**Human Reproduction** vol.12 no.2 pp.321–327, 1997

# The chromosomal constitution of embryos developing from abnormally fertilized oocytes after intracytoplasmic sperm injection and conventional in-vitro fertilization

**Catherine Staessen[1] and André C.Van Steirteghem**

Centre for Reproductive Medicine, University Hospital and Medical School, Dutch-speaking Brussels Free University (Vrije Universiteit Brussel), Belgium

[1]To whom correspondence should be addressed at: Centre for Reproductive Medicine, Academisch Ziekenhuis, Vrije Universiteit Brussel, Laarbeeklaan 101, B-1090 Brussels, Belgium

**The aim of this study was to analyse the chromosomal constitution of embryos developing from mono- (1PN) and tripronuclear (3PN) oocytes, after in-vitro fertilization (IVF) and after intracytoplasmic sperm injection (ICSI) into oocytes, by means of the fluorescent in-situ hybridization (FISH) technique with specific probes for the chromosomes X, Y and 18. FISH analysis was carried out on embryos from 3PN oocytes: 106 after ICSI and 71 after conventional IVF. In the 3PN embryos after ICSI, equal ratios of XXX and XXY were observed and no XYY embryos were present. This shows the digynic origin of such 3PN embryos. On the other hand, after conventional IVF, the XYY status indicative of dispermic fertilization was observed in some embryos. After IVF, only 12.7% of the 3PN oocytes developed into embryos with uniformly triploid blastomeres, compared with 55.7% after ICSI ($P < 0.001$). On the other hand, after ICSI only 16.0% of the embryos developing from 3PN oocytes were mosaic, compared with 42.3% after conventional IVF ($P < 0.001$). FISH was also carried out on embryos from 1PN oocytes: 61 after ICSI and 115 after conventional IVF. In 35.6% of IVF embryos developing from 1PN oocytes Y-specific hybridization signals were observed. This indicates that in 70–75% of such cases a spermatozoon had penetrated the oocyte and that only 25–30% of them were parthenogenetic. A significantly higher proportion ($P < 0.001$) of embryos developing from 1PN oocytes were diploid after IVF (48.7%) than after ICSI (27.9%); equal ratios of XX and XY embryos were observed in the two groups. Formation of a single pronucleus in an embryo subsequently shown to be diploid indicates that normal fertilization was followed by asynchronous formation of pronuclei. A significantly ($P < 0.001$) higher proportion of 1PN oocytes developed into haploid embryos after ICSI (31.2%) than after conventional IVF (13.1%). In both groups most of the haploid embryos were X-bearing (IVF, 93.3%; ICSI, 84.2%) and only a few were Y-bearing (IVF, 6.7%; ICSI, 15.8%). A contribution of normal fertilization and androgenetic activation thus led to 1PN oocytes. Gynogenetic and/or parthenogenetic activation, both leading to indistinguishable chromosomal distributions, also contributed to the formation of 1PN oocytes after ICSI and IVF.**

*Key words:* FISH/ICSI/IVF/monopronuclear oocytes/tripronuclear oocytes

## Introduction

In in-vitro fertilization (IVF), normal fertilization is determined by the presence of two distinct pronuclei. Two main aberrations of the number of pronuclei have been reported after conventional IVF and after intracytoplasmic sperm injection (ICSI): monopronuclear (1PN) oocytes, which are generally considered to be due to parthenogenetic activation, and tripronuclear (3PN) oocytes or triploidy. Most of the 1PN and 3PN oocytes obtained after conventional IVF and after ICSI are capable of undergoing subsequent early cleavage divisions. These cleaved embryos have the same morphological appearance as embryos originating from bipronuclear (2PN) oocytes.

Triploidy does not only occur *in vitro*, since after in-vivo conception triploid embryos resulting in abortions or in some exceptional non-viable live births have been observed (Uchida and Freeman, 1985). After in-vivo conception, dispermy was the most common cause of this phenomenon, although some digynic triploids have been reported (Lauritsen *et al.*, 1979; Uchida and Freeman, 1985). Analysis of triploid embryos after conventional IVF has indicated that 86% of them originate from dispermic penetration of the oocyte (Plachot *et al.*, 1989). After ICSI of a single spermatozoon into a metaphase II oocyte, however, three distinct pronuclei have been observed in the surviving oocytes (Palermo *et al.*, 1993). Since only one spermatozoon is injected, the additional pronucleus must be of maternal origin, probably as a result of the non-extrusion of a second polar body.

It is generally accepted that 1PN human oocytes after IVF result from parthenogenetic activation. However, as regards the ploidy status of embryos developing from 1PN oocytes, the proportional incidence of haploidy and diploidy varies between the different reports (Plachot *et al.*, 1987, 1989; Plachot and Crozet, 1992; Staessen *et al.*, 1993). Demonstration of the presence of a Y chromosome, undeniable evidence differentiating parthenogenetic activation from a fertilization-related phenomenon, was not carried out systematically in the earlier cytogenetic investigations.

The aim of this study was first to determine the ploidy status and to identify the sex chromosomes in the cleaving embryos originating from 1PN and 3PN oocytes after ICSI and after IVF by means of the fluorescent in-situ hybridization (FISH) technique. Such analysis of the sex chromosomes in the embryos developing from 3PN oocytes can indicate dispermic origin after conventional IVF and monospermic digynic origin

after ICSI. Secondly, the cell division and the chromosomal segregation patterns in both types of 3PN embryos were analysed and compared. Another goal of this study was to answer the question of whether 1PN oocytes obtained after IVF or ICSI are indicative of parthenogenetic activation or of a failure in the fertilization process.

## Materials and methods

### *IVF and ICSI treatment*

Ovarian stimulation was performed by a desensitizing protocol using the gonadotrophin-releasing hormone agonist buserelin acetate (Suprefact; Hoechst, Brussels, Belgium) in association with human menopausal gonadotrophin (Humegon from Organon, Oss, The Netherlands or Pergonal from Serono, Brussels, Belgium). Ovulation was induced by administering 10 000 IU of human chorionic gonadotrophin (HCG; Pregnyl from Organon or Profasi from Serono) (Smitz *et al.*, 1988, 1992). Oocyte retrieval was carried out by ultrasound-guided puncture 36 h after HCG administration.

The cumulus–corona–oocyte complexes (CCOC) were placed in 25 µl droplets of Ménézo B2 medium (BioMérieux, Montalieu Vercieu, France) covered by lightweight paraffin oil and incubated in an humidified 37°C incubator (5% $CO_2$, 5% $O_2$ and 90% $N_2$).

For conventional IVF, 2–6 h after oocyte collection 2000–3000 progressively motile spermatozoa were added to each drop of medium containing a CCOC (Staessen *et al.*, 1989). For ICSI, after removing the surrounding cumulus and corona cells, the nuclear maturation of the oocytes was assessed under an inverted microscope. Oocytes which had extruded their first polar body, i.e. metaphase-II oocytes, were then injected with a single spermatozoon into the ooplasm (Van Steirteghem *et al.*, 1993a,b, 1995). Further culture of injected oocytes was performed in 25 µl droplets of Ménézo B2 medium under lightweight paraffin oil and the fertilization was checked 16–18 h later (Nagy *et al.*, 1995).

For conventional IVF as well as after ICSI, fertilization was confirmed by the presence of two distinct pronuclei and polar bodies (often difficult to visualize in the CCOC in conventional IVF) at ×400 magnification using an inverted microscope. In our centre, only the embryos derived from oocytes with two distinct pronuclei with nucleoli were transferred. After conventional IVF, 1PN oocytes were re-evaluated 4 h after the first observation (Staessen *et al.*, 1993). If only one pronucleus was still present or no nucleus was visible, the embryos were not replaced.

1PN and 3PN oocytes were further cultured for another 24 or 48 h and the embryos were spread as a whole or the blastomeres were spread separately. In the latter case, the zona was removed by exposure to acid Tyrode's solution for 1–2 min. Separate blastomeres were obtained by transferring a zona-free embryo into sodium- and calcium-free phosphate-buffered saline (PBS) with 0.125% trypsin. The individual blastomeres or the whole embryo were then transferred in a 1–2 µl droplet of 0.01 N HCl/0.1% Tween 20 solution (Coonen *et al.*, 1994) onto a slide. A small circle was etched on the back of the slide to facilitate localization of the nuclei after fixation and hybridization. The embryos or blastomeres were observed constantly during spreading by using an inverted phase-contrast microscope (Olympus CK2 with ×5, ×10, ×40 lenses; Olympus Optical Co, Tokyo, Japan). After spreading, the slides were left to dry, washed in PBS for 5 min and dehydrated by means of an ethanol series.

### *FISH procedure*

The FISH procedure as described in 1994 by Coonen *et al.* was used. Briefly, the nuclei were digested with pepsin (from porcine stomach mucosa: 100 µg/ml; Sigma) in 0.01 N HCl for 20 min at 37°C. The slides were rinsed in MilliQ UF System water (Millipore, Saint-Quentin, France) and 1× PBS and fixed for 10 min in 1% paraformaldehyde in PBS at 4°C. After fixation, the slides were first rinsed in 1× PBS and then in MilliQ water and then dehydrated by means of an ethanol series.

The triple-target FISH technique was performed using directly labelled DNA probes for chromosomes X, Y and 18. The X [Vysis GmbH (Stuttgart-Fasanenhof, Germany), Alpha Satellite DNA probe, spectrum Green] and Y (Vysis, Alpha Satellite DNA probe, spectrum Orange) probes were used for gender determination and the autosome 18 probe (Vysis, Alpha Satellite DNA probe, 1:1 mixture Green/Orange spectrum) to determine ploidy status. The hybridization mixture was added to the slide under a coverslip, and the nuclear and probe DNA were denatured simultaneously for 4 min at 75°C. The slides were then incubated in a moist chamber at 37°C for 30–60 min to allow hybridization of the DNA probes. After hybridization, the slides were washed for 5 min with 60% formamide/2× SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7) and 5 min with 2× SSC at 42°C, followed by an additional 5 min wash at room temperature with 4× SSC/0.05% Tween 20 solution. The slides were dehydrated by means of an ethanol series and mounted in the antifade medium Vectashield (Vector Laboratories, CA, USA) containing 1.25 ng/ml 4′,6-diamidino-2-phenylindole (DAPI) to counterstain the nuclei. The nuclei were examined using a Zeiss Axioskop fluorescence microscope (Zeiss, Oberkochen, Germany) with the relevant filter set: filter 10 (FITC), filter 02 (DAPI) and filter Omega (FITC/Texas red). All the slides were observed and interpreted by two independent observers.

### *Statistics*

To analyse differences in distribution between the two groups, $\chi^2$ tests were performed. $P < 0.05$ was considered significant.

## Results

In one year of conventional IVF, we observed pronuclei in 6135 oocytes: 88.6% of oocytes had two pronuclei, 7.7% had one pronucleus and 4.2% had three pronuclei. Over the same period in the ICSI programme, we observed 6963 oocytes with pronuclei: 88.5% had two pronuclei, 5.0% had one pronucleus and 6.5% had three pronuclei. The total of abnormally fertilized oocytes was similar after ICSI and after IVF, but the relative proportion of oocytes with one or three pronuclei was significantly different in the two assisted reproduction techniques ($P < 0.01$).

### *Tripronuclear oocytes*

The early development of the 3PN oocytes obtained after IVF and ICSI is summarized in Table I. The overall cleavage rate of 3PN oocytes after ICSI and IVF was similar to the development of normally fertilized oocytes. After ICSI, the speed of development of 3PN oocytes was slower than the speed of development of 2PN oocytes and there were more 3-cell embryos developing from the 3PN oocytes compared to the 2PN oocytes ($P < 0.001$). In contrast, 3PN oocytes after IVF cleaved more rapidly than 2PN oocytes; there were also more 3-cell embryos developing from the 3PN oocytes as compared to the 2PN oocytes ($P < 0.001$). When we compared the cleavage of 3PN oocytes obtained after ICSI and IVF, we observed no difference in percentage of 3-cell embryos, but

**Table I.** Cleavage characteristics of embryos developing from bi- and tripronuclear oocytes obtained after intracytoplasmic sperm injection (ICSI) and conventional in-vitro fertilization (IVF). Values in parentheses are percentages

| Procedure | Oocytes | | Cleaved embryos on day 2 | | | | | |
|---|---|---|---|---|---|---|---|---|
| | No. of pronuclei | Number (A) | Total number (B) | % of A | Cleavage stage | | | |
| | | | | | 2-cell (% of B) | 3-cell (% of B) | 4-cell (% of B) | 5- to 8-cell (% of B) |
| ICSI | 2 | 2827 | 2434 | 86.1 | 254[a] (10.4) | 286[a] (11.8) | 1360[a] (55.9) | 534[a] (21.9) |
| ICSI | 3 | 643 | 536 | 83.4 | 137[b,e] (25.6) | 113[b,e] (21.1) | 196[b,e] (36.6) | 90[b,e] (16.8) |
| IVF | 2 | 900 | 798 | 88.7 | 147[c] (18.4) | 98[c] (12.3) | 384[c] (48.1) | 169[c] (21.2) |
| IVF | 3 | 323 | 288 | 89.2 | 56[d,f] (19.4) | 58[d,f] (20.1) | 77[d,f] (26.7) | 97[d,f] (33.7) |

[a–f]Contingency table: a versus b, $P < 0.001$; c versus d, $P < 0.001$; e versus f, $P < 0.001$.

**Table II.** Results of fluorescent in-situ hybridization analysis for the embryos developing from tripronuclear oocytes obtained after intracytoplasmic injection (ICSI) and conventional in-vitro fertilization (IVF)

| | ICSI ($n$ = 106) | IVF ($n$ = 71) |
|---|---|---|
| Uniformly triploid | | |
| XXX181818 | 28 | 4 |
| XXY181818 | 31 | 5 |
| XYY181818 | 0 | 0 |
| | 55.7%[a] | 12.7%[a] |
| Triploid mosaics | | |
| with XXX181818 | 6 | 5 |
| with XXY181818 | 7 | 5 |
| with XYY181818 | 0 | 5 |
| | 12.3%[b] | 21.1%[b] |
| Uniformly diploid | | |
| XX1818 | 9 | 10 |
| XY1818 | 5 | 5 |
| | 13.2%[c] | 21.1%[c] |
| Uniformly haploid | | |
| X18 | 0 | 1 |
| Uniformly tetraploid | | |
| XXXX18181818 | 1 | 0 |
| Haploid/diploid | | |
| $n$/2$n$ | 2 | 1 |
| Complex mosaic | 17 | 30 |
| | 16.0%[d] | 42.3%[d] |

Distribution between different categories for IVF and ICSI: $P < 0.001$.
[a]$P < 0.001$, [b]$P < 0.01$, [c]$P < 0.01$, [d]$P < 0.001$ (contingency table).

after IVF we observed more embryos at the 5- to 8-cell stage than after ICSI ($P < 0.001$).

From the ICSI and conventional IVF programmes respectively, 106 and 71 cleaving embryos originating from 3PN oocytes were analysed (Table II). From these 177 embryos, a total of 798 blastomeres were counted before spreading, i.e. a mean of 4.5 per embryo. In all, 673 interphase nuclei were observed and analysed, i.e. a mean of 3.8 per embryo. The difference between the number of spread blastomeres and the number of analysed nuclei is partially due to the loss of some nuclei and partially to the presence of anucleated blastomeres. Since most of the embryos were spread as a whole, we cannot distinguish between the two explanations.

For the FISH analysis, we grouped the results in Table II as follows: (i) uniformly triploid embryos in which all the blastomeres analysed clearly showed three sex chromosomes and three chromosomes 18, (ii) embryos composed of uniformly triploid blastomeres together with blastomeres showing different chromosome mosaicisms, (iii) uniformly diploid embryos, (iv) uniformly haploid embryos, (v) uniformly tetraploid embryos, (vi) embryos with a combination of haploid and diploid blastomeres and (vii) complex mosaic embryos in which all nuclei have a different chromosome constitution. A significantly different distribution of these categories was observed in embryos obtained after ICSI and after IVF ($P < 0.001$).

In the 3PN embryos obtained after ICSI, 59 out of the 106 embryos (55.7%) were uniformly triploid. Of these, 28 (47.5%) revealed an XXX and 31 (52.5%) an XXY pattern for the sex chromosomes; no XYY were found. Moreover, in 13 (12.3%) embryos we observed triploid nuclei XXX181818 or XXY181818 together with mosaic nuclei, indicative of irregular division. This shows that at least the first mitotic division was regular. Here too, no XYY181818 nucleus was found. On the other hand, after IVF, only nine (12.7%) out of 71 analysed embryos were uniformly triploid; of these, four (44.5%) were XXX and five (55.5%) were XXY. In another 15 embryos we found triploid nuclei together with mosaic nuclei, and here the XYY181818 pattern was observed in five embryos. The first mitotic division could be considered normal in 72 ICSI embryos (68.0%), which was a significantly higher ($P < 0.001$) proportion than in the IVF embryos ($n$ = 24, 33.8%). The number of uniformly diploid embryos was significantly lower ($P < 0.001$) after ICSI ($n$ = 14, 13.2%) than after IVF ($n$ = 15, 21.1%). Complex mosaic embryos, indicative of chaotic division from the first mitotic division, were more frequently observed after IVF (42.3%) than after ICSI (16.0%). The results from the separate fixation of blastomeres indicated that multinuclear blastomeres contributed to the mosaicism of the nuclei observed. In addition, one complete haploid embryo was observed after regular IVF and one tetraploid embryo after ICSI. Two embryos after ICSI and one after IVF were composed of a combination of haploid and diploid cells.

The FISH results for ICSI embryos were also analysed with regard to the status of the polar bodies at the time of fertilization

**Table III.** Results of fluorescent in-situ hybridization analysis for the embryos developing from tripronuclear oocytes obtained after intracytoplasmic injection (ICSI) in relation to the polar body status at the time of fertilization assessment. There were no significant differences within ploidy groups

| | Polar body (PB) status | | |
|---|---|---|---|
| | One PB | Two PB | Fragmented PB |
| Uniformly triploid | | | |
| XXX181818 | 14 | 2 | 11 |
| XXY181818 | 14 | 0 | 13 |
| XYY181818 | 0 | 0 | 0 |
| | 50.9% | 50.0% | 60.0% |
| Triploid mosaics | | | |
| with XXX181818 | 4 | 0 | 2 |
| with XXY181818 | 2 | 0 | 3 |
| with XYY181818 | 0 | 0 | 0 |
| | 10.9% | | 12.5% |
| Uniformly diploid | | | |
| XX1818 | 5 | 1 | 3 |
| XY1818 | 4 | 0 | 1 |
| | 16.4% | 25.0% | 10.0% |
| Uniformly tetraploid | | | |
| XXXX18181818 | 0 | 0 | 1 |
| | | | 2.5% |
| Haploid/diploid | | | |
| *n*/2*n* | 1 | 0 | 1 |
| | 1.8% | | 2.5% |
| Complex mosaic | 11 | 1 | 5 |
| | 20.0% | 25.0% | 12.5% |

**Table IV.** Results of fluorescent in-situ hybridization analysis for the embryos developing from monopronuclear oocytes obtained after intracytoplasmic injection (ICSI) and conventional in-vitro fertilization (IVF)

| | ICSI ($n$ = 61) | IVF ($n$ = 115) |
|---|---|---|
| Diploid | | |
| XX1818 | 10 | 31 |
| XY1818 | 6 | 25 |
| YY1818 | 1 | 0 |
| | 27.9%[a] | 48.7%[a] |
| Haploid | | |
| X18 | 16 | 14 |
| Y18 | 3 | 1 |
| | 31.2%[b] | 13.1%[b] |
| Triploid | | |
| XXX181818 | 0 | 1 |
| Mosaicisms | | |
| X18/XX1818 | 6 | 10 |
| X18/XXX181818 | 2 | 2 |
| X18/XXX181818/XXXX18181818 | 1 | 0 |
| | 14.8% | 10.4% |
| Complex mosaics | | |
| with Y | 12 | 15 |
| without Y | 4 | 16 |
| | 26.2% | 27.0% |

Distribution between different categories for IVF and ICSI: $P < 0.001$.
[a]$P < 0.001$, [b]$P < 0.001$.

assessment (Table III). The status of the PB was recorded at the moment of fertilization assessment for 99 out of the 106 embryos analysed. In these 3PN oocytes we observed one polar body (PB) in 55% of the oocytes, fragmented PB in 40% of the oocytes and two PB in only 4% of the oocytes. The results of the 3PN analysis were grouped in relation to the status of the PB and are given in Table III. No difference in the distribution among the different categories was observed for either one PB, fragmented PB or two PB. The PB status of the oocytes obtained in the conventional IVF programme was not recorded as it is not reliably defined, owing to the presence of remaining cumulus cells.

The relationship between the presence of triploid cells and the number of blastomeres at the first embryo evaluation was assessed. From the embryos developing from the 3PN oocytes obtained after IVF and showing a 2-cell stage at the first embryo evaluation, 42.5% were uniformly triploid or contained triploid cells. This figure was 6% for those showing a 3-cell stage, 43% for those showing a 4-cell stage, and 16% for those showing more than four cells ($P < 0.05$) at the first embryo evaluation. After ICSI, 70% of the 3PN oocytes showing a 2-cell stage were uniformly triploid or contained triploid cells. This figure was 62.5% for the 3-cell stage, 70% for the 4-cell stage and 68% for the oocytes showing more than four cells (no statistically significant difference) at the first embryo evaluation.

### *Monopronuclear oocytes*

A total of 176 embryos derived from 1PN oocytes were analysed successfully by the FISH technique: 115 originating from conventional IVF and 61 from the ICSI programme. For the FISH analysis, we grouped the results shown in Table IV as follows: (i) uniformly diploid embryos in which all the blastomeres analysed clearly showed two sex chromosomes and two chromosomes 18, (ii) uniformly haploid embryos, (iii) uniformly triploid embryos, (iv) embryos with a combination of haploid and diploid blastomeres, or a combination of haploid and triploid, or a combination of haploid, triploid and tetraploid blastomeres, (v) complex mosaic embryos in which all nuclei had a different chromosome constitution; the distinction between mosaics with or without a Y chromosome was made. Globally, a significantly different distribution of these categories was observed in embryos obtained after ICSI and after IVF ($P < 0.001$).

Among the 1PN embryos obtained after ICSI, a significantly lower ($P < 0.001$) incidence of diploid embryos was observed: 27.9% after ICSI compared to 48.7% after IVF. The distribution of XX- and XY-bearing embryos was similar between the two groups: among the diploid embryos a Y chromosome was present in 41.2% (7/17) of the cases after ICSI and in 44.6% (25/56) of the cases after IVF. One interesting case was observed after ICSI: an 8-cell stage 'embryo' with five informative nuclei all had a YY1818 chromosome status, indicating penetration of the oocyte by a diploid spermatozoon or haploid spermatozoon with diploidization afterwards, and no involvement of the oocyte nuclear material.

Haploid embryos were observed in 31.2% (19 out of 61) of the cases after ICSI and 13.1% (15 out of 115) of the cases after IVF ($P < 0.001$). For both IVF and ICSI, in addition to a majority of haploid X-bearing embryos (IVF: 93.3%; ICSI: 84.2%), some haploid Y-bearing embryos indicative of the male nucleus were found (IVF: 6.7%; ICSI: 15.8%). After ICSI in nine embryos (14.8%) and after IVF in 12 (10.4%),

different cell lines were observed. Moreover, after IVF and after ICSI, in respectively 26.2 and 27.0% of the embryos, complex mosaicism was observed.

Finally, the results of the FISH analysis of embryos generated from 1PN oocytes demonstrated that after IVF in 41 out of the 115 embryos (35.6%) and after ICSI in 22 out of 61 embryos (36.1%) a Y-specific hybridization signal was present.

## Discussion

### *Tripronuclear oocytes*

One of the major objectives of this study was to find the origin of the extra pronucleus in 3PN oocytes obtained after ICSI and after conventional IVF. The examination of the sex chromosomes can indicate the origin of this extra pronucleus. In cases of dispermy, the theoretical distribution for the gonosomes is 25% XXX, 50% XXY and 25% XYY. Digyny would result in 50% XXX and 50% XXY. In the embryos from 3PN oocytes after ICSI, equal ratios of XXX and XXY were observed; none of the embryos had an XYY status. This observation clearly demonstrated the digynic origin of the 3PN oocytes after ICSI, as was expected, since only one spermatozoon was injected and most of the 3PN oocytes had only one polar body. It is the non-extrusion of the second polar body which leads to the formation of the extra, third, pronucleus. After conventional IVF, a digynic origin of the 3PN oocytes cannot be excluded. In this study, a distribution of nine XXX, 10 XXY and five XYY was found in the embryos from 3PN oocytes, which is not significantly different from the expected distribution for dispermic fertilization. The sex chromosome pattern XYY was previously reported in 3PN embryos after conventional IVF (Pieters *et al.*, 1992; Jamieson *et al.*, 1994).

One of the further obvious findings from comparing chromosome complements in embryos derived from 3PN oocytes after ICSI and after conventional IVF was the high incidence after ICSI of uniformly triploid blastomeres, indicative of regular chromosome segregation, in contrast with the low incidence of uniformly triploid blastomeres after conventional IVF. Furthermore, some embryos after IVF and ICSI also showed some mosaic blastomeres besides uniformly triploid cells, which is indicative of mosaicism originating at the second or third mitotic division and implies that the first division was regular. This demonstrates that for IVF as well as for ICSI, all 69 chromosomes of the 3PN oocytes can gather on the metaphase plate of the first division and divide regularly into daughter cells with a full triploid complement. After in-vivo conception, 1–2% of all clinically confirmed conceptions are triploid (Boué *et al.*, 1975; Jacobs *et al.*, 1978). The further developmental potential of these embryos may depend on the frequency of real triploid cells. For this reason, the early recognition of 3PN oocytes is of considerable clinical significance in IVF, and especially after ICSI, where a majority of 3PN embryos developing into uniform triploid blastomeres are observed.

Furthermore, the comparison of chromosome complements in embryos derived from 3PN oocytes demonstrated a low incidence of mosaic blastomeres after ICSI, in contrast with the high incidence after conventional IVF. The mosaics observed were of complex origin, indicative of a chaotic chromosome segregation from the very first division onwards. This may imply the presence of an aberrant mitotic spindle.

Traditional cytogenetic studies performed by metaphase analysis in embryos developing from 3PN oocytes obtained after conventional IVF have reported incidences of mosaicism ranging from 14 to 29% (Michelmann *et al.*, 1986; Kola *et al.*, 1987; Angell, 1989; Pieters *et al.*, 1992; Ma *et al.*, 1995). Analysis by FISH (Cohen *et al.*, 1994; Palermo *et al.*, 1995) showed that, in embryos developing from 3PN oocytes obtained after conventional IVF, 89% were mosaic. With conventional cytogenetic analysis, only a limited number of metaphases for each embryo could be analysed, probably resulting in an underestimation of the incidence of these mosaicisms (Ma *et al.*, 1995). In embryos developing from 3PN oocytes obtained after ICSI, Cohen *et al.* (1994) reported that the incidence of mosaicism determined by FISH was 14%. The major difference between the two types of 3PN oocytes is that after conventional IVF it is dispermic fertilization and after ICSI monospermic fertilization combined with non-extrusion of the second polar body that results in the appearance of the third pronucleus. It has been demonstrated that human centrioles are paternally derived from the mid-pieces of spermatozoa (Sathananthan *et al.*, 1991). Moreover, the enucleation experiments by Palermo *et al.* (1994) provided evidence that the human sperm centrosome controls the first mitotic division after fertilization. When two spermatozoa enter the oocyte, two centrosomes are present and a tripolar spindle is formed. The presence of a tripolar spindle leading to cleavage into three cells was also demonstrated by Plachot and Crozet (1992) in a cytological study using both anti-tubulin antibodies and Hoechst dye. The tripolar spindle will be responsible for disturbed chromosome distribution at the first division. The bipolar spindle present in the 3PN embryos after ICSI leads to a more regular cleavage pattern.

The presence of diploid nuclei in these embryos may be partly explained by the presence of a misleading vacuole, although the incidence remains quite high in view of the fact that experienced embryologists can make a distinction between a vacuole and a pronucleus, which contains clearly distinct nucleoli. Another possible explanation for the presence of diploid cells is the extrusion and subsequent degeneration of a haploid nucleus followed by a normal division involving a normal bipolar spindle. The presence of a tetraploid embryo after ICSI can be explained by an additional error of the first meiotic division or penetration by a diploid spermatozoon.

In spite of the bipolar spindle, mosaic embryos are also present after ICSI. This is not remarkable because in embryos obtained after normal fertilization 17–28% are mosaic, although few are found arising from the first cleavage onwards (Munné *et al.*, 1994). During metaphase II, the nuclear membrane disappears and the spindle microtubules are connected to the chromosomes. The microtubules of the meiotic spindle are mostly located under the polar body, and care is taken to have the first polar body at the 12 o'clock or 6 o'clock position in relation to the holding pipette and to inject right into the

middle of the egg at the 3 o'clock position. Damage to the microtubules during injection, resulting in the formation of an extra nucleus and subsequent chromosome segregation errors leading to mosaicism, cannot, however, be excluded as an explanation for the presence of mosaicisms. Different reports (Balakier, 1993a; Van Blerkom *et al.*, 1984) have demonstrated that 19–23% of the embryos developing from 3PN oocytes after IVF are indeed multinucleated. Multinucleation in one blastomere contributes to the mosaicisms observed in some of these embryos after ICSI and IVF.

The absence of a relationship between polar body status and chromosome complement indicates that spontaneous fragmentation of the first polar body or cleavage into two polar bodies may be quite a normal phenomenon.

An unusual type of division has been reported for about half of triploid oocytes: they divided first into three cells and then into six cells, whereas diploid oocytes divide into two and then into four cells (Kola *et al.*, 1987; Kola and Trounson, 1989; Plachot *et al.*, 1989). We also found in both conventional IVF and ICSI a higher proportion of 3-cell embryos originating from 3PN oocytes at the first evaluation, as compared to 2PN oocytes. 3PN oocytes after conventional IVF dividing into 3-cell embryos have a lower proportion of uniformly triploid cells than those dividing into 2- or 4-cell embryos, thus confirming the presence of a tripolar spindle leading to irregular chromosome distributions. It was rather surprising that after ICSI too we had a higher proportion of 3-cell stage embryos, but there was no relationship here with the chromosomal constitution. However, the cell stage at the moment of observation is only a single observation in a dynamic developmental process. No distinction can be made between the cleavage to a 3-cell embryo on the one hand and the asynchronous cleavage from a 2-cell to a 4-cell embryo which may also result in the temporary observation of a 3-cell embryo.

### *Monopronuclear oocytes*

One of the purposes of this study was to determine the ploidy status and to identify the sex chromosomes of embryos resulting from 1PN oocytes. The persistent observation of a monopronucleus after IVF may be considered the result of parthenogenetic activation. After ICSI, the situation is somewhat different. It is inherent to the ICSI procedure that a single spermatozoon is injected into the oocyte. If a single pronucleus is observed after ICSI, gynogenetic or androgenetic activation of the oocyte has to be considered.

Monopronuclear human oocytes resulting from parthenogenetic activation by calcium ionophore of failed fertilized human oocytes were all found, by DNA measurements, to have indeed a haploid DNA content (Taylor and Braude, 1994).

In this study, 48.7% of the embryos derived from 1PN oocytes after conventional IVF were diploid, with an equal ratio of XX- and XY-bearing embryos. The presence of a Y chromosome is undeniable evidence of fertilization. The observation of a monopronucleus in these embryos assumes a normal fertilization process followed by asynchronous pronuclear formation (Staessen *et al.*, 1993). In a previous study, in 25% of the cases where one pronucleus was observed, a second pronucleus could be identified if the oocytes were examined a few hours later, which indicates asynchronous pronucleus formation (Staessen *et al.*, 1993). As it became routine clinical practice in IVF to re-evaluate 1PN oocytes, in order to dispel confusion with asynchronous pronucleus formation, the incidence of diploid embryos fell from 80 to 50% (Staessen *et al.*, 1993).

After ICSI, diploid embryos developing from 1PN oocytes were also observed: 27.9% were diploid, with equal proportions showing XX or XY signals. These results indicate, however, that some of the 1PN oocytes observed 16–18 h after injection do indeed result from a normal fertilization process. At 8–10 h after injection, the asynchronous appearance of pronuclei has been observed (Nagy *et al.*, 1994); however 16–18 h after injection it was not observed. The systematic introduction of a second observation of 1PN oocytes could also be considered in the ICSI programme.

Of particular interest is the observation of an 8-cell embryo after ICSI with two Y- and two 18-specific signals and, probably, a 46,YY chromosome status. This 'embryo' originated from the development of an oocyte involving only the male genome, which was initially diploid or underwent subsequent diploidization. The female genome was completely extruded or inactivated.

After ICSI and after IVF, 2- to 8-cell embryos developing from monopronucleated oocytes are all found to be uniformly haploid; the incidence of this, however, was significantly higher after ICSI (31.2%) than after IVF (13.1%). For both groups of embryos, 2- to 8-cell embryos with all blastomeres containing only one Y- and one 18-specific signal or, probably, with a 23,Y chromosome status were observed. This again indicates an androgenetic origin: the sperm nucleus had been activated and was involved in the monopronucleus formation while the female genome was completely excluded. Some of the 23,X embryos may be paternal in origin; however, proportionally more 23,X were found, indicative of a higher proportion of activation of the oocyte genome.

Monopronuclear oocytes obtained after ICSI ($n = 21$) and after IVF ($n = 21$) and cleaving into embryos were analysed by Sultan *et al.* (1995) using the FISH technique, giving the following results: after IVF, 14.3% were found to be haploid and 61.9% were diploid, of which about half were XY. After ICSI, the majority (66.7%) of the embryos were found to be haploid and only 9.5% were diploid. Also, one haploid and one diploid embryo contained a Y chromosome. Our results for IVF are quite similar to those of Sultan *et al.* (1995), although for ICSI Sultan *et al.* reported a higher proportion of haploid cases in their small sample.

The presence of 23,X/46,XX mosaics can be explained by activation of the oocyte followed by spontaneous diploidization after the first cleavage. This may also lead to the other observed mosaicisms, i.e. X18/XXX181818 and X18/XXX181818/XXXX18181818.

Complex mosaic embryos, with and without the presence of a Y chromosome, have been observed. After normal fertilization, irregular chromosome segregations leading to mosaicisms have also been reported, although at lower incidences and seldom from the first division onwards (Munné *et al.*, 1994), indicating that 1PN-derived embryos are more unstable.

In all, ~36 % of the analysed embryos originating from 1PN oocytes after conventional IVF and ICSI contained a Y-specific signal. Taking into account that ~50% of the paternal set contains an X chromosome, we can assume that in ~70–75% of the 1PN oocytes after conventional IVF a spermatozoon has penetrated the oocyte, excluding parthenogenetic activation. This is in agreement with the findings by Balakier *et al.* (1993b), indicating that at least 50% of embryos developing from 1PN oocytes originate from sperm-penetrated oocytes. For ICSI, a spermatozoon is always introduced into the cytoplasm of the oocyte, indicating that the male pronucleus is involved in 70–75% of the embryo formation after observation of one pronucleus. In the remaining 25–30%, for IVF as well as for ICSI, activation of the oocyte is assumed.

The morphological observation of a monopronucleus may be indicative of different mechanisms. After ICSI and IVF, apparently 1PN oocytes develop into diploid embryos as a result of asynchronous formation of the pronuclei. The results indicate furthermore, that the proportion of parthenogenetically activated oocytes after IVF is quite low, since in 70–75% of the cases a spermatozoon has penetrated the oocyte. Androgenetic embryos have been found in this study after ICSI as well as after IVF. Gynogenetic and or parthenogenetic activation, both leading to indistinguishable chromosomal distributions, also contribute to the formation of 1PN oocytes.

Since after IVF and after ICSI 50 and 70% respectively of the obtained embryos developing from monopronucleated oocytes were found to be chromosomally abnormal, no risks can be taken and these embryos are always excluded from transfer.

## Acknowledgements

The authors thank the clinical, scientific, nursing and technical staff of the Centre for Reproductive Medicine and especially the colleagues of the microinjection and IVF laboratory. We are grateful to Mrs Sigrid Nerinckx for technical assistance with fluorescence in-situ hybridization. We thank Mr Frank Winter of the Language Educational Centre for correcting the English text. Grants from the Belgian Fund for Medical Research are gratefully acknowledged.

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*Received on May 23, 1996; accepted on October 23, 1996*