Human Reproduction vol.11 no.11 pp.2502–2506, 1996

Undocumented embryos: do not trash them, FISH them

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Pronuclei formation is routinely assessed 16–20 h after oocyte insemination in in-vitro fertilization (IVF). Occasionally, the pronuclei disappear before this time, rendering them as 'undocumented'. Since the number of pronuclei detected is used to distinguish normal from abnormal embryos in the context of ploidy, the diploidy of undocumented embryos is questionable, and therefore they are routinely discarded. The introduction of fluorescent in-situ hybridization (FISH) technology allows the assessment of ploidy status in undocumented embryos that continue to cleave to form blastomeres. In this study, we used FISH to analyse the chromosomal status of 23 undocumented embryos obtained from 10 patients. Biopsied blastomeres were fixed and probed for five chromosomes (X, Y, 13, 18, 21). Diploidy was confirmed in 13 (57%) embryos while the remaining 10 embryos displayed various chromosomal anomalies. Six of the diploid embryos were transferred subsequently to the patients. One ongoing pregnancy was achieved following transfer of an undocumented, analysed embryo, which was already cleaved when assessed 20 h after insemination. We suggest that accelerated dismantling of the pronuclear membrane and subsequent cleavage do not necessarily indicate abnormal chromosomal content and may result in normal pregnancy. In a patient with a small number of embryos, FISH may be used to ascertain diploidy of undocumented embryos, thereby increasing the number of available embryos for transfer.

Key words: embryo FISH/ ploidy/preimplantation diagnosis/ pronuclei/undocumented embryo

Introduction

The morphological correlate of normal fertilization of the human oocyte is the appearance of two pronuclei which can be detected as early as 5 h post-insemination (Trounson and Osborn, 1993). The pronuclear membranes break down 20–22 h post-insemination, resulting in their disappearance, and allowing subsequent cleavage. The time frame of pronuclei appearance and disappearance is of utmost importance in an in-vitro fertilization (IVF) procedure, since their number is taken to reflect the ploidy of the zygote: two pronuclei suggest diploidy, while three or more pronuclei indicate polyploidy. Such polyploid embryos are discarded in an effort to minimize the risk of abnormal pregnancy. Unipronuclear zygotes have been the subject of intense investigation, in which fluorescent in-situ hybridization (FISH) technology has been used (Staessen *et al.*, 1993; Palermo *et al.*, 1995; Sultan *et al.*, 1995).

Routine IVF protocols dictate pronuclei assessment 12-20 h after insemination (Trounson and Osborn, 1993); however, occasionally the pronuclei disappear before they can be assessed. Since the chromosomal composition of these embryos is questionable, most IVF programmes will define them as 'undocumented embryos' and choose not to transfer them.

The exact frequency of early pronuclei disappearance is not known, but can be estimated based on data regarding timing of first cleavage. Cummins *et al.* (1986) have shown that first cleavage division occurs 33.6 h after insemination. Osborn (1991) presented data on accelerated first cleavage. Specifically, 1.1, 3.5 and 16% of zygotes undergo first cleavage division at 20–22, 22–24 and 24–26 h after insemination respectively. In a series of 1364 inseminated oocytes, Trounson and Osborn (1993) have identified accelerated first cleavage in eight embryos (0.6%). We have evaluated 2000 inseminated oocytes in our programme. In 20 fertilized oocytes (1%) the pronuclei disappeared before their first assessment, 16–20 h post-insemination (unpublished data).

A single undocumented embryo in a batch of multiple, well-documented embryos constitutes a minor challenge, since discarding this embryo will hardly influence the chances of achieving pregnancy in a given treatment cycle. In contrast, a situation in which a patient has only a small number of embryos, of which one (or more) is undocumented, presents a clinical dilemma. The development of FISH technology, and its implementation in blastomere pre-implantation diagnosis, offers a potential solution to these situations. FISH has been used for preimplantation diagnosis and in the chromosomal assessment of abnormally developing IVF embryos (Griffin *et al.*, 1994; Munne *et al.*, 1994a,b, 1995; Harper *et al.*, 1994, 1995a,b). The aim of the present communication is to report our experience with FISH analysis of undocumented embryos. Our data demonstrate that undocumented embryos, or in the broader sense, embryos displaying accelerated cleavage, are not necessarily abnormal.

Materials and methods

Patients and embryos

Ten IVF patients were enrolled in this study. Ovarian stimulation with daily injections of human menopausal gonadotrophin was

Table I. Summary of IVF treatment cycles

Patient no.	Age (years)	Oocytes retrieved	IVF/ICSI[a]	Assessment time[b] (h)	2 PN zygotes	Undocumented embryos	No. of cells[e]	Embryo grade
1[c]	31	11	ICSI(6)	18	2	3[d]	6,8	4,4
1	31	8	ICSI(4)	20	2	1	8	5
2	35	14	IVF	19	10	1	7	2+
3	35	27	IVF	21	20	1	4	5
4	35	11	ICSI(7)	18	6	1	3	3
5	31	16	ICSI(14)	18	1	10	2–6	2 (all)
6	32	4	IVF	18.5	2	1	7	2+
7	40	5	IVF	21	1	3	6–7	2+, (all)
8	34	23	IVF	18	15	2	3-6	2+, 4
9	26	25	IVF	18.5	7	1	7,8	2+
10	29	32	ICSI(29)	18	14	4	9–12	3 (all)

[a]The number of metaphase II oocytes as determined in stripped oocytes prepared for ICSI is given in parentheses.
[b]Hours post-insemination.
[c]Patient no. 1 underwent two treatment cycles.
[d]Only two embryos could be analysed.
[e]At the time FISH was performed.

initiated after pituitary down regulation in six patients [mid-luteal phase decapeptyl® CR 3.75 mg (D-Trp_6-luteinizing hormone-releasing hormone (LHRH), Ferring, Malmo, Sweden)], or on cycle day 3 in four patients. Patients were monitored by daily serum oestradiol and progesterone measurements, and serial transvaginal sonography. Ovulation was triggered by human chorionic gonadotrophin (HCG) (10 000 IU, Chorigon, Teva, Petach Tikva, Israel) when the leading follicle was >16 mm in diameter. Oocyte retrieval was performed 36 h later under vaginal ultrasound guidance. All 10 patients had undocumented embryo(s) in at least one IVF treatment cycle. An undocumented embryo was defined as an embryo in which no pronuclei could be identified 16–20 h after insemination. Six patients had regular IVF, while intracytoplasmic sperm injection (ICSI) was performed in five treatment cycles due to severe male infertility. In one patient (no. 5), ICSI was performed because of multiple undocumented embryos in previous regular IVF cycles, during which FISH analysis could not be performed, as the embryos were totally fragmented by day 3. In the subsequent ICSI cycle, 10 of the 16 embryos had already cleaved when assessed for pronuclei, six of which could be analysed. Patient no. 7 had three undocumented embryos out of four. Due to her age (40 years), the FISH procedure was performed on all four embryos.

The patients gave informed consent for the FISH analysis on their undocumented embryos.

A total of 23 embryos was analysed and morphologically graded according to Veeck (1986). Seven embryos had already undergone cleavage when first assessed 18–20 h post-insemination. In five additional embryos, first cleavage was noted 22–25 h post-insemination.

Blastomere biopsy and fixation

Each embryo was held gently by a holding micropipette (20 micron diameter aperture). A 10 micron diameter aperture micropipette filled with acid Tyrode's (pH 2.4; Sigma Chemical Co., St Louis, MO, USA), was used to drill the zona pellucida. The hole so created was slightly smaller than the blastomere size (~40 microns). A 40 micron micropipette filled with medium was inserted through this opening, and the nearest blastomere(s) was aspirated. Each blastomere was then fixed individually on a glass slide according to Tarkowski's technique (Tarkowski, 1966) with minor modification. Each blastomere was held for 2–4 min in a drop of hypotonic solution [0.5% sodium citrate in water, 3 mg/ml bovine serum albumin (Sigma)], and then transferred onto a slide with minimal volume of the hypotonic solution. A fixative (methanol:acetic acid, 3:1) was dripped onto the blastomere, with an additional 2–3 drops, as necessary, to achieve complete dissolution of the cytoplasm.

FISH technique

FISH was performed as described by Munne *et al.* (1995), with some modifications. Blastomeres were probed for five chromosomes: direct chromosome enumerator probe (CEP) (Vysis; Stuttgart - Fasanenhof, Germany) was used for chromosomes X,Y and 18; indirect alpha satellite (Oncor, Gaithersburg, MD, USA), labelled with digoxigenin was used to probe for chromosomes 13 and 21.

A 6 ml aliquot of the probe mixture was applied to each slide, and the coverslip was mounted and sealed with rubber cement. The slides were heated to 80°C for 5 min, followed by 135 min of incubation for hybridization in a humidified chamber (37°C). The slides were washed with 50% formamide/2×sodium chloride, sodium citrate (SCC), 2×SCC/0.1% Nonidet P-40 (NP-40, Sigma) and phosphate buffer detergent (PBD, Oncor) for 6 min, 4 min and 2 min (twice) respectively. After applying 4,6 diamidino-2-phenylindole (DAPI, Oncor) with antifade, the slides were analysed under a fluorescence microscope (Olympus BX-70) for chromosomes X,Y and 18. The slides were then washed with PBD for 5 min until the coverslips fell off. The signals for chromosomes 13 and 21 were amplified and detected as follows: a 15 min incubation period with each of 15 µl rodamine-labelled anti-digoxigenin, rabbit anti-sheep antibody-I, and anti-rabbit antibody-II. Each incubation period was followed by two washes with PBD. The 13/21 probe was used initially on normal human lymphocytes, of which 90% gave four signals. The rest showed a mixture of three and four signals, with a ratio of 3:2 respectively (D.Manor, unpublished observation). Therefore, blastomeres with three signals were considered normal.

Results

The clinical data describing the IVF cycles that resulted in undocumented embryos are given in Table I. In three patients (nos 1, 5 and 7) the number of undocumented embryos was higher than the number of two pronuclei embryos.

In all, 34 blastomeres from 23 embryos were analysed, of which 20 (59%) yielded normal signals (Table II).

A total of 13 embryos (57%) were diagnosed to be diploid, six of which were transferred to three patients, resulting in

Table II. Summary of blastomere biopsies from undocumented embryos

Patient no.	Cells	Blastomeres biopsied	Results	Transferred
1	6	1	X,X, 18,18, 21/13 × 4[a]	yes
1	8	1	X,X, 18,18, 21/13 × 4[g]	yes
1	8	2	X,Y,Y, 18,18, 21/13 × 3 X,Y,Y, 18, 18, 21/13 × 3	no
2	7	2	X,X, 18,18, 21/13 × 4[g] X,X, 18,18, 21/13 × 4	no[b]
3	4	1	X,Y, 18,18, 21/13 × 3[g]	no[c]
4	3	1	X,Y, 18,18, 21/13 × 4	no[c]
5	4–5	2	X × 5, Y × 2, 18 × 5[d,h] X,X, 18 × 4	no
5	5	1	X × 6, 18 × 5[h]	no
5	4–5	1	X,X, 18 × 3[h]	no
5	4–5	1	X,X, 18 × 3[h]	no
5	3–4	1	X,Y, no 18[h]	no
5	3–5	1	X × 4, Y × 4, 18 × 4[h]	no
6	7	1	X,Y, 18,18[c]	yes
7	7	1	X,X, 18,18, 21/13 × 4[h]	yes[f]
7	6	1	X,Y, 18 × 4, 21/13 × 8	no
7	6	1	X,Y, 18,18, 21/13 × 4	yes
8	4	4	X,X, 18,18, 21/13 × 4 X,X, 18,18, 21/13 × 4 X,X, 18,18, 21/13 × 4 X,X, 18,18, 21/13 × 4	no[c]
8	2	2	X × 4, 18 × 4, 21/13 × 8[g] X × 4, 18 × 4, 21/13 × 8	no
9	8	1	X,Y, 18,18[c]	yes
10	10	2	X,X, 18,18, 21/13 × 4 X,X, 18,18, 21/13 × 4 X,X, 18,18, 21/13 × 4	frozen
10	8	2	X,Y, 18,18, 21/13 × 4 X,Y, 18,18, 21/13 × 4	no[c]
10	12	2	X,X, 18,18, 21/13 × 4 X,X, 18,18, 21/13 × 4	no[c]
10	9	2	X,X,X, 18 × 3, 21/13 × 6 X,X, 18 × 3, 21/13 × 6	no

[a]Detection of three or four signals of 21/13 is interpreted as normal (Weier and Gray, 1992).
[b]Absence of subsequent cleavage precluded transfer of this embryo.
[c]The patients chose not to use these embryo after FISH analysis; therefore, they were left for observation, and subsequently frozen if they progressed to the blastocyst stage.
[d]Further analysis with 21/13 was not attempted in embryos with abnormal signals for X, Y, or 18 chromosomes.
[e]The signal for 21/13 was not detected because of a technical problem.
[f]This embryo has produced an ongoing pregnancy.
[g]Cleaved at 22–25 h post-insemination.
[h]Cleaved at 18–20 h post-insemination.

one ongoing pregnancy. Of special interest was patient no. 7, who had all her four embryos analysed. FISH revealed 4PN mosaicism in her only embryo that was 'documented'. In contrast, two of her three undocumented embryos proved to be normal and were transferred (in April, 1996), resulting in an ongoing pregnancy. A normal female fetus (46 XX) was documented by amniocentesis. This specific embryo was found to have cleaved when first assessed 20 h after insemination.

Illustrations of normal and abnormal blastomeres are given in Figure 1.

Discussion

To the best of our knowledge, this is the first report describing FISH analysis of biopsied undocumented embryos with subsequent transfer of normal embryos. Our preliminary experience indicates that early dismantling of the pronuclei membranes and cleavage do not necessarily reflect abnormal embryo development, and probably should be regarded as an intriguing biological variation. Our data also document the possibility of achieving normal pregnancy from accelerated–cleaved embryos. Increasingly, as the mean age of our IVF patients increases, we face patients with only a few oocytes and embryos. In these cases, all efforts should be made to maximize the number of transferred embryos, including chromosomal analysis of undocumented embryos.

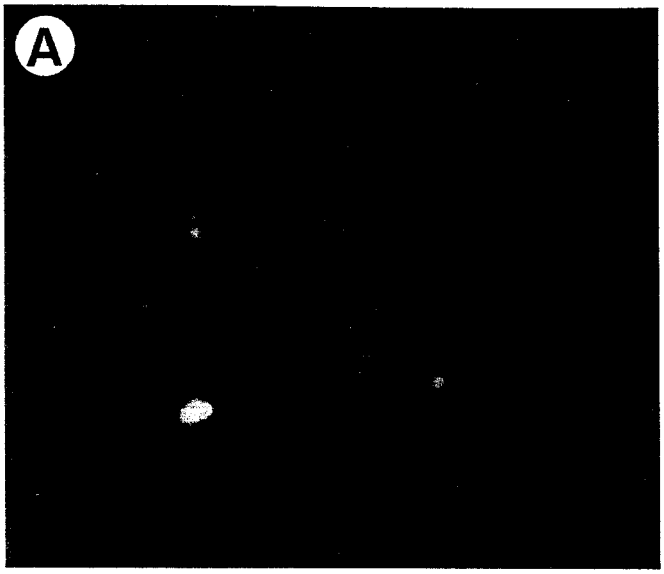
The literature on this entity is quite limited, probably due to the lack of tools to analyse these embryos. Harper *et al.* (1994) have used FISH to document fertilization in embryos that underwent accelerated cleavage. While fertilization was ascertained in all five embryos assessed, a wide range of X chromosome signals were observed. The authors speculate that early oocyte activation may stem from penetration by multiple spermatozoa. Since 13 out of 23 embryos (57%) in our series were found to be diploid, our data document the possibility that accelerated disappearance of the pronuclear membrane following either regular IVF or ICSI, does not necessarily reflect aneuploidy. Furthermore, the mean age of the study patients (32.8) is comparable to the mean age of our general patient population, suggesting that age is not a significant contributory factor in the context of the occurrence of undocumented embryos.

Of interest is the patient who had repeatedly produced embryos that showed accelerated cleavage, during both IVF and ICSI cycles. It is tempting to speculate that this phenomenon was imprinted in her oocytes, which upon fertilization entered an accelerated sequence of development. It will be of interest to see if her embryos can produce a viable pregnancy.

Also worthy of note is the patient who had four embryos, three of which were undocumented. FISH showed that the seemingly 'normal' embryo (which upon initial assessment had two pronuclei) was, in fact, tetraploid mosaic. On the other hand, two undocumented embryos were found to be normal. The pregnancy achieved by one of these two embryos (currently ongoing) establishes the ability of undocumented embryos to implant.

While FISH is an efficient and accurate method (Delhanty *et al.*, 1993), based on a previous report, covering 555 blastomeres (Munné *et al.*, 1995), it should be emphasized that successful FISH analysis can be expected in ~90% of the blastomeres assessed. In addition, Munné *et al.* (1995) raise the possibility of normal diploid embryos in combination with one to three polyploid cells. Hence, in using this technique for assessment of undocumented embryos, patients should be counselled with regard to these potential limitations. The above notwithstanding, the extensive literature on the use of FISH in preimplantation diagnosis (Munné *et al.*, 1994b; Harper *et al.*, 1995a), has driven the technology to a stage of selected clinical use.

Our preliminary experience is still premature to draw conclusions concerning the percentage of undocumented embryos which are genetically normal, and can result in normal pregnancy. However, our data strongly suggest that accelerated dismantling of the pronuclear membrane may constitute a



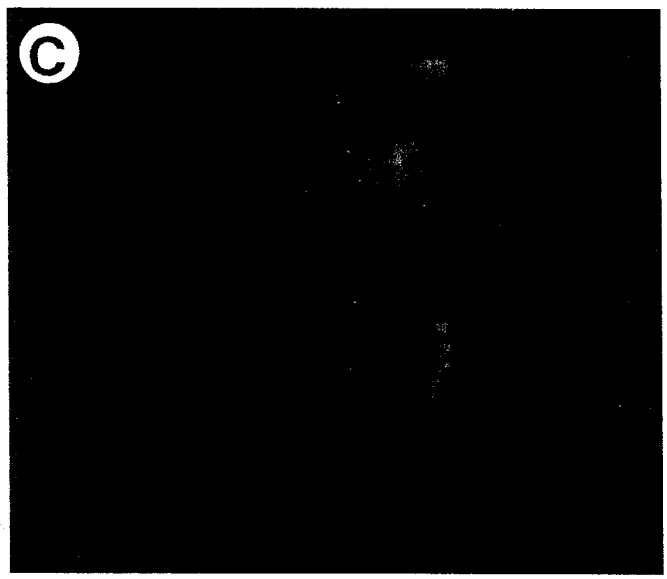


Figure 1. (**A**) A normal blastomere (X,Y, 18,18) before applying the 21/13 probe. The blue and red signals denote the Y and X chromosomes respectively. The two green signals denote the pair of chromosomes 18. (**B**) A normal blastomere (X,X, 18,18, 21/13×4) after applying the 21/13 probe. The two green signals representing the two chromosomes 18 are seen, together with six red signals representing the two X and four 21/13 chromosomes. (**C**) An abnormal blastomere (X,X, 18,18,18) before applying the 21/13 probe. The two red signals represent the X chromosomes, while three green signals reflect the three chromosomes 18.

biological variation that, although rare, does not necessarily reflect abnormal chromosomal composition.

References

Cummins, J.M., Breen, T.M., Harrison, K.L. *et al.* (1986) A formula for scoring human embryo growth rates in *in vitro* fertilization: its value in predicting pregnancy and in comparison with visual estimates of embryo quality. *J. In Vitro. Fertil. Embryo Transfer,* **3,** 284–295.

Delhanty, J.D.A., Griffin, D.K., Handyside, A. H. *et al.* (1993) Detection of aneuploidy and chromosomal mosaicism in human embryos during preimplantation sex determination by fluorescent *in situ* hybridisation, (FISH). *Hum. Mol. Genet.*, **8,** 1183–1185.

Griffin, D.K., Handyside, A.H., Harper, J.C. *et al.* (1994) Clinical experience with preimplantation diagnosis of sex by dual fluorescent *in situ* hybridisation. *J. Assist. Reprod. Genet.*, **11,** 132–143.

Harper, J.C., Robinson, F., Duffy, S. *et al.* (1994) Detection of fertilization in embryos with acceletared cleavage by fluorescent *in situ* hybridization (FISH). *Hum. Reprod.*, **9,** 1733–1737.

Harper, J.C., Coonen, E., Handyside, A.H. *et al.* (1995a) Mosaicism of autosomes and sex chromosomes in morphologically normal, monospermic, preimplantation human embryos. *Prenatal Diag.*, **15,** 41–50.

Harper, J.C., Dawson, K., Delhanty, J.D.A. and Winston, R.M.L. (1995b) The use of FISH for the analysis of IVF embryos: a diagnostic tool for the infertile couple. *Hum Reprod.*, **10,** 3255–3258.

Munné, S., Tang, Y.X., Grifo, J. *et al.* (1994) Sex determination of human embryos using the polymerase chain reaction and confirmation by fluorescence *in situ* hybridization. *Fertil. Steril.*, **61,** 111–117.

Munné, S., Sultan, K.M., Weier, H.-U. *et al.* (1995a) Assessment of numeric abnormalities of X, Y, 18, and 16 chromosomes in perimplantation human embryos before transfer. *Am. J. Obstet. Gynecol.*, **172,** 191–201.

Munné, S., Alikani, M., Grifo, J. and Cohen, J. (1994b) Chromosome abnormalities in arrested human preimplantation embryos: a multiple probe fluorescent *in situ* hybridisation (FISH) study. *Am. J. Hum. Genet.*, **55,** 150–159.

Osborn, J.C. (1991) Asynchronous pronuclear development in human embryos fertilized *in vitro*. *Proc. Xth Ann. Sci. Meet. Fertil. Soc.* #94.

Palermo, G.D., Munne, S., Colombero, L. *et al.* (1995) Genetics of abnormal fertilisation . *Hum. Reprod.*, **10,** 120–121.

Staessen, C., Janssenswillen, C., Devroey, P. and Van Steirteghem, A.C. (1993) Cytogenetic and morphological observations of single pronucleated human oocytes after *in vitro* fertilization. *Hum. Reprod.*, **8,** 221–223.

Sultan, K.M., Munne, S., Palermo, G.D. *et al.* (1995) Chromosomal status of uni-pronuclear human zygotes following IVF and intra-cytoplasmic sperm injection (ICSI). *Hum. Reprod.*, **10,** 132–136.

Tarkowski, A.K. (1966) An air-drying method of chromosome preparations from mouse eggs. *Cytogenetics*, **5,** 394–400.

Trounson, J.C. and Osborn, J. (1993) In vitro fertilization and embryo development. In Trounson, A. and Gardner, D.K. (eds), *Handbook of In Vitro Fertilization.* CRC Press, Boca Raton, Florida, pp. 57–84.

Veeck L.L. (1986) *Atlas of the Human Oocyte and Early Conceptus*. Williams and Wilkins, Baltimore

Weier, H.-U. and Gray, J.W. (1992) A degenerate alpha satellite probe, detecting a centromeric deletion on chromosome 21 in an apparently normal male, shows limitations of the use of satellite DNA probes for interphase ploidy analysis. *Anal. Cell. Pathol.*, **4,** 81–86.

Received on June 10, 1996; accepted on September 20, 1996