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The Relationship of Pronuclear Stage Morphology and Chromosome Status at Cleavage Stage

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***Purpose*:** Infertile couples undergoing *in vitro* fertilization-embryo transfer (IVF-ET) program were included to study the relationship of pronuclear stage morphology and chromosome status at cleavage stage.

***Methods*:** Eighteen to twenty-one hours after fertilization, zygotes were checked for pronuclear morphology with modified Scott Z-score system. After embryo transfer on day 3, arrested or non-transferred 2 PN embryos were spread for fluorescence *in situ* hybridization (FISH) staining of probes to chromosomes 18, X and Y.

***Results*:** Ninety-eight embryos were successfully fixed and stained. The chromosome status were recorded in each 2 PN score group: 7 (54%) of 13 embryos in Z2 group, 14 (35%) of 40 in Z3 group and 10 (36%) of 28 in Z4 group being normal diploid. Z1 group has 12 (71%) of 17 embryos being normal diploid, which is significantly more than Z3 ($p = 0.020$) and Z4 group ($p = 0.033$).

***Conclusion*:** Our results demonstrated a high probability to get normal diploid embryos if good morphology at pronuclear stage was used as selection criteria, especially for Z1 score embryos.

KEY WORDS: Chromosome status; fluorescence *in situ* hybridization (FISH); pronuclear morphology; zygote scoring.

INTRODUCTION

The efficiency of human in vitro fertilization (IVF) and embryo transfer (ET) is quite low, with only below 30% of transferred embryos ever achieving their full developmental potential (1). Multiple embryos have to be replaced to compensate this low implantation rate. However, the risk of high-order multiple pregnancy and its morbidities have often been the problems. On the other hand, methods have been investigated to predict and select "good-potential" embryos to transfer. Good selection criteria are so important that the pregnancy rate will not be compromised with lowering the number of embryos to be transferred. Recently, a scoring system on pronuclear stage embryos has been suggested for selection of good potential embryos and used successfully for pronuclear embryo transfer by Scott and Smith (2). The comparison of the result from pronuclear stage scoring and blastocyst culture showed both equally effective in selecting viable embryos (1).

The original pronuclear scoring system was set to correlate the morphology of the zygote with the outcome using the criteria of pronuclear size; nucleolar position; alignment of nucleoli and the appearance of the cytoplasm. The rate at which nuclear membrane broke down and progressed to the first cleavage division was also considered (2). Tesarik and Greco (3) reported a modified grading system by a single static observation with the criteria based on the number and distribution of nucleolar precursor bodies in

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each pronucleus. A strong correlation was noted between implantation and the equality of nucleoli within each nucleus of the pronuclear embryos from which the transferred embryos arose. Scott (4) later revised his zygote scoring system as Z-score, which was also based on the distribution and size of nucleoli within each nucleus. In his series, the morphology of pronucleus was positively related to blastocyst development and implantation. Some of the following reports favored the use of pronuclear scoring system with good results from transfer of embryos with good morphology at pronuclear stage (5–7), although the optimism has been questioned (8).

Nucleoli form within the nucleus at areas known as the "nucleolus-organizing regions". Nucleoli comprise chromatin and ribosomal nuclear protein. The nucleolus-organizing regions are located on the chromosomes where the genes coding for ribosomal RNA are located and are the sites where pre-rRNA is synthesized. The number and location of nucleolus-organizing regions are species specific but the nucleoli differ according to the cell type, cellular activity, and the stage of development in any one species (9). Using spectral karyotyping and analyzing via *in situ* hybridization with probes to major satellite DNA and telomeric DNA sequences, Dozortsev *et al.* (10) have demonstrated that pronuclear nucleoli in a pronuclei-stage mouse embryo are represented by α-satellite sequences of interconnecting chromosomes that hold all chromosomes together during interphase. Their observation suggested that satellite association ensures normal chromosome division between cells. Chromosomes remain associated throughout metaphase and this may ensure equal chromosomal division even if an individual chromosome becomes detached from the spindle. In view of this, one might expect a good-looking zygote to go mitosis rather normal and has mistakes less than a poor-looking zygote, although many factors influence a good process of mitosis. Recent evaluation of the efficiency of pronuclear scoring system has made this suggestion reasonable. Since the pronuclear scoring system is comparable to the blastocyst culture, probably the scoring system can be used to select an embryo with less possibility of chromosomal abnormality, and therefore with better potential of development. Kahraman *et al.* (11) investigated the relationship between pronuclei morphology scoring and chromosomal complement of the embryos in couples with severe male infertility undergoing ICSI. Chromosomal status of one blastomere per embryo was evaluated through embryo biopsy. They found pronuclear morphology scoring system might be effectively used in prediction of postzygotic fate and further embryo development. Our study was aimed on this basis to investigate the relation of pronuclear scoring and chromosomal status of whole embryos at cleavage stage. Arrested and nontransferred 2PN embryos in IVF programs were spread and chromosomal status were checked using multicolor FISH. The result was correlated to the pronuclear scoring system.

MATERIALS AND METHODS

Patients

This study included 40 infertile couples undergoing IVF–ET program between April and October 2002. Elaborate counseling was given to couples before treatment and the study consent form was filled.

Ovarian Stimulation and Oocytes Retrieval

The main stimulation protocol used was long protocol. A gonadotropin-releasing hormone (usually leuprolide acetate, Lupron; TAP Pharmaceuticals, Chicago, IL) was given since day 21 of menstrual cycle. When menses came, patients received examinations of vaginal ultrasound and serum estradiol. Once ultrasound showed ovarian quiescence with no ovarian cysts and estradiol level was below 30 pg/mL, administration of gonadotropins was initiated with recombinant FSH 150 IU (Gonal F; Serono laboratories, Randolph, MA) per day. The daily dose was adjusted by individual response. Oocyte retrieval using transvaginal ultrasound was carried out 34–36 h after administration of human chorionic gonadotrophin (HCG, Profasi; Serono, Italy) when two or more follicles reached 18 mm mean diameter.

Oocyte Collection, Insemination, and Embryo Culture

Oocytes were collected in HEPES-buffered human tubal fluid medium (HTF). After serial wash, each oocyte recovered was maintained at 37°C in a separate drop of IVF-50 culture medium (Universal IVF, MediCult) equilibrated with 5% CO_2 in air. Conventional IVF or intracytoplasmic sperm injection was carried out using motile spermatozoa prepared by swim-up procedure. The zygotes were checked for the presence of PN and polar bodies 18–21 h after the insemination or microinjection. Pronuclear morphology was described using the criteria in the following section. Zygotes with 2 PN were cultured at 37°C in a separate drop of IVF-50 culture medium (Universal IVF,

Medi-Cult) equilibrated with 5% CO_2 in air till day 3. Through all subsequent steps of insemination and culture, the oocytes or embryos were maintained in a separate drop of medium, that made it possible to associate each particular type of pronuclear stage morphology with the subsequent developmental fate and chromosomal ploidy status. All oocytes and embryos handling were performed under oil.

Pronuclear Embryo Scoring (Modified from Z-Score) (4)

At 18–21 h postinsemination, the remaining cumulus–coronal cells were stripped from the proembryos using a washed, sterile, pulled Pasteur pipette to allow visualization of their nuclei. The proembryos were placed in individual 10 μL drops of medium under oil and scored on an inverted microscope with Hoffman differential contrast optics at magnifications of $\times$400. Image documentation of the embryos was performed during this inspection and used later for embryo scoring purposes. To assess accurately, the alignment plane of pronuclear junction should be perpendicular to the stage of inverted microscope. Sometimes, it was helpful to manipulate the embryo with a pulled glass pipette or to shake the culture dish slightly. The pronuclear scoring system used in this study is modified from that of Scott *et al.* (4) with the difference of *Z*2 definition (see Table I and Fig. 1). There is no limitation of number of nucleoli so long as the number in each pronucleus is equal. In the original Scott system, the number of nucleoli of *Z*2 zygotes was between three and seven.

Day 3 Scoring and Embryo Transfer

On day 3, embryos were scored as grades 1–5 according to certain morphological criteria. Grade 1: the size of blastomere being equal and no fragmentation. Grade 2: the size of blastomere being equal and little fragmentation. Grade 3: the size of blastomere being unequal and no fragmentation. Grade 3.5: the size of blastomere being unequal and little fragmentation (<20%). Grade 4: the fragmentation being more than 20% but below 50%. Grade 5: the fragmentation being more than 50%. The grading of embryo and the number of blastomeres were taken into considerations for selection of transfer. Only embryos of grading better than grade 3.5 and cell number more than 5 were considered for transferring back or cryopreservation. The rest were considered as embryos of poor quality and put into further investigation in this study. Some couples did not want to cryopreserve the spare embryos and they were also included in this study after informed consents.

Table I. Modified *Z*-Score System[a]

*Z*1	Zygotes with equal numbers of nucleoli (3–7) aligned at the pronuclear junction.
*Z*2	Zygotes with equal numbers and sizes of nucleoli that were equally scattered in the two pronuclei.
*Z*3	Zygotes with equal numbers of nucleoli of equal sizes in the same pronuclei but with one pronucleus having alignment at the pronuclear junction and the other with scattered nucleoli. Zygotes having unequal numbers (a difference of more than one nucleolus) and/or sizes of nucleoli.
*Z*4	Zygotes with pronuclei that were not aligned, were of grossly different sizes or were not located in the central part of the zygote.

[a] Modified from Scott *Z*-score system (2000).

Whole Embryo Spreading and Blastomere Preparation

Each embryo was transferred and washed in PBS at room temperature. It was then placed on a drop around 10 μL of fixatives (0.1% Tween 20, 0.01 M HCl in distilled water) on top of a poly-L-lysine-coated slide. The fixative was spread by continuous and gentle blowing until the cytoplasm dissolved. The final position of the nucleus was marked with a diamond pen. After dehydration in the serial ethanol (70, 90, and 100%), slides were dried and ready for FISH.

FISH Methods

The FISH method followed as described by Harper and Delhanty (12). The efficiency of the FISH procedure was tested in each experiment on interphase nuclei of male human leukocytes (12–14).

Probes used in this study were pBam X5, alphoid probe (insert size 2.0 kb), specific for the centromeric region of the X chromosome, cY98, specific for a 3.77kb region on the long arm of the Y chromosome and L1.84, alphoid probe, specific for the centromeric region of human chromosome 18. (These probes were kind gifts from Prof Joyce Harper and Prof Joy Delhanty in University College London, UK). All probes were labeled by nick translation with the fluorophores of fluorecein-11-dUTP (Amersham, UK) for X probe and rodamine-4-dUTP (Amersham, UK) for Y probe. Probe to chromosome 18 was labeled with both fluorecein-11-dUTP and rodamine-4-dUTP mixed in the 50:50 percentage to give a white fluorophore.

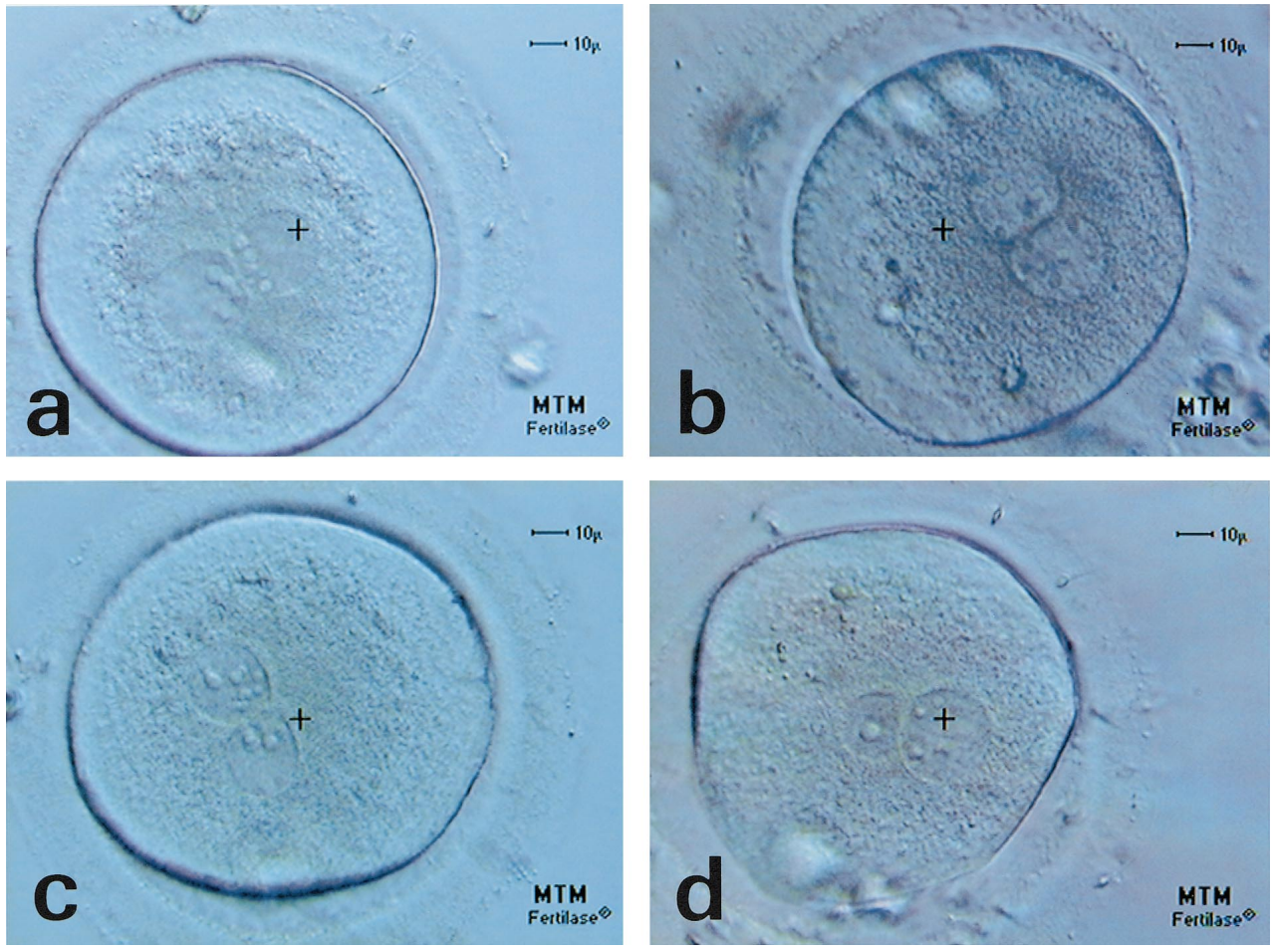

Fig. 1. Pronuclear morphology scoring system modified from that of Scott *et al.* (a) *Z*1, aligned nucleoli, equal numbers; (b) *Z*2, scattered nucleoli, equal numbers; (c) *Z*3, unequal alignment, numbers and size; (d) *Z*4, unequal sized pronuclei. Magnification: ×400.

Before the hybridization, slides were pretreated with pepsin solution for 20 min at 37°C to remove any remaining protein. Through the 5-min rinse in PBS, refixation in 1% *para*-formaldehyde in MgCl2/PBS for 5-min, brief wash in PBS and serial ethanol dehydration, nuclei were ready for denaturation and hybridization. Enough amount of probe was applied on each slide and sealed with rubber cement. Denaturation was performed at 75°C for 3 min and hybridization at 37°C for 45–60 min. After hybridization, slides were washed in 60% formamide, 2× SSC at 42°C for 5 min, 2× SSC at 42°C for 5 min and twice in 4× SSC/0.05% Tween 20 at room temperature for 5 min. The slides were then air-dried and mounted in Vectarshield (Vector Laboratories, USA) antifade medium containing 1 ng/mL 4′,6-diaminidino-2-phenyolindole (DAPI) to counterstain the nuclei. Slides were stored in the dark at 4°C until analysis under the fluorescence microscope. FISH signals was counted using criteria described by Hopman *et al.* (15).

Analysis and Statistics

The chromosomal abnormalities detected by FISH studies included aneuploidy, haploidy, and polyploidy. Embryos comprising more than two cell lines were classified as mosaic embryos (13,16). Since chromosomes 18, X and Y were used in this study, analytic criteria and classification were adopted from those of Munné *et al.* (16).

Differences in the number of nontransferred embryos and those successfully studied in each *Z*-score group were tested for significance using chi-square analysis or Fisher's exact test. The numbers of embryos with all kinds of abnormal chromosomal status

in different *Z*-score group were compared and analyzed using Fisher's exact test. They were also evaluated with chi-square test for trend. $P < 0.05$ was considered significant.

RESULTS

The mean age of these 40 females was 31 years. A total of 368 normally fertilized oocytes (2PN) were evaluated using modified *Z*-score system. Of them, 52 (14%) were *Z*1, 40 (11%) were *Z*2, 174 (47%) were *Z*3, and the rest (102; 28%) were *Z*4. The examples are shown in Fig. 1. After embryo transfer on day 3, 127 nontransferred embryos were recruited for investigation with probes to chromosomes 18, X and Y via FISH methods (Fig. 2). Most (109; 30%) of them were of poor development or arrested. The proportion of each *Z*-scoring group among these 127 embryos was similar (from 30 to 43%) and not statistically significant (see Table II). However, if only embryos of poor development or arrested were compared among different *Z*-score groups, there is a trend of increasing incidence from *Z*1-score group toward *Z*4-score group (chi-square test for trend: $p = 0.016$). In this study, only embryos having at least three blastomeres fixed were included for assessment and were deemed as successfully studied. Ninety-eight embryos (77%) fulfilled the criteria. Embryos with good pronuclear morphology had a better chance to be successfully fixed and studied by FISH than those with poor morphology, though there was no statistical significance about it. The chromosome status of embryos was classified in relation to each *Z*-score group as shown in Table III. The proportion of embryos (12/17; 71%) with normal diploid pattern in *Z*1 group was significantly more than those in *Z*3 (14/40; 35%) or *Z*4 (10/28; 36%) score groups as deduced from total abnormal embryos in each group. The *p* values are shown in Table III.

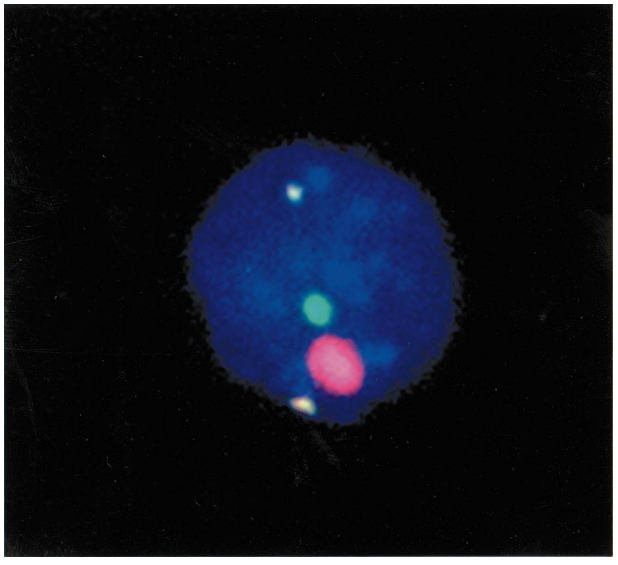

Fig. 2. A blastomere from a spread embryo with *Z*1 socre showed one green signal to chromosome X, one orange signal to chromosome Y, and two white signals to chromosome 18.

DISCUSSION

From the initial proposition of scoring system on pronuclear stage embryo by Scott and Smith (2), several modifications of the system have been done. The clinical application has been also advocated by a lot of IVF centers, although some authors preferred a conservative point of view (8). However, little study mentioned about the mechanism underlying the application. Kahraman *et al.* (11) have reviewed their 65 IVF cycles for preimplantation genetic diagnosis (PGD) and correlated the results of FISH findings with pronuclear morphology of respective embryos. Our study was also designed to clarify the benefit of pronuclear morphology scoring system. For the study by Kahraman *et al.* (11), the chromosome status was assessed by only one blastomere per embryo. Our study applied the whole embryo spreading and the information for embryos is complete. Therefore, our result could be deemed as complement and confirmatory of the study by Kahraman *et al.* (11).

The assumption of this study is based on the idea that a zygote with pronuclei of "poor morphology" will result in an embryo with some mistakes of chromosomes status, since the recent evidences have demonstrated the close correlation of nucleoli and pronuclei with chromosomes. Most of embryos during pronuclear stage are at S or G2 phase when the pronuclear morphology is evaluated during 12–21 hours postinsemination (17). The chromosomes are closely interconnected via nucleoli. Both parental pronuclei go through sequential events of cell cycle with highly synchronized pace. If there is discrepancy and disturbance involving chromosome duplication and division, the chromosome status of resulting embryos will not be normal diploid.

Our result has supported this proposition, so is the result by Kahraman *et al.* (11). They found Group II preembryos (poor pronuclear morphology)

Table II. The Numbers of Embryos with 2PN, of Those Nontransferred, and of Those Successfully Studied in Each *Z*-score Group in These 40 Cases[a]

		Nontransferred no.		
	2PN embryo no.	Suboptimal development	Poor development/arrested	Successfully studied by FISH[b]
*Z*1	52	9	10 (19%)[c,d]	17 (89%)
*Z*2	40	7	10 (25%)[c]	13 (76%)
*Z*3	174	2	51 (29%)[c]	40 (75%)
Z4	102	0	38 (37%)[c,d]	28 (74%)
Total	368	18	109 (30%)	98 (77%)

[a] The percentage is presented in parentheses.
[b] Only embryos having at least three blastomeres fixed were included for assessment and deemed as successfully studied.
[c] Chi-square test for trend: $p = 0.016$.
[d] Chi-square test, $p = 0.034$.

had higher risk of chromosomally abnormal embryos than Group I preembryos (good pronuclear morphology). Our result as shown in Table III demonstrated that the number of embryos with normal diploid pattern in *Z*1 group among nontransferred embryos is significantly larger than that in *Z*3 group or that in *Z*4 group. Besides, there is a trend of increasing percentage of embryos with diploid pattern from *Z*4 to *Z*1 group. Lack of statistical significance when comparison of *Z*2 with *Z*3 or *Z*4 group was made is probably an implication that embryos with *Z*2 score at this stage (18–21 h) are not suitable. From the previous studies using time-lapse recorders to evaluate fertilized oocytes (17,18), the pronuclei morphology with scattered nucleoli is seen earlier than that with aligned nucleoli and is present at 8–8.5 h post-ICSI. Therefore embryos with nucleoli appearing as scattered pattern at a later time have a higher probability of not being diploid and of not being good-potential embryos. Since there is only one observation and assessment

Table III. Summary of Chromosomal Status in These 98 Embryos in Relation to Each Z-Score Group

	*Z*1	*Z*2	*Z*3	*Z*4	Total
Diploid	14	11	29	22	76 (78%)
Normal	12	7	14	10	43 (44%)
Aneuploid		1	3	7	11 (11%)
Monosomy XO1818		1	1	1	3
Monosomy YO1818				1	1
Monosomy XY18O				1	1
Monosomy XY18O and mosaic				3	3
Trisomy18 and mosaic			2	1	3
Mosaics	2	3	12	5	22 (22%)
2N/N			1	3	4
2N/N/4N	1				1
2N/aneuploid/variable ploidy	1	3	11	2	17
Haploid mosaics	1	2	2	1	6 (6%)
Mosaic N/2N		1	1		2
Mosaic haploid/aneuploid	1	1	1	1	4
Polyploid:	2		9	5	16 (16%)
All cells the same ploidy			2	3	5
5N			1	1	2
6N			1	1	2
7N				1	1
Mosaic polyploids	2		7	2	11
Two ploidies (xN/yN)	1		3	0	4
Complex polyploid mosaic	1		4	2	7
Total mosaics (any ploidy)	5 (29%)	5 (29%)	23 (58%)	12 (43%)	45 (46%)
Total abnormal	5(29%)[a,b,c]	6 (46%)[a]	26 (65%)[a,b]	18 (64%)[a,c]	55 (56%)
Total abnormal other than mosaics	0	1 (8%)	3 (8%)	6 (21%)	10 (10%)

[a] Chi-square test for trend: $p = 0.013$.
[b] Fisher's exact test, $p = 0.020$.
[c] Fisher's exact test, $p = 0.033$.

of pronuclear morphology at a relatively later stage without an earlier history in our series, many fast-dividing embryos having had a *Z*1 or *Z*2 score at an earlier stage probably looked like having a *Z*3 or *Z*4 score when being assessed at a later time. This is partly reflected in that our proportions of good pronuclear morphology (*Z*1 and *Z*2 groups) are smaller than those reported in the literature. In our series, 92 (25%) of 368 embryos were belonging to good pronuclear morphology (*Z*1 or *Z*2) while Scott *et al.* (4) reported a proportion of 68% were *Z*1 or *Z*2 embryos and Tesarik and Greco (3) said that 35% of their embryos were of pattern 0 (similar to *Z*1 or *Z*2 score). Nevertheless, our result of relationship between chromosome status and pronuclear morphology may explain why recent clinical applications of such a pronuclear scoring system has higher pregnancy rate when *Z*1 embryos are selected for transferring back. Our observation also implies that if assessment is taken at an earlier stage (e.g., 12–18 h), embryos with *Z*2 score will have more chance to be normal diploid and would be selected for transfer as well. If assessment is taken as late as in our series, embryos with *Z*2 score are less preferable for transfer.

The chromosome status of embryos in our studies is evaluated with whole embryo spreading. It provided more information of the whole embryos than FISH study of one blastomere in PGD. Previous study also demonstrated that if the nucleoli were of grossly different sizes there was an increased risk of the embryo being aneuploid (19,20). Study showed 15% of embryos were mosaic for the sex chromosome and up to 46% of embryos were abnormal (14). Munné *et al.* (21) also reported that 21% of normally developing embryos were mosaics, but only 9% showed more than 3/8 abnormal cells when all the cells were analyzed. In a recent study, postzygotic errors leading to mosaicism is shown to persist throughout preimplantation development *in vitro* (22). The incidence of mosaicism at the blastocyst stage is up to 90.9%. Our result showed more mosaicism in *Z*3 or *Z*4 embryos than in *Z*1 or *Z*2 embryos though without statistical significance. Moreover, the percentage of chromosomal abnormalities other than mosaicism is increased in *Z*4 group embryos. Their implications need to be further clarified.

Good pronuclear morphology is related to good cleavage stage embryos (3–5,11). Our study showed that *Z*4 embryos resulted in more embryos with poor quality or arrested than *Z*1 embryos. There is an increasing tendency of having poor cleavage stage embryos from *Z*1-score group toward *Z*4-score group (Table II). It was shown that aneuploid cell lines may persist to the blastocyst stage from day 3 postinsemination (23). Another group has demonstrated that not all morphologically abnormal embryos are chromosomally affected and aneuploidy is not associated with morphological abnormality (24). Thus, culture to blastocyst stage might not be an effective tool to select against chromosomally abnormal embryos. Pronuclear morphology scoring system could be an additive tool to select embryos with normal diploid chromosomal status. Our study, though with only three kinds of FISH probes, provides confirmatory evidence. In the future, our proposition could be intensified by the use of more probes to chromosomes or other techniques to disclose detailed chromosome status like comparative genomic hybridization applied in embryos (25).

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