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Microtubules and parental genome organisation during abnormal fertilisation in humans

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Summary

We analysed the distribution of β-tubulins, acetylated α-tubulins and chromatin configuration in 113 human zygotes showing abnormal fertilisation, 16–18 h after conventional *in vitro* fertilisation (IVF) or intracytoplasmic sperm injection (ICSI). After a first characterisation using phase contrast microscopy, immunofluorescence staining was performed in 67 IVF and 46 ICSI zygotes that developed one ,three or more pronuclei and/or subnuclei, with or without extrusion of the second polar body. Independently of the number of pronuclei found, β-tubulins were uniformly distributed throughout the cytoplasm of the abnormal zygotes. We did not observe any kind of microtubule alteration with respect of the ploidy level and/or its origin. The most frequent abnormal fertilisation pattern found after IVF was the presence of three or four pronuclei (74.6%). On the other hand, the presence of one pronucleus (63.0%) was the main pattern found after ICSI. No differences between the two groups were seen in terms of development of subnuclei. Anamolies detected after IVF and ICSI showed different aetiologies such as parthenogenetic activation, gynogenetic or androgenetic development, as well as digynic or diandric fertilisation.

Keywords: Abnormal fertilisation, Cytoskeletal organisation, Human zygotes, Microtubules

Introduction

The concluding event of the fertilisation process in animals is the association of male and female pronuclei at syngamy. In humans, the late interaction of maternal and paternal pronuclei is characterised by a tight association of male and female pronuclear envelopes with close alignment of the nucleoli (Wright *et al*., 1990).

Fertilisation failure is a recurrent event in humans and it has traditionally been explained in terms of chromosomal alterations (Wramsby & Fredga, 1987) and more recently of sperm decondensation, oocyte activation and pronuclei migration (Asch *et al*., 1995). The direct consequence is a total arrest before the first embryo cleavage can take place. In contrast, abnormal fertilisation mainly relates to alterations in the ploidy levels as a consequence of a disorganisation of chromatin (or chromosome) arrangement leading to the formation of a variable number of pronuclei (PN) and even subnuclei (SN). Contrarily to fertilisation failure, abnormally fertilised embryos may cleave and have major effects during the peri- and postimplantation period, causing embryo loss and developmental alterations.

Abnormal fertilisation occurs at a relatively low percentage after *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI). The incidence of monopronuclear zygotes varies from 2% to 5% for inseminated oocytes after IVF (Abramczuk & Lopata, 1990) and from 5% to 27% after ICSI (Palermo *et al*., 1993; Van Steirteghem *et al*., 1993*a*, *b*). Multipronuclear human zygotes after IVF may originate from dispermic fertilisation and they represent around 4% of inseminated oocytes (Plachot & Crozet, 1992). Multipronuclear zygotes after ICSI, on the other hand, are thought to be mostly digynic in their origin (Nagy *et al*., 1995, Macas *et al*., 1996).

It is well known that monopronuclear zygote development occurs more frequently following ICSI than IVF (Abramezuk & Lopata, 1990; Palermo *et al*., 1993; Sultan *et al*., 1995), but the exact causes of abnormal fertilisation after the two techniques are still not clear.

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We describe in this report the distribution of β-tubulins, acetylated α-tubulins and chromatin configuration in mono- and multipronuclear zygotes after IVF and ICSI.

Materials and methods

Inseminated and injected human oocytes, discarded as 'abnormally fertilised' between 16 and 18 h post-insemination or sperm injection, were obtained from couples undergoing IVF or ICSI in our Assisted Fertilisation Program and who had signed an informed consent. Ovarian stimulation as well as IVF and ICSI procedures were done as previously described (Rawe *et al.*, 2000).

Cytoskeleton, sperm tail and chromatin labelling

A first characterisation on the number of pronuclei and polar body extrusion was performed under phase contrast microscopy in 113 abnormally fertilised zygotes. One PN, ≥ 3 PN and ≥ 3SN zygotes were analysed using phase contrast and epifluorescence microscopy. In all zygotes studied an incorporated sperm tail and sperm chromatin in the oocyte cytoplasm were the criteria to identify the presence of one or more spermatozoa. Abnormally fertilised zygotes were classified under phase contrast microscopy as monopronuclear, multipronuclear or subnuclear. The number of polar bodies was assessed in each case.

The material was stained using a modified protocol already described (Messinger & Albertini, 1991; Rawe *et al.*, 2000). For this purpose, the zona pellucida was removed by a brief incubation with Tyrode's acid, and denuded zygotes were fixed and permeabilised for 20 min at 37 °C in a microtubule-stabilising buffer (0.1M PIPES, pH 6.9, 5 mM $MgCl_2$.6 H_2O, 0.25 mM ethylene glycol-*bis*[β-aminoethyl ether] *N,N,N',N*-tetraacetic acid (EGTA) containing 2.0% formaldehyde, 0.5% Triton X-100 and 1 µM taxol). Fixed zygotes were blocked for at least 1 h at 37 °C with 2% bovine serum albumin (BSA), 2% powdered milk, 2% normal goat serum, 0.1 M glycine and 0.01% Triton X-100 in phosphate-buffered saline (PBS). If necessary, they were stored for up to 3 days at 4 °C in this solution. To identify the sperm tail, zygotes were incubated overnight at 4 °C with 1:100 anti α-acetylated tubulin monoclonal antibodies in PBS containing 0.1% BSA and 0.02% sodium azide (PBS + BSA), washed in blocking solution, and further incubated in 1:100 fluorescein-conjugated goat anti-mouse immunoglobulin G (IgG), for 1 h at room temperature. To analyse the cytoskeleton, the material was incubated with 1:500 anti-β-tubulin-Cy3 monoclonal antibodies in PBS + BSA for 1 h at 37 °C. Finally, both anti-α-acetylated tubulin and anti-β-tubulin treated zygotes were washed 3 times in PBS + BSA, counterstained with Hoechst 33258 (1 µg/ml) for 30 min at room temperature, washed in PBS, mounted between a slide and a coverslip in PBS + BSA, examined using an Olympus epifluorescence microscope and photographed with Ektachrome film (1600ASA). Images were processed using Adobe Photoshop 5.0 software (Adobe System). For control staining, PBS + BSA alone replaced the specific antibody solution. Monoclonal antibodies and reagents were purchased from Sigma (St. Louis, MO).

Statistical analysis was performed using the chi-square test, and differences were considered significant when $p < 0.05$.

Results

A total of 113 abnormal fertilised human zygotes that developed 1PN, ≥ 3PN or ≥ SN after 16–18 h post-insemination or sperm injection, were studied; all of them were informative. The relative abundance of each type of development is summarised in Table 1.

Independently of the number of pronuclei found, β-tubulins were uniformly distributed throughout the cytoplasm of the abnormal zygotes. We did not observe any kind of microtubule alterations with respect to the ploidy level and/or its origin (Fig. 2).

Fourteen and 29 1PN zygotes were identified after IVF and ICSI respectively (20.9% and 63.0%) (Table 1; Figs. 1*A*, 2*A*). Monopronuclear development was the pattern most commonly found after ICSI and its frequency was statistically different compared with IVF ($p < 0.01$; Table 1). One PN development can be accompanied by the extrusion of one or two polar bodies (PB). The frequency of 1PN and 1PB is not statistically different between IVF and ICSI, but the existence of a second polar body is highest after ICSI (11.93% after

Table 1 Immunofluorescence analysis of abnormal human zygotes after IVF and ICSI

Development	Nuclear content	IVF ($n = 67$)	ICSI ($n = 46$)	p
Monopronuclear	1PN, 1PB	6 (7.9)	3 (6.5)	NS
	1PN, 2PB	8 (11.9)	26 (56.5)	< 0.01
Multipronuclear	3PN, 1PB	19 (28.3)	12 (26.1)	NS
	3PN, 2PB	28 (41.8)	3 (6.5)	<0.01
	4PN, 1PB	3 (4.5)	0 (0)	
Subnuclear	≥ 3SN, 1PB	3 (4.5)	2 (4.3)	NS

PN, pronucleus; PB, polar body; SN, subnucleus.
Statistical analysis was performed using the chi-square test, and differences were considered significant when $p < 0.05$.
Values in parentheses are percentages. NS, not significant.

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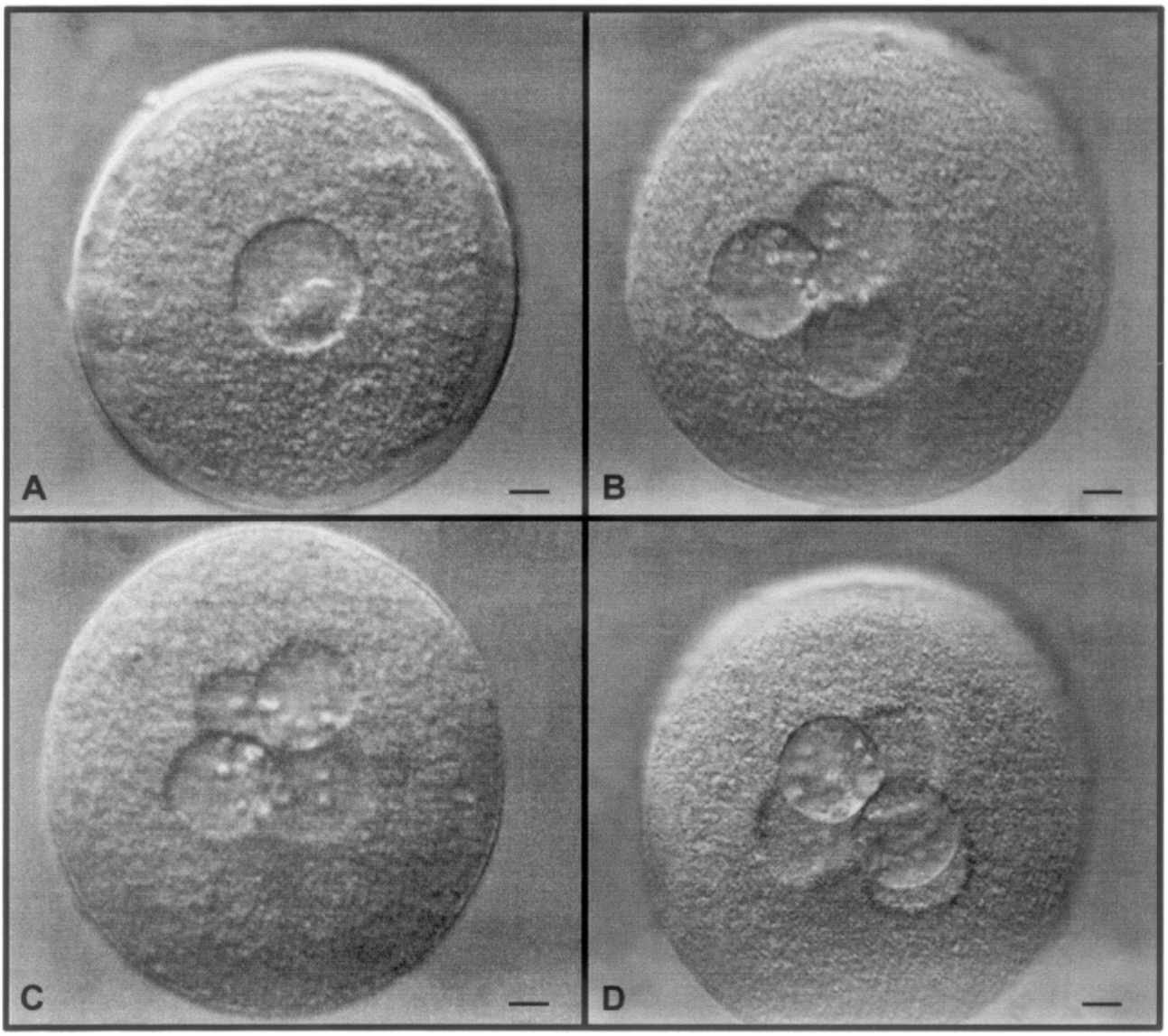

Figure 1 Phase contract micrographs of human zygotes after abnormal fertilisation. (*A*) One pronucleus (large) (1PN)/1 polar body zygote (1PB) after ICSI. In this case two nucleoli are visualised, presumably originated from the fusion of others. (*B*) A 3PN/1PB zygote after IVF. Retention of the second polar body may be the origin of this abnormal fertilisation pattern. (*C*) Development of 4PN and extrusion of 1PB after IVF. We hypothesised that retention of the second polar body and dispermic fertilisation was the cause of this pattern and this was confirmed after immunofluorescence analysis. (*D*) Pronuclear and subnuclear development after IVF. Photographs were taken after zona pellucida removal and most times polar bodies are lost. Scale bars represent 10 µm.

IVF vs 56.5% after ICSI; $p < 0.01$). In some cases, zygotes classified by light microscopy as 1PN were later found by immunofluorescence to have a different pronuclear constitution. This situation was found in statistically different proportions after IVF (4%) and ICSI (36%: $p < 0.01$).

Multipronuclear development occurred in 76.6% of the abnormal zygotes after IVF and in 32.6% after ICSI (Figs. 1*B–D*, 2*B*, *C*). This difference was statistically significant ($p < 0.01$) and represented the most frequent pattern found after IVF.

Besides the abnormalities described above, we have observed the development of subnuclei at a much lower frequency (Table 1). Zygotes with subnuclear development after IVF and ICSI represented 4.5% and 4.3% of the abnormal zygotes, respectively (Fig. 2*D*).

Discussion

The results obtained here indicate the existence of different mechanisms in the origin of abnormal

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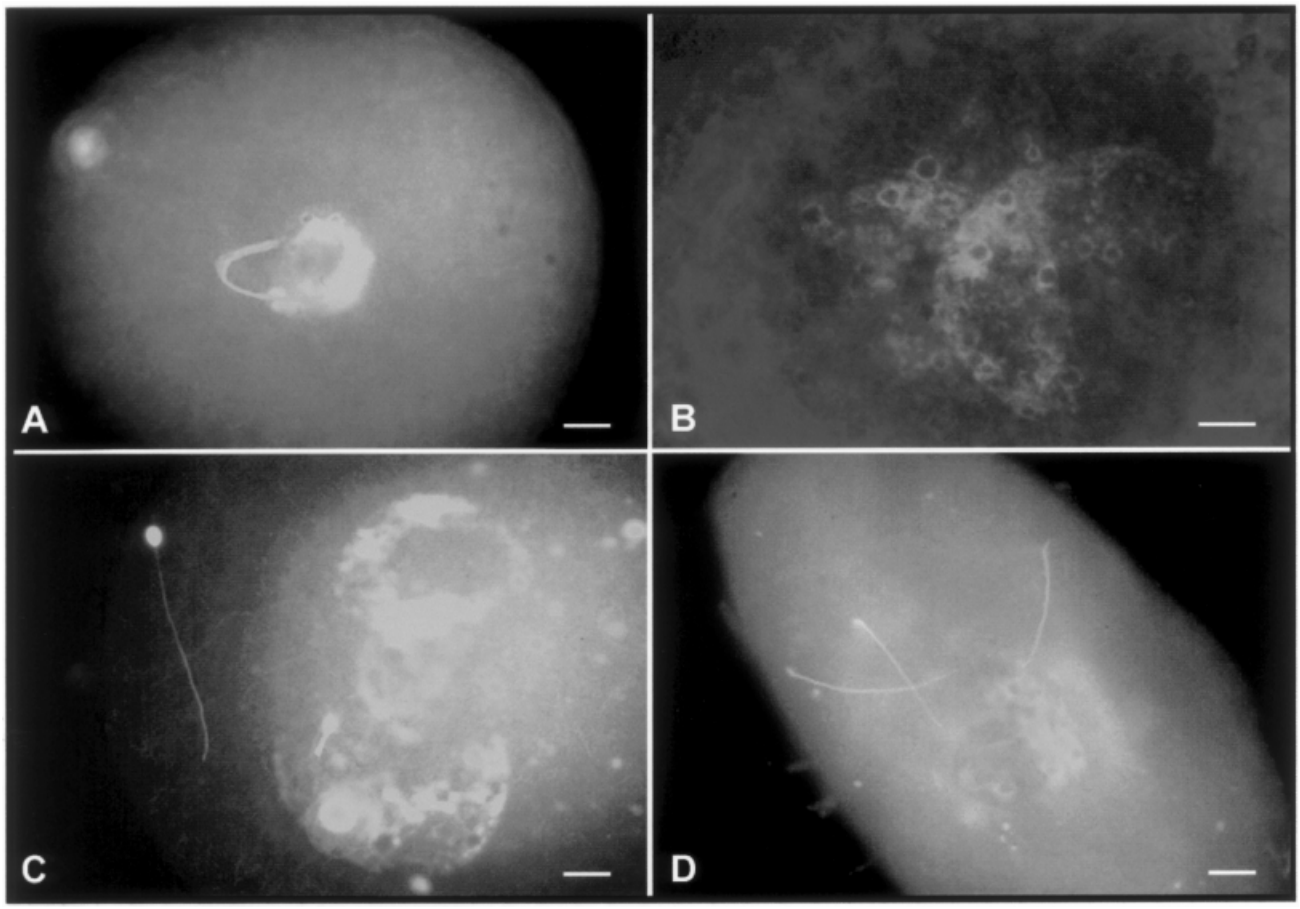

Figure 2 Immunofluorescence analysis of abnormal fertilised zygotes. (*A*) One PN development after ICSI. Female chromosomes are located out of the focal plane. Note the sperm tail associated with the only pronucleus in the centre of the cell. (*B*) Three PN development after IVF. (*C*) Detail of 3PN formation after IVF. Three interphase pronuclei can be seen. (*D*) Polyspermic fertilisation after IVF. The zygote had developed 4PN seen by phase contrast microscopy. Note the three sperm tails in the ooplasm. Scale bar represents: in *A*, *C* and *D*, 10 μm; in *B*, 5 μm.

fertilisation after IVF and ICSI. The frequency of formation of abnormally fertilised zygotes after IVF and ICSI was comparable to that in previous reports (Sultán *et al*., 1995).

Microtubule distribution found in abnormal fertilised zygotes was similar to that observed in normal fertilised human oocytes (unpublished observations), and is completely different to the patterns observed during 'fertilisation failure'. During the latter event, microtubules are not homogeneously distributed and they localise in specific areas depending on the cause of fertilisation failure (Rawe *et al*., 2000).

The elevated proportion of 1PN zygotes after ICSI may be the consequence of incomplete activation failing to lead to appropriate pronuclear development. Oocyte activation abnormalities are suspected to be the cause of abnormal or incomplete fertilisation processes after ICSI (Tesarik & Sousa, 1995). Swann (1990) and Dozortzev *et al.* (1995) have shown that the sperm contains a temperature-sensitive cytosolic factor, namely oscilin, that when injected into the oocyte can generate oocyte activation. Abnormal oocyte activation may be explained by the lack of this factor or its activity in abnormal sperm, such as those used in ICSI procedures. At the same time, the ICSI technique itself can act as a 'strong activator agent', bypassing the supposed alterations in the oscilin. In fact, it was more frequent to find 1PN/2PB zygotes than 1PN/1PB zygotes after ICSI in our study. Some of the 1PN embryos cleave normally and, as was clearly demonstrated, they can be diploid (Staessen *et al*., 1993). Asynchronous pronuclear development (Staessen *et al*., 1993) or pronuclear fusion, a phenomenon found in primitive species such as the sea urchin (Wilson, 1925) or even in humans (Tesarik & Kopecny, 1989), can be the causes of diploid embryos derived from monopronuclear zygotes.

On the other hand, oocyte activation during IVF depends on gamete interaction and, as a consequence, the incidence of monopronuclear zygotes with one or two polar bodies are very similar (Table 1).

As expected, multipronuclear development was

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more frequent after IVF than ICSI (Table 1). The formation of 3PN and 4PN is more commonly the product of diandric triploidy as a consequence of dispermic fertilisation. Digynic triploidy is less frequent and is the consequence of the retention of the second polar body within the cytoplasm. After ICSI, this situation is the most frequent cause of multipronuclear development (Table 1).

In general, pronuclear number and size depend on the number of chromosomes involved and whether they remain in groups or not (Austin, 1969). Some of the pronuclei observed after *in vitro* fertilisation can be the product of subnuclei development, also called pseudomultipronuclei (Van Blerkom *et al.*, 1987). This is a rather infrequent event (~4% of abnormal fertilised zygotes) that occurs when the development of some small pronuclei takes place instead of one or more bigger pronuclei. Its frequency was similar after IVF and ICSI, and a possible explanation for this resides in a dispersion of chromosomes before fertilisation, followed by the formation of a nuclear membrane around them (Kaufman, 1983). The incidence of subnuclei development in mammals seems to be genetically determined (Braden, 1957). Nevertheless, this percentage can reach higher values when the oocytes are old or they are exposed to an activating stimulus such as colchicine (Edwards, 1958*a*, *b*) or heat (Austin & Barden, 1954; Komar, 1973). Abnormally fertilised zygotes after both assisted reproduction techniques were more commonly the product of retention of the second polar body and the presumptive split of one or more pronuclei within the cytoplasm. Independently of the fertilisation technique used, the ability to initiate a subnuclear development seems to be related to oocyte quality.

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