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Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques

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The predictive value of sperm chromatin integrity for pregnancy outcome following in-vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) was studied in 24 men attending a university-based assisted reproductive techniques laboratory using the flow cytometric sperm chromatin structure assay (SCSA). The SCSA is a measure of the susceptibility of sperm DNA to low pH-induced denaturation *in situ*. The mean percentage of spermatozoa in the neat sample demonstrating DNA denaturation was significantly lower in the seven men that initiated a pregnancy (15.4 ± 4.6, $P = 0.01$) than in the 14 men who did not initiate a pregnancy (31.1 ± 3.2). No pregnancies resulted if ≥27% of the spermatozoa in the neat semen sample showed DNA denaturation. These data demonstrate that SCSA parameters are independent of conventional semen parameters. Furthermore, the SCSA may allow physicians to identify male patients for whom IVF and ICSI will be unlikely to result in pregnancy initiation.

Key words: chromatin structure/human fertility/in-vitro fertilization/intracytoplasmic sperm injection/SCSA

Introduction

Identification of sperm parameters that predict the outcome of assisted reproductive techniques would allow physicians to counsel patients on their chance of pregnancy following IVF and intracytoplasmic sperm injection (ICSI). The value of this information has been the impetus for numerous studies designed to identify a single parameter or group of parameters that have significant predictive value for assisted reproductive techniques' (ART) outcomes. Sperm strict morphology criteria have been used effectively to develop thresholds that are predictive of fertilization and pregnancy rates following IVF (Kruger *et al*., 1986; Vawda *et al*., 1996). In addition, better embryo quality has been associated with normal sperm morphology (Van der Zwalmen *et al*., 1991; Parinaud *et al*., 1993; Janny and Ménézo, 1994). Many investigators (Claassens *et al*., 1992; Sukcharoen *et al*., 1995; Hammadeh *et al*., 1997; Parinaud *et al*., 1997) have shown the importance of optimizing conventional sperm parameters, including morphology for IVF success. However, the importance of sperm morphology for ART's success has become increasingly ambiguous with the advent of ICSI which overrides many inherent boundaries for natural selection (Hoshi *et al*., 1996).

The ability of ICSI to bypass oocyte-associated boundaries has facilitated the successful treatment of males with severe oligozoospermia, teratozoospermia, azoospermia and immotile sperm cells (Engel *et al*., 1996). Some studies indicate that fertilization, embryo cleavage and pregnancy following ICSI are independent of conventional sperm parameters (Hammadeh *et al*., 1996; Oehninger, 1996). However, other studies have reported that impaired sperm quality, characteristic of ICSI patients, leads to a lower percentage of embryos that form blastocysts (Shoukir *et al*., 1998), poor blastocyst quality (Janny and Ménézo, 1994) and high abortion rates (Sanchez *et al*., 1996). Janny and Ménézo (1994) showed the strong paternal influence in preimplantation embryo development, reporting that both the overall number of blastocysts obtained and the number of patients having at least one blastocyst were reduced in patients with severely impaired sperm quality.

The influence(s) of sub-optimal sperm chromatin integrity on post-embryonic development is the subject of intense investigation. Spermatozoa from infertile men have a higher frequency of chromosomal abnormalities (Moosani *et al*., 1995), lower resistance to sodium dodecyl sulphate (SDS)-induced decondensation (Colleu *et al*., 1988), poor DNA packing quality (Hofmann and Hilscher, 1991; Bianchi *et al*., 1996; Filatov *et al*., 1999), increased DNA strand breaks (Lopes *et al*., 1998; Irvine *et al*., 2000) and susceptibility to acid-induced DNA denaturation *in situ* (Evenson *et al*., 1980; Evenson, 1999; Evenson *et al*., 1999; Spano *et al*., 2000). It is unclear if ART are effective in compensating for poor chromatin packaging and/or DNA damage or if sub-optimal chromatin integrity may contribute to the poor implantation rate (<20%) in the majority of ART patients (Edwards and Beard, 1999). Understanding of the effect of the paternal genome becomes more critical as we become less discriminatory with the maturity and quality of paternal nuclear material which is introduced into the oocyte (Sakkas, 1999).

The sperm chromatin structure assay (SCSA) holds promise for determining the importance of chromatin structure in ART outcomes. SCSA is an unbiased, quantitative assessment of sperm chromatin integrity defined as susceptibility of DNA to acid-induced denaturation *in situ*. SCSA parameters are stable within individuals over time if men are not exposed to

Table I. Mean and SE of semen analysis values for neat and washed samples of men who did and did not initiate pregnancy

	Neat		Washed	
	No pregnancy[a]	Pregnancy[b]	No pregnancy[c]	Pregnancy[d]
Concentration ($\times 10^6$/ml)	74.6 ± 15.8	101.1 ± 22.3	34.1 ± 7.0	47.1 ± 9.8
Motility (%)	48.0 ± 4.4	57.6 ± 6.2	75.8 ± 6.8	92.1 ± 9.6
Forward progression	80.7 ± 3.7	81.6 ± 5.3	82.9 ± 5.1	84.1 ± 7.3
Qualitative strict morphology (%)	4.7 ± 0.9	5.3 ± 1.6	–	–
Quantitative strict morphology (%)[e]	6.2 ± 0.71	8.3 ± 0.99	–	–

[a]$n = 14$, [b]$n = 7$, [c]$n = 10$, [d]$n = 5$.
[a,b,c,d]Data for three patients without transfer not included.
[c,d]Data for six patients with inadequate washed samples not included.
[e]$n = 15$. Two of the 17 men with quantitative strict morphology assessed were patients without transfer due to abnormal fertilization.

reproductive stressors (Evenson *et al*., 1991). In addition, SCSA parameters are correlated with DNA strand breaks (Sailer *et al*., 1995; Aravindan *et al*., 1997) and decreased fertility *in vivo* (Evenson *et al*., 1980, 1999). However, SCSA parameters are not strongly correlated with World Health Organization (WHO) parameters including concentration, motility and morphology (Evenson *et al*., 1991). Therefore, SCSA parameters are independent and may have predictive value beyond WHO parameters for patient success in ART. The objective of this study was to examine the relationship between neat and washed semen sample SCSA parameters and fertilization, embryo grade and pregnancy outcome following ART.

Materials and methods

Semen analysis

Semen samples were collected by masturbation from 24 men (mean age of 35 ± 4.8 years) after 2–7 days of abstinence and on the day of their partner's (mean age of 34 ± 5.1 years) oocyte aspiration for IVF or ICSI at the University of Nebraska Medical Center. Semen analysis was performed following WHO (1992) guidelines. Means for sperm concentration ($\times 10^6$/ml), motility (%), forward progression (%) and qualitative morphology (%) of neat and washed samples are shown in Table I. Strict morphology of neat samples was also assessed for 17 of the patients during a semen analysis before the day of IVF or ICSI (Table I). No individuals with varicocele, obstructive and non-obstructive azoospermia, or underlying reproductive tract pathologies were included in the study population. Of the original sample of 24 men, six suffered from male factor infertility, two of whom achieved pregnancy.

Sperm separation

A double-layered discontinuous Isolate© (Irvine Scientific, Santa Ana, CA, USA) gradient technique was used to prepare spermatozoa for IVF or ICSI. To form the gradient, 1 ml of the 'lower layer' Isolate© (~90%) was pipetted into the bottom of a 15 ml conical centrifuge tube, and 1 ml of 'upper layer' Isolate© (~45%) was pipetted gently over the bottom layer. Liquefied semen was placed on the top of the gradient using a sterile pipette, and the tube centrifuged at 200 *g* for 25 min. The supernatant was removed and discarded, and the sperm pellet suspended in 1 ml fresh HEPES-buffered human tubal fluid (HTF) containing 1% human serum albumin (h-HTF) (In Vitro Care Inc., San Diego, CA, USA). The suspension was centrifuged at 300 *g* for 3–5 min, and the supernatant aspirated and discarded. The sperm pellet was resuspended in h-HTF and a post-wash sperm analysis performed for concentration, motility and forward progression.

Sperm chromatin structure assay

Following the procedure(s) of Evenson and Jost (Evenson and Jost, 1994), the SCSA evaluated chromatin integrity of spermatozoa in neat and washed semen. 200 µl of sperm samples ($1–2\times 10^6$ spermatozoa/ml) were treated for 30 s with 400 µl of a pH 1.2 solution containing 0.1% Triton X-100, 0.15 mol/l NaCl and 0.08 N HCl. Triton X-100 permeabilizes sperm cell membranes providing greater accessibility of acridine orange (AO) to DNA. The low pH solution partially denatures DNA in spermatozoa with abnormal chromatin structure. Spermatozoa with normal chromatin structure do not demonstrate DNA denaturation. After the 30 s acid treatment, 1.20 ml of AO staining buffer (6 µg AO/ml, 37 mmol/l citric acid, 126 mmol/l Na_2HPO_4, 1 mmol/l disodium EDTA, 0.15 mol/l NaCl, pH 6.0) was added to the cells before analysing by flow cytometry. AO that intercalates into double-stranded DNA (native; normal) fluoresces green (515–530 nm) while AO that associates with single-stranded (denatured) DNA fluoresces red (⩾630 nm) when excited by a 488 nm light source (Darzynkiewicz *et al*., 1975).

The extent of sperm DNA denaturation was quantified using an Ortho Diagnostic Cytofluorograf II (Becton Dickinson, Westwood, MA, USA) with a closed quartz flow cell and a 100 mW argon ion laser operated at 35 mW power that was interfaced to a Cicero unit with PC-based Cyclops Software (Cytomation, Fort Collins, CO, USA). This system measured the amount of red and green fluorescence emitted from individual sperm cells flowing at ~200/s and calculated the αt [red/(red+green) fluorescence] distribution and associated parameters for each sample. Sperm populations with normal chromatin structure have a small mean α_t ($X\alpha_t$), $SD\alpha_t$, and percentage of cells outside the main population ($COMP\alpha_t$, i.e. percentage of cells with denatured DNA). SCSA also identifies immature sperm nuclei by the percentage of cells with high green fluorescence (HGRN), reflecting uncondensed chromatin that is more accessible to the AO stain. Mature ejaculated spermatozoa have a 5-fold lesser DNA stainability than round spermatids (Evenson and Melamed, 1983).

Assisted reproductive techniques

Oocyte retrieval and incubation

Oocytes were retrieved from women, <40 years of age, undergoing ovarian stimulation [leuprolide acetate, FSH, and/or FSH/LH as detailed by Roy *et al*. (1998)] using ultrasound-guided transvaginal follicular aspiration. Oocytes were cultured in synthetic HTF-con-

taining 1% human serum albumin (In Vitro Care Inc.) in a 5% CO_2, 37°C, humidified incubator for ~16–18 h in the presence of washed spermatozoa (50 000 to 100 000 motile spermatozoa/ml) or after ICSI. ICSI was done using established techniques and only on mature oocytes (Palmero *et al*., 1992; Van Steirteghem *et al*., 1993).

Fertilization assessment and embryo culture

Oocytes were assessed for fertilization 16–18 h post-insemination or ICSI. For inseminated oocytes, cumulus cells were removed by repeated pipetting through a sterile, small bore, glass pipette. For inseminated and ICSI oocytes, fertilization was considered normal if two pronuclei and two polar bodies were identified. Oocytes without visible pronuclei were considered to be unfertilized. Oocytes with a single pronucleus or more than two pronuclei were considered to be abnormally fertilized and discarded. Patients were allocated to one or more of the following fertilization categories: normal (⩾45% oocytes fertilizing normally), unfertilized (100% oocytes not fertilized), abnormal (⩾20% oocytes fertilizing abnormally). Fertilized oocytes were transferred by pipette to a dish containing fresh culture medium (HTF plus 5% human serum albumin) and incubated for an additional 2 days.

Embryo grading

Assessment of embryo quality was made 2 days after fertilization using a modification of Veeck (1986) and the grading system was as follows. Grade 1: pre-embryo with blastomeres of equal size; no cytoplasmic fragments. Grade 2: pre-embryo with blastomeres of equal size; minor cytoplasmic fragments or blebs. Grade 3: pre-embryo with blastomeres of distinctly unequal size; few or no cytoplasmic fragments or pre-embryo with blastomeres of equal or unequal size; significant cytoplasmic fragmentation or pre-embryo with few blastomeres of any size; severe or complete fragmentation.

Embryo transfer

Tubal embryo transfer and uterine embryo transfer procedures were performed 3 days after fertilization. In general, tubal embryo transfer was used for women with normal Fallopian tubes ($n = 11$) and IVF and embryo transfer for women with tubal factors ($n = 10$).

Pregnancy outcome

Ultrasound detection for fetal sac was used to confirm a positive clinical pregnancy. One patient with only a positive quantitative β-human chorionic gonadotrophin (βHCG) was also included as a positive pregnancy.

Statistical analysis

χ^2-Analysis was used to test the significance of SCSA parameter thresholds chosen for positive pregnancy. This analysis was applied to SCSA parameters of neat and washed samples separately. Each sample was analysed by SCSA twice, the replicate run immediately following the first. χ^2-Analysis was completed using the mean of the replicate SCSA values. Three patients who did not have embryos transferred due to abnormal fertilization and/or fertilization failure were not included in χ^2-analysis of pregnancy outcome. Four washed samples were removed from the statistical analysis because the amount and pattern of DNA degradation indicated delayed sample freezing. Two further washed samples were not included in SCSA analysis because of insufficient volume. If a relationship was identified through exploratory data analysis, regression analysis was used to determine the amount and significance of the correlation between SCSA parameters, WHO parameters and ART outcomes (Table II) by means of the SAS program (SAS, 1988).

Table II. Parameters used in statistical analysis

SCSA parameters	WHO parameters	Assisted reproductive technique outcomes
$X\alpha_t$ (channels)	Concentration ($\times 10^6$/ml)	No fertilization (%)
$SD\alpha_t$ (channels)	Motility (%)	Normal fertilization (%)
$COMP\alpha_t$ (%)	Forward progression	Abnormal fertilization (%)
HGRN (%)	Qualitative morphology (%)	Embryo grade (1–3)
	Quantitative morphology (%)	Pregnancy/oocyte transfer (%)
		Pregnancy outcome (+/–)

SCSA = sperm chromatin structure assay; WHO = World Health Organization; HGRN = high green fluorescence.
For definitions of $X\alpha_t$, $SD\alpha_t$ and $COMP\alpha_t$ see text.

Results

Mean SCSA values are shown for neat and washed samples in Table III. Spermatozoa in the neat semen of the seven men initiating pregnancy were significantly less susceptible to DNA denaturation than the 14 men not initiating pregnancy following embryo transfer (Table III). SCSA thresholds ($X\alpha_t = 300$, $P < 0.01$; $SD\alpha_t = 200$, $P = 0.01$; and $COMP\alpha_t = 27\%$, $P < 0.01$) for the susceptibility to low pH-induced denaturation in the neat sperm suspensions were predictive of no pregnancy (Table III). Pregnancy rate per oocyte transferred was 24% in patients with <27% ($COMP\alpha_t$) of the spermatozoa in the neat sample showing acid-induced DNA denaturation compared to 0% pregnancy rate in patients with ⩾27% denaturation (Table IV). The percentage of immature (HGRN) spermatozoa in the neat sample also appeared to have a threshold (17%) for pregnancy success; however, the confidence level was not significant ($P = 0.19$). No SCSA thresholds for the neat sample were identified for normal fertilization or embryo development (data not shown). However, four of the five patients with ⩾20% abnormally fertilizing oocytes had SCSA parameters that exceeded the thresholds for successful pregnancy (Table IV). The mean percentage of spermatozoa showing dentauration ($COMP\alpha_t$) in these five patients was 33% compared to 23% in the 19 patients with <20% abnormal fertilization.

SCSA parameters of the washed sample were not predictive of fertilization, embryo development or pregnancy outcome (Figure 1). No patient's washed sample exceeded the neat $X\alpha_t$ or $SD\alpha_t$ thresholds, and only one patient's washed sample exceeded the neat $COMP\alpha_t$ threshold. In addition, no significant relationships between the SCSA parameters (neat or washed) and WHO parameters, fertilization or embryo development were identified by regression analysis.

Discussion

The SCSA was predictive of negative pregnancy outcome when ⩾27% ($COMP\alpha_t$) of the spermatozoa in the neat sample showed acid-induced DNA denaturation. The percentage of cells with DNA damage was significantly ($P = 0.01$) greater in the 14 men with failed pregnancy outcome (31.1 ± 3.2) than in the seven men initiating pregnancy following IVF or ICSI (15.4 ± 4.6). Predictive thresholds for two other SCSA parameters [$X\alpha_t$ (300), $SD\alpha_t$ (200)] were established for

Table III. Threshold values of neat samples for pregnancy outcome and mean and SE of sperm chromatin structure assay values for neat and washed samples of men who did and did not initiate pregnancy

	Threshold	Neat		Washed	
		No pregnancy[a]	Pregnancy[b]	No pregnancy[c]	Pregnancy[d]
$\chi\alpha_t$	300	325.2[e] ± 14.6	254.3[f] ± 20.6	209.5 ± 10.7	193.7 ± 15.1
$SD\alpha_t$	200	216.6[e] ± 9.2	164.3[f] ± 13.0	91.2 ± 11.2	88.8 ± 15.8
$COMP\alpha_t$	27	31.1[e] ± 3.2	15.4[f] ± 4.6	7.8 ± 2.6	4.1 ± 3.7
% HGRN	17	14.8 ± 2.4	10.1 ± 3.4	4.3 ± 0.9	3.2 ± 1.3

[a]$n = 14$, [b]$n = 7$, [c]$n = 10$, [d]$n = 5$.
[a,b,c,d]Data for three patients without transfer not included.
[c,d]Data for six patients with inadequate washed samples not included.
[e,f]Pairs are significantly different ($P \leqslant 0.01$).
HGRN = high green fluorescence. For other abbreviations, see text.

Table IV. Assisted reproductive technique outcomes related to $COMP\alpha_t$ threshold of neat sample

Assisted reproductive technique outcome	Total no. of patients	$COMP\alpha_t$ <27	$COMP\alpha_t$ ⩾27
Fertilization[a]	24	14	10
Normal: ⩾45% of oocytes	19	12	7
Unfertilized: 100% of oocytes	2	2	0
Abnormal: ⩾20% of oocytes	5[b]	1	4
Pregnancy outcome[c]			
No. of patients with transfer	21	12	9
No pregnancy	14	5	9
Pregnancy initiated (+ βHCG)	7	7[d]	0[e]
Mean pregnancy/oocyte transferred (%)		24 ± 8.7	0 ± 0

[a]$n = 24$ patients.
[b]The oocytes of two patients had both ⩾20% abnormal fertilization and ⩾45% normal fertilization, so the sum of the fertilization categories was 26, i.e. two more than the total number of patients included in the study.
[c]Outcome for three patients without transfer due to abnormal fertilization ($n = 1$; $COMP\alpha_t = 41\%$) and total fertilization failure ($n = 2$; $COMP\alpha_t = 23$ and 7%) not included in analysis.
[d,e]Results were significantly different ($P = 0.005$; χ^2-analysis).
For definition of $COMP\alpha_t$, see text.

negative pregnancy outcome, indicating the potential value of SCSA analysis in an assisted reproduction treatment programme. Evenson *et al*. (1999) reported a similar threshold of 30% damaged spermatozoa ($COMP\alpha_t$) for in-vivo fertility (Evenson *et al*., 1999), while Spano *et al*. (2000) reported that fecundity became small when >40% of the cells showed denaturation (Spano *et al*., 2000). Therefore, the SCSA parameters appear to provide an unbiased, quantitative assessment of sperm chromatin structure that is an independent predictor of in-vivo as well as in-vitro fertility potential, i.e. pregnancy. In contrast to flow cytometric SCSA results, Angelopoulis *et al*. (1998) reported that microscopic examination of AO fluorescence in unprocessed spermatozoa does not predict pregnancy outcomes following IVF cycles (Angelopoulis *et al*., 1998). The difference may be due to high intra- and interspecimen coefficients of variation reported for the microscopic AO method, making it less sensitive and less specific than the flow cytometric SCSA (Claassens *et al*., 1992).

There was no relationship between the susceptibility to DNA denaturation, assessed by the SCSA, and normal fertilization and early embryo development (Table IV); however there was a trend for increased abnormal fertilization when DNA denaturation exceeded the established threshold. It was reported (Twigg *et al*., 1998) that DNA strand breaks did not affect the rate of fertilization. Similarly, spermatozoa with high amounts of DNA denaturation penetrated zona-free hamster eggs (Ibrahim and Pedersen, 1988), and spermatozoa with genetic-based defects fertilized normally (Engel *et al*., 1996). However, chromatin abnormalities appear to influence later embryonic development as demonstrated by the absence of clinical pregnancy in patients with SCSA parameters exceeding the thresholds established for neat samples.

SCSA parameters improved following density-gradient preparation as reported previously (Golan *et al*., 1997; Larson *et al*., 1999). However, the SCSA parameters of the prepared spermatozoa were not predictive of pregnancy outcome, indicating that elevated SCSA values in neat semen reflected chromatin abnormalities within the entire sperm population that were not eliminated by sperm preparation techniques. Other studies have shown that improved sperm concentration, motility and morphology after washing are not concomitant with increased ICSI fertilization and cleavage rates (Liu *et al*., 1994; De Vos *et al*., 1997). The current study demonstrated that ICSI leads to pregnancy in patients with poor sperm morphology and motility. For example, two couples with ⩽4% morphologically normal spermatozoa became pregnant following ICSI. Although the prognoses of these patients was poor based on conventional semen parameters, SCSA parameters of the neat semen were below the threshold for DNA damage (12 and 20% of spermatozoa showed DNA denaturation; i.e. $COMP\alpha_t$), indicating that the chromatin integrity of the sperm population was adequate to support a viable pregnancy. These patients illustrate the importance of the additional, independent information provided by SCSA analysis. Other assessments of sperm chromatin structure including chromomycin A_3 and acidic aniline blue staining are significantly associated with sperm morphology (Franken *et al*., 1999) and therefore may not provide the additional, independent information of the SCSA.

If applied clinically, the SCSA may assist the clinician in providing informed consent to patients, for example if donor spermatozoa should be considered. The SCSA will provide

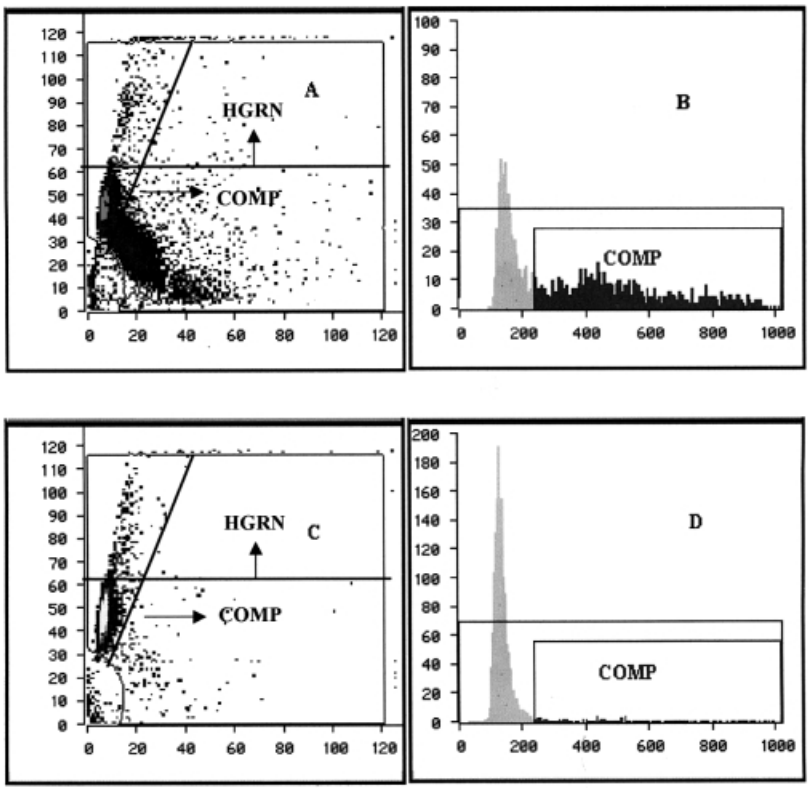

Figure 1. Cytograms (**A** and **C**) and corresponding alpha$_t$ (α_t) histograms (**C** and **D**) comparing denaturation of 5000 acridine orange-stained spermatozoa from the neat (**A** and **B**) and washed (**C** and **D**) samples from a patient with failed pregnancy following intracytoplasmic sperm injection. Each point on the cytograms represents the green (double-stranded DNA; *y*-axis) versus red (denatured single-stranded DNA; *x*-axis) fluorescence of a single spermatozoon while the histogram illustrates the α_t [red/(red + green) fluorescence] frequency distribution of spermatozoa within the suspension. Cells outside the main population (COMP) have a larger ratio of red:green fluorescence, indicative of abnormal chromatin structure. High green (HGRN) fluorescence indicates immature spermatozoa with incomplete chromatin condensation. For this patient, the neat sample had values that exceeded sperm chromatin structure assay (SCSA) thresholds whereas the corresponding washed sample values were below the thresholds, illustrating that the SCSA values of the neat sample, not the washed sample, were predictive of negative pregnancy outcome.

additional information, beyond conventional semen analysis, that may identify DNA damage that will not lead to viable pregnancies following transfer even though the spermatozoa may be capable of initiating fertilization and early embryo development.

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