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Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection

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In this study we investigated whether morphology and chromatin anomalies in human spermatozoa can influence fertilization after intracytoplasmic sperm injection (ICSI). We examined unfertilized oocytes, using the fluorochrome Hoechst 33342, to determine whether a relationship exists between failure of fertilization and sperm chromatin quality. Sperm chromatin packaging quality was assessed using the chromomycin A_3 (CMA_3) fluorochrome, and the presence of DNA damage in spermatozoa, using in-situ nick translation. Normal males present sperm parameters with a normal morphology of >20%, CMA_3 fluorescence of <30% and exhibit endogenous nicks in <10% of their spermatozoa. When patients were separated according to these values no difference was observed in their fertilization rates after ICSI. When the unfertilized ICSI oocytes were examined, we found that patients with CMA_3 fluorescence of <30% and nicks in <10% of their spermatozoa had only 17.5 and 21.6% respectively of their unfertilized oocytes containing spermatozoa that remained condensed. In contrast, patients with higher CMA_3 and nick values had a significantly higher number, 41.2 and 48.9%, of their unfertilized oocytes containing condensed spermatozoa. Sperm morphology did not show any such pattern. The percentage of spermatozoa which had initiated decondensation in unfertilized oocytes was not influenced by morphology, CMA_3 fluorescence or nicks. In light of these results we postulate that poor chromatin packaging and/or damaged DNA may contribute to failure of sperm decondensation after ICSI and result in failure of fertilization.

Key words: fertilization/intracytoplasmic sperm injection/male infertility/sperm chromatin/sperm nuclear decondensation

Introduction

Since the report of the initial pregnancies using intracytoplasmic sperm injection (ICSI) (Palermo *et al.*, 1992) a dramatic improvement in the treatment of cases of severe male factor infertility has been witnessed. Consequently, ICSI has now superseded previous micromanipulation techniques, used for treating male factor couples, such as partial zona dissection (PZD) and sub-zonal insemination (SUZI) (Palermo *et al.*, 1993; Van Steirteghem *et al.*, 1993; Trounson, 1994). It is now widely applied to couples that have failed to achieve fertilization in conventional in-vitro fertilization (IVF) cycles, in which the husband has sperm parameters limiting or ruling out the use of IVF or in which the husband has undergone surgery to recover epididymal or testicular spermatozoa. The use of ICSI in these patients has led to a surprisingly high success rate in both fertilization and pregnancy rates regardless of the severity of the sperm defect (Van Steirteghem *et al.*, 1993; Payne *et al.*, 1994; Tournaye *et al.*, 1994; Nagy *et al.*, 1995; Silber *et al.*, 1995). This success has subsequently caused debate on the possibility that using ICSI to force fertilization by abnormal spermatozoa may have hidden consequences in the normality of embryos and the resulting fetus (Cummins *et al.*, 1994; Benagiano and Rowe, 1995; Ménézo and Dale, 1995; Tarín and Cano, 1995; Seamark and Robinson, 1995). Initial data thankfully suggest that the normality of the babies delivered has not been jeopardized (Van Steirteghem *et al.*, 1993; Bonduelle *et al.*, 1994). While it may be argued that a resulting baby may have ensued as a consequence of the selection of the most capable spermatozoon giving rise to a normal embryo, the question arises as to the fate and quality of the spermatozoa that failed to achieve fertilization.

ICSI in the majority of cases is applied to couples in which the male has a severe sperm defect. These defects can manifest themselves in numerous ways: low sperm number, poor motility, abnormal morphology or combinations of these parameters. In contrast to these normally accepted parameters spermatozoa from certain subfertile men may have defects in their surface proteins and/or in their chromatin organization. Numerous groups have shown that male factor infertility patients possess hidden anomalies in the composition of their sperm nuclei, displaying higher levels of loosely packaged chromatin and damaged DNA (Evenson *et al.*, 1980; 1986; Foresta *et al.*, 1992; Sailer *et al.*, 1995). In our own studies we have used two methods to assess sperm chromatin quality: (i) the guanine–cytosine specific fluorochrome chromomycin A_3 (CMA_3) which provides evidence of poor packaging quality of chromatin in human spermatozoa, as it allows an indirect visualization of protamine-deficient, nicked and partially-denatured DNA; and (ii) in-situ nick translation, not preceded by endonuclease treatment, to evaluate the presence of endogenous nicks in the DNA of ejaculated spermatozoa (Bianchi *et al.*, 1993, 1996; Manicardi *et al.*, 1995).

The fertilization potential of spermatozoa with abnormal chromatin organization in conventional IVF has been difficult to ascertain as results may be influenced by the initiation of

the acrosome reaction and sperm membrane interactions with the oocyte. In light of this we have shown in a recent study that semen with high CMA_3 positivity leads to significantly lower fertilization rates when using SUZI (Bianchi *et al.*, 1996). When using ICSI the sperm membrane–oocyte interaction is of limited importance placing more emphasis on the quality of the sperm chromatin and the ability of the oocyte to initiate decondensation and pronuclear formation. The presence of spermatozoa containing damaged DNA (Bianchi *et al.*, 1993; Manicardi *et al.*, 1995; Sailer *et al.*, 1995) and the use of ICSI to force fertilization in these patients may cause further uneasiness as to the fate of fertilized ICSI eggs.

In this study we have therefore assessed the CMA_3 fluorescence and the presence of DNA damage in spermatozoa of patients treated by ICSI. We have investigated whether the quality of a patient's spermatozoa in terms of chromatin anomalies can influence the outcome of ICSI. In addition we have examined oocytes in which no pronuclei were observed after ICSI, using the fluorescent dye Hoechst 33342, to determine whether a relationship exists between failure of fertilization and sperm chromatin quality.

Materials and methods

Patient selection and treatment

Patients undergoing infertility treatment using IVF or ICSI, from September, 1994 to August, 1995 were included in this study. The stimulation protocol used by our group has been previously published (Urner *et al.*, 1993; Sakkas *et al.*, 1994).

IVF and ICSI procedures

Oocytes were collected by ultrasound guided transvaginal follicular aspiration ~34 h after administration of human chorionic gonadotrophin (HCG). They were cultured in 25 μl droplets of T6 medium containing 10% maternal serum at 37°C in an atmosphere of 5% CO_2 in air. All cultures were grown using 35 mm Falcon Petri dishes (Becton Dickinson, Plymouth, UK) under oil (Light White Mineral Oil, Sigma Pharmaceuticals, Buchs, Switzerland). For normal IVF, the oocytes were inseminated with 50 000 motile spermatozoa obtained by either swim-up migration or mini-Percoll gradient centrifugation (Ord *et al.*, 1990). In cases where the normal sperm morphology of the patient was 15–20 or <15%, insemination was performed with 150 000 or 300 000 motile spermatozoa, respectively.

ICSI was used in couples in whom the male partner had a very poor semen quality and/or who were unable to achieve fertilization in previous IVF cycles. For the ICSI procedure pipettes were prepared using the apparatus previously described (Sakkas *et al.*, 1992). The ICSI injection pipette was prepared with an internal diameter of 5–7 μm with a 30° bevel, no spike was placed on the tip. The cumulus cells were removed ~2–3 h after aspiration of the oocytes, by pipetting the oocytes in HEPES-buffered T6 medium containing 1 mg/ml Type IV hyaluronidase (Sigma). The injection procedure was carried out on a glass slide with a depression. All manipulations were carried out on a warming stage and under oil. Oocytes were maintained in HEPES-buffered T6 medium during the procedure. Spermatozoa were selected from a droplet of culture medium containing 10% polyvinylpyrrolidone (PVP-360; Sigma). Following immobilization of the spermatozoa, by stroking the mid-piece-tail region (Van Steirteghem *et al.*, 1993), the spermatozoon was injected into the cytoplasm of the oocyte. The oocyte was maintained with the polar body in the 12 o'clock or 6 o'clock position. After injection, the oocytes were examined for any damage, washed carefully and placed in culture medium; <5% of the oocytes injected were damaged during the procedure.

The presence of pronuclei was recorded ~14–18 h after insemination or ICSI. The remaining non-fertilized oocytes were subsequently observed twice a day until the day of embryo transfer, using an Olympus IMT-2 microscope. Embryos were transferred into the uterus of the patients 2 days after routine IVF or ICSI.

Preparation of unfertilized oocytes

After embryo transfer had been performed the non-degenerated unfertilized MII oocytes were prepared for staining with Hoechst 33342 (Sigma) and the fluorescent chromatin patterns in the oocytes classified as previously described (Urner *et al.*, 1993). Unfertilized oocytes were taken only in cycles where more than two oocytes could be fixed. Of the 55 IVF cycles performed, unfertilized oocytes were examined from 19 IVF cycles. In cycles where a high number or all of the oocytes failed to fertilize after conventional IVF, the oocytes were injected by ICSI the following day (reinjection). In these cases unfertilized oocytes were fixed one day later than for conventional IVF or ICSI. Unfertilized oocytes were fixed in 42 cycles after conventional ICSI.

In this study the fate of the injected spermatozoa was of most interest. The chromatin of the spermatozoa in the unfertilized oocytes was scored as either: (i) condensed; (ii) decondensing or decondensed in the cytoplasm of the oocyte and the maternal chromatin was between metaphase II (one polar body) and telophase II stage (two polar bodies); or (iii) both the maternal and paternal chromatin formed pronuclei.

Sperm assessment

Semen samples were collected by masturbation and allowed to liquefy. Once liquefied one slide was immediately prepared for assessment of sperm morphology. Morphology was assessed according to World Health Organization criteria (1992). The major part of the sample was used for the respective treatment of the couple and a small part used for preparing slides for the study. In a number of cases of severe oligozoospermia, none or only one or two slides were prepared, so as not to jeopardize the patients' treatment and as the preparation would not have allowed sufficient sperm cells to be counted on the slides. To prepare slides for CMA_3 staining and in-situ nick translation the semen aliquot was first washed in Dulbecco's Ca-Mg free phosphate-buffered saline (PBS) and centrifuged at 170 *g* for 10 min. The spermatozoa were washed again and then fixed in methanol/glacial acetic acid (3:1) at 4°C, for 5 min and then spread on clean slides.

CMA_3 staining was performed as previously described (Bianchi *et al.*, 1993). Briefly, each slide was treated for 20 min with 100 μl of CMA_3 solution (Sigma) (0.25 mg/ml in McIlvaine buffer, pH 7.0, containing 10 mM $MgCl_2$). Slides were then rinsed in buffer and mounted with buffered glycerol. Positivity to the fluorochrome was examined using a Zeiss photomikroskop III using a combination of exciter: dichroic: barrier filters of BP 436/10: FT 580: LP 470. A total of 100–150 spermatozoa were counted on each slide for morphology and CMA_3 positivity.

To examine for the presence of endogenous nicks in sperm DNA, in-situ nick translation was performed by omitting the endonuclease treatments, since the DNA polymerase I by virtue of its 5'-3' exonucleotic activity can catalyse movement of the nicks along the double helix (Sambrook *et al.*, 1989). The nick translation procedure was performed according to Sumner *et al.* (1990), except that streptavidin–fluorescein isothiocyanate was used as a label instead

Table I. Fertilization and pregnancy rates of patients treated by routine in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) during the study. Figures in parentheses are percentages

	IVF	ICSI
Cycles	55	42
Mean age[a] (years)	34.7 ± 4.3	33.5 ± 4.4
Oocytes	382	342
Two pronuclei (2PN) (% 2PN/oocyte)	231 (60.5)	155 (45.3)
Transfers	48	42
Pregnancies (pregnancies/transfer)	13[b] (27.1)	7[b] (16.7)

[a]± SD.
[b]One biochemical pregnancy.

Table II. Distribution of the semen characteristics, sperm concentration and motility, in treatment cycles where unfertilized oocytes were fixed from in-vitro fertilization (IVF), including routine IVF + reinjection, and intracytoplasmic sperm injection (ICSI)

Sperm concentration ($\times 10^6$/ml)	Sperm motility (%)					
	0–20		21–50		>50	
	IVF	ICSI	IVF	ICSI	IVF	ICSI
0–5	0	13	0	3	0	2
6–20	0	2	1 + 2	14	5 + 1	2
>20	0	1	2 + 4	0	11 + 12	5

of streptavidin alkaline phosphatase to show biotinylated d-UTP incorporation. The methods have been previously described (Bianchi *et al.*, 1993; Manicardi *et al.*, 1995). A total of 100–150 spermatozoa were counted on each slide.

Results

Patient characteristics

Patients were divided according to those who had undergone routine IVF, those who had a low fertilization rate after IVF and had ICSI the following day (reinjection) and those in whom ICSI was performed on the day of oocyte retrieval. The clinical results obtained during the period of the study are summarized in Table I. Unfertilized oocytes were fixed from 42 cycles of ICSI and from 19 cycles each of conventional IVF and reinjection. The fertilization rate after reinjection was 48 out of 121 (39.7%); however, no pregnancies were obtained after transfer of embryos arising only from this procedure. The poor viability of rescued oocytes is in agreement with a recent report by Tucker *et al.* (1995). The sperm concentration and forward motility of the patients treated by IVF and ICSI in which unfertilized oocytes were taken from are shown in Table II.

Sperm characteristics: morphology, chromomycin A_3 fluorescence and endogenous nicks

When assessing the parameters of the patients undergoing IVF and ICSI distinct differences were evident in all their semen parameters. The mean percentage of spermatozoa presenting normal morphology, CMA_3 fluorescence and endogenous nicks for the IVF patients were all significantly different to the ICSI patients (Table III). When examining these three parameters it could be presumed that males with acceptable sperm parameters would present a normal morphology of >20%, CMA_3 fluorescence <30% and exhibit endogenous nicks in <10% of their spermatozoa.

Table III. Mean percentage of spermatozoa presenting normal morphology, CMA_3 fluorescence and endogenous nicks for the in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) patients

Assessment	IVF		ICSI	
	No. of patients	Mean (±SEM)	No. of patients	Mean (± SEM)
Morphology	36	33.2 ± 2.6[a]	42	15.7 ± 2.1[a]
CMA_3	32	29.2 ± 3.3[b]	38	44.3 ± 3.5[b]
Nicks	30	8.6 ± 1.1[c]	36	17.1 ± 2.0[c]

[a,b,c]Similar letters are all significantly different $P < 0.01$. The means were transformed using an arcsin square root transformation and analysed using one-way analysis of variance and Scheffé's F-test.

Table IV. Sperm chromatin patterns in assumed unfertilized oocytes after intracytoplasmic sperm injection (ICSI), reinjection after failed in-vitro fertilization (IVF) and IVF

	ICSI (%)	Reinjection (%)	IVF (%)
Cycles	42	19	19
Oocytes examined	133	55	51
Oocytes with sperm inside	112	40	-*
Spermatozoa			
condensed	32 (28.5)	13 (32.5)	-
decondensed	47 (42.0)	17 (42.5)	1 (2.0)
Two pronuclei	13 (11.6)	4 (10.0)	4 (7.8)
Others**	20 (17.8)	6 (15.0)	3 (5.9)

*43 (84.3%) of the oocytes showed no indications of sperm penetration.
**See Results.

Oocyte chromatin patterns

A total of 51 unfertilized oocytes were examined after conventional IVF. Only five of the oocytes showed any signs of sperm penetration, four of them containing pronuclei which were not observed under bright field microscopy and one other containing a decondensing spermatozoon (Table IV). Three more oocytes were classified as 'others', two were showing signs of degeneration and not able to be interpreted and another displayed micronuclei. These results are in agreement with a previous report by our own group (Urner *et al.*, 1993).

Of the oocytes subjected to micromanipulation (ICSI and reinjection) there were no differences in the sperm chromatin patterns (Table IV). Of all the oocytes examined a proportion did not show any presence of spermatozoa inside, 21 out of 133 (15.8%) after ICSI and 15 out of 55 (27.3%) after reinjection. This compares favourably to the results of Flaherty *et al.* (1995) who reported that 19% of their oocytes examined had ejected the spermatozoa after ICSI. The ejection of the spermatozoa is most likely due to a technical error during the ICSI procedure. In the remainder of the oocytes examined ~30% contained spermatozoa that remained condensed while ~42% presented spermatozoa either decondensing or decondensed (Table IV). A further 10–12% of the oocytes

Table V. Sperm chromatin patterns in assumed unfertilized oocytes after intracytoplasmic sperm injection (ICSI) in relation to chromomycin A_3 (CMA_3) positivity and the presence of endogenous nicks in the patients' spermatozoa. Figures in parentheses are percentages

	CMA_3 <30%	CMA_3 >30%	Nicks <10%	Nicks >10%
Cycles	13	25	13	23
Oocytes examined containing spermatozoa	40	51	37	47
Condensed spermatozoa	7[a] (17.5)	21[a] (41.2)	8[b] (21.6)	23[b] (48.9)
Decondensed spermatozoa	26 (65.0)	26 (51.0)	21 (56.8)	20 (42.6)
Others	7 (17.5)	4 (7.8)	8 (21.6)	4 (8.5)

[a–a]Significantly different ($\chi^2 = 4.84$; $P = 0.03$).
[b–b] Significantly different ($\chi^2 = 5.51$; $P = 0.02$).
*Includes sperm decondensing, decondensed and pronuclei.

Table VI. Sperm chromatin patterns in assumed unfertilized oocytes after ICSI in relation to the percentage of normal morphology of the patients' spermatozoa

	Morphology		
	<10%	10–20%	>20%
Cycles	17	9	16
Oocytes examined containing spermatozoa	59	20	33
Condensed spermatozoa	17 (28.8)	7 (35.0)	8 (24.2)
Decondensed spermatozoa*	32 (54.2)	11 (55.0)	17 (51.6)
Others (%)	10 (17.0)	2 (10.0)	8 (24.2)

*Includes sperm decondensing, decondensed and pronuclei.

possessed two pronuclei which had not been visualized by bright field microscopy. Although the appearance of the pronuclei may have been missed during the routine analysis, they are most likely indicative of an abnormality as by the second day after fertilization they would be expected to be cleavage stage embryos.

Of the oocytes classified as 'others': in the reinjection group, two were not interpretable, one was parthenogenetically activated, one contained micronuclei and two others appeared as 2-cell embryos with unequal blastomeres. In the ICSI group, nine were not interpretable, three were parthenogenetically activated, two contained micronuclei, two showed signs of premature chromatin condensation and four others appeared as 2-cell embryos with unequal blastomeres.

Oocyte chromatin patterns according to sperm characteristics

To ascertain whether a relationship existed between the sperm chromatin parameters of the patients and the ability of spermatozoa to fertilize after ICSI we separated the patients according to their morphology, CMA_3 fluorescence and the presence of endogenous nicks. Our own results (Manicardi *et al.*, 1995; present study) indicate that in a normal semen sample CMA_3 fluorescence and endogenous nicks can be taken as <30 and <10% respectively, while levels above these values would be indicative of a semen sample containing spermatozoa with abnormal chromatin. When the ICSI patients were separated according to these criteria no overall difference was observed in their ability to achieve fertilization; patients with less than and greater than 30% CMA_3 fluorescence had a fertilization rate of 56 out of 130 (43.1%) and 86 out of 175 (49.1%) respectively, while those with <10 and >10% endogenous nicks had a fertilization rate of 53 out of 122 (43.4%) and 78 out of 163 (47.8%) respectively.

When the same assessment was performed on the fate of the spermatozoa in the unfertilized oocytes after ICSI, a significant difference was observed in the percentage of spermatozoa which remained condensed (Table V). In patients who had a lower percentage of anomalies in their chromatin (<30% CMA_3 fluorescence and <10% nicks) a significantly lower number of spermatozoa remained condensed. In contrast, patients who had a high percentage of anomalies in their chromatin (>30% CMA_3 fluorescence and >10% nicks) had more than double the number of unfertilized oocytes containing spermatozoa that had remained condensed. Once sperm decondensation had been initiated no significant difference was observed between the patient groups.

When the patients were separated according to their sperm morphology no difference was observed in fertilization rates: normal morphology of <10%, 80 out of 171 (46.8%); 10–20%, 23 out of 51 (45.1%) and >20%, 52 out of 120 (43.3). The percentage of unfertilized oocytes containing spermatozoa that had remained condensed did not differ in relation to the patients morphology (Table VI).

In the six clinical pregnancies after ICSI three of the fathers possessed spermatozoa which had >30% CMA_3 fluorescence and >10% nicks. In the other three pregnancies, CMA_3 fluorescence was <30% in two, while the presence of endogenous nicks was <10% in all three.

Discussion

In this study we have analysed unfertilized oocytes after ICSI to investigate the fate of the spermatozoa in these oocytes. Dozortsev *et al.* (1994) previously reported that 41 and 51% of unfertilized oocytes after ICSI contained intact and swollen sperm heads respectively, while Flaherty *et al.* (1995) reported figures of 10 and 71% respectively. The results reported in this study fall in between these studies. In addition, our study indicates that aged oocytes retain the ability to decondense spermatozoa after ICSI in a similar manner to that observed in fresh oocytes. Interestingly, the overall percentage of sperm heads remaining condensed and decondensing in unfertilized oocytes examined after reinjection was similar to that of the fresh oocytes. Of paramount interest in this study, however, was to investigate whether certain sperm characteristics, in particular sperm chromatin anomalies, could lead to a decrease in fertilization after ICSI.

ICSI has now been accepted as a successful treatment for male factor infertility patients, in that it results in high fertilization and pregnancy rates (Palermo *et al.*, 1992; Van Steirteghem *et al.*, 1993). Regardless of whether the male suffers from extreme oligoasthenoteratozoospermia or has had a failure in conventional IVF, fertilization and pregnancy rates do not seem to differ (Van Steirteghem *et al.*, 1993). The only ultimate criterion for successful ICSI appears to be at least

one living spermatozoon in the semen sample (Nagy *et al.*, 1995). When examining sperm count, motility and morphology Nagy *et al.* (1995) found that there was no important influence from either the type or extent of sperm impairment on the outcome of ICSI. Furthermore, normal fertilization and pregnancy rates can also be achieved in obstructive and non-obstructive azoospermia where ICSI is combined with epididymal or testicular sperm extraction (Devroey *et al.*, 1994; Silber *et al.*, 1994, 1995). The success of ICSI using spermatozoa from males with varying semen parameters, and especially those with a high percentage of abnormal spermatozoa, brings into question the importance of the sperm contribution during fertilization, embryo development and fetal viability.

The results of this study indicated that spermatozoa from patients with high CMA_3 fluorescence, which is indicative of poorly-packaged chromatin, and a higher level of endogenous DNA nicks are not limited in their ability to achieve fertilization using ICSI when compared to patients exhibiting low levels of these two parameters. Concurrently, pregnancies were also obtained using spermatozoa from patients with high CMA_3 fluorescence and a high level of endogenous DNA nicks. In contrast, the unfertilized oocytes examined using Hoescht 33342 showed distinct differences in sperm chromatin patterns indicating that certain differences may exist. Unfertilized oocytes from patients exhibiting high values of CMA_3 positivity and endogenous nicks gave rise to unfertilized oocytes that contained a higher percentage of spermatozoa that remained condensed. As oocytes were obtained from women whose average age and hormonal stimulation were similar, differences observed in these groups cannot be easily attributed to the quality of the oocytes. In addition, as the ICSI procedure effectively transcends membrane interactions between the gametes, the onus for this difference falls on a relationship between the sperm chromatin organization and the ooplasm. Griveau *et al.* (1992) found that asthenozoospermic men displayed a high percentage of spermatozoa with nuclear abnormality, and, using cytoplasmic extracts from unfertilized *Xenopus laevis* oocytes to induce decondensation, demonstrated that their sperm chromatin decondensed slowly and partially compared to normozoospermic men. In contrast, Perreault *et al.* (1987) reported that hamster sperm nuclei treated with the S–S bond-reducing agent dithiothreitol (DTT) decondensed more rapidly when microinjected into hamster oocytes. This result in the hamster suggests that spermatozoa with a lower chromatin packaging quality should transform into pronuclei more readily and possibly more rapidly. The question however remains as to the type of chromatin packaging anomaly that is present in human spermatozoa. Artificially decondensing the nuclei of a homogeneous population of normal hamster spermatozoa with DTT may not be indicative of the type of decondensed nuclei observed in ejaculated human spermatozoa. Chromatin packaging anomalies in human spermatozoa arise because of defects in the sperm nuclei condensation mechanism in certain patients. This implies a more intrinsic problem, as evidenced by disturbances in aberrant protamine 1/protamine 2 ratios (Balhorn *et al.*, 1988; Belokopytova *et al.*, 1993; de Yebra *et al.*, 1993) and the presence of nicks in the DNA (Bianchi *et al.*, 1993; Gorczyca *et al.*, 1993; Manicardi *et al.*, 1995; Sailer *et al.*, 1995). These anomalies may also make these spermatozoa more susceptible to exogenous factors leading to further damage. We propose that the differences observed in the percentage of condensed spermatozoa in unfertilized oocytes after ICSI arise because abnormal chromatin of some spermatozoa could either fail to decondense due to a physical or mechanical inability or it may fail to trigger regulatory mechanisms leading to sperm decondensation.

One of the major factors leading to chromatin packaging problems in ejaculated human spermatozoa could be due to faulty protamine deposition during spermatogenesis. A number of studies have shown that human spermatozoa from infertility patients are more likely to exhibit sperm chromatin anomalies related to the deposition of protamines (Balhorn *et al.*, 1988; Belokopytova *et al.*, 1993; de Yebra *et al.*, 1993). We have previously suggested that mature human spermatozoa with underprotaminated regions can also be naturally nicked and/or partially denatured (Bianchi *et al.*, 1993). A correlation between abnormal sperm chromatin packaging and the presence of DNA strand breaks has been shown to exist (Manicardi *et al.*, 1995; Sailer *et al.*, 1995) and in both these studies it has been hypothesized that these anomalies may arise due to faults in the mechanisms that package and protect the sperm chromatin during spermatogenesis. While spermatogenesis must require many gene products, no human mutations specifically disrupting spermatogenesis have been defined at the molecular level. Schlicker *et al.* (1994) failed to detect any mutation in the genes which code for protamine 1 and 2 and transition protein 1 in infertile men whose spermatozoa showed a positive reaction to Aniline Blue, which is an indicator of disturbed sperm chromatin condensation. Genetic causes linked to anomalies in the male reproductive tract are evident. For example, mutations in the gene complex controlling cystic fibrosis are associated with congenital bilateral absence of the vasa deferentia (Bienvenu *et al.*, 1993) while recently it has also been shown that 12 out of 89 males with azoospermia presented a deletion on the long arm of their Y chromosomes (Reijo *et al.*, 1995).

As previously stated, the advent of ICSI has removed numerous barriers of sperm selection that were necessary to choose the fittest spermatozoon. Whereas previous micromanipulation techniques, such as PZD and SUZI, still depended on gamete membrane interaction, ICSI bypasses even this step and forcibly introduces the spermatozoon into the ooplasm where the onus falls largely on the capabilities of the oocyte to initiate sperm decondensation. ICSI has therefore provided the tool to achieve fertilization and in doing so it is also more likely to succeed in completing the genetic project of any spermatozoon, regardless of the quality of its genome. Cummins *et al.* (1994) have already warned that the application of reproductive techniques such as micromanipulation should proceed cautiously as there may well be adverse genetic consequences for the children born. A paternal effect on human embryo development has already been noted *in vitro* (Ron El *et al.*, 1991; Janny and Ménézo, 1994).

The apparent unimpaired embryo viability after the use of

ICSI indicates that a selection process may transpire at the time of fertilization. The oocytes fertilized may have resulted from spermatozoa that had normal or only slightly abnormal chromatin. A high level of abnormalities in the chromatin of a spermatozoon selected for ICSI may impede the completion or initiation of decondensation therefore leading to a failure of fertilization. This may occur even though the oocyte possesses the necessary mechanism to initiate decondensation. Although we do not postulate that the failure of fertilization is entirely due to a sperm defect it seems likely that poor chromatin packaging and/or damaged DNA may contribute to a failure in the decondensation process. It remains to be answered, however, if sperm chromatin anomalies, whether in the form of underprotamination or more seriously when apparent as endogenous DNA nicks, have a genetic basis. A greater understanding of the molecular basis of male infertility is therefore needed to avoid the inappropriate use of these micromanipulation techniques.

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