Article

Blastocyst development after sperm selection at high magnification is associated with size and number of nuclear vacuoles

Pierre Vanderzwalmen entered the field of embryology in 1978 at the University of Liege after graduating as engineer in biotechnology and biochemistry. With Professor Massip, he reported in 1986 the first live birth after vitrification of mouse and cow embryos. From 1989 to 2003, he worked at the Schoysman Infertility Management Foundation, obtaining the first TESE baby in the world. As Senior Clinical Embryologist, he now coordinates the scientific IVF activities of the Institute for Reproductive Medicine and Endocrinology in Bregenz and the Centre Hospitalier Inter Regional Cavell (CHIREC) in Brussels. His current research interests are focused on vitrification of oocytes and embryos, on sperm selection and on dynamic embryo culture techniques.

Pierre Vanderzwalmen

Pierre Vanderzwalmen[1–3,7,8], Antje Hiemer[1,7], Paul Rubner[1], Magnus Bach[1], Anton Neyer[1], Astrid Stecher[1], Petr Uher[2], Martin Zintz[1], Bernard Lejeune[3], Sabine Vanderzwalmen[3], Guy Cassuto[4], Nicolas H Zech[6]
[1]Institute for Reproductive Medicine and Endocrinology, Bregenz, Austria; [2]Institute for Reproductive Medicine and Endocrinology, Pilsen, Czech Republic; [3]Centre Hospitalier Inter Régional Cavell (CHIREC), Braine l'Alleud, Bruxelles, Belgium; [4]Laboratoire Drouot, Paris, France; [6]Reproductive Genetics Institute, 2825 N Halsted, Chicago, IL 60657, USA and Department of Obstetrics, University Hospital Zurich, Switzerland
[7]Contributed equally to this paper.
[8]Correspondence: e-mail: pierrevdz@hotmail.com

Abstract

Spermatozoa selection at high magnification before intracytoplasmic sperm injection seems to be positively associated with pregnancy rates after day 3 embryo transfers. The aim was to demonstrate an association between the presence of vacuoles in sperm nuclei and the competence of embryos to develop to day 5. Grading of spermatozoa at ×6000–×12,500 magnification: grade I, no vacuoles; grade II, ≤2 small vacuoles; grade III, ≥1 large vacuole; grade IV, large vacuoles with other abnormalities. The outcome of embryo development in a group of 25 patients after sibling oocyte injection with the four different grades of spermatozoa showed no significant difference in embryo quality up to day 3. However, the occurrence of blastocyst formation was 56.3 and 61.4% with grade I and II spermatozoa respectively, compared with 5.1% with grade III and 0% with grade IV respectively ($P < 0.001$). Spermatozoa selection at high magnification using Nomarski interference contrast is useful to identify more precisely the size and the number of nuclear vacuoles that greatly exert a negative effect on embryo development to the blastocyst stage. These observations confirm previous studies pointing to possible 'early and late paternal effects', both of which may have an impact on early embryonic development.

Keywords: *blastocyst, ICSI, IMSI, sperm nuclei, vacuole*

Introduction

With the introduction of a new concept for observing spermatozoa called 'motile-sperm organelle-morphology examination' (MSOME), it is now possible to examine the fine nuclear morphology of motile spermatozoa in real time at a magnification of up to ×6600 using Nomarski differential interference contrast (Bartoov *et al.*, 2001). Bartoov *et al.* (2001, 2002) report on the benefit of selecting spermatozoa using such a technique. As a consequence, they established a new intracytoplasmic sperm injection (ICSI) procedure called intracytoplasmic morphologically selected sperm injection (IMSI). It is now well accepted that, unlike IMSI, the classic method of selecting spermatozoa at ×400 magnifications has severe limitations: a large panel of malformations that may negatively influence the outcome of embryo development go undetected.

Several publications report that the selection of spermatozoa with normal nuclear shapes at high magnification is positively associated with pregnancy rates after day-3 embryo transfers in couples with previous and repeated implantation failures

(Bartoov *et al*., 2002, 2003; Junca *et al*., 2004; Berkovitz *et al*., 2006a) and in patients with an elevated degree of DNA fragmented spermatozoa (Hazout *et al*., 2006).

If no normal spermatozoa can be found, the only alternative is to choose those that are morphologically second best. Berkovitz *et al*. (2005, 2006a) report low fertilization rates and a low percentage of top quality embryos on day 3 after IMSI with spermatozoa exhibiting a large panel of nuclear malformations in terms of shape, size and the presence of vacuoles.

In a recent paper, Berkovitz *et al*. (2006b) analysed more specifically the impact of nuclear vacuoles in the head of spermatozoa on pregnancy outcome. They concluded that spermatozoa of normal nuclear shape and with large vacuoles negatively influence pregnancy and implantation rates as compared with a control group containing spermatozoa of normal nuclear shape and content. Furthermore, they demonstrated an association between defective spermatozoa and higher early abortion rates, despite no apparent decrease in embryo quality on day 3.

If no apparent early paternal effects on embryo development up to day 3 can be observed when oocytes are fertilized by spermatozoa with large vacuoles in the sperm head, an intriguing question arises: does the presence of such nuclear vacuoles, which cannot be detected with conventional ICSI at ×200 or ×400 magnification, influence the embryo's capacity to develop to the blastocyst stage? This would suggest the presence of a late paternal effect that impacts embryo development after the onset of paternal DNA content contribution to embryonic development, which starts around day 3 after fertilization (Tesarik *et al*., 2005).

One of the most frequent questions with regard to IMSI relates to its indications and if IMSI can be seen as a useful tool in addition to conventional ICSI in certain cases. It is generally acknowledged that for patients with previous failure of implantation or for patients with high level of DNA fragmentation, IMSI is a good option (Bartoov *et al*., 2003; Berkovitz *et al*., 2006a; Hazout *et al*., 2006). There is a great heterogeneity between all the semen samples, so that the frequency by which good spermatozoa can be selected varies greatly from one patient to the other. As a consequence, the fundamental question to elucidate concerns the probability with which a normal spermatozoon can be selected with the classical ICSI method under ×400 magnification.

In order to clarify these issues, a study was designed to determine whether the existence of vacuoles in the nuclei of spermatozoa affects embryo development to the blastocyst stage.

In answer to this question, preliminary results are presented in which the percentage of selected normal spermatozoa was analysed after classical ICSI in relation to the percentage of normal forms present in the sample and detected using the high magnification methodology IMSI.

Materials and methods

The study was conducted between September and December 2006 on couples entering the ICSI programme as a result of male factor infertility. In order to minimize the influence of female factor infertility, only those couples were eligible to enter the study in which the woman was younger than 40 years [mean age 36.7 ± 2.1 (SD)] and had at least eight oocytes available upon oocyte retrieval.

The couples were informed that their oocytes would be injected after selecting spermatozoa with the help of IMSI instead of ICSI.

A sperm washing procedure was performed after centrifugation on three layers gradient of pure sperm (Nidacon, Sweden), as described previously (Vanderzwalmen *et al*., 1991).

For IMSI, different droplets were placed into glass-bottomed dishes (Willco wells, Amsterdam, The Netherlands). An elongated polyvinylpyrrolidone (PVP) drop (Ferti pro, Bornem, Belgium) was placed adjacent to a maximum of 2 drops of human tubal fluid (HTF)–HEPES (IVFonline, Canada), each containing one oocyte. A smaller drop of HTF medium contain the spermatozoa was placed close to the PVP drop. The drops were covered with sterile mineral oil (Cryo Biosystems, l'Aigle, France).

At the start of IMSI, spermatozoa were inserted into the small drop and a small bridge was created to the PVP drop in order to allow the motile spermatozoa to swim to the PVP drop.

A first selection of motile spermatozoa in the PVP drop was made at ×630–1000 magnification under a Normarski interferential Leica AM 6000 inverted microscope (Leica, Germany). If possible, spermatozoa that were selected displayed a normal oval head shape as well as absence of both cytoplasmic extrusions and tail defects. Using a variable zoom lens (HC VarioC-mount; Leica,), it was possible to evaluate, after immobilization, the morphology on the monitor at magnifications ranging between ×6600 and ×12,000 and photo-documented them for subsequent classification. The primary intention was to choose spermatozoa without vacuoles for injection into the oocytes. Depending on the degree of sperm morphology impairment, the time required to select the best sperm ranged between 2 and 15 min. When it became obvious after 15 min that spermatozoa of a normal appearance could not be found, the second-best spermatozoa with the least number of vacuoles and/or other abnormalities was selected for injection. In such situations, it is difficult to decide when to stop the search for a normal spermatozoon. It can take 15 min and longer, and this also depends on the number of oocytes that have to be injected.

Since the influence of the size and number of vacuoles on the outcome of embryo development was unknown, it was necessary to establish a new grading system. Small (<4% of the head volume) or big vacuoles were defined according to the analysis made by Bartoov and colleagues (2003). The spermatozoa were graded and classified into four groups according to the presence or size of vacuoles: grade I, absence of vacuoles (**Figure 1A**); grade II, maximum of two small vacuoles (**Figure 1B**,**C**); grade III, more than two small vacuoles (**Figure 1D**) or at least one large vacuole (**Figure 1E**); grade IV, large vacuoles in conjunction with abnormal head shapes or other abnormalities (**Figure 1F**).

After selecting the best spermatozoon with IMSI and immobilization, injection was performed as described

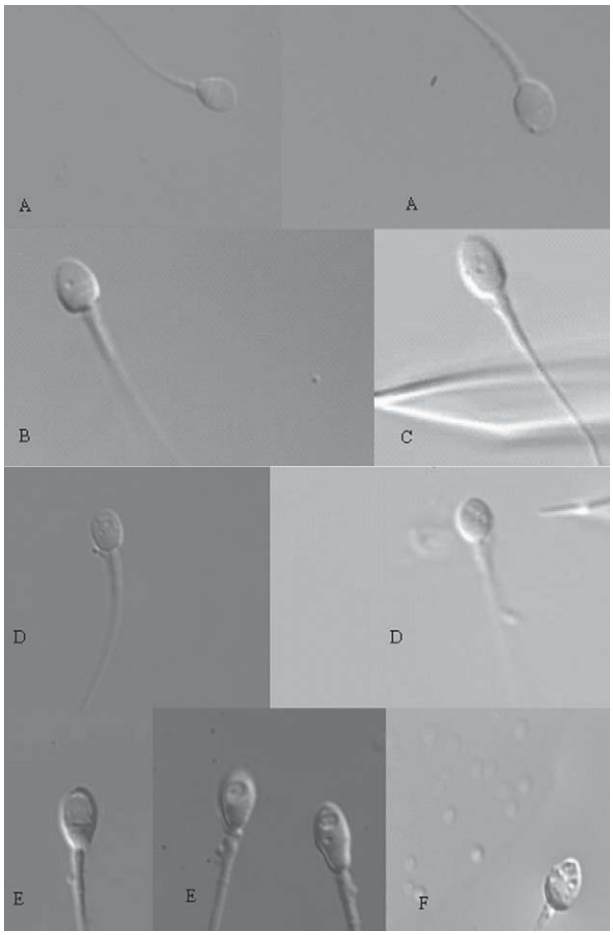

Figure 1. Grading of spermatozoa into four groups according to the presence or size of the vacuoles. Grade I: normal form and no vacuoles (**A**). Grade II: normal form and ≤ 2 small vacuoles (**B**, **C**). Grade III: normal form, >2 small vacuoles or at least one large vacuole (**D**, **E**); Grade IV: large vacuole and abnormal head shapes or other abnormalities (**F**). Original magnification ×6600.

previously for conventional ICSI (Vanderzwalmen *et al.*, 1996) at ×200 and ×400 magnification. Oocytes were placed into the dish one at a time to minimize the amount of time outside the incubator.

Fertilized oocytes were cultured individually in 4-well multidishes (Nunc), each well containing 800 µl of non-sequential Global medium (LifeGlobal, Ontario, Canada) supplemented with 7.5% human serum albumin (LifeGlobal) at 37°C in a humidified atmosphere of 6% CO_2 in air. Sixteen to 20 h post-ICSI, all oocytes were checked for the presence of two pronuclei. On day 3 of culture, the quality of the embryos was evaluated. They were classified into one of three groups according to the number and size of blastomeres, the percentage of fragmentation and the presence of multinucleated blastomeres. The 'good' quality group consisted of embryos with six or eight cells, no fragmentation and even-sized blastomeres. The 'moderate' quality group consisted of embryos with six to eight cells, 30–50% fragmentation or uneven-sized blastomeres. Embryos with fewer than six blastomeres and/or more than 50% fragmentation and uneven-sized blastomeres were classified as 'poor' quality embryos.

On day 5, embryo quality was recorded and assessed according to the degree of blastocoele expansion and the quality of both the inner cell mass (ICM) and the trophectoderm. Blastocysts and expanded blastocysts containing a lot of tightly packed ICM and many cells forming a cohesive epithelium were classified as 'excellent'. Early blastocysts or blastocysts and expanded blastocysts with a small amount of tightly packed ICM and/or large cells forming the epithelium were classified as 'moderate'.

The likelihood of selecting a normal spermatozoon, at ×400 magnification, before oocyte injection was analysed on 15 semen samples (**Figure 2**).

In a first step, a spermocytogram was established in real time at magnification on the screen at ×6600 or at ×1000 magnification under the objectives (**Figure 3A**,**B**). A total of 100 spermatozoa were analysed and the percentage of grade I and II spermatozoa was recorded. In a second step, 30 morphologically normal spermatozoa were selected in the PVP drop using the conventional ICSI approach at ×400 or ×1000 magnification under Hoffman modulation contrast (**Figure 3C**,**D**). After this first selection, the spermatozoa were aspirated into a needle and moved from the plastic Petri dish into a glass-bottomed dish and immobilized. The percentage of grade I and II spermatozoa was analysed at higher magnification under differential interference contrast of Normarski. In the last phase of the experiment, 30 grade I and II spermatozoa were searched using the IMSI procedure at minimal ×6600 magnification on the screen or at ×1000 magnification under the objectives.

Statistical analysis

The chi-squared test was used to analyse differences in embryo development after oocyte injection within all four categories of spermatozoa qualities. A multiple comparison analysis with Bonferroni adjustment of α was done to analyse differences pairwise. A two-tailed Student's *t*-test was performed to exclude or show statistically significant differences concerning age and number of oocytes per patient. *P*-values below 0.05, or below 0.009 after Bonferroni adjustment were considered as statistically significant.

Results

During a 4-month period a total of 67 couples were involved in 67 IMSI-ICSI cycles. That resulted in 442 metaphase II (MII) oocytes becoming available for injection (**Figure 4**). For each IMSI–ICSI attempt, the selection policy consisted of attempting to select the best spermatozoa out of the prepared semen sample. However, the quality of the spermatozoa selected differed greatly from one attempt to the next, so that even after extensive searching it was impossible, in some semen samples, to find spermatozoa free of abnormalities. Only 7.0% (n = 31) of spermatozoa selected were completely free of any abnormalities (grade I). The majority of the spermatozoa presented small (grade II, 59.7%; n = 264) or large (grade III,

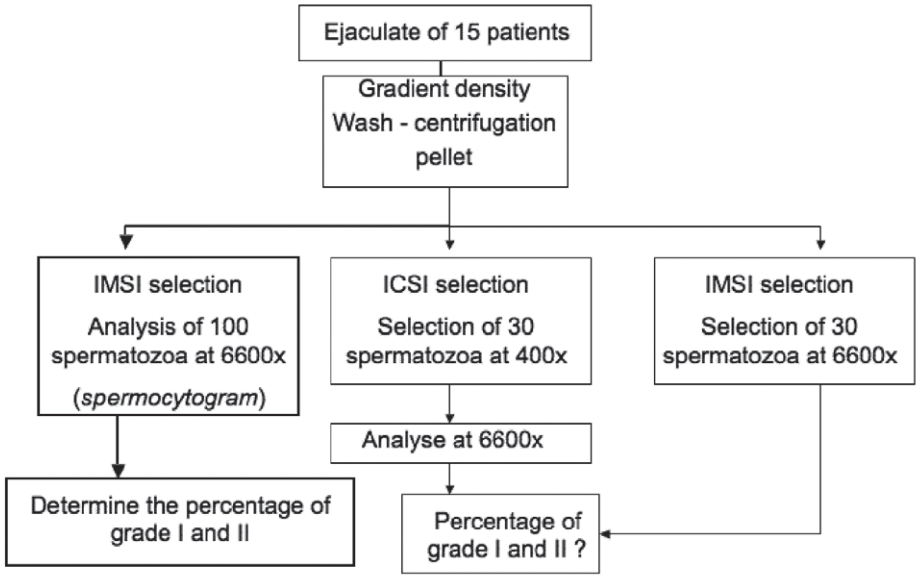

Figure 2. Experimental design. Probability of selecting 30 grade I and II spermatozoa at ×400 or ×6600 magnification in relation to the percentage of normal spermatozoa present in the sample and assessed at ×6600. ICSI = intracytoplasmic sperm injection;IMSI=intracytoplasmic morphologically selected sperm injection.

20.4%; *n* = 90) nuclear vacuoles. The remaining spermatozoa contained in addition to vacuoles other abnormalities (grade IV, 12.9%; *n* = 57).

According to the grade of spermatozoa selected, three groups of patients were defined (**Figure 5**). In the first group of 25 patients (37%), oocytes were injected with different quality grades of spermatozoa because insufficient numbers of grade I and II spermatozoa were available. In the second group with 34 (51%) different patients, it was possible to find and inject all 209 oocytes with grade I and II spermatozoa. In a third group of eight patients (12%), even after extensive searching as described above, only grade III and IV spermatozoa were found and selected for injection in 69 oocytes.

Table 1 illustrates the rate of zygote, day-3 embryo and blastocyst formation in a group of 25 patients after oocyte injection of grade I, II, III and IV spermatozoa. When all groups were analysed together, no statistically significant difference could be found between the four groups concerning the number zygotes and embryo development to day 3, including the subgroup analysis. On the other hand, the four groups differ highly significantly in their development to blastocysts and good quality blastocysts. In the subgroup analysis, no statistically significant difference could be shown between groups I and II and between groups III and IV concerning the development to blastocysts. Therefore, it was permissible in this study to combine groups I and II (*n* = 86) and groups III and IV (*n* = 78) for further analysis (**Table 2**). There was no statistically significant difference in the number of injected oocytes per patient. Even in the combined groups there is no difference concerning the embryo development to day 3 (group I/II: 88.4% versus group III/IV: 82.1%). However, blastocyst formation rate was associated with the grade of injected spermatozoa (**Table 2**). After injection with grade I and grade II spermatozoa, 60.5 and 37.2% of the injected oocytes developed to the blastocyst and good blastocysts stage respectively. Conversely, after IMSI with compromised spermatozoa (grade III and grade IV), only 3.8 and 1.3% of embryos developed to the blastocyst and good quality blastocyst stage ($P < 0.001$).

The same trend was observed when the data between the other two groups of patients (34 versus 8) with different grades of spermatozoa injected (**Table 3**) were analysed. No significant difference in the number of oocytes per patient was observed. Although a significantly higher number of MII oocytes/patient ($P < 0.01$) were injected with grade III and IV spermatozoa, the percentage of blastocysts (10.1%) was significantly lower as compared with MII oocytes injected with grade I and II spermatozoa (43.5%) ($P < 0.001$). Similarly, the rate of good quality blastocysts was significantly reduced when grade III/IV (2.9%) spermatozoa were injected as compared with the use of grade I/II spermatozoa (19.1%) ($P < 0.01$).

Outcome and probability of selecting grade I/II spermatozoa with ICSI or IMSI approach are shown in **Table 4**. According to the percentage of grade I and II spermatozoa observed at high magnification under Nomarski optics, sperm samples were classified into three categories (category I: >40% normal sperm forms; category II: between 20 and 40% normal sperm forms and category III: <20% normal sperm forms) (**Figure 2**).

As shown in **Table 4**, even though it was possible to capture grade I/II spermatozoa to some extent with ICSI pre-selection, the success rate was much lower than when working directly with the IMSI selection approach. In category I, the likelihood of selecting grade I/II spermatozoa using the ICSI selection procedure ranged from 50 to 67%. However, when the selection was performed directly at high magnification (IMSI), it was possible to select normal spermatozoa in 100% of cases. In category III it was possible to harvest on average 55% of grade I/II spermatozoa using the IMSI selection approach compared with 22% when applying the ICSI selection approach.

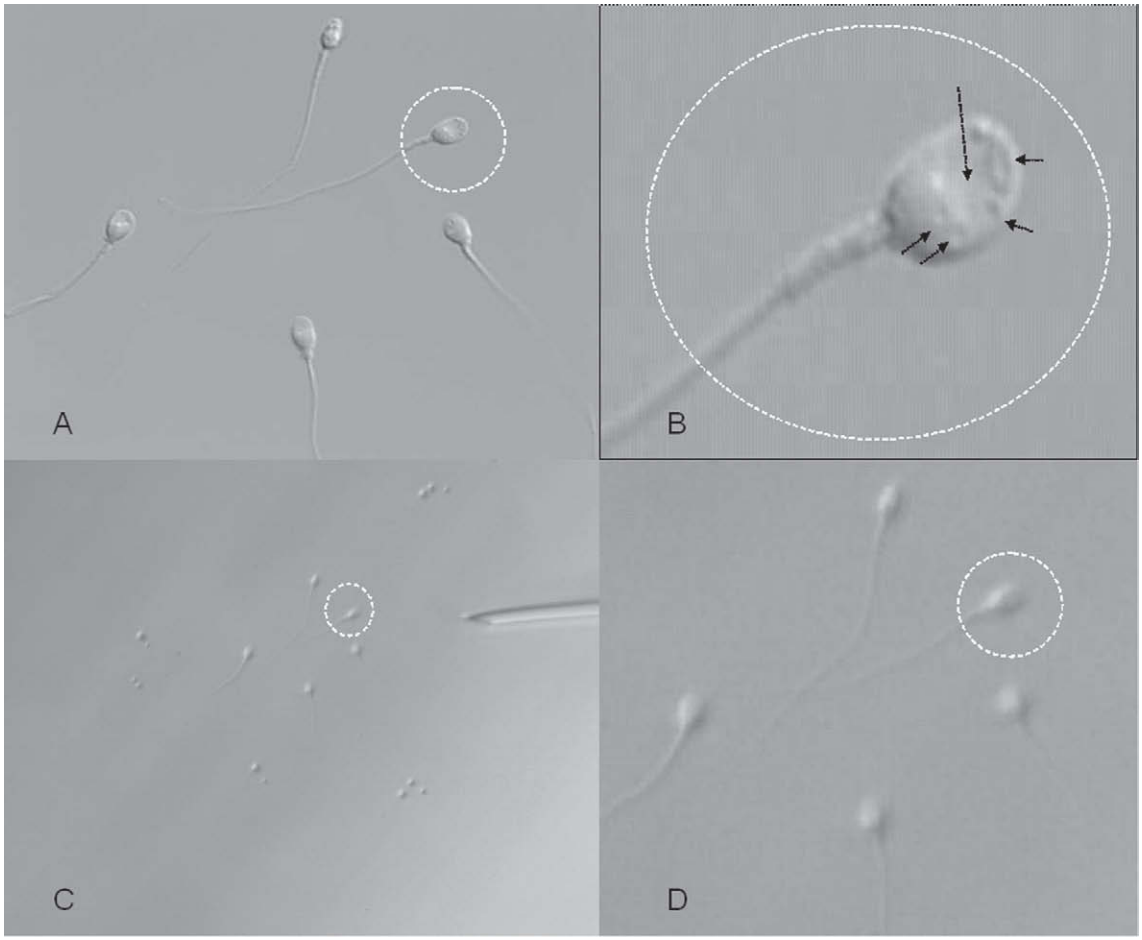

Figure 3. Spermatozoa with Nomarski and Hoffman optic systems at different magnifications. (**A**) Nomarski differential interference contrast at ×1000 magnification (×100 DIC objective). (**B**) Nomarski differential interference contrast at ×12,000 magnification (×100 DIC objective plus VarioC-mount Zoom). (**C**) Hoffman modulation contrast at ×400 magnification (×40 HMC objective). (**D**) Hoffman modulation contrast at ×1000 magnification (×40 HMC objective plus VarioC-mount Zoom). Short arrow: small vacuoles; long arrow: large vacuole.

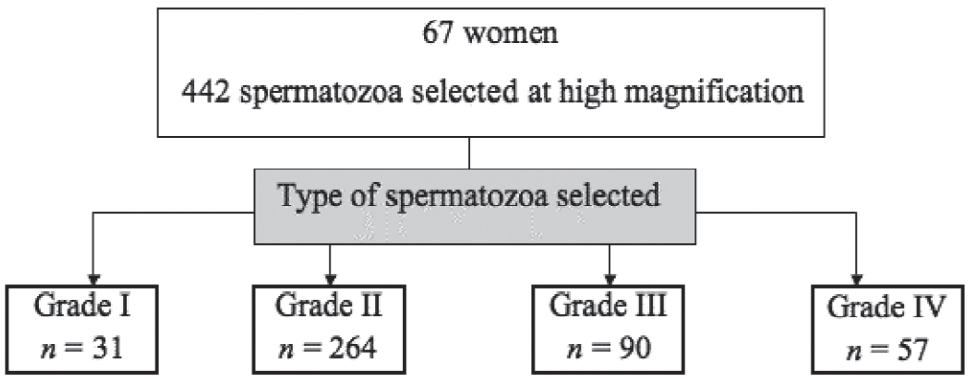

Figure 4. Distribution of selected spermatozoa using the intracytoplasmic morphologically selected sperm injection (IMSI) approach.

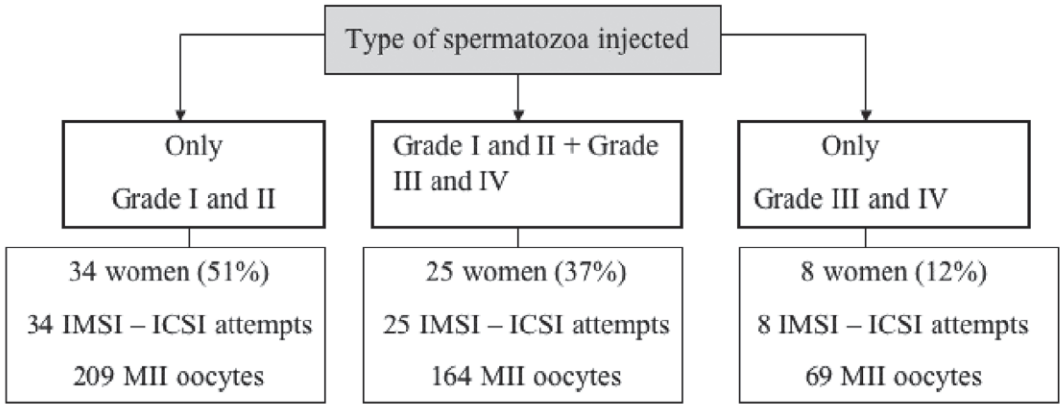

Figure 5. Experimental design: spermatozoa grading and groups of patients according to the types of spermatozoa selected at high magnification. ICSI = intracytoplasmic sperm injection; IMSI = intracytoplasmic morphologically selected sperm injection; MII = metaphase II.

Table 1. (**a**) Outcome of embryo development in a group of 25 patients after sibling oocytes were injected with grade I, grade II, grade III and grade IV spermatozoa. (**b**) Chi-squared test results.

a

Type of injected spermatozoa	*Grade I*	*Grade II*	*Grade III*	*Grade IV*
No. injected oocytes	16	70	59	19
Percentage (no.) of embryos per injected oocyte				
Zygotes	87.5 (14)	90.0 (63)	94.9 (56)	52.6 (10)
Day-3 embryos	87.5 (14)	88.6 (62)	93.2 (55)	47.4 (9)
Good quality day-3 embryos	43.8% (7)	42.9 (30)	33.9 (20)	21.1 (4)
Blastocysts	56.3% (9)	61.4 (43)	5.1 (3)	0 (0)
Good quality blastocysts	37.5% (6)	37.1 (26)	1.7 (1)	0 (0)

b

Chi-squared-test	*All groups*	*Multiple comparison analysis (P-values)*					
Grade of spermatozoon		*I versus II*	*I versus III*	*I versus IV*	*II versus III*	*II versus IV*	*III versus IV*
Injected oocytes versus							
Zygotes	NS	NS	NS	NS	NS	NS	NS
Day-3 embryos	NS	NS	NS	NS	NS	NS	NS
Good quality day-3 embryos	NS	NS	NS	NS	NS	NS	NS
Blastocysts	<0.001	NS	<0.001	0.003	<0.001	0.001	NS
Good quality blastocysts	<0.001	NS	<0.001	NS	<0.001	NS	NS

Significance level $P < 0.009$ after Bonferroni adjustment, NS = not statistically significant.

Table 2. Outcome of embryo development in a group of 25 patients after sibling oocytes were injected with grade I/II and grade III/IV spermatozoa.

Characteristics	*Value*		
No. of patients	25		
Women's age (years, mean ± SD)	36.2 ± 2.5		
No. of oocytes (mean ± SD)	247 (9.9 ± 1.6)		
No. of MII oocytes (mean ± SD)	198 (7.9 ± 1.8)		
No. of MII oocytes for injection: (mean ± SD)	164 (6.6 ± 1.4)		
Results			
Type of injected spermatozoa	*Grade I/II*	*Grade III/IV*	*P-value*
No. of injected oocytes (mean ± SD)	86 (3.4 ± 0.9)	78 (3.12 ± 1.0)	NS
Percentages (no.) of embryos per injected oocyte			
Zygotes	89.5 (77)	84.6 (66)	NS
Day-3 embryos	88.4 (76)	82.1 (64)	NS
Good quality day-3 embryos	43.0 (37)	30.8 (24)	NS
Blastocysts	60.5 (52)	3.8 (3)	<0.001
Good quality blastocysts	37.2 (32)	1.3 (1)	<0.001
No. of embryo transfers		25	
No. embryos transferred (mean)		34 (1.4)	
No. of deliveries (%)		5 (20.0)	
Implantation rate %		20.6[a]	

[a]Two twins.
MII = metaphase II; NS = not statistically significant.

Table 3. Outcome of embryo development in a group of 34 and eight patients in which only grade I/II or grade III/IV spermatozoa respectively were available for injection.

Characteristics	*Grade I/II*	*Grade III/IV*	*P-value*
No. of patients	34	8	–
Women's age (mean ± SD)	37.0 ± 1.8	37.0 ± 1.9	NS
No. of oocytes (mean ± SD)	310 (9.1 ± 1.2)	85 (10.6 ± 1.4)	NS
No. of MII oocytes (mean ± SD)	244 (7.2 ± 0.9)	74 (9.2 ± 1.0)	<0.01
No. MII oocytes for injection (mean ± SD)	209 (6.1 ± 1.0)	69 (8.6 ± 1.1)	<0.01

Results *Type of injected spermatozoa*	*Only grade I/II*	*Only grade III/IV*	*P-value*
No. of injected oocytes	209	69	–
Percentages (no.) of embryos per injected oocytes			
Zygotes	88.5 (185)	79.7 (55)	NS
Day 3 embryos	87.1 (182)	66.7 (46)	NS
Good quality day-3 embryos	31.1 (65)	17.4 (12)	NS
Blastocysts	43.5 (91)	10.1 (7)	<0.001
Good quality blastocysts	19.1 (40)	2.9 (2)	<0.01
No. of embryo transfers	34	8	–
No. of embryos transferred (mean)	52 (1.6)	9 (1.1)	–
No. of deliveries (%)	15 (44)	1 (12.5)	–
Implantation rate (%)	34.6[a]	11.1	–

[a]Three twins.
MII = metaphase II; NS = not statistically significant.

Table 4. Outcome of selecting 30 grade I and II spermatozoa at ×400 or ×6600 magnification in relation to the percentage of normal spermatozoa present in the sample and assessed at ×6600 magnification.

Semen sample evaluated by spermozytogram	*Percentage (no.) of grade I and II after:*		
	Selection of spermatozoa and IMSI evaluation (spermozytogram approach)	*Pre-selection at ×400 followed by IMSI evaluation (ICSI selection approach)*	*Direct IMSI selection and evaluation (IMSI selection approach)*
1	47 (100)	50 (30) 15	100 (30) 30
2	48 (100)	73 (30) 22	100 (30) 30
3	47 (100)	67 (30) 20	100 (30) 30
Average	*47*	*63*	*100*
4	26 (100)	40 (30) 12	100 (30) 30
5	33 (100)	47 (30) 14	100 (30) 30
6	29 (100)	70 (30) 21	100 (30) 30
7	38 (100)	43 (30) 13	93 (30) 28
8	39 (100)	43 (30) 13	53 (30) 16
Average	*33*	*49*	*89*
9	19 (100)	30 (30) 9	67 (30) 20
10	16 (100)	23 (30) 7	40 (30) 12
11	13 (100)	27 (30) 8	67 (30) 20
12	11 (100)	33 (30) 10	53 (30) 16
13	7 (100)	20 (30) 6	63 (30) 19
14	3 (100)	13 (30) 4	57 (30) 17
15	3 (100)	7 (30) 2	40 (30) 12
Average	*10*	*22*	*55*
Total	25 (379/1500)	39 (176/450)	76 (340/450)

ICSI = intracytoplasmic sperm injection; IMSI = intracytoplasmic morphologically selected sperm injection.
Semen samples 1–3: >40% normal sperm forms; semen samples 4–8: between 20 and 40% normal sperm forms and semen samples 9–15 <20% normal sperm forms.

Discussion

The introduction of the Normarski optic system permits the fine nuclear morphology of motile spermatozoa to be observed in real time at high magnification. This meticulous approach towards sperm selection allows identification of vacuole(s) in the sperm head that are otherwise not evident to detect at ×400 magnification with Hoffman modulation contrast. However, as noticed already earlier by Berkovitz *et al.* (2005, 2006a, b), in spite of having a more precise selection method at hand, it is not always possible even with this tool to find and select morphologically completely normal appearing spermatozoa for fertilization. Thus, in such situations, second choice spermatozoa with the least vacuoles and/or abnormal shape have to be used.

The policy of embryo culture to the blastocyst stage gives the opportunity to ascertain whether vacuoles in the spermatozoa have a negative effect on embryo development, and whether such an effect occurs before or after the genomic transition that takes place around 72 h after fertilization. So far as is known, this is the first report that describes a negative impact of the presence of one large nuclear vacuole or several small ones in the head of spermatozoa on the competence of embryos to develop to blastocysts. Moreover, in addition to a reduction of the proportion of good quality embryos that develop to the blastocyst stage, the presence of vacuoles in the nuclei of spermatozoa is also associated with diminished pregnancy and implantation rates.

The present data and those of Berkovitz *et al.* (2006b) and Hazout *et al.* (2006) are further substantiated by studies on several species (bulls, stallions, rabbits) indicating increased rates of early embryonic death and reduced fertility after fertilization of oocytes with spermatozoa showing nuclear vacuoles (Barth and Oko 1988; Thundathil *et al.*, 1998). Although the negative effects of large nuclear vacuoles or several small ones on embryo development after day 3 are becoming more and more evident, the underlying cause is still not understood. As a consequence, one of the top priorities for future studies has to be to investigate their origin and meaning, how they influence embryo development and what effects they have on the health of the progeny.

Some studies argue that there is a correlation between chromatin defects and the presence of vacuoles. A significant negative correlation between the size of nuclear vacuoles and chromatin stability is reported by Berkowitz *et al.* (2005) using the sperm chromatin structure assay. Gopalkrishnan *et al.* (2000) found that poor semen quality is associated with repeated early pregnancy loss. In their study, the chromatin material of spermatozoa from men whose partners presented with early

pregnancy loss was often found to be either compact or partially compact with irregular nuclear borders and larger vacuoles. Hazout *et al*. (2006) suggest that the presence of vacuoles may reflect molecular defects responsible for abnormal chromatin remodelling during sperm maturation.

Recently, two papers have reinforced the concept that an association between DNA damage and the presence of nuclear vacuoles exist. Garolla *et al*. (2008) observed significantly better mitochondrial function, chromatin status and less aneuploidy rates when nuclear vacuoles were absent. Franco *et al*. (2008) demonstrated an association between large vacuoles in the sperm and DNA damage. They postulate that the high levels of denatured DNA in sperm with large nuclear vacuoles point to precocious decondensation and desegregation of sperm chromatin fibres.

The allocation of vacuoles to errors at the level of the DNA or chromatin has to be done in future studies. In addition, the morphological aspect of vacuoles itself and their localization has to be evaluated and related to the nuclear integrity of the spermatozoa. It would be very important to execute such studies in the near future. As it becomes more and more evident that there might be an association between DNA damage and the presence of nuclear vacuoles, the attitude towards and the follow-up of patients who show semen samples carrying 100% large vacuoles might have to change.

Irreparable abnormalities of the paternal genome may affect blastocyst development (Jones *et al*., 1998). The argument that the oocyte supplies all the primary material (proteins and RNA) and that the spermatozoa and their DNA only play a secondary role most probably needs to be revised (Krawetz, 2005). Spermatozoa contain almost 3000 different kinds of mRNA, some of which contain the code for proteins needed for early embryo development. The present observations reinforce previous studies pointing to possible 'early and late paternal effects', both of which may have an impact on early embryonic development (Vanderzwalmen *et al*., 1991; Shoukir *et al*., 1998; Tesarik *et al*., 2004; Tesarik, 2005).

An unwanted high degree of sperm decondensation (disruption of disulphide bridges) can result in asynchronous chromosome condensation, and may lead to cytoplasmic fragments in the embryo (Ménézo *et al*., 2007). It is now generally accepted that abortive apoptosis during meiosis I or faulty chromatin remodelling during spermiogenesis and oxidative stress may cause sperm DNA fragmentation that is related to embryo development to the blastocyst stage (Seli *et al*., 2004; Virro *et al*., 2004; Muriel *et al*., 2006) and with pregnancy outcome (Larson *et al*., 2000; Nasr-Esfahani *et al*., 2005). According to Hammadeh *et al*. (2000), the integrity of the sperm chromatin may play an as yet underestimated role for embryo development, particularly after fertilization with ICSI, where most of the natural selection mechanisms are bypassed. The transfer of embryos resulting from the fertilization of oocytes with spermatozoa showing high DNA fragmentation indices have low implantation rates (Virro *et al*., 2004; Nasr-Esfahani *et al*., 2005). However, although the implantation rate is low with such spermatozoa, deliveries are nonetheless observed. Berkovitz *et al*. (2007) recently reported that the chance of having a healthy child following IMSI treatment is higher compared with conventional ICSI treatment.

Aitken *et al*. (2007) already outlined the possible negative influences of sperm DNA fragmentation both on pregnancy outcome and the next generation. Furthermore, Fernandez-Gonzalez *et al*. (2008) showed in the murine model that oocytes fertilized with DNA-fragmented spermatozoa can generate effects that emerge during later life, such as aberrant growth, premature ageing, abnormal behaviour and mesenchymal tumours. Thus, based on clinical/laboratory findings of the repercussions of possible DNA damage for offspring (Carrell, 2008) and considering that sperm nuclear vacuoles are evaluated more precisely by MSOME, it can be anticipated that such selection approaches will most probably substitute ICSI in the near future.

It was possible to demonstrate that, even after extensive search with IMSI the frequency of detecting spermatozoa of normal morphological appearance or at least ones with a maximum of two small vacuoles greatly varies according to semen samples. In more than 50% of cases, selection of spermatozoa without any abnormalities (grade I) was not possible (personal observation). The results of the present study, which analysed the frequency of finding a normal spermatozoon and showed no abnormalities, reinforce the usefulness of IMSI. It can be assumed that if selection had been performed on the same sperm population using the classic ICSI approach, the likelihood of selecting spermatozoa with a large nuclear vacuole or multiple ones as well as concomitant effects on embryo development would have been very high.

Initially IMSI was proposed to patients with several implantation failures (Bartoov *et al*., 2002, 2003; Junca *et al*., 2004; Berkovitz *et al*., 2006a) and high degree of DNA fragmentation (Hazout *et al*., 2006). However, in light of the present findings, it may be justifiable to also offer IMSI to those patients where a previous spermozytogramme at high magnification reveals a high percentage of vacuoles. Prior screening of semen samples for the presence of vacuoles would therefore be useful in order to recommend ICSI or IMSI to patients. The establishment of new classification criteria, based on an assessment system, seems a valuable approach to determine a threshold limit for making the right therapeutic decision (Cassuto *et al*., 2007).

Several articles (Sakkas *et al*., 1998; Shoukir *et al*., 1998; Miller *et al*., 2001; Zech *et al*., 2002) show that ICSI embryos have a significantly lower chance of developing to the blastocyst stage compared with those that result from routine IVF. The present results may explain why the percentage of blastocysts may be lower after ICSI using normal spermatozoa as compared with the IVF group. In fact, the probability of injecting spermatozoa containing vacuoles is high when using normal ICSI. The zona pellucida (ZP) acts as a selective biological barrier to abnormal spermatozoa, so that in most cases only normal spermatozoa are able to fertilize an oocyte. In a recent article, Liu and Baker (2007) reported that sperm binding to human ZP is highly selective for double-stranded DNA. They conclude that the sperm–ZP binding process plays an important role in the selection of sperm with normal motility and morphology as well as of spermatozoa with normal chromatin DNA. During ICSI, the ZP barrier is breached and a subjective selection of spermatozoa by the biologist at ×400 magnification may increase the probability of injecting cells containing vacuoles. Although some morphological abnormalities may be detected at ×400 magnification, others can only be detected using

special optical equipment. Berkovitz *et al*. (2006a,b) report a dramatic decrease in the ongoing pregnancy rate after performing ICSI if all the spermatozoa within the ejaculate exhibit large nuclear vacuoles.

This study confirms that IMSI is a powerful research tool for investigating spermatozoa carrying several abnormalities that cannot be detected during a conventional ICSI procedure.

Evaluating the presence of vacuoles may significantly upgrade the prognostic information provided by conventional ICSI. It shows that the present morphological criteria used on days 2 or 3 to select embryos, especially in the case of single embryo transfer, are of limited value. Prolonging embryo culture until day 5 may be a better strategy with which to correctly identify and select from a cohort those embryos with a higher overall probability of successful implantation, especially in the case of single blastocyst transfer (Papanikolaou *et al*., 2006; Zech *et al*., 2007).

This study has shown that vacuoles exert a negative effect on embryo development. The next step will be to research their origin and identify the conditions under which the frequency of such vacuoles increases.

References

Aitken R 2007 Origins and consequences of DNA damage in male germ cells. *Reproductive BioMedicine Online* **14**, 727–733.

Barth AD, Oko RJ 1988 Defects of the sperm head. In: Barth AD, Oko RJ (eds) *Abnormal Morphology of Bovine Spermatozoa*. IS Iowa State University Press, Ames, pp. 130–192.

Bartoov B, Berkovitz A, Eltes F *et al*. 2003 Pregnancy rates are higher with intracytoplasmic morphologically selected sperm injection than with conventional intracytoplasmic injection. *Fertility and Sterility* **80**, 1413–1419.

Bartoov B, Berkovitz A, Eltes F *et al*. 2002 Real-time fine morphology of motile human sperm cells is associated with IVF–ICSI outcome. *Journal of Andrology* **23**, 1–8.

Bartoov B, Berkovitz A, Eltes F 2001 Selection of spermatozoa with normal nuclei to improve the pregnancy rate with intracytoplasmic sperm injection. *New England Journal of Medicine* **345**, 1067–1068.

Berkovitz A, Eltes F, Paul M *et al*. 2007 The chance of having a healthy normal child following intracytoplasmic morphologically-selected sperm injection (IMSI) treatment is higher compared to conventional IVF–ICSI treatment. *Fertility and Sterility* **88**, S20.

Berkovitz A, Eltes F, Lederman H *et al*. 2006a How to improve IVF–ICSI outcome by sperm injection? *Reproductive BioMedicine Online* **12**, 634–638.

Berkovitz A, Eltes F, Ellenbogen A *et al*. 2006b Does the presence of nuclear vacuoles in human sperm selected for ICSI affect pregnancy outcome? *Human Reproduction* **21**, 1787–1790.

Berkovitz A, Eltes F, Yaari S *et al*. 2005 The morphological normalcy of the sperm nucleus and pregnancy rate of intracytoplasmic injection with morphologically selected sperm. *Human Reproduction* **20**, 185–190.

Carrell D 2008 Symposium: Genetic and epigenetic aspects of assisted reproduction. Contributions of spermatozoa to embryogenesis: assays to evaluate their genetic and epigenetic fitness. *Reproductive BioMedicine Online* **16**, 474–484.

Cassuto NG, Plouchart JM, Balet R *et al*. 2007 Interest of a new morphological classification of human spermatozoa for ICSI allowing to obtain a better blastocyst score. *Human Reproduction* **22** (Suppl. 1), i113.

Fernandez-Gonzalez R, Nuno Moreira P, Perez-Crespo M *et al*. 2008 Long term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring. *Biology of Reproduction* **78**, 761–772.

Franco Jr JG, Baruffi RLR, Mauri AL *et al*. 2008 Significance of large nuclear vacuoles in human spermatozoa: implications for ICSI. *Reproductive BioMedicine Online* **17**, 42–45.

Garolla A, Fortini D, Menegazzo M *et al*. 2008 High-power microscopy for selecting spermatozoa for ICSI by physiological status. *Reproductive BioMedicine Online,* in press.

Gopalkrishnan K, Padwal V, Meherji PK *et al*. 2000 Poor quality of sperm as it affects repeated early pregnancy loss. *Archives of Andrology* **45**, 111–117.

Hammadeh M, Nkemayim D, Georg T *et al*. 2000 Sperm morphology and chromatin condensation before and after semen processing. *Archives of Andrology* **44**, 221–226.

Hazout A, Dumomt-Hassant M, Junca AM *et al*. 2006 High-magnification ICSI overcomes paternal effect resistant to conventional ICSI. *Reproductive BioMedicine Online* **12**, 19–25.

Jones G, Trounson A, Lolatgis N *et al*. 1998 Factors affecting the success of human blastocyst development and pregnancy following in vitro fertilization and embryo transfer. *Fertility and Sterility* **70**, 1022–1029.

Junca AM, Cohen-Bacrie P, Hazout A 2004 Improvement of fertilisation and pregnancy rate after intracytoplasmic fine morphology selected sperm injection. *Fertility and Sterility* **82** (Suppl. 2), S173.

Krawetz S 2005 Paternal contribution: new insights and future challenges. *Nature Reviews Genetics* **6**, 633–642.

Larson K, DeJonge C, Barnes A *et al*. 2000 Relationship of assisted reproductive technique (ART) outcomes with sperm chromatin integrity and maturity as measured by the sperm chromatin structure assay (SCSA). *Human Reproduction* **15**, 1717–1722.

Liu D, Baker H 2007 Human sperm bound to the zona pellucida have normal nuclear chromatin as assessed by acridine orange fluorescence. *Human Reproduction* **22**, 1597–1602.

Ménézo Y, Hazout A, Panteix G *et al*. 2007 Antioxidants to reduce sperm DNA fragmentation: an unexpected adverse effect. *Reproductive BioMedicine Online* **14**, 418–421.

Miller JE, Smith TT 2001 The effect of intracytoplasmic sperm injection and semen parameters on blastocyst development in vitro. *Human Reproduction* **16**, 918–924.

Muriel L, Garrido N, Fernandez JL *et al*. 2006 Value of the sperm deoxyribonucleic acid fragmentation level, as measured by the sperm chromatin dispersion test, in the outcome of in vitro fertilization and intracytoplasmic sperm injection. *Fertility and Sterility* **85**, 371–383.

Nasr-Esfahani MH, Salehi M, Razavi S *et al*. 2005 Effect of sperm DNA damage and sperm protamine deficiency on fertilization and embryo development post-ICSI. *Reproductive BioMedicine Online* **11**, 198–205.

Papanikolaou EG, Camus M, Kolibianakis EM *et al*. 2006 In vitro fertilization with single blastocyst-stage versus single cleavage-stage embryos. *New England Journal of Medicine* **354**, 1139–1146.

Sakkas D, Urner F, Bizzaro D *et al*. 1998 Sperm nuclear DNA damage and altered chromatin structure: effect on fertilization and embryo development. *Human Reproduction* **13**, 11–19.

Seli E, Gardner DK, Schoolcraft WB *et al*. 2004 Extent of nuclear damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. *Fertility and Sterility* **82**, 378–383.

Shoukir Y, Chardonnens D, Campana A *et al*. 1998 Blastocyst development from supernumerary embryos after intracytoplasmic sperm injection: a paternal influence. *Human Reproduction* **13**, 1632–1637.

Tesarik J 2005 Paternal effects on cell division in the preimplantation embryo. *Reproductive BioMedicine Online* **10**, 370–375.

Tesarik J, Greco E, Mendoza C 2004 Late but not early paternal effect on human embryo development is related to sperm DNA fragmentation. *Human Reproduction* **19**, 611–615.

Thundathil J, Palasz A, Barth A *et al*. 1998 Fertilization characteristics and in vitro embryo production with bovine sperm containing

multiple vacuoles. *Molecular Reproduction and Development* **50**, 328–333.

Vanderzwalmen P, Bertin G, Lejeune B *et al*. 1996 Two essential steps for a successful intracytoplasmic sperm injection: injection of immobilized spermatozoa after rupture of the oolemma. *Human Reproduction* **11**, 540–547.

Vanderzwalmen P, Bertin-Segal G, Geerts L *et al*. 1991 Sperm morphology and IVF pregnancy rate: comparison between Percoll gradient centrifugation and swim-up procedures. *Human Reproduction* **6**, 581–588.

Virro MR, Larson-Cook KL, Evenson DP 2004 Sperm chromatin structure assay (SCSA) parameters are related to fertilization, blastocyst development, and ongoing pregnancy in in vitro fertilization and intracytoplasmic sperm injection cycles. *Fertility and Sterility* **81**, 1289–1295.

Zech NH, Lejeune B, Puissant F *et al*. 2007 Prospective evaluation of the optimal time for selecting a single embryo for transfer: day 3 versus day 5. *Fertility and Sterility* **88**, 244–246.

Zech I, Vanderzwalmen P, Zech N 2002 Entwicklung von Embryonen nach intrazytoplasmatischer Spermieninjektion (ICSI) bei 'non-male factor' Infertilität in Relation zur Injektionstechnik. *Journal für Reproduktionmedizin und Endokrinologie* **12**, 13–18.

Declaration: The authors report no financial or commercial conflicts of interest.

Received 2 May 2008; refereed 12 June 2008; accepted 16 July 2008.