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# Multinucleation in cleavage stage embryos

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**BACKGROUND: The aim was to analyse multinucleation in relation to its incidence in time and in the population, and its correlation with clinical variables, with other morphological characteristics and with the implantation rate of cleavage stage embryos. METHODS: Retrospective analysis of 10 388 cleaved embryos from 1395 consecutive IVF/ICSI cycles in 700 patients between January 1, 1999 and June 30, 2002. RESULTS: Multinucleation was observed in 3491 (33.6%) embryos in 1107 cycles (79.4%) of 609 (87%) patients, more frequently on day 2 than on day 3: 2848 (27.4%) versus 1567 (15.1%) [relative risk (RR) = 1.82; 95% confidence interval (CI) = 1.72–1.92]. Its incidence increased with fragmentation: 31.0, 34.4 and 36.5% for fragmentation ⩽10%, 10–20% or 20–30%. It was increased in stimulation cycles that were shorter (34.9 versus 32.0%, RR = 1.09; 95% CI = 1.03–1.15), required a higher FSH dose (34.6 versus 32.0%, RR = 1.08; 95% CI = 1.02–1.14) and yielded more oocytes (34.5 versus 29.7%, RR = 1.16; 95% CI = 1.08–1.25). Four-cell embryos on day 2 showed minimal multinucleation (16.8%) as well as 8-cell embryos on day 3 (15.5%). Embryos counting both 4 blastomeres on day 2 and 8 on day 3 showed minimal multinucleation (11.6%). Multinucleated embryos had a decreased implantation rate: 4.3% in single and 5.7% in double embryo transfers. CONCLUSIONS: Multinucleation is a frequently observed phenomenon. It is associated with impaired cleavage and increased fragmentation and is compromising the ongoing implantation rate. Multinucleation should be part of embryo assessment.**

*Key words:* cleavage stage embryos/embryo assessment/fragmentation/implantation rate/multinucleation



## Introduction

Abnormalities in the DNA synthesis of multinucleated blastomeres (MNB) in cleaving human embryos were demonstrated as early as 1987 (Tesarik *et al*., 1987). Not surprisingly multinucleation has been correlated with an increased rate of aneuploidy and chromosomal abnormalities (Munné and Cohen, 1987; Pickering *et al*., 1993; Kligman *et al*., 1996; Laverge *et al*., 1997).

In daily practice, multinucleation in normally fertilized embryos has been associated with a lower implantation rate. Hardy *et al*. (1993) found multinucleation in 17% of 2–4-cell embryos. From estimations of the size of these multinucleated cells and comparing them with normal mononuclear cells, they concluded that these blastomeres arise from a failure of cytokinesis and that they contribute to cleavage stage arrest *in vitro*. More recently concordant observations were made (Hardarson *et al*., 2001): embryos with uneven-sized blastomeres displayed a much higher multinucleation as well as aneuploidy rate and this eventually resulted in a lower implantation rate. This lower implantation rate of multinucleated embryos has been documented by others (Levy *et al*., 1997; Pelinck *et al*., 1998; Van Royen *et al*., 2001). In extended culture systems, multinucleation was associated with a significantly lower blastocyst formation rate (Alikani *et al*., 2000).

Multinucleation of one or more blastomeres has been reported in 31% of the embryos examined and in 74% of all cycles. Because of this high incidence of multinucleation, its relative ease of detection using light microscopy, and its association with diminished embryo growth potential, lower implantation, clinical pregnancy and live birth rate, it has been advocated that the evaluation of the nuclear status was to be included in the embryo scoring system (Jackson *et al*., 1998).

This retrospective analysis consists of four main parts: (i) data on the incidence of multinucleation over time and in the population; (ii) analysis of factors that might have an impact on multinucleation; (iii) multinucleation in relationship to other morphological characteristics of early cleaving embryos and (iv) impact of multinucleation on the ongoing implantation rate in an IVF/ICSI programme.

## Materials and methods

### *Patients*

Female age ranged from 20 to 45 years of age (mean 32.52 ± 4.33). Main causes of infertility were exclusively male-related in 684 cycles

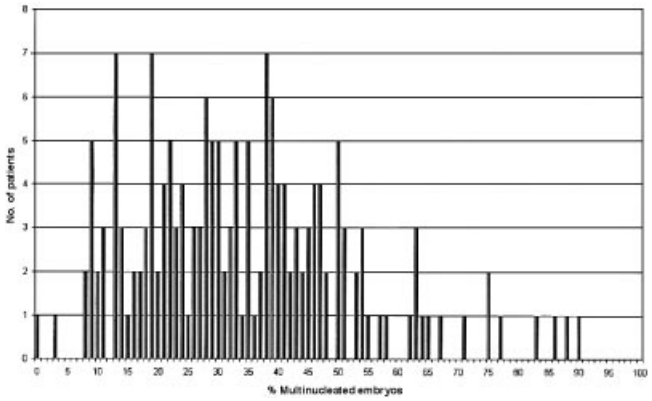


**Figure 1.** Distribution of the percentage of multinucleation over the population for patients with ⩾20 embryos in one or more cycles.

(47.2%): 519 cycles with oligoteratoasthenozoospermia, 112 cycles with obstructive and 13 with non-obstructive azoospermia and 40 cycles with a male immunological factor. Main causes of infertility were female-related in 333 cases (23.0%): 120 tubal, 64 tuboperitoneal, 86 endometriosis, 11 immunological, 32 polycystic ovary syndrome, eight chronic anovulation and 12 oocyte donation cycles. In 172 cycles (11.9%) there was a combined male and female pathology and in 214 cycles (14.8%) the clinical diagnosis was idiopathic infertility. Forty-seven cycles (3.2%) were originally planned as ovulation stimulation with intrauterine insemination, but were converted to IVF because of an unacceptably high number of maturing follicles.

### *Stimulation protocol*

Patients were treated with the long protocol for ovarian stimulation. Desensitization was initiated in the mid-luteal phase with buserelin acetate (Suprefact®; Hoechst, Germany) six times 100 µg per day intranasally. For follicular stimulation, urinary gonadotrophins (Humegon; Organon, The Netherlands) were used in 7% of cycles, purified FSH (Metrodin HP; Serono, Switzerland) in 70% of cycles and recombinant FSH (rFSH; Gonal-F; Serono) in 23% of cycles. When ⩾3 follicles reached a size of ⩾18 mm in diameter, hCG (Profasi; Serono) 10 000 IU i.m. was administered. A transvaginal ultrasound-guided ovum retrieval was performed 37 h later.

### *Laboratory procedures*

Standard IVF/ICSI procedures were used. Culture medium on the day of oocyte retrieval was Ménézo B2 in 25 µl (Laboratoire C.C.D., France) droplets under oil (Sigma no. M8410; Sigma–Aldrich, Belgium). Oocytes were inseminated, each in an separate droplet with 20 000 sperm having a linear motility >22 µm/s in case of IVF. In the case of ICSI, up to 10 injected oocytes were incubated together in a 10 µl Ménézo B2 droplet under oil. On day 1, oocytes were examined for the appearance of two pronuclei and up to 10 fertilized oocytes were cultured together in a 10 µl droplet of Ménézo B2 under oil. On day 2, embryos were rinsed and transferred to individual 10 µl droplets of Medi-Cult M3 medium (Medi-Cult, Denmark) under oil in order to follow their further individual development. All transfers were performed on day 3 after insemination/injection.

All embryos were scored for three parameters on day 2 (41–44 h after insemination/injection) and again on day 3 (66–71 h post insemination/injection): (i) fragmentation, F1: ⩽10% of anucleated fragments, F2: 10–20% of anucleated fragments, F3: 20–30% anucleated fragments, etc.; (ii) number of blastomeres and (iii) number of MNB. The embryos in which, either on day 2 or on day 3 or on both days, one or more MNB were observed will be referred to as multinucleated embryos.

In this study, fragmentation was considered on day 3 exclusively because this offers the most relevant information just prior to transfer.

All observations were made with a Leitz Labovert microscope (Wild Leitz GmbH, Germany) using modulation contrast (Modulation Optics Inc., USA) at ×400 magnification.

### *Outcome analysis*

An ongoing pregnancy was defined as a pregnancy with fetal heartbeat which was ongoing past the first trimester. For calculating the ongoing implantation rate, only concepti reaching the second trimester were considered. Confidence interval analysis (Gardner and Altman, 1986) was used for statistical analysis.

## Results

### *Observed incidence of multinucleation*

#### *Day of observation*

From January 1, 1999 to June 30, 2002 a total of 1450 ovum retrievals in 700 couples resulted in 16 917 oocytes. In 1395 cycles (94%) there was fertilization and a total of 10 388 (61.4%) cleaved embryos was obtained. Of these, 1924 (18.5%) showed multinucleation on day 2 exclusively, 643 (6.2%) on day 3 exclusively whereas 924 (8.9%) showed multinucleation on both days. Therefore, in a total of 3491/10 388 (33.6%) embryos the phenomenon was observed. It was observed in 2848 (27.4%) embryos on day 2 and in 1567 (15.1%) embryos on day 3. This means significantly more observations were made on day 2 [relative risk (RR) = 1.82, 95% confidence interval (CI) = 1.72–1.92].

Multinucleation in one or more embryos was identified in 1107/1395 cycles (79.4%).

#### *Distribution in the patient population*

In order to analyse the distribution, all embryos obtained in different cycles from the same couple were grouped. Multinucleation was seen in 609/700 patients (87%). Considering patients having five or more embryos this ratio increased to 543/585 (93%), for patients with ⩾10 embryos it was 382/394 (97%) and for patients with ⩾20 embryos it even reached 170/171 (99%).

The distribution of the incidence of multinucleation among these 170 patients with ⩾20 embryos is shown in Figure 1. The embryos of each patient originated from one or several cycles. Of these 170 patients there were seven with ⩾75% multinucleated embryos. Incidence of multinucleation was remarkably stable among these patients: in 25/26 cycles there was >50% multinucleation and the only cycle with <50% showed two multinucleated embryos out of five. Ovulation induction was not accelerated in these cycles: the median duration of stimulation was 15 days which is exactly the same as in the overall population.

### *Factors that might have an impact on multinucleation*

#### *Multinucleation and method of fertilization*

Of the 10 388 cleaved embryos, 5284 resulted from regular IVF and 5104 from ICSI. Multinucleation was seen in 1728 (32.7%)

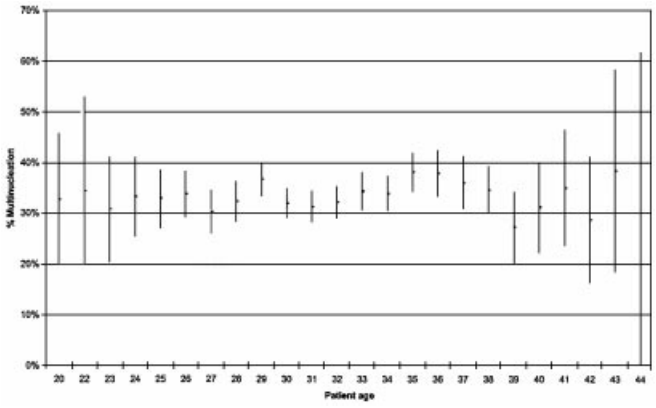


**Figure 2.** Relationship between the age of the patients and the percentage of multinucleation. The graph represents the mean percentage ±1 SD per year of age.

**Table I.** Multinucleation for cycles stratified in groups according to the number of oocytes retrieved

| Oocytes per cycle | No. of cycles | No. of embryos | No. of multinucleated embryos | Range |
|---|---|---|---|---|
| 1–4 | 143 | 296 | 93 (31.4)[a] | 0–4 |
| 5–9 | 395 | 1712 | 503 (29.4)[b] | 0–6 |
| 10–14 | 431 | 3223 | 1134 (35.2)[c] | 0–10 |
| 15–19 | 253 | 2642 | 939 (35.5)[d] | 0–16 |
| ⩾20 | 173 | 2515 | 822 (32.7)[e] | 0–15 |

Values in parentheses are percentages. Range: the minimum and maximum number of multinucleated embryos per group.
[a]versus[b]: not significantly different (RR = 1.07, 95% CI = 0.89–1.28).
[b]versus[c]: significantly different (RR = 1.19, 95% CI = 1.10–1.31).
[b]versus[d]: significantly different (RR = 1.21, 95% CI = 1.11–1.32).
[b]versus[e]: significantly different (RR = 1.11, 95% CI = 1.01–1.22).

of IVF and in 1763 (34.5%) of ICSI embryos. The odds for multinucleation in ICSI versus IVF is 1.06 (95% CI 1.00–1.11).

*Multinucleation and male and/or female factor*

When both a male and a female factor were present, 474/1471 embryos (32.2%) were multinucleated; when only a male factor was involved 1751/5049 embryos (34.7%) and when only a female factor was the cause of infertility 789/2413 embryos (32.7%) showed multinucleation. None of these incidences was significantly different, nor were they different from the observed incidence of multinucleation in cycles of patients with idiopathic infertility, with 473/1439 embryos (32.9%) showing multinucleation.

*Multinucleation and stimulation*

*Duration of stimulation*. The median time interval between the start of stimulation and the oocyte retrieval was 15 days. Stimulations of ⩽15 days resulted in 5700 embryos of which 1991 were multinucleated (34.9%) whereas stimulations of >15 days yielded 4688 embryos with 1501 multinucleated (32.0%) (RR = 1.09, 95% CI = 1.03–1.15).

*Number of oocytes retrieved*. The median number of oocytes retrieved per cycle was 11. Multinucleation was calculated for cycles with respectively 1–4, 5–9, 10–14, 15–19 and >19 oocytes (Table I). Cycles with nine oocytes or less had significantly less multinucleated embryos 596/2008 (29.7%) compared with those with ⩾10 oocytes: 2895/8380 (34.5%) (RR = 1.16, 95% CI = 1.08–1.25).

**Table II.** Multinucleation rate in relation to cleavage rate on day 2

| No. of blastomeres | No. of embryos | No. of multinucleated embryos | % multinucleation |
|---|---|---|---|
| 2 | 771 | 440 | 57.1 |
| 3 | 380 | 190 | 50.0 |
| 4 | 1799 | 302 | 16.8 |
| 5 | 490 | 138 | 28.2 |
| 6 | 189 | 59 | 31.2 |
| ⩾7 | 91 | 25 | 27.5 |

**Table III.** Multinucleation rate in relation to cleavage rate on day 3

| No. of blastomeres | No. of embryos | No. of multinucleated embryos | % multinucleation |
|---|---|---|---|
| 2 | 46 | 31 | 67.4 |
| 3 | 68 | 48 | 70.6 |
| 4 | 578 | 276 | 47.8 |
| 5 | 394 | 184 | 46.7 |
| 6 | 400 | 157 | 39.3 |
| 7 | 497 | 141 | 28.4 |
| 8 | 1187 | 184 | 15.5 |
| 9 | 311 | 75 | 24.1 |
| 10 | 164 | 49 | 29.9 |
| ⩾11 | 112 | 31 | 27.7 |

*Gonadotrophins used for stimulation*. Urinary gonadotrophin (Humegon) stimulation resulted in 605 embryos of which 189 were multinucleated (33.4%). Purified urinary FSH (Metrodin HP) was the medication in most cycles resulting in 7321 embryos of which 2447 were multinucleated (33.4%). rFSH (Gonal F) stimulation yielded 2462 embryos of which 855 were multinucleated (34.7%). None of these incidences of multinucleation was significantly different.

*Total dose of gonadotrophin used per stimulation*. The median dose per stimulation was 2400 IU. Stimulations requiring <2400 IU led to 1596/4993 embryos with multinucleation (32.0%) whereas those requiring ⩾2400 IU resulted in 1866/5395 embryos with multinucleation (34.6%) (RR = 1.08, 95% CI = 1.02–1.14). Stimulations requiring more ampoules were associated with a significant increase of multinucleation.

*Multinucleation and female age*. Female age seems to have no impact on the incidence of multinucleation (Figure 2).

### *Multinucleation in relationship with other morphological characteristics of early cleaving embryos*

*Multinucleation and fragmentation*

There were 3720, 3066 and 1652 embryos with fragmentation of type F1, F2 and F3 respectively, of which 1154 (31.0%), 1055 (34.4%) and 603 (36.5%) were multinucleated. There is a statistically significant difference between the incidence of multinucleation in embryos with fragmentation of type F1 versus F2 (RR = 1.11, 95% CI = 1.04–1.19) and between

**Table IV.** Multinucleation rate for embryos with fragmentation F1-categorized according to the number of blastomeres on day 2 and day 3. Only categories with at least 20 embryos were retained

| | No. of multinucleated/no. embryos (% multinucleated) | | | | |
|---|---|---|---|---|---|
| No. of blastomeres on day 2 | 2 | 3 | 4 | 5 | 6 |
| No. of blastomeres on day 3 | | | | | |
| 2 | 17/25 (68) | | | | |
| 3 | 25/32 (78) | | | | |
| 4 | 213/348 (61) | 30/65 (46) | 29/156 (19) | | |
| 5 | 82/137 (60) | 58/95 (61) | 23/106 (22) | 14/44 (32) | |
| 6 | 51/89 (57) | 34/61 (56) | 53/189 (28) | 12/37 (32) | 7/22 (32) |
| 7 | 25/56 (45) | 26/51 (51) | 53/274 (19) | 29/90 (32) | |
| 8 | 19/73 (26) | 17/63 (27) | 104/896 (12) | 25/97 (26) | 12/39 (31) |
| 9 | | | 18/101 (18) | 31/139 (22) | 11/34 (32) |
| 10 | | | 11/43 (26) | 18/56 (32) | 11/38 (29) |
| 11 | | | | | 6/23 (26) |

**Table V.** Multinucleation rate for embryos with fragmentation F2-categorized according to the number of blastomeres on day 2 and day 3. Only categories with at least 20 embryos were retained

| | No.of multinucleated/no. embryos (% multinucleated) | | | | | |
|---|---|---|---|---|---|---|
| No. of blastomeres on day 2 | 2 | 3 | 4 | 5 | 6 | 7 |
| No. of blastomeres on day 3 | | | | | | |
| 2 | 31/43 (72) | | | | | |
| 3 | 40/53 (75) | 21/27 (78) | | | | |
| 4 | 166/287 (58) | 29/75 (39) | 29/154 (19) | | | |
| 5 | 78/98 (80) | 66/118 (56) | 36/135 (27) | 14/54 (26) | | |
| 6 | 31/63 (49) | 53/88 (60) | 51/175 (29) | 26/74 (35) | 9/30 (30) | |
| 7 | 16/27 (59) | 28/56 (50) | 54/222 (24) | 28/89 (31) | 9/35 (26) | |
| 8 | 6/27 (22) | 13/43 (30) | 45/371 (12) | 31/108 (29) | 13/50 (26) | |
| 9 | | | 10/50 (20) | 21/112 (19) | 16/52 (31) | 4/23 (17) |
| 10 | | | | 7/37 (19) | 6/30 (20) | 4/21 (19) |

embryos with fragmentation of type F1 versus F3 (RR = 1.18, 95% CI = 1.09–1.27) but not between embryos with fragmentation of type F2 versus F3 (RR = 1.06, 95% CI = 0.98–1.15).

*Multinucleation and cleavage rate*

*Day 2*, *F1 embryos*. Multinucleation reaches a minimum of 16.8% for the ideal cleavage pattern of 4 blastomeres. All other cleavage patterns (Table II) are associated with significantly higher multinucleation rates. Both 3- and 5-cell embryos on day 2 show significantly more multinucleation than embryos with the ideal cleavage pattern of 4 cells (RR = 2.98, 95% CI = 2.58–3.44 and RR = 1.68, 95% CI = 1.41–2.00 respectively).

*Day 3*, *F1 embryos*. On day 3 the ideal cleavage pattern (8 cells) equally coincides with a minimal incidence of multinucleation of 15.5% (Table III). All other cleavage patterns have a significantly higher multinucleation rate, e.g. 7-cell embryos as well as 9-cell embryos show significantly more multinucleation (RR = 1.83, 95% CI = 1.51–2.22 and RR = 1.56, 95% CI = 1.23–1.97 respectively).

*Multinucleation and specific cleavage pattern in F1 embryos*. In order to analyse in further detail the impact of multinucleation on each cleavage pattern (number of cells on day 2 and number of cells on day 3) for embryos with a fragmentation of $\leqslant$10%, the ratio of multinucleated embryos to the total number was calculated (Table IV). Because cleavage types containing too few embryos would produce irrelevant ratios, only those observations comprising $\geqslant$20 embryos were considered. The ideal cleavage pattern of 4 blastomeres on day 2 and 8 blastomeres on day 3 coincides with a minimal multinucleation rate of 104/896 (11.6%). This minimal multinucleation is significantly different from that calculated for all other cleavage patterns except for the multinucleation rate of embryos developing from 4 cells on day 2 and 9 on day 3. The odds for multinucleation in F1 embryos 'near' the optimal cleavage pattern are as follows: 4–7 cells versus 4–8 cells: RR = 1.67, 95% CI = 1.23–2.25; 4–9 cells versus 4–8 cells: RR = 1.54, 95% CI = 0.97–2.42; 3–8 cells versus 4–8 cells: RR = 2.32, 95% CI = 1.49–3.63; 5–8 cells versus 4–8 cells: RR = 2.22, 95% CI = 1.51–3.26.

*Multinucleation and specific cleavage pattern in F2 embryos*. The same method of analysis was used for embryos with fragmentation F2 (Table V). Here as well the ideal cleavage pattern of 4 blastomeres on day 2 and 8 on day 3 coincides with minimal multinucleation of 45/371 (12%). This minimal multinucleation is significantly different from all other cleavage patterns except for those embryos developing from 4 cells on day 2–9 cells on day 3. The odds for multinucleation in the cleavage patterns nearest to the ideal pattern are as follows: 4–7 cells versus 4–8 cells: RR = 2.01, 95% CI = 1.40–2.87; 4–9 cells versus 4–8 cells: RR = 1.65,

**Table VI.** Ongoing implantations after single embryo transfer of either, one top embryo, one non-top embryo without multinucleation or one multinucleated embryo. Transfers were stratified according to the age category of the patient: <25, 25–29, 30–34, 35–39 and ⩾40 years of age

| Age (years) | Single embryo transfers | | | | | |
|---|---|---|---|---|---|---|
| | One top embryo | | One non-top embryo no multinucleation | | One embryo with multinucleation | |
| | No. of transfers | No. of implantations | No. of transfers | No. of implantations | No. of transfers | No. of implantations |
| <25 | 11 | 3 | 0 | 0 | 2 | 0 |
| 25–29 | 116 | 50 | 17 | 4 | 3 | 0 |
| 30–34 | 150 | 50 | 24 | 12 | 10 | 1 |
| 35–39 | 62 | 23 | 16 | 2 | 6 | 0 |
| ⩾40 | 2 | 0 | 3 | 0 | 2 | 0 |
| Total | 341 | 126 (37.0) | 60 | 18 (30.0) | 23 | 1 (4.3) |

Values in parentheses are percentages.

95% CI = 0.89–3.06; 3–8 cells versus 4–8 cells, RR = 2.49; 95% CI = 1.47–4.24; 5–8 cells versus 4–8 cells, RR = 2.37; 95% CI = 1.58–3.55.

### *Impact of multinucleation on the ongoing implantation rate*

*Analysis of single embryo tranfers*

In the period covered (January 1, 1999 to June 30, 2002) there were 424 single embryo transfers. Most of these (341) were transfers of top quality embryos, i.e. embryos with fragmentation of type F1 or F2, 4 or 5 blastomeres on day 2 and ⩾7 on day 3 and no multinucleation ever (Van Royen *et al.*, 1999). Some (83) were compulsory transfers of a single non-top quality embryo.

These transfers with a documented 1:1 relationship between embryo characteristics and outcome offered a first study group to examine the impact of multinucleation on the chance to obtain an ongoing implantation. Table VI shows the ongoing implantations after single embryo transfers. The ongoing implantation rates of top quality embryos, non-top quality embryos without multinucleation and embryos with multinucleation were 37.0, 30.0 and 4.3% respectively. The odds of obtaining an ongoing implantation were significantly different between the first and the last group (RR = 8.50, 95% CI = 1.24–58.08) but not between the first and the second (RR = 1.23, 95% CI = 0.82–1.86) and just failed to be significantly different between the second and the last group (RR = 6.90, 95% CI = 0.98–48.76). The only multinucleated embryo that resulted in an ongoing implantation showed ⩽10% fragmentation, 4 cells on day 2 and 8 on day 3 and only 1 MNB on day 2.

*Analysis of double embryo transfers*

A larger group to study the impact of multinucleation on ongoing implantation is the double embryo transfer (DET) group. There were 770 DET; these were stratified into three groups according to the number of embryos with multinucleation: group I ($n$ = 608) in which both embryos were without multinucleation, group II ($n$ = 118) with one multinucleated and one non-multinucleated embryo and group III ($n$ = 44) in which both embryos were multinucleated (Table VII). In group I there were 299 ongoing pregnancies: 143 singletons, 73 twins and five dizygotic triplets. In calculating the ongoing implantation rate these triplets were considered as double implantations: both embryos led to (at least) one ongoing implantation. In group II there were 19 singleton and five twin pregnancies and in group III there were only five singletons. This led to an ongoing implantation rate of 24.6, 12.3 and 5.7% respectively. The implantation of the group without multinucleation was significantly higher than both other groups (group I–II: RR = 2.00, 95% CI = 1.40–2.85; group I–III: RR = 4.33, 95% CI = 1.84–10.20). In the five double transfers of group III that led to an ongoing implantation, eight of the embryos involved showed multinucleation exclusively on day 2, and two both on day 2 and on day 3.

## Discussion

Day 2 after insemination/injection seems to be the most rewarding for nuclear observations: multinucleation was discovered in 27.4% of the embryos on this day versus only 15.1% on day 3. This may be partly due to the larger dimensions of day 2 cells and also their better optical accessibility (less overlap) due to the smaller number of cells. It also has been reported (Staessen and Van Steirteghem, 1998) that 30% of embryos with multinucleation in the 2-cell stage did not show multinucleation in the 3–8-cell stage. The percentages reported here as well as in other papers only reflect multinucleation of the blastomeres at the interphase stage when the nucleus is visible. Consequently these observations will be an underestimation of the actual multinucleation rate.

Multinucleation is a common phenomenon: it was seen in 79.4% of all cycles which is comparable with the 74% reported by Jackson *et al.* (1998). These authors found 31% of their embryos to be multinucleated, we found 33.6%. When considering the spread amongst the population, our results indicate that 87% of all patients had at least one multinucleated embryo. This is substantially more than the 44% reported by Balakier and Cadesky (1997) but these authors identified only 14.5% of the embryos as being multinucleated. In 99% of our patients with ⩾20 embryos multinucleation was recorded.

Multinucleation is not characterized by a normal distribution in the population. This is demonstrated by analysing its incidence in the population. All embryos originating from different cycles in the same couple were grouped and only patients having a large number of embryos (⩾20) were

**Table VII.** Ongoing implantation rates (IR) after double embryo transfer of either 2 embryos without multinucleation, 1 multinucleated and 1 non-multinucleated embryo or 2 multinucleated embryos. Transfers were stratified according to the age category of the patient: <25, 25–29, 30–39, 35–39 and ⩾40 years of age

| Age (years) | Double embryo transfers | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Two embryos without multinucleation | | | | One embryo with and one without multinucleation | | | Two embryos with multinucleation | |
| | No. of transfers | Singleton | Twin | Triplet | No. of transfers | Singleton | Twin | No. of transfers | Singleton |
| <25 | 24 | 6 | 3 | 1 | 3 | 1 | 0 | 1 | 1 |
| 25–29 | 172 | 47 | 24 | 3 | 32 | 5 | 2 | 13 | 0 |
| 30–34 | 289 | 62 | 31 | 0 | 45 | 8 | 2 | 22 | 4 |
| 35–39 | 113 | 28 | 15 | 1 | 30 | 5 | 1 | 7 | 0 |
| ⩾40 | 10 | 0 | 0 | 0 | 8 | 0 | 0 | 1 | 0 |
| Total | 608 | 143 | 73 | 5 | 118 | 19 | 5 | 44 | 5 |
| Ongoing IR (%) | 24.6 | | | | 12.3 | | | 5.7 | |

analysed (Figure 1). In the case of a normal distribution, there would be a typical bell-shaped curve. Instead, Figure 1 shows a peak around the mean incidence of 33.6%, but there is a very wide distribution with incidences scattered between 0 and 90%. Seven patients had ⩾75% multinucleation and in these patients this high incidence showed a repetitive pattern in subsequent cycles. This suggests a patient-linked predisposition to multinucleation in these patients. They did not show any accelerated ovulation induction response, a factor that has been correlated with increased multinucleation in our data as well as in others' (Jackson *et al*., 1998).

Embryos obtained by ICSI were 34.5% multinucleated and those fertilized after IVF only 32.7%; this difference just failed to be statistically significant. No significant impact on multinucleation could be demonstrated by the clinical category to which the patients belongs.

In accordance with Jackson *et al*. (1998) a significant increase in multinucleation was demonstrated in cycles with a faster ovulation induction response. Like these authors we also found a significantly increased multinucleation rate in cycles with an increased number of oocytes.

No impact of the type of drugs used for stimulation of the cycle could be demonstrated, whether these were urinary gonadotrophins, purified urinary FSH or rFSH. However, the total dose of FSH used proved to be correlated: cycles requiring a higher dose are correlated with a higher multinucleation rate than cycles requiring a lower dose. This observation together with the increased incidence in shorter cycles may have a common explanation in the higher number of premature follicles at the moment of ovulation triggering. These oocytes may reach metaphase II and become fertilized but are unable to undergo proper nuclear cleavage. Such oocytes are obviously more likely to be generated in very short cycles but also in cycles requiring a large FSH dose: there will be a lack of synchronicity due to recruitment of follicles over a period of increased stimulation. Both types of stimulation will produce an increased number of follicles with suboptimal maturity at the time of oocyte recovery and these might lead to embryos with multinucleation. This hypothesis is supported by the findings of Nogueira *et al*. (2000). These authors used germinal vesicle oocytes obtained from FSH/hMG-stimulated cycles. These oocytes were further matured *in vitro*. They found a high incidence of multinucleation in these embryos: only 2/30 (6.7%) were completely mononuclear. If this hypothesis proves to be correct it might offer an opportunity to reduce the incidence of multinucleation by introducing more gentle stimulation regimens and by extending the period of stimulation before starting ovulation induction. Especially patients with a high multinucleation rate might benefit from this approach.

Female age seems to have no effect on multinucleation rates.

In contrast with others, for example Jackson *et al*. (1998) who found no difference in multinucleation rates between different average fragmentation scores, we found a significant relationship between multinucleation and fragmentation: embryos with a minor degree of fragmentation (F1) showed significantly less multinucleation than embryos with type 2 fragmentation (F2) or with type 3 fragmentation (F3). This is remarkable because a higher fragmentation rate makes it more difficult to detect multinucleation due to possible visual obstruction by fragments, thus disabling a clear observation of the nuclei.

Most importantly, there appears to be a clear relationship between multinucleation and the cleavage pattern. To investigate this in further detail we concentrated on F1 and F2 embryos exclusively because these were the largest groups, the groups with better visibility of the nuclei and also the groups containing the bulk of implanting embryos (Van Royen *et al*., 2001). On day 2 the minimal incidence of multinucleation coincided with the optimal cleavage pattern and both the embryos with a lower as well as those with a higher than optimal number of blastomeres showed a significantly increased multinucleation rate. The same is applicable to day 3 embryos: the embryos with the optimal blastomere number of 8 showed the lowest incidence of multinucleation. Not only the types of embryos with a lower but also those with a higher than optimal number of blastomeres show a higher frequency of multinucleation. Until now it has been postulated (Balakier and Cadesky, 1997; Jackson *et al*., 1998) that multinucleated embryos may display a reduced cleavage rate because they contain chromosomally abnormal blastomeres (Hardy *et al*., 1993). Our data seem to prove these authors correct for a large

proportion of the embryos, but there also seems to be another group of embryos where an increased multinucleation rate is associated with an increased cleavage rate. It should also be stressed that 16.8% of 4-cell embryos on day 2 and 15.5% of 8-cell embryos on day 3, i.e. the embryos with a normal cleavage rate, exhibit multinucleation. For these embryos multinucleation seems to have had no impact on the cleavage rate. This is a strong argument in favour of a systematic screening of the nuclear status of all embryos: a considerable fraction of the normal embryos and an even more important fraction of the fast-cleaving embryos may exhibit multinucleation. This means that the cleavage rate alone offers no basis for eliminating these embryos for transfer or cryopreservation.

When embryos are stratified, first according to the level of fragmentation (F1 in Table IV and F2 in Table V) and second according to the number of blastomeres on day 2 and on day 3, then the minimal incidence of multinucleation coincides with the optimal cleavage pattern (4 cells on day 2 and 8 cells on day 3) both for F1 as for F2. In other words the maximum incidence of mononucleated embryos coincides with the optimal cleavage pattern. This maximum is predictively correlated with the maximal 'implanted fraction' (for mononucleated embryos) as calculated earlier (Van Royen *et al*., 2001).

The severe impact of multinucleation on ongoing implantation has been demonstrated earlier (Pelinck *et al*., 1998; Van Royen *et al*., 2001). In the present study, it has been possible to calculate the implantation rate of exclusively multinucleated embryos in two different groups of embryo transfers and to compare them with embryos without observed multinucleation: the patients having a single multinucleated embryo transferred and the patients having a double transfer of two multinucleated embryos. In the first group the ongoing implantation rate was 1/23 (4.3%) and in the second group 5/88 (5.7%). In single embryo transfer, despite a 6.9-fold lower chance for a multinucleated embryo to result in an ongoing implantation compared with a non-top quality embryo, confidence interval analysis just failed significance because of the small sample size. In double embryo transfer, two multinucleated embryos resulted in a significantly 4.3-fold lower implantation rate compared with transfers of non-multinucleated. This means multinucleation is a factor with a highly discriminatory power between embryos with a high and a low implantation rate.

The reason why multinucleation has such an impact on implantation is probably to be found in the observations made by Kligman *et al*. (1996) and later by Hardarson (2001). Analysing embryos with MNB for chromosomal abnormalities, they found that 40/47 (85%) and 6/7 (86%) of embryos with multinucleation respectively, were chromosomally abnormal in >50% of their constitutive blastomeres. The number of abnormal blastomeres exceeded by far the number in which multinucleation was observed. This means that it is to be expected that in an embryo where multinucleation is observed in one or more blastomeres the genetic quality of other blastomeres is also compromised.

Embryos with documented multinucleation had an overall ongoing implantation rate of 6/111 (5.4%). This can be explained by a further proliferation of the unaffected blastomeres. Of these six ongoing implantations, five have resulted in the birth of a healthy baby, one led to a late miscarriage. The birth of a healthy baby originating from a multinucleated embryo has been reported before (Balakier and Cadesky, 1997; Jackson *et al*., 1998).

We can conclude that multinucleation is a widely spread phenomenon but it is unevenly distributed over the population. It was observed in more than one-third of all embryos. Its incidence was positively correlated with factors such as shorter than average stimulations, higher than average number of oocytes retrieved, higher than average FSH dose for stimulation. Because of these findings we can hypothesize that multinucleation is due to a developmental failure of the oocyte. This developmental failure may either be patient-linked (intrinsic oocyte quality) or it may be stimulation-linked (the consequence of submaturity at the time of fertilization). There is a significant relationship between multinucleation and other morphological characteristics of early cleaving embryos like fragmentation and cleavage rate and the lowest incidence of multinucleation coincides with minimal fragmentation and optimal cleavage rate. Finally it was shown that multinucleation is a serious handicap for ongoing implantation. It is hoped that these data may contribute to an improvement in embryo selection.

## References


Alikani, M., Calderon, G., Tomkin, G., Garrisi, J., Kokot, M. and Cohen, J. (2000) Cleavage anomalies in early human embryos and survival after prolonged culture in-vitro. *Hum. Reprod.*, **15**, 2634–2643.

Balakier, H. and Cadesky, K. (1997) The frequency and developmental capability of human embryos containing multinucleated blastomeres. *Hum. Reprod.*, **12**, 800–804.

Gardner, M.J. and Altman, D.G. (1986) Confidence intervals rather than P values: estimation rather than hypothesis testing. *Br. Med. J. (Clin. Res.)*, **292**, 746–750.

Hardarson, T., Hanson, C., Sjogren, A. and Lundin, K. (2001) Human embryos with unevenly sized blastomeres have lower pregnancy and implantation rates: indications for aneuploidy and multinucleation. *Hum. Reprod.*, **16**, 313–318.

Hardy, K., Winston, R.M. and Handyside, A.H. (1993) Binucleate blastomeres in preimplantation human embryos in vitro: failure of cytokinesis during early cleavage. *J. Reprod. Fertil.*, **98**, 549–558.

Jackson, K.V., Ginsburg, E.S., Hornstein, M.D., Rein, M.S. and Clarke, R.N. (1998) Multinucleation in normally fertilized embryos is associated with an accelerated ovulation induction response and lower implantation and pregnancy rates in in vitro fertilization–embryo transfer cycles. *Fertil. Steril.*, **70**, 60–66.

Kligman, I., Benadiva, C., Alikani, M. and Munné, S. (1996) The presence of multinucleated blastomeres in human embryos is correlated with chromosomal abnormalities. *Hum. Reprod.*, **11**, 1492–1498.

Laverge, H., De Sutter, P., Verschraegen-Spae, M.R., De Paepe, A. and Dhont, M. (1997) Triple colour fluorescent in-situ hybridization for chromosomes X, Y and 1 on spare human embryos. *Hum. Reprod.*, **12**, 809–814.

Levy, R., Benchaib, M., Cordonier, H., Souchier, C. and Guerin, J.F. (1998) Laser scanning confocal imaging of abnormal or arrested human preimplantation embryos. *J. Assist. Reprod. Genet.*, **15**, 485–495.

Munné, S. and Cohen, J. (1993) Unsuitability of multinucleated human blastomeres for preimplantation genetic diagnosis. *Hum. Reprod.*, **8**, 1120–1125.

Nogueira, D., Staessen, C., Van de Velde, H. and Van Steirteghem, A. (2000) Nuclear status and cytogenetics of embryos derived from in vitro-matured oocytes. *Fertil. Steril.*, **74**, 295–298.

Pelinck, M.J., De Vos, M., Dekens, M., Van der Elst, J., De Sutter, P. and Dhont, M. (1998) Embryos cultured in vitro with multinucleated

blastomeres have poor implantation potential in human in-vitro fertilization and intracytoplasmic sperm injection. *Hum. Reprod.*, **13**, 960–963.

Pickering, S.J., Taylor, A., Johnson, M.H. and Braude, P.R. (1995) An analysis of multinucleated blastomere formation in human embryos. *Hum. Reprod.*, **10**, 1912–1922.

Staessen, C. and Van Steirteghem, A. (1998) The genetic constitution of multinuclear blastomeres and their derivative daughter blastomeres. *Hum. Reprod.*, **13**, 1625–1631.

Tesarik, J., Kopecny, V., Plachot, M. and Mandelbaum, J. (1987) Ultrastructural and autoradiographic observations on multinucleated blastomeres of human cleaving embryos obtained by in-vitro fertilization. *Hum. Reprod.*, **2**, 127–136.

Van Royen, E., Mangelschots, K., De Neubourg, D., Valkenburg, M., Van de Meerssche, M., Ryckaert, G., Eestermans, W. and Gerris, J. (1999) Characterisation of a top quality embryo, a step towards single-embryo transfer. *Hum. Reprod.*, **14**, 2345–2349.

Van Royen, E., Mangelschots, K., De Neubourg, D., Laureys, I., Ryckaert, G. and Gerris, J. (2001) Calculating the implantation potential of day 3 embryos in women younger than 38 years of age: a new model. *Hum. Reprod.*, **16**, 326–332.

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