

A logistic regression model including DNA status and morphology of spermatozoa for prediction of fertilization *in vitro*

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To determine predictive values of routine semen analysis, sperm morphology evaluation using strict criteria and DNA status for in-vitro fertilization (IVF), 66 consecutive couples undergoing IVF in a university hospital IVF programme were prospectively investigated. Semen samples from 66 men were evaluated by routine semen analysis, morphology evaluation using strict criteria and acridine orange staining for determination of DNA status. A new technique is described for acridine orange scoring which consisted of evaluation of two smears per case, with and without heat treatment. Resistance to heat-provoked denaturation was determined by the difference between two evaluations. A logistic regression model was built and receiver operating characteristic curves were constructed to determine the threshold values and to compare diagnostic properties. Morphology evaluation using strict criteria and concentration of progressively motile spermatozoa were found to be the principal parameters determining the sperm fertilizing capacity *in vitro*. The logistic regression model composed of morphology evaluation using strict criteria and acridine orange score had a powerful diagnostic capability for prediction of fertilization *in vitro*.

Key words: acridine orange score/fertilization *in vitro*/morphology/strict criteria

Introduction

Sperm concentration, motility and morphology have been the traditional criteria used for assessment of semen quality (Menkveld *et al.*, 1990). When used in combination, morphology evaluation using strict criteria and concentration of progressively motile spermatozoa have been reported to be good predictors of in-vitro fertilization (IVF) (Enginsu *et al.*, 1992). Other studies have also confirmed the prognostic value of morphology evaluation using strict criteria in IVF (Menkveld *et al.*, 1990; Enginsu *et al.*, 1991; Ombelet *et al.*, 1994).

Evaluation of DNA status and its relation to fertility has been a focus of research (Evenson *et al.*, 1980; Ibrahim and Pedersen, 1988). Evenson *et al.* (1980) first reported differential staining of human semen samples with acridine orange, based on the amount of denatured DNA in spermatozoa; with this

technique, spermatozoa from infertile men displayed increased red fluorescence when compared to those from fertile men (Evenson *et al.*, 1980). Golan *et al.* (1996) demonstrated that spermatozoa obtained from the more distal parts of the epididymis were more mature with regard to chromatin condensation by means of flow cytometric analysis (Golan *et al.*, 1996). In addition, a flow cytometric method for evaluating the degree of sperm chromatin condensation was developed recently, which also identified some specific chromatin abnormalities that may in future be related to some specific clinical entities (Golan *et al.*, 1997). Another method for simplified evaluation of sperm DNA status was developed by Tejada *et al.* (1984). Smears of semen with high proportions of sperm heads with denatured DNA displayed red fluorescence and were associated with decreased fertility. However, smears from fertile men had a high proportion of mature spermatozoa with normal DNA content, as reflected by green fluorescence. In parallel with this finding, another study demonstrated that spermatozoa from fertile donors had less denatured DNA content as shown by acridine orange staining, when compared with that of infertile men (Peluso *et al.*, 1992). Moreover, a recent study has shown a strong relationship between the results of acridine orange staining of smears in acid denaturing conditions only and the fertilizing ability of spermatozoa *in vitro* (Hoshi *et al.*, 1996). However, resistance to heat denaturation of sperm DNA was proposed as a more sensitive technique than direct staining with acridine orange in acid denaturing conditions, for the assessment of fertility potential (Roux and Dadoune, 1989).

One of the current uses of IVF is to evaluate putative tests of human sperm function (Liu *et al.*, 1988). The aims of this study were to investigate the values of routine semen analysis, sperm morphology evaluation using strict criteria and DNA status for the prediction of fertilization *in vitro*. We also aimed to construct a logistic regression model with a good predictive value for fertilization *in vitro*.

Materials and methods

A total of 66 consecutive couples undergoing their initial IVF cycle was included in the study, following approval for human experimentation by our local institution. Indications for IVF included tubal factor ($n = 37$), unexplained infertility ($n = 15$), male factor ($n = 12$) and endometriosis ($n = 2$).

Ovarian stimulation was undertaken using a short flare-up gonadotrophin-releasing hormone (GnRH) analogue and human menopausal gonadotrophin (HMG) protocol. Buserelin nasal spray (Suprefact™, Hoechst AG, Frankfurt am Main, Germany) was initiated as 100 µg/qid on the first day of the menstrual bleed and continued until the administration of human chorionic

gonadotrophin (HCG). After confirmation of the absence of cystic ovarian structures >10 mm in diameter by transvaginal ultrasonography performed on day 3 of the cycle, HMG was commenced (Humegon™, Organon, Istanbul, Turkey). The dosage of HMG was individualized according to follicular response as assessed by transvaginal ultrasonography and daily serum oestradiol concentrations. HCG was administered (10 000 IU, Pregnyl™, Organon, Istanbul, Turkey) when two or more follicles reached or exceeded a mean diameter of 18 mm with serum oestradiol concentrations >500 pg/ml (1800 pmol/l). Follicular aspiration for oocyte collection was performed with transvaginal sonographic guidance 36 h following the administration of HCG. Minimal essential medium with Earle's salts and L-glutamine (Gibco BRL, Life Technologies Ltd, Paisley, Scotland; catalogue no. 31095029) supplemented with female partner's serum in 10–15% concentrations was used for insemination and culture media respectively. Standard laboratory procedures were employed for handling of gametes and embryos (American Fertility Society, 1991). Semen was provided within 2 h after the oocyte collection. For each sample, a suspension of motile spermatozoa was prepared by a standard swim-up technique (Mahadevan and Baker, 1984). Liquefied semen was mixed with an equal volume of modified human tubal fluid (HTF; Irvine Scientific Co., Irvine, CA, USA) and centrifuged at 350 g for 5 min and the supernatant was discarded. Modified HTF (0.5 ml) was added to the pellet, mixed and the dense sperm suspension was placed at the bottom of a small test tube containing 2.0 ml modified HTF. After being incubated for ~2 h, the sperm suspension near the bottom of the tube was removed. The remaining suspension was centrifuged at 230 g for 5 min. The sperm pellet was then resuspended and diluted in the modified HTF. At the fifth hour of oocyte collection, 50 000 motile spermatozoa were inseminated to each oocyte. Embryo transfer was performed 48 h after oocyte retrieval if fertilization had occurred. A maximum of four embryos was transferred.

Conventional methods were used to evaluate the volume of semen, sperm concentration and motility (World Health Organization, 1992). According to the motility pattern, two groups of spermatozoa were recognized: slow, sluggish movement or movement of the spermatozoa hampered by an obvious, clearly visible morphological defect (group A) and rapid and linearly progressive movement (group B). The total number of the spermatozoa per unit volume in group B was defined as the concentration of progressively motile spermatozoa.

For morphology evaluation using strict criteria, a smear from each semen sample was prepared. The slide was air-dried for ~3 min, fixed for 15 s in Diff-Quik fixative prior to staining with Diff-Quik solution 1 for 10 s and with Diff-Quik solution 2 for 5 s (Diff-Quik™, Dade Diagnostics, Miami, FL, USA). Sperm morphology was evaluated as outlined by Kruger *et al.* (1988). At least 200 spermatozoa per patient were evaluated at a magnification of ×1000.

After insemination, the rest of the sample was used for acridine orange scoring as follows: 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 10 min with freshly prepared acridine orange stain (0.19 mg/ml). The remaining slide was stained similarly after being incubated in 20 ml of tamponade solution (80 mM citric acid + 15 mM Na₂HPO₄, pH = 2.5) at 87°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; Zeiss, Germany). In all, 300 spermatozoa per smear were

evaluated at a magnification of ×1000. The duration of evaluation was limited to 40 s per field. Spermatozoa with normal DNA content displayed a distinct green fluorescence whereas spermatozoa with an abnormal DNA content gave 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 (Tejada *et al.*, 1984). Pre-incubation percentage of green cells was calculated and referred to as pre-incubation acridine orange staining as previously defined by Tejada *et al.* (1984). Acridine orange scoring was thereafter calculated by subtracting post-incubation percentage of green cells from pre-incubation percentage of green cells for each pair of smears (Equation 1), as follows:

Equation 1: Determination of acridine orange score (AOS)

$$\text{AOS} = [\% \text{Green cells}]_{\text{preincubation}} - [\% \text{Green cells}]_{\text{postincubation}}$$

All data were processed by Statistical Program for Social Sciences (SPSS Inc., Chicago, IL, USA) on an IBM compatible personal computer. Pearson's correlation coefficients were calculated between individual test results and fertilization rates. A logistic regression analysis was done to assess the values of semen parameters to predict fertilization *in vitro* and to build up a model with a high predictive value.

Patients were divided into two groups according to whether or not fertilization had occurred. Receiver operating characteristic (ROC) curves, which are useful graphic means for comparing diagnostic tests, were constructed and area under the curves was calculated for the assessment and comparison of the effectiveness of each parameter to predict fertilization (Metz, 1978; Griner *et al.*, 1981; Hanley and McNeil, 1983). Optimal cut-off points that best predicted total IVF failure for each parameter were determined by ROC analysis. Odds ratios (OR) for each parameter, predicting total fertilization failure were assessed. Cohen's kappa statistics (κ) were calculated to assess the agreement beyond chance between diagnostic tests and fertilization *in vitro*; a κ value of <0.4 was accepted as poor and 0.4–0.75 as good agreement beyond chance. Finally, likelihood ratios (LR) were calculated to express the likelihood of fertilization over that of non-fertilization, given normal test result (fertilization) as negative LR (LR⁻) and abnormal test result (total fertilization failure) as positive LR (LR⁺). The likelihood ratio expresses the odds that the test results occur in patients with infertility versus those without infertility. Thus, there is one likelihood ratio for a positive test and another for a negative test. Likelihood ratios are much more stable for changes in prevalence than are sensitivity and specificity (Sackett *et al.*, 1985). A positive likelihood ratio of <2 was considered to be inoperative, whereas 2–5 indicated fair and 5–10 good agreement. Similarly, a negative likelihood ratio of >0.5 was considered to be inoperative, whereas 0.2–0.5 indicated fair and 0.1–0.2 good agreement.

Results

Forward stepwise logistic regression analysis was performed to compare the predictive values of various semen parameters for total fertilization failure. The semen parameters evaluated were sperm concentration, motility, progressive motility, concentration of progressive motile spermatozoa, morphology evaluation using strict criteria and acridine orange score. When the likelihood criterion was used, acridine orange score and morphology evaluation using strict criteria (MEUSC) were found to be the only two significant

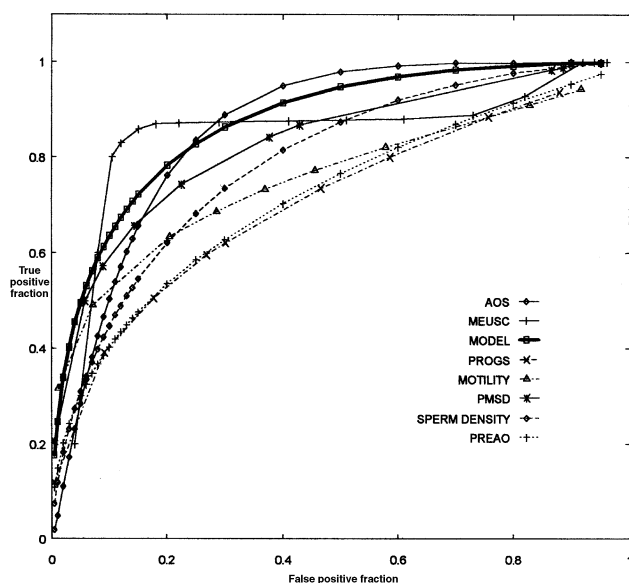


Figure 1. Receiver operating characteristic (ROC) curves of semen parameters and the logistic regression model. AOS, acridine orange score; MEUSC, morphology evaluation using strict criteria; MODEL, logistic regression model; PROGS, progressive motility; PMSD, progressive motile sperm density; PREAO, pre-incubation acridine orange staining.

parameters to be included in the model ($-2 \log \text{likelihood} = 46.801$, $\chi^2 = 23.946$ with 2 df, $P < 0.0001$). The model is formulated in Equation 2:

Equation 2: Formulation of the logistic regression model

$$\text{Probability of fertilization} =$$

$$1$$

$$1 + \exp[-(0.5238 + 0.3388 \times \text{MEUSC} - 0.0808 \times \text{AOS})]$$

ROC curves of various semen parameters and the model were constructed and areas under the curves were calculated for the assessment, and comparison was made of the effectiveness of each parameter to predict fertilization (Figure 1). The area under the ROC curve of the logistic regression model was the closest to 1 and therefore appeared to be the best predictor of fertilization. When maximum likelihood estimation was used to compare the area under the ROC curves, the only significant difference appeared to be between that of the model and the progressive motility. The results of ROC analysis for each parameter and model are summarized in Table I.

The logistic regression model had the highest OR to predict total fertilization failure (47.0), followed by morphology evaluation using strict criteria (40.9), acridine orange score (28.0) and progressive motile sperm concentration (20.6). Pre-incubation acridine orange staining had a lower OR (16.4).

Kappa values and likelihood ratios were calculated for the optimum cut-off points of various semen parameters and the model, in order to assess agreement with fertilization *in vitro* (Table II). The model with a cut-off point of 0.57 appeared to have the best agreement with fertilization *in vitro* beyond chance ($\kappa = 0.70498$), morphology evaluation using strict criteria the second ($\kappa = 0.65263$), followed by progressively

Table I. Results of ROC analysis of various semen parameters and the logistic regression model to predict fertilization *in vitro*

	Area under the curve	SE (95% CI) ^a
Acridine orange score	0.807	0.072 (0.690–0.894)
Pre-incubation acridine orange staining	0.767	0.062 (0.646–0.862)
Motility	0.725	0.068 (0.601–0.828)
MEUSC	0.834	0.051 (0.722–0.914)
Progressive motility	0.695 ^b	0.071 (0.569–0.802)
Progressively motile sperm concentration	0.829	0.052 (0.716–0.910)
Sperm concentration	0.756	0.064 (0.634–0.853)
Model	0.867 ^b	0.045 (0.761–0.938)

^aStandard error (95% confidence intervals).

^b $P < 0.05$.

MEUSC = morphology evaluation using strict criteria.

motile sperm concentration ($\kappa = 0.58801$). Pre-incubation acridine orange staining, motility and AOS had lower κ values (0.51852, 0.45679 and 0.43590 respectively). However, at ROC curve analysis, AOS appeared to be superior to pre-incubation acridine orange staining and motility to predict fertilization *in vitro* (Table I).

To predict total fertilization failure, positive likelihood ratio values again showed the model to have the highest predictive value, followed by progressively motile sperm concentration and morphology evaluation using strict criteria. These parameters were similarly followed by motility, pre-incubation acridine orange staining and acridine orange score. In predicting fertilization, acridine orange score, however, had the best negative likelihood ratio, followed by morphology evaluation using strict criteria, the model and pre-incubation acridine orange staining. The other parameters, including motility, had inoperative negative likelihood ratio values (Table II).

The Pearson's correlation coefficients between various semen parameters and fertilization rates were also given in Table II. In this study, among the various semen parameters evaluated, the best correlation with fertilization rate was observed with morphology evaluation using strict criteria, followed by acridine orange score (negative correlation). The negative value for the correlation coefficient of acridine orange score with fertilization rate means increasing DNA denaturation is associated with decreasing fertilization rate. Acridine orange score had a stronger correlation with fertilization rate than pre-incubation acridine orange staining.

As a general summary of the data and clinical outcome, the number of fertilized oocytes, fertilization rate, number of pregnancies, number of embryo transfers, pregnancy rate per cycle and embryo transfer were listed in Table III.

Discussion

The predictive value of sperm DNA status without heat-provoked denaturation (pre-incubation acridine orange staining) for fertilization *in vitro* is still controversial. It has been previously reported to be a less predictive parameter than morphology evaluation using strict criteria, to predict fertilization failure *in vitro* (Claassens *et al.*, 1992). Another study, aiming to build up a model for the prediction of IVF outcome,

Table II. Kappa values and likelihood ratios for the optimum cut-off points of various semen parameters and the model, as well as correlation between various semen parameters and fertilization rates

	Cut-off point	κ^a	LR+ ^b	LR- ^c	$r (P)^d$
Acridine orange score	11%	0.43590	2.80	0.10	-0.5831 (< 0.001)
Pre-incubation acridine orange staining	53%	0.51852	4.08	0.25	0.5235 (< 0.001)
Motility	58%	0.45679	5.44	0.52	0.4401 (< 0.001)
MEUSC	5%	0.65263	6.31	0.15	0.6015 (< 0.001)
Progressive motility	44%	0.34386	2.78	0.51	0.2248 (0.07)
Progressively motile sperm concentration	15×10^6	0.58801	7.92	0.38	0.4089 (0.001)
Sperm concentration	92×10^6	0.27273	1.90	0.33	0.3774 (0.002)
Model	0.57	0.70498	10.20	0.22	-

^a κ : Cohen's kappa (<0.4: poor agreement; 0.4–0.75: good agreement).

^bLR+: likelihood of fertilization failure when the test result is abnormal (positive likelihood ratio).

^cLR-: likelihood of fertilization failure when the test result is normal (negative likelihood ratio).

^d $r (P)$: Pearson's correlation coefficient between semen parameter and fertilization rate, and probability.

MEUSC = morphology evaluation using strict criteria.

Table III. The outcome of IVF in relation to the cut-off points of acridine orange score (AOS) and morphology evaluation using strict criteria (MEUSC)

Parameter	Number of fertilized oocytes	Fertilization rate (%)	Number of pregnancies	Number of embryo transfers	Pregnancy/cycle (%)	Pregnancy/transfer (%)
AOS < 11	34 ^a	97.2	4	32 ^b	11.4	12.5
AOS \geq 11	18 ^a	56.3	3	18 ^b	9.4	16.7
MEUSC < 5	13 ^c	54.2	2	13 ^a	8.3	15.4
MEUSC \geq 5	39 ^c	90.7	5	37 ^a	11.6	13.5

^a $P < 0.0001$.

^b $P = 0.002$.

^c $P = 0.02$.

reported no significant contribution of acridine orange fluorescence of spermatozoa to its fertilizing potential. In contrast, the in-vitro fertilizing ability of human spermatozoa was found to be related to sperm morphology (WHO criteria, 1992), attributes of sperm movement (linearity and average path velocity) and reactive oxygen species production (Sukcharoen *et al.*, 1996). According to a recent study, however, only spermatozoa stained green by acridine orange fluorescence were able to bind efficiently to the zona pellucida. In addition, the spermatozoa which fertilized oocytes *in vitro* were those whose nuclei exhibited green acridine orange fluorescence (Hoshi *et al.*, 1996). To increase the sensitivity of the test for detection of sperm cells with chromatin abnormalities, combined use of acridine orange staining and heat treatment was proposed (Roux and Dadoune, 1989). This new technique was thought to be promising but detailed data have not been reported (Roux *et al.*, 1993). In our study, when acridine orange score was normal (<11%), likelihood of fertilization was found to be superior to that of all other parameters including pre-incubation acridine orange staining (Table II). This indicates that the more stable the sperm chromatin, the higher is the likelihood of fertilization. In contrast, acridine orange score does not appear to be a good predictor of total fertilization failure.

This fact can also be seen from the results shown in Table III. Fertilization rate was highest for the group with acridine orange score <11%, whereas the number of fertilized oocytes was lowest for the group with morphology evaluation using strict criteria <5%, which indicated a higher predictivity for fertilization failure. Clinical pregnancy rate while this study

has been undertaken was abnormally low for our centre. The reason for this was later found to be due to a non-sterile batch of embryo transfer catheters.

One of the drawbacks of acridine orange staining is the subjective interpretation of the fluorescent colour shades. The distinction between the colours may even decrease when the examination per field exceeds 40 s. Another consideration may be the possible fading of acridine orange fluorescence on smears after heat treatment. With a flow cytometer (Evenson *et al.*, 1980) or a fluorescence activated cell sorter (Claassens *et al.*, 1992), more objective interpretation can be made, albeit at higher cost and greater specialization.

In our study, morphology evaluation using strict criteria appeared to be the best individual parameter with respect to Cohen's kappa (0.65263) for the prediction of in-vitro fertilization, followed by progressively motile sperm concentration. There is a paucity of data on the combined effect of individual semen parameters on fertilization *in vitro*. Enginsu *et al.* (1992) combined the results of morphology evaluation using strict criteria and progressively motile sperm concentration to predict IVF outcome, and noted that fertilization was uniformly absent when morphology evaluation using strict criteria gave <5% normal morphology and progressively motile sperm concentration was < 3×10^6 /ml; whereas the highest fertilization rate was achieved when morphology evaluation was carried out using strict criteria >5% normal morphology and progressively motile sperm concentration > 3×10^6 /ml. In our study, fertilization *in vitro* was predicted most reliably by the model composed of acridine orange score and morphology evaluation using strict criteria.

In conclusion, acridine orange score is a simple, inexpensive method for sperm DNA assessment. It has better predictive capacity than the previously reported pre-incubation acridine orange staining for fertilization *in vitro*. When combined with morphology evaluation using strict criteria in a logistic regression model, a higher predictive value is attained. This new model may be used as a patient selection criterion for standard IVF and assisted fertilization.

References

- American Fertility Society (1991) Guidelines for in vitro fertilization, gamete intrafallopian transfer and related procedures. *Fertil. Steril.*, **56**, 194–197.
- Claassens, O.E., Menkveld, R., Franken, D.R. *et al.* (1992) The acridine orange test: determining the relationship between sperm morphology and fertilization *in vitro*. *Hum. Reprod.*, **7**, 242–247.
- Enginsu, M.E., Dumoulin, J.C.M., Pieters, M.H.E.C. *et al.* (1991) Evaluation of human sperm morphology using strict criteria after Diff-Quik staining: correlation of morphology with fertilization *in vitro*. *Hum. Reprod.*, **6**, 854–858.
- Enginsu, M.E., Pieters, M.H.E.C., Dumoulin, J.C.M. *et al.* (1992) Male factor as determinant of in vitro fertilization outcome. *Hum. Reprod.*, **7**, 1136–1140.
- Evenson, D.P., Darzynkiewicz, Z. and Melamed, M.R. (1980) Relation of mammalian sperm chromatin heterogeneity to fertility. *Science*, **210**, 1131–1133.
- Golan, R., Cooper, T.G., Oschry, Y. *et al.* (1996) Changes in chromatin condensation of human spermatozoa during epididymal transit as determined by flow cytometry. *Hum. Reprod.*, **11**, 1457–1462.
- Golan, R., Shochat, L., Weissenberg, R. *et al.* (1997) Evaluation of chromatin condensation in human spermatozoa: a flow cytometric assay using acridine orange staining. *Mol. Hum. Reprod.*, **3**, 47–54.
- Griner, P.F., Mayewski, R.J., Mushlin, A.I. and Greenland, P. (1981) Selection and interpretation of diagnostic tests and procedures: principles and applications. *Ann. Int. Med.*, **94**, 553–592.
- Hanley, J.A. and McNeil, B.J. (1983) A method for comparing the areas under receiver operator characteristic curves derived from the same cases. *Radiology*, **148**, 839–843.
- Hoshi, K., Katayose, H., Yanagida, K. *et al.* (1996) The relationship between acridine orange fluorescence of sperm nuclei and the fertilizing ability of human sperm. *Fertil. Steril.*, **66**, 634–639.
- Ibrahim, M.E. and Pedersen, H. (1988) Acridine orange fluorescence as male fertility test. *Arch. Androl.*, **20**, 125–129.
- Kruger, T.F., Acosta, A.A., Simmons, K.F. *et al.* (1988) Predictive value of abnormal sperm morphology in in vitro fertilization. *Fertil. Steril.*, **49**, 112–117.
- Liu, D.Y., Du Plessis, Y.P., Nayudu, P.L. *et al.* (1988) The use of in vitro fertilization to evaluate putative tests of human sperm function. *Fertil. Steril.*, **49**, 272–277.
- Mahadevan, M. and Baker, G. (1984) Assessment and preparation of semen for in vitro fertilization. In Wood, C. and Trounson, A. (eds), *Clinical In Vitro Fertilization*. Springer-Verlag, Berlin, p. 83.
- Menkveld, R., Stander, F.S.H., Kotze, T.J.W. *et al.* (1990) The evaluation of morphological characteristics of human spermatozoa according to stricter criteria. *Hum. Reprod.*, **5**, 586–592.
- Metz, C.E. (1978) Basic principles of ROC analysis. *Semin. Nucl. Med.*, **8**, 283–298.
- Ombelet, W., Fourie, Fle.R., Vandeput, H. *et al.* (1994) Teratozoospermia and in-vitro fertilization: a randomized prospective study. *Hum. Reprod.*, **9**, 1479–1484.
- Peluso, J.J., Luciano, A.A. and Nulsen, J.C. (1992) The relationship between alterations in spermatozoal deoxyribonucleic acid, heparin binding sites, and semen quality. *Fertil. Steril.*, **57**, 665–670.
- Roux, C. and Dadoune, J.P. (1989) Use of the acridine orange staining on smears of human spermatozoa after heat-treatment: evaluation of the chromatin condensation. *Andrologia*, **21**, 275–281.
- Roux, C., Joanne, C., Clavequin, M.C. *et al.* (1993) Use of acridine orange staining on smears of human spermatozoa after heat-treatment: results in IVF. Presented in: *Andrology in the Nineties. International Symposium on Male Infertility and Assisted Reproduction*, April 21–24, 1993, Ombelet, W., Vandeput, H., Bosmans, E. *et al.* (eds). Abstract book, Wyeth, Genk, Belgium.

Sackett, D.L., Haynes, R.B. and Tugwell, P. (eds) (1985) *Clinical Epidemiology*. Little Brown, Boston.

Sukcharoen, N., Keith, J., Irvine, D.S. and Aitken, R.J. (1996) Prediction of the in-vitro fertilization potential of human spermatozoa using sperm function tests: the effect of the delay between testing and IVF. *Hum. Reprod.*, **11**, 1030–1034.

Tejada, R.I., Mitchell, J.C., Norman, A. *et al.* (1984) A test for the practical evaluation of male fertility by acridine orange (AO) fluorescence. *Fertil. Steril.*, **42**, 87–91.

World Health Organization (1992) *WHO Laboratory Manual for the Examination of Human Semen and Sperm Cervical Mucus Interactions*, 3rd edn. Cambridge University Press, Cambridge.

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