

# **Sperm nuclear DNA damage and altered chromatin structure: effect on fertilization and embryo development**

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**In the first part of this report we investigate whether chromatin anomalies in human spermatozoa can influence fertilization after intracytoplasmic sperm injection (ICSI). We have examined the sperm chromatin packaging quality using the chromomycin A<sub>3</sub> (CMA<sub>3</sub>) fluorochrome and the presence of DNA damage in spermatozoa using in-situ nick translation. When comparing the spermatozoa of patients undergoing in-vitro fertilization (IVF) and ICSI distinct differences are evident in that ICSI males have a higher CMA<sub>3</sub> fluorescence, indicating spermatozoa with loosely packed chromatin, and more spermatozoa containing endogenous DNA nicks. When examining the unfertilized oocytes of ICSI patients we found that men who had a high percentage of anomalies in their chromatin, i.e. >30% CMA<sub>3</sub> fluorescence and >10% nicks, had more than double the number of unfertilized oocytes containing spermatozoa that had remained condensed. The observation that failed fertilized oocytes, injected with spermatozoa from patients with a higher percentage of sperm nuclear anomalies, contain more condensed spermatozoa indicates that a selection process against these spermatozoa may be in place at the time of fertilization. In the second part of the study we show that spare ICSI embryos have significantly lower rates of development to the blastocyst stage compared with those developed after routine IVF. These results show that a greater understanding of the molecular basis of male infertility is therefore needed to broaden our knowledge on the effect that abnormal spermatozoa have on fertilization and embryo development.**

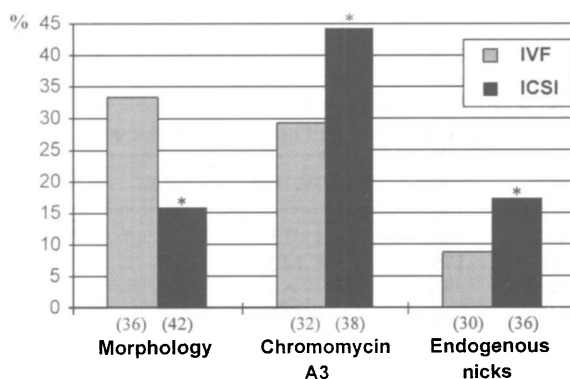
*Key words:* fertilization/intracytoplasmic sperm injection/male infertility/sperm chromatin/sperm nuclear decondensation

## Introduction

The treatment of severe male factor infertility has seen a dramatic advancement since the report of the initial pregnancies using intracytoplasmic sperm injection (ICSI) (Palermo *et al.*, 1992). Consequently, ICSI 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 and in which the husband has undergone surgery to recover epididymal or testicular spermatozoa. The surprisingly high success rate in both fertilization and pregnancy rates after ICSI regardless of the severity of sperm defect (Van Steirteghem *et al.*, 1993; Payne *et al.*, 1994; Tournaye *et al.*, 1994; Nagy *et al.*, 1995; Silber *et al.*, 1995) has subsequently caused debate on the possibility that using ICSI to force fertilization by abnormal spermatozoa may have hidden consequences for the normality of embryos and the resulting fetus (Cummins *et al.*, 1994; Ménézo and Dale, 1995; Seamark and Robinson, 1995; Tarín and Cano, 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) even though some studies report an increase in sex chromosome anomalies (Bonduelle *et al.*, 1996; Meschede and Horst, 1997).

ICSI in the majority of cases is applied to couples in which the male has a severe sperm defect. These defects are distinguishable as low sperm numbers, poor motility, abnormal morphology or combinations of these parameters. In addition to these normally accepted parameters, spermatozoa from certain sub-fertile men also display hidden defects in their surface proteins and/or in their chromatin organization. We and others have shown that male factor infertility patients possess 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; Golan *et al.*, 1997). In our own studies we have used two methods to assess sperm chromatin quality: (i) the guanine–cytosine specific fluorochrome, chromomycin A<sub>3</sub> (CMA<sub>3</sub>), which evidences 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 evidence the presence of endogenous nicks in the DNA of ejaculated spermatozoa (Bianchi *et al.*, 1993; 1996; Manicardi *et al.*, 1995; Bizzaro *et al.*, 1998).

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 that semen with high CMA<sub>3</sub> positivity leads to significantly lower fertilization rates when using sub-zonal sperm injection (SUZI) (Bianchi *et al.*, 1996). When using the ICSI technique all sperm membrane–oocyte interactions are superseded placing more importance on the quality of the sperm nucleus and the ability of the oocyte to initiate decondensation and pronuclear formation. The presence of spermatozoa containing damaged



**Figure 1.** The mean percentage of spermatozoa presenting normal morphology, chromomycin A<sub>3</sub> fluorescence and endogenous nicks for the patients undergoing in-vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). The number of patients assessed for each group is shown in parentheses. \*Significantly different ( $P < 0.01$ ) from the IVF value. The means were transformed using an arcsin square root transformation and analysed using one-way analysis of variance and Scheffé's  $F$ -test.

DNA (Bianchi *et al.*, 1993; Gorczyca *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. Here we therefore discuss whether the quality of a patient's spermatozoa, in terms of chromatin anomalies, can influence the outcome of ICSI and present results comparing embryo development to the blastocyst stage of patients undergoing routine IVF and ICSI.

### Comparing the morphology, chromomycin A<sub>3</sub> fluorescence and endogenous nicks of ejaculated human spermatozoa from patients undergoing routine IVF and ICSI

When assessing the spermatozoa of patients undergoing IVF and ICSI distinct differences are evident in the mean percentage of spermatozoa presenting normal morphology, CMA<sub>3</sub> fluorescence (an indicator of the packaging quality of sperm chromatin) and endogenous nicks (an indicator of DNA damage in the sperm nucleus) (Figure 1).

When examining the above three parameters it could therefore be presumed that males with acceptable sperm parameters would present a normal morphology of >20%, CMA<sub>3</sub> fluorescence of <30% and exhibit endogenous nicks in <10% of their spermatozoa (Figure 1). The question therefore arises as to whether the above anomalies may influence fertilization after ICSI.

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 sperm morphology, CMA<sub>3</sub> fluorescence, and the presence of endogenous nicks. When ICSI patients were separated according to these criteria no overall difference was observed in their ability to achieve fertilization (Table I).

These results indicate that spermatozoa from patients with poor morphology,

**Table I.** Fertilization rates after intracytoplasmic sperm injection (ICSI) in male factor patients separated according to their percentage of normal morphology, chromomycin A<sub>3</sub> positivity and the presence of endogenous nicks

No. of:	Chromomycin A <sub>3</sub>		Endogenous nicks		Morphology	
	<30%	>30%	<10%	>10%	<20%	>20%
Cycles	13	25	13	23	17	25
Oocytes injected	130	175	122	163	222	120
2PN <sup>a</sup>	56	86	53	78	103	52
(%)	(43.1)	(49.1)	(43.4)	(47.8)	(46.4)	(43.3)

<sup>a</sup>Fertilization rates are reduced as the study only involved the analysis of cycles in which more than two unfertilized oocytes were retrieved to investigate whether sperm had initiated decondensation after intracytoplasmic sperm injection (ICSI).

2PN = two pronuclear.

high CMA<sub>3</sub> fluorescence, and/or a higher level of endogenous DNA nicks are not limited in their ability to achieve fertilization using ICSI when compared with those from patients exhibiting low levels of these parameters. This may however mean that spermatozoa selected for ICSI were among those that did not possess anomalies in the sperm nucleus. What however is the fate of the spermatozoa selected for ICSI in oocytes that failed to fertilize?

When failed fertilized oocytes are examined after ICSI, using Hoechst 33342, and the status of the injected spermatozoa investigated, the sperm chromatin in the unfertilized oocyte can be 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. The relationship between the sperm chromatin patterns in the unfertilized oocytes after ICSI and the patients morphology, CMA<sub>3</sub> fluorescence and presence of endogenous nicks is shown in Table II. In patients who had a lower percentage of anomalies in their chromatin (<30% CMA<sub>3</sub> 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<sub>3</sub> fluorescence and >10% nicks) had more than double the number of unfertilized oocytes containing spermatozoa that had remained condensed. The percentage of unfertilized oocytes containing spermatozoa that had remained condensed did not differ in relation to the patients morphology. Once sperm decondensation had been initiated, no significant difference was observed between the patient groups.

### Embryo development

Although the above evidence suggests that spermatozoa with abnormal chromatin may influence the process of fertilization, the question of whether the development of an ensuing embryo is adversely affected is not known. A paternal effect has

**Table II.** Sperm chromatin patterns in assumed unfertilized oocytes after intracytoplasmic sperm injection (ICSI) in relation to morphology, chromomycin A<sub>3</sub> positivity and the presence of endogenous nicks in the patients spermatozoa (adapted from Sakkas *et al.*, 1996)

No. of:	Chromomycin A <sub>3</sub>		Endogenous nicks		Morphology	
	<30%	>30%	<10%	>10%	<20%	>20%
Cycles	13	25	13	23	17	25
Oocytes examined	40	51	37	47	59	53
Sperm condensed (%)	7 <sup>a</sup> (17.5)	21 <sup>a</sup> (41.2)	8 <sup>b</sup> (21.6)	23 <sup>b</sup> (48.9)	17 (28.8)	15 (28.3)
Sperm decondensed <sup>c</sup> (%)	26 (65.0)	26 (51.0)	21 (56.8)	20 (42.6)	32 (54.2)	28 (52.8)
Others (%)	7 (17.5)	4 (7.8)	8 (21.6)	4 (8.5)	10 (16.9)	10 (18.9)

Values with same superscripts were significantly different using Fisher's exact test, <sup>a</sup>*P* = 0.02 and <sup>b</sup>*P* = 0.01.

<sup>c</sup>Includes spermatozoa decondensing, decondensed and pronuclei.

been associated with the development of human embryos to the blastocyst stage (Janny and Ménézo, 1994). These authors showed that frozen spermatozoa and abnormal spermatozoa gave rise to embryos with a significantly lower cleavage rate and potential to form blastocysts. Prior to this study, a number of other authors also suggested that semen quality and embryo development may be related (Ron-El *et al.*, 1991; Chan *et al.*, 1993; Parinaud *et al.*, 1993). The previous studies that have indicated a paternal effect have however been based on using routine IVF insemination procedures where cleavage rates and embryo quality may be influenced by the timing of fertilization.

In six clinical pregnancies after ICSI, three of the fathers possessed spermatozoa which had >30% CMA<sub>3</sub> fluorescence and >10% nicks, while in another three pregnancies CMA<sub>3</sub> fluorescence was <30% in two, while the presence of endogenous nicks was <10% in all three. The establishment of pregnancies might not however be a good parameter to measure the effect of spermatozoa on the developmental potential of an embryo after ICSI. Any problems in development would be evident in the earlier stages of embryo development, in particular that of blastocysts. In our own clinic we have examined the development of supernumerary embryos after ICSI and IVF to the blastocyst stage. Embryos were cultured to the blastocyst stage using the Vero cell co-culture system (Sakkas *et al.*, 1994). Interestingly, the development of blastocysts after ICSI is significantly less than that after routine IVF (Table III). How this relates to a sperm defect however needs further investigation.

### **The consequences of anomalies in sperm chromatin packaging or damaged DNA on fertilization and embryo development**

In procedures such as ICSI, membrane interactions between the gametes are effectively transcended, hence the onus for the completion of successful decon-

**Table III.** The development of spare human embryos to the blastocyst stage after routine in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI)

	ICSI	IVF
No. of cycles	60	90
No. of cycles with blastocysts (%)	22 (36.7)	70 (77.7)*
No. of spare embryos	168	423
No. of blastocysts (%)	45 (26.8)	200 (47.3)*

<sup>a</sup>Significantly different from ICSI ( $P < 0.001$ , Fisher's exact test).

densation of the spermatozoa falls largely on a relationship between sperm chromatin organization and the ooplasm. How anomalies in the sperm nuclear structure can influence decondensation during fertilization is not established. 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. In addition, Burruel *et al.* (1996) showed that mouse spermatozoa with grossly misshapen heads could be injected into oocytes and contribute to the development of normal fertile adults.

The question of whether DNA damaged spermatozoa can impair the process of fertilization is not clear, however the alarming studies by the group of Robaire (see below) have indicated that damage to sperm DNA may be linked to an increase in early embryo death. They have shown that treatment of male rats with cyclophosphamide had little effect on the male reproductive system but caused single strand DNA breaks in the cauda-epididymal spermatozoa (Qiu *et al.*, 1995a) and altered the decondensation potential of spermatozoa (Qiu *et al.*, 1995b). More disturbingly, similar treatment protocols using cyclophosphamide produce an increase in postimplantation loss and malformations (Trasler *et al.*, 1985; 1986; 1987) and are transmissible to the next generation (Hales *et al.*, 1992). A number of studies have indicated that the oocyte has the capability to repair the damaged DNA of spermatozoa. For example, Matsuda and Tobar (1988) showed that newly fertilized eggs were capable of repairing some of the deliberately damaged DNA of mouse spermatozoa irradiated with UV or treated with alkylating agents. Although there may be inbuilt mechanisms to guard against the incorporation of damaged DNA the results from the group of Robaire are highly indicative that these systems may not be foolproof.

The observation that failed fertilized oocytes injected with spermatozoa from patients with higher rates of sperm nuclear anomalies contain more condensed

spermatozoa indicates that a selection process against these spermatozoa may transpire at the time of fertilization. A high level of abnormalities in the chromatin of a spermatozoa 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. Furthermore, lower rates of development to the blastocyst stage by ICSI embryos compared to those developed after routine IVF indicate that a further selection may occur during the preimplantation stage. A greater understanding of the molecular basis of male infertility, is therefore needed to broaden our knowledge on the effect that abnormal spermatozoa have on fertilization and embryo development and to avoid the inappropriate use of ICSI.

## References

- Bianchi, P.G., Manicardi, G.C., Bizzaro, D. *et al.* (1993) Effect of DNA protamination on fluorochrome staining and *in situ* nick-translation of murine and human mature spermatozoa. *Biol. Reprod.*, **49**, 1038–1043.
- Bianchi, P.G., Manicardi, G.C., Urner, F. *et al.* (1996) Chromatin packaging and morphology in ejaculated human spermatozoa: evidence of hidden anomalies in normal spermatozoa. *Mol. Hum. Reprod.*, **2**, 139–144.
- Bizzaro, D., Manicardi, G.C., Bianchi, P.G. *et al.* (1998) In-situ competition between protamine and fluorochromes for sperm DNA. *Mol. Hum. Reprod.*, **4**, 127–132.
- Bonduelle, M., Legein, J., Buysse, A. *et al.* (1994) Comparative follow-up study of 130 children born after ICSI and 130 children born after IVF. *Hum. Reprod.*, **9** (Suppl. 1), 38.
- Bonduelle, M., Legein, J., Buysse, A. *et al.* (1996) Prospective follow-up study of 432 children born after ICSI. *Hum. Reprod.*, **11**, 1558–1564.
- Burrue, V.R., Yanagimachi, R. and Whitten, W.K. (1996) Normal mice develop from oocytes injected with spermatozoa with grossly misshapen heads. *Biol. Reprod.*, **55**, 709–714.
- Chan, S.Y.W., Tucker, M.J., Leung, C.K.M. and Leong, M.K.H. (1993) Association between human *in vitro* fertilization rate and pregnancy outcome: a possible involvement of spermatozoal quality in subsequent embryonic viability. *Asia-Oceania J. Obstet. Gynaecol.*, **19**, 357–373.
- Cummins, J., Jequier, A. and Kan, R. (1994) Molecular biology of human male infertility: links with ageing, mitochondrial genetics and oxidative stress. *Mol. Reprod. Dev.*, **37**, 345–362.
- Evenson, D.P., Darzynkiewicz, Z. and Melamed, M.R. (1980) Relation of mammalian sperm chromatin heterogeneity to fertility. *Science*, **240**, 1131–1133.
- Evenson, D., Darzynkiewicz, Z., Jost, L. *et al.* (1986) Changes in accessibility of DNA to various fluorochromes during spermiogenesis. *Cytometry*, **7**, 45N53.
- Foresta, C., Zorzi, M., Rossato, M. and Varotto, A. (1992) Sperm nuclear instability and staining with aniline blue: abnormal persistence of histones in spermatozoa in infertile men. *Int. J. Androl.*, **15**, 330–337.
- Golan, R., Schochat, L., Weissenberg, R. *et al.* (1997) Evaluation of chromatin condensation in human spermatozoa: a flow cytometric assay using acridine orange staining. *Mol. Reprod. Dev.*, **3**, 47–54.
- Graczyka, W., Traganos, F., Jesionowska, H. and Darzynkiewicz, Z. (1993) Presence of DNA strand breaks and increased sensitivity of DNA *in situ* to denaturation in abnormal human sperm cells: Analogy to apoptosis of somatic cells. *Exp. Cell Res.*, **207**, 202–205.

- Griveau, J.F., Charbonneau, M., Blanchard, Y. *et al.* (1992) Decondensation of human sperm nuclei and HP1 protamine degradation from normospermia and asthenospermia in *Xenopus* egg extracts. *Arch. Androl.*, **29**, 127–136.
- Hales, B.F., Crosman, K. and Robaire, B. (1992) Increased postimplantation loss and malformations among the F<sub>2</sub> progeny of male rats chronically treated with cyclophosphamide. *Teratology*, **45**, 671–678.
- Janny, L. and Ménézo, Y.J.R. (1994) Evidence for a strong paternal effect on human preimplantation embryo development and blastocyst formation. *Mol. Reprod. Dev.*, **38**, 36–42.
- Manicardi, G.C., Bianchi, P.G., Pantano, S. *et al.* (1995) Underprotamination and nicking of DNA in ejaculated human spermatozoa are highly related phenomena. *Biol. Reprod.*, **52**, 864–867.
- Matsuda, Y. and Tobari, I. (1988) Chromosomal analysis in mouse eggs fertilized *in vitro* with sperm exposed to ultraviolet (UV) light and methyl and ethyl methansulfonate (MMS and EMS). *Mutat. Res.*, **198**, 131–144.
- Ménézo, Y. and Dale, B. (1995) Paternal contribution to successful embryogenesis. *Hum. Reprod.*, **10**, 1326–1328.
- Meschede, D. and Horst, J. (1997) Sex chromosomal anomalies in pregnancies conceived through intracytoplasmic sperm injection: a case for genetic counselling. *Hum. Reprod.*, **12**, 1125–1127.
- Nagy, Z., Liu, J., Joris, H. *et al.* (1995) The result of intracytoplasmic sperm injection is not related to any of the three basic sperm parameters. *Hum. Reprod.*, **10**, 1123–1129.
- Palermo, G., Joris, H., Devroey, P. and Van Steirteghem, A.C. (1992) Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. *Lancet*, **340**, 17–18.
- Parinaud, J., Mieusset, R., Vieitez, G. *et al.* (1993) Influence of sperm parameters on embryo quality. *Fertil. Steril.*, **60**, 888–892.
- Payne, D., Flaherty, S.P., Jeffrey, R. *et al.* (1994) Successful treatment of severe male factor infertility in 100 consecutive cycles using intracytoplasmic sperm injection. *Hum. Reprod.*, **9**, 2051–2057.
- Perreault, S.D., Naish, S.J. and Zirkin, B.R. (1987) The timing of hamster sperm nuclear decondensation and male pronucleus formation is related to sperm nuclear disulfide bond content. *Biol. Reprod.*, **36**, 239–244.
- Qiu, J., Hales, B.F. and Robaire, B. (1995a) Damage to rat spermatozoal DNA after chronic cyclophosphamide exposure. *Biol. Reprod.*, **53**, 1465–1473.
- Qiu, J., Hales, B.F. and Robaire, B. (1995b) Effects of chronic low dose cyclophosphamide on the nuclei of rat spermatozoa. *Biol. Reprod.*, **53**, 33–40.
- Ron-El, R., Nachum, H., Herman, A. *et al.* (1991) Delayed fertilization and poor embryonic development associated with impaired semen quality. *Fertil. Steril.*, **55**, 338–344.
- Sailer, B.L., Jost, L.K. and Evenson, D.P. (1995) Mammalian sperm DNA susceptibility to In Situ denaturation associated with the presence of DNA strand breaks as measured by the terminal deoxynucleotidyl transferase assay. *J. Androl.*, **16**, 80–87.
- Sakkas, D., Jacquenoud, N., Campana, A. and Leppens, G. (1994). Comparison of results after *in vitro* fertilized human embryos are cultured in routine medium and in coculture on Vero cells: a randomized study. *Fertil. Steril.*, **61**, 521–525.
- Sakkas, D., Urner, F., Bianchi, P.G. *et al.* (1996) Sperm chromatin anomalies can influence decondensation after intracytoplasmic sperm injection (ICSI). *Hum. Reprod.*, **11**, 837–843.
- Seamark, R.F. and Robinson, J.S. (1995) Potential health problems stemming from assisted reproduction programmes. *Hum. Reprod.*, **10**, 1321–1322.
- Silber, S.J., Van Steirteghem, A.C. Liu, J. *et al.* (1995) High fertilization and pregnancy rate after intracytoplasmic sperm injection with spermatozoa obtained from testicle biopsy. *Hum. Reprod.*, **10**, 148–152.
- Tarín, J.J. and Cano, A. (1995) Effects of manipulation *in vitro* of human oocytes and embryos on the birthweight of the resultant babies. *Hum. Reprod.*, **10**, 1322–1324.
- Tournaye, H., Devroey, P., Liu, J. *et al.* (1994) Microsurgical epididymal sperm aspiration and intracytoplasmic sperm injection: a new effective approach to infertility as a result of congenital bilateral absence of the vas deferens. *Fertil. Steril.*, **61**, 1045–1051.
- Trasler, J.M., Hales, B.F. and Robaire, B. (1985) Paternal cyclophosphamide treatment causes fetal loss and malformations without affecting male fertility. *Nature*, **316**, 144–146.



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- Trasler, J.M., Hales, B.F. and Robaire, B. (1986) Chronic low dose cyclophosphamide treatment of adult male rats: Effect on fertility, pregnancy outcome and progeny. *Biol. Reprod.*, **34**, 276–283.
- Trasler, J.M., Hales, B.F. and Robaire, B. (1987) A time course study of chronic paternal cyclophosphamide treatment of rats: effects on pregnancy outcome and the male reproductive and haematologic systems. *Biol. Reprod.*, **37**, 317–326.
- Van Steirteghem, A.C., Liu, J., Joris, H. *et al.* (1993) Higher success rates by intracytoplasmic sperm injection than by subzonal insemination. Report of a second series of 300 consecutive treatment cycles. *Hum. Reprod.*, **8**, 1055–1060.