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Correlation between DNA defect and sperm-head morphology

Nino G Cassuto ^{a,*}, André Hazout ^a, Ibrahim Hammoud ^c, Richard Balet ^b,
Dominique Bouret ^a, Yona Barak ^a, Sonia Jellad ^a, Jean Marie Plouchart ^a,
Jacqueline Selva ^c, Chadi Yazbeck ^{d,e}

^a Art Unit, Drouot Laboratory, 19 Rue Drouot, 75009 Paris, France; ^b Reproductive Medicine Department, Hopital des Bluets, 4 Rue Lasson, 75012 Paris, France; ^c Reproductive Medicine Department, CHR, 78300 Poissy, France; ^d Obstetrics, Gynecology and Reproductive Medicine Department, Bichat Claude, Bernard University Paris VII, Paris, France; ^e INSERM Unit 780, Epidemiology and Biostatistics, Villejuif, France
* Corresponding author. E-mail address: guycassuto@labodrouot.com (NG Cassuto).



Nino Guy Cassuto is the head of the Drouot laboratory. Since 1995 he has also led the assisted reproduction units of two hospitals in Paris. His research fields are embryo development and male infertility. He has published national and international articles, particularly concerning sperm morphology. He continues to pursue his interests in early embryo development with a particular emphasis on the role of spermatozoa.

Abstract The utility of sperm DNA testing remains controversial. However, it may be helpful in couples with unexplained failures of multiple assisted reproductive techniques and/or recurrent abortions. This study analysed 10,400 spermatozoa of 26 patients for sperm-head morphology with high-magnification microscopy, DNA fragmentation and sperm chromatin decondensation. A significant negative correlation was demonstrated between sperm-parameters and abnormal sperm-head morphology as assessed by high magnification (score 0 according to this study's classification): concentration ($r = -0.41$; $P = 0.03$), motility ($r = -0.42$; $P = 0.03$), morphology ($r = -0.63$; $P = 0.0008$). No correlation was found with DNA fragmentation. However, the sperm chromatin-decondensation rate of score-0 spermatozoa was twice as high as the controls (19.5% versus 10.1%; $P < 0.0001$). This observation suggests that score-0 spermatozoa should not be selected for intracytoplasmic sperm injection. 

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Introduction

Infertility is a common condition affecting approximately 10% of the population. In half of these cases, a male factor is involved, making defective sperm function the largest single

cause of human infertility. Intracytoplasmic sperm injection (ICSI) in humans was first introduced in 1992 (Palermo et al., 1992). Since then, the use of ICSI for the treatment of men with poor-quality spermatozoa has improved knowledge of sperm biology and has clarified several negative factors.

The development of a new tool using a high-power ($\times 1500$) inverted light microscope with a zoom up to $\times 6100$ or higher (Bartoov et al., 2001) has permitted the more precise real-time selection of normal motile spermatozoa. The selection of spermatozoa for ICSI with normal head morphology and size at high magnification is associated with higher blastocyst (Cassuto et al., 2009; Vanderzwalmen et al., 2008), implantation and pregnancy rates and lower miscarriage rates (Bartoov et al., 2002, 2003; Berkovitz et al., 2006; Hazout et al., 2006; Antinori et al., 2008) In fact, some spermatozoa that appear morphologically normal at routine magnification ($\times 200$ or $\times 400$) present abnormalities when examined at high magnification.

The capacity of human spermatozoa to fertilize and produce a good-quality embryo with a high implantation and development potential depends on its DNA integrity (Miller et al., 2010). During spermatogenesis, deleterious effects could occur that affect the chromatin condensation and DNA integrity.

Some recent follow-up studies have reported increased intrauterine growth retardation and lower birthweights in singleton assisted-conception babies compared with natural conception (Bonduelle et al., 2005; Hansen et al., 2002). Increased perinatal mortality, congenital anomalies and epigenetic abnormalities have been reported to be associated with IVF/ICSI (Allen et al., 2008; Barratt et al., 2010; Carrell and Hammoud, 2010; Steel et al., 2009). The causes of these anomalies are still unknown but alterations in the epigenetic state of spermatozoa from severely infertile men undergoing ICSI have been proposed as potential contributors (de Boer et al., 2010; Lim et al., 2009; Manipalviratn et al., 2009; Miller et al., 2010).

A previous work (Cassuto et al., 2009) showed that oocytes microinjected with a high-magnification-score-0 spermatozoon, with an abnormal head, the presence of one or several vacuoles and an abnormal base (Figure 1), do not reach the expanded blastocyst stage. Since it is not easy to detect microvacuoles in the nucleus before ICSI by standard examination procedures, and considering that an

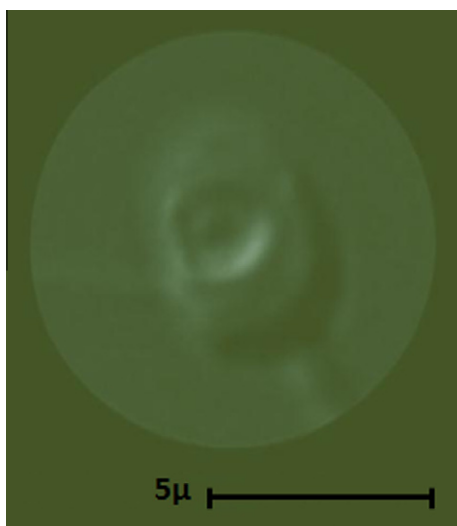


Figure 1 High-magnification-score-0 spermatozoon, showing an abnormal head with a vacuole and an abnormal base. Bar = 5 μm .

intact paternal genome is required for proper embryo development and outcome, the aim of the present study was to investigate whether chromatin damage, and particularly sperm chromatin decondensation (SCD), was linked with sperm-head abnormalities identified at high magnification.

Materials and methods

Study subjects and sperm preparation

This study evaluated a population of 26 infertile men with oligoasthenoteratospermia and IVF failures with a variety of sperm profiles who were referred for ICSI to the study centre. Ejaculates were collected in sterile containers by masturbation after 2–4 days of sexual abstinence. Only fresh ejaculates were used for the study; epididymal, testicular and cryopreserved sperm sample were not considered.

Semen samples were liquefied for 15 min at room temperature, then sperm parameters were analysed according to the World Health Organization guidelines (WHO, 1999). Teratozoospermia were defined as $<50\%$ of spermatozoa with typical morphology.

Sperm migration was performed with a bilayer gradient. In a tube, 1 ml total spermatozoa was prepared on a two-layer concentration gradient of 45% and 90% of isolate sperm separation medium (cat. no. 99264; Irvine Scientific, Santa Ana, CA, USA), then centrifuged at 300g for 20 min. The supernatant was discarded and the sperm pellet washed with early cleavage medium (90140; Irvine Scientific) and centrifuged at 600g for 10 min. The final pellet of the total migrated spermatozoa was resuspended in 1 ml phosphate-buffered saline (PBS) (BioMérieux, Marcy l'Etoile, France) and centrifuged at 200g for 10 min. For each patient, the resultant fresh pellet formed the control and then high-magnification-score-0 spermatozoa were extracted from the same pellet.

The study was approved by the local ethical committee. After consultation with the Institutional Review Board (IRB) it was found that IRB approval was not mandatory because the study was of a non-interventional design. Patients were informed that their semen would be observed under high magnification and that sperm DNA tests would be carried out.

Sperm-morphology assessment and sperm selection

High-magnification selection was performed under an inverted microscope (Olympus IX 71; Olympus, Rungis, France) equipped with Nomarski contrast optics. The high-power magnification was provided by a polarization light with a magnification of $\times 1500$ and a zoom up to $\times 6100$ and higher.

In a glass-bottomed culture dish (GWST 5040; Will Co, Wells BV, Amsterdam, The Netherlands) 10 μl of the fresh washed pellet was placed in early cleavage medium (90140; Irvine Scientific) under light mineral oil (cat. no. 9305; Irvine Scientific).

All motile spermatozoa examined were observed in three dimensions in a micropipette at magnification $\times 6100$. Assessment of morphology was carried out as previously described by Bartoov et al. (2002) according to the

Cassuto–Barak classification. The morphology of spermatozoa was based on the head shape, the presence of a vacuole in the nucleus and the head base. The formula for the scoring system is two points for a normal head, three points for a head without a vacuole and one point for a normal base. A range of spermatozoa with different scores is shown in **Figure 2** and grading sketches of spermatozoa with different scores are shown in **Figure 3** (Cassuto et al., 2009). A 'top' spermatozoon with a score of 6 points is shown in **Figure 4**. A score of 0 was defined by a nuclear-shape disorder with an abnormal base and/or a nuclear asymmetrical extrusion and/or invagination of the nuclear membrane and at least one large vacuole (**Figure 1**). For the purpose of this study, only score-0 spermatozoa were evaluated.

For each of the 26 men, two slides were prepared with a control droplet of PBS containing total migrated spermatozoa next to a second droplet with 100 score-0 spermatozoa. One slide was dedicated to studying DNA fragmentation study and the other slide to SCD. All glass slides were air dried and stored at -20°C . Therefore, this study examined 400 spermatozoa per patient and 10,400 spermatozoa for DNA fragmentation and SCD evaluation (**Figure 5**).

Technicians assessing the DNA assessments were blinded to the morphological grading. Correlations were studied between sperm parameters, DNA fragmentation, SCD and percentage score 0 in total migrated spermatozoa.

Mean DNA fragmentation and SCD were also compared in score-0 spermatozoa and total migrated spermatozoa, which served as the patient control.

DNA fragmentation assay

DNA fragmentation of the first batch of 26 slides was evaluated using the TdT (terminal deoxynucleotidyl transferase)-mediated dUDP nick-end labelling (TUNEL) assay (Cell Death Detection Kit; Roche Diagnostics, Milan, Italy). Fluoro-

rescein isothiocyanate (FITC)-dUTP was used as the label according to the manufacturer's instructions and counterstained with 4(,6-diamidino-2-phenylindole) (DAPI). Briefly, after preparation, spermatozoa were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. The specimens were incubated in TUNEL reaction mixture in a humidified atmosphere for 120 min at 37°C in the dark. The slides were rinsed twice for 5 min in PBS and counterstained with DAPI. Only DAPI-counterstained spermatozoa were evaluated and DNA fragmentation was considered for cells with homogenous intensive green fluorescence.

Sperm chromatin decondensation assay

The second batch of 26 slides was washed in PBS and permeabilization was achieved with 1% trisodium citrate and 10% sodium disulphate. After washing and drying, the slides were stained with 4% aniline blue solution (British Drug Houses Ltd VWR England) at pH 3.5 for 5 min, then washed with tap water and allowed to completely air dry. The slides were examined under oil immersion at magnification $\times 1000$ with bright-field illumination. Heads with high blue colouration corresponded to abnormal SCD with abundant histones.

Data analysis

Statistical data analysis was performed using SAS version 9.1.3 (SAS Institute Cary, NC, USA). Variables were evaluated for normality and outliers. Percentages of SCD and DNA fragmentation for each patient were assessed as continuous variables and were summarized by calculating the mean \pm standard deviation (SD) and interquartile range. Comparisons of means between score 0 and total migrated spermatozoa were performed by paired Student *t*-tests. Spearman correlations were obtained between score-0

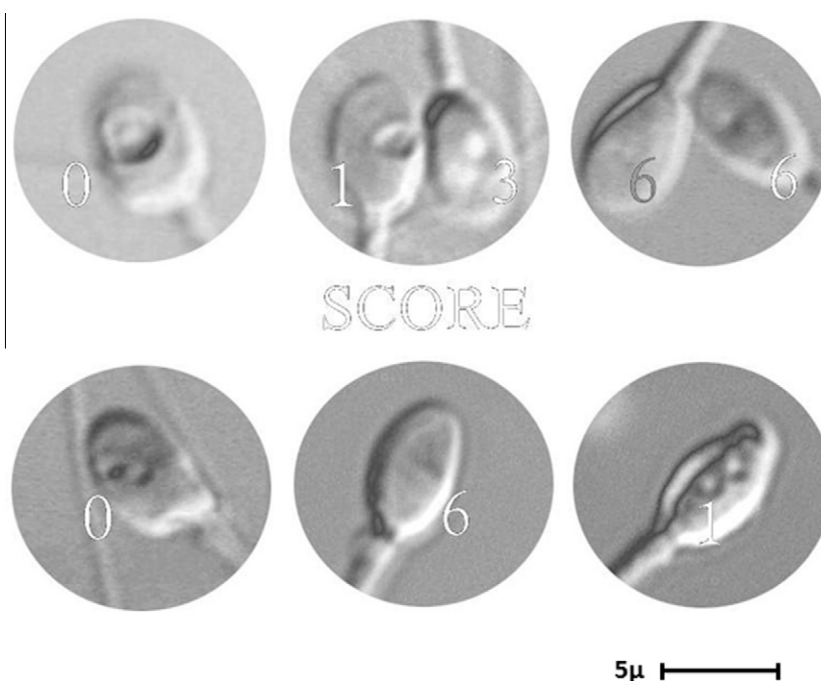


Figure 2 Spermatozoa scored at high magnification according to the Cassuto–Barak classification. Bar = $5\ \mu\text{m}$.

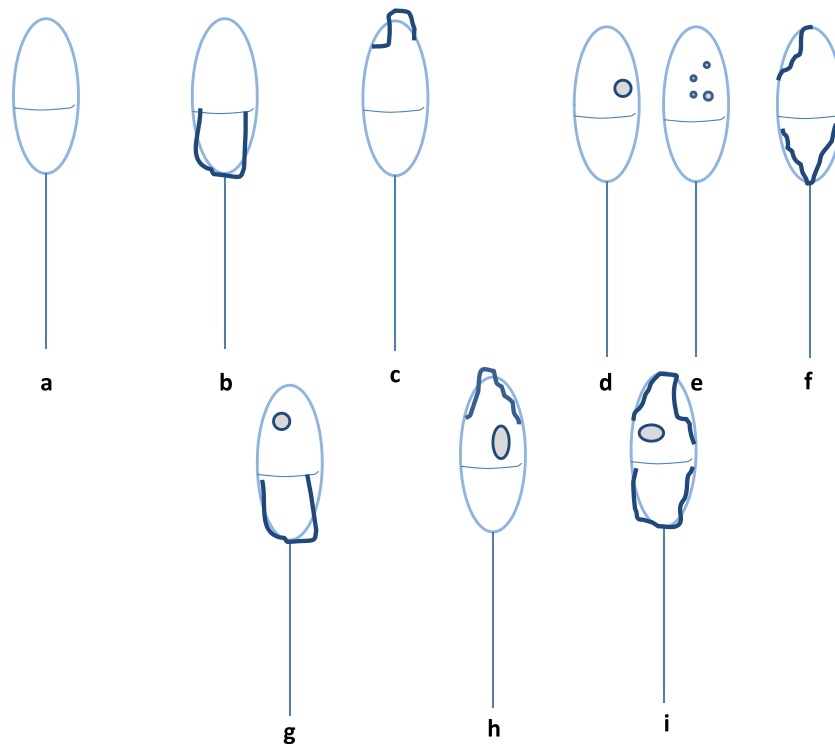


Figure 3 Sperm-scoring sketches according to the Cassuto–Barak classification: 2HN + 3VN + 1BN (BN = normal base; HN = normal head; VN = no vacuole(s)), maximum score is 6. (a) Score 6: HN + VN + BN; (b) score 5: HN + VN; (c) score 4: VN + BN; (d and e) score 3: HN + BN (f) score 3: VN; (g) score 2: HN; (h) score 1: BN; (i) score 0.



Figure 4 Score-6 spermatozoon, showing normal head without a vacuole with a normal base. Bar = 5 μ m.

spermatozoa and different sperm parameters. Regression equation was obtained by linear regression models.

Results

Table 1 illustrates baseline characteristics of the study population. The mean age was 37.1 ± 7.7 years (range

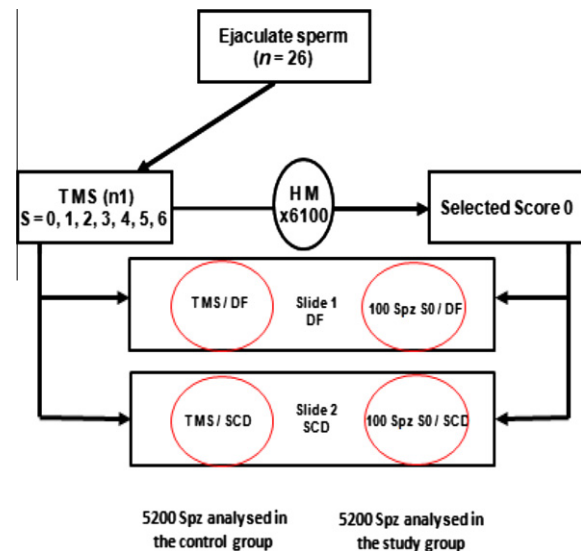


Figure 5 Study protocol for 26 men. From each semen sample, 100 score-0 spermatozoa were placed on each of two slides next to drops of 100 total migrated spermatozoa (TMS) (control). A total of 400 spermatozoa were examined per patient and 10,400 spermatozoa for DNA fragmentation (DF) and sperm chromatin decondensation (SCD). HM = high magnification; S = score; Spz = spermatozoa.

25–56 years). All men were non- smokers. The mean sperm concentration was 33.2×10^6 /ml, volume was in the normal range, motility was 41.3% and the percentage of normal

Table 1 Baseline characteristics of the study population ($n = 26$) in total ejaculated spermatozoa.

	Mean \pm SD	5–95th percentile range	IQR
Patient's age (years)	37.1 \pm 7.7	30.0–54.0	5.0
Total migrated spermatozoa volume (ml)	3.2 \pm 1.3	1.0–5.6	1.7
Count ($\times 10^6$ /ml)	33.2 \pm 30.0	2.0–90.0	40.0
Motility (%)	41.3 \pm 13.2	20.0–60.0	20.0
Normal morphology (%)	26.2 \pm 7.4	17.0–35.0	13.0
Score 0 (%)	50.2 \pm 18.8	30.0–85.0	25.0

IQR = interquartile range.

morphology spermatozoa according to the WHO, 1999 criteria was 26.2%. The mean percentage of score-0 spermatozoa in the total migrated spermatozoa was 50.2% (5–95th percentile range 30–85%).

Table 2 shows the correlations between sperm parameters of the study population and the percentage of score-0 spermatozoa in the total migrated spermatozoa. Sperm concentration, motility and morphology were negatively correlated with the percentage of score-0 spermatozoa ($r = -0.41$, $P = 0.03$; $r = -0.42$, $P = 0.03$; $r = -0.63$, $P < 0.0008$; respectively). While no correlation was found with DNA fragmentation, a slight positive but not significant correlation between the rate of score-0 spermatozoa and SCD was observed ($r = \pm 0.36$).

Table 2 Correlation between percentage of score-0 spermatozoa in total migrated spermatozoa and sperm parameters in 26 patients.

Sperm parameter	Correlation with score 0 (r)	P-value
Concentration ($\times 10^6$ ml)	-0.41	0.03
Motility (%)	-0.42	0.03
Morphology (%)	-0.63	0.0008
DNA fragmentation (%)	-0.23	NS
SCD (%)	0.36	NS

NS = not statistically significant; SCD = sperm chromatin decondensation.

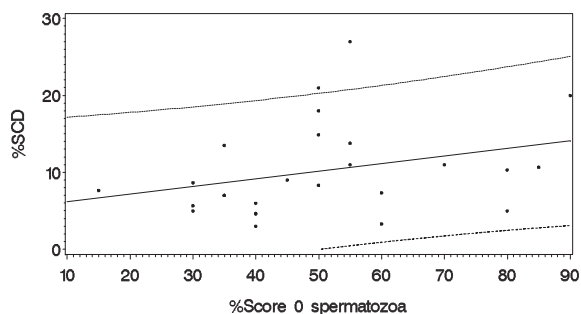
**Figure 6** Regression of sperm chromatin decondensation (SCD) with score-0 spermatozoa in total migrated spermatozoa ($n = 26$). Regression equation: $\%SCD = 5.16 + 0.10 \times (\%score\ 0)$. 90% confidence intervals are shown.

Figure 6 illustrates the regression equation ($\%SCD = 5.16 + 0.10 \times (\%score\ 0)$) between score-0 spermatozoa and SCD. The percentage of SCD was significantly higher in score-0 spermatozoa than in total migrated spermatozoa (19.5% versus 10.1%, $P \leq 0.0001$). The percentage of DNA fragmentation was comparable between groups (**Table 3**). These results showed a strong link between spermatozoon head abnormalities detectable at high magnification and SCD. We did not find a difference in DNA fragmentation in abnormally condensed spermatozoa in comparison with spermatozoa that had undergone normal histone removal/protamine substitution.

The comparison of DNA fragmentation between score-0 spermatozoa and total migrated spermatozoa did not show any significant difference (**Table 3**). This result demonstrated that a high-magnification score 0 could not distinguish spermatozoa with normal as opposed to abnormal DNA fragmentation but only abnormal spermatozoa linked to failure of chromatin condensation.

Discussion

Sperm-morphology assessment is an integral part of the semen analysis work-up in cases of infertility. Kruger et al. (1986; 1988) were the first to propose sperm classification on the basis of strict morphological criteria. In a meta-analysis of IVF outcome, severe teratozoospermia with $<4\%$ normal forms in the semen sample was associated with poorer fertilization outcome (Grow et al., 1994). Another study analysing ICSI outcome in a subset of patients with severe teratozoospermia demonstrated that sperm morphology had little predictive value for fertilization and pregnancy outcome in men undergoing ICSI (McKenzie et al., 2004).

In fact, the role of paternal factors in embryonic development, as well as the effect of the ICSI procedure itself, remains controversial. The present study demonstrates, as far as is known for the first time, a link between DNA compaction and sperm-head morphology as assessed by high magnification for three specific sperm morphologies: abnormal head shape, presence of a not-particularly large vacuole and abnormal base. These head abnormalities are invisible at low magnification (**Figures 7 and 8**). Recent interest in the integrity of sperm DNA (Aitken and De luliis, 2007) has raised concerns that, while spermatozoa may appear motile, those with fragmented or decondensed

Table 3 Comparison of sperm DNA damage (DNA fragmentation, SCD) between score-0 spermatozoa and total migrated spermatozoa in 26 patients.

Sperm DNA damage	Score-0 spermatozoa		Total migrated spermatozoa (control)		P-value
	Mean \pm SD	P5–P95	Mean \pm SD	P5–P95	
DNA fragmentation (%)	4.2 \pm 5.5	0.0–20.0	3.7 \pm 6.7	0.0–24.0	NS
SCD (%)	19.5 \pm 8.7	7.0–36.0	10.1 \pm 6.0	3.3–21.0	<0.0001

P5–P95 = 5–95th percentile range; SCD = sperm chromatin decondensation.

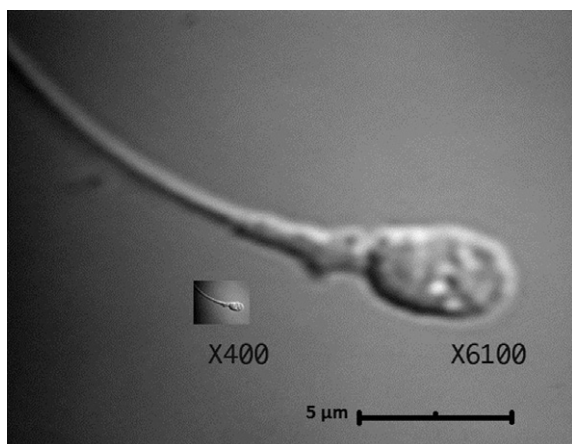


Figure 7 Score-0 spermatozoon at high and low magnification. Bar = 5 μ m.

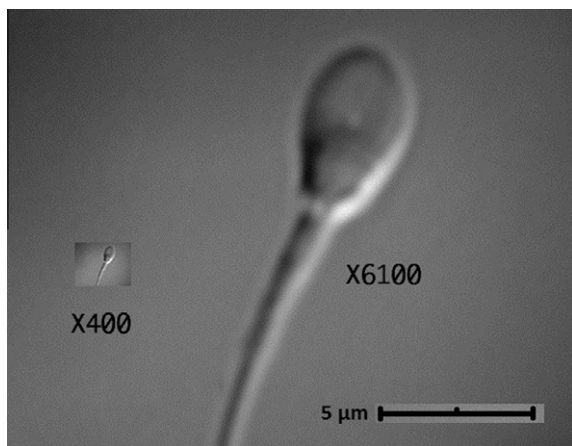


Figure 8 Score-5 spermatozoon with an abnormal base at high and low magnification. Bar = 5 μ m.

DNA may still be selected for ICSI, and they have been linked to poor embryonic development and an increased risk of miscarriage (Borini et al., 2006; Evenson and Wixon, 2006, 2008; Sakkas et al., 1996).

The current report of 26 patients found a significant correlation between high-magnification sperm-head morphology score 0 and sperm motility. No significant correlation was found between the percentage of score-0 spermatozoa and the percentage of DNA fragmentation: DNA fragmentation was comparable in the score-0 spermatozoa versus

total migrated spermatozoa, suggesting that score-0 head morphology is not useful for DNA fragmentation analysis. These results are apparently not in accordance with a previous report by Zini et al. (2009) where sperm morphology, according to the Kruger classification, was significantly correlated with DNA fragmentation and high DNA stainability (HDS; a measure of nuclear chromatin compaction) as assessed by the sperm chromatin structure assay (SCSA) with both DNA fragmentation index and HDS. In the present study, DNA fragmentation was assessed by TUNEL which identifies DNA breaks by labelling 3'-OH termini and is therefore a measure of existing DNA damage, whereas SCSA measures single-stranded DNA after acid treatment and therefore includes potential DNA damage.

High-magnification sperm-head examination in this study provided a more precise evaluation of head defects that are invisible in routine ICSI and demonstrated an association between score-0 spermatozoa and SCD. A strong significance between rate of score-0 spermatozoa and SCD was not found in total migrated spermatozoa, probably because of the small number of samples. A previous work (Cassuto et al. 2009) studying and following the outcome of each embryo after injection of a spermatozoa scored at high magnification was not able to identify the reason why oocytes microinjected with a score-0 spermatozoon did not reach the blastocyst stage. This particular sperm morphology score with a sperm-head vacuole has a negative impact on early embryo development.

Some authors have reported the relationships between the presence of large nuclear vacuoles and failure of chromatin condensation (Franco et al., 2008; Garolla et al., 2008). More recently, Perdrix et al. (2011) showed in a selected teratozoospermic population that sperm vacuoles were exclusively nuclear; and aneuploidy and chromatin condensation defects were the main alterations observed in spermatozoa with large vacuoles. This large vacuole appears to be a nuclear 'thumbprint' linked to failure of chromatin condensation (Boitrelle et al., 2011).

Chromatin condensation is a crucial step in protecting the paternal genome during the transit from the male to the oocyte prior to fertilization (Ward, 2010). Rousseaux et al. (2010) demonstrated a new key element in DNA compaction in humans and murines; they postulated that histones are replaced by transitional proteins called bromodomains before protamination. Early embryogenesis is a critical time for epigenetic regulation and this process is sensitive to environmental factors (Aitken and De luliis, 2007; Koppers et al., 2008). Epigenetic patterns are usually faithfully maintained during development. However, this

maintenance sometimes fails, resulting in the disturbance of epigenetic and human disorders. It is known that, prior to histone replacement by protamines, the nucleosomes are destabilized by hyperacetylation and DNA methylation levels rise (Miller et al., 2010; Palermo et al., 2008). These potential epigenetic mechanisms could be implied in chromatin condensation failures.

Whereas an oocyte may partially repair sperm DNA damage, it is postulated that more extensive defects and less DNA repair will result in the introduction of mutations and these have been associated with poor fertilization or embryo development rates and recurrent miscarriages (Bungum et al., 2007; Zini et al., 2008). High-magnification sperm selection could be an important step and a new tool for the clinician, who could decide to discard the score-0 spermatozoa with a high risk of abnormal chromatin and select the best spermatozoa for ICSI (Hazout et al., 2006). Sperm chromatin-packaging quality seems to have a major impact on sperm morphology and early embryo development.

In summary, this study suggests that score-0 spermatozoa selected by high magnification are associated with a high level of SCD, which may be deleterious for early embryo development and outcome. Consequently, the use of high magnification prior to ICSI may be helpful to discard score-0 spermatozoa at least and improve IVF outcome.

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