

# Sperm DNA quality predicts intrauterine insemination outcome: a prospective cohort study

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**BACKGROUND:** We aimed to investigate whether sperm DNA quality may predict intrauterine insemination (IUI) outcome. **METHODS:** The study was designed in a prospective cohort fashion, at a tertiary centre for reproductive medicine. A total of 119 patients underwent 154 cycles of IUI. Parameters related to demography, cycle management and semen sample used for IUI were evaluated. Conventional semen parameters, morphology (strict criteria), sperm DNA fragmentation and stability [evaluated by terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL) and acridine orange staining under both acid and acid + heat denaturing conditions respectively] were measured. The main outcome measure was clinical pregnancy, defined as ultrasonographic visualization of intrauterine gestational sac(s). **RESULTS:** Logistic regression analyses were done on six sets of data, including all cycles combined, cycles with washed samples, first cycle of each couple, first cycle of each couple with washed samples, cycles stimulated with gonadotrophins and finally gonadotrophin-stimulated cycles with washed samples. The number of pre-ovulatory follicles on day of hCG, the age of the woman and the percentage of sperm with acid- + heat-resistant DNA were the parameters that predicted IUI outcome in most of these data subsets. For the gonadotrophin-stimulated cycles, age of the man appeared as a predictor as opposed to that of the woman; and for the cycles within this subgroup, where the semen sample was washed, sperm DNA fragmentation and age of the man were the only two parameters to predict IUI outcome. No samples with >12% of sperm having DNA fragmentation resulted in pregnancy. **CONCLUSIONS:** The number of follicles, age of the woman/man and sperm DNA quality may predict IUI outcome.

*Key words:* acridine orange/DNA fragmentation/DNA stability/intrauterine insemination/sperm

## Introduction

Intrauterine insemination (IUI) with husband's sperm has been widely used for the treatment of infertility with a variety of indications, such as non-severe male factor infertility, unexplained infertility, cervical mucus hostility and ovulatory disturbances. In contemporary practice of infertility management, IUI often precedes the costly and rigorous options of assisted reproductive technologies, such as IVF with or without ICSI. To obtain a higher likelihood of achieving pregnancy, IUI is usually synchronized with ovulation, either in a natural or a stimulated cycle. It also involves fractionating and/or washing of motile sperm, before being injected into the uterine cavity.

The overall success of IUI varies, with pregnancy rates between 5 and 66% per cycle (Allen *et al.*, 1985). Several prognostic factors for IUI outcome have been proposed, including the age of the woman (Campana *et al.*, 1996; Stone *et al.*, 1999; Hendin *et al.*, 2000), endometrial thickness and follicle number by the time of ovulation (Tomlinson *et al.*, 1996; Stone *et al.*, 1999; Khalil *et al.*, 2001), aetiology and duration of infertility (Tomlinson *et al.*, 1996; Hendin *et al.*,

2000; Khalil *et al.*, 2001), presence and type of ovarian stimulation (Khalil *et al.*, 2001), time and number of inseminations (Silverberg *et al.*, 1992; Ragni *et al.*, 1999; Khalil *et al.*, 2001), percentage of sperm with normal morphology (Lindheim *et al.*, 1996; Ombelet *et al.*, 1997), type and percentage of sperm motility (Tomlinson *et al.*, 1996; Shulman *et al.*, 1998; Stone *et al.*, 1999; Hendin *et al.*, 2000) and total number of motile sperm inseminated (van der Westerlaken *et al.*, 1998; Khalil *et al.*, 2001).

We planned this prospective study to investigate whether the degree of sperm DNA fragmentation and stability, as well as conventional semen parameters, can predict IUI outcome. Sperm DNA fragmentation was evaluated by terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL), which identifies sperm with fragmented DNA. In addition, stability of sperm DNA was tested by acridine orange staining under both acid and acid + heat denaturing conditions.

## Materials and methods

The study was performed upon approval by the Institutional Review Board of Eastern Virginia Medical School.

### Cycle management

Semen was obtained from male partners of couples who were undergoing IUI for treatment of infertility. Only couples who agreed to participate and signed a consent form were included in the study, which comprised a total of 154 cycles out of 119 couples. Cycles were either natural or stimulated. Clomiphene citrate and/or gonadotrophins (FSH or hMG) were used for controlled ovarian stimulation. For clomiphene citrate-stimulated cycles, 100 mg clomiphene citrate was given between days 3 and 7. For clomiphene citrate + gonadotrophin stimulation, 100 mg clomiphene citrate was given between days 3 and 7, followed by 150 IU of gonadotrophins added by day 9. For cycles managed by gonadotrophins only, stimulation was started on day 3 with 75–150 IU daily. Follicle maturation was monitored by serial transvaginal ultrasonography and plasma estradiol ( $E_2$ ) levels. Timing of spontaneous pre-ovulatory LH surge was monitored by urine LH kits in 24 cycles, whereas the remaining 130 received either 10 000 IU of hCG or 250  $\mu$ g of recombinant hCG when the diameter of leading follicle(s) was  $>18$  mm. A single IUI was performed 36 h after hCG injection. Luteal phase was supported by daily vaginal administration of 50 mg progesterone suppositories. Plasma  $\beta$ -hCG levels were measured routinely, 2 weeks after IUI. Clinical pregnancy was defined as transvaginal ultrasonographic visualization of intra-uterine gestational sac(s).

### Semen analysis and handling for IUI

All semen parameters were evaluated for every ejaculate, i.e. for couples who underwent more than one cycle, every single sperm parameter (including morphology and DNA parameters) was assessed for that given ejaculate. Semen samples were allowed to liquefy for 30 min at 37°C, followed by assessment of sperm parameters. Sperm concentration and motion parameters were assessed using the HTM-IVOS semen analyser (version GS 771; Hamilton Thorne Research, Beverly, MA, USA) and manually monitored (Oehninger *et al.*, 1990). Motion parameters were examined by mixing the sperm suspension and loading a 5  $\mu$ l aliquot into a Makler chamber. The chamber was then transferred to the HTM, where it was maintained at 37°C for 2 min before starting data collection, which was conducted on randomly selected fields. Sperm morphology was assessed using strict criteria after slide staining with Diff-Quik (Dade AG, Dudinger, Switzerland).

Sperm in samples containing round cells  $<1 \times 10^6$ /ml ( $n = 123$ ) were washed in human tubal fluid (HTF; Irvine Scientific, Santa Ana, CA, USA) supplemented with 0.2% human serum albumin (HSA; Irvine), centrifuged at 400  $g$  for 10 min and re-suspended with 0.6 ml HTF + HSA. On the other hand, sperm in samples containing  $\geq 1 \times 10^6$ /ml round cells ( $n = 31$ ) were isolated by density gradient separation by centrifuging (DGC, by a single layer of 90% ISolate™) at 400  $g$  for 20 min. Purified populations of highly motile sperm were recovered, washed in HTF + HSA as described above and re-suspended with 0.6 ml HTF + HSA. An aliquot of 100  $\mu$ l from the final suspension was taken for acridine orange staining and TUNEL assay. The remaining 500  $\mu$ l was used for insemination.

### Acridine orange staining

For acridine orange staining, two smears were prepared from each sample. The smears were air-dried and then fixed overnight in Carnoy's solution (methanol:acetic acid, 3:1). Once air-dried again, one slide was stained for 5 min with freshly prepared acridine orange stain (0.19 mg/ml). The remaining slide was stained similarly after being incubated in tamponade solution (80 mmol/l citric acid + 15 mmol/l  $Na_2HPO_4$ , pH 2.5) at 75°C for 5 min to induce heat-

provoked DNA denaturation. Coverslips were then applied and sealed. Slides were evaluated on the same day using a fluorescence microscope (490/530 nm excitation/barrier filter; Nikon, Tokyo, Japan). In all, 300 sperm per smear were evaluated at a magnification of  $\times 1000$ . The duration of evaluation was limited to 40 s per field. Sperm with normal DNA content displayed a distinct green fluorescence whereas sperm with an abnormal DNA content emitted fluorescence in a spectrum varying from yellow–green to red. The spectrum of fluorescence other than distinct green colour was classified as abnormal, since DNA denaturation had begun. Preincubation percentage of green cells was calculated and referred to as preincubation acridine orange staining ( $AO_{pre}$ ) as previously defined (Tejada *et al.*, 1984). Acridine orange score (AOS) was thereafter calculated as the absolute value after subtracting post-incubation percentage of green cells ( $AO_{post}$ ) from preincubation percentage of green cells for each pair of smears (Duran *et al.*, 1998). It was noted that some samples exhibited an increased ratio of sperm with native DNA after heat treatment, possibly because of renaturation of already denatured DNA. The intra-observer and inter-observer variability was  $<7$  and  $<10\%$  respectively.

### TUNEL

For TUNEL assay, we used the In Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics GmbH, Mannheim, Germany) that uses fluorescein-dUTP to label sites of DNA fragmentation according to the manufacturer's instructions and as published (Duru *et al.*, 2000, 2001). Final suspensions of sperm were fixed with 4% paraformaldehyde and were permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. This was followed by incubation in the dark at 37°C for 1 h in TUNEL reaction mixture containing 0.5 IU/ $\mu$ l of calf thymus terminal deoxynucleotidyl transferase, fluorescein-dUTP and propidium iodide. Negative (omitting the enzyme terminal transferase) and positive (using deoxyribonuclease I, 1 mg/ml for 20 min at room temperature) controls were performed in each experiment. A total of 300 cells was randomly analysed per slide. Each sperm was assigned to contain normal (red nuclear fluorescence, due to propidium iodide) or fragmented DNA (intense green nuclear fluorescence). Final percentage of sperm with fragmented DNA is referred to as TUNEL (%). The intra-observer and inter-observer variability was  $<8$  and  $<7\%$  respectively.

### Statistical analysis

Statistical analysis was performed using SPSS™ 9.0 on a personal computer. Parametric (Student's *t*-) and non-parametric (Mann–Whitney *U*-) tests were applied to compare variables as appropriate. Pearson correlation coefficients were calculated for several parameters. Several logistic regression models were built to evaluate the contribution of various parameters to the outcome, using different sets of data.

### Results

The mean age of women included in the study was 34.6 years (range 22–45), whereas the mean age of the male partner was 36.5 years (range 21–55). The aetiology of infertility was unexplained for 49 couples (41%), oligo-ovulation for 27 couples (23%), male factor for 22 couples (19%), endometriosis for 19 couples (16%), hypothalamic for one couple (1%) and corrected intrauterine adhesions for one couple (1%). Basal endocrine evaluation, as well as several other parameters related to cycle are listed in Table I. Seven cycles (4.5%) were natural cycles, whereas 94 (61.0%) used clomiphene citrate,

**Table I.** Cycle parameters of the study population

Parameter	<i>n</i>	Mean ± SD
Day 3 FSH (mIU/ml)	113	7.6 ± 0.4
Day 3 LH (mIU/ml)	109	5.5 ± 0.3
Day 3 estradiol (pg/ml)	110	64.4 ± 2.7
Total gonadotrophin ampoules used	53	12.5 ± 1.6
Day of intrauterine insemination	154	15.1 ± 0.2
No. of follicles	154	2.3 ± 0.1

**Table II.** Correlations between various sperm-related parameters

Parameters correlated	<i>r</i> <sup>*</sup>	<i>P</i>
TUNEL morphology	-0.62	0.049
TUNEL AO <sub>pre</sub>	-0.330	0.00003
TUNEL % motility in semen	-0.328	0.00003
TUNEL % rapid sperm in semen	-0.268	0.001
TUNEL AO <sub>post</sub>	-0.220	0.006
TUNEL concentration of round cells in semen	0.201	0.012
TUNEL AOS	-0.175	0.030
AOS morphology	0.170	0.038

<sup>\*</sup>Pearson's correlation coefficient.

TUNEL = terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling; AO = acridine orange; AOS = acridine orange score.

37 (24.0%) used clomiphene citrate + gonadotrophins (FSH or HMG) and 16 (10.3%) used gonadotrophins alone.

Post-processing sperm concentration (150 versus 58 × 10<sup>6</sup>/ml, *P* < 0.00001) and total motile sperm recovered (49 versus 23 × 10<sup>6</sup>, *P* < 0.00001) were significantly higher in washed samples (*P* < 0.00001) than in samples prepared by DGC. Ejaculates processed by DGC had a higher percentage of motility (76.7 versus 61.2%, *P* < 0.00001), rapid sperm (66.8 versus 46.6%, *P* < 0.00001) and DNA fragmentation after recovery (19.1 versus 12.0%, *P* = 0.01) than in washed samples.

Significant correlations between various sperm-related parameters are listed in Table II. Of those, correlations between morphology and DNA parameters are noteworthy.

Of the total 154 cycles, 13 cycles resulted in pregnancy, with a pregnancy rate of 8.4% per cycle and 10.9% per patient. One cycle resulted in ectopic pregnancy, which later resolved spontaneously with expectant management. Two out of the 13 pregnancies were twin gestations, whereas two others ended with spontaneous miscarriage.

Various sperm parameters of cycles that resulted in conception were compared with those of failed cycles. Some of these parameters are listed in Table III. Briefly, in cycles resulting in pregnancy, the degree of DNA fragmentation after preparation was significantly lower than in those that did not result in pregnancy (7.3 ± 3.5 versus 13.9 ± 10.8 respectively, *P* = 0.044). No other sperm parameter exhibited a heterogeneous distribution among cycles likewise, although AO<sub>post</sub> tended to be higher for cycles resulted in pregnancy.

Various logistic regression analyses were performed in a forward stepwise fashion using likelihood ratios, on different sets of data. A brief summary of models built from those analyses and parameters included in those models are summarized in Table IV. Number of follicles, age of the woman and AO<sub>post</sub> were the most common parameters present in most of the models. When all cycles were included in the logistic regression analysis, the stimulation protocol (gonadotrophins > clomiphene citrate + gonadotrophins > clomiphene citrate > natural), preparation method (wash > DGC), number of follicles, age of woman (negative impact), motility before preparation, AO<sub>post</sub> and total motile sperm count inseminated were the included determinants of outcome, as ordered by their contribution. However, when the data were broken into smaller subsets, the number of determinants were also decreased. Briefly, analyses were performed in following data sets: (i) only the cycles, where the sample was washed; (ii) only the first cycle of each couple; (iii) only the first cycle of each couple, where the sample was washed; (iv) only the cycles stimulated by gonadotrophins; (v) only the cycles stimulated by gonadotrophins, where the sample was washed.

Number of follicles, age of the woman (negative impact) and AO<sub>post</sub> were the determinants of outcome respectively for the first three data sets. However, for gonadotrophin-stimulated cycles (fourth data set), age of the woman was replaced by age of the man (negative impact), with the other parameters and their order being preserved. When this group was further broken down to include only washed samples (fifth data set), only age of the man and TUNEL were included in the model respectively, both with negative impacts on the outcome.

## Discussion

Sperm DNA damage may occur by at least three putative mechanisms: (i) defective chromatin condensation during spermiogenesis; (ii) initiation of apoptosis during spermatogenesis or transport of sperm through male or female genital tract; (iii) by oxidative stress mainly resulting from reactive oxygen species (ROS) produced internally or externally. Endogenous nicks in DNA have been shown to be present at specific stages of spermatogenesis in rodents, during the replacement of histones by protamines (McPherson and Longo, 1992). It is postulated that an endogenous nuclease, topoisomerase II, creates and ligates nicks to provide relief of torsional stress and to aid chromatin rearrangement during protamination (McPherson and Longo, 1993). Therefore, the existence of endogenous nicks in ejaculated sperm may indicate incomplete maturation. Other researchers also confirmed this hypothesis by observing a correlation between poor chromatin packaging and the presence of DNA damage (Gorczyca *et al.*, 1993; Manicardi *et al.*, 1995; Sailer *et al.*, 1995).

Apoptosis is a major regulatory mechanism during normal spermatogenesis in several species, including humans (Hikim *et al.*, 1995; Lue *et al.*, 1997; Hikim *et al.*, 1998). Unlike rat, mouse or hamster testes, human testes exhibit spontaneous occurrence of germ cell apoptosis involving all three classes of germ cell, including spermatogonia, spermatocytes and spermatids (Sinha Hikim and Swerdloff, 1999). There is

**Table III.** Comparison of sperm parameters in cycles that resulted in pregnancy or failure

Parameter	Pregnancy <sup>a</sup>	Failure <sup>a</sup>	P
Before preparation			
Sperm concentration ( $\times 10^6/\text{ml}$ )	64 $\pm$ 47	62 $\pm$ 45	NS
Motility (%)	64.7 $\pm$ 12.1	57.5 $\pm$ 18.2	NS
Morphology (%)	6.8 $\pm$ 4.5	6.4 $\pm$ 4.4	NS
After preparation			
Sperm concentration ( $\times 10^6/\text{ml}$ )	110 $\pm$ 83	130 $\pm$ 136	NS
Motility (%)	66.6 $\pm$ 12.9	64.2 $\pm$ 19.5	NS
Total motile recovered ( $\times 10^6/\text{ml}$ )	39 $\pm$ 30	44 $\pm$ 48	NS
AO <sub>pre</sub> (%)	46.1 $\pm$ 17.3	38.9 $\pm$ 18.8	NS
AO <sub>post</sub> (%)	36.4 $\pm$ 13.9	27.7 $\pm$ 16.1	0.052 (NS)
TUNEL (%)	7.3 $\pm$ 3.5	13.9 $\pm$ 10.8	0.044

<sup>a</sup>Values are presented as mean  $\pm$  SD.

**Table IV.** Results of logistic regression analyses according to various cycle classifications

	All cycles (n = 154)	Washed cycles (n = 123)	First cycles of each couple (n = 119)	First + washed cycles (n = 95)	Gonadotrophin- stimulated cycles (n = 53)	Gonadotrophin- stimulated + washed cycles (n = 42)
Pregnancies (rate, %)	13 (8.4)	12 (9.8) <sup>a</sup>	12 (10.1) <sup>b</sup>	11 (11.6) <sup>c</sup>	9 (17.0) <sup>d</sup>	8 (19.0) <sup>e</sup>
Model coefficients						
-2 log likelihood	43.688	61.035	58.244	49.614	32.069	25.353
$\chi^2$	41.867	14.076	15.896	15.034	15.070	14.226
D.f.	10	3	3	3	3	2
P	0.00001	0.003	0.001	0.002	0.002	0.001
Parameters						
AO <sub>post</sub>	+	+	+	+	+	-
Age of the woman	+	+	+	+	-	-
Number of follicles	+	+	+	+	+	-
Preparation method	+	NA <sup>f</sup>	-	NA	-	NA
Motility in semen	+	-	-	-	-	-
TMCI <sup>g</sup>	+	-	-	-	-	-
Stimulation protocol	+	-	-	-	NA	NA
TUNEL	-	-	-	-	-	+
Age of the man	-	-	-	-	+	+

<sup>a</sup>Not significantly different from that of samples prepared by density gradient separation (3.2%)

<sup>b</sup>Not significantly different from that of other cycles (2.9%).

<sup>c</sup>Not significantly different from that of first cycle, prepared by density gradient separation (4.2%).

<sup>d</sup>Significantly different from clomiphene citrate-stimulated cycles (4.3%,  $P = 0.01$ ).

<sup>e</sup>Not significantly different than that of samples prepared by density gradient separation among gonadotrophin-stimulated cycles (9.0%).

<sup>f</sup>Not applicable.

<sup>g</sup>Total motile sperm count inseminated.

evidence showing that the apoptotic machinery is also present in later stages of germ cell development, including sperm. Fas receptors have been demonstrated on ejaculated sperm of both fertile and oligozoospermic samples, the latter showing a higher ratio of positivity (Sakkas *et al.*, 1999). Furthermore, we have shown that caspase-3 is also present in ejaculated sperm, in relatively small amounts compared with leukocytes (Weng *et al.*, 2002). However, the presence of the apoptotic machinery does not necessarily mean that it is active and efficient. Besides, it is less likely for apoptosis to be initiated or advanced in mature sperm, because of its unique cytoplasmic and nuclear organization. Nevertheless, these findings suggest

that there may at least be an abortive apoptotic process, which extends until the latest stage of spermatogenesis. Fragmented DNA in some sperm may be an indicator of such an abortive apoptotic process.

It has been demonstrated that ROS can lead to oxidative stress in sperm. White blood cells in the ejaculate, as well as immature germ cells and mature sperm, may contribute to production of ROS (Krausz *et al.*, 1992; Ollero *et al.*, 2001). Independent of the origin of ROS, it is now well established that they cause fragmentation of DNA in sperm (Lopes *et al.*, 1998; Barroso *et al.*, 2000; Duru *et al.*, 2000). Given this fact, it is not surprising to find a correlation between the

concentration of round cells and DNA fragmentation in our findings. It is also reflected as a higher percentage of DNA fragmentation among samples prepared by DGC, where the selection of preparation was entirely based upon round cell concentration in semen. Several other researchers have also reported the presence of negative correlations between the extent of sperm DNA fragmentation and morphology, as well as parameters related to motility similar to those we found (Taylor *et al.*, 1999; Muratori *et al.*, 2000; Ramos and Wetzels, 2001; Zini *et al.*, 2001). The correlation we found between the extent of DNA fragmentation and denaturation, however, is not as high as the ones that have been reported (Sailer *et al.*, 1995; Aravindan *et al.*, 1997; Zini *et al.*, 2001). This may be due to the methodology used, as flow cytometry is more accurate than microscopic evaluation of acridine orange-stained slides. No influence of AOS on IUI outcome has been detected, although the mean AO<sub>post</sub> (percentage of sperm with heat- and acid-resistant DNA) tended to be lower among cycles that resulted in failure, than those that resulted in pregnancy (Table III).

Several studies have shown that sperm DNA quality had robust power to predict fertilization *in vitro* (Sun *et al.*, 1997; Duran *et al.*, 1998; Larson *et al.*, 2000; Chan *et al.*, 2001). In a recent report, the only parameter that showed a significant difference between pregnant and non-pregnant groups by IVF was the percentage of sperm with DNA damage after preparation, as assessed by in-situ nick translation. It was significantly higher in those patients that did not establish a pregnancy (Tomlinson *et al.*, 2001). Furthermore, sperm-derived effects have recently been reported to condition human embryo development (Tesarik *et al.*, 2002). The authors designed the study as a shared donor oocyte programme and included males, whose sperm yielded poor zygote quality consistently in previous attempts. They compared them with control males, who proved normal zygote quality in preceding cycles, with a similar semen sample in terms of basic semen analysis. Zygotes obtained from two consequent ICSI cycles using the sperm of those males with a poor history of zygote quality (patient group) had a significantly lower quality, when compared with the controls, who shared oocytes from the same donor. This difference in zygote quality was also followed by poor embryo quality, cleavage and implantation rates, in the patient group. Although the origin of this paternal effect needs further clarification, sperm DNA quality is clearly one of the possible candidates.

In addition, to predict fertilization *in vitro*, sperm DNA stability—as assessed by sperm chromatin structure assay (SCSA)—has been documented as a powerful diagnostic and prognostic tool in a human fertility clinic (Evenson *et al.*, 1999). The SCSA measures susceptibility to DNA denaturation *in situ* in sperm exposed to acid for 30 s, followed by acridine orange staining. Utilization of flow cytometry in SCSA increases its dependability. Consequently, among couples who were trying to conceive over 12 months, sperm with denatured DNA was the best predictor for whether or not a couple would achieve a pregnancy. Furthermore, SCSA data predicted 39% of miscarriages.

Ours is the first report to claim sperm DNA quality to be a predictor of fertilization and pregnancy *in vivo*, as a result of IUI for treatment of human infertility. All of the logistic regression analyses performed herein, including the one assessing all cycles as well as others for five different subsets of data, contained a sperm DNA parameter related to either stability or fragmentation (as assessed by AO<sub>post</sub> and TUNEL respectively), sometimes being the only male parameter to predict outcome. In addition, no woman inseminated with a sample having >12% of sperm with fragmented DNA achieved a pregnancy. Furthermore, the two patients who miscarried were inseminated with the samples containing the highest degree of DNA fragmentation among all cases who became pregnant (TUNEL = 12 and 10%).

Washing may seem a superior method for sperm preparation for IUI, based on the logistic regression analysis for all cycles. However, since the method of sperm preparation was selected according to the number of round cells in the ejaculate, the result may simply reflect its consequences, indirectly. Thus, based on our findings, one should not conclude washing to be a better sperm preparation technique, with a higher likelihood of pregnancy.

Other parameters we report to predict pregnancy after IUI are compatible with those published in the literature, especially those studies with a larger sample size (Campana *et al.*, 1996; Stone *et al.*, 1999; Goverde *et al.*, 2000; Hendin *et al.*, 2000; Khalil *et al.*, 2001). The two parameters with the strongest impact were related to female factors, indicating the importance of the number of follicles and age of the woman for successful reproductive outcome. These parameters are indicators of the quality and availability of the female gamete, which is also the major determinant of successful outcome in assisted reproduction treatment. The sample size of our study is also large enough to show the importance of the female gamete for successful reproductive outcome under *in-vivo* conditions. However, gonadotrophins seem to compensate for the negative effects of advanced maternal age, at least to a certain degree, when used for ovarian stimulation (Table IV). In this case, the age of the man appears to be a negative confounding variable instead. In some men, fertility may be decreased in the absence of evidence of disruption in testis morphology or semen production, although it is usually maintained up to a very high age. Reasons for this decrease in fertility may include defects in sperm maturation; age-related increases in germ cell mutations, impairment of DNA repair mechanisms and apoptotic processes (Rolf and Nieschlag, 2001). Our results support the existence as well as importance of age-dependent problems with sperm DNA, especially when female factors are corrected for.

In conclusion, the number of follicles, the age of the woman/man and the quality of sperm DNA may predict IUI outcome. Whether it is a result of suboptimal maturation, apoptosis, an oxidative hazard or other causes, the stability of the sperm DNA, as well as the extent of its fragmentation is an indicator of poor IUI outcome. Further assessment of sperm DNA status and different types of damage is warranted to establish their overall importance in the efficiency of human reproduction. We suggest that tests analysing sperm DNA quality should be

a part of the routine semen analysis for patients suffering infertility, regardless of the type of treatment they undergo.

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## References

- Allen, N.C., Herbert, C.M., III, Maxson, W.S., Rogers, B.J., Diamond, M.P. and Wentz, A.C. (1985) Intrauterine insemination: a critical review. *Fertil. Steril.*, **44**, 569–580.
- Aravindan, G.R., Bjordahl, J., Jost, L.K. and Evenson, D.P. (1997) Susceptibility of human sperm to in situ DNA denaturation is strongly correlated with DNA strand breaks identified by single-cell electrophoresis. *Exp. Cell Res.*, **236**, 231–237.
- Barroso, G., Morshedi, M. and Oehninger, S. (2000) Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa. *Hum. Reprod.*, **15**, 1338–1344.
- Campana, A., Sakkas, D., Stalberg, A., Bianchi, P.G., Comte, I., Pache, T. and Walker, D. (1996) Intrauterine insemination: evaluation of the results according to the woman's age, sperm quality, total sperm count per insemination and life table analysis. *Hum. Reprod.*, **11**, 732–736.
- Chan, P.J., Corselli, J.U., Patton, W.C., Jacobson, J.D., Chana, S.R. and King, A. (2001) A simple comet assay for archived sperm correlates DNA fragmentation to reduced hyperactivation and penetration of zona-free hamster oocytes. *Fertil. Steril.*, **75**, 186–192.
- Duran, E.H., Gurgan, T., Gunalp, S., Enginsu, M.E., Yarali, H. and Ayhan, A. (1998) A logistic regression model including DNA status and morphology of spermatozoa for prediction of fertilization in vitro. *Hum. Reprod.*, **13**, 1235–1239.
- Duru, K.N., Morshedi, M. and Oehninger, S. (2000) Effects of hydrogen peroxide on DNA and plasma membrane integrity of human spermatozoa. *Fertil. Steril.*, **74**, 1200–1207.
- Duru, N.K., Morshedi, M.S., Schuffner, A. and Oehninger, S. (2001) Cryopreservation–thawing of fractionated human spermatozoa is associated with membrane phosphatidylserine externalization and not DNA fragmentation. *J. Androl.*, **22**, 646–651.
- Evenson, D.P., Jost, L.K., Marshall, D., Zinaman, M.J., Clegg, E., Purvis, K., de Angelis, P. and Claussen, O.P. (1999) Utility of the sperm chromatin structure assay as a diagnostic and prognostic tool in the human fertility clinic. *Hum. Reprod.*, **14**, 1039–1049.
- Gorczyca, 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.
- Goverde, A.J., McDonnell, J., Vermeiden, J.P., Schats, R., Rutten, F.F. and Schoemaker, J. (2000) Intrauterine insemination or in-vitro fertilisation in idiopathic subfertility and male subfertility: a randomised trial and cost-effectiveness analysis. *Lancet*, **355**, 13–18.
- Hendin, B.N., Falcone, T., Hallak, J., Nelson, D.R., Vemullapalli, S., Goldberg, J., Thomas, A.J. and Agarwal, A. (2000) The effect of patient and semen characteristics on live birth rates following intrauterine insemination: a retrospective study. *J. Assist. Reprod. Genet.*, **17**, 245–252.
- Hikim, A.P., Wang, C., Leung, A. and Swerdloff, R.S. (1995) Involvement of apoptosis in the induction of germ cell degeneration in adult rats after gonadotropin-releasing hormone antagonist treatment. *Endocrinology*, **136**, 2770–2775.
- Hikim, A.P., Wang, C., Lue, Y., Johnson, L., Wang, X.H. and Swerdloff, R.S. (1998) Spontaneous germ cell apoptosis in humans: evidence for ethnic differences in the susceptibility of germ cells to programmed cell death. *J. Clin. Endocrinol. Metab.*, **83**, 152–156.
- Khalil, M.R., Rasmussen, P.E., Erb, K., Laursen, S.B., Rex, S. and Westergaard, L.G. (2001) Homologous intrauterine insemination. An evaluation of prognostic factors based on a review of 2473 cycles. *Acta Obstet. Gynecol. Scand.*, **80**, 74–81.
- Krausz, C., West, K., Buckingham, D. and Aitken, R.J. (1992) Development of a technique for monitoring the contamination of human semen samples with leukocytes. *Fertil. Steril.*, **57**, 1317–1325.
- Larson, K.L., DeJonge, C.J., Barnes, A.M., Jost, L.K. and Evenson, D.P. (2000) Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. *Hum. Reprod.*, **15**, 1717–1722.
- Lindheim, S.R., Barad, D.H., Zinger, M., Witt, B., Amin, H., Cohen, B., Fisch, H. and Barg, P. (1996) Abnormal sperm morphology is highly predictive of pregnancy outcome during controlled ovarian hyperstimulation and intrauterine insemination. *J. Assist. Reprod. Genet.*, **13**, 569–572.
- Lopes, S., Jurisicova, A., Sun, J.G. and Casper, R.F. (1998) Reactive oxygen species: potential cause for DNA fragmentation in human spermatozoa. *Hum. Reprod.*, **13**, 896–900.
- Lue, Y., Hikim, A.P., Wang, C., Bonavera, J.J., Baravarian, S., Leung, A. and Swerdloff, R.S. (1997) Early effects of vasectomy on testicular structure and on germ cell and macrophage apoptosis in the hamster. *J. Androl.*, **18**, 166–173.
- Manicardi, G.C., Bianchi, P.G., Pantano, S., Azzoni, P., Bizzaro, D., Bianchi, U. and Sakkas, D. (1995) Presence of endogenous nicks in DNA of ejaculated human spermatozoa and its relationship to chromomycin A3 accessibility. *Biol. Reprod.*, **52**, 864–867.
- McPherson, S.M. and Longo, F.J. (1992) Localization of DNase I-hypersensitive regions during rat spermatogenesis: stage-dependent patterns and unique sensitivity of elongating spermatids. *Mol. Reprod. Dev.*, **31**, 268–279.
- McPherson, S.M. and Longo, F.J. (1993) Nicking of rat spermatid and spermatozoa DNA: possible involvement of DNA topoisomerase II. *Dev. Biol.*, **158**, 122–130.
- Muratori, M., Piomboni, P., Baldi, E., Filimberti, E., Pecchioli, P., Moretti, E., Gambera, L., Baccetti, B., Biagioli, R., Forti, G. and Maggi, M. (2000) Functional and ultrastructural features of DNA-fragmented human sperm. *J. Androl.*, **21**, 903–912.
- Oehninger, S., Acosta, R., Morshedi, M., Philput, C., Swanson, R.J. and Acosta, A.A. (1990) Relationship between morphology and motion characteristics of human spermatozoa in semen and in the swim-up sperm fractions. *J. Androl.*, **11**, 446–452.
- Ollero, M., Gil-Guzman, E., Lopez, M.C., Sharma, R.K., Agarwal, A., Larson, K., Evenson, D., Thomas, A.J. Jr and Alvarez, J.G. (2001) Characterization of subsets of human spermatozoa at different stages of maturation: implications in the diagnosis and treatment of male infertility. *Hum. Reprod.*, **16**, 1912–1921.
- Ombelet, W., Vandeput, H., Van de, P.G., Cox, A., Janssen, M., Jacobs, P., Bosmans, E., Steeno, O. and Kruger, T. (1997) Intrauterine insemination after ovarian stimulation with clomiphene citrate: predictive potential of inseminating motile count and sperm morphology. *Hum. Reprod.*, **12**, 1458–1463.
- Ragni, G., Maggioni, P., Guermendi, E., Testa, A., Baroni, E., Colombo, M. and Crosignani, P.G. (1999) Efficacy of double intrauterine insemination in controlled ovarian hyperstimulation cycles. *Fertil. Steril.*, **72**, 619–622.
- Ramos, L. and Wetzels, A.M. (2001) Low rates of DNA fragmentation in selected motile human spermatozoa assessed by the TUNEL assay. *Hum. Reprod.*, **16**, 1703–1707.
- Rolf, C. and Nieschlag, E. (2001) Reproductive functions, fertility and genetic risks of ageing men. *Exp. Clin. Endocrinol. Diabetes*, **109**, 68–74.
- 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., Mariethoz, E. and St John, J.C. (1999) Abnormal sperm parameters in humans are indicative of an abortive apoptotic mechanism linked to the Fas-mediated pathway. *Exp. Cell Res.*, **251**, 350–355.
- Shulman, A., Hauser, R., Lipitz, S., Frenkel, Y., Dor, J., Bider, D., Mashiach, S., Yogev, L. and Yavetz, H. (1998) Sperm motility is a major determinant of pregnancy outcome following intrauterine insemination. *J. Assist. Reprod. Genet.*, **15**, 381–385.
- Silverberg, K.M., Johnson, J.V., Olive, D.L., Burns, W.N. and Schenken, R.S. (1992) A prospective, randomized trial comparing two different intrauterine insemination regimens in controlled ovarian hyperstimulation cycles. *Fertil. Steril.*, **57**, 357–361.
- Sinha Hikim, A.P. and Swerdloff, R.S. (1999) Hormonal and genetic control of germ cell apoptosis in the testis. *Rev. Reprod.*, **4**, 38–47.
- Stone, B.A., Vargyas, J.M., Ringler, G.E., Stein, A.L. and Marrs, R.P. (1999) Determinants of the outcome of intrauterine insemination: analysis of outcomes of 9963 consecutive cycles. *Am. J. Obstet. Gynecol.*, **180**, 1522–1534.
- Sun, J.G., Jurisicova, A. and Casper, R.F. (1997) Detection of deoxyribonucleic acid fragmentation in human sperm: correlation with fertilization in vitro. *Biol. Reprod.*, **56**, 602–607.

- Taylor, Z., Molloy, D., Hill, V. and Harrison, K. (1999) Contribution of the assisted reproductive technologies to fertility in males suffering spinal cord injury. *Aust. NZ J. Obstet. Gynaecol.*, **39**, 84–87.
- Tejada, R.I., Mitchell, J.C., Norman, A., Marik, J.J. and Friedman, S. (1984) A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. *Fertil. Steril.*, **42**, 87–91.
- Tesarik, J., Mendoza, C. and Greco, E. (2002) Paternal effects acting during the first cell cycle of human preimplantation development after ICSI. *Hum. Reprod.*, **17**, 184–189.
- Tomlinson, M.J., Amissah-Arthur, J.B., Thompson, K.A., Kasraie, J.L. and Bentick, B. (1996) Prognostic indicators for intrauterine insemination (IUI): statistical model for IUI success. *Hum. Reprod.*, **11**, 1892–1896.
- Tomlinson, M.J., Moffatt, O., Manicardi, G.C., Bizzaro, D., Afnan, M. and Sakkas, D. (2001) Interrelationships between seminal parameters and sperm nuclear DNA damage before and after density gradient centrifugation: implications for assisted conception. *Hum. Reprod.*, **16**, 2160–2165.
- van der Westerlaken, L.A., Naaktgeboren, N. and Helmerhorst, F.M. (1998) Evaluation of pregnancy rates after intrauterine insemination according to indication, age and sperm parameters. *J. Assist. Reprod. Genet.*, **15**, 359–364.
- Weng, S.-L., Taylor, S.L., Morshedi, M., Schuffner, A., Duran, E.H., Beebe, S. and Oehninger, S. (2002) Caspase activity and apoptotic markers in ejaculated human sperm. *Mol. Hum. Reprod.*, **8**, 984–991.
- Zini, A., Bielecki, R., Phang, D. and Zenzes, M.T. (2001) Correlations between two markers of sperm DNA integrity, DNA denaturation and DNA fragmentation, in fertile and infertile men. *Fertil. Steril.*, **75**, 674–677.

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