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ORIGINAL ARTICLE

Sperm chromatin structure assay results after swim-up are related only to embryo quality but not to fertilization and pregnancy rates following IVF

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The aim of this study was to investigate whether the sperm chromatin structure assay (SCSA) results after swim-up are related to fertilization rates, embryo quality and pregnancy rates following *in vitro* fertilization (IVF). A total of 223 couples undergoing IVF in our hospital from October 2008 to September 2009 were included in this study. Data on the IVF process and sperm chromatin structure assay results were collected. Fertilization rate, embryo quality and IVF success rates of different DNA fragmentation index (DFI) subgroups and high DNA stainability (HDS) subgroups were compared. There were no significant differences in fertilization rate, clinical pregnancy or delivery rates between the DFI and HDS subgroups. However, the group with abnormal DFI had a lower good embryo rate. So, we concluded that the SCSA variables, either DFI or HDS after swim-up preparation, were not valuable in predicting fertilization failure or pregnancy rate, but an abnormal DFI meant a lower good embryo rate following IVF.
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INTRODUCTION

The integrity of chromosomal DNA in a spermatozoon is a prerequisite for normal fertilization and transmission of paternal genetic information.[1] Under normal conditions, DNA in a mature spermatozoon is condensed and compact in structure. Up to 85% of DNA binds to protamine to form DNA–protamine complexes, which are more compacted than DNA–histone complexes in somatic cells.[2] By forming these condensed and insoluble complexes, normal sperm DNA becomes highly resistant to physical or chemical stimulation and has a doughnut-shaped configuration, which can prevent DNA damage during sperm transport.[3] DNA damage may be caused by numerous factors, not all of which are known.

Many tests are currently available for the measurement of sperm DNA fragmentation,[4,5] including terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate fluorescein nick end labelling assay,[6] *in situ* nick translation assay, comet assay,[7] chromomycin A3 test,[8] DNA breakage detection-fluorescence *in situ* hybridisation technique[9] and sperm chromatin structure assay (SCSA).[10,11] Among these methods, the most frequently used assay in clinical studies is SCSA, which measures the stability of sperm chromatin in acid media with acridine orange. The proportion of sperm containing fragmented DNA is determined by flow cytometric analysis and is expressed as the DNA fragmentation index (DFI) and high DNA stainability (HDS).

In fact, an increasing number of studies suggest that DNA fragmentation could be used as a marker of semen quality and a predictor of outcome in assisted reproductive technology (ART).[1] It has been reported that about 10%–20% of ejaculated spermatozoa have DNA fragmentation and that apoptosis is more prevalent in oligozoospermic samples.[12] Two time-to-pregnancy studies showed that SCSA test results were significantly associated with the probability of pregnancy in normal couples who had discontinued the use of contraconceptive measures.[9,13] However, the associations between sperm DNA integrity and fertilization or pregnancy rates were seldom investigated in *in vitro* fertilization (IVF) and intracytoplasmic sperm injection (ICSI) studies.[2,14,15] On the other hand, most of the published SCSA–ART studies, which found associations between SCSA parameters and ART success rate, are based on the analysis of neat and unprepared semen. Spermatozoa used for ART are, in the vast majority of cases, prepared by density gradient centrifugation (DGC) or by a swim-up to favour the isolation of motile and morphologically normal spermatozoa. Several studies have shown that both sperm separation methods are effective in isolating spermatozoa with nicked DNA and poorly condensed chromatin as evaluated by SCSA, though various levels of efficiency were reported.[16–21] Among these, three SCSA–ART studies[15,17,19] were based on DGC preparation of sperm and concluded that DFI and HDS results after sperm preparation were not predictive for the outcome of IVF and ICSI.

In this study, we aimed to investigate the efficiency of swim-up to isolate spermatozoa with damaged DNA and poorly condensed chromatin and to assess the relationship between SCSA results with this preparation method and the outcomes of IVF.

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MATERIALS AND METHODS

Patients

A total of 223 couples undergoing IVF treatment at Ruijin Hospital (Affiliated to the Medical School of Shanghai Jiao Tong University (Shanghai, China) were included in this study from October 2008 to September 2009. Informed consents were received from all participants. All experimental procedures and sample procurements were approved by the Institutional Review Board of Ruijin Hospital.

All female partners were <40 years of age and had day 3 follicle-stimulating hormone levels <10 IU l^{-1}. Cases with factors adversely affecting implantation, such as hydrosalpinx, uterine synechia, adenomyosis, myoma and uterine abnormality, were excluded. Only cases with complete SCSA data of both neat semen and sperm post-preparation for IVF were included in this study.

Semen preparation

Semen samples were collected from the 223 men (on the day of oocyte retrieval for IVF) by masturbation after 2–5 days of abstinence. All samples were prepared by swim-up. Raw ejaculates were diluted 1 : 1 (v/v) with Sperm Medium (MediCult, Jyllinge, Denmark). Samples were then pelleted at 400*g* for 10 min, and the supernatants were discarded. Then, 0.5–1.0 ml of fresh medium was added without disturbing the pellet and incubated for 45 min with tubes at a 45° inclination. After this procedure, the upper 0.1– 0.5 ml was removed for IVF. Two semen aliquots (100 μl) that were taken from each semen sample before and after sperm swim-up were diluted to 1 : 5 with TNE buffer (0.01 mol l^{-1} Tris-HCl, 0.15 mol l^{-1} NaCl, 1 m mol l^{-1} EDTA, pH 7.4) at room temperature and then immediately frozen at −80 °C for subsequent SCSA analysis.

IVF procedures

Ovarian stimulation, oocyte retrieval, embryo culture, embryo transfer and pregnancy outcome follow-ups were performed as previously described.[22]

Fertilization and embryo morphology-quality assessment

Recovered oocytes were inseminated by conventional IVF procedures. Fertilization was assessed after 18 h (day 1), and embryo cleavage was assessed 24 h thereafter (day 2). Fertilization was considered normal if two pronuclei and two polar bodies were identified. Oocytes without visible pronuclei were considered unfertilized. Embryo morphology was evaluated on day 3 by taking into account the number and symmetry of blastomeres and the percent of fragmentation. Embryos were characterized as viable by the presence of at least five blastomeres after insemination, the absence of multinucleated blastomeres and <30% cellular fragments. A good embryo was defined as having no fewer than eight blastomeres with ⩽10% cellular fragmentation. Embryo transfers were performed 72 h after oocyte retrieval, and only viable embryos were selected for transfer.

Sperm chromatin structure assay

The SCSA was performed as previously described.[11] On the day of analysis, stored samples were thawed in a 37 °C water bath for 30 s and analysed immediately. A total of 1×10^6–2×10^6 cells were treated for 30 s with a detergent solution (pH 1.2) containing 0.1% Triton X-100, 0.15 mol l^{-1} NaCl and 0.08 mol l^{-1} HCl and then stained with 6 mg l^{-1} purified acridine orange (Sigma, St Louis, MO, USA) in a phosphate citrate buffer (pH 6.0). Stained cells were analysed by a fluorescence activated cell sorter scan flow cytometer (Cell Lab Quanta SC; Beckman Coulter, Fullerton, CA, USA) and a minimum of 10 000 events was accumulated for each measurement. After excitation with a 488-nm light source, acridine orange bound to double-strain DNA emits green fluorescence and acridine orange bound to single-strain DNA emits red fluorescence. These results are displayed as red (fragmented DNA) versus green (DNA stainability) 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. High DNA stainability (% HDS) was calculated based on the percentage of sperm with high levels of green fluorescence, which are thought to represent immature spermatozoa with incomplete chromatin condensation.

Statistical analysis

Data are presented as mean±s.d. To determine whether higher or lower values of DFI affected pregnancy, two categories of DFI intervals (⩽27% and >27%) were defined based on published results.[23] HDS intervals were defined as ⩽15% and >15%. Differences in reproductive outcomes (e.g., pregnancy rate, fertilization rate, embryo quality and abortion rate) between the two DFI and HDS groups were compared using Chi-square test, independent *t*-test and one-way ANOVA test. Statistical analysis was performed using SPSS 14.0 for Windows (SPSS Inc., Chicago, IL, USA). The term 'statistically significant' was used to denote a two-sided test with a *P* value of <0.05.

RESULTS

Descriptive statistics of sperm parameters evaluated for neat and post-swim-up are shown in **Table 1**. In the present study, the percentage of motile cells increased from 49.5%±12.7% to 87.8%±6.4%, and the majority of samples showed improvement in DFI and HDS after swim-up preparation. There were 44 cases that showed DFI >27% and 39 cases with HDS >15% in neat semen samples. However, the corresponding figures post-swim-up were 21 and 29 cases for the DFI >27% and HDS >15%, respectively. For 256 treatment cycles, mean DFI (mean±s.d.) decreased from 14.3%±11.2% to 4.9%±3.1% after swim-up, whereas the corresponding figures for HDS were 9.1%±4.2% and 4.6%±3.0%, both the difference are statistical significant ($P<0.05$) (**Table 1**).

Descriptive analyses of data for couples receiving IVF are summarized in **Tables 2** and **3**. In these couples undergoing IVF, no statistically significant differences were found between the two DFI groups and the two HDS groups regarding male or female age, years of infertility and main diagnosis of infertility ($P>0.05$) (**Table 2**). No statistically

Table 1 Semen analysis parameters before and after swim-up preparation

Parameters	*Neat*	*Swim-up*
Concentration ($\times10^6$ ml^{-1})		
Mean±s.d.	64.2±33.6	29.3±27.4
Median (range)	66.3 (24.2–210.0)	27.8 (21.5–40.6)
Motility (%)		
Mean±s.d.	49.5±12.7	87.8±6.4
Median (range)	47.9 (34.2–73.1)	88.5 (79.4–93.0)
DFI (%)		
Mean±s.d.	14.3±11.2	4.9±3.1*
Median (range)	12.3 (1.1–71.6)	2.6 (0.2–54.3)
HDS (%)		
Mean±s.d.	9.1±4.2	4.6±3.0*
Median (range)	8.4 (0.8–34.2)	4.2 (0.1–30.6)

Abbreviations: DFI, DNA fragmentation index; HDS, high green stain.
*$P<0.05$, compared with corresponding Neat.

Table 2 Patients' background characteristics according to DFI and HDS after swim-up preparation

Patients' background characteristics	*DFI*		*HDS*	
	⩽27%	*>27%*	*⩽15%*	*>15%*
No. of cycles	235	21	227	29
Age, year (mean±s.d.)				
Female	31.5±3.6	31.9±4.0	31.4±3.7	31.9±4.0
Male	35.2±4.2	34.9±4.4	35.0±4.3	35.3±4.2
Years of infertility	4.8±2.9	4.3±2.4	4.7±2.7	4.6±3.0
Main infertility diagnosis, *n* (%)				
Tubal	102 (43.5)	9 (42.9)	97 (42.7)	14 (48.2)
Endometriosis	25 (10.5)	2 (9.6)	26 (11.5)	1 (3.5)
Ovulation failure	6 (2.6)	1 (4.7)	5 (2.2)	2 (6.9)
Asthenospermia	74 (31.5)	8 (38.1)	72 (31.7)	10 (34.5)
Unexplained	28 (11.9)	1 (4.7)	27 (11.9)	2 (6.9)

Abbreviations: DFI, DNA fragmentation index; HDS, high green stain.

Table 3 Comparison of IVF outcomes according to DFI and HDS after swim-up preparation

IVF outcomes	*DFI*		*HDS*	
	⩽27%	*>27%*	*⩽15%*	*>15%*
No. of oocyte retrieved (mean±s.d.)	12.4±4.9	13.1±5.8	12.4±5.1	12.9±5.4
Fertilization rate (%)	65.9	61.4	64.2	63.8
Cleavage rate (%)	97.5	98.1	97.8	97.4
Viable embryo rate (%)	64.2	61.8	64.8	61.4
Good embryo rate (%)	27.5*	13.2	25.2	22.8
Embryo transfer (*n*)	199	19	193	25
No. of embryo transferred (mean±s.d.)	2.0±0.1	2.1±0.2	2.0±0.1	2.0±0.1
Biochemical pregnancies rate (% per ET)	37.2 (74/199)	36.8 (7/19)	37.8 (73/193)	32.0 (8/25)
Clinical pregnancies rate (% per ET)	35.7 (71/199)	36.8 (7/19)	37.3 (72/193)	24.0 (6/25)
Implantation rate (%)	22.1 (88/398)	22.5 (9/40)	23.2 (90/388)	14.0 (7/50)
Abortion rate (%)	7.0 (5/71)	14.3 (1/7)	6.9 (5/72)	16.7 (1/6)
Ongoing pregnancies/ birth rate (% per ET)	33.2 (66/199)	31.6 (6/19)	34.7 (67/193)	20.0 (5/25)

Abbreviations: DFI, DNA fragmentation index; ET, embryo transfer; HDS, high green stain; IVF, *in vitro* fertilization.
* $P<0.05$, compared with the DFI >27% group.

significant differences in IVF outcomes regarding number of oocytes retrieved, fertilization rate, viable embryo rate, clinical pregnancy rate and abortion rate were observed between the two DFI groups and the two HDS groups ($P>0.05$). One difference noted between the two DFI groups was that the good embryo rate in the DFI ⩽27% group was significantly higher than in the DFI >27% group (27.5% *vs.* 13.2%; $P<0.05$) (**Table 3**).

In addition, the relationship between sperm DNA fragmentation and fertilization rate was also analysed. No difference in percentage of patients with low, moderate-low and normal fertilization rate intervals (<30%, 30%–65% and >65%) between the two DFI groups and the two HDS groups was observed ($P>0.05$)(**Table 4**). However, the percentage of embryos with a fragmentation percentage of 11%–30% was

Table 4 Percentages of couples with different fertilization rates (<30%, 30–65% and >65%) in DFI and HDS groups after swim-up preparation

Fertilization rate	*DFI*		*HDS*	
	⩽27%	*>27%*	*⩽15%*	*>15%*
No. of cycles	235	21	227	29
Low (<30%), *n* (%)	20 (8.5)	2 (9.5)	18 (7.9)	4 (13.8)
Moderate (30%–65%), *n* (%)	52 (22.1)	3 (14.3)	47 (20.7)	8 (27.6)
Normal (>65%), *n* (%)	163 (69.4)	16 (76.2)	162 (71.4)	17 (58.6)

Abbreviations: DFI, DNA fragmentation index; HDS, high green stain.

Table 5 Comparison of embryo development according to DFI and HDS after swim-up preparation

	DFI		*HDS*	
	⩽27%	*>27%*	*⩽15%*	*>15%*
No. of embryo fragmentation at different levels (%), *n* (%)				
Total No.	1752	225	1685	292
0	442 (25.2)	45 (20.0)	420 (24.9)	67 (22.9)
1%–10%	652 (37.2)	73 (32.4)	607 (36.1)	118 (40.4)
11%–30%	448 (25.6)*	80 (35.6)	451 (26.7)	77 (26.4)
31%–50%	90 (5.5)	14 (6.2)	92 (5.5)	12 (4.1)
>50%	120 (6.8)	13 (5.82)	115 (6.8)	18 (6.2)
No. of embryo fragmentation at different development stages, *n* (%)				
8–10 cells	619 (35.3)	74 (32.9)	586 (34.8)	107 (36.6)
6–7 cells	608 (34.7)	82 (36.4)	588 (34.9)	102 (34.9)
5 cells	344 (19.6)	49 (21.8)	343 (20.4)	50 (17.1)
<5 cells	181 (10.4)	20 (8.9)	168 (9.9)	33 (11.4)

Abbreviations: DFI, DNA fragmentation index; HDS, high green stain.
* $P<0.05$, compared with DFI >27% group.

significantly higher in the DFI >27% group than in the ⩽27% group (35.6% *vs.* 25.6%, $P<0.05$) (**Table 5**). Nevertheless, no association between SCSA parameters and number of blastomeres was observed.

DISCUSSION

In this study, we focused on the possible predictive value of SCSA analysis for sperm after swim-up. The main conclusion that can be drawn from the current study is that assessment of sperm DNA damage after swim-up preparation can only predict the quality of embryos but not the outcome of IVF.

DNA strand breaks in human spermatozoa are indicators of deteriorated functions. Sperm DNA damage may occur due to the following mechanisms: first, protamine deficiency, a genetic disorder that is more common in infertile men, in which the protective function of DNA is diminished;[24] second, oxidative stress due to leucocytosis or varicocele, which is also associated with sperm DNA damage;[25] and third, increased apoptotic activity in older men and in those with abnormal semen parameters may also contribute to DNA damage.[26] Systems for DNA repair are less active in the later stages of spermatogenesis, which allow sperm with DNA strand breaks to enter the ejaculate.[13]

Sperm chromatin structure assays have been used to assess the extent of sperm DNA fragmentation and to predict the outcome of ART. Some studies have demonstrated the threshold for negative

pregnancy outcomes after ART.[15,27,28] However, discrepancies exist in the association between the fraction of defective sperm and fertilization rate, embryo quality and pregnancy rate and whether the technique should be used in the context of IVF or ICSI.[29–32] According to a systematic review involving 2162 cycles of IVF/ICSI treatment,[30] there was a lack of strong evidence of a clinical indication for the routine use of SCSA in the evaluation of infertile men, and it should be determined which subgroup of infertile couples may benefit from this test. In addition, several authors have claimed that it is necessary to conduct studies aimed at clarifying the predictive value of processed semen in ART.[18,33,34]

In the present study, the majority of the samples showed an improvement in DFI and HDS after swim-up preparation, which suggests that the preparation removed a significant amount of spermatozoa with DNA breaks as measured by SCSA. Several papers have demonstrated that sperm preparation may initially decrease the fraction of defective sperm in neat semen though with different net performances.[18,35–39] On the other hand, Zini *et al.*[18,40] and Tomsu *et al.*[41] also reported either no change or a decrease in sperm quality.

To date, several published SCSA–ART studies used prepared semen for SCSA analysis,[15,17,19] and all concluded that SCSA parameters with DGC or swim-up samples were not predictive for pregnancy outcomes, though there are no published data regarding the relationship between SCSA parameters and embryo quality. In the present study, which included 223 patients, the quality of embryos that were developed from high or low DFI sperm showed differences in cell fragmentation, though no relationship between SCSA parameters after swim-up and low fertilization rate and pregnancy outcomes in IVF was observed. Among a total of 225 embryos that originated from spermatozoa with DFI $>$27%, the percentage of embryos with blastomere fragmentation $\leqslant$10% (normally classified as good embryos) was lower than that from normal DFI sperm, though no statistically significant difference was observed. However, the percentage of embryos with fragmentation in 11%–30% on day 3, which is the lowest criterion to be classified as 'viable', was significantly higher in the high DFI group than in the normal DFI group (35.6% *vs.* 25.6%). Thus, the viable embryo frequency was the same in the different DFI interval groups, but the good embryo frequency in the high DFI group decreased significantly. Therefore, our results suggest that although sperm DNA damage after swim-up did not preclude fertilization and embryo development, it did contribute significantly to the failure of good embryo development.

The embryonic genome is activated on day 3, and the transcriptional products may supersede the regulatory control by maternal messages stored in the oocyte. Although oocytes and embryos can repair sperm DNA damage, there is a threshold beyond which sperm DNA cannot be repaired, the effects of which may influence embryo quality. Seli *et al.*[26] reported that blastocyst stage embryo development shows a significant negative correlation with the extent of nuclear DNA fragmentation in ejaculated spermatozoa used in IVF. Larson *et al.*[17] suggested that elevated DFI in neat semen may reflect chromatin or other abnormalities within the entire sperm population; this can interfere with the fertilizing ability of the sperm but is not completely eliminated by DGC or swim-up. According to a study by Avendaño *et al.*,[42] DNA fragments among morphologically normal sperm after swim-up preparation were more meaningful than total sperm (morphologically normal and abnormal). They found that based on a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate fluorescein nick end labelling (TUNEL) assay, motile spermatozoa selected by the swim-up technique containing more than 17.6% of morphologically normal sperm with fragmented DNA are associated with a higher possibility of generating poor quality embryos and are less likely to result in pregnancy.

Based on the results of current study, we conclude that neither DFI nor HDS SCSA variables after swim-up preparation were valuable in predicting fertilization failure and pregnancy rate, although our data support a clear relationship between DFI and good embryo rate on day 3 that may result in a decreased cumulative pregnancy rate. It should be noted that SCSA is a generalized test of sperm chromatin stability, but it still cannot precisely detect the type of sperm DNA breaks (single/double-strand breaks). We cannot exclude the possibility that an unknown type of damage may also exist in the sperm, although DNA denaturation does not occur during the SCSA procedure. Future research should focus on the development of the appropriate tests to specifically identify types of DNA defects and quantity threshold of damaged DNA that may affect fertility. In addition, future studies are needed to identify and isolate spermatozoa with intact DNA for ART use.

CONCLUSIONS

The SCSA variables, either DFI or HDS after swim-up preparation, were not valuable in predicting fertilization failure and pregnancy rate; however, abnormal DFI is indicative of a lower rate of good embryos following IVF.

AUTHOR CONTRIBUTIONS

ZHN participated in the design of the study, data analysis, interpretation and drafting of the manuscript. AJZ and YJS helped drafting the manuscript. HJS, HQZ and YF helped critical review and approval of article. All authors contributed extensively to the work presented in this paper.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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