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The sperm chromatin structure assay as a diagnostic tool in the human fertility clinic

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BACKGROUND: Sperm DNA integrity has been shown to be necessary for achieving and sustaining embryo development. The objective was to evaluate the sperm chromatin structure assay (SCSA) as a diagnostic tool in clinical practice for intrauterine insemination (IUI), in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) treatments. METHODS: A total of 385 semen samples from 234 couples were frozen for SCSA, and smears were prepared for morphology: 48 IUI, 139 IVF and 47 ICSI. The main SCSA variables were DNA fragmentation index (DFI), standard deviation of DFI (SD-DFI) and high DNA stainability (HDS), and the reproductive outcomes were biochemical pregnancy (BP), clinical pregnancy (CP) and implantation ratio (IR). RESULTS: The results showed no significant difference in the fertility variables BP, CP and IR when <27% DFI was used between the IVF and ICSI groups. A low number of patients received IUI with low success rate, and statistical analysis was therefore not performed. Ongoing pregnancy was achieved for both IVF and ICSI couples with DFI levels >27%, and six couples in ICSI treatment achieved CP full-term. DFI >27% had a high prognostic power for predicting no CP for IVF patients, with a specificity of 97%. Couples diagnosed with male infertility had a significantly higher level of DFI compared to couples with idiopathic fertility. Sperm head morphology showed low but significant correlations with the SCSA variables. CONCLUSION: SCSA is a useful tool in andrological diagnosis and contributes with a prognosis for the fertility outcome of conventional IVF. Although full-term pregnancy can be achieved with assisted reproductive techniques with a DFI >27%, the probability of a successful pregnancy may be reduced.

Key words: male infertility/morphology/pregnancy/sperm DNA fragmentation/spermatozoa

Introduction

During the last steps of spermatogenesis, the sperm nucleus condenses, and the characteristic shape of the sperm head is formed. Approximately 85% of the DNA-incorporated histones are exchanged with transitional proteins, which are then exchanged with protamines during spermiogenesis. The result is a highly packaged chromatin structure, with apparently no DNA transcription or RNA translation taking place. The capacity of the maturing sperm cells for DNA repair is greatly reduced, and their ability to respond to damage by undergoing programmed cell death is progressively lost (Aitken *et al*., 2004). Therefore, breaks in the DNA that may have escaped repair prior to packing or damage occurring after packing has been completed, can be delivered to the oocyte and may result in early embryonic loss. However, mouse oocytes have been shown to have the capability of repairing DNA damage in the paternal DNA but only to a certain level (Ahmadi and Ng, 1999).

Evenson and coworkers (1980) described the sperm chromatin structure assay (SCSA) in the early 1980s, and the assay has since been refined (Evenson *et al*., 2002) and used for both animal and human semen (Evenson, 1999). The SCSA utilizes the metachromatic properties of the fluorescent stain acridine orange (AO), and the extent of DNA denaturation after an acidic treatment is determined by measuring the shift from green fluorescence (double-stranded, native DNA) to red fluorescence (single-stranded, denatured DNA) using flow cytometry. Correlations between SCSA and other types of assays that determine DNA integrity in the sperm have been shown (Evenson *et al*., 2002; Erenpreiss *et al*., 2004). At present, the SCSA is widely used in the human fertility clinic (Evenson *et al*., 1999; Spano *et al*., 1999; Larson *et al*., 2000; Bungum *et al*., 2004) and has also proven useful in semen analysis in domesticated species (Evenson, 1999), such as the boar (Evenson *et al*., 1994) and the bull (Ballachey *et al*., 1988).

Several studies have shown the relationship between SCSA variables and fertility after intrauterine insemination (IUI), IVF and ICSI, and some studies have established statistical threshold levels for these variables with regard to fertility. These studies

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have shown that a DNA fragmentation index (DFI) above 27% (Larson *et al*., 2000), 30% (Evenson *et al*., 1999) and 40% (Spano *et al*., 2000) was related to male sub- or infertility. A recent report found that a DFI level >27% is compatible with ongoing pregnancy and delivery after IUI, IVF (Bungum *et al*., 2004) and ICSI (Bungum *et al*., 2004; Gandini *et al*., 2004), but at a lower probability. The 'Tip of the Iceberg' theory states that although a relatively small percentage (30%) of the sperm is included in the DFI population, the remaining sperm also have defects although undetectable, under the exact physical and chemical conditions of the SCSA, resulting in a negative reproductive outcome (Evenson and Jost, 2000).

The available artificial reproductive techniques (ART) in the human fertility clinic—IUI, IVF and ICSI—make it possible for the sperm to bypass the natural barriers of fertilization to a variable degree. Whether certain ART procedures are able to compensate for high levels of sperm DNA fragmentation and result in ongoing pregnancies is a question of great importance in the human fertility clinic (Larson-Cook *et al*., 2003; Virro *et al*., 2004).

The objective of this study was to evaluate the SCSA as a diagnostic tool in clinical practice for IUI, IVF and ICSI treatments.

Materials and methods

A total of 234 couples contributing with 385 ejaculates, from three Danish fertility clinics, were included in the study. The distribution of couples between the three fertility clinics was 188 from the public clinic and 46 from the two private clinics. Couples attending the clinics were enrolled from 1 December 2002 to 1 April 2004. The Local Scientific Ethics Committees approved the study (KF 01-043/02). The couples gave their written consent to participate, and the women fulfilled the following criteria: born after 1 January 1970, a normal hormonal status, FSH on day 2–3 was <10mIU/ml and anatomically normal [women with polycystic ovaries (PCO) and endometriosis were excluded from the study]. The couples were divided into the following groups: A, closed fallopian tubes; B, oligozoospermia, sperm concentration $<20 \times 10^6$ sperm/ml; C, idiopathic infertility; and D, closed fallopian tubes and oligozoospermia.

Each man delivered between one and four ejaculates, which were either used in the clinic diagnostic work or in the treatment of the couple. The samples were collected by masturbation either at the clinic or at home into a clean wide-mouthed plastic container and delivered to the laboratory within an hour of collection. The sperm concentration was determined using a Makler Chamber (SEFI Medical Instruments, Haifa, Israel). After liquefaction, an aliquot of 100 µl was withdrawn and diluted 1 : 5 with TNE buffer (0.01 M TrisCl, 0.15 M NaCl, 1 mM EDTA disodium, pH 7.4) at room temperature. The sample was mixed carefully, and two coded 0.23 ml plastic straws (IMV, L'Aigle cedex, France), printed with the identity of the patient, were manually filled and sealed. The straws were frozen, stored and transported in liquid nitrogen until SCSA analysis.

Prior to the study, an ejaculate from a donor was chosen for the SCSA reference sample. The ejaculate was diluted in TNE buffer to a concentration of 2×10^6 sperm/ml. Aliquots of 500 µl of the reference sample were then stored at –80°C in 2 ml microcentrifuge tubes until SCSA analysis.

Preparation of smears

Smears of semen were made from all patient samples. A small aliquot of the raw semen sample (20 µl) was diluted 1 : 2 with 3% sodium citrate and mixed carefully. If the concentration of sperm was below 6×10^6 sperm/ml, 20 µl was applied to two slides. If the concentration was above 6×10^6 sperm/ml, 5 µl was used. The slides were allowed to air dry and then fixed in 96% ethanol for 5 min. The slides were then placed at 5°C until further staining and morphological analysis.

Sperm chromatin structure assay

The SCSA was performed according to the procedure described by Evenson and coworkers (Evenson and Jost, 2000; Evenson *et al*., 2002) with minor modifications (Boe-Hansen *et al*., 2005). The test samples were analysed in random order. One straw from each ejaculate was thawed in a water bath at 37°C for 30 s. The content was emptied into a microcentrifuge tube, which was immediately placed on ice. After 5 and 10 min of incubation on ice, an aliquot of the thawed semen sample was diluted to a concentration of 2×10^6 sperm/ml in TNE buffer to a total of 200 µl in a Falcon tube (BD Biosciences, San Jose, CA, USA). Immediately, 0.40 ml of acid detergent solution (0.08 M HCl, 0.15 M NaCl, 0.1% v/v Triton X-100, pH 1.2) was added to the Falcon tube. After exactly 30 s, 1.20 ml of Acridine Orange (AO)-staining solution was added, containing 6 µg AO (chromatographically purified; Polysciences, Warrington, PA, USA) per ml buffer (0.037 M citric acid, 0.126 M Na_2HPO_4, 1.1 mM EDTA disodium, 0.15 M NaCl, pH 6.0). Owing to low concentration of some of the test samples, it was not possible to do a second analysis at 10 min incubation. In addition, for a few samples of very low sperm concentration, it was not possible to get event rates in the optimal range of 170–300 events/s during the data acquisition. A few samples contained large amounts of gel or had high viscosity, which made pippeting exact volumes from these samples difficult. For every six test samples, one reference sample, frozen in 2 ml micro centrifuge tubes, was thawed for 2 min at 37°C until the ice had disappeared and analysed to ensure instrument stability. The reference samples were incubated on ice for 5 and 10 min before staining and flow cytometric analysis.

Flow cytometric measurements

The samples were analysed using a FACScan flow cytometer (BD Immunocytometry Systems, San Jose, CA, USA) with an air-cooled argon laser operated at 488 nm and a power of 15 mW. The green fluorescence (FL1) was collected through a 515–545 nm bandpass filter, and the red fluorescence (FL3) was collected through a 650 nm longpass filter. The sheath/sample was set on 'low', adjusted to a flow rate of 200 events/s when analysing a sample containing 2×10^6 sperm/ml. Immediately after the addition of the AO-staining solution, the sample was placed in the flow cytometer and run through the flow system. Data acquisition of 5000 events was initiated exactly 3 min after the addition of acid detergent solution and collected in list mode using the BD CellQuest™ Pro version 4.0 software (BD Biosciences). The laboratory technician manually recorded the *X*-mean (red fluorescence), *Y*-mean (green fluorescence) values and the event rate for each sample. A solution of 0.05% v/v Triton X-100 was used as sheath fluid, and a tube with AO equilibration buffer (400ul of acid detergen solution and 1.20ml of AO-staining solution) was run between every new sample to ensure AO equilibration conditions in the tubes of the flow cytometer.

The flow cytometric setup for the reference sample was *X*-mean 100 ± 5 and *Y*-mean 410 ± 5.

List mode data were analysed with the software program SCSASoft (SCSA Diagnostics, Brookings, SD, USA). The SCSASoft calculated the percentage of sperm with an abnormally high DNA stainability (HDS), the level of DNA fragmentation index (DFI) as the percentage of sperm with denatured DNA, high DFI and moderate DFI. The standard deviation of the DFI (SD-DFI) was calculated as the SD of the values of DFI on a 0–1024 scale.

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Morphology

One slide from each ejaculate was stained using the Papanicolaou stain, in batches of 20–50 slides. Harris haematoxylin (Sigma Diagnostics, St. Louis, MO, USA), Orange G6 and EA 50 Papanicolaou stain (CellPath, Newtown, UK) were used (World Health Organization, 1999). The slides were allowed to dry overnight, mounted and kept in a dark and cool place until analysis. A total of 100 sperm cells were scored per slide for normality according to the Krüger Strict criteria (Kruger *et al*., 1987, 2000). In smears with very low sperm concentration, all assessable sperm in the slide were evaluated. Each sperm was classified according to the type of head defects: shape, vacuoles and acrosome.

Fertility

Fertility data were collected retrospectively from the clinical records of all the couples. The type of treatment (IUI, IVF and ICSI), number of embryos transferred, biochemical pregnancy (BP) and clinical pregnancy (CP), including the number of foetuses, were recorded for each of the ejaculates used for treatment and included in the study.

For IVF and ICSI treatments, BP was determined by assessing human chorionic gonadotrophin in serum (s-HCG) (MiniVIDAS, bioMerieux, Durham, NC, USA) 14 days after embryo transfer. A s-HCG value above 10 IU/l was scored as positive and above 100 IU/l as strongly positive. When s-HCG was positive, an ultrasound examination was carried out 3 weeks later. A weakly positive s-HCG (10–99 IU/l) may be caused by a transitory increase in s-HCG due to the presence of BP or ectopic pregnancy. Therefore, in patients with weak positive s-HCG, a repeated s-HCG determination was performed. In IUI patients, s-HCG was replaced by urine-HCG determination 16 days after insemination. In the present study, BP is used to define all pregnancies with a positive s-HCG or u-HCG. With the ultrasound examination, it was possible to clarify if a CP was obtained, and the numbers of intrauterine gestation sacs with a heartbeat were registered. The implantation ratio (IR) was calculated as the numbers of gestation sacs divided by the numbers of embryos transferred. The statistically correct term IR, and not implantation rate, is used in the present study.

Statistical analysis

The effect of incubation time (5 and 10 min) on the SCSA variables was evaluated by an analysis of variance using a general linear mixed model. The outcome SCSA variables were DFI, high DFI, moderate DFI, SD-DFI and HDS. The assumption for a normal distribution could not be fulfilled (using Shapiro–Wilks test), and the outcome variables were therefore rank transformed. Incubation time and age (22–30, 31–32, 33–35, 35–50 years or 22–32 and 33–50 years), clinic (*x*, *y* and *z*), treatment (IUI, IVF and ICSI) and diagnosis, defined as the man contributing to the infertility (groups B and D versus groups A and C), were included as fixed effects in the model. Owing to confounders, diagnosis and treatment could not be included in the same model. The random effect of couples was included in the models. Differences in morphology due to age, clinic, treatment and diagnosis were evaluated by an analysis of variance using a general linear model. The percentage of normal sperm, percentage of head defects (1 or 2) and the percentage of specific head defects in combination (shape, acrosome and vacuoles) were the outcome variables for morphology. The fixed effects of age, clinic, treatment and diagnosis were included in the models. The outcome variables were rank transformed because the assumption of a normal distribution could not be fulfilled (using Shapiro–Wilks test). The correlation between the SCSA variables and morphology was determined using Spearman's correlation coefficients. The effect of treatment (IUI, IVF and ICSI) on the binary ART outcomes (BP and CP) was evaluated by a logistic analysis using a generalized linear model. The association between ART outcomes (BP and CP) and the DFI groups (DFI ≤27%, >27%) was evaluated using Fisher's exact test. Differences in IR between DFI and HDS groups (DFI ≤27%, >27%; HDS ≤15%, >15% and HDS ≤10%, >10%) for the different treatments were evaluated by a logistic analysis, using a generalized linear model with DFI group or HDS group as the single fixed effect. To identify the clinical utility of the SCSA, diagnostic measures were calculated for DFI and HDS with BP and CP as the gold standard. For DFI, a threshold value of 27% was used and for HDS 10 or 15% were used. The validation was performed for the two treatments IVF and ICSI, and the diagnostic measures were sensitivity, specificity, negative predictive value (NPV) and positive predictive value (PPV). The statistical analyses were performed using the Statistical Analysis System SAS, version 8.2 (SAS institute). A 5% significance level was used throughout the study.

Results

Sperm chromatin structure assay

Aliquots from 385 ejaculates were assessed using the SCSA. A significant effect of incubation time on ice was found. Significant higher values were found for 5 min compared to 10 min for DFI ($P < 0.001$), high DFI ($P = 0.022$) and moderate DFI ($P < 0.001$). There were no significant effects of incubation time on SD-DFI ($P = 0.054$) and HDS ($P = 0.59$). The further analysis was therefore performed on the data obtained from the sample incubated 5 min on ice when these were available; otherwise the data obtained after 10 min incubation was used for nine of the samples.

We did not find a significant effect of the age of the man on the SCSA variables, when divided into age groups of below and above 32 years, and when using the age groups 22–30, 30–32, 32–35 and 36–50 years. Diagnosis, defined as the man contributing to the infertility (groups B and D), was found to have a significant effect on each of the SCSA variables, and higher values were found for all five SCSA variables ($P < 0.001$). Treatment group also had a significant effect on each of the five SCSA variables ($P < 0.001$). We found no significant differences between the clinics in the SCSA variables.

Pearson's correlation coefficient was determined between the three SCSA variables, showing 0.834 ($P < 0.0001$) for DFI and SD-DFI, 0.834 ($P < 0.0001$) for SD-DFI and HDS and 0.226 ($P < 0.0001$) for HDS and DFI. Further statistical analysis using the SD-DFI was not conducted.

Morphology and concentration

Smears from the 385 ejaculates were assessed. Some smears contained large amounts of debris and others no sperm at all. Therefore, it was only possible to score 374 of the ejaculates for morphology. There was no significant effect of the age of the man (22–30, 30–32, 32–35 and 36–50 years) on the percentage of normal sperm. However, an effect was seen on the percentage of normal sperm when dividing the men into age below and above 32 years ($P = 0.023$). Age had no effect on sperm concentration. Diagnosis, defined as the man contributing to the infertility (groups B and D), was found to have a significant effect on sperm concentration ($P < 0.001$) but not on the percentage of normal sperm cells ($P = 0.16$). An association between treatment group and sperm concentration ($P < 0.001$), and the percentage of normal sperm cells ($P = 0.033$),

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was detected. We found a significant difference in sperm concentration between the clinics ($P < 0.001$) but not in the percentage of normal sperm cells ($P = 0.13$).

SCSA and morphology

The defects were grouped into the number of head defects observed as one defect, two defects and three defects. The correlations between the SCSA variables and the morphological variables are shown in Table I. Significant negative correlations were observed between one defect and all the SCSA variables. For three defects, a positive correlation to all SCSA variables except moderate DFI was found. A Spearman's correlation coefficient of 0.44 ($P < 0.001$) between HDS and the category with abnormal head shape and abnormal acrosome was found. This morphological category also correlated positively with high DFI [0.36 ($P < 0.001$)] and SD-DFI [0.34 ($P < 0.001$)].

SCSA and fertility data

A total of 234 of the collected samples were used in IUI, IVF or ICSI treatments. Treatment had a significant effect on successes for BP and CP ($P < 0.001$ and $P = 0.006$). We found no significant difference between the successes for BP for couples receiving IVF treatment (39.9%) and for couples receiving ICSI treatment (31.9%). Furthermore, no significant difference was found in the percentage of couples achieving a CP for IVF (28.3%) and ICSI (29.8%). The percentage of IUI treatments resulting in BP and subsequent CP was only 8.3%. For IUI, only two of the 48 couples had DFI values >27%, and none of these resulted in BP. A total of 11 IUI couples had >15% in HDS, and these inseminations did not result in BP. No further statistical analyses were preformed on data from the IUI couples due to the low success of the treatments and the low number of couples in the DFI >27% group.

Table I. Correlation between the sperm chromatin structure assay (SCSA) variables—DNA fragmentation index (DFI) as total percentage of sperm with elevated levels of DNA fragmentation, moderate DFI, high DFI, standard deviation of DFI (SD-DFI) and high DNA stainability (HDS)—and head morphology variables ($n = 374$)

	DFI	Moderate DFI	High DFI	SD-DFI	HDS
Normal	NS	NS	NS	NS	–0.18***
One-head defect	–0.18***	–0.16**	–0.12*	–0.15**	–0.27***
Two-head defects	0.14**	0.14**	NS	NS	0.26***
Three-head defects	0.12*	NS	0.20***	0.21***	0.13*
Shape[a]	NS	–0.12*	NS	NS	NS
Acrosome[b]	0.13*	NS	0.26***	0.23***	0.28***
Vacuoles[c]	–0.22***	–0.14**	–0.25***	–0.25***	–0.33***
Shape[a] and acrosome[b]	0.23***	NS	0.36***	0.34***	0.44***
Acrosome[b] and vacuoles[c]	NS	0.13*	NS	NS	NS
Shape[a] and vacuoles[c]	–0.12**	NS	–0.19***	–0.17**	–0.21**

NS = not significant ($P > 0.05$), *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
[a]Abnormal head shape.
[b]Abnormal acrosome.
[c]Vacuoles in sperm head.

Descriptive analysis of data for couples receiving IVF is summarized in Table II. The percentage of BP in the ≤27% and >27% DFI groups was 41.2 versus 14.3%, respectively ($P = 0.24$). Descriptive analysis of data for couples receiving ICSI treatment is shown in Table III. The percentage of BP in the ≤27% and >27% DFI groups was 31.0 versus 33.3%, respectively ($P = 1.0$).

There were no statistical differences between the IRs achieved after IVF compared to those achieved after ICSI treatment. The IRs for both IVF and ICSI treatments were lower for the >27% DFI than the ≤27% DFI group, but statistical significance was not found within each treatment group ($P > 0.05$). We did not find a significant difference between the IRs for >15% HDS and the ≤15% HDS for IVF ($P = 0.15$) and for ICSI ($P = 0.056$). When using 10% HDS as the threshold level, a significant difference between IRs for IVF was seen, with the >10% HDS resulting in a significantly higher IR ($P = 0.045$).

For IVF and ICSI, BP and CP were achieved in seven cases where levels of DFI were above 27%. For one couple receiving IVF with an actual DFI of 27.1%, a positive s-HCG resulting in CP with two gestation sacs and heartbeats was seen. One of the two children died due to premature labour, week 26 of gestation. A total of six positive s-HCGs was seen in ICSI patients with DFI >27%, all of which resulted in CP. All pregnancies went to term, and six babies without remarks regarding health were born. The actual DFI in the six semen samples had a mean DFI of 37.4%, ranging from 30.7 to 47.9%.

Table II. Descriptive analysis of the different measures—DNA fragmentation index (DFI) and high DNA stainability (HDS)—in the sperm chromatin structure assay (SCSA) for couples receiving IVF treatment

	DFI ≤ 27%	DFI > 27%	HDS ≤15%	HDS >15%
Couples (*n*)	132	7	112	27
Male age (years)	32.65	34.19	32.81	32.39
Embryo transferred	209	11	174	46
Sperm concentration (x 10^6/ml)	81.20	70.14	88.77	46.92
Biochemical pregnancy (%)	54 (41.2)	1 (14.3)	44 (39.3)	11 (42.3)
Clinical pregnancy (%)	38 (29.0)	1 (14.3)	29 (25.9)	10 (38.5)
Implantation ratio (%)	47/209 (22.5)	2/11 (18.2)	35/174 (20.1)	14/46 (30.4)

Table III. Descriptive analysis of the different measures—DNA fragmentation index (DFI) and high DNA stainability (HDS)—in the sperm chromatin structure assay (SCSA) for couples receiving ICSI treatment

	DFI ≤ 27%	DFI > 27%	HDS ≤15%	HDS > 15%
Couples (*n*)	29	18	26	21
Male age (years)	33.61	34.25	34.81	32.67
Embryo transferred	42	29	42	29
Sperm concentration (x 10^6/ml)	41.04	14.21	51.70	4.84
Biochemical pregnancy (%)	9 (31.0)	6 (33.3)	11 (42.3)	4 (19.0)
Clinical pregnancy (%)	8 (27.6)	6 (33.3)	10 (38.5)	4 (19.0)
Implantation ratio (%)	12/42 (28.6)	6/29 (20.7)	14/42 (33.3)	4/29 (13.8)

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Diagnostic measures of the SCSA

When a threshold level of 27% DFI was used, the specificity was estimated at 91% for BP and 88% for CP. When combining the two SCSA variables, DFI (>27%) and HDS (>10%), using a parallel test, the specificity was 92 and 89% for BP and CP, respectively. However, when distinguishing between treatment type (IVF and ICSI), the SCSA applied on the IVF samples had a higher specificity for BP (98%) compared to the ICSI samples (60%). For CP, the figures were 97 and 57%, respectively (Table IV). The specificity for BP was higher for the HDS (>15%) for IVF compared to ICSI (80 and 73%, respectively) and that for CP was 74 and 71%, respectively.

Diagnosis

In Table V, descriptive data for the four diagnosis groups are shown. Semen samples from men with diagnosed male infertility (B) have a significantly higher DFI, moderate DFI and high DFI, SD-DFI and HDS compared to the idiopathic infertility group (C). A significant lower number of BP was seen in couples with diagnosed male infertility compared to the tubal factor group (A) (P = 0.038) and idiopathic infertility (C; P = 0.007). However, a significant difference was not detected in CP and IR.

Table IV. Validation of the SCSA variables—DNA fragmentation index (DFI) and high DNA stainability (HDS)—as diagnostic test for infertility, measured as clinical pregnancy for ICSI and DFI, with lower and upper 95% confidence levels. Given as negative predictive value (NPV), positive predictive value (PPV), sensitivity and specificity.

Threshold value	DFI >27%	HDS >10%	HDS >15%
ICSI			
NPV	28 (13–47)	35 (14–62)	38 (20–59)
PPV	67 (41–87)	73 (54–88)	81 (58–95)
Sensitivity	36 (20–55)	67 (48–82)	52 (33–69)
Specificity	57 (29–82)	43 (18–71)	71 (42–92)
IVF			
NPV	29 (21–38)	21 (13–32)	26 (18–35)
PPV	86 (42–100)	63 (50–75)	62 (41–80)
Sensitivity	6 (2–13)	39 (30–50)	16 (10–25)
Specificity	97 (87–100)	41 (26–58)	74 (58–87)

Discussion

The biological impact of sperm DNA damage may depend on the combined effects of the level of the sperm DNA damage and the competence of the oocyte or early embryo to repair the DNA damage. In the present study, SCSA analysis was performed on semen aliquot taken from the ejaculate used for ART in younger women (<34 years). A study determining the diagnostic value of the SCSA in couples with younger women has, to our knowledge, not previously been conducted. Two of the 48 couples receiving IUI had DFI levels above 27%, and treatment did not result in pregnancy in either case. However, the IUI group with DFI level above 27% was too small to draw any statistical conclusions. In a previous study (Bungum *et al*., 2004), no significant difference between the BP rate for IUI in the >27% DFI group (4.3%) and the <27% DFI group (20.2%) was found, and the delivery rates in the two groups did not differ statistically.

In the present study, we found that ongoing pregnancy was achieved for only one sample with a DFI >27% and undergoing IVF treatment. The sample was only marginally above the 27% threshold level. In comparison, in couples receiving ICSI treatment, six samples with DFI above the 27% threshold level resulted in ongoing pregnancies, and the highest value was 47.9%. The diagnostic validation of the SCSA showed a higher specificity for predicting both BP and CP for IVF than ICSI, but a significant difference was not observed between the IRs for IVF and ICSI, when DFI was >27%. Previous reports have found that no CPs were achieved using IVF or ICSI when the DFI exceeded 27% (Larson *et al*., 2000; Larson-Cook *et al*., 2003) or 28% (Saleh *et al*., 2003), and an increased miscarriage rate has been associated with high levels of DFI (Virro *et al*., 2004; Check *et al*., 2005). However, recent studies have shown successful fertilization and ongoing full-term pregnancies despite high levels (>27%) of DFI in the particular sample used for ICSI (Bungum *et al*., 2004; Gandini *et al*., 2004; Virro *et al*., 2004). It has been hypothesized that the sperm DNA integrity becomes particularly relevant when fertilization occurs under more natural circumstances or in conventional IVF (Gandini *et al*., 2004). This was also reported by Bungum and coworkers who showed that semen samples with DFI above 27% were more likely to result in BP using ICSI, compared with the

Table V. Descriptive analysis for the four patient groups (A, B, C and D) for the SCSA and fertility variables

	A	B	C	D
Ejaculates (*n*)	71	92	213	9
Male age (years)	32.03[b]	32.84[ab]	32.87[b]	36.38[a]
Sperm concentration (x 10^6/ml)	86.01[a]	9.41[b]	78.10[a]	23.56[b]
DFI	12.14[c]	23.64[a]	14.22[b]	22.45[a]
HDS	10.95[c]	18.66[a]	11.72[b]	9.27[bc]
High DFI	3.96[b]	8.92[a]	4.17[b]	8.43[a]
Moderate DFI	8.20[c]	14.78[a]	10.07[b]	14.07[a]
SD DFI	124.27[b]	176.50[a]	127.33[b]	166.44[a]
Treated couples (*n*)	40	52	134	7
Biochemical pregnancy (%)	14 (35.0)[a]	11 (21.2)[b]	48 (35.8)[a]	1 (14.3)[ab]
Clinical pregnancy (%)	9 (22.5)	10 (19.2)	37 (27.6)	1 (14.3)
Implantation ratio (%)	11/61 (18.0)	11/62 (17.7)	48/160 (30.0)	1/8 (12.5)

A, closed fallopian tubes; B, oligozoospermia, sperm concentration $<20 \times 10^6$ sperm/ml; C, idiopathic infertility; D, closed fallopian tubes and oligozoospermia. The SCSA variables are DNA fragmentation index (DFI), high DNA stainability (HDS), high DFI, moderate DFI and standard deviation of DFI (SD-DFI). Different letter superscripts (a and b) indicate a significant difference ($P < 0.05$).

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traditional IVF method. However, differences in IRs were not found (Bungum *et al*., 2004). Furthermore, a recent study showed a significantly larger IR after ICSI using sperm obtained from the testes compared to ejaculated sperm, when a high DNA fragmentation was detected by the TUNEL assay in the ejaculated sperm (Greco *et al*., 2005). Likewise, others have shown no association between spermatozoa with DNA strand breaks and fertilization rates after ICSI using the TUNEL assay (Høst *et al*., 2000). It has recently been proposed to aspirate sperm from the testes for ICSI as a treatment option for men whose fertility is compromised by sperm DNA damage (Greco *et al*., 2005). The present study and the above-cited studies (Bungum *et al*., 2004; Greco *et al*., 2005) suggest that ICSI treatment provides better pregnancy results with samples with a high level of DNA fragmentation.

In the present study, the association of a low percentage of immature cells (HDS <10%) with a higher pregnancy rate was not observed. On the contrary, a significantly lower IR was detected in the IVF group for samples with a HDS of <10%. This difference was not found when using 15% as the threshold value. Evenson and coworkers (Evenson *et al*., 1999; Larson *et al*., 2000; Larson-Cook *et al*., 2003) were the first to point out that the percentage of immature (HDS) sperm in a sample appeared to have a threshold for pregnancy success. In a recent study, it was found that samples with a HDS smaller or equal to 10% was associated with a higher proportion of CPs and births for IUI treatment, but the difference was not statistically significant (Bungum *et al*., 2004). Virro and coworkers (2004) did not find a significant relationship between HDS and blastocyst rates, BP and ongoing pregnancy and spontaneous abortions, but a relationship between HDS >15% and low fertilization rates in IVF was found. On the basis of our observations, we cannot recommend a HDS threshold level of 10%. However, when the SCSA variables HDS and DFI were used in parallel with threshold values of 15 and 27%, respectively, versus DFI with a threshold value of 27% alone, an increase in specificity for both BP and CP was detected for IVF/ICSI treatment. The NPVs and sensitivities of the DFI and HDS thresholds were low, reflecting that several other factors are important for fertility. Future studies should be conducted to establish a relationship between HDS and fertility and determine whether HDS adds significant information related to fertility and the SCSA analyses. In selecting sperm for ICSI, other sperm parameters may be of greater importance for the success of the embryo, and this bypassing of normal selection procedure may have detrimental effects on the offspring. In the present study, we showed that the DFI in the group of patients diagnosed with male infertility (B) had a significantly higher level of DFI compared to the other groups. Also a significantly higher number of patients receiving ICSI had high levels of DFI >27%, compared to patients receiving IVF. Furthermore, we found low, but significant correlations between morphology and the SCSA variables. The highest correlation was found between HDS and the morphology categories, sperm head shape and acrosome defect, which may reflect that the cells in the HDS region could be larger and immature sperm with increased native DNA-binding sites. Studies have reported a low correlation between the traditional semen variables such as sperm concentration, motility, strict morphology and the SCSA variables (Evenson *et al*., 1999; Larson *et al*., 2000; Giwercman *et al*., 2003), and most of these studies were conducted on selected materials from heterogeneous groups of infertility patients from fertility clinics (Evenson *et al*., 1999; Larson *et al*., 2000). Our findings support previous findings, reporting that a significant higher percentage of sperm from poor-quality semen samples have fragmented DNA (Sun *et al*., 1997). Therefore, spermatozoa possessing damaged DNA are inevitably being used for assisted reproduction and especially for ICSI. Further research must be conducted to confirm ICSI as a safe procedure and with regard to the use of the technique in cases where semen samples show high level of DNA fragmentation. This includes the effect of imprinting disease and childhood cancer in the offspring (Fraga *et al*., 1996; Ji *et al*., 1997), which may be related to genetic damage in paternal gametes (Sorahan *et al*., 1997).

Previous reports have shown an improvement in the SCSA variables comparing neat semen samples and prepared samples for ART (Golan *et al*., 1997; Larson *et al*., 1999, 2000; Gandini *et al*., 2004). The SCSA variables of the prepared washed semen have shown not to be as predictive of pregnancy outcome as the neat samples (Larson *et al*., 2000). It has previously been suggested that tests detecting DNA damage, like the SCSA, have greater strength in prediction of the outcome of natural conception compared to ART, where the sperm is prepared and the fraction of DNA damaged sperm may be reduced (Seli and Sakkas, 2005). Semen samples of variable quality are used for different ART and therefore exposed to different microenvironments, in the form of preparation technique, but also media and time before fertilization. Very little is known about the DNA integrity of the sperm that actually fertilize the oocyte. We therefore hypothesize that additional DNA damage perhaps caused by reactive oxygen species in the oviduct and fertilizing media is more extensive in IUI and conventional IVF, compared to when the sperm is directly injected into the oocyte. Perhaps therefore ICSI is more lenient to the DNA integrity, compared to the other reproductive techniques.

The SCSA protocol described by Evenson and coworkers (Evenson and Jost, 2000) with slight modification (Boe-Hansen *et al*., 2005) was used. Our results showed small but significantly higher values for the SCSA variable DFI in samples after incubation for 5 min on ice post-thawing compared to those after 10 min incubation on ice post-thawing, prior to staining and analysis. This observation is in agreement with our previous results; however, a statistical difference in DFI between the two time points was not found (Boe-Hansen *et al*., 2005). This stresses the importance of keeping a short and standardized incubation time post-thawing and prior to staining in the SCSA analysis with meticulous adherence to the SCSA protocol.

In conclusion, the present study confirms that high levels of DNA fragmentation are compatible with ongoing pregnancy and delivery, using ART, especially ICSI. Levels as high as 47.9% in DFI could result in ongoing pregnancy and delivery of an apparently healthy child using ICSI. Future studies may show whether a specific or statistical threshold exists for sperm

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DNA fragmentation, which is incompatible with pregnancy using ICSI, and perhaps why increased DNA fragmentation of the paternal gamete apparently is of less importance in ICSI compared to natural conception or conventional IVF treatment. Finally, the question whether ICSI can be considered a safe treatment for couples with male infertility caused by DNA fragmentation should be addressed.

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