Six Morphological Parameters (Max Values; Logistic Regression Analysis)

Observed	Predicted		
	Not pregnant	Pregnant	Percentage correct
Not pregnant	46	5	90.2
Pregnant	9	25	73.5
Total	55	ˉ30	83.5

Table V. Pregnancy Rate [% (No.)] Related to $ZPTV_{max}$, VMB_{max}, and the Parameters Combined (Total)

ZPTV (%)	VMB (No)		
	0	≥1	Total
<15	0 (0/14)	12.5 (1/8)	0.5 (1/22)
15–20	21.4 (3/14)	26.7 (4/15)	24.1 (7/29)
>20	25.0 (2/8)	92.3 (24/26)	76.5 (26/34)
Total	13.9 (5/36)	59.2 (29/49)	40.0 (34/85)

pregnancy in a homogeneous group of women who underwent their first IVF treatment cycles. ZPTV and VMBs as well as shape and size of the blastomeres were analyzed on video recordings. The preembryos had been selected for replacement by assessing EDR and fragmentation. Still EDR, fragmentation, and shape and size of blastomeres did not correlate with pregnancy (Table III).

ZPTV in individual preembryos has been proposed to be a marker of normal human preembryo development because a relationship was observed between ZPTV and implantation of thawed as well as fresh preembryos (10,14,15). Their conclusion was based on the assumption that the "best" of the group of one to three preembryos that were replaced was also the one that implanted. Our results showed that, using the cumulative or average score of the embryos replaced, ZPTV had as high a predictive value as the highest value of the preembryos replaced.

High ZPTV is consistent with a thinner portion of the zona pellucida most likely necessary for appropriate hatching during the blastocyst stage. Low ZPTV, on the other hand, means an even thickness of the zona pellucida. The implantation potential of such preembryos has been enhanced by assisted hatching (16). ZPTV should therefore be a useful tool to determine when assisted hatching should be used. The mechanisms by which the zona pellucida is regulated and formed are not clear. Nevertheless, our data show that the zona pellucida should be focused on more when scoring preembryos in IVF treatment.

VMB was strongly associated with pregnancy and had a predictive strength comparable to that of ZPTV. In contrast to ZPTV, which can always be measured, the prerequisite for using VMBs is one blastomere being in the phase of the cell cycle where the nucleus is visible. Still, our results indicate that the occurrence of one mononucleated blastomere is a firm indicator of developmental potential. Because the blastomeres are often cleaving asynchronously (13), the highly predictive value of one blastomere with a visible nucleus would allow us to hypothesize that asynchronous cleavage is not a negative sign. In a search of the literature we did not find any report on mononucleated blastomeres as a parameter in preembryo scoring. However, our first experience with VMBs indicates that this parameter should be analyzed further.

Regularity of blastomere shape and equality of blastomere size did not show predictive strength in other studies (5,6), which is in accordance with our findings. EDR and fragmentation are widespread morphological parameters for preembryo assessment in IVF programs (5,6,17). We found a low pregnancy-predicting impact of these parameters. However, the preembryos were already selected to have the highest EDR and lowest rate of fragmentation. Thus, based on the present results, it is not possible to rank the importance of EDR or fragmentation compared to ZPTV of VMBs, although the latter characteristics had the ability of further prediction. EDR has been found to have some value in assessing preembryo quality (18,19), while the role of fragmentation has been questioned (20). Nevertheless, a high rate of fragmentation seemed to impair embryo viability and correlated with a high rate of chromosomal aberrations (21). In cases in which several preembryos are available, a prolonged culture time, up to 4 or 5 days, has been proposed as a method to identify high-quality preembryos (22,23). It would seem that ZPTV and VMBs should be tested as alternative predictors that could be applied earlier in the culture process.

At present many IVF treatment cycles result in more preembryos than should be replaced if one wants to reduce the risk of multiple pregnancies. From this point of view accurate criteria for selecting preembryos with the highest developmental potential are necessary. Our present data support the idea of using two criteria reflecting two qualities of the preembryo. We hypothesize that ZVPT could represent hatching ability, while VMBs could represent blastomere developmental potential.

When more elaborate scoring systems are developed, the selection of preembryos and the number to be replaced should be more accurate and individualized and could, in some cases, be reduced to one (24). In this context preembryo cryopreservation will be even more important, while, on the other hand, milder ovarian stimulation protocols can be used.

In conclusion, ZPTV and VMBs seem to be strong predictors of pregnancy in IVF treatment and thus important indicators of good embryo quality. However, further studies are needed before they can be used in the routine scoring of IVF preembryos.

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