

Clinical Studies

Semen DNA Fragmentation Index, Evaluated with Both TUNEL and Comet Assay, and the ICSI Outcome

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Abstract. *Background:* In intracytoplasmic sperm injection (ICSI), there is always a risk of using spermatozoa with damaged DNA. The purpose of this study was to evaluate the percentage of spermatozoa with DNA fragmentation in processed semen samples used in ICSI cycles and to investigate the relationship between the DNA fragmentation index (DFI) and the ICSI outcome. *Patients and Methods:* Fifty-six couples undergoing ICSI treatment were included. DFI was evaluated, by both terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL) and single cell gel electrophoresis (Comet) assays, in the processed semen samples used for ICSI. *Results:* Of the processed semen samples 17.85% had $\geq 10\%$ spermatozoa with fragmented DNA. There was no correlation between DFI and the ICSI outcome. DFI assessed by the TUNEL assay was negatively correlated with sperm concentration, progressive motility and sperm morphology. *Conclusion:* A considerable proportion of processed semen samples used for ICSI have a high DFI. However, DFI of the processed semen samples does not seem to be related to the ICSI outcome.

The integrity of nuclear DNA of spermatozoa is an indicator of cell health and there is an increasing awareness of its importance in assisted reproduction and particularly in intracytoplasmic sperm injection (ICSI) cycles.

ICSI is an *in vitro* fertilization (IVF) method that bypasses mechanisms of natural selection and overcomes factors which otherwise could hinder fertilization. During ICSI, a spermatozoon is immobilized, aspirated by means of

the injecting pipette and it is injected into the cytoplasm of a metaphase II (MII) oocyte, in a position distant from the first polar body. ICSI can be applied either with ejaculated spermatozoa or with spermatozoa of epididymal origin (1, 2), with testicular spermatozoa (3-5) or even with spermatozoa retrieved from post-ejaculatory urine, in cases of retrograde ejaculation (6). The selection of the spermatozoon to be injected into the oocyte is based on the gross morphology and motility. However, sperm morphology and motility do not always go together with DNA integrity (7, 8). Consequently, in ICSI, there is always the risk of inadvertently using spermatozoa with damaged DNA. In cases of semen of poor quality, the risk is probably higher, as it has been reported that semen of poor quality has an increased proportion of spermatozoa with DNA fragmentation (9). The percentage of spermatozoa with nuclear DNA fragmentation, referred to as the DNA fragmentation index (DFI), has been associated with fertilization failure (10, 11) or possible damage to the foetus (12). For the above reasons, the study of DNA fragmentation in semen samples used in ICSI, particularly in semen samples of poor quality, is of special interest.

The strand breaks in the DNA of human spermatozoa can be detected by different methods such as the nick translation assay, the terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling (TUNEL) assay, the single cell gel electrophoresis (Comet) assay and the sperm chromatin structure assay (SCSA).

In the nick translation assay, an exogenous DNA polymerase is used, whereas terminal deoxynucleotidyl transferase is used in the TUNEL assay. The TUNEL assay identifies double and single DNA strand breaks by enzymatic labelling of the free 3'OH end of the DNA with a fluorescent substrate. TUNEL positive cells are identified either microscopically or by fluorescence-activated cell sorting. Several investigators have used TUNEL assay to detect DNA strand breaks in human spermatozoa (9, 11, 13-15).

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The Comet assay, extensively used in somatic cells to measure genotoxic damage, has been modified to investigate DNA damage in spermatozoa and it measures double strand breaks (16-20). SCSA measures the susceptibility of sperm nuclear DNA to *in situ* denaturation, induced by heat or acid followed by staining with acridine orange (20). This technique measures the population of spermatozoa with DNA fragmentation and altered chromatin structure.

The object of this study was to evaluate the percentage of spermatozoa with nuclear DNA fragmentation in semen samples prepared for ICSI and to investigate the possible association between DFI and the ICSI outcome. The evaluation of DFI was made by both TUNEL and Comet assays.

Patients and Methods

The study group consisted of 56 couples undergoing ICSI treatment in the Department of Obstetrics and Gynecology, University of Schleswig-Holstein, Campus Lübeck, Germany. Couples with genetic disorders, as well as ICSI cycles combined with testicular sperm extraction or micro-epididymal sperm aspiration were not included. All couples gave verbal consent and did not receive any monetary compensation for participating in the study.

Controlled ovarian hyperstimulation (COH) followed the multidose antagonist protocol ("Lübeck protocol") (21, 22). Briefly, the ovarian stimulation was made with human menopausal gonadotropins (hMG) (Menogon; Ferring Arzneimittel GmbH, Kiel, Germany) and recombinant follicle stimulating hormone (recFSH) (Gonal-F; Serono International S.A., Geneva, Switzerland). Pituitary suppression was achieved with the daily use of 0.25 mg of cetrorelix (Cetrotide; Serono International S.A.) from the sixth day of ovarian stimulation until the day of the induction of ovulation. The ovulation was induced with 10,000 IU of human chorionic gonadotropin (hCG) (Choragon; Ferring Arzneimittel GmbH). Oocytes were retrieved by ultrasound-guided follicular puncture, then denuded and washed in culture medium.

Semen samples were collected by masturbation after 3 days of sexual abstinence. Routine semen analysis was carried out to evaluate sperm concentration, motility and morphology using light microscopy. The "swim up" technique was used for the preparation of the semen samples for ICSI.

The oocytes were assessed for the presence of pronuclei 16-18 hours after ICSI (day 1). Embryo transfers were performed on day 2. Up to three embryos were replaced in each patient, according to the German Embryo Protection Law. Before the embryo transfers, paying attention to the degree of fragmentation and regularity of blastomeres, each embryo was graded as 1, 2 or 3 (modified grading according to Veeck (23)). The grade of each embryo was multiplied by its number of blastomeres to produce a quality score.

The DFI was evaluated by both Comet and TUNEL assays. The evaluation of the DFI was made in the remaining aliquot of each semen sample that had already been processed with "swim up" to be used for ICSI.

Methodology for the Comet assay. Spermatozoa were cast in agarose on comet assay slides, purchased from Trevigen (Gaitersburg, MD, USA) and hardened at 4°C. Then, they were lysed (at 4°C for a

minimum of 30 minutes) to degrade the membranes, release DNA associated proteins and allow the DNA to unwind. The lysis buffer (2.5 M NaCl, 10 Mm Tris-Base and 100 mM EDTA, pH 10) contained 1% lauryl-sarcosine and 1% Triton X-100. The gels were then equilibrated in electrophoresis buffer (TBE) and electrophoresed (15 min at 15V) in neutral buffer (1xTBE). DNA migrated from the nucleus towards the anode. Then, the slides were submerged in 70% ethanol, dried, stained with SYBR Green DNA binding fluorescent dye and viewed using a Zeiss Axiovert 135 epifluorescent microscope (Zeiss, Jena, Germany). The slides were viewed under 100x magnification. Damaged DNA formed characteristic pear-shaped comets. A total of 500 nuclei per slide were evaluated, the comets were counted and their percentage was calculated. The final percentage of spermatozoa with fragmented DNA is referred to as DFI-Comet (%).

Methodology for the TUNEL assay. Air-dried sperm samples were used to determine DNA breaks with the TUNEL assay (Cell Death Detection Kit, Roche Biochemicals, Mannheim, Germany) following the manufacturer's specifications with minor modifications.

Air-dried slides were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature, rinsed with PBS, pH 7.4, and then permeabilised with 2% Triton X-100. The terminal deoxynucleotidyl transferase (TdT)-fluorescent-labelled nucleotide mix was added to the slides and the labelling reaction was carried out for 1 hour in the dark at 37°C in a humidified chamber. After labelling, slides were rinsed twice in PBS and then counterstained with 10 mg/ml of 4,6 diamidino-2-phenylindole (DAPI). Controls were included in every experiment: for negative control the TdT was omitted from the nucleotide mix. The positive controls were prepared by incubating the sperm cells for 20 minutes at room temperature with 50 units/ml DNase I (Boehringer Mannheim, Mannheim, Germany).

In each slide, the total number of spermatozoa and the percentage of cells with fragmented DNA were determined by analysing each microscope field using both light and fluorescence microscopy. At least 500 cells in each sample were counted. Every spermatozoon was assigned to contain normal (blue nuclear fluorescence due to DAPI) or fragmented DNA (intense green nuclear fluorescence). The final percentage of spermatozoa with fragmented DNA is referred to as DFI-TUNEL (%).

Statistical analysis. The statistical analysis was performed with Statistica for Windows 6.0 (StatSoft Inc., Tulsa, OK, USA). It included descriptive statistics and analysis of correlation with the Spearman Rank test. Comparisons between the groups were performed with two non-parametric tests, the Mann-Whitney *U*-test and Kolmogorov-Smirnov. The two-tailed significance level was set at $p < 0.05$. All values are given as mean \pm standard deviation.

Results

The age of the male patients was 38.07 ± 5.08 years and ranged from 26 to 55. The results of the routine semen analysis of the male population, as well as of the DFI evaluation are given in Table I. According to World Health Organization (WHO) guidelines, the semen samples from male partners were abnormal in 96.42% ($n=54$) and normal in 3.57% ($n=2$) of the infertile couples.

Table I. Results of the conventional semen analysis according to WHO guidelines and DFI evaluation by TUNEL and Comet assays.

Semen analysis	Mean±standard deviation	Range (min-max)
Concentration (x10 ⁶)	41.45±41.63	1-200
Motility (%)	43.14±24.24	5-80
Progressive motility (%)	24.65±23.07	0-80
Normal morphology (%)	6.28±6.98	0-30
DFI-TUNEL (%)	5.932±6.498	0.2-30
DFI-Comet (%)	4.505±4.727	0-17.5

DFI: DNA fragmentation index.

The DFI was $\geq 4\%$ in 44.64% of the cases by TUNEL and in 41.07% of the cases by Comet assay. The results with both assays showed that 17.85% of the patients had semen samples with $\geq 10\%$ spermatozoa with fragmented DNA.

The age of female patients was 33.39 ± 4.522 years and ranged from 25 to 42 years. COH was successful resulting in oocyte retrieval, without cases of hyperstimulation syndrome. The number of oocytes retrieved per patient was 8.00 ± 4.11 and 6.07 ± 3.56 of them were at metaphase II (MII). The fertilization rate was 54.84%. The grading of the embryos gave an embryo score of 11.97 ± 4.88 . The number of embryos transferred was 1.98 ± 0.88 . Fourteen pregnancies were achieved, identified by ultrasonographic examination.

No significant relationship between male patients' age and DFI was found by both assays ($r = -0.127$, $p = 0.369$ for TUNEL, and $r = 0.178$, $p = 0.899$ for Comet assay). There was no correlation between the DFI assessed by the Comet assay and any of the conventional semen parameters (concentration, percentage of motile spermatozoa and percentage of morphologically normal spermatozoa) (Table II). However, a slight negative correlation was found between the DFI assessed by the TUNEL assay and the sperm concentration, the percentage of progressive motile spermatozoa (a+b according to WHO), as well as the percentage of morphologically normal spermatozoa (Table II).

Neither the fertilization rate nor the embryo score was found to be correlated with the DFI detected by Comet or TUNEL assay ($r = -0.047$, $p = 0.727$ for fertilization rate and $r = 0.214$, $p = 0.126$ for embryo score by TUNEL assay and $r = -0.205$, $p = 0.128$ for fertilization rate and $r = -0.02$, $p = 0.846$ for embryo score by Comet assay).

There were no statistically significant differences between the cycles that resulted in pregnancy and those that did not regarding the following parameters: age of males, sperm concentration, percentage of motile or progressively motile spermatozoa, percentage of morphologically normal spermatozoa, DFI evaluated by TUNEL and DFI evaluated by Comet assay.

Table II. Analysis of the correlation between DFI, evaluated by TUNEL- and Comet assays and conventional semen parameters.

	Spearman R	p-value
DFI-TUNEL		
Concentration	-0.350	0.008*
Motility	-0.186	0.016
Progressive motility	-0.278	0.039*
Normal morphology	-0.294	0.027*
DFI-Comet		
Concentration	-0.042	0.754
Motility	0.042	0.754
Progressive motility	-0.207	0.127
Normal morphology	-0.095	0.483

DFI: DNA fragmentation index. *Statistically significant, n=56.

Discussion

The evaluation of sperm characteristics is the first step in male partner examination. The clinical diagnosis and management of male infertility is usually based on conventional semen parameters. Though conventional semen analysis is a mandatory test when counselling an infertile couple, it lacks information about DNA integrity and it is not always sufficient in the assessment of sperm function and male infertility. Moreover, it does not assess the outcome in terms of fertility or of a healthy conceptus. To ascertain if a male's sperm is competent new methods of diagnosis are needed to investigate spermatozoa at different levels.

It has been reported that male infertility is associated with poor sperm DNA integrity (24), and a high percentage of endogenous DNA nicks in ejaculated sperm has been correlated with reduced fertility (25-27). The studies performed in patients attending infertility clinics, either by Comet or TUNEL assay, showed that these patients have a considerable percentage of sperm with fragmented DNA (24, 28-30). The DFI in ejaculated human sperm had an incidence of 0.1% to 10% (31) but this rate was between 15% and 20% in cases of male factor infertility (11, 32-33). Sakkas *et al.* (34) found that almost 60% of oligozoospermic men exhibited $>10\%$ TUNEL positive cells.

In our study, both assays gave similar results. The DFI evaluated by TUNEL was slightly higher than the DFI evaluated by Comet. The results of both assays showed that 17.85% of the patients had a $DFI \geq 10\%$. In the present study, processed semen samples were evaluated after "swim-up" and taking into consideration that "swim-up" increases the quality of the semen in terms of DNA integrity (35), the DFI in the native samples could have been even higher.

The DNA damage in ejaculated spermatozoa determined by TUNEL or Comet assay has been negatively correlated with fertilization and pregnancy in conventional *in vitro* fertilization (IVF) cycles (15, 36). Recently, Henkel *et al.* reported that IVF patients with a DFI > 36.5% had a pregnancy rate of 18%, much lower than the pregnancy rate (34%) of those patients with DFI < 36.5% (37).

Nevertheless, in ICSI cycles, no consensus exists regarding the impact of DFI on the outcome. Host *et al.* (38), as well as Henkel *et al.* (37), reported no correlation between DNA fragmentation of spermatozoa and fertilization, embryo quality or pregnancy rates, in ICSI cycles. Recently, Abu-Hassan *et al.* (19) also did not find any correlation between the percentage of spermatozoa with fragmented DNA, evaluated by Comet assay, and fertilization rate or embryo quality after ICSI. In contrast, Lopes *et al.* (11) reported a negative correlation between the percentage of spermatozoa with DNA fragmentation and fertilization rate in ICSI cases. In another study, the percentage of spermatozoa with DNA damage was positively associated with impairment of post-fertilization embryo cleavage in ICSI cycles (39).

In our study, the results confirmed that both fertilization and embryo quality in ICSI cycles were not correlated with the DFI of processed semen samples. It seems that in ICSI, the selected motile and morphologically normal spermatozoa from a processed semen sample have a high possibility of containing intact nuclear DNA.

Apart from fertilization rate, embryo quality and pregnancy rate, it remains questionable as whether semen having a high DFI can give healthy children. In other words, it could be postulated that the use of semen samples with a high DFI may be correlated with the incidence of congenital abnormalities especially after ICSI. As evidenced in long-term follow-up studies, children born after ICSI have a higher major congenital malformation rate (8.7%) compared with those born after spontaneous conception (6.1%) (40). However, it is not known whether sperm DNA fragmentation counts for these abnormalities.

Several attempts have been made to link abnormal integrity of the male genome with conventional sperm parameters such as concentration, morphology and motility. Most of the relative studies have evaluated DNA fragmentation of spermatozoa by TUNEL assay, reporting a negative correlation between the incidence of spermatozoa with DNA fragmentation and concentration (15, 24, 33, 39, 41), motility and morphology (11, 15, 24, 39, 41).

In our study, the results with TUNEL assay showed a significant negative correlation between the DFI of processed semen samples and sperm concentration, progressive motility and the percentage of spermatozoa with normal morphology. However, no such correlations were found with the Comet assay. It could be suggested that this is an indication that TUNEL is more sensitive

than the Comet assay, although further investigation is needed to confirm this notion.

Several studies have reported an association between DFI and age. Singh *et al.* (42) studying semen samples from men attending infertility clinics as well as the general population with Comet assay, reported that the percentage of spermatozoa with highly damaged DNA was statistically significantly higher in men aged 36-57 years than in those aged 20-35 years. Similarly, the incidence of spermatozoa with DNA damage was positively associated with age in the study of Morris *et al.* (39). In contrast, in our study, where the DFI was evaluated in semen samples after "swim-up", the results of both assays were not correlated with the age of the male patients. It seems that the "swim-up" method negated the influence of age on DFI.

In summary, this study showed that a considerable proportion of processed semen samples used for ICSI had a DFI > 10%. However, the DFI of processed semen samples was not correlated with fertilization rate, embryo quality and the achievement of pregnancy in ICSI cycles. The DFI of processed semen samples evaluated by TUNEL assay was negatively correlated with the parameters of conventional semen analysis, namely sperm concentration, progressive motility and sperm morphology.

In our experience, TUNEL was much simpler, faster and probably of higher sensitivity than the Comet assay. The availability of the commercial kits also makes this assay easier to use. The main disadvantage, which creates a great handicap in its routine use, is the expense and the special equipment required in the procedure.

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