

Sperm-Preparation Techniques for Men with Normal and Abnormal Semen Analysis

A Comparison

Cemal Tamer Erel, M.D., Levent Mehmet Senturk, M.D., Tulay Irez, Ph.D., Leyla Ercan, M.D., Koray Elter, M.D., Umur Colgar, M.D., and Erdogan Ertungealp, M.D.

OBJECTIVE: To compare two commonly used sperm-preparation techniques, density gradient centrifugation and swim-up procedures, with respect to their effects on acrosome reaction (AR), hypoosmotic swelling (HOS) and nuclear maturity in men with abnormal and normal semen analyses.

STUDY DESIGN: In accordance with World Health Organization criteria, 23 men with abnormal (group I) and 20 men with normal (group II) semen analyses

were included in a prospective, controlled study. Each semen specimen was divided into aliquots in order to assess AR, HOS and nuclear maturity, determined with acridine orange staining, in both raw and processed semen samples using the density gradient centrifugation and swim-up techniques.

RESULTS: Initial semen samples in group I revealed di-

minished AR, HOS and nuclear maturity rates in comparison to those in group II. In group I, density gradient centrifugation improved AR, HOS and nuclear maturity rates more than did swim-up. However, in group II it improved only the AR; HOS rates were better than with swim-up. There was a significant positive correlation between sperm concentration and HOS rate in raw semen samples from group I. In the same group, motility and morphology correlated with

the nuclear maturity rate but not with AR and HOS rates. Semen samples with better motility (>20%) or morphology (>25%) showed better nuclear maturity rates (>50%) in men with abnormal semen analyses. Motility had a sensitivity of 77% and specificity of 90% in predicting nuclear maturity. Morphology had similar sensitivity but lower specificity (70%).

[D]ensity gradient centrifugation is superior to the swim-up procedure in selecting spermatozoa with good AR, HOS and nuclear maturity rates in men with abnormal semen analyses.

From the Division of Reproductive Endocrinology, Department of Obstetrics and Gynecology, Cerrahpasa School of Medicine, Istanbul University, Istanbul, Turkey.

Drs. Erel and Senturk are Assistant Professors.

Drs. Irez, Colgar and Ertungealp are Professors.

Drs. Ercan and Elter are Fellows.

Presented at the 16th World Congress on Fertility and Sterility and 54th Annual Meeting of the American Society for Reproductive Medicine, San Francisco, October 4–9, 1998.

Address reprint requests to: Cemal Tamer Erel, M.D., Kasaneler Sk. Nigarhanim Apartments, No. 28/11 Erenkoy, 81070, Istanbul, Turkey (tamererel@superonline.com).

Financial Disclosure: The authors have no connection to any companies or products mentioned in this article.

CONCLUSION: Density gradient centrifugation is superior to the swim-up technique in improving AR, HOS and nuclear maturity rates in men with abnormal semen analyses. However, when only nuclear maturity rate is taken into account, the swim-up technique seems to be sufficient for selecting spermatozoa in men with normal semen analyses. The nuclear maturity rate also correlates with sperm morphology and motility. (J Reprod Med 2000;45:917–922)

Keywords: infertility, male; sperm; semen.

Introduction

Density gradient centrifugation and the swim-up procedure are the two most commonly used sperm-preparation techniques for selecting the best spermatozoa.

We suggest performing...density gradient centrifugation in men with abnormal semen analyses.

Which sperm-preparation procedure yields better pregnancy rates is still being debated.^{1,2} It was recently reported that these two techniques equally improved sperm motility, morphology, nuclear maturity rates and the fertilizing ability of human spermatozoa.³

Which sperm parameter or sperm function test has the highest predictive value, enough to be the gold standard in determining the fertilizing ability of human spermatozoa, has not been agreed on. Although sperm morphology, according to strict criteria, is well known as the most significant determining factor in predicting *in vitro* fertilization outcome, sperm motility is claimed to be as important as morphology in predicting fertilization and pregnancy.^{4,5} In addition, acrosome reaction (AR), hypoosmotic swelling (HOS) and nuclear maturity, determined with acridine orange (AO) staining, can give valuable information on the fertilizing potential of human spermatozoa.^{6–10} Some authors have studied the correlation between semen parameters and those sperm tests.^{3,11–13}

We evaluated the effects of the two commonly used methods of sperm preparation, density gradient centrifugation and the swim-up procedure, on AR, HOS and nuclear maturity in men with abnormal and normal semen analyses. We also investigated if there was any correlation between AR,

HOS, nuclear maturity rates and conventional semen parameters.

Materials and Methods

Subjects

After informed consent was obtained, men from 43 couples were recruited for the study. Semen samples were obtained by masturbation into a sterile plastic container after abstinence of two to four days and were allowed to liquefy at room temperature. Each semen sample was analyzed using World Health Organization (WHO) guidelines.¹⁴ Sperm concentrations were determined using a Makler counting chamber, and sperm motility was assessed at room temperature. Sperm morphology was determined on Papanicolaou-stained smears from the raw semen samples. According to the WHO criteria, semen samples from 23 men were abnormal (group I), and 20 men were normal (group II). These 20 men had children and thus had proven fertility. In addition to semen analysis, the initial, raw semen sample and two sperm fractions prepared by Percoll (Sigma Chemical Co., St. Louis, Missouri) density gradient centrifugation and swim-up were divided into aliquots in order to assess AR with triple staining after calcium induction, HOS and nuclear maturity with AO staining. All sperm-preparation techniques and sperm tests were performed by a single person (T.I.).

Swim-up Procedure

In the swim-up procedure, 0.5 mL of semen was washed twice with 2.0 mL of sterile minimum essential medium (MEM) (Gibco BRL, Grand Island, New York) and then centrifuged at 300 g for 10 minutes. After the supernatant was discarded, the pellet was resuspended in 1.0 mL of MEM and incubated at 37°C for 45 minutes at an angle of 45° in a 5% CO₂ incubator. The top 0.5 mL of each tube was used for assessment of AR, HOS and nuclear maturity rates.

Density Gradient Centrifugation Procedure

Isotonic Percoll was prepared by combining one part 10× MEM with nine parts 100% Percoll. The desired final 90% and 60% Percoll concentrations were achieved by mixing MEM solution with isotonic Percoll solution at ratios of 1:9 and 4:6. Discontinuous Percoll density gradient was prepared by overlaying 1.0 mL of the 90% Percoll solution with 1.0 mL of the 60% Percoll solution. A 0.5-mL raw semen sample following liquefaction was lay-

ered over the uppermost part of the Percoll layers. After centrifugation at 300 g for 30 minutes, the pellet was removed, washed with 2.0 mL of MEM solution and centrifuged at 300 g for 10 minutes. The sperm pellet was then resuspended with 1.0 mL of MEM solution and used for assessment of AR, HOS and nuclear maturity rates.

Acrosome Reaction

The percentage of sperm undergoing AR was estimated with the use of the triple-staining technique.¹⁵ This technique allows determination of the viability of human spermatozoa. Viability is identified using the vital stain trypan blue. Acrosomal and postacrosomal regions are differentiated using Bismarck brown and Rose Bengal. Bismarck brown stains the postacrosomal region light brown, and Rose Bengal stains the acrosomal region of acrosome-intact spermatozoa pink.

Aliquots of raw and processed semen samples (after swim-up and Percoll density gradient centrifugation) were exposed to 10 mM calcium ionophore (Sigma) for three hours at 37°C. After induction of AR with a calcium ionophore, the sperm suspension was incubated with a 2% trypan blue solution with a ratio of 1:1 at 37°C for 15 minutes. After washing, the sperm were fixed for 30 minutes in 2.5% glutaraldehyde (0.1 M cacodylate buffer, pH 7.4) at 4°C. The glutaraldehyde was removed by centrifuging the sperm and resuspending the pellet in deionized water.

Fixed sperm were air dried on glass slides. These sperm were stained with 0.8% Bismarck brown in deionized water at 37°C for 15 minutes and then were washed in deionized water. Slides were stained with 0.8% Rose Bengal solution in 0.1 M Tris (tris[hydroxymethyl]aminomethane) buffer at room temperature for 40 minutes. After dehydration in 90% ethanol, the slides were examined at 1,000× magnification under a light microscope. At least 200 sperm were counted at random on each slide, and light brown spermatozoa were considered to have undergone AR. The results were reported as a percentage of acrosome-reacted spermatozoa.

Hypoosmotic Swelling Test

The hypoosmotic solution was prepared by mixing 7.35 g of sodium citrate and 13.51 g of fructose in 1.0 L distilled water.¹¹ An aliquot of 0.1 mL of semen sample was added to 1.0 mL of this hypoosmotic solution. After incubation for 30 minutes at 37°C in

5% CO₂, the swelling of the sperm tail was evaluated under a phase-contrast microscope. At least 200 sperm were evaluated. The results were reported as the percentage of spermatozoa that showed typical morphologic changes as described by Jeyendran et al.¹¹

AO

Nuclear maturity was assessed with the AO fluorescence method described by Tejada et al.¹⁶ After air drying, the sperm smear was fixed with Carnoy's solution (one part glacial acetic acid and three parts 100% methanol) overnight. The slides were then washed in distilled water and allowed to dry before staining. The sperm smear was stained with the AO staining solution (10 mL of 1.0% AO in distilled water added to a mixture of 40 mL of 0.1 M citric acid and 2.5 mL of 0.3 M Na₂HPO₄·7H₂O, pH 2.5) for five minutes. Then the slide was gently rinsed and mounted with distilled water. The percentage of spermatozoa with normal DNA was determined by counting at least 200 spermatozoa under a fluorescence microscope (AH-3, Olympus, Tokyo, Japan) at 400× (oil lens) magnification with excitation at 450–490 nm. Spermatozoa with normal double-stranded DNA gave green and those with denatured or single-stranded DNA gave red or yellow fluorescence.

Statistical Analysis

Distributions of the data were assessed with the Kolmogorov-Smirnov test. The paired *t* test was used for intragroup comparisons. Student *t* and Fisher's exact tests were used to assess the statistical significance of intergroup differences. Correlations between the parameters were determined by using Pearson's correlation test. For the correlations observed between sperm parameters and the results of function tests, sensitivity and specificity of the sperm parameter for the function test were calculated as follows: the number of samples with low rates of both the parameter and function test was divided by the total number of samples with poor sperm function, and the number of samples with high rates of both the parameter and function test was divided by the total number of samples with good sperm function, respectively. A *P* value < .05 was considered significant.

Results

The mean ages of groups I and II were 32.3 ± 3.1 (range, 25–41) and 31.0 ± 3.0 (range, 27–36), respec-

Table I Results of Semen Analyses in Both Groups

Parameter	Group		P
	I (n=23)	II (n=20)	
Sperm concentration ($\times 10^6/\text{mL}$)	10.8 \pm 5.9	40.6 \pm 14.0	<.001
Motility (%)	20.9 \pm 10.9	53.5 \pm 11.3	<.001
Normal morphology (%)	26.8 \pm 12.4	59.7 \pm 11.0	<.001

All values are mean \pm SD.

tively. Average sperm concentration, percentages of motility and normal morphology were higher in group II than in group I (Table I).

AR, HOS and the nuclear maturity rates in the initial and processed semen (after swim-up and Percoll density gradient centrifugation) in both groups are shown in Table II. The men in group II had better AR, HOS and nuclear maturity rates than did those in group I in the raw semen samples ($P = .001$, $P < .01$, $P < .001$, respectively). Following swim-up and the Percoll density gradient centrifugation procedure, the AR, HOS and nuclear maturity rates significantly improved in comparison to the initial semen samples in both groups ($P < .001$). In addition, Percoll density gradient centrifugation gave better results for AR, HOS and nuclear maturity rates than did the swim-up procedure in men with abnormal semen analyses ($P < .001$, $P < .05$ and $P = .001$, respectively). However, while Percoll density gradient centrifugation yielded better AR and HOS rates ($P < .01$ and $P < .001$, respectively), the two sperm-preparation techniques were comparable in terms of the nuclear maturity rate in the group of men with normal semen analyses ($P > .05$).

In the group of men with abnormal semen analyses, a significant positive correlation was observed between the sperm concentration and HOS rate in

the initial semen samples ($r = .6$, $P < .01$). Sperm motility and morphology correlated with the nuclear maturity rate ($r = .6$, $P < .01$ and $r = .8$, $P < .001$, respectively). Initial semen samples with $> 20\%$ motility or with $> 25\%$ normal morphology showed higher nuclear maturity rates ($> 50\%$) in the group of men with abnormal semen analyses (group I) ($P < .01$ and $P < .05$, respectively) (Table III). Motility had a sensitivity of 77% and a specificity of 90% to predict nuclear maturity. Morphology had similar sensitivity but lower specificity (70%).

Discussion

A variety of sperm-preparation procedures are in use for selecting the best spermatozoa to increase fertilization and pregnancy rates.¹⁻³ The improvement in sperm after these procedures has been shown mainly in morphology and motility.^{17,18} In this study, we found a significant improvement in AR, HOS and the nuclear maturity rates following swim-up and Percoll density gradient centrifugation. Among studies comparing the two procedures, some could not show a difference between these two semen-preparation procedures and reported the superiority of the swim-up procedure to Percoll density gradient centrifugation in selecting spermatozoa with good AR, HOS and nuclear maturity rates.^{3,19-21} However, most findings are consistent with ours that Percoll density gradient centrifugation yielded better results for AR, HOS and nuclear maturity than did the swim-up procedure in men with abnormal semen analyses.²²⁻²⁶

The nuclear maturity rate is higher in men who proved their fertility than in men having infertility problems.²⁷ We showed that the two sperm-preparation techniques were comparable in selecting spermatozoa with mature nuclei in men having normal semen analyses but not in men having infertility problems (Table II). This finding is support-

Table II Results of Sperm Function Tests of Initial Semen and Samples After the Swim-up and Percoll Density Gradient Procedures in Groups I and II

Parameter	Group I			Group II		
	Initial semen	Swim-up	Percoll	Initial semen	Swim-up	Percoll
AR (%)	5.1 \pm 1.0	14.1 \pm 1.7 a	17.7 \pm 2.0 a	9.8 \pm 0.8	22.9 \pm 1.1 b	24.9 \pm 1.1 b
HOS (%)	37.3 \pm 4.6	57.3 \pm 5.5 c	63.5 \pm 5.6 c	56.9 \pm 5.3	67.1 \pm 4.7 d	72.1 \pm 4.4 d
Normal morphology (%)	45.9 \pm 5.0	52.4 \pm 5.3 e	56.3 \pm 5.6 e	87.2 \pm 1.4	93.0 \pm 1.0	94.7 \pm 1.0

All values are mean \pm SE.

Pairs of letters a, b, c, d and e denote results that are significantly different ($P \leq .001$ for a, d and e; $P < .01$ for b; $P < .05$ for c). Note that there is only one pair for each letter and that the elements of the pairs are significantly different from each other. (For example, "a" denotes that Percoll density gradient centrifugation improved the AR rate in group I better than did swim-up [$P < .001$].) Also, note that only Percoll and swim-up are compared in this table. As mentioned in the text and as can be seen in this table, both procedures improved sperm function significantly over that in the initial semen.

Table III Nuclear Maturity with Motility and Morphology

Nuclear maturity	Motility		Morphology	
	≤20	>20	≤25	>25
≤50	10	3	10	3
>50	1	9	3	7

ed by Angelopoulos et al, who recently claimed that the nuclear maturity of sperm from normozoospermic men was improved equally after both sperm preparation procedures.³ This might be explained by the following: (1) reactive oxygen species (ROS), which damage sperm DNA, have not been detected in semen from normal men; on the contrary, it has been detected in semen from 40% of men having infertility problems²⁸⁻³⁰; (2) oligozoospermic semen samples showed a higher rate of ROS production than normozoospermic semen samples³¹; and (3) spermatozoa selected by the Percoll gradient procedure produce less ROS than do those selected by swim-up.²³

The success of Percoll density gradient centrifugation in selecting good spermatozoa is not completely understood. Several mechanisms have been suggested. One is that the procedure separates sperm on the basis of density, selecting those with good nuclear maturity, which are more dense.²⁶ Mature sperm with good morphology enter the Percoll gradient in the head-down position because of the influence of gravity. This is true as long as their specific gravity is higher than that of the surrounding medium. This technique mainly eliminates the head abnormalities rather than the tail abnormalities.^{22,26} Therefore, Percoll allows recovery of a sperm fraction with significantly reduced chromatin defects.^{3,26} However, nuclear maturity correlates not only with sperm morphology but also with sperm motility.³ Another mechanism is the presence of defective spermatozoa and leukocytes in the semen, which are potential sources of ROS and cause not only a disturbance in membrane function related to lipid peroxidation but also damage to DNA.^{28,32} They are removed by Percoll density gradient centrifugation.²³

Since spermatozoa with normal DNA should have normal morphology and motility,³ we specifically investigated whether there was a correlation between conventional semen parameters and the nuclear maturity rate in men with abnormal semen analyses. Tejada et al reported that infertile men

had spermatozoa with nuclear maturity rates <50%.¹⁶ It was confirmed by another study, indicating the successful fertilizing ability of human sperm exhibiting >50% green fluorescence (mature nuclei).¹⁰ Therefore, we chose the cutoff value of 50% for the nuclear maturity rate and found that in men with abnormal semen analyses, sperm with >20% motility or with >25% normal morphology showed higher nuclear maturity rates (>50%). Reports on correlation of nuclear maturity with semen parameters have been inconsistent. Most studies could not find any correlation between nuclear maturity and sperm concentration or motility.^{3,10,16,33} We observed that motility has a sensitivity of 77% and a specificity of 90% to predict nuclear maturity. Morphology was found to have similar sensitivity but lower specificity (70%). Some studies also have shown a correlation with sperm morphology,^{3,16,33} while others have not.¹⁰ Angelopoulos et al reported that 90% of sperm with normal morphology displayed mature nuclei.³ Dadoune et al reported a similar rate (80%) in the population of morphologically normal spermatozoa versus 40% in the population of abnormal forms.³⁴ The relationship between morphology and nuclear maturity might be explained by the fact that some morphologic abnormalities of sperm cells are due to the loss of supercoiling of DNA, resulting in easier access of AO to DNA.¹⁶

In conclusion, Percoll density gradient centrifugation is superior to the swim-up procedure in selecting spermatozoa with good AR, HOS and nuclear maturity rates in men with abnormal semen analyses. However, when only nuclear maturity is taken into account, the swim-up technique seems to be sufficient for selecting spermatozoa in men with normal semen analyses. Therefore, we suggest performing Percoll density gradient centrifugation in men with abnormal semen analyses.

References

- Carrell DT, Kuneck PH, Peterson M, et al: A randomized, prospective analysis of five sperm preparation techniques before intrauterine insemination of husband sperm. *Fertil Steril* 1998;69:122-126
- Dodson WC, Moessner J, Miller J, et al: A randomized comparison of the methods of sperm preparation for intrauterine insemination. *Fertil Steril* 1998;70:574-575
- Angelopoulos T, Moshel YA, Lu L, et al: Simultaneous assessment of sperm chromatin condensation and morphology before and after separation procedures: Effect on the clinical outcome after in vitro fertilization. *Fertil Steril* 1998;69:740-747

4. Donnelly ET, Lewis SE, McNally JA, et al: In vitro fertilization and pregnancy rates: The influence of sperm motility and morphology on IVF outcome. *Fertil Steril* 1998;70:305-314
5. Kruger TF, Menkveld R, Stander FS, et al: Sperm morphologic features as a prognostic factor in IVF. *Fertil Steril* 1986;46:1118-1123
6. Abu-Musa A, Takahashi K, Kitao M: Correlation between postswim-up hypoosmotic swelling test and in vitro fertilization results. *Int J Fertil* 1993;38:113-116
7. Wang C, Lee GS, Leung A, et al: Human sperm hyperactivation and acrosome reaction and their relationships to human in vitro fertilization. *Fertil Steril* 1993;59:1221-1227
8. Pampiglione JS, Tan SL, Campbell S: The use of the stimulated acrosome reaction test as a test of fertilizing ability in human spermatozoa. *Fertil Steril* 1993;59:1280-1284
9. Van der Ven HH, Jeyendran RS, Al-Hasani S, et al: Correlation between human sperm swelling in hypoosmotic medium (hypoosmotic swelling test) and in vitro fertilization. *J Androl* 1986;7:190-196
10. Hoshi K, Katayose H, Yanagida K, et al: The relationship between acridine orange fluorescence of sperm nuclei and the fertilizing ability of human sperm. *Fertil Steril* 1996;66:634-639
11. Jeyendran RS, Van der Ven HH, Perez-Pelaez M, et al: Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J Reprod Fertil* 1984;70:219-228
12. Singer R, Fish B, Levinsky H, et al: Separation of human semen on Percoll gradients: Effect on percentage of motile and morphologically normal sperm and proportion of acrosome reacted sperm. *Int J Fertil Menopausal Stud* 1995;40:161-166
13. Calvo L, Dennison-Lagos L, Banks SM, et al: Characterization and frequency distribution of sperm acrosome reaction among normal and infertile men. *Hum Reprod* 1994;9:1875-1879
14. World Health Organization: WHO Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction. Cambridge, Cambridge University Press, 1992
15. Talbot P, Chacon RS: A triple-stain technique for evaluating normal acrosome reactions of human sperm. *J Exp Zool* 1981;215:201-208
16. Tejada RI, Mitchell JC, Norman A, et al: A test for the practical evaluation of male fertility by acridine orange fluorescence. *Fertil Steril* 1984;42:87-91
17. Prakash P, Leykin L, Chen Z, et al: Preparation by differential gradient centrifugation is better than swim-up in selecting sperm with normal morphology (strict criteria). *Fertil Steril* 1998;69:722-726
18. Moohan JM, Lindsay KS: Spermatozoa selected by a discontinuous Percoll density gradient exhibit better motion characteristics, more hyperactivation, and longer survival than direct swim-up. *Fertil Steril* 1995;64:160-165
19. Smith S, Hosid S, Scott L: Use of postseparation sperm parameters to determine the method of choice for sperm preparation for assisted reproductive technology. *Fertil Steril* 1995;63:591-597
20. Check JH, Katsoff D, Kozak J, et al: Effect of swim-up, Percoll and Sephadex sperm separation methods on the hypoosmotic swelling test. *Hum Reprod* 1992;7:109-111
21. Morales P, Vantman D, Barros C, et al: Human spermatozoa selected by Percoll gradient or swim-up are equally capable of binding to the human zona pellucida and undergoing the acrosome reaction. *Hum Reprod* 1991;6:401-404
22. Colleu D, Lescoat D, Gouranton J: Nuclear maturity of human spermatozoa selected by swim-up or by Percoll gradient centrifugation procedures. *Fertil Steril* 1996;65:160-164
23. Griveau JF, Le Lannou D: Effects of antioxidants on human sperm preparation techniques. *Int J Androl* 1994;17:225-231
24. Sanchez R, Villagran E, Risopatron J, et al: Evaluation of nuclear maturity in human spermatozoa obtained by sperm preparation methods. *Andrologia* 1994;26:173-176
25. Sharma RK, Seifarth K, Agarwal A: Comparison of single- and two-layer Percoll separation for selection of motile spermatozoa. *Int J Fertil Womens Med* 1997;42:412-417
26. Le Lannou D, Blanchard Y: Nuclear maturity and morphology of human spermatozoa selected by Percoll density gradient centrifugation or swim-up procedure. *J Reprod Fertil* 1988;84:551-556
27. Johannison E, Eliasson R: Ethidium bromide uptake by spermatozoa from fertile and infertile men. *Int J Androl (suppl)* 1981;3:72-73
28. Steenken S: Purine bases, nucleosides and nucleotides: Aqueous solution redox chemistry and transformation reactions of their radical cations and e- and OH adducts. *Chem Rev* 1989;89:503-520
29. McKinney KA, Lewis SE, Thompson W: Reactive oxygen species generation in human sperm: Luminol and lucigenin chemiluminescence probes. *Arch Androl* 1996;36:119-125
30. Zini A, de Lamirande E, Gagnon C: Reactive oxygen species in semen of infertile patients: Levels of superoxide dismutase- and catalase-like activities in seminal plasma and spermatozoa. *Int J Androl* 1993;16:183-188
31. Zalata A, Hafez T, Comhaire F: Evaluation of the role of reactive oxygen species in male infertility. *Hum Reprod* 1995;10:1444-1451
32. Aitken RJ: Assessment of sperm function for IVF. *Hum Reprod* 1988;3:89-95
33. Liu DY, Baker HW: Sperm nuclear chromatin normality: Relationship with sperm morphology, sperm-zona pellucida binding, and fertilization rates in vitro. *Fertil Steril* 1992;58:1178-1184
34. Dadoune JP, Mayaux MJ, Guihard-Moscato ML: Correlation between defects in chromatin condensation of human spermatozoa stained by aniline blue and semen characteristics. *Andrologia* 1988;20:211-217