The predictive value of sperm chromatin structure assay (SCSA) parameters for the outcome of intrauterine insemination, IVF and ICSI

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INTRODUCTION: Sperm chromatin integrity assessment has been suggested as a fertility predictor. The aim of this study was to examine the relationship between the results of sperm chromatin structure assay (SCSA) and the outcome of IVF, ICSI and intrauterine insemination (IUI). METHODS: A total of 306 consecutive couples undergoing assisted reproduction were included. IUI was performed in 131, IVF in 109 and ICSI in 66. SCSA results were expressed as DNA fractionation index (DFI) and highly DNA stainable (HDS) cell fractions. Reproductive outcome parameters were biochemical pregnancy (BP), clinical pregnancy (CP) and delivery (D). RESULTS: For IUI, the chance of pregnancy/delivery was significantly higher in the group with DFI <27% or HDS <10% than in patients with DFI >27% or HDS >10%. The odds ratios (ORs) (95% confidence intervals) were 20 (2.3–117), 16 (1.9–137) and 14 (1.6–110) for BP, CP and D, respectively. No statistical difference between the outcomes of IVF versus ICSI was observed in the group with DFI <27%. In the DFI >27% group, however, the results of ICSI were significantly better than those of IVF. Comparing ICSI with IVF, the OR (95% CI) for BP was 26 (1.9–350). CONCLUSIONS: SCSA is a useful method for prediction of the outcome of assisted reproduction.

Key words: ART/DNA damage/pregnancy rate/sperm chromatin

Introduction

Sperm nuclear condensation is a complex process that involves a series of critical events including rearrangements of chromosomes, transition of DNA-binding proteins and alteration of transcription (Ward, 1994; Kramer and Krawetz, 1997). Disturbances in this process may have a negative impact on male fertility as the highly organized and compact sperm chromatin is known to have a crucial influence on the fertilizing process (Twigg et al., 1998a,b; Evenson et al., 1980, 1999, 2002; Larson et al., 2000; Spano et al., 2000; Braun, 2001; Agarwal and Said, 2003; Perreault et al., 2003; Sakkas et al., 2003). Infertile men are reported to have a higher fraction of sperm with chromatin defects and DNA breaks than fertile controls (Evenson et al., 1980, 1999; Lopes et al., 1998; Host et al., 1999; Gandini et al., 2000; Irvine et al., 2000; Larson et al., 2000; Carrell and Liu, 2001; Hammadeh et al., 2001; Zini et al., 2001, 2002; Sakkas et al., 2002; Saleh et al., 2002, 2003; Erenpreisa et al., 2003; Muratori et al., 2003). Moreover, sperm DNA defects may have a possible negative impact on the outcome of assisted reproductive techniques (ARTs) (Lopes et al., 1998; Larson et al., 2000; Evenson and Jost, 2000; Duran et al., 2002; Morris et al., 2002; Tomatsu et al., 2002; Benchaib et al., 2003; Larson-Cook et al., 2003; Saleh et al., 2003). Furthermore, it has been questioned whether or not ART is able to compensate for poor DNA quality (Twigg et al., 1998b; Evenson et al., 1999; Larson et al., 2000; Larson-Cook et al., 2003).

The sperm chromatin structure assay (SCSA), first described by Evenson et al. (1980), is a flow cytometric (FCM) technique which identifies the spermatozoa with abnormal chromatin packaging defined as susceptibility to acid-induced DNA denaturation in situ. Acridine orange (AO) staining is used, after a low pH challenge, to distinguish between denaturated (red fluorescence = single stranded) and native (green fluorescence = double stranded) DNA regions in sperm chromatin, the former being a result of DNA breaks and/or of derailments in protamine quantity and composition and/or of an insufficient level of disulfide groups (Sakkas et al., 1999; Irvine et al., 2000; Aitken and Krausz, 2001). The level of DNA breaks is conveniently expressed by the DNA fragmentation index (DFI) (Larson et al., 2000; Evenson et al., 2002; Larson-Cook et al., 2003), which is the ratio of red to total (red plus green) fluorescence intensities in the flow cytometric analysis. In two independent studies, DFI levels >30–40% were incompatible with fertility in vivo, whatever sperm concentration, morphology and motility (Evenson et al., 1999; Spano et al., 2003).
et al., 2000). Furthermore, although based on limited numbers of patients, no pregnancy has been reported after in vitro ART procedures, both standard IVF and ICSI, when the DFI in raw semen was >27% (Larson et al., 2000; Larson-Cook et al., 2003; Saleh et al., 2003). As a consequence, the SCSA method has been recommended for routine use in ART programmes in order to predict a couple’s probability of obtaining a pregnancy (Evenson and Jost, 2000). Before recommending SCSA as routine prior to ART, however, the predictive value of this method needs to be tested in larger scale studies where other parameters of importance for the success of the treatment (e.g. sperm concentration and motility, age of the female and number of previous unsuccessful ART treatments) are taken into consideration.

The present study was set up to examine the relationship between sperm chromatin defects evaluated by the SCSA and the outcome of IVF, ICSI and intrauterine insemination (IUI), respectively.

### Materials and methods

#### Patients and study design

The study was based on a cohort of consecutive infertile couples undergoing ART treatment at the Fertility Clinic, Skive Hospital during the period April 2002–March 2003. Only cycles with a sperm concentration of at least 1 x 10^6/ml in raw semen were included in the study. All patients were to take part in our ART programme. The inclusion criteria for the female partner was: (i) female age ≤40 years; (ii) female body mass index (BMI) <30 kg/m²; and (iii) female baseline FSH <12 IU/l. For each couple, only one treatment cycle during the study period was included in the analysis. The study was approved by the Ethics Committee of Viborg County.

A total of 306 patients were included in the study: 131 couples underwent IUI, 109 IVF and 66 ICSI. All demographic data including female BMI, pre-treatment levels of FSH and number of previous ART treatments for all the three categories of treatments are given in Table I.

#### Sperm collection and semen analysis

Semen samples were collected by masturbation at the day of oocyte retrieval or insemination. After semen analysis, within 1 h from the time of ejaculation, 100 µl of the raw semen sample was frozen at –80°C for later SCSA analysis. Semen analysis was performed according to the World Health Organization guidelines (World Health Organization, 1999).

### Table I. Background characteristics for 306 patients undergoing IUI or IVF/ICSI

<table>
<thead>
<tr>
<th>IUI</th>
<th>DFI &lt;27%</th>
<th>DFI &gt;27%</th>
<th>IVF/ICSI</th>
<th>DFI &lt;27%</th>
<th>DFI &gt;27%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Couples included (n)</td>
<td>108</td>
<td>23</td>
<td>140</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Female age, median (range) (years)</td>
<td>30.2 (24.4–40.4)</td>
<td>32.1 (25.7–37.6)</td>
<td>31.2 (22.3–40.0)</td>
<td>32.1 (25.2–39.6)</td>
<td></td>
</tr>
<tr>
<td>BMI, median, (range) (kg/m²)</td>
<td>23.8 (16.5–30.0)</td>
<td>23.6 (18.1–30.0)</td>
<td>23.7 (17.3–30.0)</td>
<td>22.7 (18.6–30.0)</td>
<td></td>
</tr>
<tr>
<td>Couples undergoing first or second ART treatment (%)</td>
<td>76.0</td>
<td>70.0</td>
<td>71.4</td>
<td>60.0</td>
<td></td>
</tr>
<tr>
<td>bFSH, median (range) (IU/l)</td>
<td>6.8 (1.1–12.0)</td>
<td>7.0 (2.4–12.0)</td>
<td>6.6 (1.7–12.0)</td>
<td>6.8 (2.1–12.0)</td>
<td></td>
</tr>
<tr>
<td>Male age, median (range) (years)</td>
<td>31.1 (24.3–55.3)</td>
<td>32.4 (27.0–56.7)</td>
<td>31.2 (23.5–47.6)</td>
<td>34.9 (28.0–49.5)</td>
<td></td>
</tr>
</tbody>
</table>

### Sperm chromatin structure assay (SCSA)

Sperm DNA damage was measured by SCSA following the procedure as described elsewhere (Spano et al., 2000; Evenson et al., 2002). On the day of analysis, the samples were quickly thawed and analysed immediately. A cell suspension (1–2 x 10^6) was treated with a low-pH (pH 1.2) detergent solution containing 0.1% Triton X-100, 0.15 mol/l NaCl and 0.08 mol/l HCl for 30 s, followed by staining with 6 mg/l purified AO (Molecular Probes, Eugene, OR) in a phosphate-citrate buffer, pH 6.0. Cells were analysed using a FACSort flow cytometer (Becton Dickinson, San Jose, CA), equipped with an air-cooled argon ion laser. A total of 5000 events were accumulated for each measurement. Under these experimental conditions, when excited with a 488 nm light source, AO intercalated with double-stranded DNA emits green fluorescence and AO associated with single-stranded DNA emits red fluorescence. Thus, sperm chromatin damage can be quantified by the FCM measurements of the metachromatic shift from green (native, double-stranded DNA) to red (denatured, single-stranded DNA) fluorescence and displayed as red (fragmented DNA) versus green (DNA stainability) fluorescence intensity cytogram patterns. For the flow cytometer set-up and calibration, reference samples were used from a normal donor ejaculate sample retrieved from the laboratory repository. Off-line analysis of the flow cytometric data was carried out using dedicated software (List View, Phoenix Flow Systems, San Diego, CA). Computer gates are used to determine the proportion of spermatozoa with increased levels of red fluorescence (denatured single-stranded DNA) and green fluorescence (native double-stranded DNA). We have expressed the extent of DNA denaturation in terms of the DFI, which is the ratio of red to total (red plus green) fluorescence intensity, i.e. the level of denatured DNA over the total DNA (Evenson et al., 2002). The DFI value was calculated for each sperm cell in a sample, and the resulting DFI frequency profile was obtained (Figure 1, upper panel). Most sperm form a unimodal distribution representing the normal population of sperm with no detectable DNA damage. Sperm with higher red fluorescence, falling in the histogram area beyond the curve of normal sperm, represent the population of abnormal sperm with detectable DFI. Additionally, we have also considered the fraction of high DNA stainable (HDS) cells. HDS represents another distinct population in semen that characterizes immature spermatozoa with incomplete chromatin condensation. The percentage of HDS cells was calculated by setting an appropriate gate on the bivariate cytogram (Figure 1, lower panel) and considering as immature spermatozoa those events which exhibit a green fluorescence intensity higher than the upper border of the main cluster of the sperm population with a non-detectable DFI.

All SCSA measurements have been performed on raw semen cryopreserved prior to the preparation for IUI, IVF or ICSI. SCSA studies addressing the outcome of ART were based predominantly on
Predictive value of SCSA for ART outcome

**IVF and ICSI**

Hormonal treatment. IVF/ICSI patients underwent pituitary down-regulation and hormonal treatment as previously described (Bungum et al., 2003). In brief, patients were downregulated with a GnRH agonist (Suprefact, Hoechst, Hørsholm, Denmark), using a long or a short protocol. Ovarian stimulation was performed with rec-FSH (Gonal F; Serono Nordic) (using an individualized dose of between 100 and 375 IU s.c. per day according to age, ovarian volume, baseline FSH and BMI). The ovarian response was monitored by ultrasound examination starting on day 8 of stimulation, and the dose of rec-FSH administered was adjusted if necessary.

Oocyte retrieval and sperm preparation. When at least one follicle had reached a diameter of ≥17 mm, 10 000 IU of HCG (Profasi; Serono Nordic) was administered to induce final follicular maturation. Oocyte retrieval was performed 35 h later by vaginal ultrasound-guided follicle aspiration. Gamete™ (Vitrolife, Gothenburg, Sweden), a HEPES-buffered medium, was used to rinse the oocytes.

A standard density gradient centrifugation method, PureSperm, 45 and 90% (Nidacon Ltd) diluted in SpermRinse™ (Vitrolife) was used for sperm preparation. The washing procedure and dilution was performed in IVF-100™ (Vitrolife).

**IVF.** In cases of conventional IVF, spermatozoa with a final concentration of 150 000/ml were added to the oocytes. After incubation in a humidified incubator with a gas phase of 6% CO₂, 5% O₂ and 89% N₂ for 90 min, the oocytes were washed three times before further culture in IVF-100™ until time of assessment for fertilization.

**ICSI.** In cases of ICSI, denudation of cumulus cells was performed by exposure of the oocytes to HYASE™ (Vitrolife) for a maximum of 30 s. Denudation of cumulus cells was performed by the use of glass denuding pipettes (SweMed Lab, Billdal, Sweden) immediately before injection. The oocytes were washed four times in Gamete™ after denudation. ICSI was performed in Gamete™ covered by OVOIL™ (Vitrolife) using commercially available ICSI pipettes (Cook, Brisbane, Australia). A 5 µl ultra-microdroplet of polyvinylpyrrolidone (PVP), ICSI-100™ (Vitrolife) was spread out in a thin layer on the Petri dish. A 1 µl aliquot of the sperm preparation was introduced to the centre of the PVP droplet. After injection, the oocytes were washed twice in G.1.2™ (Vitrolife) medium before further culture in G.1.2™

**Culture media and procedure for culturing**

IVF-100™ was used in the first step of culture, from oocyte retrieval to fertilization, after which the oocytes were rinsed twice in G.1.2™ and cultured in G.1.2™ until the morning of day 3. In cases of blastocyst culture, G.2.2™ (Vitrolife) was used from the 8-cell stage on day 3 until embryo transfer.

A maximum of six fertilized oocytes were cultured in 20 µl media droplets under oil (G.300™). In cases with fewer than six oocytes, 10 µl droplets were used. A gas phase of 6% CO₂, 5% O₂ and 89% N₂ was used in a humidified incubator.

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**Intrauterine insemination (IUI), hormonal treatment and sperm preparation**

All IUI patients had a diagnosis of unexplained infertility. Normal tubal patency was confirmed by either a hysterosalpingography (HSG) or by laparoscopy. IUI was only performed in cases with at least 2.5 × 10⁶ spermatozoa per ml after gradient centrifugation. The IUI patients were stimulated with clomiphene (Pergotone; Serono Nordic, Copenhagen, Denmark) on cycle days 3–7 and 75 IU of recombinant FSH (rec-FSH) (Gonal F; Serono Nordic) on cycle days 7–9. When 1–2 follicles had reached a diameter of ≥17 mm, 10 000 IU of HCG (Profasi; Serono Nordic) was administered to induce final follicular maturation. After 36 h, a fresh semen sample was prepared by a standard density gradient centrifugation method. PureSperm, 40 and 80% (Nidacon Ltd, Gothenburg, Sweden) diluted in SpermRinse™ (Vitrolife, Gothenburg, Sweden) was used for sperm preparation. The washing procedure and dilution was performed in IVF-100™ (Vitrolife, Gothenburg, Sweden). A 1 ml aliquot of the prepared semen sample was used for insemination with a Cashmed catheter (catalogue no. IU118; Cashmed, Surrey, UK). No luteal phase support was given.

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Figure 1. Upper panel: frequency distribution histogram of the DNA fragmentation index (DFI). The area located to the right of the main peak (which includes normal sperm with non-detectable DFI) represents the region where the sperm with detectable levels of fragmented DNA accumulate. Lower panel: SCSA scattergram of red (fragmented DNA, x-axis) versus green (double-stranded DNA, y-axis) fluorescence intensity of the same semen sample. Cytogram dots represent single spermatozoa with dual-parameter green and red fluorescence values acquired at 10-bit resolution (1024 dots represent single spermatozoa with dual-parameter green and red fluorescence values acquired at 10-bit resolution (1024 channels) on the flow cytometer. Debris (bottom left corner) were excluded from the analysis. The region for calculating the fraction of immature sperm with high DNA stainability (HDS) is indicated by the box. The long dashed line indicates the threshold for HDS (channels 550 on the y-axis).

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*data derived from raw semen (Larson et al., 2000; Larson-Cook et al., 2003; Saleh et al., 2003).*
Assessment of fertilization, embryo morphology classification, cryopreservation and embryo transfer

Fertilization was determined 18–20 h after the IVF/ICSI procedure. The oocytes were fertilized when two distinct pronuclei were visible. Cleavage and classification of morphology was assessed 24 h later by criteria set up by Ziebe S, Groendal ML, Agerholm I and Erb K (personal communication). Strict criteria for cryopreservation were used. Only embryos with at least seven blastomeres and <20% intracellular fragments were cryopreserved on day 3. On day 5, embryos were assessed according to scoring criteria for blastocysts (Gardner and Schoolcraft, 1999). Only expanded blastocysts were cryopreserved.

A maximum of two embryos were transferred on day 2, 3 or 5 after retrieval. Embryo transfer was performed in G2.2™. All embryo transfers were performed with a Cook Soft 5000 catheter (Cook, Brisbane, Australia).

Luteal phase support and pregnancy test

IVF/ICSI patients received luteal phase support in the form of daily vaginal administration of micronized progesterone, either 400 mg twice a day (Cyclogest; Hoechst, Copenhagen, Denmark) or 90 mg once a day (Crinone 8%; Serono Nordic) starting on the day following oocyte retrieval and continuing until the day of the pregnancy test (i.e., day 12 after embryo transfer). A positive pregnancy test was defined by a plasma βHCG concentration >10 IU/l. A clinical pregnancy was defined as an intrauterine gestational sac with a heart beat 3 weeks after a positive HCG test. The implantation rate was calculated as the ratio of gestational sacs determined by ultrasound after 7 weeks in relation to the total number of embryos transferred. Birth or abortion was recorded for all patients.

Statistical methods

Statistical analysis was performed using the SPSS 11.0 software (SPSS Inc., Chicago, IL). To test inter-group differences in sperm concentration, percentage progressive motile sperm, DFI and HDS, the Mann–Whitney test was applied. Binary correlations were assessed by calculating Spearman’s r. The reproductive outcome measures were treated as discrete variables (±fertilization; ±biochemical pregnancy; ±clinical pregnancy; ±delivery). Since a DFI of 27% previously was reported to be the cut-off level for achieving a pregnancy by *in vitro* ART (Larson-Cook et al., 2003), the couples were grouped according to whether the DFI in raw semen sample was ≤27% or >27%. Furthermore, having assessed the results of HDS analysis in relation to the reproductive outcomes, a cut-off level of 10% was defined for this parameter. By the use of Fischer’s exact test, the chance of a positive reproductive outcome was calculated for the different treatment groups (IUI, IVF and ICSI) divided according to the DFI and HDS as described above (DFI: ≤27% and >27%; HDS: ≤10% and >10%). To compare the results of IVF and ICSI, in the group with DFI >27%, odds ratios (ORs) and the 95% confidence intervals (95% CIs) were calculated by the use of binary logistic regression analysis. Fischer’s exact test and OR calculations were also applied to compare the reproductive outcomes, i.e., biochemical and clinical pregnancy in two groups defined as follows: group 1: DFI ≤27% and HDS ≤10%; and group 2: DFI >27% or HDS >10%. A P-value ≤0.05 was considered as statistically significant.

Results

**IUI**

The median (range) age of women was 30.2 (24.4–40.4) years in the group with DFI ≤27% and 32.1 (25.7–37.6) years in the group with DFI >27%. The median (range) age of men was 31.1 (24.3–55.3) years in the group with DFI ≤27%, whereas the median (range) age was 32.4 (27.0–56.7) years in the group with DFI >27% (Table I). When comparing the groups with and without clinical pregnancy, there were no statistically significant differences regarding DFI (medians: 12.1 versus 15.7; P = 0.14) or HDS (medians: 6.3 versus 6.7; P = 0.12). With biochemical pregnancy as a grouping parameter, lower DFI and HDS values were found in couples with positive reproductive outcome, the difference not being statistically significant for DFI [12.9% (4.3–33.7) versus 15.4% (3.0–48.7); P = 0.25] but close to the level of statistical significance for HDS [6.2% (3.6–17.8) versus 6.7% (2.7–20.2); P = 0.06].

There was a statistically significant negative correlation between DFI and the percentage of progressively motile spermatozoa (ρ = −0.25; P = 0.004), but not between DFI and sperm concentration. HDS correlated in a negative manner with both sperm concentration (ρ = −0.36; P < 0.0005) and percentage progressively motile sperms (ρ = −0.25; P = 0.004).

**Predictive value of DFI >27%**. One biochemical pregnancy was observed in the 23 cycles with males having DFI >27% (4.3%), whereas in the ≤27% group, 24 of the 108 cycles resulted in a positive pregnancy test (22.2%) (P = 0.07; Table II). The clinical pregnancy and delivery rates were 4.5 versus 20.2% (P = 0.12) and 4.5 versus 17.6% (P = 0.20), respectively. In the above-mentioned case where the DFI was >27%, the actual value being 33.7%, there was a positive biochemical and subsequently even clinical pregnancy and delivery. When the cut-off level was increased to a DFI of 34%, there were no statistically significant differences with regard to any of the reproductive outcomes.

### Table II. Comparison of reproductive outcomes in the 306 couples undergoing assisted reproduction, according to the treatment type and the DFI

<table>
<thead>
<tr>
<th>Treatment Type</th>
<th>DFI &lt;27%</th>
<th>DFI &gt;27%</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cycles started, n</strong></td>
<td>108</td>
<td>23</td>
<td>–</td>
</tr>
<tr>
<td><strong>Embryo transfer (ET), n (% per started cycle)</strong></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Biochemical pregnancies, n (% per ET)</strong></td>
<td>24 (22.2%)</td>
<td>1 (4.3%)</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Clinical pregnancies, % per ET or IUI</strong></td>
<td>22 (20.2%)</td>
<td>1 (4.5%)</td>
<td>0.20</td>
</tr>
<tr>
<td><strong>Implantation rate, %</strong></td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><strong>Deliveries, % per started cycle</strong></td>
<td>19 (17.6%)</td>
<td>1 (4.5%)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Note: One ectopic pregnancy.

NS: P > 0.05.
Predictive value of HDS>10%. Only one biochemical and clinical pregnancy, and birth was seen when the HDS exceeded the value of 10%. An HDS of 17.8% was found in the sample having a DFI of 33.7%, as mentioned above. There were 24 biochemical and 21 clinical pregnancies, respectively, among those 115 having a HDS of ≤10%. Nineteen of the clinical pregnancies resulted in a delivery. The difference between the two groups defined according to the HDS level was not statistically significant.

Predictive value of combined use of DFI and HDS. When the patients were divided into two groups, group 1, with semen samples with DFI ≤27% and HDS ≤10%, and group 2, with either DFI >27% or HDS >10%, 24 biochemical pregnancies, 21 clinical pregnancies and 19 deliveries were observed among the 95 couples in group 1, whereas only one of the 36 couples in group 2 experienced a biochemical as well as a clinical pregnancy and, finally, delivery. These differences in reproductive outcomes between group 1 and group 2 were statistically significant, the P-values being 0.002 for biochemical pregnancy, 0.008 for clinical pregnancy and 0.01 for delivery rate. The OR for biochemical pregnancy in group 1, with group 2 as reference, was 12 (95% CI 1.5–96). This value increased to 20 (95% CI 2.3–117) when sperm concentration, percentage progressively motile, female age and treatment number were included in the model. For clinical pregnancies, the ORs in group 1 were 9.9 (95% CI 1.3–77) and 16 (95% CI 1.9–137), without and including the above-mentioned covariates, respectively. For delivery as an outcome variable, the ORs were 8.7 (95% CI 1.1–68) and 14 (95% CI 1.6–110), respectively, with and without inclusion of sperm concentration, percentage progressively motile spermatozoa, female age and treatment number in the model.

IVF/ICSI

There was no difference in sperm concentration, percentage of progressively motile sperm, DFI or HDS when couples with positive and negative reproductive outcome were compared, regardless of whether biochemical or clinical pregnancy was used as a criterion. This lack of a statistically significant difference was true for the whole group of IVF/ICSI-treated couples and also when the two treatment types were analysed separately (data not shown).

Similarly to the findings in the IUI group, there was a statistically significant negative correlation between both DFI and HDS and the percentage of progressively motile sperm, and additionally between HDS and sperm concentration (data not shown).

There was a considerable overlap between DFI and HDS values when couples who achieved a pregnancy were compared with those who did not (Figure 2).

The likelihood of obtaining a clinical pregnancy was the same in the group of patients with DFI >27% (38.2%) and those with DFI ≤27% (38.2%). The implantation rates were also comparable, 31.4 versus 34.3%. Forty-four women (31.4%) in the DFI ≤27% group and 12 of the women (34.3%) in the group with DFI >27% (P = 0.8) all delivered healthy children. Two pregnancies in the DFI ≤27% group and one in the DFI >27% group were ectopic (Table III).

Predictive value of DFI >27%. In the DFI >27% group, the results of ICSI were significantly better than those of standard IVF regarding biochemical (70.6 versus 22.2%; P = 0.007) and clinical pregnancies (52.9 versus 22.2%; P = 0.09). For delivery rates, the percentages were 47.1% in the ICSI and 22.2% in the standard IVF group (P = 0.16) (Table III), with an OR of 7.8 (95% CI 1.7–36) for a positive HCG test, 3.9 (95% CI 0.9–17.0) for clinical pregnancy and 3.1 (95% CI 0.72–13) for delivery rate. After inclusion of sperm concentration, percentage progressively motile sperm, female age and treatment number in the model, the ORs for successful reproductive outcome when ICSI was compared with standard IVF increased to 26 (95% CI 1.9–350), 7.7 (95% CI 0.84–70) and 7.0 (95% CI 0.75–65), with biochemical pregnancy, clinical pregnancy and delivery as dependent variables, respectively. Significantly higher DFI (P = 0.014) (medians 18.0 versus 16.61) and HDS (medians 7.3 versus 6.81) (P = 0.033) were found in the ICSI group compared with IVF.

Discussion

Two major conclusions can be drawn from this, to our knowledge, largest ever reported study on the predictive value of SCSA in relation to the outcome of IUI, IVF and ICSI. First, combining the two SCSA parameters, DFI and HDS gave a high predictive value regarding the outcome of IUI treatments. Secondly, contrary to previous reports (Larson et al., 2000; Larson-Cook et al., 2003; Saleh et al., 2003), we found that a
Table III. Comparison of the reproductive outcomes between couples undergoing IVF and ICSI, allocated according to the DFI

<table>
<thead>
<tr>
<th>Cycles started, n</th>
<th>DFI &lt;27%</th>
<th>DFI &gt;27%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IVF</td>
<td>ICSI</td>
</tr>
<tr>
<td>Embryo transfer (ET), n ( % e started cycle)</td>
<td>91</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>82 (90.1%)</td>
<td>41 (83.7%)</td>
</tr>
<tr>
<td>Biochemical pregnancies, n IVF/ICSI ( % per ET)</td>
<td>42 (51.2%)</td>
<td>21 (51.2%)</td>
</tr>
<tr>
<td></td>
<td>4 (22.2%)</td>
<td>4 (22.2%)</td>
</tr>
<tr>
<td>Clinical pregnancies, % per ET</td>
<td>30 (36.6%)</td>
<td>17 (41.5%)</td>
</tr>
<tr>
<td></td>
<td>4 (22.2%)</td>
<td>4 (22.2%)</td>
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<tr>
<td>Implantation rate, n ( %)</td>
<td>47/141 (33.3%)</td>
<td>24/76 (31.6%)</td>
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<td></td>
<td>6/31 (19.4%)</td>
<td>12/32 (37.5%)</td>
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<tr>
<td>Deliveries, % pr started cycle</td>
<td>27 (29.7%)</td>
<td>17 (34.7%)</td>
</tr>
<tr>
<td></td>
<td>4 (22.2%)</td>
<td>8 (47.1%)</td>
</tr>
</tbody>
</table>

*aTwo ectopic pregnancies.
*bOne ectopic pregnancy.
NS: P > 0.05.

DFI level >27% is compatible with pregnancy and delivery after both IVF and ICSI. Thus it seems that in vitro ART is able to compensate for the impairment of sperm chromatin integrity, in particular if ICSI is chosen as a fertilization method.

In couples treated with IUI, clinical pregnancy was seen in only one out of 23 cases when the DFI exceeded 27% and/or a HDS >10% was found in the raw ejaculate prepared and used for insemination. The OR was 16 for clinical pregnancy and 14 for delivery when both these parameters were below the cut-off levels given above. This finding is in accordance with previously published in vivo studies in which the time to pregnancy (TTP) for couples with no previous infertility background has been studied (Evenson et al., 1999; Spano et al., 2000). These studies indicated a DFI level of 30–40% as a cut-off for no pregnancy. Two other recently published studies (Duran et al., 2002; Saleh et al., 2003) reached a similar conclusion. Saleh et al. (2003), using the SCSA, found significantly higher DFI levels in the couples who failed to obtain a pregnancy after IUI. This study was, however, based on 11 IUI couples only. Using the TUNEL assay indicative for DNA fragmentation, Duran et al. (2002) found that men with a DNA fragmentation level >12% had a decreased chance of fathering a pregnancy following IUI.

Apart from slightly lower (close to the level of statistical significance) HDS in men of those IUI-treated couples who achieved a biochemical pregnancy, there was no statistically significant difference in SCSA parameters between the groups with a negative and a positive reproductive outcome. The SCSA parameter, HDS, is believed to correlate to the level of immature spermatozoa and the extent of chromatin packaging (Evenson and Jost, 2000). Although previous studies indicated that high HDS could be a pejorative factor for in vivo fertility impairment (Evenson et al., 1999, 2002), in the available materials, the associations did not reach a level of statistical significance. In our study, when a relatively large number of couples were included, an HDS of ≈10% was associated with a higher proportion of clinical pregnancies and births, although the statistical significance was borderline. It is therefore to be considered, as a novel finding, that the predictive value of SCSA increases when both DFI and HDS are used to define groups with high and low chances of obtaining a pregnancy after IUI. Recently, we have shown that both DFI and HDS are independently and negatively associated with sperm motility (Giwercman et al., 2003). Motility is essential for fertilization in vitro. Impairment of the ability of spermatozoa to reach the ovum might at least partly explain the association between SCSA parameters and the probability of obtaining a pregnancy after IUI. An additional explanation could be the fact that spermatozoa with damaged DNA could be more likely to undergo apoptosis during the transport through the genital tract than spermatozoa with normal DNA integrity (Edwards, 2003).

The present study demonstrates that even men with high levels of DNA damage can become biological fathers, when DFI exceeded 27%; however, significantly higher clinical pregnancy rates (52.9 versus 22.2%) and delivery rates (47.1 versus 22.2%) were seen after ICSI compared with IVF. When DFI exceeded 27%, the OR for a positive reproductive outcome after ICSI compared with standard IVF was 8 for biochemical pregnancy, 4 for clinical pregnancy and 3 for delivery, respectively. These ORs increased after inclusion of sperm concentration, the percentage of progressively motile sperm, number of treatments and the age of the female as co-variates. The increase in OR was statistically significant (95% CIs not including the value of 1) for biochemical pregnancy, but not for the two other reproductive outcomes, probably due to the limited number of couples included in this study. None of the previous studies dealing with this issue (Larson et al., 2000; Larson-Cook et al., 2003; Saleh et al., 2003) have taken into consideration the age of the female and the number of previously failed ART treatments, parameters known to be associated with the success rate of the treatment. Therefore, our findings show that DFI as assessed by means of SCSA is an independent predictor of male fertility potential in vitro.

This is the first study showing that semen samples with high rates of DNA breaks as assessed by the SCSA method are more likely to result in pregnancy in ICSI procedures, compared with traditional IVF. The finding is novel in relation to the SCSA parameters, but confirms the results of a recent study by Host et al. (2000) using the TUNEL assay.

The safety of the ICSI procedure has been questioned (Schultz and Williams, 2002), and the findings from the present study may give further reason for concern. ICSI enables fertilization to take place in spite of severely compromised semen characteristics and damaged DNA. Aitken and Krausz (2001) proposed that sperm DNA damage is promutagenic and
can give rise to mutations after fertilization, as the oocyte attempts to repair DNA damage prior to the initiation of the first cleavage. Mutations occurring at this point will be fixed in the germline and may be responsible for the induction of pathology, such as infertility (Aitken and Krausz, 2001), childhood cancer in the offspring (Fraga et al., 1996; Ji et al., 1997; Aitken and Krausz, 2001) and imprinting diseases (Cox et al., 2002; DeBaun et al., 2003). So far, however, follow-up studies of children born after ICSI compared with children born after conventional IVF have not been conclusive regarding the risks of congenital malformations and health problems in general (Bonduelle et al., 1996, 1998, 2003; Kurinczuk and Bower, 1997; Sutcliffe et al., 1999; Wennerholm and Berg., 2000; Hansen et al., 2002). It has been reported previously that the highest level of sperm DNA damage usually is found in men with the poorest semen quality, i.e. ICSI candidates (Aitken and Krausz, 2001). In the present study, the men in the ICSI group had significantly higher DFI levels compared with the men in the IVF group (median 18.0 versus 15.0%, respectively). It gives reason for concern that the most efficient ART techniques are used to treat males with the highest level of sperm DNA damage. SCSA parameters, however, refer to the DNA status of the whole cell population, not excluding the presence of a subpopulation with no significant DNA damage. Host et al. (2000), suggested that the technician who performs ICSI attempts to select spermatozoa with normal morphology and thereby reduces the risk of introducing spermatozoa with strand breaks. This statement may, however, be questioned, since the traditional sperm parameters such as sperm count, motility and morphology have been proven to be poorly correlated to the SCSA parameters (Evenson et al., 1991, 1999; Spano et al., 1998, 2000). A future challenge will be to develop methods to identify and select spermatozoa with intact DNA during the ICSI procedure.

We selected a DFI level of 27% as a cut-off for calculation of ORs in relation to the reproductive outcome. This level was defined previously in reports where the SCSA measurement was performed in other laboratories. One could ask whether each laboratory performing the SCSA analysis should have its own reference levels or not. Apart from being subject to a very limited intra-laboratory variation (Giwercman et al., 1999), however, the SCSA analysis was shown to be very robust to variation between laboratories. In an external quality control based on >180 samples, a high (r = 0.8) correlation was found between the values obtained by our laboratory and those from a control laboratory. Furthermore, not only was there a high level of correlation between the results reported by two independent laboratories that strictly followed the SCSA protocol, but the absolute DFI values obtained at two different places, using different equipment, did not on average differ by >1% (Giwercman et al., 2003). Furthermore, in the present study, viewing the distribution of DFI values between those who became pregnant and those who did not, 27% was confirmed to be an appropriate cut-off level.

The results from this study confirm previous reports indicating a low chance of obtaining in vivo pregnancy when the DFI exceeds the level of 27%. Contrary to previous reports, the present study has shown that a DFI level >27% is not incompatible with pregnancy and delivery following IVF and ICSI. ICSI seems to be the most efficient method in cases with high levels of sperm DNA breaks. Additional large-scale trials are needed to confirm the results of the present study and to investigate further the value of SCSA in relation to other available pregnancy predictors in vivo or in vitro. Methods for selection of spermatozoa with intact DNA should be developed in order to avoid the potential risk of using gametes with chromatin breaks for ICSI treatment.

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