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ASSISTED REPRODUCTION TECHNOLOGIES

The combination of pronuclear and blastocyst morphology: a strong prognostic tool for implantation potential

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Abstract
Purpose To (i) investigate a possible association between different features of pronuclear (PN) morphology and different features of blastocyst morphology, (ii) evaluate the combination of PN and blastocyst morphologies as a predictive factor for ICSI outcomes and (iii) identify possible contributing factors to poor PN morphology.
Methods This study included 908 normally fertilised zygotes reaching full blastocyst stage, obtained from 350 patients undergoing ICSI cycles, in which the implantations rates were 0 % or 100 %. The influence of PN morphology on blastocyst morphology and on the rates of pregnancy and miscarriage was investigated. Embryos were graded and split into three groups, taking into consideration both the PN and the blastocyst status. The pregnancy rate was compared among these groups.
Results Inner cell mass (ICM) alterations were correlated with the number of nucleolar precursor bodies (NPB), while trophectoderm alterations were correlated with the size of the pronuclei and the distribution of the NPB. The distribution of the NPB had an impact on the chances of pregnancy. A significant difference was observed among the groups regarding the pregnancy rate. The maternal age, number of aspirated follicles and number of retrieved oocytes influenced the incidence of PN defects.
Conclusions These findings suggest that a lower oocyte yield may lead to higher-quality PN zygotes. In addition, different PN features may influence further embryo development, especially the quality of the blastocyst. Moreover, the association between PN and blastocyst morphology may be used as a prognostic tool for implantation.

Keywords Pronuclear · Blastocyst · Embryo · Morphology · Pregnancy · Implantation

Capsule The association between pronuclear and blastocyst morphology may be used as a prognostic tool for implantation competence.

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Introduction

The necessity to decrease the multiple pregnancies incidence in assisted reproduction has become a health, economic and legal issue in many countries [1]. Meanwhile, the approach of transferring a single embryo is still challenging [2], since the identification of embryos with high implantation potential remains difficult. The evaluation of the cleavage stage embryo morphology at the time of the embryo transfer has been used as a tool to select the best embryo for transfer. However, these morphological features do not correlate sufficiently with the embryo implantation potential. A number of other strategies have thus been proposed, including evaluating oocyte morphology [3, 4], scoring pronuclear (PN) stage zygotes [5, 6], selecting early cleaving embryos [7] and culturing embryos up to the blastocyst stage [8].

The most frequently observed PN zygote features include the cytoplasmic halo, the position of the PN and the number and distribution of nucleolar precursor bodies (NPB) in the pronuclei. However, several authors have reported different results regarding the prognostic value of each PN feature. Some studies have reported a strong association between the PN orientation [9–11] or the number and distribution of NPB [12–16] and the implantation potential; others have suggested that an increased chance of implantation exists when the cytoplasmic halo is present in zygotes [2, 17–21].

Whether there is a correlation between early stage embryo morphology and blastocyst morphology is still unknown.

Graham and colleagues [22] showed that fewer than half of the embryos that were selected on day three for transfer were reselected on day five. Rjinders and Jansen [23] also reported that only 51 % of the embryos transferred on day five had been selected for transfer on day three. Chen and Katerra [24] demonstrated that there is no difference, in terms of implantation and pregnancy, between whether PN morphology or early cleavage is used to select embryos for transfer on day three.

The goals for the present study were: (i) to investigate a possible association between different features of PN morphology and different features of blastocyst morphology, (ii) to identify the predictive value of the combination of PN and blastocyst morphology for ICSI clinical outcomes and (iii) to identify possible contributing factors to poor PN morphology.

Materials and methods

Study design

This retrospective observational study included 908 normally fertilised zygotes reaching full blastocyst stage (blastocyst with a blastocoel completely filling the embryo), obtained from 350 patients undergoing ICSI cycles, in which the implantations rates were 0 % or 100 %. All of the embryos were evaluated at 16–18 h post-ICSI and on days two, three and five of development. All cases of severe spermatogenic alteration, including frozen and surgically retrieved sperm, were excluded from the study.

The influence of PN morphology on blastocyst morphology and on the rates of pregnancy and miscarriage was investigated. The predictive value of the combination of PN and blastocyst morphologies on pregnancy and implantation rates was also evaluated. Embryos were graded and split into three groups, taking into consideration both the PN status, according to Tesarik and Greco [13], and the blastocyst status, according to the Istanbul consensus workshop on embryo assessment [25].

The embryos' groups were as follows: embryos with high PN quality and high blastocyst quality (score 1), embryos with high PN quality and low blastocyst quality (score 2); embryos with low PN quality and high blastocyst quality (score 3) and embryos with low PN quality and low blastocyst quality (score 4). The pregnancy rate was compared among these groups.

Finally, the influence of the following variables on the incidence of poor PN morphology was evaluated: (i) maternal age, (ii) total dose of FSH administered for ovarian stimulation, (iii) level of serum 17β-estradiol on the ovulation trigger day, (iv) number of aspirated follicles, (v) number of retrieved oocytes, (vi) retrieved oocytes rate (number of retrieved oocytes per number of aspirated follicles) and (vii) immature oocytes rate.

Fertilisation was defined as the presence of both two clearly distinct pronuclei and two polar bodies; abnormal fertilisations, such as the presence of only one or more than two pronuclei, were excluded. Pregnancy was defined as the presence of foetal heart activity by ultrasound at 6 to 7 weeks of gestation, and implantation was defined as the presence of a gestational sac, as visualised by ultrasound at 4 to 6 weeks after the embryo transfer. Miscarriage was defined as the spontaneous loss of a pregnancy before the 24th week of gestation occurred.

A written informed consent was obtained, in which patients agreed to share the outcomes of their own cycles for research purposes, and the study was approved by the local institute review board.

Controlled ovarian stimulation & laboratory procedures

Controlled ovarian stimulation was achieved by pituitary blockage using a GnRH antagonist (Cetrotide, Serono, Geneva, Switzerland), and ovarian stimulation was performed using recombinant FSH (Gonal-F; Serono, Geneva, Switzerland).

Follicular growth was followed by a transvaginal ultrasound examination, starting on day four of gonadotropin administration. When adequate follicular growth and serum E2 levels were observed, recombinant hCG (Ovidrel; Serono, Geneva, Switzerland) was administered to trigger the final follicular maturation. Oocytes were collected 35 h after hCG administration by transvaginal ultrasound ovum pick-up.

The recovered oocytes were assessed for their nuclear status, and those in metaphase II were submitted to ICSI following routine procedures [26].

Embryo morphology evaluation

Embryo morphology was assessed at 16–18 h post-ICSI and on the mornings of days two, three and five of embryo development, using an inverted Nikon Diaphot microscope (Eclipse TE 300; Nikon, Tokyo, Japan) with a Hoffmann modulation contrast system under 400X magnification.

For the PN morphology, the following features were recorded: the presence of a cytoplasmic halo, the size and position of the PN and the number and distribution of NPB in the PN.

For the blastocyst stage morphology, the following characteristics were recorded: the size and compactness of the ICM and the cohesiveness and number of TE cells. The blastocysts were graded according to the Istanbul consensus workshop on embryo assessment [25].

Regarding the pronuclear stage, were considered of low quality embryos presenting abnormal patterns such as: (i) Big difference in the number of NPB in both pronuclei; (ii) small number of NPB without polarization in at least one pronucleus, (iii) large number of NPB with polarization in at least one pronucleus; (iv) very small number of NPB in at least one

pronucleus; and (v) polarized distribution of NPB in one pronucleus and non-polarized in the other.

Embryos with high blastocyst quality presented a tightly packed ICM with many cells and a trophectoderm with many cells forming a cohesive epithelium, any embryo lacking one of these characteristics were considered of low quality.

Statistical analyses

Binary logistic regressions were performed to study the influence of PN morphological characteristics on blastocyst morphological parameters, pregnancy rates and miscarriage rates.

To evaluate the value of the combination of PN and blastocyst morphology for the prediction of pregnancy chi squared was used, respectively.

Finally, the effect of the variables was also investigated through linear logistic regression analyses: (i) maternal age, (ii) total dose of FSH administered for ovarian stimulation, (iii) serum 17β-estradiol level on the ovulation trigger day, (iv) number of aspirated follicles, (v) number of retrieved oocytes, and immature oocytes rate on the PN morphology.

The regression analyses were adjusted for maternal and paternal age, the number of retrieved oocytes, endometrium thickness and FSH dose, as these would be considered potential confounders of the association between the factors evaluated and the ICSI outcomes.

Results were expressed as odds ratios (OR) and their 95 % confidence intervals (CI) or regression coefficients (RC), and p value. Results were considered to be significant at the 5 % critical level ($p<0.05$). Data analysis was carried out using the Minitab (version 14) Statistical Program.

Results

The patients' characteristics are described as the mean ± standard deviation: maternal age: 32.9±10.3; paternal age: 37.4±18; total dose of FSH administered for ovarian stimulation: 2055 IU±825; serum 17β-oestradiol levels on the ovulation trigger day: 2575.9±1368; number of aspirated follicles: 10.8±12.7 and; number of retrieved oocytes: 9.3±10.3. The pregnancy rate was 44.3 % (155/350) and the multiple pregnancy rate was 21.3 % (33/155).

The effect of PN morphological characteristics on blastocyst morphological parameters

ICM alterations were correlated with the number of NPB, while TE alterations were correlated with both the size of the pronuclei and the distribution of NPBs, but not with any other PN factor (Table 1).

Table 1 Binary regression analysis of the pronuclear morphological characteristics that may affect the blastocyst morphological factors

Response variable	Predictor variable	P	OR	CI: Lower	CI: Upper
ICM alteration	Halo normality	0.513	2.33	0.19	9.25
	PN size	0.562	0.46	0.03	6.3
	PN position	0.649	1.63	0.2	3.28
	Number of NPB	**0.035**	2.51	1.2	3.34
	Size of NPB	0.997	1.76	0.65	3.43
	Distribution of NPB	0.185	0.18	0.01	2.27
TE alterations	Halo normality	0.434	1.43	0.58	3.51
	PN size	0.127	2.27	0.79	6.51
	PN position	**0.049**	2.38	1.31	4.05
	Number of NPB	0.196	0.86	0.21	1.55
	Size of NPB	0.236	0.754	0.34	1.14
	Distribution of NPB	**>0.001**	6.03	2.31	15.77

Bold numbers are statistically significant

PN pronuclear, *NPB* nucleolar precursor bodies, *ICM* Inner cell mass, *TE* trophectoderm cells, *OR* odds ratio, and *CI* confidence interval

ICM and TE graded according to the Istanbul consensus workshop on embryo assessment [25]

PN and blastocyst morphological characteristics on clinical outcomes

The distribution of the NPB had an impact on the chances of pregnancy, however, the miscarriage rate was not affected by any PN factor (Table 2).

Table 2 Binary regression analysis of the pronuclear morphological characteristics that may affect pregnancy and miscarriage rates

Response variable	Predictor variable	P	OR	CI: Lower	CI: Upper
Pregnancy rate	Halo normality	0.56	0.89	0.59	1.33
	PN size	0.865	0.95	0.56	1.63
	PN position	0.084	1.61	0.94	2.75
	Number of NPB	0.217	0.83	0.61	1.12
	Size of NPB	0.842	1.09	0.46	2.60
	Distribution of NPB	0.003	1.86	1.24	2.81
Miscarriage rate	Halo normality	0.321	0.52	0.14	1.89
	PN size	0.355	0.36	0.04	3.12
	PN position	0.495	1.55	0.44	5.44
	Number of NPB	0.912	0.95	0.35	2.55
	Size of NPB	0.988	1.01	0.25	4.10
	Distribution of NPB	0.834	1.14	0.33	3.91

PN pronuclear, *NPB* nucleolar precursor bodies, *OR* odds ratio, and *CI* confidence interval

When the embryos were categorised into four groups, according to PN and blastocyst morphology, a significant difference among the groups was observed regarding the pregnancy rate (Score 1: 63.1 %, Score 2: 54.5 %, Score 3: 42.5 % and Score 4: 33.3 %, $p<0.001$) where the rate is higher when embryos with high PN and high blastocyst morphology were transferred and lower when embryo with low PN and low blastocyst morphology were transferred. The pregnancy rate did not differ among embryos with Scores 2 and 3.

Contributing factors to the incidence of poor pronuclear morphology

The incidence of low-quality PN embryos was not influenced by the total dose of FSH used for ovarian stimulation, the 17β-estradiol levels, the retrieved oocytes rate or the immature oocytes rate. However, the maternal age, the number of aspirated follicles, and the number of retrieved oocytes influenced the incidence of PN defects (Table 3).

Discussion

In the present study, individual PN morphological characteristics were evaluated, and their correlation with blastocyst morphology and ICSI outcomes was investigated. Our results showed that the number and distribution of NPBs had an important influence on the ICM defects and TE defects, while the PN position was significantly correlated only with TE alterations. In addition, the distribution of the NPB had an impact on the chances of implantation.

It is well-known that the dynamic sequence of morphological changes occurring in the PN zygote plays a critical role for subsequent embryo development. In our study, we noted that the PN size may influence the TE morphology, but neither the PN size nor its orientation had any impact on any other blastocyst feature or on the clinical outcomes. Several authors have reported inconsistent results regarding the influence of PN orientation on embryo development and implantation [9–11]. According to Senn and colleagues [2], PN position is not correlated with successful implantation, but the NPB patterns are relevant indicators of implantation, which is in keeping with our findings.

Previous time-lapse observations evaluating the pronuclear morphology [27], suggests that the number and distribution of NPBs does not predict the quality of the zygote, in terms of live birth potential. Despite this, these morphological features have been chosen as a quality indicator of the fertilized oocyte. In fact, it has been described that a minimum number of NPB in each PN, and the same distribution in the two PNs, have both been positively correlated with pregnancy [14–16]. In addition, zygotes presenting polarised NPB appear to have a better implantation potential [12, 28]. Nevertheless, the function of NPB fusion and polarization are at present not explainable. This strongly suggests further studies are required to clarify the role and dynamics of NPB before using these characters in embryo selection.

Abnormal or absent cytoplasmic halos have been thought to indicate changes in organelle localisation, which is of critical importance for subsequent implantation. Many studies have confirmed this importance of the cytoplasmic halo, showing an increased implantation rate when the cytoplasmic halo is present in zygotes [12, 17–21]. However, our study failed to find any such association, most likely because most of the embryos evaluated here showed a normal cytoplasmic halo pattern.

Balaban et al. [29] indicate that the pronuclear pattern of the zygote is closely related to blastocyst formation and quality. In fact, blastocysts derived from high quality PN zygotes had a higher potential for implantation. It was also described by other authors that the early embryo assessment can be used as an indicator of subsequent good blastocyst development [20, 30].

However, other studies highlighted the limited prognostic value of the pronuclear score in assisted reproduction outcome [31–34].

Because PN morphology does not consistently identify embryos with high implantation potential, a graduated embryo score combining PN morphology, first cleavage and day three morphology was developed by Fish and colleagues [35].

In this study, embryos with high PN scores had significantly better blastocyst formations than embryos with lower scores. However, although embryo morphologies on days two and three are still evaluated in our centre, manipulation of the embryos outside of the incubator should be avoided as much as possible. Therefore, we proposed a combination of observing embryo morphology at the PN zygote stage, when fertilisation is checked, and at the day five blastocyst stage, when embryo transfers are usually performed in blastocyst transfer programs.

Our results should indicate that the association between PN and day five blastocyst morphology is a powerful tool for predicting the embryo's implantation competence. Indeed, in our study, when embryos were categorised into three groups,

Table 3 Regression analysis of factors contributing to low pronuclear morphology with the following variables: maternal age, total dose of FSH, serum 17β-estradiol level, number of aspirated follicles, number of retrieved oocytes, retrieved oocytes rate, and immature oocytes rate

Response variable	Predictor variable	P	RC
Pronuclear morphologys	Maternal age	**0.033**	−5,243
	FSH dose	0.098	−4.659
	17β-estradiol level	0.765	−1.239
	Aspirated follicles	**0.042**	−2.435
	Retrieved oocytes	**0.023**	−7.654
	Retrieved oocytes rate	0.879	1.239
	Immature oocytes rate	0,987	2.987

Bold numbers are statistically significant

RC regression coefficient

according to PN and blastocyst morphology, significantly higher rate of pregnancy was noted when embryos with high PN and high blastocyst morphology were transferred.

In the present study, the pronuclear morphology was evaluated at 16–18 h after ICSI. Recent data using time-lapse imaging demonstrated that the pronuclear morphology changes over time, indicating that the single light microscopy observation approach is deficient in comparison to time-lapse [27]. Therefore, our findings must be confirmed by future trials, if possible using time-lapse imaging technology.

When possible factors for affecting the PN morphology were evaluated, it was demonstrated that neither the total dose of FSH, nor the serum 17β-estradiol level, nor the retrieved oocytes rate nor immature oocytes rate affected the PN morphology. The maternal age, the number of aspirated follicles, and the number of retrieved oocytes, however, appeared to play a role with these factors.

Despite advanced maternal age, no clearly established factor has been described for the development of embryos with poor morphology. For a woman over 40 years old undergoing IVF treatment, it is typical for more than half of the retrieved oocytes to have abnormal chromosomes [36]. This is in agreement with our findings, which demonstrated that maternal age influences PN defects.

While the FSH dose was not related to embryonic morphology, a negative influence on the oocyte yield was noted. It has been previously demonstrated that mild ovarian stimulation, leading to a lower oocyte yield, is associated with a decrease in the proportion of aneuploid embryos [37]. A previous study also showed that mild stimulation resulted in high-quality embryos for transfer, as indicated by good embryo morphology [38].

In summary, the results presented here suggest that the association of PN and blastocyst morphology may be useful as a strong prognostic tool for implantation. Moreover, our evidence suggests that a lower oocyte yield may represent a more appropriate response to ovarian stimulation, leading to the formation of high-quality zygotes.

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