Detection and clinical significance of DNA fragmentation in human sperm

A brief overview of the literature

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This overview is produced by SPZ Lab A/S which performs SCSA®-analyses on a commercial basis. It has been our goal to present the scientific literature in the field as unbiased as possible. Don’t hesitate to contact us, if you have comments to the overview or would like to receive a copy of some of the cited literature.
Summary
During the latest decades, the amount of literature has increased significantly with regard to the clinical significance of DNA fragmentation in human sperm. Due to the amount of new information, it can be very difficult to keep up to date. A high number of publications show that DNA fragmentation has an impact on the rate of success achieved after different types of fertility treatments. In other words, information regarding sperm DNA fragmentation appears to be important to select the most optimal fertility treatment.

For research purposes several different methods have been used for detection of DNA fragmentation in retrospective studies. Although these studies have provided valuable information, an important limitation of the used methods are lack of precision and/or use of a subjective evaluation. For clinical use, the ideal test is objective and precise and enables the user to obtain results which can be used to guide couples to the most effective treatment. The SCSA®-method and TUNEL are the only methods which can be combined with flow cytometry and thus has the precision and objectivity needed for clinical use.

In spring 2007, the SCSA®-method has been implemented in the South region of Sweden as a part of the public fertility programme. An SCSA®-analysis will determine whether a couple can receive intra uterine insemination (IUI) or will be advised to intra cytoplasmic sperm injection (ICSI). The political decision is based on the recent publication by Bungum et al. (2007) which shows that approximately every fourth couple is experiencing infertility due to DNA fragmentation. It is expected that the Swedish decision will reduce the public expenses and at the same time will lead to a more efficient treatment for couples suffering from infertility.

Introduction
The aim of the present overview is to describe the different methods used for detection of DNA fragmentation as well as their potential for assessment of male fertility in vivo, after intra uterine insemination (IUI), in vitro fertilization (IVF) and intra cytoplasmic sperm injection (ICSI). Differences between methods have made it more difficult to interpret the scientific results. In addition, conflicting information in some publications regarding the clinical significance of DNA fragmentation has been caused by too small size of the clinical trials.

The compact structure of chromatin and the close association between DNA and protamin in the sperm nucleus makes detection of single- and double stranded DNA breaks (so called DNA “strand breaks” or “DNA fragmentation”) more difficult than in somatic cells. Moreover, utilization of new technologies during the recent decades has contributed to an increase in the amount of information regarding DNA fragmentation. The amount of information makes it necessary to understand differences between these methods as well as the limitations they possess as a diagnostic tool in the clinic.
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It is essential to be able to trust the results of a test when the purpose is to obtain a clinical diagnosis and use the result for fertility counselling of a couple. The method needs to have high precision, to be objective (independent of technician), and it should be possible to establish a threshold value which can be used to give the couple advice with regard to the most effective treatment. TUNEL and SCSA® are the only methods which by the use of flow cytometry produces a precise and objective result. The latest version of the WHO manual (WHO 1999) mentions the number of sperm assessed (counting error) and the sample variation as the two most important sources of variation. Subjective differences between observers are impacting tremendously on qualitative assessment of microscopic smears. In addition, a significant variation is often due to variations from one slide to another or variations within a slide. Fading of fluorescent smears and the time needed to conduct the assessment increases variation even more (Boe-Hansen 2005, Chohan et al. 2006). As a consequence of these problems, several of the methods used for research (retrospective studies) are unsuitable for clinical use.

Methods for detection of DNA fragmentation

TUNEL (terminal deoxynucleotidyl transferase-mediated dUDP nick-end labelling)

This method was developed for somatic cells and was later adapted to use for sperm (Gorczyza et al. 1993, Sailer et al. 1995). The principle of the method is to use terminal deoxynucleotidyl transferase to facilitate binding of a labelled nucleotide to the free 3'-OH group where the break is in the DNA. Several commercial kits are available on the market and the assessment is usually quantified by brightfield microscopy (Høst et al. 2000, Benchaib et al. 2007) or fluorescence microscopy (Duran et al. 2002, Carrell et al. 2003, Henkel et al. 2003, Henkel et al. 2004). As mentioned in the introduction, the main problems for microscopic assessments are counting error (too few cells assessed) and the subjective differences between observers. Differences between thresholds for TUNEL with regard to fertility are likely to be due to the subjective evaluation. However, some variation may also be due to differences between the different commercial kits with regard to the binding affinity of the probe and the penetration of the probe into sperm DNA (Høst et al. 2000, Henkel et al. 2003, Benchaib et al. 2007). Only few publications have combined TUNEL and flow cytometry (Seli et al. 2004). TUNEL is technical demanding as well as time consuming and the use of flow cytometry limits this technique to very few laboratories worldwide.

The SCSA®-method (Sperm Chromatin Structure Assay)

The SCSA®-method was developed for analysis of sperm (Evenson et al. 1980). The principle is based on denaturation of sperm DNA using low pH and subsequent staining by acridine orange. Due to the metachromatic nature of this dye, denatured single stranded DNA will emit a red fluorescence signal while intact and double stranded DNA will emit a green signal. The method provides an indirect measure of DNA strand breaks since such damage are occurring in the areas
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where DNA can be denatured. With the SCSA®-method the fluorescence signals is measured by use of flow cytometry which allows detection of intensities of red and green signals 500 times more accurate than with the human eye. In addition the precision of the analysis is very high since 5000 sperm are assessed routinely according to the SCSA®-protocol which is in contrast to the 100 to 200 sperm usually assessed by microscopic techniques.

The DNA fragmentation level is expressed by a DNA Fragmentation Index, also called the DFI value. A higher DFI value implies that a higher percentage of the sperm contains fragmented DNA. With the SCSA®-method calculation of the DFI values is based on the amount of red fluorescence in the individual cell divided by the total fluorescence (red + green) from the same cell. In the end of the 90’ties when several research groups became interested in the SCSA®-method, some publications reported DFI values which were calculated incorrectly with a software that could not determine the red fluorescence in comparison to the total fluorescence. In most of those publications a simple ratio between red and green fluorescence was calculated. In order to standardize the method, SCSA Diagnostics Inc. (Brookings, SD, USA) developed a specific software (SCSASoft®) and described the correct protocol for the SCSA®-method (Evenson og Jost 2000).

To obtain correct results with the SCSA®-method it is important to be aware of the different factors which may influence the result (Boe-Hansen et al. 2005). One particular important factor is that acridine orange is an equilibrium dye and that the relationship between dye molecules and sperm can result in variation if it is not kept within a certain limit. Precise estimation of sperm concentration is therefore necessary prior to the SCSA® analysis. It is furthermore important to use a reference sample for every six analyses to ensure that the instrument is stable.

Other methods for detection of DNA fragmentation

Neither the TUNEL by use of microscopic assessment nor the acridine orange test (AOT) which also is based on fluorescence microscopy is suitable for diagnostic use (Chohan et al. 2006, Evenson og Wixon 2006). Use of other dyes such as aniline blue or toudine blue does not solve the problem with a subjective evaluation (Erenpreiss et al. 2004). The Comet method is based on single cell electrophoresis with migration of small DNA fragments into a gel and formation of a “comet”. This method is also based on a microscopic assessment. Although data analysis can be carried out with a special software, the number of cells (usually 100) is far to low to satisfy the need for good counting statistics. The method is time consuming and the precision is very low (Boe-Hansen 2005, Evenson og Wixon 2006). The sperm chromatin dispersion test (SCD) examines the migration of small DNA fragments into agarose and uses visualisation with the DAPI dye and evaluation by fluorescence microscopy. This method is time consuming, subjective and lacks precision (Chohan et al. 2006). The “in situ nick translation assay” detects single stranded DNA breaks by incorporation of biotynilated dUTP using DNA polymerase I. The method quantifies endogenous DNA damage, but has up to now not showed significant relationship to fertility (Irvine et al. 2000).
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**Detection of apoptosis**

Several publications have used the term apoptosis synonymously with DNA fragmentation. However, it is important to understand the difference between these two terms. Programmed cell death (apoptosis) is important for the control of production of male gametes (Blanco-Rodriguez og Martinez-Garcia 1996). Apoptosis is an active process which is dependent on synthesis of proteins. During formation of the sperm the ribosomes are lost and classical apoptosis is therefore not possible in the late stages of spermiogenesis. However, apoptotic markers (i.e. Fas, Sakkas et al. 1999) can be found on ejaculated sperm and are thought to be due to a uncompleted apoptotic process (Cayli et al. 2004). Some authors (i.e. Barroso et al. 2000) have used Annexin V for identification of phosphatidylserine as an indicator of apoptosis. However, the new research in the field indicates that the same changes in the membranes are caused during capacitation of the sperm (Birck 2007).

Use of the term apoptosis synonymously with DNA strand breaks (Høst et al. 2000, Henkel et al. 2003) is incorrect when the detection method is TUNEL. To avoid confusion the term apoptosis should be reserved for programmed cell death. Single and double stranded DNA breaks in sperm can be created after spermiation as a result of so called “reactive oxygen species” (ROS). ROS is produced in all respirating cells as a consequence of intracellular redox activities. In the late stages of spermatogenesis and the early stages of maturation in the epididymis, the sperm are particular sensitive to the harmful effects of ROS. The reason is that the compaction of the DNA in the sperm has not been completed and that DNA repair mechanisms at the same time have been turned off (Golan et al. 1996, Aitken et al. 1998).

**Clinical significance of DNA fragmentation**

The term DNA fragmentation index (DFI) can lead to confusion since it is used for the SCSA®-method (Evenson og Jost 2000) but also have been used by some authors for TUNEL (i.e. Benchaib et al. 2007). For the SCSA®-method, a DFI value of 25 to 30 % results in a significant reduction in the possibility to obtain pregnancy by intercourse or IUI. This threshold is also important for selection of the most effective fertility treatment. With the TUNEL method, different thresholds has been reported and varies from 4 % to 36 % in the different studies (Høst et al. 2000, Henkel et al. 2003, Evenson og Wixon 2006). As mentioned before, subjective differences in the microscopic assessments as well as differences between the kits used, explains some of the variation between in threshold values. Altogether the results show that increased DNA fragmentation has a negative effect on male fertility in vivo and for the effectiveness of different types of fertility treatments.

Several studies have been based on a limited number of patients. For couples starting up in a fertility treatment programme, approximately every fifth man has a DFI value above 30 % with the SCSA®-method and every fourth has a DFI value above 25 % (A. Giwercman, personal communication). When a study compares 2 or more treatment protocols, the basis for comparisons is very limited if the study
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only includes for example a total of 100 couples. Another very important issue is that a clinical study needs to clarify any infertility factor in the female which may affect the fertility for the couple.

Significance of DNA fragmentation for in vivo fertility

The two largest studies where the SCSA®-method was used in combination with in vivo fertility are Evenson et al. (1999) and Spano et al. (2000), where 165 and 215 couples were followed while they tried to achieve pregnancy by normal intercourse, respectively. Evenson et al. (1999) observed that 85 % of the couples who had a DFI value below 15 % achieved pregnancy within 3 months. Only 50 % of the couples who achieved pregnancy between 4 and 12 months had a corresponding low DFI value. Furthermore, Evenson et al. (1999) observed that a DFI value above 30 % resulted in a longer time to achieve pregnancy or that no pregnancy was achieved. Spano et al. (2000) followed 215 couples until they achieved pregnancy or for a maximum of 2 years. Data acquired across 1301 cycles showed that the probability to achieve pregnancy was 13.3 % per cycle when the DFI value was below 20 % and 7.5 % when the DFI value was between 20 and 40 %. If the DFI value was above 40 %, the chance for achieving pregnancy was only 1.7 % per cycle.

Significance of DNA fragmentation for intra uterin insemination (IUI)

The largest study with the SCSA®-method and IUI include 387 cycles (Bungum et al. 2007). The percentage of children born per cycle was 19.0 % when the DFI value was below 30 % (n=321), while the group with a DFI value above 30 % only had a success rate of 1.5 %. Boe-Hansen et al. (2006) used the SCSA®-method in a study with 48 couples receiving IUI treatment. Only two of these couples had a DFI value above 30 % and none in this group achieved pregnancy. Saleh et al. (2003) performed a small study where 12 of 19 couples had a DFI value above 28 % and all of these were unable to achieve pregnancy.

The TUNEL method has been used in a retrospective study with 119 couples (154 cycles) by Duran et al. (2002). It was found that pregnancy could not be achieved when DNA fragmentation was present in more than 12 % of the sperm.

Significance of DNA fragmentation for IVF and ICSI treatments

The first studies describing the relationship between SCSA® results and IVF or ICSI treatments was too small with regard to the number of patients who actually had a DFI value above 30 %. The conclusions made by Larson et al. (2000) and Larson-Cook et al. (2003) have resulted in controversy regarding used of the SCSA®-method to predict the outcome of IVF or ICSI treatments. It is unclear why these conclusions was not disclaimed by Virro et al. (2004), which was a large collaboration between Virro, Larson-Cook and Evenson.

Larson-Cook et al. (2003) conducted a study based on 89 couples receiving IVF/ICSI treatments and only 10 of the couples had a DFI value above 30 %. The
study by Larson et al. (2000) was even smaller with only 24 men of which 10 had DFI values above 27 %. In these two studies, none achieved pregnancy if the DFI value was above the thresholds (27 or 30 %). Since this results was almost identical to the threshold observed in the in vivo studies (Evenson et al. 1999, Spanno et al. 2000), Larson et al. (2000) og Larson-Cook et al. (2003) concluded that IVF/ICSI treatments was likely to be unsuccessful if the DFI value was above 27 %.

In 2004 two larger studies concerning the SCSA®-method and IVF/ICSI treatments were published. Virro et al. (2004) and Bungum et al. (2004) based their studies on 249 and 306 couples, respectively. Virro et al. (2004) followed the couples until 3rd pregnancy month and concluded that a DFI value above 30 % did not appear to affect the process of fertilization itself but that blastocystrate and the possibility of achieving pregnancy was reduced. Virro et al. (2004) did not deny the threshold suggested by Larson et al. (2000) og Larson-Cook et al. (2003). On the contrary Virro et al. (2004) suggested that use of donor semen was considered when the DFI value was above 30 %.

Bungum et al. (2004) concluded that the authors could not confirm a threshold of 27 % in DFI for IVF or ICSI treatments. The conclusion was furthermore that IVF or ICSI treatments appeared to be ideal when the DFI value was above 27 %. Boe-Hansen et al. (2006) confirmed that a DFI value above 27 % does not prevent successful treatment by IVF or ICSI. Similar observations have since been made by Gandini et al. (2004), Payne et al. (2005) and Check et al. (2005), but these studies have been base on a much smaller clinical material.

The overall conclusion is that increased DNA fragmentation has an negative impact on the ability to achieve pregnancy after IVF and ICSI treatments. A long list of publications have documented lower blastocystrate and increased risk of abortion (Carrell et al. 2003, Henkel et al. 2004, Seli et al. 2004, Virro et al. 2004, Check et al. 2005, Benchaib et al. 2007). However, when the DFI value is above 25 to 30 % and pregnancy cannot be achieved in vivo or by insemination, successful treatment is possible by use of IVF or ICSI treatments.

**Is ICSI treatment more effective than IVF, when the DFI value is above 30 %?**

Bungum et al. (2004) observed, that ICSI treatment is more likely to result in pregnancy that IVF if the DFI value was above 30 %. This important observation for the SCSA®-method is new and confirms the results achieved by Høst et al. (2000) with the TUNEL method. The same tendency has been observed in other studies (e.g. Boe-Hansen et al. 2006), however all these studies have been too small with regard to number of patients to achieve a statistical significance and draw firm conclusions.

Bungum et al. (2007) confirmed their observation from 2004 and reports that the likelihood of achieving pregnancy is 1.6 times higher for ICSI in comparison to IVF when the DFI value is above 30 %. The theoretical explanation could be that ICSI
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treatment removes the oxidative stress that the sperm are exposed to during passage through female reproductive tract, due to hyperactivation and acrosome reaction as well as penetration of the zona pellucida. Accordingly, ICSI treatment will lead to a better chance of pregnancy than after IUI or IVF. Other possible explanations could be that the zygote has a better ability to repair DNA fragmentation after ICSI than IVF and that females receiving ICSI treatments is slightly younger than those receiving IVF treatment (30.8 years vs. 32.1 years, respectively; Bungum et al. 2007).

Controversies regarding DNA fragmentation

The studies by Larson et al. (2000) and Larson-Cook et al. (2003) have resulted in a significant discussion of the basis for routine implementation of the SCSA®-method in fertility treatments. As mentioned above, Virro et al. (2004) recommended that the use of donor semen should be considered when the DFI is above 27 %. Other studies (Gandini et al. 2004, Check et al. 2005, Payne et al. 2005) rejected this suggestion and pointed to the fact that IVF/ICSI appears to be the only efficient methods when the DFI value are above threshold. However, some of the conclusion by Payne et al. (2005) were incorrect and just added more confusion to the discussion. For instance, Payne et al. (2005) concluded that the lowest pregnancy rate was achieved when the husband had a low DFI value. Payne et al. (2005) did not review their data critically with regard to female factors (i.e. PCO, endometriosis) which may result in a low overall fertility even when the DFI value is low (Evenson 2006).

The latest report from The Practice Committee of the American Society for Reproductive Medicine (ASRM Practice Committee, 2006) is to some extend based on the confusion and conflicting reports in the field. Further more the report mentions that several newer techniques for assessment of DNA fragmentation is poorly validated and lacks the precision needed for clinical practice. (Schlegel og Paduch 2005, Fernandez et al. 2005). Additionally, the report mention that publications regarding treatment of the man with antioxidants to reduce DNA fragmentation or use of testicular sperm (Greco et al. 2005a, 2005b) is too few to warrant a clinical application.

Clinical use of the SCSA®-method in South Sweden

As mentioned above it is important that the SCSA®-analysis is carried out according to the Evenson protocol and that SCSAsoft® is used for data analysis (Evenson og Jost 2000, Boe-Hansen et al. 2005).

Approximately every fourth man entering fertility treatment will have a DFI value above 25 % (A. Giwercman, personal communication). A high DFI value can also be observed in patients with normal classical parameters (volume, concentration, motility and morphology, Saleh et al. 2002). Accordingly, the SCSA®-analysis should be performed on all patients in the beginning of a fertility treatment programme.
If the DFI value is raised (above e.g. 20 %) it is important to check for factors which may be the cause of the high DFI value. Age of the patient is important (Wyrobek et al. 2006) as is smoking (Potts et al. 1999), leucocytospermia (Alvarez et al. 2002), varicocele (Saleh et al. 2003) and any factors which may increase the temperature in the scrotum (fewer, hot tubs, heat from a laptop, tight clothing etc., Banks et al. 2005). Recent publications indicate that a high BMI (Kort et al. 2006) and/or diabetes/hyperinsulinaemia (Agbaje et al. 2007) can cause sperm DNA fragmentation. Correction of one or more factors could result in a lower DFI value after a period of approximately 3 months and may very well improve the chance for a successful treatment.

In March 2007 the Council of the South Swedish region decided to implement the SCSA®-method routinely for all couples entering public fertility treatment (Södre Regionvårdsnämnden, 2007).

The rules which have been used from 1st april 2007 regarding the SCSA®-method, which is performed at Reproduktions Medicinsk Centrum at Malmo Universitety Hospital is as follows:

If the DFI-value is below 25%:
Treatment by insemination with husbands semen is offered as a part of the public health care.

If the DFI-value is above 25%:
The probability to achieve pregnancy by insemination (IUI) is almost zero (Bungum et al. 2007), and it is therefore recommended that the couple recieve treatment by intra cytoplasmic sperm injection (ICSI). ICSI is recommended rather than IVF, since Bungum et al. (2007) have showed that the chance of achieving pregnancy with a DFI value above 30 % is 1.6 times higher with ICSI in comparison to IVF.

It is expected that the Swedish implementation of the SCSA®-method will reduce the public expenses and at the same time will lead to a more efficient treatment for couples suffering from infertility (A. Giwercman, personal communication).
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**Literature**


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Evenson DP. Letter to the Editor of Fertility and Sterility. 2006;85:810.


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Larson-Cook KL, Brannian JD, Hansen KA, Kasperson KM, Aamold ET, Evenson DP. Relationship between the outcomes of assisted reproductive techniques and sperm DNA fragmentation as measured by the sperm chromatin structure assay. Fertil Steril 2003;80:895-902.
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