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The probability of abnormal preimplantation development can be predicted by a single static observation on pronuclear stage morphology

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This retrospective study was undertaken to determine whether further developmental progression of two-pronucleated (2PN) zygotes can be predicted by a single, non-invasive examination of pronuclei, with the use of criteria based on the number and distribution of nucleolar precursor bodies in each pronucleus. The normal range of pronuclear variability was defined by analysis of zygotes giving rise to embryos transferred in 100%-implantation cycles (pattern 0). Morphological patterns differing from pattern 0 were classified as patterns 1–5. The frequency of developmental arrest of pattern 0 zygotes was only 8.5% as compared with 31.6, 21.9, 30.0, 20.5 and 24.1% for patterns 1–5 respectively. Relationships of pronuclear patterns with blastomere multinucleation and cleaving embryo morphology were also noted. Clinical pregnancy was achieved in 22 of 44 (50%) treatment cycles in which at least one pattern 0 embryo was transferred, but only in two of 23 (9%) cycles in which only pattern 1–5 embryos were transferred. These data present new evaluation criteria which can be used to predict the developmental fate of human embryos as early as the pronuclear stage, without requiring repeated observations or an exact timing of pronuclear zygote inspection. Further prospective study is needed for clinical validation of these criteria.

Key words: nucleolar precursor bodies/preimplantation development/pronuclear development/zygote selection

Introduction

In most of the current in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) programmes, fertilized oocytes are kept in culture for 1 or more days after the assessment of fertilization and are not transferred back to the mother until they undergo cleavage. This makes it possible to perform an elective embryo transfer whereby embryos supposed to be of the best quality, as assessed by diverse criteria based on blastomere regularity, the presence of anucleate fragments and cleavage speed (Edwards *et al.*, 1984; Cummins *et al.*, 1986; Claman *et al.*, 1987; Puissant *et al.*, 1987; Hill *et al.*, 1989; Steer *et al.*, 1992; Shulman *et al.*, 1993; Giorgetti *et al.*, 1995; Ziebe *et al.*, 1997; Sakkas *et al.*, 1998), are selected for transfer from the available cohort. Implantation rates after elective embryo transfer are significantly higher as compared to non-elective embryo transfer, which makes it possible to reduce the number of transferred embryos and thus to reduce the risk of multiple pregnancies (Waterstone *et al.*, 1991; Staessen *et al.*, 1993; Tasdemir *et al.*, 1995).

One of the major drawbacks of embryo transfer policies involving the selection of embryos at the early cleavage stages is the problem of the fate of those embryos that have not been selected for immediate transfer but still appear to be viable. Ethical, administrative and technical concerns regarding the eventual cryostorage and immediate or delayed destruction of such embryos have been extensively discussed from various points of view (Evans and Evans, 1996; Schafer *et al.*, 1996; Edwards and Beard, 1997a).

To overcome the problem of supernumerary embryos, some centres suggest the selection of embryos for later transfer to be performed as early as the pronuclear stage, when the genomes originating from both gametes are still physically separated within the respective pronuclei. Pronuclear zygotes can thus hardly be considered as embryos because they lack genetic integrity, one of the characteristics often evoked to characterize an individual. In fact, genomes coming from both gametes can still be separated at the pronuclear stage by a relatively simple micromanipulation procedure.

Moreover, pronuclear zygotes show good survival rates after freezing and thawing, and pregnancy rates after transfer of embryos developing from frozen/thawed zygotes resulting from conventional IVF or ICSI do not appear to be inferior to those with embryos frozen at cleavage stages (Veeck *et al.*, 1993; Al-Hasani *et al.*, 1996; Hoover *et al.*, 1997). However, clear and easily applicable criteria, based on non-invasive examination, for the selection of the most viable pronuclear zygotes, are still lacking, although previous work has suggested a relationship between pronuclear morphology and the ability of an embryo to implant and continue developing (Wright *et al.*, 1990; Balakier *et al.*, 1993; Van Blerkom *et al.*, 1995; Payne *et al.*, 1997; Scott and Smith, 1998). If this last obstacle is overcome, the current embryo transfer policies can be replaced with those employing pronuclear zygote cryostorage and allowing further development of only those zygotes that have been previously selected for transfer.

There are many morphological transformations during the development of human pronuclear zygotes, many of which involve phenomena that can be easily assessed in living zygotes, by a simple, non-invasive microscopical observation. Among these transformations, an early phase of nucleologenesis, consisting of the assembly, growth and mutual fusion of nucleolar precursor bodies (NPB) has a well-known morphological picture and timing in human zygotes (Tesarik and

Kopecny, 1989a) and is known to be dependent on an early wave of pronuclear transcriptional activity (Tesarik and Kopecny, 1989b, 1990) and on the presence of ooplasmic factors appearing during oocyte maturation (Tesarik and Kopecny, 1989c). All these features make the development of NPB a good candidate for a potential non-invasive morphological marker of pronuclear zygote quality.

In this study, an attempt is made to establish a relationship between the pronuclear morphology, with a special attention to NPB, on the one hand, and preimplantation embryo development on the other hand. The relative contribution of different pronuclear irregularities to cleavage arrest and blastomere multinucleation is also evaluated.

Materials and methods

Study design

The definitions of normal and abnormal patterns of pronuclear morphology were basically derived from comparisons between 100%-implantation cycles (ongoing clinical pregnancies with the number of embryonal sacs equal to the number of embryos transferred) and the other treatment cycles in which either only part of the transferred embryos implanted or no pregnancy was achieved. This kind of evaluation was made possible by a strict separation of each individual oocyte, zygote and embryo throughout the period from oocyte recovery to embryo transfer or cryopreservation, such that pronuclear morphology could be related to further embryonic development in each individual zygote.

Any type of pronuclear morphology found in zygotes that were subsequently transferred in the 100%-implantation cycles was considered to be associated with zygote viability. Configurations different from those detected in the above group of zygotes were classified into five different patterns (see Results). The frequency of embryos with different developmental fates (cleavage arrest, blastomere multinucleation, good morphology) developing from each category of pronuclear zygotes was calculated. No selection of embryos for transfer according to the criteria described in this study was performed.

Patients

Patients enrolled in this study were 61 couples suffering from different types of infertility and treated by conventional IVF (11 couples) or by ICSI (50 couples). The ICSI treament was repeated twice in six of these couples, giving the total number of 67 treatment cycles. All the couples gave their informed consent with the additional examinations performed in this study. These examinations were completely non-invasive and prolonged the standard time of observation by no more than 10–20 s for each zygote.

Interventions

Ovarian stimulation, oocyte and sperm recovery, IVF and ICSI were carried out using a standard protocol (Rienzi *et al.*, 1998). Each oocyte recovered was maintained at 37°C in a separate drop of IVF-50 culture medium (Scandinavian IVF Science, Gothenburg, Sweden) equilibrated with 5% CO_2 in air throughout all subsequent steps, which made it possible to associate each particular type of pronuclear stage morphology with the subsequent developmental fate of each embryo. Zygotes were checked for pronuclear morphology 12–20 h after in-vitro insemination or ICSI, using a ×20 objective and an inverted microscope equipped with Hoffman modulation contrast optics. Cleavage speed and morphology were assessed during two sequential observations on the following 2 days. The interval between these two observations was 23–25 h. During these examinations, the eventual presence of multinucleated blastomeres (MNB) was also noted. Embryos that had the same number of blastomeres at the two sequential times of observation, together with those zygotes that remained blocked at the pronuclear stage, were considered as developmentally arrested. Morphology of cleaving embryos was evaluated with particular attention to the size regularity of individual blastomeres, to the relative volume occupied by cell fragments and to the optical transparency of blastomere cytoplasm. Embryos with equal-sized blastomeres, with <10% of intrazonal space occupied by fragments and with clear, non-granulated cytoplasm in all blastomeres are referred to as good-morphology embryos throughout this study. The only exception to the application of the above criteria was the three-cell stage at which embryos with one bigger and two smaller, equal-sized blastomeres were considered as good-morphology embryos, whereas those with three equal-sized blastomeres were not.

Assessment of pronuclear morphology

The morphological parameters evaluated in this study include the number of NPB and their distribution in each pronucleus (polarized versus non-polarized). This assessment required the changing of focus during observation until the whole volume of both pronuclei was inspected. Before this examination, zygotes were rotated by repeated movements of the culture dish until both pronuclei were clearly visible and there was no optical superimposition of both abutted pronuclei (Figure 1). Zygotes in which the totality of both pronuclei was not clearly visible in this position (e.g. because of residual corona radiata cells) were excluded from this study. The distribution of NPB was considered polarized when all NPB present in a pronucleus were present in the pronuclear hemisphere whose pole was the point of contact with the other pronucleus (Figure 1b); it was considered non-polarized when at least one NPB was found in the opposite hemisphere (Figure 1a). The relative size of both pronuclei (equal or unequal) and their position with regard to one another (in apposition versus at distance) were also noted. The size of NPB was not considered as a separate parameter because it was inversely related to NPB number in a given pronucleus (Figures 1 and 2), in agreement with the previous electron microscopic observation that larger NPB arise through fusion of smaller ones (Tesarik and Kopecny, 1989a).

These evaluations only concerned two-pronucleated (2PN) zygotes; one-pronucleated and three-or-more-pronucleated eggs were not taken into account.

Quantitative evaluations

For each embryo involved in this study, the following data were recorded during sequential observations: pronuclear morphology on the day following IVF or ICSI, embryo morphology on the 2 consecutive days following the day of pronuclear assessment, and the presence of MNB detected during each of these two observations. Zygotes that did not cleave and cleaved embryos that showed the same number of blastomeres on the 2 consecutive days following the detection of pronuclei were considered as arrested embryos.

Six patterns of pronuclear morphology (patterns 0–5) were distinguished, based on the number and distribution of NPB (see Results). The percentages of 2PN zygotes developing into good-morphology embryos, of those yielding embryos with MNB and of those undergoing developmental arrest were calculated for each pattern of pronuclear morphology.

Statistical analysis

The frequency of individual developmental fates (developmental arrest, development of MNB and development into good-morphology embryos) was compared between groups of zygotes with different

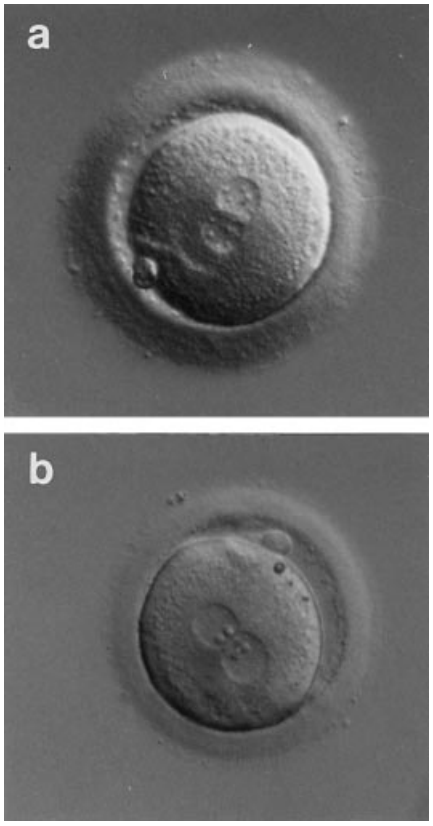

Figure 1. Micrographs showing the size and distribution of nucleolar precursor bodies (NPB) in pronuclei of human zygotes at different phases of pronuclear development. (**a**) Relatively early phase of pronuclear development, characterized by a high number of NPB in both pronuclei (only part of them is visible at this focal level). The NPB are relatively small at this phase and are distributed randomly in the pronuclei. (**b**) Later phase of pronuclear development, characterized by a low number of NPB in both pronuclei. The NPB are larger at this phase and show a polarized distribution, with accumulation near that pole of each pronucleus at which this pronucleus makes contact with the other one. (Hoffman modulation contrast, original magnification ×200.)

pronuclear morphology. These comparisons were made by using the χ^2-test with continuity correction (StatView, Abacus Concepts, Berkeley, CA, USA).

Results

General observations

Out of 446 2PN zygotes observed, the one pronucleus was at least two-fold larger than the other in six zygotes (1.3%), and equal-sized pronuclei were located at a distance from one another in four other zygotes (0.9%). All these 10 zygotes either did not cleave or developed into embryos that were later cleavage arrested. Further analysis only deals with the remaining 436 zygotes (97.8%) that had equal-sized pronuclei, in apposition with each other.

The maximal and the minimal number of NPB observed in one pronucleus was 12 and 1 respectively. In pronuclei with larger numbers of NPB, the NPB were usually distributed randomly throughout the pronucleus (Figure 1a), whereas NPB present in lower numbers tended to have a polarized distribution, accumulating near the pole at which the pronucleus was attached to the other one (Figure 1b). The number and distribution of NPB was similar in both pronuclei in some zygotes, but interpronuclear differences were observed in others (see below).

Table I. Definition of presumptive abnormal patterns of pronuclear stage morphology

Pattern	Description
1	Big difference (>3) in the number of NPB in both pronuclei
2	Small number (<7) of NPB without polarization in at least one pronucleus
3	Large number (>7) of NPB with polarization in at least one pronucleus
4	Very small number (<3) of NPB in at least one pronucleus
5	Polarized distribution of NPB in one pronucleus and non-polarized in the other

NPB = nucleolar precursor bodies.

Definition of patterns of pronuclear morphology

The analysis of pronuclear morphology in 10 zygotes that developed into embryos capable of implantation after uterine transfer (transferred embryos in 100%-implantation cycles) showed a relatively high variability in the number and distribution of NPB. Six of these zygotes had fewer than seven large NPB per pronucleus, accumulated in the area of interpronuclear contact in each pronucleus. However, the other four zygotes had more than seven small NPB per pronucleus, randomly distributed throughout the pronuclear volume in each pronucleus. There were some features that were shared by all those zygotes. First of all, the number of NPB did not show big differences between both pronuclei in this category of zygotes; in fact, the number of NPB in both pronuclei never differed by more than three. Further, NPB were always polarized when they were fewer than seven and never polarized when they were more than seven in a pronucleus. The number of NPB in a pronucleus was never fewer than three. Finally, the distribution of NPB was either polarized or non-polarized in both pronuclei but never polarized in one pronucleus and non-polarized in the other.

Based on these observations, zygotes that did not conform to this morphological picture were considered as potentially abnormal and were classified into five patterns according to distinct specific features whereby they differed from the zygotes in the 100%-implantation group (Table I, Figure 2). These patterns were defined so as to exclude all zygotes from which transferred embryos in the 100%-implantation cycles developed and to include all the other zygotes in at least one of these patterns. In some cases, two or more patterns could be attributed to a single zygote. Zygotes lacking any of the features characterizing these five presumably abnormal patterns, and thus conforming to the 100%-implantation zygotes, were classified as pattern 0 (Figure 2).

Relationship between the pattern of pronuclear morphology and subsequent embryonic development

The developmental fate of 436 2PN zygotes falling into different categories according to the pattern of pronuclear

morphology is summarized in Table II. It is evident from Table II that the frequencies of spontaneously arrested embryos, of embryos showing MNB and of good-morphology embryos were distributed unevenly among different categories of embryos distinguished according to the pattern of pronuclear morphology detected in the zygotes from which these embryos had developed. Most notably, the proportion of arrested embryos developing from pattern 0 zygotes was markedly reduced as compared to any other pattern (Table II). Developmental arrest was most frequently associated with patterns 1 (31.6%) and 3 (30.0%).

As compared with the developmental arrest, differences in the occurrence of blastomere multinucleation between individual categories of embryos were less striking. In any case, patterns 1 and 5 developed MNB almost twice as frequently as pattern 0 (Table II). For pattern 2 there was a trend towards a higher frequency of blastomere multinucleation as compared with pattern 0, but this difference was only at the limit of statistical significance.

Finally, the development into good-morphology embryos was significantly more frequent for pattern 0 zygotes as compared to patterns 1 and 2 (Table II), although the differences were not as dramatic as for the frequency of developmental arrest.

When two or more presumptive pronuclear abnormalities occurred simultaneously in a single zygote, the combination of patterns 1 and 2 appeared to signal a bad prognosis, as nine of 20 such embryos (45%) were arrested and another one (5%) showed MNB. The incidence of developmental arrest for the coincidence of pattern 1 with pattern 5 (28 zygotes) and of pattern 2 with pattern 5 (57 zygotes) was 32.1 and 22.1% respectively. As to other combinations, their frequency was too low to allow quantitative analysis.

Even though the criteria described in this study were not taken into account for embryo selection for transfer, retrospective analysis of successful (in terms of establishing a pregnancy) and unsuccessful treatment cycles suggested that embryos developing from pattern 0 zygotes implant more easily as compared with the other patterns. In fact, out of 44 embryo transfers involving at least one pattern 0 embryo, 22 (50%) resulted in a clinical pregnancy of which two miscarried in the first trimester. This compared favourably with the 23 transfers without any pattern 0 embryo, which yielded one biochemical pregnancy and only two (9%) ongoing clinical pregnancies. These data show that the patterns of pronuclear morphology considered as abnormal in this study are not necessarily linked to embryo demise but rather signal irregularities of zygote development that are often, but not always, irreversible.

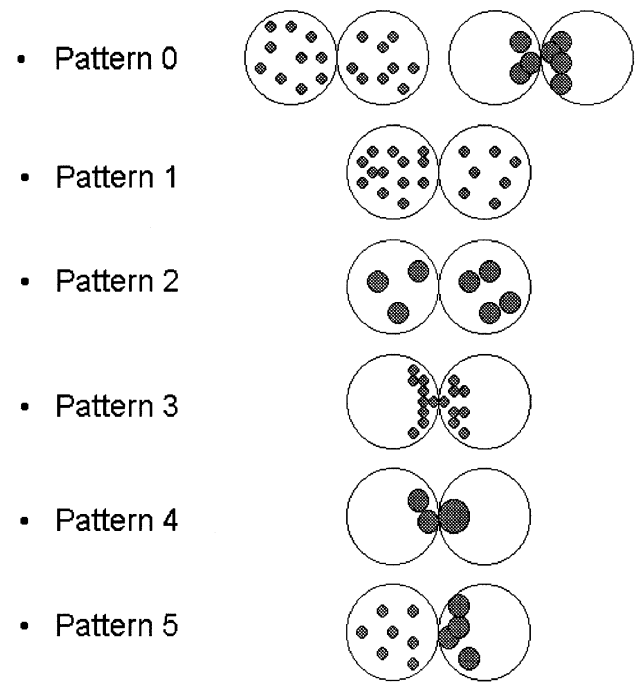

Figure 2. Schematic representation of pronuclear morphology corresponding to individual patterns defined in this study. Pattern 0 corresponds to normal zygotes that developed to embryos that implanted after embryo transfer. Patterns 1–5 represent irregularities described in Table I.

Discussion

It has been suspected for a long time that major abnormalities of pronuclear development, such as the lack of pronuclear apposition or the failure of pronuclear growth, are incompatible with further normal development of the zygote. The former abnormality is now known to result from an abnormal function

Table II. Developmental fate of zygotes showing different patterns of pronuclear stage morphology

	No. (%) of embryos with different developmental fate			
Pattern	Arrested embryos	Embryos with MNB	Good-morphology embryos	Total[a]
0	13 (8.5)[b]	10 (6.5)[b]	57 (37.3)[b]	153 (100)
1	18 (31.6)[c]	7 (12.3)[c]	11 (19.3)[c]	57 (100)
2	37 (21.9)[d]	16 (9.5)[bc]	47 (27.8)[d]	169 (100)
3	9 (30.0)[cd]	2 (6.7)[b]	8 (26.7)[bcd]	30 (100)
4	8 (20.5)[d]	2 (5.1)[b]	13 (33.3)[bd]	39 (100)
5	26 (24.1)[d]	13 (12.0)[c]	33 (30.6)[bd]	108 (100)

[a]The total of this column (556) is higher than the total number of zygotes involved in this study (436) because of coincidence of different patterns in some of the zygotes (see main text).
[b,c,d]Percentages within columns with no common superscript differ significantly ($P < 0.05$).
MNB = multinucleated blastomeres.
Note: Embryos unaccounted for in this table are poor-morphology embryos that were neither arrested nor multinucleated.

of sperm-derived centriole and its associated microtubule-organizing region (Schatten, 1994), whereas the latter is relatively often seen after fertilization with immature sperm cells (Ogura *et al.*, 1994; Tesarik and Mendoza, 1996) and may thus be related to incomplete nuclear protein transition in the male gamete. Even though this study has confirmed the developmental failure of such zygotes, their occurrence appears to be very low (2.2% in this study) when mature spermatozoa are used for IVF or ICSI. That is why this study focused on subtle deviations from the normal course of pronuclear morphogenesis that have not been, up to now, considered as an anomaly. Zygotes that gave rise to embryos transferred in 100%-implantation cycles were used as a reference standard for the definition of these subtle anomalies. This strategy made it possible to avoid the bias of unknown uterine receptivity which can influence pregnancy outcomes regardless of the quality of transferred embryos.

As pointed out by Edwards and Beard (1997b), polarization of both chromatin (Van Blerkom *et al.*, 1995) and NPB (Payne *et al.*, 1997) reflects chromatin rotation in developing pronuclei and represents an important step in the establishment of embryonic axes, which is fundamental to subsequent cell determination in preimplantation embryos (Edwards and Beard, 1997b). However, polarization of NPB is not evident from the very beginning of pronuclear development and appears progressively with time, together with coalescence of NPB giving rise to fewer but larger structures (Tesarik and Kopecny, 1989a). This explains why the morphological pattern 0, defined in this study on the basis of observations on embryos that implanted after uterine transfer, accommodated both polarized and non-polarized forms of NPB distribution. Because zygotes were inspected only once, those pattern-0 zygotes with non-polarized NPB at the time of observation are likely to undergo NPB polarization later in development. Interpronuclear synchrony is thus more important than the actual polarity in this kind of evaluation.

The morphological patterns (1–5) corresponding to presumptive deviations from the normal (pattern 0) are basically characterized by asynchrony of developmental progression between both pronuclei or by dissociation of differentiation events that are normally linked with each other. Hence, these criteria are not dependent on the time elapsed between sperm–egg fusion (or oocyte activation) and the observation of pronuclei. This time would be difficult to assess with sufficient precision, especially in the case of conventional IVF. Moreover, the speed of pronuclear development is likely to be variable even in zygotes with excellent developmental potential, and the cut-off values of this variability remain to be determined. In contrast, the criteria described in this study can be applied at any phase of pronuclear development, once the apposition of both pronuclei has occurred. However, in the case of only a single observation, the most suitable time of this observation appears to be between 14 and 18 h after in-vitro insemination or ICSI, a period during which the proportion of normally fertilized oocytes in which pronuclei can actually be observed is the highest (Nagy *et al.*, 1994, 1998). Asynchrony of pronuclear development, observed in this study by a single static observation, may correspond to prolongation of the interval between the appearance of the male and the female pronucleus detectable in some human zygotes by time-lapse video microcinematography (Payne *et al.*, 1997). It may be pertinent that good-morphology embryos develop from zygotes with significantly shorter interval between the appearance of both pronuclei as compared to poor-morphology embryos (Payne *et al.*, 1997). The possibility of improving predictive value by differing the time of pronuclear observation remains to be examined.

One of the working hypotheses of this study was that, rather than the absolute speed, asynchrony of pronuclear development is more harmful to embryonic viability. This hypothesis is actually corroborated by the present data. So, the probability of future developmental arrest for pattern 0 (presumably normal) zygotes was 2.4–3.7 times lower as compared with each of the patterns 1–5, representing diverse pronuclear irregularities. Moreover, the risk of blastomere multinucleation, a condition associated with the failure of embryonic genome activation between the four-cell and the eight-cell stage of human preimplantation development (Tesarik *et al.*, 1987) invariably leading to developmental arrest of the respective blastomeres, was nearly twice lower for pattern 0 zygotes as compared with patterns 1 and 5. On the other hand, significantly more pattern 0 zygotes developed into embryos with good morphology as compared with patterns 1 and 2.

Most of the embryos involved in this study came from ICSI treatment cycles, and only relatively few resulted from conventional IVF. It remains to be determined whether there is any difference in the overall patterns of pronuclear morphology between ICSI and conventional IVF embryos. The eventual effect of the source of ICSI spermatozoa and the type of male infertility is another open question.

Globally, the power of prediction with the approach described in this study is relatively high for developmental arrest but low for blastomere multinucleation and embryo morphology. Other criteria of pronuclear zygote quality evaluation were proposed (Payne *et al.*, 1997; Scott and Smith, 1998), but they were not related to blastomere multinucleation and embryo morphology either. Further study is needed to determine whether those criteria, eventually in combination with ours, would improve the prediction of these abnormalities of early embryo development. After appropriate standardization, the criteria described in this and the other studies can be applied to select the presumably viable zygotes destined for elective transfer at a later stage of embryonic development. This might improve results of embryo transfer in those programmes in which the decision of which embryos will be transferred as fresh and which embryos will be frozen is taken as early as the pronuclear stage. A prospective study using these criteria is needed to confirm this expectation. It also remains to be determined whether the evaluation of pronuclei can be made more informative by the inclusion of some specific criteria for zygotes resulting from ICSI as compared to those resulting from conventional IVF.

Moreover, the approach described in this study may be useful with any embryo-transfer policy as it adds further criteria with which the evaluation of embryo viability can be refined. In particular, a question arises about the developmental potential of those embryos that ultimately attain a good

morphology during cleavage but develop from zygotes with irregular pronuclear development. Work is in course to determine whether the subtle pronuclear anomalies, as described in this study, impact on implantation and on the postimplantation development of good-morphology embryos.

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