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Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome

M.Bungum[1,2,5], P.Humaidan[1], A.Axmon[3], M.Spano[4], L.Bungum[1], J.Erenpreiss[2] and A.Giwercman[2]

[1]Fertility Clinic, Viborg Hospital (Skive), Skive, Denmark, [2]Fertility Centre, Scanian Andrology Centre, Malmö University Hospital, Malmö, [3]Division of Occupational and Environmental Medicine and Psychiatric Epidemiology, Lund University, Lund, Sweden and [4]Section of Toxicology and Biomedical Sciences, BIOTEC-MED, ENEA Casaccia Research Center, Rome, Italy

[5]To whom correspondence should be addressed at: Fertility Centre, Scanian Andrology Centre, Malmö University Hospital, Malmö, Sweden. E-mail: mona.bungum@med.lu.se

BACKGROUND: The sperm chromatin structure assay (SCSA) has been suggested as a predictor of fertility *in vivo* as well as *in vitro*. The available data however, have been based on limited numbers of treatments. We aimed to define the clinical role of SCSA in assisted reproduction. METHODS: A total of 998 cycles [387 intrauterine insemination (IUI), 388 IVF and 223 ICSI] from 637 couples were included. SCSA results were expressed as DNA fragmentation index (DFI) and high DNA stainable (HDS) cell fractions. Outcome parameters were biochemical pregnancy (BP), clinical pregnancy (CP) and delivery (D). RESULTS: For IUI, the odds ratios (ORs) for BP, CP and D were significantly lower for couples with DFI >30% as compared with those with DFI ≤30%. No statistical difference between the outcomes of ICSI versus IVF in the group with DFI ≤30% was seen. In the DFI >30% group, the results of ICSI were significantly better than those of IVF. CONCLUSIONS: DFI can be used as an independent predictor of fertility in couples undergoing IUI. As a result, we propose that all infertile men should be tested with SCSA as a supplement to the standard semen analysis. When DFI exceeds 30%, ICSI should be the method of choice.

Key words: ICSI/IUI/IVF/SCSA/sperm DNA

Introduction

Infertility is a common condition. Approximately one in six couples seeks medical help at some time during their reproductive life due to infertility (Hull *et al*., 1985). Although prevalence of infertility is high and as many as 50% of the infertility problems are predominantly or partly due to a male factor (World Health Organization, 1987), the diagnostic tools in male fertility are insufficient (Jequier, 2004), being mainly based on the evaluation of sperm concentration, motility and morphology (World Health Organization, 1999). These parameters are, however, poorly standardized (Jorgensen *et al*., 1997), subjective (Auger *et al*., 2000) and not powerful predictors of fertility (Bonde *et al*., 1998; Guzick *et al*., 2001).

With the development of assisted reproductive technology (ART), the demand for treatment has increased substantially. ART covers intrauterine insemination (IUI), where fertilization occurs through *in vivo* and *in vitro* methods: IVF and ICSI, the latter developed to treat cases with impaired semen quality (Palermo *et al*., 1992). In Europe, the annual number of ART treatments has passed 270 000 (Andersen *et al*., 2005) whereas in 2002 the United States reported about 115 000 treatments (US Department of Health and Human Services, 2004). Numbers are expected to rise further because of delayed childbearing (Stephen and Chandra, 1998) and possibly declining sperm counts (Carlsen *et al*., 1992; Auger *et al*., 1995). Although the use of ART is well established, its costs are high (Garceau *et al*., 2002) seen in relation to the low take-home baby rates (20–30%) (Andersen *et al*., 2005). So far, except for female age (Hull *et al*., 1996), no other factor of significant prognostic value for the outcome of ART has been identified. Although the traditional sperm characteristics (World Health Organization, 1999) are poor fertility markers, they are used when deciding the type of treatment to be given to a couple. Therefore, patients may undergo expensive IVF/ICSI therapies where no such treatment is really indicated or on the contrary be treated with IUI or IVF where ICSI should have been performed.

Normal sperm chromatin structure is essential for a correct transmission of paternal genetic information, and it is well documented that there is a negative correlation between defective sperm chromatin structure (DNA breaks) and fertility, *in vivo* (Evenson *et al*., 1999; Spano *et al*., 2000) and *in vitro* (Evenson and Jost, 2000; Larson *et al*., 2000; Larson-Cook *et al*., 2003; Saleh *et al*., 2003; Bungum *et al*., 2004; Gandini *et al*., 2004; Virro *et al*., 2004; Check *et al*., 2005; Evenson and Wixon, 2006). However, although 30% of patients seeking ART have high rates of sperm DNA breaks (Bungum *et al*., 2004), very

few clinics, so far, have implemented routine DNA integrity testing. In one of these tests, the sperm chromatin structure assay (SCSA) DNA fragmentation index (DFI) is used to get an estimate of DNA breaks, a parameter suggested as an independent predictor of fertility (Evenson *et al*., 1999). The available SCSA studies, however, have been based on few subjects and can therefore only be seen as indicative (Evenson and Jost, 2000; Larson *et al*., 2000; Larson-Cook *et al*., 2003; Saleh *et al*., 2003; Bungum *et al*., 2004; Gandini *et al*., 2004; Virro *et al*., 2004; Check *et al*., 2005). To improve the diagnostics and therapeutic interventions for infertile couples, we initiated this prospective study. The aim was to test whether SCSA parameters can be used as independent predictors of ART outcome and to investigate whether the risk of early pregnancy loss is increased in pregnancies achieved by the use of semen samples with high DFI.

Materials and methods

Patients

The study was based on a cohort of consecutive infertile couples undergoing ART at Viborg Hospital during the period April 2002–December 2003. A total of 998 cycles (387 IUI, 388 IVF and 223 ICSI) from 637 couples were included. Male partners had a sperm concentration of at least 1×10^6/ml in raw semen. The inclusion criteria for the female partner were: (i) age <40 years; (ii) BMI <30 kg/m^2 and (iii) baseline FSH (b-FSH) <12 IU/l. Regarding demographic data including male/female age, female b-FSH and BMI, number of previous ART treatments and sperm parameters, no differences between the categories of treatments or DFI groups were seen (Table I). The choice of fertilization method was based upon infertility diagnosis. Whereas couples diagnosed with unexplained infertility were referred to IUI, the IVF group mainly consisted of couples with female factor infertility. The criteria for performing ICSI was a total sperm count of <500 000 after gradient centrifugation.

The study was approved by the Ethics Committee of Viborg County, and all patients provided written informed consent.

Semen collection and analysis

Semen samples were collected by masturbation on the day of oocyte retrieval or insemination. Sperm concentration was assessed by use of a Makler-chamber, and motility was scored according to the World Health Organization guidelines (World Health Organization, 1999). Sperm morphology was not assessed.

SCSA

The principles and procedure of measuring sperm DNA damage by flow cytometry (FCM) SCSA are described in details elsewhere (Evenson *et al*., 1999; Spano *et al*., 2000; Bungum *et al*., 2004). SCSA is based on staining of sperm nuclei with acridine orange, to evaluate the ratio of single and double stranded DNA (following acid exposure which causes denaturation of double stranded DNA in sperm with an impairment of their chromatin structure). Sperm chromatin damage was 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 versus green fluorescence intensity cytogram patterns. The extent of DNA denaturation is expressed as the DFI, which is the ratio of red to total fluorescence intensity, i.e. the level of denatured DNA over the total DNA. Additionally, we have considered the fraction of cells with high DNA stainable (HDS) cells, which are thought to represent immature spermatozoa with incomplete chromatin condensation. The intra-laboratory coefficient of variation was found to be 4.5% for DFI and 10% for HDS, respectively.

ART procedures

In IUI patients, all hormone stimulation and insemination procedures were performed as previously described (Bungum *et al*., 2004). In IVF/ICSI patients, hormonal treatment, oocyte retrieval, gamete handling, culture and embryo transfer were performed as previously described (Bungum *et al*., 2004).

Table I. Demographic data on 998 assisted reproductive techniques cycles divided according to the type of treatment; IUI, IVF and ICSI

	IUI		IVF		ICSI	
DFI ≤30%	DFI ≤30%	DFI >30%	DFI ≤30%	DFI >30%	DFI ≤30%	DFI >30%
Cycles included (*n*)	321	66	326	62	150	73
Female age (median in range) (years)	29.9 (21.2–40.6)	32.1 (23.7–38.9)	31.9 (22.7–40.6)	33.1 (25.2–40.4)	30.9 (22.4–40.4)	30.7 (24.4–40.4)
Female BMI (median in range) (kg/m^2)	23.9 (16.5–30.0)	23.7 (18.1–30.0)	24.0 (17.1–30.0)	23.7 (17.7–30.0)	24.5 (17.6–30.0)	23.8 (18.0–30.0)
Female b-FSH (median in range) (IU/l)	6.7 (1.1–12.0)	7.0 (2.4–10.0)	6.6 (1.7–12.0)	6.7 (1.1–12.0)	6.6 (2.0–12.0)	6.5 (2.6–12.0)
Number of previous treatments (median in range)	2 (1–6)	2 (1–8)	2 (1–6)	2 (1–6)	2 (1–6)	2 (1–5)
Oocytes retrieved, (median in range) (*n*)	–	–	8 (1–25)	8 (2–20)	7 (1–25)	8 (1–20)
Oocytes fertilized (2 pronuclei) median (range) (*n*)	–	–	4 (0–20)	5 (0–18)	4 (0–13)	3 (0–10)
Embryo transfer (*n*) (%/started cycle)	–	–	275 (84.4%)	55 (88.7%)	128 (85.3%)	65 (89.0%)
Embryos transferred, (median in range) (*n*)	–	–	2 (0–2)	2 (0–2)	2 (0–2)	2 (0–2)
Implantation rate (%)	–	–	28.7	27.0	34.1	40.5
Male age, (median in range) (years)	31.1 (23.3–56.7)	33.1 (26.2–46.2)	33.1 (23.7–62.3)	35.4 (25.0–56.3)	33.0 (23.1–50.0)	32.0 (25.3–49.5)
Sperm concentration, (median in range) (million/ml)	58.0 (20.0–345.0)	57.0 (20.0–190.0)	64.5 (2.0–250.0)	65.5 (1.0–250.0)	26.5 (1.0–210.0)	9.0 (1.0–120.0)
Progressive sperm motility, (median in range) (%)	3 (2–4)	3 (2–4)	3 (1–4)	3 (1–4)	2 (1–4)	2 (1–3)
DFI, (median in range) (%)	15.2 (0.4–30.0)	39.5 (30.1–95.0)	14.4 (2.3–30.0)	35.1 (30.1–67.5)	19.3 (2.6–29.9)	41.3 (30.1–79.9)
High DNA stainable, (median in range) (%)	8.4 (2.5–31.6)	9.1 (4.4–22.1)	8.4 (2.5–31.6)	8.8 (4.0–19.6)	9.6 (3.9–33.7)	11.1 (2.8–48.3)

IUI, intrauterine insemination; DFI, DNA fragmentation index.

Pregnancy outcomes

A biochemical pregnancy (BP) was defined as a plasma β-HCG concentration of >10 IU/l, 12 days after embryo transfer. A clinical pregnancy (CP) was defined as an intrauterine gestational sac with a heart beat 3 weeks after the β-HCG. Finally, delivery (D) was included as an outcome variable. 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. Early pregnancy loss was defined as pregnancies lost before gestational week 12.

Statistical analysis

All couples were dichotomized based on DFI in raw semen. In the main analyses, 30% DFI was used to separate 'low DFI' from 'high DFI'. The rationale for using this limit was based on previous reports in which the SCSA was performed (Evenson and Jost, 2000; Bungum *et al*., 2004). However, further analyses included the use of different thresholds (5%, 10%, 15%, etc.) to establish the possible presence of a threshold effect. As 231 couples contributed with more than one cycle, a sensitivity analysis, where only the first cycle from each couple was included, was performed.

For each of the three treatment groups (IUI, IVF and ICSI), odds ratios (ORs) with 95% confidence intervals (CIs) for pregnancy and birth were estimated for high DFI (>30%) compared with low DFI (≤30%), using logistic regression. Furthermore, couples treated with ICSI were compared with those treated with IVF with respect to BP, CP and D. This was done for all cycles and restricted on different thresholds for DFI (5%, 10%, 15%, etc.).

Male and female age, male and female BMI, smoking habits, sperm concentration and percentage motile and treatment number were considered as potential confounders, all tried in the model according to the change-in-estimate method suggested by Greenland (1989), using a 10% change for inclusion and a 5% change for exclusion. The same factors, dichotomized at their respective medians, were also tested as effect modifiers, using the Breslow–Day test for homogeneity. Statistical analysis was performed using SPSS 11.5 for Windows (SPSS Inc., Chicago, USA). The term 'statistically significant' is used to denote a two-sided *P* value <5%.

Results

Semen parameters

Data concerning sperm parameters are summarized in Table I. In 17% of IUI, 16% of IVF and 32% of the ICSI patients, DFI was >30%.

For all end-points and treatment groups, results of sensitivity tests where only the first cycle from each couple was included did not differ from the results reported for all 998 cycles; hence, only data including all the cycles are presented.

IUI, pregnancy and delivery

There was a lower fraction of BP in couples with a DFI >30% than in couples with a DFI ≤30% (Table II and Figure 1a). Also a significantly lower chance of obtaining a CP was seen in the group with a DFI >30% compared with the group with a DFI ≤30%. A similar pattern was seen for D (Table II). The risk estimates changed only slightly when introducing different potential confounders (data not shown). Furthermore, none of the variables tested showed any significant effect modification.

When trying different thresholds to define 'low DFI' and 'high DFI', no change in effect was found when threshold

Table II. Data on pregnancy, delivery, implantation and pregnancy loss in 387 IUI, 388 IVF and 223 ICSI cycles divided according to DFI ≤30% versus DFI >30%

	IUI			IVF			ICSI		
DFI	DFI ≤30%	DFI >30%	OR (95% CI)	DFI ≤30%	DFI >30%	OR (95% CI)	DFI ≤30%	DFI >30%	OR (95% CI)
Cycles started (n)	321	66	–	326	62	–	150	73	–
Biochemical pregnancies n (% per started cycle)	77 (24.0%)	2 (3.0%)	0·10 (0.02–0.41)	127 (39.0%)	21 (33.9%)	0.74 (0.41–1.4)	64 (42.7%)	42 (57.5%)	1.9 (1.0–3.4)
Clinical pregnancies (n) (% per started cycle)	76 (23.7%)	2 (3.0%)	0.10 (0.02–0.42)	110 (33.7%)	18 (29.0%)	0.80 (0.44–1.5)	56 (37.3%)	35 (47.9%)	1.6 (0.88–2.7)
Deliveries (n) (% per started cycle)	61 (19.0%)[a]	1 (1.5%)	0.07 (0.01–0.48)	94 (28.8%)[a]	16 (25.8%)	0.86 (0.46–1.6)	53 (35.3%)?	31 (42.4%)	1.4 (0.76–2.4)
Early pregnancy loss (*n*) (% per biochemical pregnancy)	14 (18.2%)	0	4.4 (0.26–75)	31 (24.4%)	4 (19.0%)	0.79 (0.17–3.8)	10 (15.6%)	10 (23.8%)	3.5 (0.60–20)

DFI, DNA fragmentation index; IUI, intrauterine insemination; OR, odds ratio.
[a]One ectopic pregnancy.

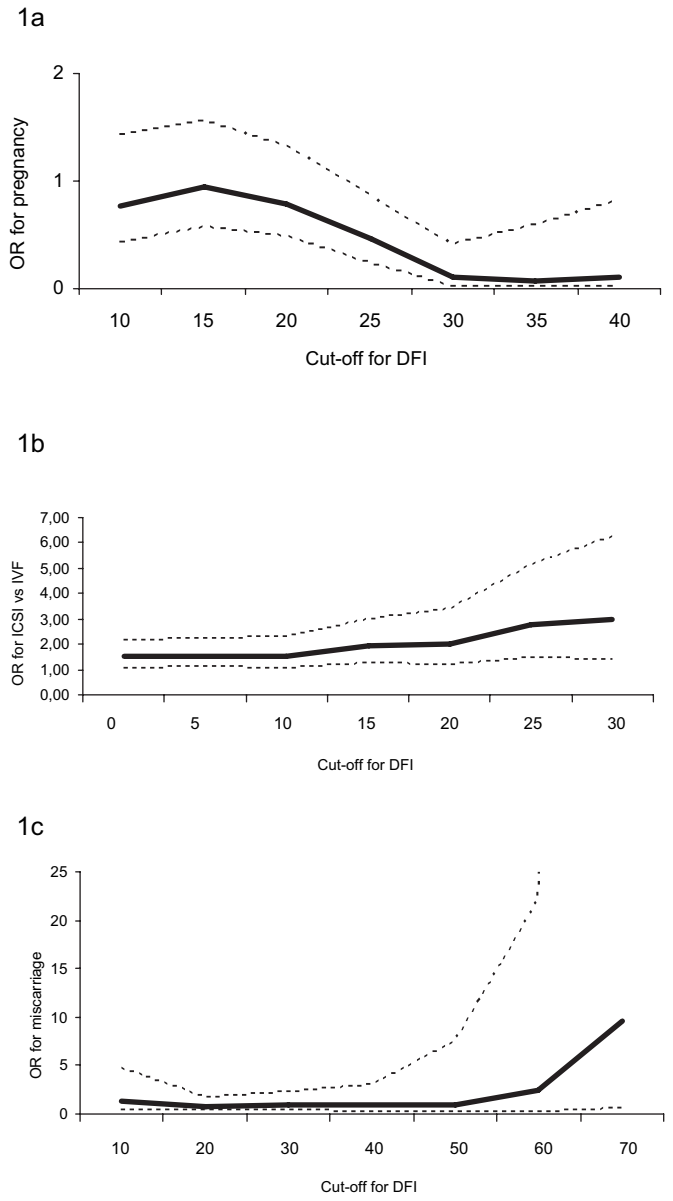

Figure 1. Odds ratios (ORs) for different outcomes of assisted reproduction treatment in relation to threshold level for the DNA fragmentation index (DFI). (**a**) Biochemical pregnancy (BP) following intrauterine insemination (IUI); (**b**) BP: ICSI compared with standard IVF; (**c**) Pregnancy loss, all three treatment categories, IUI, IVF and ICSI combined. Data are OR (±95% CI).

exceeded 30% whereas the OR diminished for threshold below 30% approaching 1 at the level of 20% (Figure 1a).

HDS was found not to be of predictive value for the outcome of IUI, alone or in combination with DFI (data not shown).

IVF/ICSI, pregnancy and delivery

Among IVF and ICSI couples, no statistically significant differences were seen between low and high DFI groups in respect to BP, CP and D (Table II).

No statistically significant difference was seen between the outcomes of ICSI versus IVF in the group with DFI ≤30% (data not shown). In the DFI >30% group, however, the results of ICSI were significantly better than those of IVF (Figure 1b). The ORs for BP, CP and D were 2.96 (1.40–6.23), 2.25 (1.10–4.60) and 2.17 (1.04–4.51), respectively. Neither sperm concentration nor motility could predict the treatment outcome.

Analyses were also performed using thresholds other than 30% for DFI. These results indicated that 30% was indeed a suitable threshold point to use for the main analyses (data not shown). None of the risk estimates changed more than marginally when including the potential confounders described previously. Furthermore, none of the variables tested showed any significant effect modification.

No statistically significant differences were seen in fertilization or embryo quality between the groups, neither about fertilization method nor to DFI (Table I). Implantation rates did not differ between the DFI groups within same treatment category (IVF or ICSI). However, implantation rate in ICSI group with DFI >30% seemed to be higher than in any other subgroup (Table I).

HDS was found not to be of predictive value for the outcome of IVF or ICSI, alone or in combination with DFI (data not shown).

Early pregnancy loss

No statistically significant difference in early pregnancy loss was seen for low and high DFI levels when DFI of 30% was used as threshold. This was the case for all treatment categories (Table II and Figure 1c). However, for DFI >60%, the OR for pregnancy loss seemed to increase to 2.4 although this increase was not statistically significant (95% CI: 0.26–22), possibly because of low numbers of subjects (Figure 1c).

Discussion

Three 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, and most importantly, we have identified a new factor, predictive for the outcome of ART. DFI can be used as an independent predictor of pregnancy and birth in couples undergoing IUI. Second, we can confirm that *in vitro* ART is able to bypass the impairment of sperm chromatin, in particular if ICSI is chosen as a fertilization method. A high DFI does not exclude successful treatment by IVF, but the OR for BP was three times higher using ICSI as compared with IVF when the DFI exceeded a level of 30%. Third, for all three treatment categories, the study demonstrated that sperm DNA damage is not associated to an increased risk of early pregnancy loss, at least when DFI of 30% is used as threshold value.

This study based on a study population of about 1000 ART cycles allows us to define SCSA as a valuable diagnostic tool in selecting the most appropriate procedure for an infertile couple undergoing ART. All men seeking infertility workup and treatment should be tested with SCSA as a supplement to the standard semen analysis. When DFI exceeds 30%, ICSI should be the method of choice, even in cases where traditional sperm parameters are normal. This study has shown that in almost 20% of the patients DFI was >30%, although the other sperm characteristics fulfil the criteria for either IUI or IVF.

The results regarding the IUI treatments fit with previous *in vivo* studies on time to pregnancy (TTP) for couples with no infertility problems (Evenson *et al*., 1999; Spano *et al*., 2000). These studies indicated a DFI level of 30–40% as a statistical threshold for a longer TTP or no pregnancy.

Two other recently published studies (Saleh *et al*., 2003; Bungum *et al*., 2004) reached a similar conclusion. Saleh *et al*. (2003) 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. In our previous study (Bungum *et al*., 2004) including 131 IUI patients, the chance of pregnancy and delivery was significantly higher in the group with DFI ≤27% and HDS ≤10% than in patients with DFI >27% or HDS >10%. In this study, the ORs for BP, CP and D in IUI were significantly lower in the group with DFI >30% as compared with those with DFI ≤30%. However, in contrast to the previous report (Bungum *et al*., 2004), here, we were unable to detect any upper or lower limit for HDS and this parameter does not seem to be of predictive value for the outcome of ART, neither alone nor in combination with DFI.

We found that ICSI was a more efficient treatment method than IVF when DFI exceeded a level of 30% and for ICSI there was even a tendency towards higher pregnancy rates with a DFI >30% versus DFI <30%. Previously the efficacy of these two methods has been found to be equal in cases of non-male factor infertility (Bhattacharya *et al*., 2001). The biological explanation behind the superior results of ICSI in cases of high DFI needs to be elucidated; however, one could ask whether ICSI women, on average, produce healthier oocytes with a better DNA repair capacity than IVF women, as in the ICSI group infertility is mainly caused by male factor. This superiority of ICSI oocytes might be most pronounced at high DFI levels at which natural conception is not possible despite excellent fertility status of the female. The higher efficiency of ICSI, at high DFI levels, as compared with IVF might also be due to two different culture environments used for these two techniques. While IVF oocytes were exposed to spermatozoa for 90 min, in ICSI the spermatozoon were injected directly into the oocyte. In ICSI the oocyte could, therefore, be less exposed to reactive oxygen species (ROS) than in IVF. Recently, Saleh *et al*. (2003) demonstrated a positive correlation between DFI levels and the concentration of ROS in the seminal plasma.

In contrast to previous reports showing an increased risk of embryonic loss in pregnancies achieved by the use of semen samples with high rates of DNA breaks (Carrell *et al*., 2003; Virro *et al*., 2004), this study showed no statistically significant association between high DFI and early pregnancy loss. However, we could not exclude the fact that DFI levels >60% are associated with higher risk of early pregnancy loss, an issue that should be addressed in additional studies.

None of the classical semen parameters including sperm concentration and motility were found to be predictive for the outcome of the ART treatment. Morphology was not assessed, but the correlation between this sperm characteristic and SCSA parameters was previously shown to be low to moderate (Evenson *et al*., 1999; Spano *et al*., 2000). Moreover, data regarding the predictive value of sperm morphology in relation to ART have been conflicting (Lundin *et al*., 1997; Coetzee *et al*., 1998).

The SCSA is a very easy and reproducible test. In addition to being subject to a very limited intra-laboratory variation, the test was shown to be very robust to variation between laboratories. In an external quality control where close to 300 semen samples were analysed, a high correlation ($\rho = 0{\cdot}8$) was found between our laboratory and a control laboratory. Furthermore, the absolute DFI values obtained at two different places, and using different equipment did not on average differ from another by >1% (Giwercman *et al*., 2003). It means that our threshold levels will be applicable to other laboratories performing the SCSA standard protocol (Evenson *et al*., 1999). However, due to an intra-individual variation in the level of DFI (Erenpreiss *et al*., in press), selection of proper treatment requires that SCSA is performed before each ART procedure.

Our findings may also give reason for concern as we have shown that semen samples with high rates of DNA breaks are more likely to result in pregnancy in ICSI than in IVF. The safety of ICSI has often been questioned as the natural selection barriers during fertilization are bypassed. DNA damaged sperm in the ejaculate may be responsible for the induction of pathology such as infertility (Aitken and Krausz, 2001), childhood cancer (Fraga *et al*., 1996; Ji *et al*., 1997; Aitken and Krausz, 2001) and imprinting diseases (Fraga *et al*., 1996; Ji *et al*., 1997; Cox *et al*., 2002; Orstavik *et al*., 2003), which may not be expressed until the child reaches puberty or adulthood. The most recent epidemiological studies report a 2-fold higher risk on infant malformations and the occurrence of syndromes related to errors in imprinting after ICSI (Hansen *et al*., 2002, 2005; Schieve *et al*., 2002; Boundelle *et al*., 2005). However, so far, no follow-up study of children born after ART where sperm DNA damage has been taken into account has been performed. We strongly recommend such studies to be initiated.

This study is the largest ever-reported study on the predictive value of SCSA in relation to the outcome of ART demonstrating that DFI can be used as an independent predictor of pregnancy and birth in couples undergoing IUI. Furthermore, the study demonstrates that the odds ratio for BP is three times higher by ICSI than by IVF when the DFI exceeded the level of 30%. Thus, when DFI exceeds 30%, ICSI should be the preferred method. Further studies are needed to investigate whether treatment modalities including administration of antioxidants (Greco *et al*., 2005) to men with high DFI can play a role in infertility treatment. Finally, to investigate possible consequences of using sperm with compromised DNA, new studies focusing on the health of children born after ART when DFI levels have been high, should be initiated.

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