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# Cytogenetic abnormalities of unfertilized oocytes generated from in-vitro fertilization and intracytoplasmic sperm injection: a double-blind study

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**In the present study we have assessed the cytogenetic abnormalities of unfertilized oocytes from in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) programmes during a one year period (July 1995 to July 1996) with the cytogenetic analysis being carried out in a double-blind manner. A total of 88 unfertilized ICSI and 85 unfertilized IVF oocytes were used for the study and of these 51 and 62 oocytes, in each respective group, were suitable for analysis. The haploidy, diploidy and aneuploidy rates between ICSI (62.7, 7.8 and 5.9%) and IVF (61.3, 9.7 and 14.5%) groups were similar. A significant inter-patient variation in the incidence of hypohaploidy was observed within the IVF group. Chromosomal fragmentation or breakage was observed at a similar rate in both groups of unfertilized oocytes (23.5 and 14.5% for ICSI and IVF respectively). A significantly higher proportion of ICSI oocytes contained sperm nuclei (27/51, 52.9%) than did IVF oocytes (20/62, 32.3%, $P < 0.01$). The distribution and state of sperm head chromatin in relation to oocyte chromosomal complement was studied in both groups. ICSI oocytes contained decondensed or swollen sperm nuclei in association with haploid oocyte chromosomes (12/27, 44.4%) or condensed sperm heads in oocytes showing no chromosomal complements (7/27, 25.9%). In IVF oocytes sperm heads were either arrested in the condensed state (5/20, 25%), metaphase stage (3/20, 15%) or had undergone premature chromosome condensation (PCC; 6/20, 30%) in association with haploid oocyte chromosomes. The incidence of PCC was similar in the two groups. A marked variation in the incidence of total chromosomal abnormality was observed between patients within both ICSI (0–75%) and IVF (0–71%) groups indicating a possible similarity in oocyte quality between the majority of male factor and tubal infertility patients. The type of sperm used in the two fertilization procedures showed an increased incidence of chromosomal breakage with ICSI–MESA (microepididymal sperm aspiration) spermatozoa (4/6, 67%) compared to the ICSI–ejaculated (6/35, 17.1%; $P < 0.05$), ICSI–testicular biopsy (2/10, 20%) and IVF–normospermic (9/62, 14.5%; $P < 0.01$) spermatozoa. Chromosomal fragmentation may be associated with the degree of difficulty experienced at sperm injection, especially with sperm retrieved from the reproductive tract. Thus chromosomal fragmentation in ICSI may need further investigation using a larger sample size in order to assess the possible causative factors.**

*Key words:* chromosomes/ICSI/IVF/unfertilized oocytes

## Introduction

Intracytoplasmic sperm injection (ICSI) has been the most significant development in the 1990s for the treatment of male factor infertility. With ICSI, it is possible to achieve fertilization with almost any type of spermatozoon, ejaculated (Van Steirteghem *et al*., 1993), epididymal (Silber *et al*., 1994; Devroey *et al*., 1995a), testicular (Devroey *et al*., 1995b; Nagy *et al*., 1995; Silber *et al*., 1995) or with gross morphological abnormalities (Hall *et al*., 1995; Silber 1995). Thus it has been questioned whether ICSI could increase the potential for genetic abnormalities in the offspring (de Kretser, 1995).

The standard in-vitro fertilization (IVF) technique involves the insemination of oocytes with 50 000–100 000 spermatozoa, usually from normozoospermic men, to achieve fertilization. In the ICSI technique a single spermatzoon is selected by the operator, usually from severe male factor patients, and injected in to the vitellus of the oocyte following the breakage of the oolemma but avoiding any damage to the metaphase chromosomes which lie immediately below the first polar body. This procedure bypasses the physiological events such as acrosome reaction of the spermatozoa, sperm–egg fusion, membrane hyperpolarization, calcium discharges and activation of the egg that usually take place during normal fertilization (Edwards, 1995). Furthermore, it is clear that sperm selection by the operator for injection rather than the involvement of physiological processes that normally take place at fertilization, as well as type of spermatozoa used, and the invasiveness of the injection procedure, increases the possibility that abnormalities could occur in the subsequent development process of the spermatozoon, interaction with the oocyte or the resulting embryo. Some researchers are concerned about this aspect of ICSI (Cummins and Jequier, 1995) and some are keen to see more research carried out even in an animal model to confirm safety of the technique (Ng *et al*., 1995; Yanagimachi, 1995).

So far, information available on the chromosomes of embryos resulting from ICSI is limited (reviewed by Plachot *et al*., 1996). However, abnormally fertilized zygotes (one-pronuclear) or unfertilized oocytes have been studied to assess the oocyte/sperm nuclear aspects following the ICSI procedure either using cytogenetic studies (Bergere *et al*., 1995; Wall *et al*., 1996), nuclear staining techniques such as Hoechst 33342

(Flaherty *et al*., 1995) or in-situ hybridization (Sultan *et al*., 1995; Wall *et al*., 1996). Flaherty *et al*. (1995) found that an increased number of one-pronuclear oocytes contained swollen sperm heads (80%), whereas in the study carried out by Sultan *et al*. (1995) only 9.5% of one-pronuclear oocytes showed Y-specific labelling. The behaviour of spermatozoa following ICSI has been studied by Dozortsev *et al*. (1994) and the majority of unfertilized and one-pronuclear ICSI oocytes have been shown to contain intact sperm heads or swollen sperm nuclei; further research has indicated the release of a heat-sensitive, intracellularly active factor by the injected spermatozoon (Dozortsev *et al*., 1995a). In order to accumulate further information on the ICSI technique, we have used unfertilized ICSI oocytes to study the incidence of cytogenetic abnormalities and the findings have been compared with unfertilized IVF oocytes analysed during the same period of the IVF/ICSI programme in a double-blind study.

## Materials and methods

For this study, oocytes which failed to show pronuclei (one or more), contained one polar body or fragmented polar body and remained uncleaved 2 days after insemination were considered unfertilized. Unfertilized oocytes from 39 patients who were undergoing IVF or ICSI between July 1995 and July 1996 were used for this study. The patients were superovulated using leuprolide acetate (Lucrin; Abbott, Sydney, Australia) either on a down-regulation (LDR) or a flare-up regimen (LF), mostly in combination with pure follicle stimulating hormone (FSH, Metrodin; Serono, Sydney, NSW, Australia). One patient was treated with Zoladex (Zeneca Ltd, Macclesfield, Cheshire, UK) for endometriosis prior to ovarian stimulation with Metrodin. Ovulation was triggered with human chorionic gonadotrophin (HCG, Pregnyl; Serono) and oocytes were collected after 35 h.

The culture medium used for oocyte/embryo culture was human tubal fluid (HTF) medium supplemented with 10% heat-inactivated patient's serum. The semen samples were collected 2–3 h after the oocyte retrieval. The sperm counts (mean ± SD) of IVF patients were: total count (TC), 77 ± 49 $\times 10^6$/ml; motile count (MC), 41 ± 29$\times 10^6$ ml; progressively motile count (PC), 25 ± 21 $\times 10^6$/ml (WHO, 1992). In the ICSI group, ejaculated spermatozoa was used for 16 patients and the sperm counts for these patients were: TC, 14 ± 26$\times 10^6$/ml (range 0.01 to 109 $\times 10^6$/ml); MC, 5 ± 11 $\times 10^6$/ml (range 0.001 to 46 $\times 10^6$/ml); PC, 3 ± 7 $\times 10^6$/ml (range 1000 to 29 $\times 10^6$/ml). The motile spermatozoa were isolated mainly using the Percoll gradients consisting of 95 and 47.5%. For some IVF–ICSI patients, due to poor sperm numbers in the ejaculate, testicular sperm extracts or epididymal sperm aspirations, the entire sperm sample was pooled, mixed with culture medium and washed twice prior to use in ICSI. In IVF all oocytes were inseminated with 100 000 spermatozoa/ml of culture medium in tubes, whereas in ICSI only the metaphase II oocytes were microinjected 4–6 h after the oocyte retrieval. Narishige micromanipulators (Model MN-108 and MO-108) attached to a Nikon Diaphot inverted microscope (Tokyo, Japan) were used for the ICSI procedure. As described previously, polyvinylpyrrolidone (PVP) was not used in the immobilization of spermatozoa prior to ICSI (Edirisinghe *et al*., 1997). Eighteen to 20 h after insemination or ICSI the oocytes were checked for fertilization. Oocytes which contained two pronuclei and two polar bodies were considered normally fertilized. The one- and three-pronuclear oocytes were considered as abnormally fertilized. Embryo transfer was performed the next day ~48–50 h after oocyte retrieval. At this time the unfertilized oocytes were processed for cytogenetic studies.

**Table I.** Details of patients and the treatment outcomes for intracytoplasmic sperm injection (ICSI) and in-vitro fertilization (IVF) groups

| | ICSI | IVF |
|---|---|---|
| No. patients | 21 | 18 |
| Age (mean ± SD) (years) | 35.5 ± 5.3 | 34.2 ± 4.7 |
| Stimulation regimen | | |
| LF | 15 | 12 |
| LDR | 6 | 6 |
| Peak oestradiol (pmol/l) (mean + SD) | 11 087 ± 7404 | 9782 ± 7518 |
| Total no. oocytes collected | 328 | 348 |
| No. metaphase II oocytes injected | 300 | – |
| No. fertilized normally, 2PN (%) | 185 (61.7) | 186 (53.4) |
| No. fertilized abnormally | | |
| 1PN | 11 (3.7) | 11 (3.2) |
| 3PN | 3 (1.0) | 5 (1.4) |
| No. oocytes damaged | 21 (7.0) | – |
| Overall pregnancy rate (%)[a] | 7/21 (33.3) | 6/18 (33.3) |
| Implantation rate (%)[b] | 8/56 (14.3) | 4/39 (10.3) |
| Pregnancy outcome | | |
| | Live births | 8 (1× twin)3 |
| Biochemical | 2 | – |
| Ectopic | – | 1 |

None of the differences between the two patient groups was significant.
LF = Lucrin flare-up regimen with Metrodin; LDR = Lucrin down-regulation with Metrodin; PN = pronuclear.
[a]Pregnancies achieved from embryos generated in the study cycle and transferred either fresh or frozen–thawed.
[b]Sacs/no. embryos transferred.

The unfertilized oocytes, especially the IVF oocytes, were drawn in and out of a pipette slightly bigger than the oocytes to remove spermatozoa attached to the zona, and the oocytes from both groups were exposed to a hypotonic solution of 1% sodium citrate for 10 min. They were then fixed on to glass microscope slides by the addition of a few drops of the fixative which consisted of 3 parts methanol and 1 part acetic acid (Tarkowski, 1966). The slides were stained with 10% Giemsa and coded for analysis by the cytogeneticist in a double-blind manner. The preparations were examined under an oil immersion objective lens.

Student's *t*-test was used for comparing the peak oestradiol concentrations between the two patient groups. The significance of the incidence of various cytogenetic abnormalities between unfertilized ICSI and IVF oocytes was tested using $\chi^2$-analysis.

## Results

Unfertilized oocytes from 21 ICSI and 18 IVF patients were used in the study. As shown in Table I, the mean age, peak oestradiol level, stimulation regimens, fertilization rate, pregnancy rate, implantation rate and pregnancy outcome for both IVF and ICSI patients were similar. The incidence of cytogenetic abnormality in unfertilized ICSI and IVF oocytes is given in Table II. A total of 88 unfertilized oocytes from ICSI and 85 from IVF patients were used. Of these, 51 ICSI and 62 IVF oocytes showed metaphase plates. Some metaphase plates could be counted accurately (29%) and the others were analysable only at the ploidy level. A similar rate of haploid chromosomal complement was observed in unfertilized ICSI (32/51, 62.7%) and IVF oocytes (38/62, 61.3%). Both groups of unfertilized oocytes showed a similar rate of diploidy. No significant difference in the incidence of aneuploidy was observed between the two groups; 3.9 and 14.5% for hypohaploidy and 2.0 and 0% for hyperhaploidy in ICSI and

**Table II.** Chromosomal abnormality rate and the incidence of sperm chromosomes among unfertilized intracytoplasmic sperm injection (ICSI) in-vitro fertilization (IVF) oocytes

| | | | Oocyte chromosomes (%) | | | | | | Sperm chromosomes (%) | |
|---|---|---|---|---|---|---|---|---|---|---|
| Patient group/number | No. oocytes used | No. with metaphases | Haploid | Fragmented haploid | Hypo-haploid | Hyper-haploid | Diploid | Fragmented diploid | PCC | Sperm nuclei or chromosome |
| ICSI | | | | | | | | | | |
| 21 | 88 | 51 (58.0) | 32 (62.7) | 11 (21.6) | 2 (3.9) | 1 (2.0) | 4 (7.8) | 1 (2.0) | 6 (11.8) | 21 (41.2) |
| IVF | | | | | | | | | | |
| 18 | 85 | 62 (72.9) | 38 (61.3) | 6 (9.7) | 9 (14.5) | – | 6 (9.7) | 3 (4.8) | 8 (12.9) | 12 (19.4) |

[a]ICSI versus IVF, $P < 0.01$.
PCC = premature chromosome condensation.

**Table III.** Distribution and the state of sperm chromosomes in relation to the oocyte chromosomes

| | | Stage of sperm nucleus | | | |
|---|---|---|---|---|---|
| Oocyte chromosomes | Total no. of oocytes nuclei | Condensed swollen nuclei | Decondensed or chromosomes | Metaphase | PCC |
| ICSI | | | | | |
| Haploid | 16 | – | 12 | 1 | 3 |
| Diploid | 2 | 1 | – | – | 1 |
| Not seen | 9 | 7 | – | - | 2 |
| IVF | | | | | |
| Haploid | 14 | 5 | – | 3 | 6 |
| Diploid | 4 | 2 | – | 1 | 1 |
| Not seen | 2 | 1 | – | – | 1 |

ICSI = intracytoplasmic sperm injection; IVF = in-vitro fertilization.

IVF oocytes respectively. A slightly higher incidence of hypohaploidy seen in unfertilized IVF oocytes was due to two patients (3/6 and 3/11) in the group and the incidence of hypohaploidy in these two patients was significantly higher (6/17, 35.3%) than in the remaining IVF patients (3/45, 6.7%; $P < 0.01$ with Yates' correction). Chromosomal fragmentation was observed to a similar extent in both groups of unfertilized oocytes and this abnormality was seen in either haploid or diploid chromosomal complements.

Premature chromosome condensation (PCC) of chromosomes at G1 phase was observed in both groups of unfertilized oocytes (11.8 and 12.9% respectively) and the incidence was not significantly different between the groups. A significantly larger number of unfertilized ICSI oocytes showed the presence of sperm nuclei (27/51, 52.9%) in the ooplasm than the IVF oocytes (20/62, 32.3%; $P < 0.01$) (Table II). The distribution and the state of the sperm head chromatin in relation to the oocyte chromosome complement are given in Table III. Of the 27 spermatozoa found in unfertilized ICSI oocytes, a high proportion of these was found in the decondensed state (12/27, 44.4%) in association with a haploid complement of oocyte chromosomes. Condensed sperm heads were found in oocytes which did not contain any chromosome complements. In the IVF oocytes the majority of spermatozoa were in the condensed state (5/20, 25%) or at metaphase stage (3/20, 15%) or had undergone PCC (6/20, 30%) in association with haploid oocyte chromosomes.

The data were further analysed to study the possible factors which could affect the incidence of chromosomal abnormality. The between-patient variability in the incidence of chromosomal abnormality was studied and patients who had four or more unfertilized oocytes analysed were included. As shown in Figure 1a there was a marked variation in the chromosomal abnormality rate between patients in both IVF (0–71%) and ICSI (0–75%) groups. Three patients, two in IVF and one in ICSI, had normal chromosomal complements in all five, six and seven oocytes for each analysed and details of these patients are given in the figure legend. The second variable studied was the effect of different sperm types [ejaculated, testicular biopsy (TB spermatozoa) and epididymal spermatozoa collected from microepididymal sperm aspiration (MESA spermatozoa)] used in ICSI on the incidence of chromosomal fragmentation. As shown in Figure 1b, a high rate of chromosomal fragmentation (4/6, 67%) was observed in six unfertilized oocytes obtained from three patients who had ICSI–MESA spermatozoa and this incidence was higher than that observed in the other groups [ICSI–ejaculated spermatozoa = 6/35, 17.1% ($P < 0.05$); ICSI–TB spermatozoa = 2/10, 20%; and IVF–normozoospermic spermatozoa = 9/62, 14.5% ($P < 0.01$)]. With ICSI–TB spermatozoa, the difference did not reach a statistically significant level due to small numbers. Thus the data for ICSI–TB spermatozoa and ICSI–MESA spermatozoa were pooled (6/16, 37.5%) and compared with other groups in order to assess whether the retrieved spermatozoa had any influence on the incidence of chromosomal fragmentation of oocytes. It was found that the chromosomal fragmentation rate was significantly higher with the retrieved spermatozoa only when compared to the IVF–normozoo-

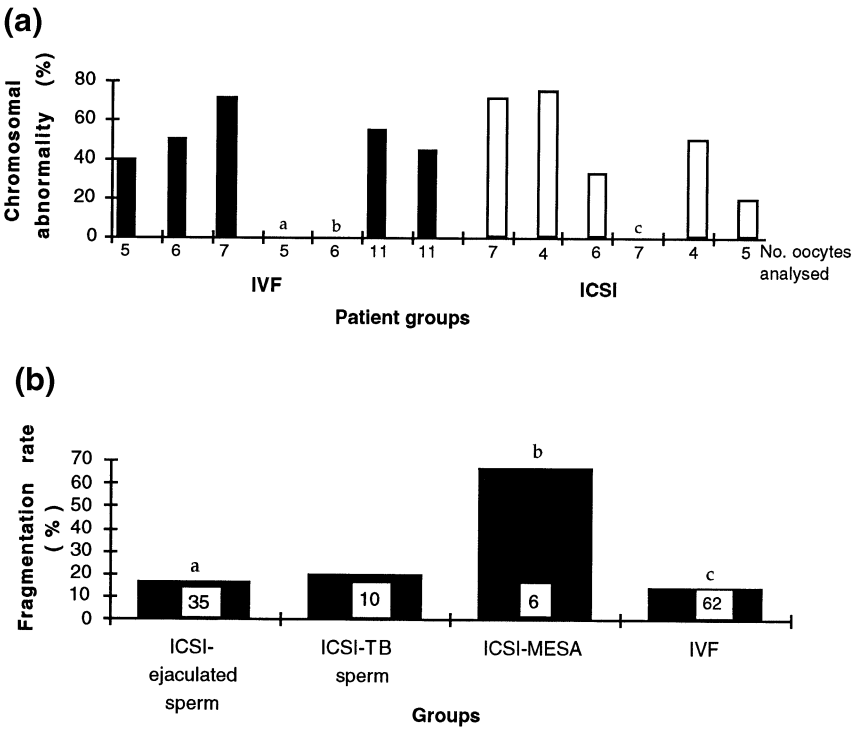


**Figure 1.** Possible factors affecting the incidence of chromosomal abnormality in unfertilized intracytoplasmic sperm injection (ICSI) and in-vitro fertilization (IVF) oocytes. (**a**) Patient variation versus chromosomal abnormality rate. a = 0% chromosomal abnormality, 0% sperm nuclei, patient aged 34 years, tubal infertility, on Lucrin down-regulation regimen + Metrodin, peak oestradiol of 21 500 pmol/l, 36 oocytes collected and 26 fertilized, three embryos transferred, pregnant. b = 0% chromosomal abnormality, 0% sperm nuclei, patient aged 34 years, tubal infertility, on Lucrin flare-up regimen + Metrodin, peak oestradiol of 5240 pmol/l, 17 oocytes collected and seven fertilized, three embryos transferred, not pregnant. c = 0% chromosomal abnormality, 28.6% sperm nuclei, patient aged 32 years, male factor infertility, on Lucrin down-regulation regimen + Metrodin, peak oestradiol of 32 000 pmol/l, 36 oocytes collected and 5/18 fertilized, three embryos transferred in a frozen embryo transfer cycle, pregnant. (**b**) Type of spermatozoa used in ICSI versus chromosomal fragmentation rate. a versus b, $P < 0.05$; b versus c, $P < 0.01$. TB = testicular biopsy.

**Table IV.** Intracytoplasmic sperm injection (ICSI) results obtained using spermatozoa retrieved from the testis (TB spermatozoa) or epididymis (MESA spermatozoa)

| Sperm type/patient | Age (years) | No. fertilized ( $\times 10^6$/ml) | Sperm count | Sperm injection | No. unfertilized oocytes with sperm chromosomes | Oocyte damage | No. unfertilized oocytes with chromosome fragmentation | Pregnant |
|---|---|---|---|---|---|---|---|---|
| TB spermatozoa | | | | | | | | |
| A (1)[a] | 41 | 1/7 | 0.001/0/0[b] | Easy | 1/4 | Nil | 1/4 | No |
| B | 40 | 1/8 | 0.001/few motile/0 | Slightly difficult | 0/6 | Nil | 1/6 | No |
| MESA spermatozoa | | | | | | | | |
| C | 36 | 9/15 | 2/1.1/0.8 | Easy | 1/2 | 5/15 | 0/2 | No |
| D | 38 | 7/8 | 2/1.5/1.4 | Easy | 1/1 | Nil | 1/1 | Yes |
| A (2)[a] | 41 | 3/6 | 0.001/few motile/0 | Difficult | 1/3 | Nil | 3/3 | No |

[a]Same patient (attempt number).
[b]Total count/motile count/progressively motile count.
MESA = microepididymal sperm aspiration; TB = testicular biopsy.

spermic sperm group ($P < 0.05$). There was a slight increase in the chromosomal fragmentation with ICSI–retrieved spermatozoa in comparison to ICSI–ejaculated spermatozoa. However, the difference did not reach a significant level ($\chi^2 = 2.40$, $P > 0.05$).

In order to assess further the high incidence of chromosomal fragmentation observed with the use of retrieved spermatozoa, particularly MESA spermatozoa, various aspects of the patients and the ICSI technique were studied (Table IV). All five patients who used TB or MESA spermatozoa were >35 years of age. It was clear that with the poor quality of spermatozoa available for ICSI (immotile or a few motile, mainly twitching spermatozoa), whether TB or MESA spermatozoa, the fertilization rates achieved were poor (A: 1; B; A: 2) and the incidence of spermatozoa in the unfertilized oocytes was low. Of these patients one had only immotile spermatozoa injected and for both the other two there was difficulty injecting spermatozoa to a varying degree due to the stickiness of the spermatozoa.

Chromosomal fragmentation was observed in all three patients and furthermore all three unfertilized oocytes from the patient with whom there was most difficulty at sperm injection showed chromosomal fragmentation. With good quality spermatozoa, improved fertilization rates were achieved (C, D) and one patient achieved an ongoing pregnancy (D).

## Discussion

The fertilization technique, either ICSI or IVF, does not seem to increase the chromosomal abnormality rate in unfertilized oocytes. As there were no differences in the age, stimulation regimens and the treatment outcomes for the two patient groups, it is possible to assume that the effects of these factors, culture conditions or other laboratory factors which could affect the incidence of chromosomal abnormality were controlled for and the true effect of the fertilization technique could be studied. However, other patient factors which were not taken in to consideration in this study, i.e. weight, cause of infertility etc., may influence the quality of oocytes and thus the incidence of chromosomal abnormality (Plachot, 1995).

The incidence of chromosomal abnormality observed in this study among unfertilized IVF oocytes is much higher than that observed in our previous study carried out in 1989–1990 (Edirisinghe *et al*., 1992). This may be due to the differences in the patient population, stimulation regimen or age of the patients in the two studies. When the aneuploidy rate was considered the incidence in both ICSI (5.9%) and IVF (14.5%) unfertilized oocytes was lower than that observed by Wall *et al*. (1996) (31.6% for ICSI and 37.3% for IVF). This may be due to the differences in the patient populations or the stimulation regimens as seen with our studies. In our present study the majority of the patients were on a pituitary 'flare-up' regimen using leuprolide acetate, whereas in the study reported by Wall *et al*. (1996) the long protocol of pituitary 'down-regulation' using buserelin acetate was used. Furthermore, in-vitro culture conditions may differ between laboratories possibly resulting in variations in the environmental and external factors affecting the oocytes (Abruzzo and Hassold, 1995).

The presence of a single pronucleus indicates oocyte activation and formation of the female pronucleus. In this study oocyte activation was seen in 3.7% of ICSI and 3.2% of IVF oocytes and this incidence was similar to that observed by other groups for either ICSI or IVF (Plachot and Crozet, 1992; Flaherty *et al*., 1995). However, some researchers have observed a higher incidence of unipronuclear oocytes in ICSI than in IVF (Winston *et al*., 1991; Palermo *et al*., 1993; Sultan *et al*., 1995). Furthermore, it is concluded that the majority of oocytes exhibiting a single pronucleus in IVF programmes contain a Y chromosome and are diploid. However, those obtained following ICSI are parthenogenetically activated (Sultan *et al*., 1995). Oocyte activation is associated with an increase in the intracellular free calcium concentrations ($[Ca^{2+}]_i$) and it has been shown that there is a brief increase in $[Ca^{2+}]_i$ immediately after the penetration of the injection needle and then a second change following a lag period of 4–12 h. The latter changes are sperm-dependent and the pattern of change can be either non-oscillatory (parthenogenetic activation) or oscillatory (normal fertilization) (Tesarik *et al*., 1994). Further research involving injection of 24 h old human oocytes has confirmed the importance of calcium in oocyte activation and fertilization (Gearon *et al*., 1995).

It is clear that the three pronuclear oocytes are formed after ICSI due to the failure of second polar body extrusion. The retained polar body chromosomes then condense to form a pronucleus along with the oocyte and sperm chromosomes (Palermo *et al*., 1993; Flaherty *et al*., 1995). Three pronuclear oocytes are formed during IVF (Selva *et al*., 1991; Plachot and Crozet, 1992; Edirisinghe *et al*., 1992) and in the present study the incidence of this abnormality was similar between the two fertilization techniques. The cytogenetic analysis of multipronuclear oocytes has given greater insight into the abnormalities associated with meiotic division following sperm entry in IVF and injection of spermatozoa in ICSI (Macas *et al*., 1996). Macas *et al*. (1996) found that the multipronuclear ICSI oocytes contain a higher incidence of abnormal chromosomal complements (56.7%) than the IVF oocytes (20.0%) and suggested from these findings that the ICSI technique could interfere with regular chromosome segregation at the second meiotic division due to its possible harmful effects (changes of intracellular hydrostatic pressure or local concentrations of calcium ions) on the oocyte cytoskeleton (Macas *et al*., 1996). The low incidence of three pronuclear oocytes (1.0%) in the present study may indicate that the incidence of non-extrusion of the second polar body is low due to the quality of oocytes used, less disturbance to the positioning of the polar body in relation to the meiotic spindle due to gentle handling of oocytes during the removal of the corona radiata (Cohen *et al*., 1994; Flaherty *et al*., 1995) or perhaps the sperm injection procedure is less damaging to the microtubular system (Macas *et al*., 1996).

Chromosomal fragmentation has been observed to a similar extent in unfertilized IVF and ICSI oocytes by Wall *et al*. (1996). In the present study chromosomal fragmentation in the ICSI oocytes was slightly increased but not to a significant level compared to the IVF oocytes. When chromosomal fragmentation was further studied according to the type of spermatozoa used, unfertilized oocytes in the retrieved sperm group, especially the ICSI–MESA sperm group, showed a high incidence of this abnormality. This may be due to the sperm agglutination increasing the stickiness of spermatozoa used which required injection of increased amounts of culture medium into the oocyte with the spermatozoa, or longer exposure to adverse culture conditions during the ICSI procedure. A 45% chromosomal breakage rate was observed by Bergere *et al*. (1995) in ICSI oocytes and they suggested that the cause for the chromosomal breakage could be the ageing of oocytes as well as the microinjection procedure. Thus it is important to carry out further investigations to study chromosomal fragmentation using a larger sample size, particularly to confirm the present findings on the retrieved spermatozoa and determine whether other factors may be involved in its aetiology.

Patient variation observed in the incidence of chromosomal

abnormality, particularly the hypohaploidy rate, may be due to the scattering and loss of chromosomes during the preparation of metaphase spreads (Bongso *et al*., 1988) or it is a genuine expression of defects seen in certain patients who would give reduced fertilization with any fertilization technique. This was further supported by the fact that in the present study a significant variation in the total incidence of chromosomal abnormality was seen among the ICSI and IVF patients (0 to ~70%) even though the cause of infertility in these two groups was mainly male factor in ICSI and mainly tubal in IVF. Two tubal infertility IVF patients and one male factor infertility ICSI patient had no chromosomal abnormality in any of the unfertilized oocytes analysed. When the details of these three patients were studied they had varying levels of peak oestradiol, oocyte numbers and fertilization rates. Achievement of two pregnancies among these three patients indicates the viability of embryos resulting from good quality oocytes.

In IVF, a large number of oocytes which remained unfertilized (67.7%) showed a lack of sperm penetration. Problems associated with sperm–oocyte interaction due to defective spermatozoa or oocytes may have contributed to this failure. However, a significantly increased number of unfertilized ICSI oocytes contained spermatozoa (52.9%) and the majority of spermatozoa were found in the decondensed state (44.4%) in association with haploid oocyte chromosomes. This finding is similar to the observations made by Dozortsev *et al*. (1994) and Flaherty *et al*. (1995). Some unfertilized ICSI oocytes also contained condensed sperm heads and these were seen mainly in oocytes which had no oocyte chromosomes. It is possible that the oocyte chromosomes may have disintegrated or extruded to form extrabodies as observed in one-pronuclear oocytes by Balakier *et al*. (1993). However, unfertilized IVF oocytes contained sperm nuclei at a lower incidence than those of ICSI oocytes (present study; Schmiady and Kentenich, 1989; Selva *et al*., 1991; Plachot and Crozet, 1992) and none of the sperm heads was found arrested in the decondensed state. The sperm nuclei in these oocytes were at condensed, metaphase stage or had undergone PCC and were mainly found in oocytes with haploid chromosomes. The incidence of condensed sperm heads may have been overestimated as the zonae pellucidae were not removed using pronase (Angell *et al*., 1991; Dozortsev *et al*., 1994) even though most of the spermatozoa attached to the zonae were removed by pipetting through a drawn pipette. The presence of condensed spermatozoa in unfertilized IVF oocytes has been reported in other studies (Van Blerkom *et al*., 1994; Asch *et al*., 1995). In some oocytes two distinct metaphase chromosome sets were found and, due to the identification of the Y chromosome or the position of the metaphase in relation to the oocyte chromosomes, the extra chromosome complement was identified as the sperm metaphase. Sperm metaphases are likely to represent PCC of spermatozoa in G2 (Schmiady and Kentenich, 1989; Van Blerkom *et al*., 1994). A similar proportion of spermatozoa exhibited PCC in both unfertilized ICSI (11.8%) and IVF (12.9%) oocytes. This phenomenon has been reported previously for unfertilized IVF oocytes (Schmiady *et al*., 1986; Placot *et al*., 1987; Schmiady and Kentenich, 1989; Edirisinghe *et al*., 1992) and ICSI oocytes (Dozortsev *et al*., 1994; Flaherty *et al*., 1995). In PCC, the sperm chromosomes undergo condensation prematurely due to the availability of cytoplasmic chromosome condensing factors due to the presence of a metaphase (the oocyte's) in the same cytoplasm (Schmiady *et al*., 1986; Calafell *et al*., 1991). It is suggested that PCC is associated with oocyte immaturity (Calefell *et al*., 1991). Oocytes acquire the competence to undergo nuclear and cytoplasmic maturation independently (Eppig *et al*., 1994) and it is possible that in some oocytes, especially the ones that are retrieved from smaller follicles, complete cytoplasmic maturation at a later stage than the nuclear maturation (Flaherty *et al*., 1995). This is supported by the findings in mice that in metaphase II stage oocytes, the capacity for activation is attained gradually (Kubiak, 1989). A similar degree of PCC observed between IVF and ICSI oocytes indicate that the overall oocyte quality in our IVF programme is similar due to standard protocols followed for ovarian stimulation and timing of oocyte retrieval post HCG (35 h) and insemination/ICSI following oocyte retrieval (4–6 h). Alternatively, PCC may identify a group of physiologically immature oocytes (Flaherty *et al*., 1995) which may require longer preincubation for cytoplasmic maturation to complete before oocyte activation and normal fertilization can occur.

The differences in the distribution and the state of sperm nuclei observed in the two groups of unfertilized oocytes could be explained as follows. In ICSI, a large number of spermatozoa arrested in the oocytes, mainly in the decondensed state in association with haploid chromosomes of the oocytes; this could be due to: (i) the lack of transfer of oocyte activation factor from the spermatozoa (Edwards, 1995; Dozortsev *et al*., 1995b; Flaherty *et al*., 1995), (ii) reduced or no access to oocyte decondensation factors probably associated with intact plasma membrane (Maleszewski, 1990; Dozortsev *et al*., 1994), (iii) spermatozoa from male factor patients may have defects associated with acrosome, the plasma membrane and/or the nucleus (Moosani *et al*., 1995; Bianchi *et al*., 1996), or (iv) defective oocyte activation-dependent sperm chromatin decondensation (Dozortsev *et al*., 1995c). In unfertilized IVF oocytes, a significant oocyte problems associated with cytoplasmic maturity may be the cause for the sperm head to either arrest in a condensed state or at metaphase stage or to undergo PCC (Schmiady and Kentenich, 1989; Asch *et al*., 1995).

In this study no difference in the range or overall numbers of cytogenetic abnormalities was observed between the unfertilized ICSI and IVF oocytes. This finding is interesting because, in ICSI, only the metaphase II oocytes are selected for sperm injection and in IVF all the oocytes retrieved (germinal vesicle, metaphase I and II) are inseminated. Under these conditions it is clear that the events such as (i) sperm entry either through sperm penetration (IVF) or sperm delivery (ICSI), (ii) possible effects of sperm injection, (iii) defects associated with nuclear maturation, (iv) defects associated with cytoplasmic maturation, (v) lack of oocyte activation, and (vi) sperm decondensation defects may each have contributed to a varying degree to the overall incidence of abnormality in the two fertilization techniques. In our study the cumulative effect of these factors has given overall cytogenetic abnormality rates of 37.3%

(ICSI) to 38.7% (IVF) and non-cytogenetic abnormality rates due to the presence of spermatozoa of 52.9% (ICSI) to 32.3 % (IVF).

The efficiency of sperm delivery was assessed by the presence of spermatozoa in unfertilized ICSI oocytes. In the present study, the sperm delivery was less efficient (53%) than in Flaherty *et al*. (1995) (66%) or Wall *et al*. (1996) (72.5%). This may be associated with the difficulties experienced at sperm injection especially with sticky spermatozoa in some retrieved sperm samples, frozen–thawed sperm samples or the presence of antisperm antibodies. Perhaps the use of PVP may improve the handling of such spermatozoa during ICSI. Even though the numbers are small, the overall fertilization rates and the implantation and live birth rates achieved in the present study are similar to the other ICSI programmes where use of PVP is a standard protocol (Van Steirteghem *et al*., 1993; Payne *et al*., 1994; Fishel *et al*., 1995; Slavander *et al*., 1995).

In conclusion, in a cytogenetic study carried out in a double-blind manner using patient groups with similar age, stimulation regimens and treatment outcomes, we were able to show that the ICSI and IVF techniques do not affect the overall incidence of chromosomal abnormality in unfertilized oocytes. However, a significant patient variation was observed in the incidence of chromosomal abnormality in both the male factor (ICSI) and the tubal (IVF) infertility groups and the hypohaploidy rate was increased in certain patients. As shown in earlier studies, the present data also show that an increased number of unfertilized ICSI oocytes contain sperm nuclei in the cytoplasm compared to IVF oocytes. The causes for the arrest of sperm nuclei either at the condensed, decondensed, metaphase or PCC stage may vary between the two fertilization techniques, where in ICSI mainly a sperm factor and in IVF mainly an oocyte factor may be involved. Difficulty at sperm injection as seen with spermatozoa retrieved from the testis or epididymis may increase the incidence of chromosomal fragmentation. Further research is needed to study the chromosomal fragmentation during ICSI using a larger sample size.

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