Clinical Application of Sperm Chromatin Structure Assessment in Andrology Patients

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Clinical Application of Sperm Chromatin Structure Assessment in Andrology Patients

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CHAPTER 1

General introduction

INTRODUCTION

Infertility, defined as the inability to conceive spontaneously within one year, is a common medical problem. Traditionally, fertility investigations initially focus on the evaluation of ovulation and tubal patency in females, and on assessment of sperm quantity and quality in males. In about one third of couples with infertility abnormalities in classic semen parameters are found, like sperm concentration, motility and morphology. In another one third of patients a combination of infertility-related female and male factors are seen (WHO 1999). Although sperm quality parameters derived from classic semen analysis are frequently used to categorise male infertile patients, the prognostic and diagnostic information they provide is limited with a predictive power that is highest at the lower ranges of the spectrum (Barratt et al. 2010).

Despite many attempts of the World Health Organization (WHO) and the European Society for Human Reproduction and Embryology (ESHRE) to standardize semen analysis procedures, e.g. by providing laboratory guidelines (WHO 1999), training courses for lab technicians and external quality control programmes, semen analysis remains to be subject to great inter- and intra-observer variation. Moreover, large biological variation within individuals is observed over time (Alvarez et al. 2003; WHO 2010). Several factors may contribute to this heterogeneity, such as abstinence time, lifestyle factors (smoking or obesity), fever, or exposure to medication or gonadotoxins (Evenson et al. 2002), and probably many others.

When abnormal classic semen parameters are found, further diagnostic procedures should be undertaken. Low sperm quantity and quality might reflect testicular malfunction resulting in inadequate spermatogenesis. A multitude of different aetiologies might be involved, including a history of cryptorchidism or testicular torsion, genital infections, chronic diseases, varicoceles and genetic abnormalities. Basic andrological investigation of the infertile male is described in the WHO manual for the standardized investigation, diagnosis and management of the infertile male. This includes medical history, physical examination, evaluation of reproductive hormones, and scrotal ultrasound in addition to classic semen analysis (WHO 2000). Despite this standardized approach, male infertility remains unexplained in 30-40% of patients with abnormal semen parameters (Nieschlag et al. 2000). The molecular and or genetic basis of defective sperm function as seen in for instance varicocele, male accessory gland infection or cryptorchidism are still largely unknown.

Andrological diagnoses that contribute to infertility like cryptorchidism, testicular insufficiency and idiopathic sperm abnormalities can often not be surgically or medically modified to increase a couples chances of natural conception. Treatment focuses on the achievement of pregnancy using assisted reproduction techniques (ART). Traditional semen parameters are used to select the most suitable type of available ART i.e. intra uter-

ine insemination (IUI), in vitro fertilization (IVF) or intra cytoplasmatic sperm injection (ICSI). In azoospermic men spermatozoa can be surgically retrieved from the epididymis (percutaneous epididymal sperm aspiration PESA) and testis (testicular sperm extraction TESE) and subsequently used in an ICSI procedure. Males with impaired sperm function are able to father children using widely available IVF/ICSI. It is estimated that ARTs have accounted for over 3 million babies since the first IVF baby was born in 1978 (Carrell et al. 2010). Reports examining long term health consequences of IVF babies are limited, however, recent studies have described increased intrauterine growth restriction, lower birth weights and perinatal mortality in IVF/ICSI offspring (Steel et al. 2009). In addition, ICSI babies have a higher risk of de novo sex chromosome abnormalities and loss of imprinting genes associated with otherwise rare conditions such as Beckwith-Wiedemann and Angelman syndrome (Feng et al. 2008; Kurinczuk 2003).

It is noteworthy that normozoospermic males with partners with normal fertility parameters may be unable to conceive even when ART is used to establish a pregnancy. In these couples, available sperm function and oocyte quality assessment fails to identify deficits that may cause difficulties in gamete fusion and embryo development.

In the less frequent case of surgical treatment of male infertility, the evaluation of the effect of surgical treatment of male fertility disorders that aim to enhance sperm parameters is seriously hampered by the characteristic variation of quality in semen analysis parameters over time. In this view, the treatment of the most common abnormality seen in infertile males, the varicocele, is still controversial. Critics oppose that the increase in sperm concentration and motility following surgical treatment could also have occurred without treatment as the consequence of natural variation of semen parameters over time. This phenomenon is referred to as regression to the mean (Evers et al. 2003).

The above mentioned considerations implicate the need for more robust biomarkers for sperm function. There is need for novel functional, genomic and molecular sperm quality markers to improve treatment outcome and the selection of spermatozoa with an intact genome to guarantee safe reproduction in ART.

As long as such sperm quality markers have not been developed, traditional basic seminology will continue to be used as the sole diagnostic tool in male infertility investigations, despite its severe limitations and limited diagnostic and prognostic discriminative power. Novel markers should therefore preferably be linked to a different biological principle.

DNA integrity would possibly be a suitable candidate. Since the 1980's, abnormalities in the structural organization of sperm DNA and its negative influences on the male fertilizing potential are being investigated in both animal and human fertility clinics. Such abnormalities are considered to be a complementary, potentially independent, diagnostic tool in the management of male infertility. Initial clinical data demonstrated

that higher levels of sperm DNA damage can be found in infertile men (Zini et al. 2002) and suggested a negative impact on both natural (Evenson et al. 1999; Spano et al. 2000) and ART conception (Larson et al. 2000). These key papers have launched a variety of studies aiming to unravel the causes of sperm DNA damage, its use as an indicator for fertility in environmental epidemiological studies and most importantly, its clinical significance for spontaneous male fertilizing potential and prognostic value for clinical sperm samples used in ART.

In this introduction we will discuss current views regarding the aetiology of sperm DNA damage (1.) and the available assays to quantify sperm DNA integrity (2.). In addition, we will outline the hypotheses of this thesis (3.).

1. AETIOLOGY OF SPERM DNA DAMAGE

The term 'chromatin' refers to the macromolecular complex that contains the intranuclear DNA. The structure of chromatin is stabilized by interactions between its main compounds, DNA and DNA-binding proteins. In somatic cells histones are the dominating class of DNA-binding proteins. Sperm cell chromatin has a different composition that is characterized by an even more highly condensed structure. Condensation of sperm DNA is necessary in order to accommodate the paternal genome into the relatively small sperm cell head, to repress gene expression during spermiogenesis and to protect the paternal genetic message making it inaccessible to nucleases or mutagens (Oliva 2006). Condensation of sperm DNA takes place during the final stage of spermatogenesis, along with a remodelling of the nucleus and the loss of most of the sperm cells cytoplasm. Condensation is achieved by the sequential displacement of histones by transition proteins and then by protamines. Protamines are smaller than histones and have extremely strong DNA binding capacity. Further condensation and stabilization of sperm chromatin is obtained by the formation of disulfide cross-links between cysteines abundantly present in protamines. This final stage of chromatin organization takes place in the epididymis.

DNA strands bound to protamines are winded in circles and fall together in a doughnut-like shape (Ward et al. 1991). DNA compaction enables safe transport of paternal DNA throughout the genital tract. It is believed that the winding of sperm DNA plays an important role in early pregnancy and allows the developing embryo to easily access the genetic information and the "switch on" of genes that are needed during embryo development (Sakkas et al. 1996). It is estimated that approximately 15% of DNA in the human sperm nucleus of fertile men is bound to retained histones, whereas the amount of retained histones in infertile men can be variable (Zalensky et al. 2002). The precise packaging of the histone portion of sperm chromatin is not well understood (Barratt et

al. 2010). Recent evidence suggests that the location of histone bound DNA segments is associated with genes that are important for early embryo development (Hammoud et al. 2009).

In men the process of sperm DNA condensation is often incomplete, resulting in highly heterogenic sperm chromatin quality within one person. When sperm DNA packaging is incomplete, the DNA remains organized in loops that are more sensitive to damage. In infertile men sperm DNA packaging is even more impaired, resulting in higher amounts of abnormal sperm chromatin and consequent decreased sperm quality (Zini et al. 2002).

The aetiology of sperm DNA damage is most probably multi-factorial but compromised chromatin remodelling, oxidative stress and abortive apoptosis are commonly described theories. Sperm DNA damage may arise from combinations of all three mechanisms.

1.1. Abnormal chromatin remodelling

Firstly, abnormal chromatin remodelling can occur due to deficient protamination or abnormalities in protamine content. Lower amount of protamines and abnormal protamine content will negatively affect sperm nuclear compaction. Human chromatin consists of two types of protamines, P1 and P2. P2 has fewer binding sites to form disulphide bridges, making it less stable than P1. By comparison, animal chromatin consists only of P1 and is therefore more stable than human chromatin. In humans, P2 is synthesised as precursor, modified and activated after binding to the DNA. It was shown that in infertile males, the P2 content is decreased (Aoki et al. 2005). In some cases of infertility P2 was found to be completely absent (Carrel & Liu 2001). In infertile men P2 precursor concentration is increased as well as histon/protamine ratio. The number of disulphide groups is lower compared with fertile males (Carrell et al. 2008). Sperm DNA damage is associated with underprotamination in infertile men (Aoki et al. 2006). It remains unclear what causes the deregulation of protamine expression in male infertility. It is hypothesised that abnormal protamine expression is indicative of a general abnormality in spermatogenesis, possibly due to abnormal function of transcriptional or translational regulators (for review of candidate regulatory factors see (Carrell et al. 2007)). A second theory is that protamines may act as checkpoint regulators for spermatogenesis. In this view, abnormal protamine expression may induce apoptosis which could explain the link between underprotamination and poor sperm quality in infertile men (Carrell et al. 2007). On the other hand, incomplete protamination could make spermatozoa more vulnerable to attack by nucleases, free radicals that can cause oxidative stress or (environmental) mutagens. Chromatin remodelling may also be compromised by abnormal topoisomerase II activity. In the spermatogenesis, topoisomerase enzymes break and repair DNA during protamination, probably to prevent torsal stress and to facilitate DNA

condensation by replacement of histones by protamines. Low expression of the enzyme may result in the persistence of DNA breaks (McPherson et al. 1993). Endogenous nicks in spermatozoa are indicative for anomalies during spermatogenesis or an incomplete maturation process (Manicardi et al. 1995).

2.2. Oxidative stress

Secondly, DNA fragmentation can be caused by oxidative stress, due to free radicals, also referred to as reactive oxygen species (ROS) (Agarwal et al. 2002). ROS formation is a general physiological phenomenon that is normally balanced by anti-oxidants. Abundance of ROS can result in oxidative stress which in turn causes DNA damage such as chromatin cross-linking, chromosome deletion, mutations, DNA strand breaks, base oxidation and other lethal genetic effects (Agarwal et al. 2003b). Sperm cells are especially vulnerable for this type of damage, since they have no defence mechanism, apart from the characteristic tight packaging of the DNA and anti-oxidants present in seminal plasma. Even if DNA packaging takes place normally, oxidative stress may cause DNA damage throughout the genital tract. Leukocytospermia is considered a contributing cause of male infertility because of excessive ROS production and ROS release, although leukocyte-derived ROS are not as important as those derived from morphologically abnormal or dead sperm. Dysmature sperm produce high levels of ROS and can induce damage in mature spermatozoa in the epididymis (Ollero et al. 2001). Clinical studies demonstrated only a very weak correlation between white blood cell concentration and sperm DNA damage (Moskovtsev et al. 2007). Cigarette smoking has been associated with significantly increased levels of seminal ROS (Saleh et al. 2002c) and may decrease anti-oxidant activity in seminal plasma (Pasqualotto et al. 2008). Studies that assessed ROS, anti-oxidant capacity and biomarkers for oxidative DNA damage like 8-hydroxy-2-deoxyguanosine (8-OHdG) all indicate a strong association between ROS and sperm DNA damage (Barroso et al. 2000; Chen et al. 2004). Furthermore, studies in which sperm was exposed to artificially produced ROS demonstrated a significant increase in sperm DNA damage (Twigg et al. 1998a).

1.3. Abortive apoptosis

Finally, the frequently cited abnormal cell process that may lead to sperm DNA damage is the abortive apoptosis theory (Sakkas et al. 1999). Normal spermatogenesis is a dynamic process in which cell production and cell death are well balanced. Cells to be eliminated will enter a cascade of reactions leading to apoptotic cell death. Cutting of sperm DNA by endonucleases is one of the earliest events in this cascade, that is completed by phagocytosis and elimination of appropriately earmarked sperm cells by Sertoli cells (Sakkas et al. 2010). If this system does not operate efficiently and the process is not completed, damaged cells may escape apoptosis. Hidden DNA damage in the nucleus or aneuploidy

may prevail, despite normal differentiation to mature spermatozoa even with apparently normal sperm motility, morphology and even fertilizing capability (Sakkas et al. 2004). Men with abnormal semen parameters possess a higher percentage of spermatozoa containing damaged DNA and abnormal spermatozoa that express apoptotic markers like Fas, phosphatidylserine, Bcl-X_L and p53 (Sakkas et al. 2010).

2. ASSAYS TO QUANTIFY SPERM DNA DAMAGE

2.1. TUNEL

The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) technique is a commonly reported assay to investigate sperm nuclear integrity. Essentially, this method takes advantage of the ability of the DNA repair enzyme terminal deoxynucleotidyl transferase (TdT) to incorporate fluorescently labelled nucleotide at the 3'-end of a broken DNA strand. TUNEL does not provide direct quantification of DNA strand breaks, but individual sperm cells are scored as fragmentation positive or negative on the basis of their fluorescence intensity. Evaluation of the fluorescent intensity can be done either microscopically or by flow cytometer. TUNEL positivity is indicative of sperm DNA damage but not synonymous with apoptosis (Boe-Hansen et al. 2005), despite the frequent use of this term in the literature. The TUNEL assay is not validated for in vivo pregnancy rate in the general population.

2.2. Comet assay

The Comet assay is an electrophoretic technique in which lysed sperm cells are embedded in agar and allowed to migrate in an electric field. Intact, high molecular weight, unbroken DNA migrates slowly and will remain in the sperm head, whereas the smaller, fragmented DNA migrates out and takes on the form of a comet. The length of the comet in 200-300 individual cells is measured microscopically and sperm DNA fragmentation can thus be quantified. In the neutral Comet assay DNA is not denatured, making the assay more sensitive for the measurement of double strand breaks. Under acid or alkaline conditions double and single strand DNA breaks can be detected (Agarwal et al. 2003a). There are few studies relating the Comet assay to clinical fertility status and no clinically useful thresholds have been established.

2.3. NT assay

In situ nick translation (NT) assay quantifies the incorporation of biotinyllated-deoxyuridine triphosphate (dUTP) at single-stranded DNA breaks in a reaction that is catalysed by DNA polymerase I. The NT assay examines the presence of endogenous DNA nicks and damage. The assay has low sensitivity compared with other assays (Evenson et al. 2006).

2.4. Chromatin stains

Several tests identify packaging defects of sperm chromatin. Aniline blue is used to stain persisting histones and indicates abnormal chromatin condensation and immaturity. Toluidine blue binds more liable with loosely packed chromatin and impaired DNA. Damaged cells will stain blue and can be assessed using light microscopy. Chromomycin A3 (CMA3) is a fluorescent DNA binding compound. Binding to DNA is competitive with protamines and CMA3 fluorescence has therefore been interpreted as an indirect demonstration of decreased presence of protamine leading to poor protamination (Lolis et al. 1996).

2.5. SCD

The sperm chromatin dispersion test (SCD) is based on the principle that sperm with fragmented DNA fail to produce a characteristic halo when mixed with an aqueous agarose following acid denaturation and removal of nuclear proteins (Fernandez et al. 2005).

2.6. SCSA

Finally, DNA integrity can be assessed by the Sperm Chromatin Structure Assay (SCSA). For the work described in this thesis all measurements were done by the use of SCSA. SCSA, together with TUNEL, are the most commonly used assays for DNA damage assessment in basic research and clinical studies. Characteristics of SCSA, advantages and drawbacks in comparison with other assays will be discussed in detail in the next paragraphs.

The SCSA was developed by Evenson in the 1980's and measures the susceptibility of sperm chromatin to DNA denaturation in situ induced by low pH treatment (for review see (Evenson et al. 2000a)). The assay is based on the metachromic properties of a DNA binding fluorescent dye, acridine orange (AO). AO intercalates with DNA and emits green fluorescence when bound to intact, double strand DNA and red fluorescence when bound to single strand, fragmented, DNA. Originally, these properties were employed in a microscopic assay developed by Darzynkiewicz (Darzynkiewicz et al. 1983). The SCSA however, quantifies the fluorescent signals by flowcytometry. Upon excitation by laser light, the emitted red and green fluorescent signals from individual cells are detected by photomultiplier tubes. Routinely, 5000 individual sperm cells are analyzed in a few minutes.

The assay described by Evenson (Evenson et al. 2000a) requires strict adherence to the protocol and instrument settings to guarantee reproducible results. Either fresh or frozen samples can be used. So far, no evidence has been published to indicate that conventional freezing of samples at -80°C without cryoprotectants has detrimental effects on the sperm DNA damage assessment with SCSA. It is likely that freezing induces sperm DNA damage, but the damage probably lies below the detection limit of the

SCSA. When used in the assay, frozen samples should be quickly thawed in a 37°C water bath and diluted to a concentration of 1-2 x 10^6 sperm cells per ml with 1x TNE buffer. The diluted samples are exposed to acid detergent solution (pH 1.2) for 30 seconds. The acid detergent solution contains Triton X-100 that permeabilizes the cells membrane, allowing AO dye molecules to enter the sperm nucleus and bind to the sperm DNA. The low pH of the acid detergent solution potentially denaturates DNA in situ. DNA with abnormal chromatin structure and single or double strand breaks is more susceptible to denaturation. Immediately following acidic treatment, cells are stained with AO. The stained sample is placed on the flow cytometer and the sperm flow rate is checked. If the flow rate is too high (>300 cells/sec) a new sample with further dilution should be made to reach a flow rate of 100-200 cells/sec which allows adequate laser detection of the fluorescence pattern in the suspension. At exactly 3 minutes following the addition of AO, data acquisition should begin.

To ensure day to day standardization of the SCSA protocol and instrument performance, a reference sample, treated similarly as the study samples, has to be run prior to the actual sample measurements and is used to adjust the voltage gains of the flow cytometer photomultipliers that analyse red and green fluorescence respectively. The voltage gains have to be adjusted to obtain stable mean red (X) and mean green (Y) values of the reference sample at respectively 110 and 370 channels, with a maximum deviation of 5 channels. An aliquot of reference sample has to be run after every 5-10 samples. The voltage gains need to be re-adjusted whenever the fluorescent signal of the reference sample has drifted. Although the cause for the drifting of the voltage gains is not clearly understood, strict adherence to Evensons protocol is recommended as it was shown to lead to highly reproducible results, even between laboratories (Giwercman et al. 1999).

The fluorescent pattern of a sample is expressed in an acquisition dot plot with red fluorescent signal on the x-axis and green fluorescence on the y-axis. The main, normal cell population is easily recognisable as a cloud of clustered dots with very low red and predominantly green fluorescence. Sperm with denaturated DNA typically lie at the right of the main population in a descending 45° angle. A small percentage of sperm has increased DNA stainability and can be seen at the top end of the main population. It is believed that these cells are immature sperm containing highly compact sperm DNA (Evenson et al. 2000a). Chromatin in these cells is probably not fully condensed, thus allowing a greater accessibility by intercalating DNA dye. These cells are indicated as cells with high DNA stainabilty (HDS), calculated as the ratio between the population that starts at the 75% of the green fluorescent scale and the total number of cells. The clinical implication of the HDS parameter is not clear, although increased HDS values correlated with reduced fertilization rates and a longer time to pregnancy in vivo (Evenson et al. 1999), following IUI (Bungum et al. 2004) and IVF (Virro et al. 2004).

The presence of debris, bacteria and leukocytes may overestimate the event count during the acquisition fase and should be gated out as recommended by Evenson (Evenson et al. 2000a). The objectivity of the SCSA is not scrutinized by this subjective action, because the events other than the fluorescent signal of sperm cells can be easily identified as a straight line to the left of the main population (bacteria and leukocytes) and as events with very low green and red fluorescence (debris).

In initial low sperm concentration samples, the pattern of fluorescence shows very little red fluorescence and an abnormal, more elongated shape of the main population is seen. Untreated, not denaturated DNA stains only green and higher concentrations of seminal plasma in low concentration sperm samples that need no or little dilution to obtain a concentration of 1-2 x 10^6 sperm cells per ml interfere with the acid denaturation step. Moreover, samples with an undiluted concentration of 1-2 x 10^6 sperm cells per ml show a high flow rate, probably due to high protein concentration because of excess seminal plasma. We showed that samples need to be diluted up to 4 or 5 times with 1x TNE buffer, to ensure adequate AO staining reaction (unpublished observations). Our experiments led us to conclude that SCSA can be performed in initial low sperm concentration samples, but these samples have to be adequately diluted to prevent interference of seminal plasma with the acid denaturation step. Due to the extra dilution with consequent lower sperm cell concentration in the suspension, the elapsing acquisition time in these samples can be severely increased. To decrease the acquisition time, the total number of cells investigated may be lowered to 1500-3000 cells. It should be noticed that this number of cells examined is still at least 10 times greater than in conventional semen analysis. Seminal plasma has strong buffering capacity that interferes with the acid denaturation step required in SCSA. Incomplete acid denaturation leads to an underestimation of sperm DNA damage in these samples because the sperm DNA is not thoroughly exposed to in situ denaturation.

In the SCSA, the extent of DNA damage is expressed as the sperm DNA fragmentation index (DFI), reflecting the ratio of red fluorescence to total fluorescence. In our laboratory, Cell Quest Pro and WinList software were used to calculate the DFI of each sample. Each sperm sample was measured in duplicate and the mean values of the results were used in the analyses.

3. COMPARISON BETWEEN SCSA AND OTHER ASSAYS

In summary, SCD and the Comet assay under acid or alkaline conditions require an initial step of denaturation in order to detect DNA breaks. TUNEL, in situ-nick translation and Comet at neutral pH measure DNA single strand breaks, whereas TUNEL and neutral Comet also assess double strand breaks.

SCSA has several advantages opposed to the labour intensive classic semen analysis. Most importantly, light microscope assessment of sperm quality in classic semen analysis is most seriously restricted by objectivity and number of cells that can be analysed. Commonly, motility and morphology measurements are made on 100 to 200 sperm per sample. The SCSA makes use of a fresh or frozen-thawed semen sample and collects objective data on 5000 cells in just a few minutes. However, the SCSA protocol does have some drawbacks, mainly the requirement of strictly following the protocol as thawing time and temperature changes can negatively affect the results (Boe-Hansen et al. 2005). Moreover, the laboratory set up of the assay can be challenging because it calls for adequate software and a reliable, readily available reference sample. Finally, because SCSA requires a cell suspension of several millilitres and a denaturation step is needed that will destroy the cells, DNA integrity cannot be assessed in one cell or in a fraction of processed sperm used in ART.

SCSA is a quantitative, as opposed to a qualitative measurement like TUNEL, and has the potential to better define thresholds associated with reproductive outcome. Only SCSA has been validated for fertilizing potential in population based studies. Evenson showed in 200 presumed fertile couples attempting to conceive naturally, that men with a DFI <15% were more likely to conceive spontaneously within 3 months. In men who could not establish a pregnancy within 12 months, DFI was significantly increased compared to those who were successful within 3 months. This study served as a blueprint for the clinically relevant DFI thresholds of high fertility potential (DFI <15%), median fertility potential (DFI 15-30%) and very low fertility potential (DFI >30%) (Evenson et al. 1999). In a study by Spano et al, the clinical fertility threshold of 30% was confirmed in 215 first pregnancy Danish couples (Spano et al. 2000). Bungum et al found that IUI patients were 8.7x more likely to deliver a baby with a DFI <27% and confirmed these data in a larger data set (Bungum et al. 2007). In the following years, several studies applied the 30% or 27% DFI threshold to ART results. Although initial results were promising (Larson et al. 2000), the use of SCSA parameters to predict the outcome of ART is still under debate (Collins et al. 2008). Currently, several studies indicate a trend in increased spontaneous abortions when the DFI is >30% (Zini et al. 2008).

The major drawback of SCSA compared to TUNEL is the fact that SCSA measures in situ susceptibility to acid denaturation, whereas TUNEL can assess actual strand breaks. SCSA is often referred to as a measurement of indirect sperm DNA damage, opposed to the more direct method of TUNEL. It has been questioned whether the DNA damage detected following denaturation is of clinical significance, because intracellular pH in the oocyte is around 7.0. At neutral pH DNA strands would not dissociate and potential damage would be easier to repair by the oocyte (Sakkas et al. 2010). The TUNEL technique has been criticized as having many variations in methodology as well as the lack of standardization and normal values. The Comet assay, SCSA and TUNEL results have

been shown to be highly correlated, even if it is not clear whether they detect exactly the same types of DNA damage (Aravindan et al. 1997; Erenpreiss et al. 2004; Gorczyca et al. 1993; Sailer et al. 1995)

In 2003 when we commenced our experiments and prospective studies presented in this thesis, SCSA seemed like the most practical and clinically useful assay to assess sperm DNA damage. Compared with TUNEL, the SCSA is less labour intensive and easier to implement in a semen analysis laboratory: except for a flowcytometer and adequate protocol set up, no additional molecular analysis techniques need to be mastered. SCSA is the only assay that provides reference values that can be used in clinical studies.

OUTLINES OF THIS THESIS

The work described in this thesis aims to *validate the SCSA*. Next, we explored the *clinical validation of SCSA* by evaluating *sperm DNA damage in normozoospermic men* and exploring the role of *SCSA as a diagnostic tool in the evaluation of the infertile male*. In order to justify routine implementation of SCSA testing in clinical Andrology, issues like the *effect of possible confounders* and *the association between SCSA and pregnancy* needed to be addressed. In this thesis, we evaluated various possible *interventions*, mainly in the context of prospective controlled clinical trials.

These studies were performed with patients recruited from the Andrology outpatient clinic of the Erasmus MC, Rotterdam, The Netherlands. In this third line, academic referral clinic, Andrology is practiced to full extent, which includes diagnostic scrotal ultrasound and surgical treatment of male infertility like varicocelectomy, microsurgical vasectomy reversal and PESA, MESA and TESE. The specialised Andrology laboratory hosts national semen analysis courses and quality control programs and functions as a regional cryo semen bank. In the adjacent IVF laboratory of the Erasmus MC all modern ARTs including ICSI and prenatal genetic screening (PGS) are performed. The Andrology department is part of the Urology department and the Reproductive Centre of the Erasmus MC, Rotterdam, the Netherlands.

In **chapter 2** we assessed the variability of the results obtained by SCSA. It is well known that high intra-, and inter-laboratory variation is a major characteristic of the classic semen analysis. Variability may be of technical or biological origin or both. Technical variation is much lower in SCSA as compared to conventional semen analysis, but also the biological variation (seen as a difference in successive samples from the same individual) in semen parameters is less when obtained by flow cytometric SCSA (Evenson et al. 1991; Zini et al. 2001). To test this, we conducted a study among 100 patients in whom 2 consecutive semen samples were evaluated with SCSA. We included the clinical diagnoses of the study participants in the analyses to determine if specific

conditions e.g. oligoasthenoteratozoospermia (OAT) or varicocele were associated with increased biological variation of sperm DNA damage, bearing in mind future studies participant characteristics.

Defects in the genomic material in sperm are more prevalent in infertile men and have been attributed to common andrological disorders like varicocele (Saleh et al. 2003). The three most commonly described mechanisms that may lead to abnormal chromatin structure are most likely interrelated in Andrology patients; abnormal chromatin packaging presumably takes places in insufficient spermatogenesis and makes sperm more vulnerable to extrinsic and environmental insults that may be mediated by oxidative stress. Primary testicular factors that may disrupt spermatogenesis and spermiogenesis and subsequently lead to sperm DNA damage include ageing (Moskovtsev et al. 2006) and cryptorchidism (Sousa et al. 2009). Extrinsic factors that may cause sperm DNA damage include fever (Evenson et al. 1991), abstinence time (Evenson et al. 1991), cigarette smoking (Potts et al. 1999), male accessory gland infection (MAGI) (Saleh et al. 2002a), exposure to pesticides (Sanchez-Pena et al. 2004), air pollution (Rubes et al. 2007), obesity (Kort et al. 2006) and varicoceles (Saleh et al. 2003). It is postulated that smoking and MAGI lead to increased leukocyte derived ROS production that subsequently has a negative impact on the quality of sperm DNA. In order to consider SCSA as an additional, independent tool in the investigation of the infertile male, we aimed to determine the prevalence of sperm DNA damage, assessed by the SCSA, in a large group of andrological patients. To study the discriminative power of SCSA, the correlation of DFI with classical semen parameters and andrological diagnoses were investigated. In addition, we evaluated all currently known confounders of sperm DNA damage in a clinical setting, to assess the additive diagnostic value of SCSA in Andrology (**chapter 3**).

A mostly ignored, but amendable factor in infertility is nutrition. Several studies recently reported that B-vitamin and zinc concentrations are associated with male (in) fertility (Ebisch et al. 2007). Wong et al demonstrated an improvement in classic sperm parameters in infertile patients after suppletion of folic acid and zinc in a double-blind, randomized, placebo-controlled trial (Wong et al. 2002). In a study among 271 infertile couples we determined several B-vitamins in seminal plasma and explored possible associations with DFI, clinical and endocrinological features (**chapter 4**).

As discussed previously, classic semen analysis is not an adequate tool for the evaluation of varicocele treatment, predominantly because OAT, as seen in clinically relevant varicoceles, is subject to the regression to mean phenomenon when consecutive samples are analysed (Evers et al. 2003). A significant decrease in DFI following varicocele correction would provide better evidence for the reversal of the negative effects of the varicocele on spermatogenesis and sperm function. In a prospective study, we assessed DFI prior to and following varicocelectomy with the aim to establish whether

the reduction of sperm DNA damage after surgery was clinically relevant as revealed by the spontaneous postoperative pregnancy rates (**chapter 5**).

Patients who seek vasectomy reversal generally achieved paternity prior to their vasectomy and generally wish to conceive spontaneously with their new, often younger, partners. A successful vasovasostomy or vasoepididymostomy is defined as renewed patency of the vas deferens, with motile sperm in the postoperative ejaculate. The quality of the spermatozoa is mainly dependent on the obstructive interval (Dohle et al. 2005b), and large numbers of non-viable sperm are commonly found in postoperative semen samples. In **chapter 6** we present the results of a prospective study among 70 vasectomy reversal patients in whom postoperative DFI and its association with pregnancy rate were evaluated.

Most severe male factor infertility patients are treated with ICSI. Previous studies have shown that sperm DNA damage is not prognostic for the outcome of ICSI; normal pregnancies can be achieved with ICSI even in patients with high levels of sperm DNA damage (Gandini et al. 2004). We observed that previous studies included patients with higher sperm density and lower prevalence of sperm DNA damage than can be expected in true male factor infertility. Moreover, sperm preparation protocols used in ART can lead to iatrogenic sperm DNA damage (Zini et al. 1999), that may interfere with the prognostic value of SCSA parameters. We explored the association of sperm DNA integrity assessed in the native and processed sperm sample to be used in ICSI in a subgroup of patients with severe male factor infertility (**chapter 7**).

Fertility is commonly impaired at the time of cancer diagnosis (van Casteren et al. 2010) and cancer treatment may further affect gonadal dysfunction and induce damage to the DNA integrity. Previous studies reported contrary results regarding the association between cancer and abnormal sperm chromatin structure and the effect of cancer treatment (O'Flaherty et al. 2008; Ribeiro et al. 2008; Stahl et al. 2008). In a prospective study (**chapter 8**) we examined sperm DNA damage before and after oncological treatment in patients with testicular germ cell tumours and lymphatic disease.

An overall discussion and summary on assay characteristics and the clinical value of SCSA is given in **chapter 9**.

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CHAPTER 2

Clinical correlates of the biological variation of sperm DNA fragmentation in infertile men attending an Andrology outpatient clinic

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ABSTRACT

Determination of sperm DNA fragmentation, as assessed by the sperm chromatin structure assay (SCSA), has become an important tool for the evaluation of semen quality. The aim of the present study was to describe the biological variation of sperm DNA fragmentation in men attending an Andrology clinic and to identify clinical correlates of the biological variation of sperm DNA fragmentation.

For this study two consecutive semen samples from 100 patients attending our Andrology outpatient clinic were subjected to semen analysis, performed in parallel according to WHO guidelines and by SCSA.

A good agreement between pairs of samples was found for SCSA derived variables, as indicated by a significantly lower median coefficient of variation (CV) of the DNA fragmentation index (DFI) and the High DNA stainability (HDS) compared with WHO semen parameters. In half of the men attending our Andrology clinic, however, the individual biological variation of DFI and HDS, expressed as CV of two samples, exceeded 10%. Dysregulation of spermatogenesis, as seen as testicular insufficiency or varicocele, was not associated with increased variability of DFI or HDS. A backward multiple linear regression analysis however, indicated that the biological variation of DFI may be more profound in men with characteristics of normal spermatogenesis.

In conclusion, we confirm previous reports that sperm DNA fragmentation has a lower biological variability than classical semen parameters. We hypothesize that the sperm chromatin structure may be more influenced in patients with normal spermatogenesis, whereas in men with disturbed spermatogenesis, the chromatin structure may be already so impaired that the effect of unidentified factors leading to variability of sperm DNA fragmentation in time may not be as profound.

INTRODUCTION

The diagnosis of male infertility relies to a great extent on semen analysis. In spite of detailed technical guidelines and external quality control programs for sperm laboratories (WHO 1999), a large degree of inter- and intra-laboratory variability in World Health Organization (WHO) sperm parameters exists. In addition to the technical variation, semen analyses in consecutive samples of an individual are known to be subject to individual biological variation (Alvarez et al. 2003). Although sperm parameters like concentration, motility and morphology are used to classify men as infertile, subfertile or fertile, none of these parameters are diagnostic of infertility (Guzick et al. 2001). Both technical and biological variation lead to difficulties in the clinical interpretation of semen analysis results. Therefore, it has become clear that additional, objective methods are needed to evaluate fertility disorders and to increase the predictive value of sperm analysis for natural conception or assisted reproduction. A promising new approach is the assessment of the structural organization and packaging of sperm DNA that appears to be vital for a proper male reproductive function (Ward et al. 1991).

A useful method to assess chromatin organization and DNA integrity is the sperm chromatin structure assay (SCSA). This assay is based on the susceptibility of defective sperm DNA for acid induced denaturation. Denaturation is facilitated by the presence of DNA strand breaks (Aravindan et al. 1997) and an increased denaturation is associated with poor semen quality and reduced fertility in mammals (Evenson et al. 2000a). The assay is primarily indicative of DNA integrity but also provides information on the extent of chromatin condensation and adequate association of DNA with mature sperm nuclear proteins (Spano et al. 2000). DNA integrity is visualized with a fluorescent DNA binding dye, acridine orange (AO). When bound to double-stranded DNA, AO emits a green fluorescent signal, while AO associated with single-stranded DNA emits red fluorescence. Sperm chromatin damage, reflected by an increased proportion of red fluorescence by AO stained cells, can be objectively quantified by flow cytometric measurements (FCM). FCM SCSA parameters only moderately correlate with classic sperm parameters suggesting that SCSA might be applied as an additional sperm quality tool to be used in both clinical and research settings (Giwercman et al. 2003). Interestingly, even semen samples with normal concentration, motility and morphology can harbour abnormalities in sperm chromatin structure that are possibly related to reduced male fertility (Saleh et al. 2002b). In addition, sperm DNA integrity as measured by SCSA has shown to be a prognostic tool related to spontaneous pregnancy rates (Evenson et al. 1999; Spano et al. 2000) as well as pregnancy rates in assisted reproduction (Bungum et al. 2004).

In contrast to the technical variation that complicates classic WHO semen analysis, the FCM sperm DNA integrity assessment is objective and reproducible in and within laboratories when the guidelines for SCSA as described by Evenson are followed (Even-

son et al. 1999). It is well documented that the biological variability of a man's semen characteristics over time may be considerable due to a number of factors, such as period of abstinence, recent and present disease, use of certain medications, drug- and alcohol abuse, gonadotoxin exposure and the conditions during sample production and transport to the laboratory. Several studies have shown that the sperm DNA integrity patterns are consistent in individuals in consecutive samples and that the biological variability is significantly less for SCSA results than for classic semen parameters (Evenson et al. 1991; Zini et al. 2001).

We observed that a substantial proportion of men attending our Andrology clinic do, however, show a variation in sperm chromatin structure in different semen samples over time. Because the SCSA guidelines by Evenson were exactly followed and the coefficient of variation of repeated measurements of the same sample was low (<5%), technical variation was unlikely to have caused the observed variability in chromatin structure. Although the variability in SCSA parameters in consecutive samples was presumably caused by individual biological variation in sperm DNA damage, the cause of this variation has not yet been elucidated.

The aim of this study was to determine the biological variation of SCSA parameters in men attending an Andrology outpatient clinic. In an effort to better understand factors that influence the biological variation of sperm DNA integrity, we evaluated the clinical correlates of the biological variation observed and the relation with WHO semen parameters.

MATERIAL AND METHODS

Population and semen sampling

For this study we evaluated the semen analysis data of 100 men of whom two consecutive semen samples were analysed as part of routine andrological evaluation (WHO 2000). Patients that had undergone any form of treatment or intervention (e.g. refertilisation surgery, varicocele ligation, endocrine treatment or anabolic steroids abuse) or that reported any significant illness or infections were excluded. Patients were instructed to produce semen samples by masturbation after 3 to 5 days of sexual abstinence. Semen analysis was performed according to WHO guidelines for semen analysis (WHO 1999). An aliquot of unprocessed semen was stored at -80°C for SCSA evaluation at a later stage.

SCSA data acquisition

The SCSA was performed essentially as described by Evenson (Evenson et al. 2000a), using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). The system was equilibrated by running a buffer mixture composed of acid detergent buffer and AO

staining solution for at least 15 minutes. Frozen samples were quickly thawed in a 37°C water bath and diluted with TNE buffer (0.01 M Tris-HCl, 0.15 M NaCl, 1 mM EDTA, pH 7.4) to a concentration of 1-2 x 10^6 sperm cells per ml in a volume of 200 µl. This cell suspension was treated with 400 µl acid detergent solution (0.08 N HCl, 0.15 M NaCl, 0.1% Triton-X 100, pH 1.2) for 30 s while swirling the sample tube and then stained with 1.2 ml AO staining solution (0.1 M citric acid, 0.2 M Na2PO4, 1 mM EDTA, 0.15 M NaCl, pH 6.0, containing 0.6 µl /l AO). A reference sample, treated in the same way, was run prior to the actual measurements and used to adjust the voltage gains of the flow cytometer FL3 and FL1 photomultipliers that detected red and green fluorescence respectively. The voltage gains were adjusted to obtain stable mean red (X) and mean green (Y) values of the reference sample at 110 and 370 channels respectively, within a maximum variation of 5 channels. An aliquot of reference sample was stained and run again after every 5-10 samples. The voltage gains were adjusted whenever the mean red and mean green fluorescent signal of the reference sample had drifted. Data collection of the fluorescent pattern in 5000 cells was performed at 3 minutes after acid treatment. Each sperm sample was measured in duplicate.

SCSA data analysis

The extent of DNA damage was expressed as the DNA Fragmentation Index (DFI), reflecting the ratio of red fluorescence to total fluorescence. Cell Quest Pro and WinList software (Becton Dickinson, San Jose, CA, USA) were used to calculate the DFI of each sample. In a histogram the DFI was calculated as a percentage of counts beyond the green fluorescent peak signal. In the green versus red fluorescence dot plot, the cells with high DNA stainability (HDS) were calculated as a percentage of the total fluorescence. Cells with increased green fluorescence are considered to be immature germ cells with high DNA content. Debris, bacteria and leukocytes were gated out during acquisition.

Patient characteristics

Clinical work up comprised medical history taking with emphasis on smoking (34%), use of prescription medication (14%) and occupational exposure to chemicals or irradiation (3%). Scrotal ultrasonography was performed in 71 patients using a Toshiba Nemio 20 with a 12 Hz transducer. Testicular volume measurements, parenchymal description, scoring of testicular microlithiasis, measurements of epididymal caput diameters, vascular diameters and quality were recorded. A varicocele was diagnosed when at least 2 venous vessels with a diameter of at least 3 mm were present, in addition to reflux or diameter increase during Valsalva's manoeuvre. In the study population, 22 out of 71 patients (31%) were diagnosed with a varicocele, of which 7 out of 22 (27%) had normozoospermia. In 29 patients physical examination and scrotal ultrasound data were not available, in these patients only sperm analysis of two consecutive samples was performed.

Serum FSH and LH were determined in 72 patients with the Immulite assay (Diagnostic Products Corporation, Los Angeles, CA, USA). Total serum testosterone was determined in 77 patients by a radioimmunoassay as described previously (Verjans et al. 1973). Inhibin-B was measured in 77 patients, using kits from Serotec Ltd (Serotec Limited, Oxford, UK) (Groome et al. 1993). Mean (± SEM) reference values for FSH, LH and testosterone levels in a group of 72 normal men were 2.5 ± 0.2 IU/l, 3.6 ± 0.2 IU/l and 17.6 ± 0.8 nmol/l respectively (de Waal et al. 1995). Mean values (± SD) for Inhibin-B were reported to be 220 ± 91 ng/l in the general male Danish population (Jensen et al. 1997). The median values of LH, FSH, testosterone and Inhibin-B in our study population were respectively 3.7 IU/l (range 0.9-14.7), 6.0 IU/l (range 1.7-53.8), 13.6 nmol/l (range 1.8-29.6) and 141.0 ng/l (range 1.0-284.0).

Statistical analysis

For statistical analysis SPSS 11.5 was used (SPSS inc, Chicago, Ill.). All results are expressed as median ± range. Statistical analyses were performed using the Wilcoxon signed-rank test and the Mann-Whitney U test. Correlations were assessed using Spearman correlations. A p value <0.05 was considered to be statistically significant.

To quantify the intra-individual variation in classic semen parameters and SCSA variables, the coefficient of variation (%) of semen samples 1 and 2 in each of the 100 individuals was calculated using the formula:

CV = 100 x SD (sample 1 result, sample 2 result) / mean (sample 1 result, sample 2 result)

To illustrate the distribution of the individual biological variation of sperm parameters and SCSA parameters we calculated the percentage of patients with a median CV of more than 5%, 10% and 20%. Based on the calculated examples and because a CV threshold of 5% is often used in laboratory work, we chose a median CV of more 10% as a clinically relevant cut off for significant differences between the two consecutive measurements.

Mean values of the two consecutive semen samples for classic sperm parameters were investigated for correlations with the CV DFI and CV HDS to establish possible relationships between the sperm quality of an individual and the variability of DFI and HDS in two consecutive samples of this individual. In addition, Spearman's correlation test was performed to assess possible relationships between values of LH, Testosterone, FSH, Inhibin-B, patient age and time between analyses and the variability of the DFI and HDS parameter in individuals.

Evenson has previously reported a clinically relevant cut off level of a DFI below 30% for fertilizing potential in vivo. To evaluate the association between sperm quality expressed as the DFI and the individual variation of DFI, we compared the CV DFI in subjects with a DFI $<30\%$ to patients with a mean DFI $\geq 30\%$.

In an attempt to evaluate the biological variability of SCSA parameters in relation to the quality of spermatogenesis, patients with a sperm concentration >20 x 10^6 sperm/ml and FSH <7 U/l were categorised as normal spermatogenesis, whereas patients with a sperm concentration <10 x 10^6/ml and FSH>10 U/l where categorised as defective spermatogenesis. Differences in the biological variation of SCSA parameters, expressed as the CV DFI and the CV HDS, were tested in these sperm quality subgroups with the Mann-Whitney U test. Repeated measurements ANOVA, allowing for between- and within-individual variations, was used to assess whether the level of classic sperm parameters in the two consecutive samples measured accounted for the level of variation in DFI and HDS in the two consecutive samples.

Multiple linear regression analysis was performed using the backward stepwise method to identify independent major determinants of the variation in DFI and HDS, the latter expressed as the CV DFI and CV HDS. To obtain approximately normal distributions all outcomes were logarithmically transformed in the various regression analyses.

RESULTS

In 100 patients two consecutive semen samples were analyzed at a median interval of 1.4 months (0.5-6.9). The median values and range of the classic sperm parameters and clinically relevant SCSA data of the two semen samples are depicted in Table 1. Using the Wilcoxon signed ranks test, no statistically significant differences were found between means of all parameters measured in semen sample 1 and sample 2.

In Table 2, the median CV with range is given for each of the WHO and SCSA semen parameters. The median CV DFI was significantly lower compared to the CV's of sperm count, concentration, progressive motility and normal morphology. The same applied to the CV HDS and the CV's of WHO sperm parameters (Wilcoxon signed ranks test, all

Table 1 WHO semen parameters and SCSA parameters of two consecutive sperm samples, median (range) and *p*-value (Wilcoxon signed-ranks test)

	Median (range) sample 1	Median (range) sample 2	*p*-value*
Total sperm count (x 10^6/ml)	27.5 (0.1-785.4)	30.5 (0.2-440.8)	0.26
Concentration (x 10^6/ml)	9.3 (0.1-238)	10.5 (0.1-184.0)	0.18
Progressive motility (%)	18.0 (0-62.0)	20.0 (0-67.0)	0.67
Normal morphology (%)	2.5 (0-15.0)	3.0 (0-15.0)	0.32
DFI (%)	25.6 (3.6-93.1)	26.1 (7.5-89.2)	0.78
HDS (%)	17.5 (3.8-57.1)	16.4 (3.7-48.4)	0.15

* Wilcoxon signed-ranks test

Note: SCSA=Sperm Chromatin Structure Assay, DFI=DNA fragmentation index, HDS=high DNA stainability.

Table 2 Median (range) coefficient of variation (CV) of WHO semen parameters and SCSA variables calculated over two consecutive semen samples and the percentage of patients with a CV exceeding 10%.

	CV Median (range)		Percentage of patients CV > 10%
Total sperm count (x 10^6/ml)	43.0	(0.0-140.5)	89%
Concentration (x 10^6/ml)	32.6	(0.0-137.7)	82%
Progressive motility (%)	28.3	(0.0-141.4)	78%
Normal morphology (%)	28.3	(0.0-141.4)	69%
DFI (%)	9.2	(0.1-77.0)	47%
HDS (%)	11.8	(0.1-79.1)	58%

Note: SCSA=Sperm Chromatin Structure Assay, DFI=DNA fragmentation index, HDS=high DNA stainability.

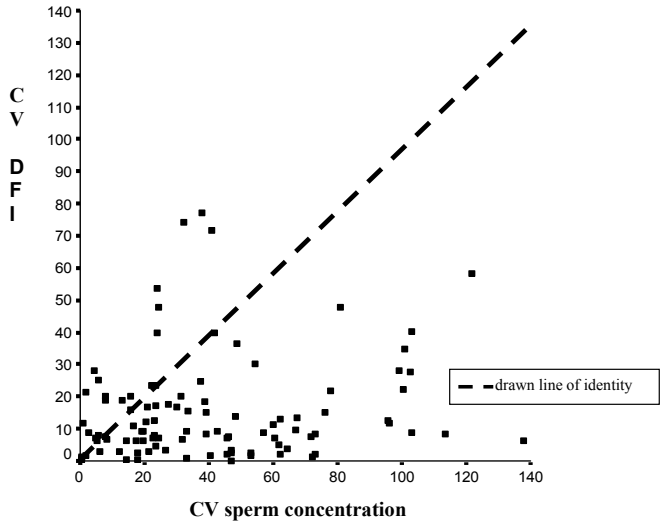

Figure 1 The association between the CV DNA Fragmentation Index (DFI) and the CV sperm concentration as calculated in two consecutive semen samples of 100 patients.

p <0.001). The association of the CV DFI with the CV sperm concentration is illustrated in Figure 1. The larger population to the right of the drawn line of identity indicates that the CV sperm concentration is significantly greater as compared to the CV DFI individuals. Of 100 patients, 47 had a CV above 10% for the two DFI measurements and in 58 patients the CV for the HDS parameter was above 10% in the two consecutive samples. For measurements of sperm concentration, motility and morphology a CV above 10% was found in respectively 82, 78 and 69% of patients.

Significant positive correlations were found between mean sperm count, concentration, progressive motility, morphology and Inhibin-B and the CV DFI in the two consecutive samples (Table 3). FSH levels were significantly negatively correlated with the CV DFI

Table 3 Spearman correlations of mean WHO semen parameters, patient characteristics and individual variability of SCSA parameters, expressed as the CV DNA Fragmentation Index (DFI) and CV High DNA stainability (HDS).

	CV DFI		CV HDS	
	r	*p-value*	*r*	*p*-value
Total sperm count (x 10^6/ml)*	0.21	0.04	0.07	0.47
Concentration (x 10^6/ml)*	0.21	0.03	0.05	0.62
Progressive motility (%)*	0.25	0.01	0.01	0.93
Normal morphology (%)*	0.27	0.01	0.07	0.50
LH (U/l)	-.20	0.10	0.06	0.53
FSH (U/l)	-.26	0.03	-.10	0.39
Testosterone (nmol/l)	0.09	0.45	0.01	0.91
Inhibin-B (ng/l)	0.23	0.04	0.12	0.30
Male age (years)	-.08	0.41	-.04	0.68
Time between samples (months)	0.04	0.73	0.07	0.52

* Mean of the two measurements

of the two consecutive samples. The variability in HDS in two consecutive samples did not correlate with any of the WHO sperm parameters, endocrine measurements, patient age or time between analyses.

The median CV DFI in subjects with a DFI <30% and patients with a DFI >30% were respectively 11.8% (0.4-77.0) and 8.2% (0.1-40.2) (p =0.040 using the Mann Whitney U test). In patients with a sperm concentration >20 x 10^6 sperm/ml and FSH <7 U/l spermatogenesis was categorized as normal, whereas patients with a sperm concentration <10 x 10^6/ml and FSH >10 U/l where categorised as defective spermatogenesis. The median CV DFI in these two subgroups was respectively 17.3% (0.7-74.0) and 7.0% (0.1-58.1) (p-value 0.042 using the Mann Whitney U test). The median CV HDS in the normal spermatogenesis category was 14.1% (0.1-79.1), compared with 10.0% (0.5-39.5) in the poor spermatogenesis subgroup (not significant).

Univariate analysis using the Mann-Whitney U test indicated that smoking, occupational exposure to gonadotoxins and the presence of a varicocele were not associated with the biological variability of DFI and HDS, expressed as the CV DFI and CV HDS. A significant decreased variability of the DFI was found in 13 patients who used some sort of medication (p-value 0.004) when compared with 77 patients who did not report medication use. It should be emphasized that the medication use reported was heterogeneous and mostly pulmonary and cardiac.

Using Repeated measurements ANOVA, sperm count, concentration, progressive motility and normal morphology at the two different semen analyses were tested in separate models for their association with respectively the DFI and HDS levels, measured in the two consecutive samples. Figure 2 illustrates the association of the log DFI level and the log

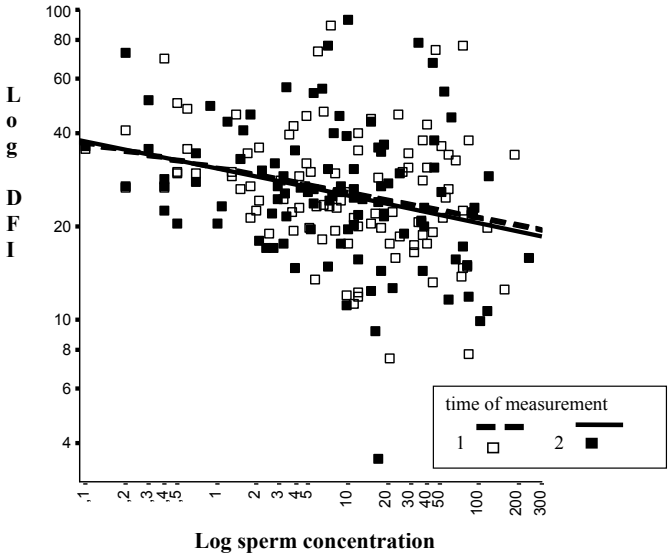

Figure 2 Illustrates the association of the DNA fragmentation index (DFI) level and the sperm concentration at the two times of measurement. Data were logarithmically transformed to obtain approximately normal data distributions. The log DFI and log sperm concentration are significantly correlated (p=0.009), and this association did not significantly differ between both measurement occasions.

sperm concentration at the two times of measurement. The log DFI and log sperm concentration are significantly correlated (p =0.009) and this association did not significantly differ between both measurement occasions. For none of the investigated associations did the association significantly differ between the two measurement occasions.

A multiple backward linear regression analysis with mean, logarithmically transformed values of sperm count, concentration, progressive motility, normal morphology, FSH and Inhibin-B as candidates for inclusion in the analysis, was performed to describe the relationship between these parameters and the log CV DFI. Mean morphology and Inhibin-B remained the only significant determinants of log CV DFI level using stepwise backward elimination. However, the predictive value was low as morphology and Inhibin-B levels could predict only about 20% of the variation (model R^2 =0.18).

DISCUSSION

Clinical interpretation of semen analysis results and the evaluation of interventional treatment are complicated by fluctuations in parameters in multiple semen samples of individuals. The variation in consecutive semen analyses within one person can be caused both by technical variation, exposure to gonadotoxic hazards or life-style factors and by the biological variation of sperm quality (Alvarez et al. 2003). The flowcytometric

SCSA is a computer-assisted, objective assay that intends to eliminate the methodological bias that hampers microscopic semen analysis. Although some studies have concluded that SCSA parameters are subject to biological variation as well, the coefficients of variation of SCSA parameters within an individual are much lower than for classic semen parameters (Evenson et al. 1991; Zini et al. 2001). Our study results are in agreement with these observations; the CV for DFI and HDS measurement were significantly lower compared with the CVs of sperm count, concentration, progressive motility and normal morphology.

It is generally accepted that SCSA is applicable in both clinical, interventional and epidemiological settings (Evenson et al. 1991; Evenson et al. 1999; Spano et al. 1998; Zini et al. 2001). A recent case report described the beneficial use of biological variability by selecting the sample with the lowest sperm DNA damage for Intra Cytoplasmatic Sperm Injection (ICSI) treatment. It was reported that about 5% of infertile men have significant unexplained fluctuations in SCSA parameters (Alvarez et al. 2004). In our experience, clinically relevant individual variation in SCSA parameters in multiple samples of men attending our Andrology outpatient clinic is more frequent. In the 100 individuals in our study we found a CV of more than 10% for the DFI parameter in 47% of patients. For the HDS parameter, 58% of patients showed a CV >10%. The variation in WHO semen parameters at the two times of measurement could not explain the level of DFI or HDS in our analyses, indicating that the variability in SCSA parameters is independent of the measured variability of WHO semen parameters. Although the mean variability in SCSA parameters is significantly lower than classic semen parameters, the individual variability expressed as the CV DFI and CV HDS is profound (>10%) in almost half of our patients. The scope of our paper is to establish relationships between the biological variation in sperm DNA fragmentation and clinical characteristics to give more insight in the origin of fluctuation in sperm DNA fragmentation over time.

Several studies have documented that conditions like abstinence time, fever, temperature alterations, seasonal changes, smoking, medication and exposure to environmental hazards can alter sperm DNA integrity (Evenson et al. 2000b; Fossa et al. 1997; Rignell-Hydbom et al. 2005; Sanchez-Pena et al. 2004; Spano et al. 1998) although no association between sperm DNA fragmentation and the length of abstinence time were observed by de Jonge et al (De Jonge et al. 2004). For our study, patients who reported changes in medication or any significant illness or infection between different semen samples were excluded. All participants were instructed equally for abstinence time. The time between analyses of both samples, ranging from 0.5 to 6.9 months, was not associated with the variability in DFI or HDS, expressed as the CV DFI and the CV HDS. Seasonal changes could not be evaluated as a possible cause of biological variation of sperm DNA fragmentation because the two samples were analysed within a median interval of 1.4 months.

Smoking is known to negatively influence sperm DNA integrity, but was not associated with the individual variability of SCSA parameters in our study. This result might be explained if smoking has a continuous negative influence on sperm quality. Only 3% of the patients reported occupational exposure to gonadotoxins, whereas 14% used some form of medication. None of these factors were related to the variability in DFI or HDS between two samples. The result that medication use was associated with lower variation in DFI in this study population is unexpected because of the heterogeneous medications used and perhaps due to an artefact based on the low number of patients.

Saleh et al previously described high levels of sperm DNA damage in infertile patients with a varicocele. The high DNA fragmentation indexes appeared to be related to high levels of reactive oxygen species and decreased antioxidant defences in seminal plasma of men with a varicocele (Saleh et al. 2003). Our results indicate that the deleterious effect of a varicocele on the sperm DNA integrity is most likely a constant factor that may continuously compromise sperm chromatin packaging, not giving rise to increased biological variation of sperm DNA fragmentation.

Our data do indicate that sperm quality is related to intra-individual variability in sperm DNA fragmentation. This observation is in contrast with a previous report by Sergerie et al who found lower within-subject standard deviations of sperm DNA fragmentation as measured by TUNEL in multiple semen samples from 6 sperm donors as compared to 10 infertility patients (Sergerie et al. 2005). In our study, mean sperm count, concentration, motility and morphology are significantly positively correlated with the intra-individual variability in DFI, expressed as the CV DFI. Also, FSH and Inhibin-B were significantly correlated with the CV DFI. Previous studies have considered DFI levels below 30% to be indicative for normal fertility. Our result that the biologicial variation of DFI, expressed as the CV DFI, was significantly increased in individuals with mean DFI levels below 30% compared with subjects with a DFI above 30% subscribes our results that an association between increased biological variation and normal sperm quality exists. When sperm concentration and FSH levels were combined to define subgroups of patients with normal and abnormal spermatogenesis quality, a significant increased CV DFI was found in patients with normal spermatogenesis quality further subscribing our previous conclusion that increased variability in sperm DNA fragmentation can be observed in patients with normal sperm quality.

Mean morphology and Inhibin-B were found to be variables with the best relation to the DFI variability in a multiple linear regression model. However, these variables were only able to predict about 20% of the observed DFI variability.

The origin of the relationship between normal sperm quality and increased biological variability of sperm DNA fragmentation remains unclear. It can only be hypothesized that in patients with a more severe disturbance in the spermatogenesis, the chromatin structure is so compromised that the deleterious effect of unknown factors causing

fluctuations in sperm DNA damage may not be as profound as in men with normal spermatogenesis and consequent normozoospermia and normal endocrine levels.

CONCLUSIONS

Because the SCSA is objective and shows high reproducibility, it may be concluded that the individual variability of SCSA parameters in consecutive samples is not caused by technical factors but is due to the biological variation of the chromatin structure. In almost half of the men attending our Andrology clinic, substantial biological variation of sperm DNA fragmentation exists. From our results, no clinical factors that periodically alter the chromatin structure could be identified. Moreover, the variation in DFI and HDS in an individual is not associated with dysregulation of spermatogenesis as seen in testicular insufficiency or varicocele. These results suggest that alterations in sperm chromatin structure over time are most likely caused by yet undetermined post testicular factors and that the biological variation of sperm DNA integrity may be more profound in men with normal, undisturbed spermatogenesis.

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CHAPTER 3

Sperm chromatin structure is associated with the quality of spermatogenesis in infertile patients

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ABSTRACT

Objective: To establish the diagnostic value of sperm chromatin structure assessment for the evaluation of male infertility, in addition to conventional andrological work-up.
Design: Cross-sectional controlled study.
Setting: A tertiary referral Andrology clinic.
Patient(s): Two hundred seventy-nine male partners of infertile couples.
Intervention(s): None.
Main outcome measure(s): The DNA fragmentation index (DFI) determined by the sperm chromatin structure assay (SCSA), semen parameters, serum levels of reproductive hormones and World Health Organization (WHO) classification of male factor subfertility.
Result(s): In all patient categories, except those including patients with hypogonadotrophic hypogonadism, sperm antibodies or normospermia, DFI was significantly higher compared with in proven fertile controls. After classification of the quality of spermatogenesis based on mean testicular volume (<10 ml vs. >15 ml), follicle stimulating hormone (FSH) (>10 U/l vs. <5 U/l) and Inhibin-B (<100 nmol/l vs. >150 nmol/l), the DFI was significantly higher in patients with poor spermatogenesis (35.9%) than in patients with normal spermatogenesis (25.9%). In a multiple regression analysis, the teratozoospermia index, sperm vitality and FSH were significant determinants of the DFI level. Male age was associated with DFI, but leukocytospermia, BMI or smoking were not confounders of DFI.
Conclusion(s): Impaired spermatogenesis, irrespective of the WHO classification of male factor subfertility, is generally associated with an increase of sperm DNA damage.

INTRODUCTION

Diagnosis and treatment of male infertility relies to a great extent on the results of semen analysis, although none of the currently used parameters accurately predict the fertilizing potential of spermatozoa (Guzick et al. 2001). Standardized diagnostic methods in the andrological work up including medical history taking, physical examination, scrotal ultrasound and hormonal analyses can categorize the majority of patients with male factor fertility disorders (Pierik et al. 2000). Still, male factor infertility is unexplained in 44-75% of patients (WHO 2000). It has been established that sperm DNA integrity plays a significant role in sperm function and fertilizing capacity (Ward et al. 1991) and that sperm DNA damage is increased in infertile couples (Zini et al. 2002). Using the Sperm Chromatin Structure Assay (SCSA) high levels of sperm DNA damage, expressed as the DNA fragmentation index (DFI) >30%, were shown to have predictive value for both spontaneous pregnancy (Evenson et al. 1999; Spano et al. 2000) and intra uterine insemination (IUI) outcome (Bungum et al. 2007). The prognostic value of SCSA testing for in vitro fertilizaton/intracytoplasmic sperm injection (IVF/ICSI outcome, however, appears to be limited (Collins et al. 2008). Because SCSA parameters are only weakly correlated with classic sperm parameters (Giwercman et al. 2003) and DFI levels above 30% can be found in 5%-8% of men from infertile couples with normal semen parameters (Erenpreiss et al. 2008; Zini et al. 2002), SCSA is a potential independent tool in the diagnosis of male infertility. The cause(s) of sperm DNA fragmentation are not entirely clear yet. Studies that evaluated the associations between sperm DNA fragmentation and leukocytospermia (Moskovtsev et al. 2007; Saleh et al. 2002a) or smoking (Saleh et al. 2002c; Sepaniak et al. 2006) in infertile men showed inconsistent results. Male age is correlated with sperm DNA damage (Moskovtsev et al. 2006; Schmid et al. 2007; Wyrobek et al. 2006) but studies on other patient characteristics, like Body Mass Index (BMI) (Kort et al. 2006), nutritional status (Vujkovic et al. 2009) and folate in seminal plasma (Boxmeer et al. 2008) yielded inconclusive or unconfirmed results. Common clinical conditions in male infertility like varicocele (Blumer et al. 2008; Saleh et al. 2003) and cryptorchidism (Sousa et al. 2009) are associated with increased levels of sperm DNA damage when compared with normozoospermic controls.

The aim of this study was to determine the prevalence of sperm DNA damage in an infertile, but otherwise heterogeneous population of men attending our outpatient Andrology clinic. All patients were stratified into andrological diagnosis groups, defined according to WHO guidelines (WHO 2000), and the possible differential prevalence of increased sperm DNA damage in particular diagnosis groups was investigated. Potential clinical confounders of increased DFI were evaluated.

MATERIALS AND METHODS

Population and semen sampling

Semen analysis was performed in 279 men attending our Andrology clinic between June 2004 and July 2005. Patients with a history of oncological treatment or vasectomy reversal were excluded. Semen analysis was performed according to WHO guidelines (WHO 1999). An aliquot of unprocessed semen was stored at -80°C for SCSA evaluation at a later stage. Twenty-two proven fertile healthy men, who donated a semen sample before vasectomy, served as controls for the SCSA measurement.

Andrological examination

Clinical work up comprised medical history taking with emphasis on cryptorchidism, urogenital infections and trauma or urogenital or inguinal surgery. Smoking habits, use of medication and occupational exposure to gonadotoxic chemicals or irradiation were recorded. Patient weight and height were measured to calculate the BMI. Testicular volume was estimated with a Prader orchidometer, testicular consistency was described as normal or weak and the position of the testes was recorded as normal or high scrotal. Scrotal ultrasound was performed using a Toshiba Nemio 20 with a 12 Hz transducer equipped with colour flow imaging. Serum FSH and LH were determined with the Immulite assay (Diagnostic Products Corporation, Los Angeles, CA, USA). Total serum testosterone (T) was determined by a radioimmunoassay as described previously (Verjans et al. 1973). Inhibin-B was measured using kits from Serotec Ltd (Oxford, UK) (Groome et al. 1993).

SCSA

The SCSA was performed essentially as described by Evenson and Jost (Evenson et al. 2000a), using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). In brief, frozen samples were quickly thawed, diluted to a concentration of 1-2 x 10^6 sperm cells per ml, exposed to acid detergent solution and stained with acridine orange. Data collection of the fluorescence pattern in 5000 cells was performed at 3 minutes after acid treatment. Debris, bacteria and leukocytes were gated out during acquisition as recommended by Evenson and Jost (Evenson et al. 2000a). The extent of DNA damage is expressed as the DFI. Cell Quest Pro and WinList software were used to calculate the DFI of each sample. All samples were measured in duplicate and the mean values of the results were used in the analysis.

Diagnosis groups

Following the WHO diagnostic classification, 11 diagnosis groups were defined. All patients were assigned a single diagnosis by exclusion in the following order: A varicocele

was diagnosed when palpable and at least 2 venous vessels with a diameter of at least 3 mm were detectable during scrotal ultrasound, in addition to reflux or diameter increase during Valsalva's manoeuvre. Patients with a history of unilateral or bilateral cryptorchidism were classified as having cryptorchidism. Male accessory gland infection (MAGI) was defined as recent epididymitis or prostatitis with or without leukocytospermia and seminal plasma pH ≥8.0. Patients who were suspected of partial ejaculatory duct obstruction (EDO) were classified as such according to low ejaculate volume (<1.0 ml) in combination with low pH (<7.0), normal FSH levels (<7 U/l), normal Inhibin B (>150 ng/l) and normal mean testicular volume (>15 ml). Patients were diagnosed with hypogonadism when T levels were <10 nmol/l and hypogonadotrophic hypogonadism when FSH and LH levels were ≤1.0 U/l. Immunological male infertility was defined as the presence of sperm antibodies as indicated by >50% IgG binding in the mixed antiglobulin reaction test. Patients were diagnosed with testicular insufficiency if none of the above diagnosis groups applied, FSH level was >7 U/l and the Inhibin-B level was <150 ng/l. Patients were diagnosed with idiopathic asthenozoospermia if sperm concentration was normal (≥20 x 10^6/ml) but sperm grade a motility was <25% and progressive motility (grades a+b) was <50%. Idiopathic oligozoospermia was defined as a sperm concentration below 20 x 10^6/ml and none of the above mentioned diagnosis categories applied. Patients without abnormalities in the medical history, physical examination, scrotal ultrasound, endocrinology and semen analysis were diagnosed as normozoospermia (Table 1).

Table 1 Prevalence of 11 diagnosis groups in 279 patients attending our Andrology outpatient clinic.

diagnosis	Number of patients (%)	Number of patients DFI >30% (%)	DFI (%)	(diagnosis) vs controls *p*-value[a]
varicocele	68 (24%)	23 (34%)	25.9 (3.8-59.1)	<0.001
testicular insufficiency	50 (18%)	32 (64%)	36.4 (3.8-74.7)	<0.001
idiopathic asthenozoospermia	38 (14%)	13 (34%)	25.7 (6.5-86.3)	<0.001
cryptorchidism	33 (12%)	16 (48%)	28.4 (10.3-69.5)	<0.001
hypogonadism	23 (8%)	10 (43%)	27.9 (5.2-90.8)	<0.001
idiopathic oligozoospermia	21 (7%)	14 (67%)	37.2 (9.7-70.0)	<0.001
normozoospermia	20 (7%)	2 (10%)	18.6 (9.4-43.1)	0.279
EDO	9 (3%)	2 (22%)	25.1 (10.7-70.7)	0.006
hypogonadotropic hypogonadism	7 (3%)	3 (43%)	22.3 (13.8-55.7)	0.103
MAGI	6 (2%)	4 (67%)	32.2 (13.6-66.8)	0.016
sperm antibodies	4 (2%)	1 (25%)	23.3 (11.1-39.3)	0.255

Note: MAGI= male accessory gland infection, EDO=partial ejaculatory duct obstruction. DFI (%) is presented as median (range). Controls are 22 proven fertile men, median DFI 15.3% (range 6.43-25.74). [a]The Mann-Whitney-U test was performed to compare DFI in the diagnosis groups with controls.

Statistical analysis

For statistical analysis SPSS 15.0 was used (SPSS inc, Chicago, Ill.). All results are expressed as median (± range). Correlations between variables were analyzed using Spearman's rank correlation coefficient. The Mann-Whitney U-test and one-way analysis of variance (ANOVA) were used to detect significant differences in SCSA parameters between the different diagnosis groups and the dichotomous parameters. A backward linear regression analysis was performed to evaluate the multivariate linear relation between significant univariate parameters and the DFI. Spearman correlations with a significant correlation coefficient of at least 0.2 were considered to be clinically relevant and were entered in the linear regression analysis model. Because of the reported confounding effects of male age and BMI on the DFI these two variables were included in the model, although univariate analysis did not demonstrate clinically relevant correlations. A p-value <0.05 was considered statistically significant.

RESULTS

Comparison of each of the diagnosis groups with the control group showed that DFI values were significantly higher in all patient categories, except for patients with hypo-

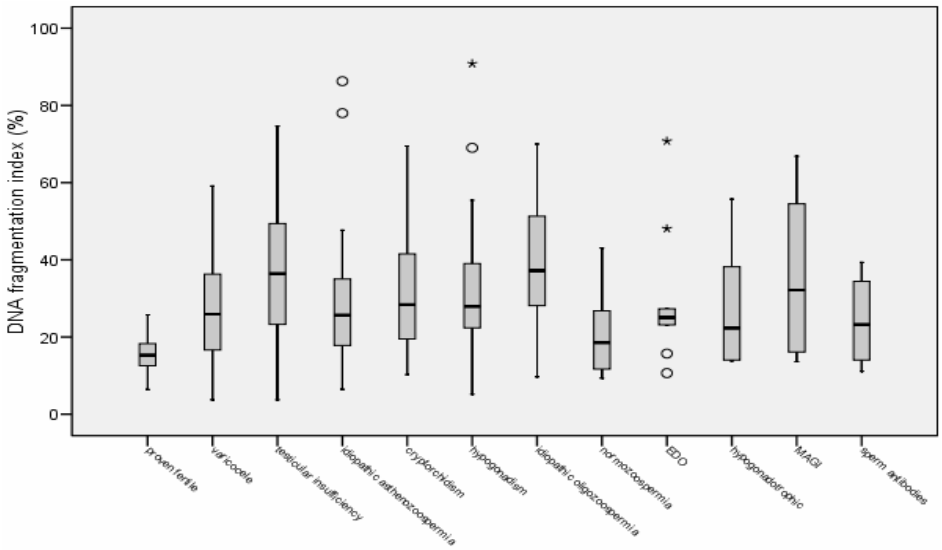

Figure 1 Boxplot graphic of the sperm DNA fragmentation index (DFI) in 11 diagnosis groups and proven fertile controls.

Note: Boxes show the mean DNA fragmentation index (DFI) with standard deviation, whiskers show the range of non-outlier DFI values, ○ represents outliers, * extremes. MAGI=male accessory gland infection, EDO=partial ejaculatory duct obstruction.

gonadotrophic hypogonadism, sperm antibodies or normozoospermia (Figure 1, Table 1). The range of DFI in all patient categories and even in proven fertile controls was substantial (Table 1). The prevalence of high DFI levels (>30%) is depicted in Table 1. Comparison of DFI levels in the six most prevalent diagnosis groups showed statistical differences in DFI levels between varicocele and testicular insufficiency (p=0.004) and between varicocele and idiopathic oligozoospermia (p=0.007). Significantly lower DFI values were seen in idiopathic asthenozoospermia compared with testicular insufficiency (p=0.008) and idiopathic oligozoospermia (p=0.009).

A Spearman correlation analysis of all continuous parameters deducted from semen analysis and andrological evaluation, showed midlevel negative correlations between the DFI and sperm count, concentration, progressive motility, normal morphology and vitality (-.309 < r < -.507). A significant positive correlation exists between DFI and the number of morphological sperm abnormalities per abnormal sperm cell, (referred to as the index of teratozoospermia or TZI index), and FSH. Significant, however weak, correlations were found for DFI and ejaculate volume, round cells, LH, male age, mean bilateral testis volume and BMI (0.131 < r < 0.196) (Table 2). To further explore male age as a confounder of DFI, all men were dichotomized based on a cut-off for male age at 45 years as previously used by Moskovtsev et al (Moskovtsev et al. 2006). DFI was significantly lower

Table 2 Spearman's rank correlation coefficient and corresponding p-value between continuous variables and the sperm DNA fragmentation index (DFI).

	DFI (%)	
	r	p-value[a]
Volume (ml)	0.194	0.001
Total sperm count (x 10^6/ml)	-.309	< 0.001
Concentration (x 10^6/ml)	-.413	< 0.001
Progressive motility (%)	-.503	< 0.001
Normal morphology (%)	-.349	< 0.001
TZI index	0.447	< 0.001
Vitality (%)	-.507	< 0.001
Round cells (x 10^6/ml)	-.131	0.029
LH (U/l)	0.174	0.008
FSH (U/l)	0.251	< 0.001
Testosteron (nmol/l)	0.097	0.115
Inhibin-B (ng/l)	-.094	0.134
Male age (years)	0.196	0.001
Mean bilateral testis volume (cc)	-.169	0.007
BMI	-.142	0.049

Note: TZI= teratozoospermia index, LH= luteinizing hormone, FSH= follicle-stimulating hormone, BMI= body mass index.

[a]Spearman's rank correlation coefficient

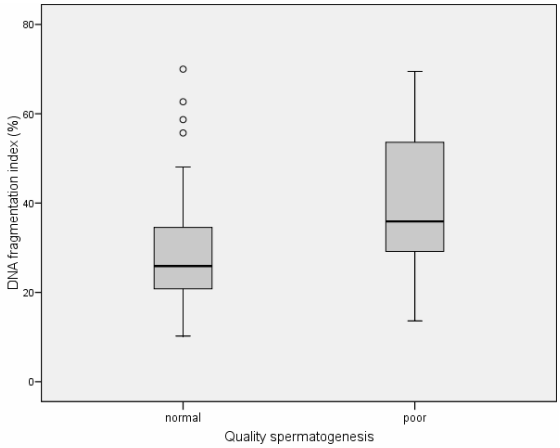

Figure 2 Boxplot graphic of the sperm DNA fragmentation index (DFI) in patients with normal quality spermatogenesis and poor quality spermatogenesis.
Note: Boxes show the mean DNA fragmentation index (DFI) with standard deviation, whiskers show the range of non-outlier DFI values, ○ represents outliers, * extremes. Normal quality spermatogenesis is defined as mean testicular volume >15 cc, FSH <5 U/l and Inhibin B >150 nmol/l. Poor quality spermatogenesis is defined as mean testicular volume <10 cc, FSH >10 U/l and Inhibin B <100 nmol/l.
[a]Median (range) DFI for good quality spermatogenesis is 25.9% (10.2-70.0) versus poor quality spermatogenesis 35.9% (13.6-69.5) (p =0.016, Mann-Whitney U test).

in younger patients (p=0.011). ANOVA analysis of patients grouped according to BMI ranges (underweight <20, normal 20-24, overweight 25-30 and obese>30) revealed no statistical differences in DFI between the BMI distribution groups.

The DFI was significantly increased in patients with a soft testicular consistency, compared with patients with normal testicular consistency (35.9% (11.65-74.4) vs. 27.2% (3.8-90.8) p=0.006). No significant differences in DFI levels were detected in relation to semen viscosity, alcohol use, medication use, occupational exposure to gonadotoxins, inguinal surgery, urogenital infection, testicular trauma, position of the testis, or reflux in the spermatic veins. Leukocytospermia was observed in 10.8% of patients. Neither leukocytospermia, nor white blood cell (WBC) concentration were significantly correlated with the DFI. Thirty-three percent of patients were smokers, who smoked 15 cigarettes a day on average, with a range of 1-60. Smoking status and the number of cigarettes smoked were not associated with increased DFI levels.

All patients were dichotomized based on the quality of spermatogenesis, using normal thresholds for testicular volume, FSH and Inhibin B and lower limits of these variables to ensure selection of patients with truly poor-quality spermatogenesis. Figure 2 shows that DFI was significantly higher in patients with poor spermatogenesis (defined as mean testicular volume <10 ml, FSH >10 U/l and Inhibin B <100 nmol/l) than in patients with normal spermatogenesis (mean testicular volume >15 ml, FSH <5 U/l and Inhibin B ≥150 nmol/l) 25.9% (10.2-70.0) vs. 35.9% (13.6-69.5) (p=0.016).

In a backward multiple linear regression analysis model, the TZI, vitality and FSH were significant determinants of the DFI level ($R^2 = 0.411$; $p<0.001$). Male age was not a significant determinant of the DFI level ($R^2 = 0.411$; $p=0.074$).

DISCUSSION

To our knowledge, this is the first study that evaluates the diagnostic value of sperm DNA damage assessment by SCSA in addition to full andrological examination according to the WHO guidelines for diagnosis and examination of the infertile couple (WHO 2000), in as many as 279 unselected subfertile men. DFI levels are nondiscriminating in the diagnosis of male infertility, but impaired spermatogenesis, irrespective of its etiology, is associated with increased levels of sperm DNA damage. For example, DFI values were similarly increased in groups as diverse as varicocele and cryptorchidism, for which the mechanism of sperm DNA damage is likely to be quite different. Remarkably, male fertility disorders presumably associated with abnormal spermatogenesis (like testicular insufficiency and idiopathic oliogozoospermia) showed the highest median levels of DFI. A diagnosis associated with presumably normal spermatogenesis like sperm antibodies had DFI levels that were not significantly different from those of controls, although the number of patients in this group was possibly too small to reach statistical significance. The univariate analysis supports our thesis because clinical measures for impaired spermatogenesis, like abnormal classical sperm parameters, high FSH and LH levels, decreased mean testicular volume and weak testicular consistency, were all significantly correlated with increased levels of DFI. In addition, in subfertile men with physical and hormonal features of poor quality spermatogenesis, the DFI was found to be significantly higher than in men with normal testicular volume and normal gonadotrophins. Finally, the multivariate analysis indicated that FSH, a strong predictor for the quality of spermatogenesis, is one of the determinants of DFI in Andrology patients.

Although our results indicate that DFI may merely reflect the quality of the spermatogenesis, the etiology of increased DFI remains unclear. Because our study indicates that sperm DNA damage is correlated with clinical hallmarks of disturbed spermatogenesis, it can be speculated that chromatin instability, and consequently DNA damage, might have been inflicted at the replacement of histones by protamines, a process taking place during spermatogenesis. Aberrant protamine isotype ratios or protamine deficiencies were indeed shown to be related to fertility status (Aoki et al. 2005). Thus, enhanced DFI might indirectly reflect generalized problems during spermatogenesis.

The substantial range of DFI in both patient diagnosis groups and controls make the search for confounding factors all the more important. We found that leukocytospermia was not associated with increased levels of DFI, which is in contrast to the results from studies by Erenpreiss et al and Saleh et al (Erenpreiss et al. 2002; Saleh et al. 2002a).

In a recent study performed in 1230 unselected patients WBC concentration correlated only very weakly with the DFI (Moskovtsev et al. 2007). Although some studies found an association between smoking and sperm DNA damage in infertile men (Sepaniak et al. 2006; Viloria et al. 2007), others could not confirm this (Belcheva et al. 2004; Saleh et al. 2002c). On the basis of our results, we conclude that in an infertile population, the additional effect of reactive oxygen species mediated risk factors like smoking and leukocytospermia on the sperm DNA integrity may not be profound.

Several studies have established Inhibin-B as a sensitive marker for both normal (Andersson et al. 2004) and impaired spermatogenesis (Pierik et al. 1998). Here we confirm previous reports that Inhibin-B levels are not associated with sperm DNA damage (Appasamy et al. 2007). In contrast to the data reported by Richthoff et al (Richthoff et al. 2002), in our study population of unselected patients, testosterone and DFI were not correlated. LH and FSH, both markers of disturbance in spermatogenesis, were positively correlated with the DFI. In our multiple linear regression analysis model, FSH was a significant determinant of the DFI level. Moreover, when endocrine and physical characteristics of impaired spermatogenesis were combined, a clear association with increased sperm DNA damage levels was shown.

Interestingly, a positive correlation was found for the DFI and the TZI, the number of abnormalities per abnormal spermatozoon. The TZI is a strong, independent determinant of the DFI in our logistic regression analysis, confirming the positive correlation of sperm DNA fragmentation and morphological abnormalities (Evenson et al. 1999). As current approaches for morphologic evaluation of sperm are quite labor-intensive and prone to subjectivity, the determination of DNA fragmentation may prove to be a valuable alternative if its effectivity can be further demonstrated in future studies.

In agreement with previous studies, high DFI levels (>30%) were found in 10% of men with normozoospermia from infertile couples (Erenpreiss et al. 2008; Zini et al. 2002). Some authors suggest that SCSA can be useful in selecting the appropriate method of assisted reproductive technologies (ART) in these couples, because unlike spontaneous pregnancy and IUI, success rates following ICSI are not associated with high DFI levels (Bungum et al. 2007). In these selected patients, SCSA may provide additional information about the nuclear integrity of sperm that cannot be diagnosed with classic WHO semen analysis and may be of some clinical use.

The apparent lack of discriminating power in the diagnosis of male infertility led us to conclude that sperm DNA fragmentation testing is not ready for routine application in the diagnostic work-up in an Andrology clinic. Our findings indicate that the quality of spermatogenesis, independent of the WHO classification of male subfertility, is associated with sperm DNA damage. In a population of Andrology patients, only increased male age is associated with increased sperm DNA damage; no other potential confounders have significant impact on DFI.

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CHAPTER 4

Low folate in seminal plasma is associated with increased sperm DNA damage

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ABSTRACT

Objective: To determine associations between vitamin B status, homocysteine (tHcy), semen parameters and sperm DNA damage.
Design: Observational study.
Setting: A tertiary referral fertility clinic.
Patient(s): Two hundred fifty-one men of couples undergoing in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) treatment, with subgroups of fertile (n=70) and subfertile men (n=63) defined according to semen concentration and proven fertility.
Intervention(s): None.
Main outcome measure(s): The DNA fragmentation index (DFI) as marker of sperm DNA damage determined using the Sperm Chromatin Structure Assay (SCSA), and semen parameters assessed according to World Health Organization (WHO) criteria; tHcy, folate, cobalamin and pyridoxine concentrations determined in seminal plasma and blood.
Result(s): In the total group of fertile and subfertile men, all biomarkers in blood were statistically significantly correlated with those in seminal plasma. No correlation was found between the biomarkers in blood and the semen parameters. In seminal plasma, both tHcy and cobalamin positively correlated with sperm count. Folate, cobalamin, and pyridoxine were inversely correlated with ejaculate volume. In fertile men, seminal plasma folate showed an inverse correlation with the DFI.
Conclusion(s): Low concentrations of folate in seminal plasma may be detrimental for sperm DNA stability.

INTRODUCTION

In general, male subfertility is of multifactorial origin. Whereas genetic causes of subfertility are difficult to modulate, other conditions such as accessory gland infections and vas deferens obstruction are potentially treatable. Environmental and lifestyle factors are also potentially amenable and thereby may cure or prevent the subfertility. Nutrition is an important modifier, but is largely ignored in the counseling and treatment of these patients. In recent years, interest has increased in the role of B vitamins as modulators of fertility outcome (Ebisch et al. 2007; Forges et al. 2007). Deficient B-vitamin concentrations cause elevated homocysteine concentrations and impair the remethylation cycle. This metabolism is involved in the methylation of phospholipids, proteins, DNA and RNA, and in the synthesis and repair of DNA. These processes are essential in spermatogenesis and therefore derangements in this pathway may be detrimental for reproduction. Recently, our group demonstrated an adverse effect of a high total homocysteine (tHcy) concentration in ejaculated sperm and follicular fluid on embryo quality (Ebisch et al. 2006). Moreover, we showed in a randomized, placebo controlled trial that during 6 months of intervention with folic acid and zinc phosphate a 74% increase of total normal sperm count in subfertile men could be achieved (Wong et al. 2002). The effect of cobalamin in reproduction is less defined, although there is some evidence that this B vitamin affects sperm parameters as well (Blair et al. 1968; Grasbeck et al. 1976; Sharp et al. 1962; Tomaszewski et al. 1963).

Conventional semen analysis consists of measuring a variety of semen parameters including volume, sperm concentration, motility and morphology. Nowadays the DNA fragmentation index (DFI) can be assessed by the Sperm Chromatin Structure Assay (SCSA), an independent measure of sperm quality with a better diagnostic and prognostic capability than the conventional semen analysis alone (Agarwal et al. 2003a; Giwercman et al. 2003). During spermatogenesis most of the cytoplasm of the spermatozoa is discarded, through which the availability of nutrients and defensive cytosolic enzymes is limited. This may result in a higher sensitivity for DNA damage. During ejaculation, the microenvironment of the spermatozoa is formed by the seminal plasma. It contains a broad spectrum of nutrients, enzymes and hormones necessary to maintain normal metabolism and function of the spermatozoa. Deficiencies or excessive concentrations may be detrimental to the spermatozoa. So far, the effect of seminal plasma tHcy and B vitamins on semen parameters have scarcely been studied. Our study investigated the associations between 1) B vitamin and homocysteine concentrations in blood and seminal plasma, 2) the biomarkers and conventional semen parameters, and 3) the biomarkers and sperm DNA damage.

MATERIALS AND METHODS

Study population

Between September 2004 and January 2007, subfertile couples undergoing in vitro fertilization (IVF) with or without intracytoplasmic sperm injection (ICSI) treatment at the Erasmus MC, University Medical Center in Rotterdam, the Netherlands were included in the prospective FOod, Lifestyle and Fertility Outcome-study (FOLFO-study). The FOLFO-project was set up to study the influence of preconception nutrition and lifestyle on fertility and pregnancy outcome. In the present analysis, the male participants are evaluated. Fertile and subfertile men were eligible for enrollment unless semen was cryopreserved or obtained by microsurgical or percutaneous epididymal sperm aspiration (MESA or PESA). Couples were invited to participate in the study at the intake visit. The study protocol was approved by the Dutch Central Committee for Human Research and the medical ethics and institutional review board of Erasmus MC, University Medical Center in Rotterdam, the Netherlands. All participants gave their written informed consent.

For standardized fertility evaluation, men visited the Andrology outpatient clinic. The fertility evaluation comprised semen analysis, blood sampling, physical examination including a scrotal ultrasound and a general questionnaire. All obtained materials were processed anonymously.

During physical examination scrotal ultrasonography was performed using a Toshiba Nemio 20 with a 12-Hz transducer (Toshiba, Tokyo, Japan). A varicocele was diagnosed when at least two venous vessels with a diameter of at least 3 mm were present, in addition to reflux or diameter increase during Valsalva's maneuver.

Furthermore, all participants filled out a general questionnaire from which the following data are extracted: medical history, body height and weight, ethnicity, and lifestyle factors, such as the use of alcohol, cigarettes and vitamin supplements.

To stratify the participants in subgroups of fertile and subfertile men, fertile men were defined by a sperm concentration of $\geq 20 \times 10^6$ cells/ml and a prior conception with the current or previous partner. Subfertile men were defined by a sperm concentration of $< 20 \times 10^6$ cells/ml and no prior conception.

Semen Collection and Analysis

Semen specimens were produced via masturbation after a demanded abstinence period of 3 to 5 days. After liquefaction, the semen parameters of volume, sperm concentration, sperm count, percentage progressive motility and percentage normal morphology were assessed according to the WHO guidelines (WHO 1999). An aliquot of unprocessed semen was stored at -80°C to determine the DFI at a later stage. Subsequently, the remainder of semen was centrifuged at 2500 x *g* for 10 minutes. The supernatant seminal plasma was frozen without preservatives and stored at -20°C until assayed.

SCSA

The principles and procedures of measuring sperm DNA damage by a FACScan flow cytometry (Becton Dickinson, San Jose, USA) SCSA have been described in detail previously (Smit et al. 2007). In short, semen samples were diluted with TNE buffer to a concentration of 1-2x10^6 sperm cells/ml in a volume of 0.20 ml. This cell suspension was mixed with 0.40 ml of acid detergent solution and then stained with 1.2 ml Acridine Orange (AO) staining solution. A reference sample treated in the same way was run before the actual measurements and was used to adjust the voltage gains of the flow cytometer FL3 and FL1 photomultipliers that detected red and green fluorescence respectively. An aliquot of reference sample was stained and run again after every 5 to 10 samples. Data collection of the fluorescent pattern in 5000 cells was performed at 3 minutes after acid treatment. Each sperm sample was analyzed twice.

The extent of DNA damage was expressed as the DNA fragmentation index (DFI), reflecting the ratio for red fluorescence to total fluorescence. Cell Quest Pro and WinList software (Becton Dickinson, San Jose, CA, USA) were used to calculate the DFI of each sample.

Determination of Biomarkers in Blood and Seminal Plasma

Concentrations of tHcy, folate, cobalamin and pyridoxine were determined in venous blood and seminal plasma samples. In blood samples red blood cell (RBC) folate, testosterone, sex hormone binding globuline (SHBG) and Inhibin B were determined as well. Venous blood was collected in dry Vacutainer tubes, Vacutainer tubes containing ethylenediamine tetra-acetate (EDTA), and Vacutainer tubes containing lithium heparin. After clotting, the blood collected in dry Vacutainer tubes was centrifuged at 2000 x *g* and the sera were stored at 4°C before being assayed. The venous blood samples collected in the EDTA-containing Vacutainer tubes were kept on ice for a maximum of 1 hour, after which the plasma was separated after centrifugation and stored at 4°C before being assayed. The venous blood that was drawn into lithium heparin containing Vacutainers was stored at 4°C before being assayed.

Concentrations of tHcy in EDTA plasma and seminal plasma, and pyridoxine as pyridoxal'5-phosphate (PLP) in whole blood and seminal plasma were determined during routine laboratory procedures using high-performance liquid chromatography with reversed phase separation and fluorescence detection (Pfeiffer et al. 1999; Schrijver et al. 1981).

For the determination of folate and cobalamin in blood and seminal plasma, an immunoelectrochemoluminescence immunoassay was used (Roche Modular E170, Roche Diagnostics GmbH, Mannheim, Germany).

For the determination of RBC folate, 100 µl blood out of one EDTA tube was hemolyzed with 2 ml freshly prepared ascorbic acid (0.05g ascorbic acid in 25 ml aqua dest) directly after blood sampling. Subsequently, the hematocrit of the EDTA-blood

was determined on a Sysmex XE-2100 (Groffin Meyvis, Etten-Leur, The Netherlands). The hemolysate was centrifuged for 10 minutes at 2000 x *g* shortly before the folate measurement. The folate concentration in the hemolysate was calculated in RBC folate using the following formula: (nmol hemolysate folate * 21) – (nmol/l serum folate * (1 – hematocrit)) / hematocrit = nmol/l RBC folate.

Testosterone concentrations were determined using a nonextraction coated tube radioimmunoassay (Coat-a-Count, Diagnostic Products Corporation, Los Angeles, USA). We used an immunometric technique on an Immulite Analyzer (Diagnostic Products) to determine the SHBG concentration, and serum Inhibin B was measured by immunoenzymometric assay (Oxford Bio-Innovation, Kidlington, Oxford, United Kingdom).

Interassay coefficients of variation for tHcy were 4.8% at 14.6 µmol/L and 3.3% at 34.2 µmol/L, folate 4.5% at 13 nmol/L and 5.7% at 23 nmol/L, PLP 1.8% at 40 nmol/L and 1.3 % at 115 nmol/L, cobalamin 3.6% at 258 pmol/L and 2.2% at 832 pmol/L, and for SHBG 6.1% at 11.6 nmol/L and 6.9% at 93 nmol/L. For testosterone these coefficients of variation were less than 7.5%, and for Inhibin B they were less than 15%. The detection limit for tHcy was 4 µmol/L, folate 1.36 nmol/L, pyridoxine 5 nmol/L, cobalamin 22 pmol/L, testosterone 0.1 nmol/L, SHBG 5 nmol/L and Inhibin B 10 ng/L. In seminal plasma, the lower detection limit of tHcy was defined as 3*SD=µmol/L.

Statistical Analysis

Biomarker concentrations were expressed in median (range), because of the skewed distributions. Consequently, these variables were log transformed before statistical analysis. Pearson correlation coefficients were calculated to determine associations between the biomarkers in serum and seminal plasma. Differences between fertile and subfertile men were tested with independent t-test or chi-square tests.

To determine associations between DFI and the semen parameters, and between the biomarkers and DFI and semen parameters, we made a causal diagram known as a directed acyclic graph (DAG) (Hernan et al. 2002). To adjust for potential confounders, multiple linear regression analyses were performed. The DAG consist of three blocks: one block with the biomarkers, one block with semen parameters (including DFI) and one block with potential confounders. Age, body mass index (BMI), smoking, alcohol use and the presence of a varicocele were considered as potential confounders.

The first step was to determine correlations between the variables within the block of biomarkers and the block of semen parameters. Second, the correlations between the two blocks were adjusted for the potential confounders and the covariates of the tested variables within each block. The covariates were included in the model at a significance level of <0.1 in a forward stepwise regression model. The analyses were performed in the total group and in the two subgroups. For statistical analysis SPSS 12.0.1 was used (SPSS Inc., Chicago, IL, USA). *p* values ≤0.05 were considered statistical significant.

RESULTS

Baseline Characteristics

The flowchart of the study is presented in Figure 1. Seventy-four percent of the eligible couples participated in the study. Semen samples could not be obtained from 27 men, and the biochemical data were missing for one individual. Thus, we evaluated the semen analyses and biochemical data of 251 men. According to our definition, 70 men were classified as fertile and 63 as subfertile. The baseline characteristics of the participating men and the subgroups are given in Table 1. The fertile men were statistically significantly older and showed statistically significantly higher Inhibin B concentrations than the subfertile men. Forty-one men (16%) were using one or more pharmacological agents for the following categories of diseases: respiratory system (10 men), gastroenteric (7 men), endocrinologic (7 men), cardiovascular (6 men), psychological (5 men), metabolic (4 men), skin (1 man) and miscellaneous (3 men). Only few of these drugs are known to affect sperm parameters or the concentrations of B vitamins and tHcy. Due to the small numbers and heterogeneity, it was not possible to perform subanalysis.

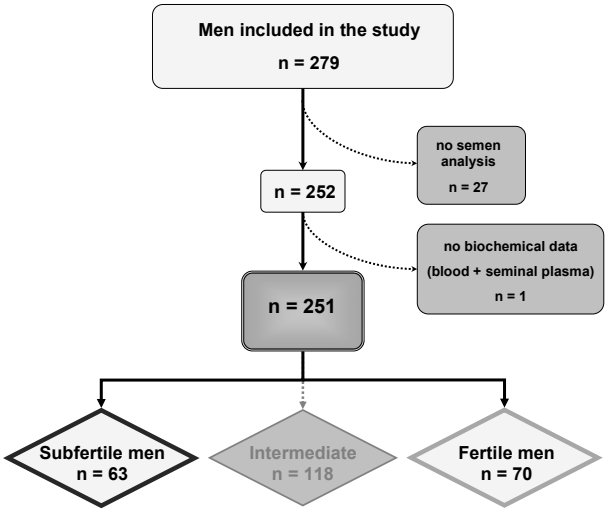

Figure 1 Flowchart of included men

Note: Fertile men: sperm concentration of equal or more than $20x10^6$ cells/ml and a prior conception with the current or previous partner. Subfertile men: a sperm concentration of less than $20x10^6$ cells/ml and no prior conception.

Correlations between Blood and Seminal Plasma

Table 2 shows the biomarkers in blood and seminal plasma. Twelve men (5%) had hyperhomocysteinemia (22.6 - 56.4 µmol/L; reference value: >22 µmol/L), and only 3 men (1%) had a mild folate deficiency (7.1, 7.5 and 7.6 nmol/L; reference value: <8 nmol/L).

Table 1 General characteristics

Characteristics	Male study population (n=251)	Fertile men (n=70)	Subfertile men (n=63)	P-value[b]
Age (years)[a]	37.0 (23.1 – 59.1)	39.1 (30.5 – 54.0)	34.0 (23.1 – 46.2)	≤ 0.001
BMI (kg/m^2)[a]	25.6 (18.4 – 38.4)	25.8 (18.8 – 38.4)	24.0 (19.6 – 37.9)	n.s.
Cause of subfertility				≤ 0.001
Male factor	88 (35.1)	12 (17.1)	42 (66.7)	≤ 0.001
Female factor	63 (25.1)	26 (37.1)	6 (9.5)	≤ 0.001
Male and female factor	17 (6.8)	2 (2.9)	9 (14.3)	≤ 0.05
Unexplained	83 (33.1)	30 (42.9)	6 (4.5)	≤ 0.001
Etnicity				n.s.
European Dutch Natives	181 (79.7)	41 (68.3)	51 (85)	
European others	16 (7.0)	5 (8.3)	3 (5)	
Non-European	30 (13.2)	14 (23.3)	6 (10)	
Smoking				n.s.
Yes	57 (25.1)	15 (25)	14 (23.3)	
No	170 (74.9)	45 (75)	46 (76.7)	
Alcohol use (units/week)[a]	4.3 (0.0 – 50.3)	2.1 (0 – 27.6)	3.5 (0 – 17.5)	n.s.
Varicocele				n.s.
Yes	37 (16.8)	6 (10.2)	12 (20.3)	
No	183 (83.2)	53 (89.8)	47 (79.7)	
Vitamin use				n.s.
Yes, with folic acid	44 (20.3)	8 (13.8)	15 (26.3)	
Yes, without folic acid	13 (6.0)	2 (3.4)	5 (8.8)	
No	160 (73.7)	48 (82.8)	37 (64.9)	
Endocrinology[a]				
Testosterone (nmol/L)	15.1 (5.8 – 36.0)	14.7 (7.6 – 25.2)	15.3 (8.8 – 34.5)	n.s.
SHBG (nmol/L)	26.1 (8.1 – 70.7)	25.7 (8.1 – 70.7)	27.8 (11.4 – 52.1)	n.s.
Inhibin B (ng/L)	160.0 (2.0 – 411.0)	186.5 (61.0 – 411.0)	126.0 (2.0 – 301.0)	≤ 0.001

Results are presented as number (%), unless otherwise indicated

[a] median (range); [b] difference between fertile and subfertile men; independent T-test or Chi^2 (two-tailed) if appropriate; n.s. = not significant

There were no statistically significant differences between the biomarker concentrations in either blood or seminal plasma in fertile and subfertile men.

Table 3 depicts a correlation matrix with the correlations between biomarkers in blood and seminal plasma and semen parameters in the total group. The tHcy concentrations in blood and seminal plasma were statistically significantly correlated (r=0.16, p≤0.05). Similarly, folate, cobalamin and pyridoxine in serum and seminal plasma were statistically significantly correlated (r =0.47, p≤0.001; r=0.36, p≤0.001; and r=0.23, p≤0.01; respectively). The tHcy and pyridoxine concentrations were statistically significantly lower in seminal plasma compared with serum (p≤0.05 and p≤0.001, respectively), and

Table 2 Concentrations of biomarkers in blood and seminal plasma and semen parameters

	Male study population (n=251)	Fertile men (n=70)	Subfertile men (n=63)	P-value[b]
Blood				
Folate (nmol/L)	15.7 (7.1 – 131.1)	15.5 (7.5 – 55.9)	15.6 (8.6 – 45.0)	n.s.
Folate RBC (nmol/L)	1025 (340 – 2329)	104 (532 – 2045)	948 (340 – 2329)	n.s.
Pyridoxine (nmol/L)	80 (39 – 310)	79 (40 – 310)	80 (51 – 310)	n.s.
Cobalamin (pmol/L)	316 (108 – 989)	337 (138 – 989)	323 (131 – 802)	n.s.
tHcy (µmol/L)	11.7 (6.8 – 56.4)	11.9 (7.7 – 56.4)	11.9 (7.0 – 34.3)	n.s.
Seminal plasma				
Folate (nmol/L)	25.3 (11.7 – 78.6)	24.5 (13.8 – 58.6)	23.9 (11.7 – 59.2)	n.s.
Pyridoxine (nmol/L)	28 (0 – 310)	29 (0 – 310)	26 (0 – 211)	n.s.
Cobalamin (pmol/L)	558 (94 – 5704)	674 (94 – 5516)	474 (111 – 4360)	n.s.
tHcy (µmol/L)	4.3 (1.2 – 35.5)	4.7 (1.7 – 30.4)	3.7 (1.2 – 35.5)	n.s.
Semen parameters				
DFI (%)	22.8 (1.3 – 74.8)	19.6 (3.7 – 74.8)	28.3 (1.3 – 65.9)	n.s.
Sperm volume (mL)	2.7 (0.2 – 8.1)	2.5 (0.2 – 6.5)	3.2 (0.2 – 6.0)	≤ 0.01
Sperm concentration (x10^6 cells/mL)	26 (0 – 278)	51 (20 – 215)	6 (0 – 19)	≤ 0.001
Sperm count (x10^6 cells)	65 (0 – 1557)	127 (10 – 690)	14.8 (0 – 95)	≤ 0.001
Sperm progressive motility (%)	34 (0 – 74)	41 (5 – 73)	26 (0 – 58)	≤ 0.001
Sperm normal morphology (%)	4 (0 – 15)	6 (1 – 14)	3 (0 – 12)	≤ 0.001

Results are presented as median (range)

[b] difference between fertile and subfertile men; independent T-test; n.s. = not significant

the folate and cobalamin concentrations in seminal plasma were statistically significantly higher compared with serum (both $p \leq 0.001$). In serum, the B vitamins were inversely correlated with tHcy and positively correlated with each other (all $p \leq 0.001$). In seminal plasma, cobalamin was positively correlated with folate and tHcy (both $r=0.26$, $p \leq 0.001$). The directed acyclic graph presents the independent correlations between the B vitamins and tHcy in seminal plasma (Figure 2). Cobalamin also correlated with tHcy after adjustment for the other B vitamins (standardized adjusted regression coefficient 0.25, $p \leq 0.01$).

Semen Parameters

The semen parameters and DFI are presented in Table 2. Ejaculate volume was statistically significantly higher and sperm concentration, sperm count, percentage progressive motility and the percentage normal morphology were statistically significantly lower in subfertile men compared with fertile men (all $p \leq 0.001$). In subfertile men, the DFI was higher compared with fertile men, albeit not statistically significantly.

Table 3 Correlation matrix of B-vitamin and homocysteine concentrations in blood and seminal plasma and semen parameters

	Blood					Seminal plasma				Semen parameters					
	tHcy	Fol	FRBC	B12	B6	tHcy	Fol	B12	B6	DFI	Vol	Conc	Count	Mot	Morp
Blood															
tHcy		-0.56[c]	-0.39[c]	-0.39[c]	-0.31[c]	0.16[a]	-0.15[a]	-0.22[c]	0.01	0.04	-0.02	-0.01	0.00	0.03	0.03
Fol	-0.56[c]		0.62[c]	0.23[c]	0.49[c]	-0.11	0.47[c]	0.01	0.12	0.01	0.03	-0.05	-0.04	0.02	-0.08
FRBC	-0.39[c]	0.62[c]		0.22[c]	0.35[c]	-0.01	0.34[c]	0.17[b]	0.02	0.03	-0.02	-0.04	-0.05	-0.02	-0.07
B12	-0.39[c]	0.23[c]	0.22[c]		0.32[c]	-0.03	0.08	0.36[c]	0.03	0.08	0.01	0.05	0.05	0.01	0.05
B6	-0.31[c]	0.49[c]	0.35[c]	0.32[c]		-0.07	0.30[c]	0.18[b]	0.23[b]	-0.05	-0.08	-0.06	-0.07	0.03	-0.08
SP															
tHcy	0.16[a]	-0.11	-0.01	-0.03	-0.07		-0.01	0.26[c]	-0.01	-0.02	-0.09	0.30[c]	0.25[c]	0.07	0.19[a]
Fol	-0.15[a]	0.47[c]	0.34[c]	0.08	0.30[c]	-0.01		0.26[c]	0.06	-0.12	-0.18[b]	0.07	0.03	-0.03	0.02
B12	-0.22[c]	0.10	0.17[b]	0.36[c]	0.18[b]	0.26[c]	0.26[c]		0.04	0.02	-0.21[b]	0.19[b]	0.13	0.01	0.00
B6	0.01	0.12	0.02	0.03	0.23[b]	-0.01	0.06	0.04		-0.04	-0.17[a]	-0.01	-0.04	0.03	-0.10
Semen															
DFI	0.04	0.01	0.03	0.08	-0.05	-0.02	-0.12	0.02	-0.04		0.21[b]	-0.24[c]	-0.17[a]	-0.49[c]	-0.25[c]
Vol	-0.02	0.03	-0.02	0.01	-0.08	-0.09	-0.18[b]	-0.21[b]	-0.17[a]	0.21[b]		-0.04	0.25[c]	0.06	0.04
Conc	-0.01	-0.05	-0.04	0.05	-0.06	0.30[c]	0.07	0.19[b]	-0.01	-0.24[c]	-0.04		0.95[c]	0.49[c]	0.63[c]
Count	0.00	-0.04	-0.05	0.05	-0.07	0.25[c]	0.03	0.13	-0.04	-0.17[a]	0.25[c]	0.95[c]		0.49[c]	0.62[c]
Mot	0.03	0.02	-0.02	0.01	0.03	0.07	-0.03	0.01	0.03	-0.49[c]	0.06	0.49[c]	0.49[c]		0.45[c]
Morp	0.03	-0.08	-0.07	0.05	-0.08	0.19[a]	0.02	0.00	-0.10	-0.25[c]	0.04	0.63[c]	0.62[c]	0.45[c]	

Note: Pearson correlation coefficients were calculated after log transformation of the parameters (n = 251) SP = Seminal plasma; Semen = Semen parameters; Fol = Folate; FRBC = Folate RBC; B12 = Cobalamin; B6 = Pyridoxine; DFI = DNA fragmentation index; Vol = Ejaculate volume; Conc = Sperm concentration; Count = Sperm count; Mot = Sperm progressive motility; Morp = Sperm normal morphology; [a] $P \leq .05$; [b] $P \leq .01$; [c] $P \leq .001$

Figure 2 shows the DAG. As can be expected, sperm count was statistically significantly correlated with volume (standardized adjusted regression coefficient 0.25, $p \leq 0.001$), the percentage progressive motility (standardized adjusted regression coefficient 0.39, $p \leq 0.001$), and the percentage normal morphology (standardized adjusted regression coefficient 0.39, $p \leq 0.001$). We found a positive correlation between ejaculate volume and DFI (standardized adjusted regression coefficient 0.23, $p \leq 0.001$); and DFI inversely correlated with the percentage progressive motility (standardized adjusted regression coefficient -0.41, $p \leq 0.001$). In the subgroup of subfertile men, DFI was also inversely correlated with the percentage of sperm cells with normal morphology (standardized adjusted regression coefficient -0.33, $p \leq 0.01$).

Correlations between the Biomarkers and Semen Parameters

The biomarkers in blood were not correlated with any of the semen parameters. In seminal plasma, both tHcy and cobalamin correlated with sperm count (standardized

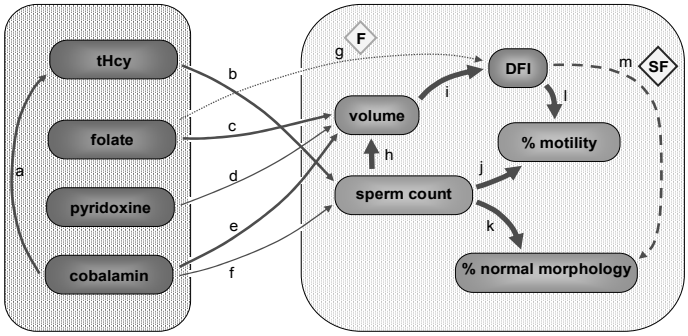

Figure 2 Correlations between B-vitamins and homocysteine in seminal plasma and semen parameters
Note: Directed Acyclic Graph (n = 251); in the left block biomarkers in seminal plasma, in the right block semen parameters. Age, body mass index (BMI), smoking, alcohol use and the presence of a varicocele were considered as potential confounders. To adjust for potential confounders, multiple linear regression analyses were performed. Only statistically significant correlations are depicted. Green arrows: positive correlations. Red arrows: inverse correlations. The strength of the *p*-values is depicted by the arrow thickness. Thin arrows: $p \leq 0.05$. Medium arrows: $p \leq 0.01$. Thick arrows: $p \leq 0.001$. F (with dotted line) = Fertile men: sperm concentration of equal or more than $20x10^6$ cells/ml and a prior conception with the current or previous partner (n = 70). SF (with dashed line) = Subfertile men: a sperm concentration of less than $20x10^6$ cells/ml and no prior conception (n = 63).
a: standardized coefficient 0.25, $p \leq 0.01$, b: standardized coefficient 0.21, $p \leq 0.01$,
c: standardized coefficient -0.20, $p \leq 0.01$, d: standardized coefficient -0.16, $p \leq 0.05$,
e: standardized coefficient -0.19, $p \leq 0.01$, f: standardized coefficient 0.15, $p \leq 005$,
g: standardized coefficient -0.36, $p \leq 0.05$ in fertile men, h: standardized coefficient 0.25, $P \leq .001$, i: standardized coefficient 0.23, $p \leq 0.001$, j: standardized coefficient 0.39, $p \leq 0.001$,
k: standardized coefficient 0.39, $p \leq 0.001$, l: standardized coefficient -0.41, $p \leq 0.001$,
m: standardized coefficient -0.33, $p \leq 0.01$ in subfertile men.

adjusted regression coefficient 0.21, $p \leq 0.01$, and 0.15, $p \leq 0.05$, respectively). Folate, cobalamin and pyridoxine concentrations in seminal plasma inversely correlated with ejaculate volume (standardized adjusted regression coefficient -0.20, $p \leq 0.01$; -0.19, $p \leq 0.01$; and -0.16, $p \leq 0.05$; respectively). In the total group of men, biomarkers were not correlated with DFI, motility or morphology. However, in the subgroup of fertile men, folate concentrations in seminal plasma were inversely correlated with DFI (standardized adjusted regression coefficient -0.36, $p \leq 0.05$).

DISCUSSION

Our study found statistically significant correlations between B vitamin and homocysteine concentrations in blood and seminal plasma. Furthermore, we have demonstrated for the first time that a low folate concentration in seminal plasma is associated with more sperm DNA damage in fertile men. This novel finding which is in line with the role of folate in DNA synthesis and with DNA and protein methylation processes. It has been

shown that folate shortage increases DNA fragility due to the misincorporation of uracil instead of thymine (Blount et al. 1997; Koury et al. 1997). During normal repair processes, when the removal of the misincorporated uracil fails, double strand breaks resulting in chromosome instability may occur (Blount et al. 1997; Koury et al. 1997; Steegers-Theunissen et al. 2000). Folate shortage also decreases the supply of methyl groups, which are important substances for the protection of DNA against harmful exposures (Blount et al. 1997).

In contrast with fertile men, we suggest that in subfertile men other much stronger causes for subfertility than folate shortage are responsible for the sperm DNA damage (Zini et al. 2006). Covariates such as testosterone, SHBG concentration, cigarette smoking, and the presence of a varicocele were not statistically significantly different between the subgroups. As expected, Inhibin B was statistically significantly lower in subfertile men. Inhibin B is strongly correlated with spermatogenesis (Jensen et al. 1997; Pierik et al. 1998), but no correlation was found between Inhibin B and DFI in a recent study (Appasamy et al. 2007). After adjustment for Inhibin B, the association between seminal plasma folate and DFI did not become statistically significant in the total group or in subfertile men, but the association remained statistically significant in the fertile men. Age has been statistically significantly correlated with sperm DNA damage (Plastira et al. 2007; Siddighi et al. 2007). Because subfertile men were statistically significantly younger than fertile men in our study population, all calculations were adjusted for age.

We determined an inverse correlation between DFI and the percentage progressive motile sperm. In the subgroup of subfertile men, DFI was also inversely correlated with the percentage sperm cells with normal morphology. These findings are in line with several studies (Appasamy et al. 2007; Giwercman et al. 2003; Spano et al. 1998), although other groups could not demonstrate associations between DNA damage, motility and morphology (Bakos et al. 2008; Khalili et al. 2006). The number and selection of men in the study may explain the different results.

In our study, cobalamin in seminal plasma correlated with sperm count. This is in line with our previous report (Boxmeer et al. 2007) and an older study by Tomaszewski et al (Tomaszewski et al. 1963). Of interest are the reports of 1960 and 1970s in which subfertile cobalamin-deficient males became fertile after treatment with cobalamin (Blair et al. 1968; Sharp et al. 1962). Both folate and cobalamin concentrations in seminal plasma were on average 1.6 and 1.8 times higher compared with the blood concentrations in the total group. This is in line with the report by Wallock et al (Wallock et al. 2001). The tHcy and pyridoxine concentrations were lower in seminal plasma than in blood. Similar differences in concentrations have been shown for zinc (Wong et al. 2002). This may suggest a passive or active transfer from blood to seminal plasma of folate and cobalamin. In future studies, it may be interesting to determine folate and vitamin B_{12}-binding

proteins in seminal plasma. It is possible that tHcy and pyridoxine either cannot pass the blood-testis barrier or are actively resorbed.

Some limitations of our study have to be addressed. Some information is missing because the questionnaires were not returned. Furthermore, we had some difficulties with the determination of the biomarkers because there was sometimes not enough seminal plasma available. Furthermore, due to the high protein content and viscosity of seminal plasma, we encountered some technical problems with the tHcy determination. Nevertheless, the missing data are differentially distributed among the subgroups, so it is not very likely that our results are significantly affected by selection bias. In addition, from our previous study we were aware off the difficulties of measuring biomarkers, in particularly tHcy, in seminal plasma (Ebisch et al. 2006). Thus, we performed dilution experiments that revealed linear dose-response curves. Furthermore, the concentrations in seminal plasma were calculated from recoveries from each individually spiked sample. We realize that these validation procedures are not the final proof but rather suggestive for the absence of matrix effects. Furthermore, we used the HPLC-method to determine tHcy, which is a generally accepted reference procedure. Although the quality of our tHcy assay has much improved, the data should be carefully interpreted.

The use of one semen sample for distinguishing fertile from subfertile men may have introduced some misclassification. For this reason, we added the criterion of a prior conception, thereby proving the fertility of the individual. A group of healthy men without a history of a fertility problem whose partners had conceived spontaneously within 1 year of regular unprotected intercourse would have been the ideal fertile group.

In the current study population, 5% of the men had a mild to severe hyperhomocysteinemia. This may be due to a polymorphism in the B vitamin metabolism and/or a deficient B vitamin intake. Previously, we had studied the associations between the MTHFR C677T polymorphism and male fertility (Ebisch et al. 2003), but it was not the aim of our current periconceptional observational study to investigate the effect of polymorphisms on semen parameters.

Seminal plasma is a mixture of the secretion of several glands, of which the Cowper and Littre glands (5%), prostate (15-30%), and seminal vesicles (60-70%) contribute the majority (Owen et al. 2005). Thus, about 90% of the seminal plasma is derived from glands outside the testicular tissue and may have a composition different from the liquid in the seminiferous tubules and epididymis where spermatogenesis and maturation take place. For this reason, the composition of the seminal plasma may not be representative for conditions during spermatogenesis. Comparison of the composition of seminal plasma with material obtained from MESA/PESA procedures would be interesting. However, due to the low volume of material derived from this technique, the invasiveness of these procedures, and medical ethics issues involved, there is limited availability of this material for research.

In our present study, the biomarkers in seminal plasma correlated with semen parameters but not with the biomarkers in blood. This might be due to the fact that active mechanisms regulate biomarker concentrations in seminal plasma in a normal range. Therefore, this study may be repeated in a population with very low and very high biomarker concentrations, through which correlations between biomarkers in blood and semen parameters may be found as well. Folic acid and cobalamin supplementation may be a useful therapy to improve male fertility, but more research should be performed to study both the efficacy and safety of supplementation dose and duration. In addition, it may be useful to establish the dietary intake of these vitamins.

Our study emphasizes the importance of the B vitamin status in spermatogenesis in humans. We found that low folate concentrations in the seminal plasma of a subgroup of fertile men was associated with increased levels of sperm DNA damage. This may suggest that low folate concentrations in the microenvironment of spermatozoa may be detrimental for sperm DNA stability. High concentrations of sperm DNA damage are associated with poor sperm cell motility and morphology. Future research is needed to determine the importance of sufficient B vitamin status in men on fertilization and subsequent pregnancy outcome.

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CHAPTER 5

Decreased sperm DNA fragmentation following surgical varicocelectomy is associated with increased pregnancy rates

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ABSTRACT

Purpose: We prospectively evaluated changes in sperm chromatin structure in infertile patients before and after surgical repair of varicocele, and the impact on the pregnancy rate.

Material and Methods: Included in the study were 49 men with at least a 1-year history of infertility, a palpable varicocele and oligospermia. World Health Organization semen analysis and sperm DNA damage expressed as the DNA fragmentation index using the sperm chromatin structure assay were assessed preoperatively and postoperatively. Pregnancy (spontaneous and after assisted reproductive technique) was recorded 2 years after surgery.

Results: Mean sperm count, sperm concentration and sperm progressive motility improved significantly after varicocelectomy from 18.3 $x10^6$ to 44.4 $x10^6$, 4.8 $x10^6$/ml to 14.3 $x10^6$/ml and 16.7% to 26.6%, respectively ($p<0.001$). The DNA fragmentation index decreased significantly after surgery from 35.2% to 30.2% ($p=0.019$). When the definition of greater than 50% improvement in sperm concentration after varicocelectomy was applied, 31 out of 49 patients (63%) responded to varicocelectomy. After varicocelectomy 37% of the couples conceived spontaneously and 24% achieved pregnancy with assisted reproductive technique. The mean postoperative DNA fragmentation index was significantly higher in couples who did not conceive spontaneously or with assisted reproductive technique ($p=0.033$).

Conclusion: After varicocelectomy sperm parameters significantly improved and sperm DNA fragmentation was significantly decreased. Low DNA fragmentation index values are associated with a higher pregnancy rate (spontaneous and with assisted reproduction technique). We suggest that varicocelectomy should be considered in infertile men with palpable varicocele, abnormal semen analysis and no major female factors.

INTRODUCTION

Varicocele is a common abnormality, found in 12% of the adult male population and in 25% of men in infertile couples (WHO 1992). The exact pathophysiology of male infertility due to varicocele is still not clear and varicocele treatment remains controversial in clinical Andrology. Although World Health Organization (WHO) data clearly indicate that varicocele is associated with semen abnormalities, and decreased testicular volume and Leydig cell function (WHO 1992), it remains to be proved whether varicocele repair also restores male fertility potential (Evers et al. 2003).

Two recent meta-analyses showed that surgical varicocelectomy significantly improves sperm concentration and motility in infertile men with palpable varicocele and abnormal preoperative semen parameters (Agarwal et al. 2007; Marmar et al. 2007) and increases the odds ratio of spontaneous pregnancy (Marmar et al. 2007).

A large body of evidence has accumulated to indicate that sperm DNA damage is increased in infertile men and high levels of damage are associated with decreased pregnancy rate, both spontaneous pregnancy (Evenson et al. 1999; Spano et al. 2000) and pregnancy after assisted reproduction technology (ART) (Bungum et al. 2004). Several groups have reported that varicocele is associated with increased sperm DNA damage (Blumer et al. 2008; Saleh et al. 2003). Since varicocelectomy may improve levels of sperm DNA damage in infertile men, (Hurtado de Catalfo et al. 2007; Zini et al. 2005a) we evaluated repair induced changes in the sperm chromatin structure and correlated postoperative DNA damage with the pregnancy rate (spontaneous and after ART).

MATERIALS AND METHODS

Patients

A total of 52 men with at least a 1-year history of infertility, a palpable varicocele, oligospermia and normal or correctable female fertility were eligible for this pilot study from November 2003 to June 2006. Andrological examination included medical history, physical examination, testicular volume measurement with a Prader orchidometer, scrotal ultrasound, endocrine analysis of serum luteinizing hormone (LH) (normal 1.5 to 8.0 IU/l), follicle stimulating hormone (FSH) (normal 2.0 to 7.0 IU/l), testosterone (T) (normal 10.0 to 30.0 nmol/l) and Inhibin B (normal 150 to 400 ng/l), and semen analysis according to WHO guidelines (WHO 1999). Sperm DNA fragmentation index (DFI) was assessed with the sperm chromatin structure assay (SCSA).

A palpable varicocele was confirmed by scrotal ultrasound done using Nemio™ 20 with a 12 Hz transducer equipped with color flow imaging when at least 1 scrotal vein had a maximum diameter of at least 3 mm and retrograde flow was observed at

rest or after the Valsalva maneuver. Grade 1 varicocele was diagnosed when reflux was measured at less than 2 seconds, grade II when reflux lasted for more than 2 seconds and grade III when reflux was noted at spontaneous respiration.

Postoperative followup at 3 months to assess the effect of varicocelectomy comprised WHO semen analysis, SCSA, scrotal ultrasound and endocrine evaluation. Patients served as their own controls since we compared the effect of varicocele repair on sperm quality with time. In 2 patients no preoperative semen sample was collected and 1 was lost to followup. A total of 49 couples were available for analysis. Since SCSA is incorporated in the standard evaluation of semen in our laboratory, medical ethical and institutional review board approval was not separately issued. In 2008 pregnancies, use of ART, the pregnancy outcome and time to pregnancy were evaluated from patients records.

Sperm DNA fragmentation Measurement

The SCSA was performed essentially as described by Evenson and Jost (Evenson et al. 2000a), using a FACScan™ flow cytometer (Becton Dickinson, San Jose, CA, USA). Briefly, frozen samples were quickly thawed, diluted to a concentration of 1 to 2 x 10^6 sperm cells per ml, exposed to acid detergent solution and stained with acridine orange. A similarly treated reference sample run prior to the actual sample measurements was used to adjust the voltage gains of the flow cytometer FL3 and FL1 photomultipliers, which analyse red and green fluorescence, respectively. An aliquot of reference sample was run after every 5-10 samples. Voltage gains were readjusted whenever the fluorescent signal of the reference sample drifted. Data collection of the fluorescent pattern in 5000 cells was done 3 minutes after acid treatment. Debris, bacteria and leukocytes were gated out during acquisition, as recommended by Evenson and Jost. The extent of DNA damage is expressed as the DFI, reflecting the ratio of red fluorescence to total fluorescence. CellQuest™ Pro and WinList™ software were used to calculate the DFI of each sample. Each sperm sample was measured in duplicate and the mean values of the results were used for analysis.

Statistical Analysis

Statistical analysis was performed using SPSS® 15.0 (SPSS Inc, Chicago, Ill.). All results are expressed as the mean ± SD. The 1-sample Kolmogorov-Smirnov Test was used to test for normality. Nonnormal distributed parameters (paired t test, total sperm count and sperm concentration) were logarithmically transformed to obtain normally distributed data. Differences between the preoperative and postoperative semen variables were analyzed with the paired samples Student t test. Correlations were calculated using Spearman's rank correlation coefficient.

All patients were divided into 2 groups based on spermatogenic response to varicocele repair. Responders were defined as patients in whom sperm concentration increased

by 50% or more following varicocelectomy (Benoff et al. 2004; Cayan et al. 2002). Preoperative differences in continuous variables between the 2 consequent data sets were analyzed with the t test and the chi-square test was used for dichotomous variables to identify possible predictors for the response to surgery. All variables with a maximum of p=0.02 were used in a backward multivariate logistic regression analysis model to evaluate determinants of surgical response. Univariate analysis using ANOVA and the test was done to evaluate possible predictors of spontaneous and ART assisted pregnancies.

RESULTS

The mean age of male patients was 34 ± 6.9 years and mean partners age at varicocele repair was 30 ± 4.9 years. Included in the analysis were 41 men diagnosed with primary infertility and 8 with secondary infertility. The mean duration of infertility was 2.7 ± 1.6 years.

Six female partners were diagnosed with irregular cycles, of whom 3 were successfully treated with clomiphene citrate and 2 couples were eventually treated with IVF. In 1 female who remained anovulotory despite treatment endometriosis was diagnosed during diagnostic laparoscopy.

The medical history revealed that 10 out of 49 patients were smokers, 1 was a farmer who was periodically exposed to pesticides, 4 men were treated for cryptorchidism at childhood, which was unilateral and bilateral in 2 each, 5 had a history of urogenital infection, 4 underwent hernia repair in childhood and 1 was treated with urethrotomia for an urethral stricture.

A left grade I varicocele was found in 16 patients, grade II and III varicoceles were present in 15 and 13, respectively, and 5 had a bilateral varicocele. Left high inguinal spermatic vein ligation (Palomo 1949) was performed in 36 men and microsurgical varicocelectomy (Goldstein et al. 1992) was done in 8. All bilateral varicoceles were treated with bilateral high inguinal ligation. Because only one urologist (GRD) at our clinic had mastered microsurgical varicocelectomy, this technique was not exclusively performed in 2003 to 2006.

In 4 of 49 patients (8%) a recurrent varicocele diagnosed during follow up was treated with high inguinal ligation for bilateral (2), and grade I (1) and III (1) varicocele.

Mean sperm count, concentration and progressive motility improved significantly after varicocelectomy but DFI significantly decreased after surgery (Table 1). A significant negative relationship was detected between the change in DFI and the change in sperm motility following varicocele surgery (see Figure 1).

When defining a positive response as greater than a 50% improvement in sperm concentration after varicocelectomy, 31 of 49 patients (63%) were responders. Analysis

Table 1 Mean ± SD values and *p*-values for sperm parameters and DFI before and after varicocelectomy

Parameter	Pre-operative	Post-operative	*p*- value
Total sperm count (x 10^6)	18.3 (± 23.3)	44.4 (± 48.0)	<0.001
Sperm concentration (x 10^6/ml)	4.8 (± 4.7)	14.3 (± 14.3)	<0.001
Progressive motility (%)	16.7 (± 12.5)	26.6 (± 15.7)	<0.001
Normal morphology (%)	2.5 (± 2.1)	2.8 (± 1.9)	0.188
DFI (%)	35.2 (± 13.1)	30.2 (± 14.7)	0.019

paired t test, total sperm count and sperm concentration were logarithmically transformed to obtain normally distributed data).

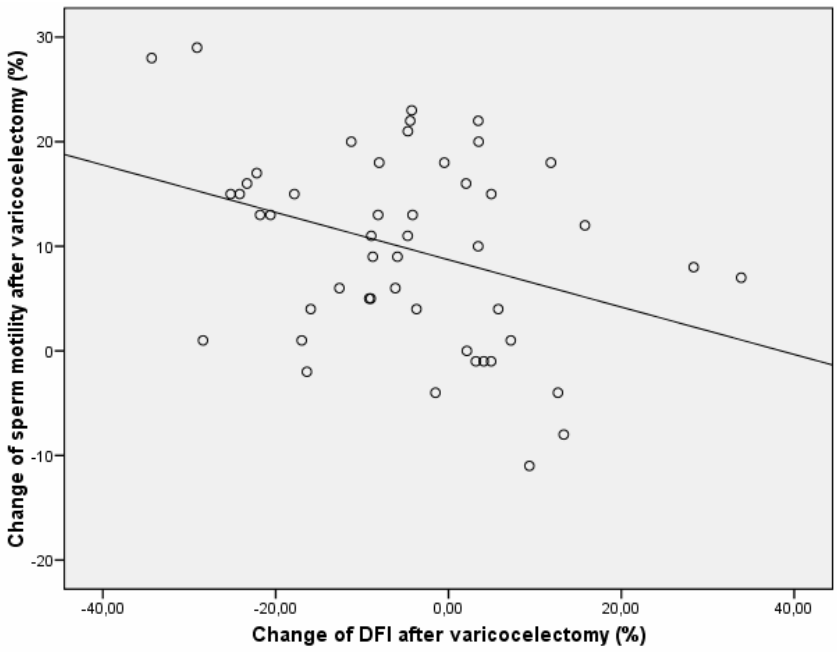

Figure 1. Change in sperm DNA fragmentation index (DFI) after varicocelectomy and subsequent change in sperm motility. Spearman correlation r -0.312, p=0.029.

of SCSA results in the responder group showed a significant decrease in DFI after surgery (Table 2). Preoperative reproductive hormones were not significantly different in the responder and nonresponder group. However, LH and FSH increased significantly after varicocele treatment in the nonresponder group.

On univariate analysis only the log preoperative sperm concentration was associated with a positive surgical outcome (p=0.039). Also, when continuous and dichotomous preoperative variables at a maximum of p=0.2 were reviewed, log preoperative sperm count (p=0.097) and preoperative LH (p=0.085) applied and were entered in a backward multivariate logistic regression analysis model. Only log preoperative sperm concentration predicted a positive outcome of surgery (OR=0.229, p=0.044). This means that a

Table 2 Mean (± SD) values and *p*-values for DFI and endocrine parameter values in the responders and non-responders groups.

	Responder n=31			Non-responder n=18		
	Pre-operative	Post-operative	*p*-value	Pre-operative	Post-operative	*p*-value
DFI (%)	35.3 (± 14.3)	28.6 (± 14.7)	0.009	35.0 (± 11.2)	33.0 (± 14.8)	0.602
LH (IU/l)	3.9 (± 1.8)	4.3 (± 1.8)	0.436	3.0 (± 1.3)	4.0 (± 1.6)	0.018
FSH (IU/l)	8.0 (± 6.2)	9.2 (± 5.6)	0.508	6.6 (± 2.8)	7.6 (± 3.6)	0.006
T(nmol/l)	14.9 (± 4.8)	15.0 (± 4.3)	0.084	14.7 (± 5.2)	15.3 (± 4.5)	0.390
Inhibin B (ng/l)	126.7 (± 48.5)	126.2 (± 65.3)	0.102	140.7 (± 60.1)	133.8 (± 63.8)	0.224

Normal values are LH 1.5-8.0 IU/l, FSH 2.0-7.0 IU/l, Testosterone 10.0-30.0 nmol/l and Inhibin B 150-400 ng/l.

Table 3 Mean(± SD) values of post-operative DFI levels in 4 pregnancy groups.

	DFI %
Spontaneous pregnancy	30.1 (± 12.2)
Failed to conceive spontaneously	37.5 (± 13.3)
Pregnancy following ART	21.3 (± 14.7)
Failed to conceive following ART	36.9 (± 15.6)

lower preoperative sperm concentration was associated with a positive response to surgery.

After varicocelectomy 18 of the 49 couples (37%) conceived spontaneously within a mean of 7.2 ± 6.7 months. In 12 of the 49 couples (24%) spontaneous pregnancy was not achieved and they decided not to proceed with ART. A pregnancy rate of 22% (11 of 49 couples) was achieved following ART within a mean of 14.6 ± 7.7 months after varicocele repair. Intrauterine insemination, IVF and intracytoplasmic sperm injection (ICSI) were performed in 8, 4 and 7 couples, respectively. Eight of the 19 couples (42%) in whom ART was used failed to conceive after 3 ICSI cycles in 5, a poor response following IVF in 1 and 6 IUI cycles in 2. The mean postoperative DFI was significantly different in couples who could not conceive spontaneously or with ART (p=0.033, Table 3). The mean DFI was significantly increased in couples with failure to conceive following ART compared to couples that achieved pregnancy with ART (p=0.041). DFI was also increased in couples who failed to conceive spontaneously vs couples who conceived after ART (p=0.014). Overall the mean DFI was significantly lower in couples who conceived spontaneously or with ART compared to that in couples with failure (26.6% ± 13.7 vs 37.3% ± 13.9, p=0.013).

DISCUSSION

In accordance with previous studies we found a significant increase in postoperative sperm count, concentration and progressive motility after surgical repair of palpable varicoceles in patients with abnormal semen parameters (Agarwal et al. 2007; Marmar et al. 2007). Since sperm quality expressed as WHO semen parameters is subject to large biological variation and semen analysis is hampered by high interobserver and intraobserver variation (Alvarez et al. 2003), it was suggested that this variability may explain the apparent differences between preoperative and postoperative semen samples after varicocele repair. Sperm DNA fragmentation provides additional information about sperm quality and the ability of a couple to conceive (Spano et al. 2000). The SCSA is a validated method for studying sperm chromatin integrity (Evenson et al. 2002). We previously reported that DFI biological variation is much lower than that of conventional semen parameters and DFI variability is not increased by varicocele.

Not all men with a varicocele have improved sperm parameters after varicocelectomy and a 50% to 70% success rate was reported (Cayan et al. 2002). We applied a strict definition of greater than 50% improvement in sperm concentration to identify clinically relevant responders to surgery (Benoff et al. 2004; Cayan et al. 2002) and found that 63% of our patients responded to varicocele repair. In this pilot study DFI decreased significantly after varicocelectomy in the whole study population and in the responder group, suggesting that varicocele repair is effective for decreasing DFI in most patients. In the nonresponders group no clear effect on sperm DNA damage was observed but the lower number of patients in that group may explain this. A limitation of our pilot study is that for practical reasons only 1 postoperative semen sample was used.

Although lower postoperative DFI was associated with a higher pregnancy rate, postoperative mean DFI was relatively high in the spontaneous pregnancy group at 30.2% when considering that fertility is reported to be limited when DFI exceeds 30% (Evenson et al. 2002). Our preliminary results require validation in a larger study of sperm DNA damage in multiple postoperative semen samples.

In the search for molecular mechanisms associated with varicocele related infertility recent research has focussed on reactive oxygen species (ROS) and apoptosis markers in testicular tissue and semen in varicocele cases (Marmar 2001). Oxidative stress and testicular apoptosis are well documented causes of increased sperm DNA fragmentation. Varicocele is associated with increased ROS production in spermatozoa and decreased antioxidant capacity in semen (Hendin et al. 1999). ROS was decreased after varicocele repair (Hurtado de Catalfo et al. 2007) even in patients in whom semen quality did not improve after varicocelectomy (Chen et al. 2008). Sperm DNA damage may be a late effect of excessive ROS, which may explain why not all infertile patients in our study showed a decrease in sperm DNA damage after varicocele repair.

Germ cell apoptosis is an inherent process in spermatogenesis but it is clearly up-regulated in a number of stress conditions, such as varicocele (Baccetti et al. 1996). Patients who responded to varicocelectomy had significant lower apoptosis levels in testicular biopsies (Benoff et al. 2004).

Because idiopathic male infertility and varicocele are linked to increased ROS, increased apoptosis and increased sperm DNA damage, one could also hypothesize that these phenomena are merely hypospermatogenesis symptoms. Abnormalities associated with hypospermatogenesis, such as improper protamination, aberrant apoptosis and the release of abnormal spermatozoa with immature chromatin status, may contribute to the generation of high ROS levels (Gil-Guzman et al. 2001). Most likely a combination of mechanisms is involved in the etiology of defective spermatogenesis in patients with a varicocele. This may be an explanation of the heterogeneous clinical presentation in men with a varicocele and the variable response to varicocelectomy.

Perhaps our population of treated patients was too heterogeneous to attribute postoperative effects only to varicocele repair. However, our patient population reflects real life practice, in which many infertility patients and their partners have multiple defects that may explain the failure to conceive. According to the second hit hypothesis introduced by Marmar "Varicocele is a secondary opportunistic lesion that contributes to infertility, the underlying cause being genetic or epigenetic factors, expressed in both testes." (Marmar 2001) Our finding that LH and FSH were significantly increased after surgery in the nonresponder group may indicate that in these patients more predominant, ongoing causes of hypospermatogenesis and sperm DNA damage, other than varicocele led to infertility.

Recently, Cayan et al reported a 38% spontaneous pregnancy rate in a meta-analysis of the best surgical technique in a Palomo series and a 42% in a microsurgical varicocelectomy series (Cayan et al. 2009). Agarwal et al previously noted no differences in sperm quality improvement after microsurgical varicocelectomy or high inguinal ligation series (Agarwal et al. 2007). Although in our study the number of patients treated with microsurgical varicocele repair was small, the spontaneous pregnancy outcome was not related to surgical technique. Microsurgical varicocele treatment seems to be the best technique with a higher spontaneous pregnancy rate and lower postoperative recurrence. Also, it is recommended by the American Urological Association best policy practice group (Jarow et al. 2002).

Ficarra et al reviewed randomized, controlled trials of varicocele repair and found a significant increase in the pregnancy rate in patients who did vs did not undergo varicocele treatment (36% vs 20%) (Ficarra et al. 2006). Marmar et al reported a 33% pregnancy rate in patients treated with surgical varicocelectomy and a 16% rate in controls with no surgery (Marmar et al. 2007). These reported pregnancy rates are comparable to the 37% spontaneous pregnancy rate after varicocelectomy in our series. Couples with lower

sperm DNA damage who do not achieve natural pregnancy after varicocele treatment have better results with ART.

CONCLUSIONS

Varicocelectomy can restore spermatogenesis, as reflected by improved sperm parameters and a significant decrease in sperm DNA fragmentation. Since lower DFI was associated with a higher pregnancy rate (spontaneous and after ART), we suggest that varicocelectomy should be considered in infertile men with a palpable varicocele, abnormal semen analysis and no major female factors.

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CHAPTER 6

Increased level of sperm DNA fragmentation in vasectomy reversal patients has no prognostic value for pregnancy rate

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ABSTRACT

Purpose: We evaluated sperm DNA fragmentation (DFI) in vasectomy reversal and its prognostic value to determine spontaneous and assisted reproductive technique pregnancy rates.

Materials and Methods: We prospectively assessed DNA fragmentation with the sperm chromatin structure assay in postoperative semen samples of 70 patients with vasectomy reversal. At a median ± SD follow up of 4.4 ± 0.5 years pregnancy rates were recorded.

Results: DNA fragmentation in patients with vasectomy reversal was significantly increased vs. that in proven fertile controls (30.2% ± 20.1 vs. 15.3% ± 5.4 $p<0.001$). Significant negative correlations were found between DNA fragmentation index and total sperm count, progressive motility, total number of progressive sperm, normal morphology and sperm vitality ($-0.325 < r < -0.805$). The obstructive interval did not correlate with DNA fragmentation. The spontaneous pregnancy rate was 46%. Significantly higher log total progressive sperm motility ($p=0.021$) and a trend toward lower female age ($p=0.064$) were detected in the spontaneous pregnancy vs. the no pregnancy group. No association was found between DNA fragmentation and the pregnancy rate.

Conclusions: Increased DNA fragmentation is present in semen samples of men after vasectomy reversal vs. fertile controls but DNA fragmentation is not associated with spontaneous or assisted reproductive technologies pregnancy rates in these patients.

INTRODUCTION

Vasectomy is commonly done as a male contraceptive procedure in The Netherlands. Currently 21% of Dutch men have a history of vasectomy. Up to 6% of men regret the vasectomy and seek professional help to have fertility restored (Goldstein 1993). Microsurgical vasovasostomy (VV) and vasoepididymostomy (VE) are considered the standard techniques of vasectomy reversal (Medicine 2008). The main prognostic factors of the surgery outcome are the interval from vasectomy to vasectomy reversal and female age (Boorjian et al. 2004; Gerrard et al. 2007). Additional factors caused by vasal obstruction, such as epididymal damage, testicular changes giving rise to decreased spermatogenesis and possibly an ongoing inflammatory reaction, may lead to the decreased fertilizing potential of spermatozoa in men with vasectomy. These obstruction mediated changes may at least in part explain the lower pregnancy rates of 30% to 70% after vasectomy reversal compared to that in the general population (Belker et al. 1991).

Classic sperm parameters such as concentration, motility and morphology are used to classify men as infertile or fertile, although none is diagnostic of infertility (Guzick et al. 2001). Recently sperm quality studies have focussed on the evaluation of sperm DNA integrity as an additional tool to assess the fertilizing potential of spermatozoa (Committee 2008). Sperm DNA damage is more prevalent in infertile patients (Zini et al. 2002) and high sperm DNA damage can even be found in 5% to 8% of normozoospermic men in infertile couples (Erenpreiss et al. 2008; Zini et al. 2002). Sperm chromatin structure assay (SCSA) determines the sperm DNA fragmentation index (DFI), which has low biological variation and only moderately correlates with classic sperm parameters (Smit et al. 2007).

In 2 large studies DFI had prognostic value for spontaneous pregnancy in healthy, first pregnancy planners (Evenson et al. 1999; Spano et al. 2000). The usefulness of sperm DNA damage assessment for assisted reproduction technologies (ART) is still under debate (Committee 2008) and contradictory results have been published regarding its prognostic value for intra uterine insemination (IUI) (Bungum et al. 2004).

We evaluated DFI in VV and VE postoperative sperm samples, and investigated its prognostic value to determine the spontaneous and ART pregnancy rates.

MATERIAL AND METHODS

Patients

Between June 2004 and July 2005 SCSA was performed in all nonazoospermic postoperative semen samples of men who underwent vasectomy reversal at our tertiary referral outpatient clinic. SCSA is integrated at our laboratory to complement semen analysis as a research tool. Patients were informed about the anonymous storage of bio-

materials for future research activity by a brochure and were given ample opportunity to object to the use of redundant biomaterial for scientific purposes. After completing traditional semen analysis according to WHO guidelines (WHO 1999) redundant material was anonymously stored at -80°C for subsequent SCSA. In accordance with institutional regulations no separate institutional review board approval was required. In February 2009 pregnancy followup data were collected by reviewing patient records. Missing information was updated by telephone inquiry. A total of 22 proven fertile healthy men who donated a semen sample before vasectomy served as SCSA controls.

A total of 70 patients met study inclusion criteria. During followup 3 couples ended the relationship or divorced, pregnancy was not pursued in 1 couple due to female partner illness and 2 patients were lost to followup. Postoperatively secondary azoospermia due to restenosis developed in 11 patients (16%), of whom 3 couples elected no treatment, while percutaneous epididymal sperm aspiration/ICSI and testicular sperm extraction/ICSI were done in 5 and 3, respectively resulting in 3 live births. Restenosis developed after VV, VE and VE with unilateral VV in 5 of 45 (11%), 1 of 8 (13%) and 5 of 11 (45%), respectively. Of the couples 53 remained available for analysis.

Median ± SD followup was 4.3 ± 0.5 years. Median time from vasectomy to vasectomy reversal was 7.0 ± 5.0 years. In 40 patients modified 1-layer VV was done (primary in 30 and repeat in 10), 6 were treated with intussusception technique VE (primary in 5 and repeat in 1), and 7 were treated with a unilateral VV and unilateral VE. Median male age at surgery was 41.6 ± 6.3 years and median female age was 31.6 ± 4.3 years. Three women had a female factor (endometriosis or relative tubal pathology).

Sperm DNA fragmentation evaluation

SCSA was done essentially as described by Evenson (Evenson et al. 2000a) using a FACScan™ flow cytometer. Briefly, samples were diluted to a concentration of 1 to 2 x 10^6 sperm cells per ml, exposed to acid detergent solution and stained with acridine orange. Data collection of the fluorescent pattern in 5000 cells was done at 3 minutes after acid treatment. Debris, bacteria and leukocytes were gated out during acquisition, as recommended by Evenson (Evenson et al. 2000a). The extent of DNA damage is expressed as the DFI, reflecting the ratio of red fluorescence to total fluorescence. Cell Quest Pro™ and WinList™ were used to calculate the DFI of each sample. Each sperm sample was measured in duplicate and the mean of the results was used in analysis.

Statistical analysis

For statistical analysis SPSS® 15.0 was used. All results are expressed as the median ± SD. All numerical variables were tested for normal distribution with the 1-sample Kolmogorov-Smirnov test. Total sperm count and total progressive sperm were logarithmically transformed to obtain the normal distribution.

Correlations between variables were analyzed using Spearman's rank correlation coefficient. Comparisons among pregnancy groups were performed with the 1-way ANOVA and the post-hoc Students t test for pairwise comparisons with *p*-value <0.05 considered statistically significant.

RESULTS

Semen analysis was done a median of 5.5 ± 3.7 months postoperatively (range 1.9 to 20.5). To explore a possible association among sperm cell vitality, DFI in the postoperative semen sample and time from vasectomy reversal to semen analysis Spearman's rank correlation analysis was used. No significant correlations were found among sperm vitality, DFI and time from vasectomy reversal to semen analysis. When analysis was repeated in the 26 patients with at least a 6-month interval between reversal surgery and semen analysis, identical results were obtained. Patients were categorized by time of postoperative semen analysis into a ≈3 month (19) and a ≈6-month group (26). No statistical differences were found in mean DFI, sperm vitality or other sperm parameters in a t test comparison between the 2 groups. In 21 patients multiple semen analyses 3 and 6 months postoperatively were available. In this limited number of patients DFI did not change significantly over time.

DFI in patients with vasectomy reversal was significantly increased vs that in proven fertile controls (30.2% ± 20.1% vs 15.3% ± 5.4%, t test p<0.001). There were significant negative correlations between DFI and total sperm count, progressive motility, total

Table 1 Semen and patient characteristics.

	Spontaneous pregnancy n=26 (46%)	ART pregnancy n=12 (21%)	No pregnancy n=15 (28%)
DFI (%)	32.9 (± 20.9)	30.8 (± 23.5)	24.3 (± 13.7)
Concentration (x 10^6/ml)	20.0 (± 21.6)	4.9 (± 7.2)	17.0 (± 12.0)
Total sperm count (x 10^6)	46.2 (± 79.4)	19.7 (± 35.3)	29.2 (± 20.2)
Progressive motility (%)	25.5 (± 15.8)	11.0 (± 16.0)	13.0 (± 15.7)
Total progressive sperm (x10^6)	12.4 (± 24.5)	2.0 (± 9.7)	5.2 (± 5.5)
Normal morphology (%)	4.0 (± 2.9)	3.0 (± 3.5)	3.0 (± 1.6)
TZI	2.0 (± 0.3)	1.8 (± 0.2)	1.9 (± 0.3)
Non-vital sperm (%)	56.0 (± 19.2)	50.0 (± 21.5)	45.0 (± 12.5)
Female age (yrs)	30.9 (± 3.9)	31.5 (± 4.7)	34.8 (± 4.4)
Male age (yrs)	40.5 (± 5.0)	44.5 (± 4.8)	41.6 (± 8.6)
Year vasectomy (yrs)	7.0 (± 4.9)	7.5 (± 5.0)	8.5 (± 5.3)

Legend Table 1 Non-vital sperm percentage was assessed in the spontaneous pregnancy group, ART pregnancy group and no pregnancy group in respectively 12, 9 and 9 patients.

number of progressively motile sperm, normal morphology and sperm vitality (-0.325 < r < -0.805). The teratozoospermia index (TZI) correlated positively with DFI (r=0.502 p<0.001). Male age did not correlate with DFI, nor did the obstructive interval between vasectomy and vasectomy reversal. The obstructive interval did not correlate with any classic sperm parameters. We noted a positive correlation between patient age and years since vasectomy (r=0.640 p<0.001).

At follow up all couples were assigned to a spontaneous pregnancy (26 or 49%), a pregnancy after ART (12 or 23%) or a no pregnancy (15 or 28%) group. Table 1 lists patient characteristics and sperm parameters. The spontaneous pregnancy rate after VV, VE and unilateral VV and VE was 43%, 67% and 71%, respectively. One-way ANOVA showed differences among the 3 pregnancy groups in sperm concentration (p=0.032) and the log total number of progressive motile sperm (p=0.043).

The t test revealed significant higher log total progressively motile sperm (p=0.021) and a trend toward lower female age (p=0.064) in the spontaneous vs no pregnancy group. No significant differences in DFI, sperm concentration or vitality, male age and obstructive interval were noted between the 2 groups. All patients were dichotomized as having low or high DFI based on a 30% threshold, as frequently described in the literature (Bungum et al. 2004; Evenson et al. 1999). DFI was less and greater than 30% in 11 (42%) and 15 (58%) of 26 patients with spontaneous pregnancy, in 5 (42%) and 7 (58%) of 12 with ART pregnancy, and in 9 (60%) and 6 (40%) of 15 with no pregnancy, respectively.

When couples with a known female factor were excluded, repeat analysis revealed identical results. In the spontaneous pregnancy group median time to pregnancy was 11.0 ± 12.8 months and it only inversely correlated with male age (r=-0.418, p=0.033). No correlation was found between time to pregnancy and DFI, sperm parameters, obstructive interval or female age.

In the spontaneous pregnancy and pregnancy after ART groups median DFI was calculated for miscarriages and ongoing live birth pregnancies. Due to the small numbers of cases no statistical analysis was done but couples with miscarriages did not have higher median DFI than men who fathered healthy children (data not shown). After complete followup 24 of 26 couples in the initial spontaneous pregnancy group reported live births, of whom 2 eventually resorted to ICSI, and 11 of 12 in the ART group achieved live births.

DISCUSSION

To our knowledge we report for the first time that DFI is increased in postoperative semen samples of patients with vasectomy reversal patients compared to those of

fertile controls. Neither the spontaneous pregnancy rate nor ART results seemed to be influenced by DFI.

After vasectomy reversal large numbers of dead sperm and poor quality spermatozoa can be seen in the ejaculate, possibly due to remnant cell debris from the formerly obstructed epididymis or to an inflammatory component after obstruction caused by vasectomy. To ensure that the interval from vasectomy reversal to semen analysis was adequate we examined the relationship between DFI and this interval. We performed additional analysis in a patient subgroup in which the interval was at least 6 months and in a subgroup in which multiple postoperative semen analyses were available. Since neither analysis showed any association between the vasectomy-reversal to semen analysis interval and vitality or DFI, we conclude that high DFI is representative of vasectomy reversal and not caused by nonviable sperm that would be washed out in the months after vasectomy reversal.

In patients with vasectomy reversal the obstructive interval is the prognostic factor with the highest impact on the pregnancy rate (Belker et al. 1991; Boorjian et al. 2004). Vasal obstruction may result in irreversible epididymal and testicular damage that may lead to a decreased pregnancy rate. Several studies show increased interstitial fibrosis in testicular biopsies obtained during vasectomy reversal. Testicular fibrosis is negatively associated with the pregnancy rate (Jarow et al. 1985; Shiraishi et al. 2002) and it worsens with an increasing obstructive interval (Raleigh et al. 2004). Two studies showed a decreased number of germ cells in the testicular tissue of men with vasectomy (Jarow et al. 1985; Shiraishi et al. 2002). A more recent study did not confirm this but showed that vasectomy results in a significant decrease in germ cells in the later stages of spermatogenesis with decreased numbers of pachytene spermatocytes and elongated spermatids (Raleigh et al. 2004). No correlation between sperm output after vasectomy reversal and testicular spermatid numbers or testicular fibrosis was detected. Our results revealed no significant correlation between the number of years that the genital tract was obstructed and the level of sperm DNA damage. The high DFI detected in patients with vasectomy reversal may originate from the damaged epididymis or testis and may be caused by the spermatogenic damage or increased testicular fibrosis induced by vasal obstruction.

Numerous studies have established an association between sperm DNA fragmentation and the reactive oxygen species (ROS) associated with male infertility. Higher ROS levels are found in patients with vasectomy reversal vs controls (Nandipati et al. 2005), which may account for the relatively lower pregnancy rate after reversal surgery (Kolettis et al. 1999). An ongoing inflammatory reaction in the genital tract may lead to high ROS levels and consequently to sperm DNA damage after vasectomy reversal (Kolettis et al. 1999; Nandipati et al. 2005). The ROS total antioxidant capacity score does not correlate with the obstructive interval (Kolettis et al. 1999). It remains unclear why no linear relationship exists between the obstructive interval and the DFI or ROS.

The spontaneous pregnancy rate after vasectomy reversal was 46% in our study, in agreement with the reported 30% to 70% in the literature (Belker et al. 1991). Based on the prognostic value of DFI in in vivo studies (Evenson et al. 1999; Spano et al. 2000), we anticipated a prognostic role for DFI in the vasectomy reversal surgery outcome. However, even men with DFI as high as 81% have the ability to achieve spontaneous pregnancy, by far surpassing the suggested 30% DFI threshold for spontaneous pregnancy (Bungum et al. 2004; Evenson et al. 1999). A potential weakness of our study is the small number of patients. In a large series Silber et al noted remarkably little difference in the pregnancy rate related to the postoperative sperm count. They observed that after the sperm count was greater than 5 million per ml there was no difference in the pregnancy rate in men with a high vs a low sperm count (Silber et al. 2004). We speculate that a similar situation exists for the prognostic value of DFI for the spontaneous pregnancy rate after vasectomy reversal. Perhaps DFI in couples with proven fertile men before vasectomy reversal and often with young wives whose fertility was investigated before reversal surgery cannot be compared to the prognostic value of DFI in the normal or even the infertile population.

CONCLUSIONS

Compared to fertile controls increased sperm DNA damage is present in semen samples 6 months after vasectomy reversal. Sperm DNA damage assessment using SCSA was not useful to predict the postoperative chance of spontaneous or ART pregnancy.

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CHAPTER 7

Sperm DNA fragmentation in severe male infertility is not associated with IVF/ICSI outcome

Submitted

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ABSTRACT

Background: This study investigates whether sperm DNA fragmentation index (DFI) in severe male infertility patients is associated with the outcome of IVF/ICSI.

Methods: In 61 couples with documented severe oligoasthenoteratozoospermia (OAT), undergoing their first cycle IVF/ICSI treatment in a tertiary referral clinic, DFI was assessed using the sperm chromatin structure assay (SCSA), both in the native sperm sample and following density gradient centrifugation (DGC).

Outcome measures: Patients were dichotomized into low and high DFI groups using a DFI threshold of 30% to analyse associations between DFI and fertilization rate, embryo quality, biochemical and ongoing pregnancies and pregnancy loss.

Results: Mean sperm concentration was 1.6 (±2.6) $x10^6$/ml. DFI levels exceeding 30% were found in the native sperm samples of 45% of the patients. DFI increased following DGC in 40% of patients. Overall IVF/ICSI treatment outcome was 67.2% fertilization rate, 41.7% biochemical pregnancy rate and 35.0% ongoing pregnancy rate. Four out of 25 biochemical pregnancies resulted in a miscarriage. Neither DFI in the native nor processed sperm sample was associated with any of the outcome measures of IVF/ICSI. A significant association was found only between DFI after DGC and embryo quality ($p=0.036$).

Conclusion: In a study population of men with severe OAT we confirmed the results from previous studies, showing that sperm DNA integrity expressed as DFI is not associated with IVF/ICSI outcome. Sperm processing results in increased DFI.

INTRODUCTION

The introduction of intracytoplasmatic sperm injection (ICSI) into *in-vitro* fertillizaton (IVF) programs has dramatically increased the treatment options for men with severe oligoasthenoteratozoospermia (OAT) (Palermo et al. 1992). In the past, it was presumed that a significant proportion of spermatozoa in subfertile patients contains fragmented DNA and hence abnormal chromatin compared to normal fertile men. Moreover, injection of oocytes in the course of an IVF/ICSI treatment with these spermatozoa could probably result in failure of sperm decondensation and subsequent fertilization. However, animal studies showed that spermatozoa with induced defective DNA can still fertilize an oocyte and produce high quality early-stage embryos, although the likelihood of a successful term pregnancy decreased as the extent of the DNA damage increased (Ahmadi et al. 1999). It has been suggested that the oocyte might be able to repair some level of sperm DNA damage. It has been demonstrated that chromatin-damaged human spermatozoa are able to form normal pronuclei in hamster oocytes after IVF/ICSI just like sperm with an intact genome (Twigg et al. 1998b). The deleterious consequences for embryo- and offspring-development arising from defective chromatin are largely unknown. Recent studies have suggested that the use of genomically compromised spermatozoa of male infertile patients may result in higher abortion rates (Sanchez et al. 1996) and higher frequency of chromosomal abnormalities in offspring following IVF/ICSI as compared to conventional IVF (Bonduelle et al. 2002). Some of these effects may be merely the result of the parental background factors that require the use of IVF/ICSI, but it may also be attributable to the technique of IVF/ICSI itself. Chromosomal anomalies and microdeletions on the Y-chromosome, that can cause severe male infertility, are five times more prevalent in the IVF/ICSI population. It is remarkable however, that even with a normal paternal karyogram and absence of Y-chromosome microdeletions, the prevalence of de novo sex chromosome abnormalities and the loss of imprinting genes associated with otherwise rare conditions are increased in IVF/ICSI offspring (Feng et al. 2008; Kurinczuk 2003).

Although the first studies that evaluated sperm DNA integrity as a prognostic factor for the outcome of assisted reproductive technologies (ART) in humans seemed promising (Larson-Cook et al. 2003; Lopes et al. 1998), more recent studies showed that even males with high extent of sperm DNA damage can successfully achieve pregnancy, especially with IVF/ICSI (Gandini et al. 2004; Lin et al. 2008; Payne et al. 2005). A recent meta-analysis of published data indicated a small but statistically significant correlation between DNA integrity and IVF and IVF/ICSI outcome. However, the predictive power of sperm DNA integrity in forecasting IVF/ICSI outcome is limited because the sensitivity and specificity values are non-discriminatory. Moreover, the likelihood ratios seem too small for abnormal or normal test results to alter the predicted probability of pregnancy

(Collins et al. 2008). Nevertheless, there is some evidence that sperm DNA damage may be associated with increased pregnancy loss following IVF and ICSI (Zini et al. 2008).

Differences in numbers of patients studied, inclusion criteria, study design and assays used to determine sperm DNA integrity have prompted many authors, including those of the meta-analyses, to conclude that future studies should determine the effects of sperm DNA damage on the outcome of ART, and of IVF/ICSI in particular, in different clinical settings and in subgroups of patients with high levels of damage.

Since higher levels of sperm DNA damage are generally found in patients with poor sperm quality (Zini et al. 2002), and because it seems more appropriate to test sperm DNA damage in post-preparation samples in relation to first cycle IVF/ICSI, we conducted a prospective study to evaluate sperm DNA damage in men with severe OAT undergoing their first IVF/ICSI cycle. The principle aims of the present study were to investigate the influence of DFI on fertilization rate, embryo quality, biochemical pregnancy, clinical pregnancy and pregnancy loss and to evaluate the prognostic value of sperm DNA fragmentation in native as well as processed sperm used for IVF/ICSI.

MATERIAL AND METHODS

Study population

Between January 2005 and December 2006, 61 couples with severe male factor infertility due to severe OAT who had a first cycle IVF/ICSI procedure at a tertiary referral fertility clinic (Erasmus MC, Rotterdam, the Netherlands) were included. Prior to the decision to proceed with IVF/ICSI treatment, all men visited the Andrology outpatient clinic for fertility evaluation comprising semen analysis according to World Health Organization (WHO) guidelines (WHO 1999), determination of reproductive hormones, physical examination and scrotal ultrasound. Men with ejaculated sperm concentrations of >0.1x 10^6/ml and at least 1% normal morphology were eligible for the study. Exclusion criteria were a history of failed IVF, oocyte donation cycles, the diagnosis of endometriosis or hydrosalpinges, and maternal age >41 years since these conditions detrimentally influence IVF/ICSI outcome. The study protocol was approved by the Institutional Review Board of Erasmus MC in Rotterdam. Participants provided written informed consent and obtained materials were processed anonymously.

Semen collection, processing and analysis

On the day of oocyte pick-up semen samples were collected by masturbation after a period of abstinence of 3 to 5 days. After liquefaction, the volume of the semen sample was measured and sperm concentration was subsequently assessed using a Makler-chamber. Finally, sperm motility was scored according to WHO guidelines (WHO 1999).

An aliquot of 100 µl unprocessed semen was stored at -80°C to assess sperm DNA integrity at a later stage.

The Sperm Chromatin Structure Assay (SCSA) was used to determine sperm DNA integrity and was performed essentially as described by Evenson (Evenson et al. 2000a) using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Because of the low sperm concentrations in this study and low volume of material collected, half of the recommended volumes of sperm and reagents were used. Samples were diluted to a concentration of at least 1-2 x 10^6 sperm cells per ml, unless initial concentrations were below 1-2 x 10^6. In the latter case samples were diluted at least twice to prevent seminal plasma from interfering with the acid denaturation and allowing adequate exposure of the diluted samples to acridine orange for staining. Data collection of the fluorescent pattern in 5000 cells was performed at 3 minutes after acid treatment. Whenever data collection was slow due to low sperm concentration, a limited number of cells, but at least 3000, was analyzed in order to prevent possible detrimental effects of extended analysis time (Boe-Hansen et al. 2005). Debris, bacteria and leukocytes were gated out during acquisition as recommended by Evenson (Evenson et al. 2000a). The extent of DNA damage is expressed as the DFI, reflecting the ratio of red fluorescence to total fluorescence. Cell Quest Pro and WinList software were used to calculate the DNA fragmentation index (DFI) of each sample. In the green versus red fluorescence dot plot, the cells with high DNA stainability (HDS) were calculated as a percentage of the total fluorescence. Cells with increased green fluorescence are considered to be immature germ cells with high DNA content. Each sperm sample was measured in duplicate and the mean value of the results was used in the analysis.

A standard Density Gradient Centrifugation (DGC) using PureSperm diluted in GZ GPO medium (Macklon et al. 2002) (Lonza, Verviers, Belgium) was applied for sperm preparation. After placing the native sperm sample on the top of a two layers PureSperm gradient (45% and 90%), the sample was centrifuged at 300 *g* for 20 minutes. The pellet was washed twice in GZ GPO and centrifuged at 1100 *g* for 7 minutes between the washing steps. The pellet was resuspended in GZ GPO, sperm count and motility were reassessed as previously described and the spermatozoa were incubated in 5% CO_2 in air, 37°C until the IVF/ICSI procedure. Remnant processed sperm after IVF/ICSI was stored at -80°C to perform SCSA at a later stage.

IVF/ICSI procedure

Thirty-five women started the ovarian stimulation treatment with daily injections of 150 IU recombinant follicle stimulating hormone (rFSH) s.c. on cycle day 2 (Puregon®, Shering-Plough, Oss, the Netherlands or Gonal-F®, Merck-Serono, Hoofddorp, the Netherlands). Administration of daily s.c. gonadotrophin releasing hormone (GnRH) antagonist (Orgalutran®, Shering-Plough or Cetrotide®, Merck-Serono) was started

when at least one follicle was ≥14 mm, as previously described (Hohmann et al. 2003). Twenty-six women were treated with a long agonist suppression protocol. After down-regulation was established with a GnRH agonist (Decapeptyl®, Ferring BV, Hoofddorp, the Netherlands), ovarian stimulation with rFSH was started in a s.c. dose of 150 IU daily. To induce final oocyte maturation, a single dose of 5 000 or 10 000 IU hCG s.c. (Pregnyl® Shering-Plough) was administered as soon as the largest follicle reached at least 18 mm in diameter and at least one additional follicle of >15 mm was observed. Oocyte retrieval was carried out 35 h after hCG injection by transvaginal ultrasound-guided aspiration of follicles. GZ GPO medium was used to rinse the oocytes. Denudation of cumulus cells was performed by exposure of the oocytes to 80 UI/ml Hyaluronidase (Sigma-Aldrich H-3757) and performed by use of glass denudation pipettes immediately before injection. The oocytes were washed in GZ GPO Hepes medium after denudation. IVF/ICSI was performed in GZ GPO Hepes covered by mineral oil using commercially available ICSI pipettes. On Day 3 after oocyte pick-up, a maximum of two embryos were transferred. Luteal phase supplementation of 600 mg/day micronized progesterone intravaginally was started on the evening following oocyte pick-up and continued 12 days thereafter.

Outcome measures

The clinical end-points of the study were fertilization rate, embryo quality, biochemical and clinical pregnancy rates in relation to SCSA parameters. Fertilization was defined as the presence of two distinct pronuclei 18-20h after the IVF/ICSI procedure. Fertilisation rate was defined as the number of fertilized oocytes divided by the total of injected oocytes. On day 3 after oocyte retrieval, embryo quality scores were assigned according to previously described criteria (Huisman et al. 2000). These scores ranged from one (superior quality) to five (underdeveloped embryo, inadequate for transfer). If two embryos were transferred, the best embryo quality score was used in the analysis. Biochemical pregnancy was determined by a urinary pregnancy test 15 days after oocyte retrieval. A clinical pregnancy was defined as an intrauterine gestational sac with heart beat 4 weeks after a positive pregnancy test. Ongoing pregnancy with live birth rate or pregnancy loss was recorded for all patients.

Statistical analysis

Statistical analysis was performed using SPSS 15.0 (SPSS Inc, Chicago, Ill.). The one sample Kolmogorov-Smirnov Test was used to test for normality. Non-normal distributed parameters were logarithmically transformed to obtain normally distributed data. All variables are given in mean (± standard deviation). Correlations between variables were calculated using Pearson's rank correlation coefficient. Paired sample T-test was performed to evaluate classic semen and SCSA parameters before and after DGC. The Mann-Whitney U test was used to detect significant differences in SCSA parameters

between the outcome measures biochemical and ongoing pregnancy and miscarriage rate. A cut-off of 30% was used to separate "low DFI" from "high DFI" as previously described (Evenson et al. 2000a). All couples were dichotomized based on DFI in native and processed sperm (DFI_{dgc}). For each of the outcome measures 2x2 tables were constructed and receiver operator characteristics (ROC) curve were made. A *p*-value of <0.05 was considered statistically significant.

RESULTS

Study population

One couple was excluded from analysis because no oocytes could be retrieved after ovarian stimulation treatment. In one couple transfer was cancelled due to inadequate embryo quality. Primary infertility was diagnosed in 40 couples, 12 couples had conceived previously, either with a previous partner (2) or spontaneously (2) or were treated with IVF/ICSI in the past resulting in their first child (8). In 3 couples the female partners had conceived spontaneously in a previous relationship, 4 men were proven fertile in a previous relationship prior to vasectomy and after subsequent vasectomy reversal and one man had been treated with IVF/ICSI in a former relationship. All IVF/ICSI procedures were indicated because of severe OAT and male infertility. These results are summarized in Table 1. All males had a normal karyotype and no evidence of Y-chromosome microdeletions. Additional female factors were diagnosed in 7 female partners and consisted of unilateral tubal pathology without hydrosalpinx, intra-abdominal adhesions or an irregular menstrual cycle being less than 21 days or exceeding 35 days in respectively 3, 2 and 2 patients. Six females and 20 males were smokers with a mean cigarette use of respectively 12 and 15 cigarettes daily. The baseline patient characteristics are given in Table 2. Single embryo transfer was performed in 54 couples, 5 couples received double embryo transfer.

Table 1 Andrological diagnosis in 60 study participants.

Andrological diagnosis	number of patients (%)
E causa incognita	15 (25%)
Varicocele	13 (21%)
Testicular insufficiency	12 (20%)
Cryptorchidism	10 (17%)
Failed vasectomy reversal	5 (8%)
Ejaculatory duct obstruction	2 (3%)
Male accessory gland infection	1 (2%)
Retrograde ejaculation	1 (2%)
Oncological treatment	1 (2%)

Table 2 Baseline patient characteristics n=60.

Patient characteristics	Mean (± SD)
Male age (yrs)	35.8 (± 5.2)
Sperm concentration (x 106/ml)	1.6 (± 2.6)
Progressive motility (%)	36.6 (± 20.9)
DFI (%)	31.6 (± 13.7)
HDS (%)	20.9 (± 11.0)
DFI_{dgc} (%)	33.4 (± 21.2)
HDS_{dgc} (%)	14.4 (± 10.3)
LH (IU/l)	4.6 (± 2.6)
FSH (IU/l)	10.8 (± 7.9)
Testosteron (nmol/l)	15.9 (± 5.1)
Inhibin B (ug/l)	108.1 (± 50.9)
Female age (yrs)	32.1 (± 3.8)
Oocytes retrieved (number)	8.2 (± 4.6)

Semen analysis

SCSA parameters were not significantly correlated with male age, endocrine values, BMI or smoking. Negative correlations were found between DFI and sperm concentration (r=-0.295 p=0.023) and progressive sperm motility (r=-0.318 p=0.015). HDS was negatively correlated with sperm count (r=-0.258 p=0.047). DFI levels exceeding 30% were found in the native sperm sample in 27/60 (45%) of patients.

Following density gradient centrifugation, progressive sperm motility increased significantly from 36.6% (± 20.9) to 47.7% (± 27.7) (p=0.007). HDS decreased significantly from 20.9% (± 11.0) to 14.4% (± 10.3) (p<0.001), but mean DFI values were not significantly different after sperm processing.

In 21/60 (35%) of patients DFI decreased with a mean percentage of 13.4% (± 6.4), in 24/60 (40%) DFI levels increased by 16.4% (± 9.5) and in 15/60 (25%) DFI levels before and after DGC remained similar (difference 0.6% (± 2.9). After DGC, DFI correlated with progressive motility in the native sample (r=-0.356 p=0.006) and progressive motility in the processed sample (r=-0.462 p<0.001). The negative correlation between HDS and total sperm count (r=-0.258 p=0.047) remained after DGC (r=-0.283 p=0.033).

Treatment outcome

Overall IVF/ICSI treatment outcome was 67.2% (± 25.7) fertilization rate, 25/60 (41.7%) biochemical pregnancy rate, 21/60 (35.0%) ongoing pregnancy rate. Four out of 25 biochemical pregnancies resulted in miscarriage. Fertilization rate was not significantly correlated with the SCSA parameters or sperm characteristics. When the 30% threshold

Table 3 2x2 tables with consequent sensitivity and specificity for outcomes measures.

Outcome	DFI<30%	DFI>30%	DFI_{dgc}<30%	DFI_{dgc}>30%
Biochemical pregnancy				
No	17	17	17	17
Yes	15	10	13	12
Sensitivity	60%		52%	
Specifity	50%		50%	
Ongoing pregnancy				
No	19	19	18	20
Yes	13	8	12	9
Sensitivity	62%		57%	
Specifity	50%		53%	
Miscarriage				
No	13	8	12	9
Yes	2	2	1	3
Sensitivity	50%		25%	
Specifity	38%		43%	

for DFI was used, fertilization rate was not statistically different in the low DFI group, compared to the high DFI group.

Only 2 embryos were considered of superior quality, 37 embryos were of good quality, 19 embryos were of lesser quality, one embryo was of poor quality and one embryo was of such poor quality that it was not transferred. None of the clinical variables assessed in both males and females, including female age, were associated with embryo quality. Significantly higher DFI following DGC (DFI_{dgc}) levels were seen in embryos with limited quality, compared to embryos with good quality (p=0.036). Embryo quality was not associated with fertilization rate, pregnancy rate or pregnancy loss.

Using the 30% DFI cut-off in both native and processed sperm, two by two tables were made and sensitivity and specificity were calculated for biochemical pregnancy, ongoing pregnancy and miscarriage rate (Table 3). Overall sensitivity and specificity was low, ranging from 25-62%. To examine the effect of DFI cut-off values on the outcome measures, ROC curves were made and depicted in Figure 1-3. None of the DFI cut-offs had any prognostic value for pregnancy rates or miscarriage rate. The area under the curve for biochemical, ongoing pregnancy and miscarriage rate was 0.451, 0.406 and 0.286 respectively.

HDS values in the native sperm sample were significantly higher in couples who achieved biochemical pregnancy, compared to those without a pregnancy (p=0.019). HDS, either in the native sperm sample or after DGC did not have a significant effect on fertilization rate, ongoing pregnancy or miscarriage rate.

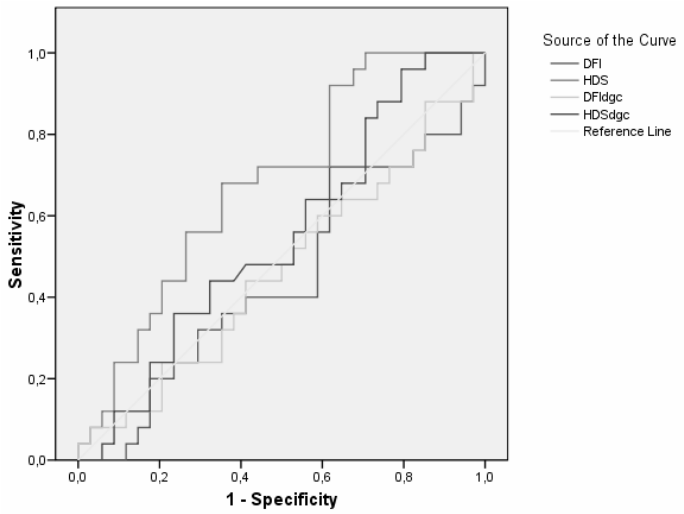

Figure 1 ROC curve biochemical pregnancy rate. Area under the curve for DFI, HDS, DFI_{dgc} and HDS_{dgc} was respectively 0.451, 0.680, 0.468 and 0.546.

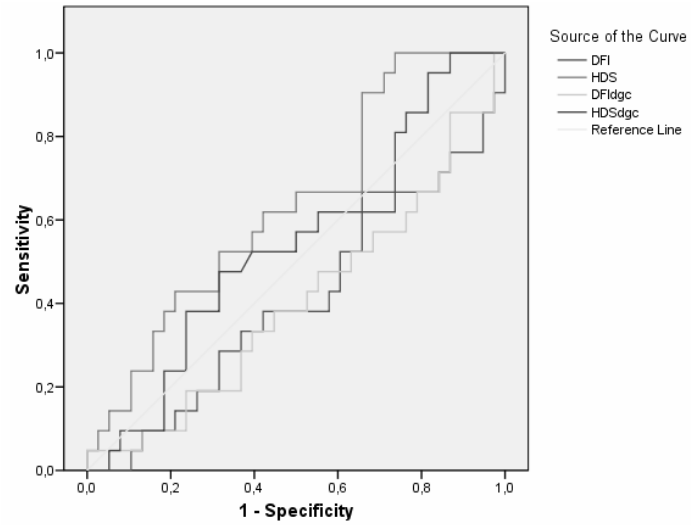

Figure 2 ROC curve ongoing pregnancy rate. Area under the curve for DFI, HDS, DFI_{dgc} and HDS_{dgc} was respectively 0.406, 0.634, 0.405 and 0.539.

Potential female confounders like age, smoking and body mass index (BMI) on IVF/ICSI outcome were evaluated. Female age was only negatively correlated with the number of oocytes fertilized (r=-0.271 p=0.036). Surprisingly, none of the other IVF/ICSI outcome parameters were associated with female age, including fertilization rate.

When the analysis was repeated in only those couples who received only one embryo transfer, identical results were obtained. All of the 21 ongoing pregnancies resulted in

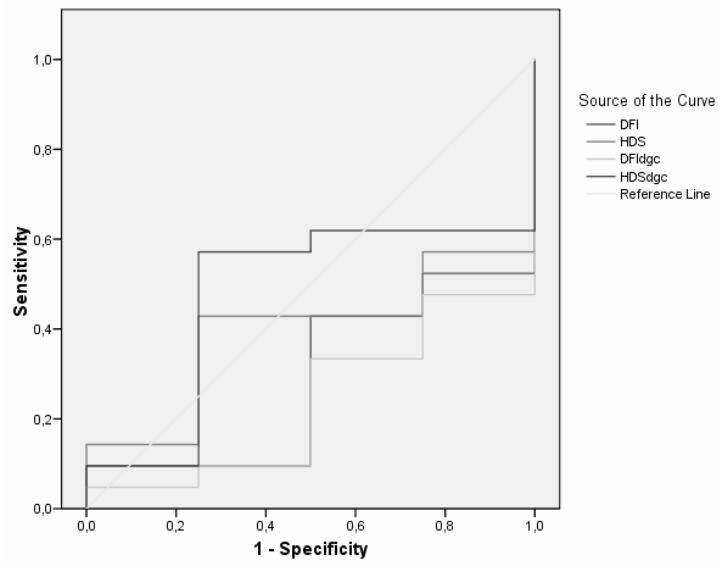

Figure 3 ROC curve miscarriage rate. Area under the curve for DFI, HDS, DFI_{dgc} and HDS_{dgc} was respectively 0.286, 0.393, 0.238 and 0.476.

healthy live births. Only one child was born prematurely possible due to an intra-uterine infection. No major or minor congenital anomalies were reported in these children.

DISCUSSION

Sperm DNA damage is prevalent in male infertility patients (Zini et al. 2002) but high levels of DFI can also be found in 5-8% of normozoospermic men from infertile couples (Erenpreiss et al. 2008; Zini et al. 2002). Previous reports on SCSA and IVF/ICSI outcome evaluated patients with far greater mean sperm concentrations than can be expected in male infertility IVF/ICSI, ranging from 14 up to 70 x 10^6/ml (Boe-Hansen et al. 2006; Gandini et al. 2004; Payne et al. 2005; Zini et al. 2005b). The fact that the indications for IVF/ICSI have expanded to couples with unexplained infertility and failed conventional IVF may explain the higher than expected sperm concentrations in patients undergoing IVF/ICSI studied. Moreover, previous studies reported highly variable prevalence of patients with high levels of DFI (>30%), ranging from only 6% up to 38% of IVF/ICSI patients. (Boe-Hansen et al. 2006; Bungum et al. 2007; Lin et al. 2008; Nicopoullos et al. 2008; Payne et al. 2005; Virro et al. 2004; Zini et al. 2005b). Finally, the majority of studies is performed in "a cohort of consecutive IVF/ICSI patients" and they did not assess DNA damage during the first IVF/ICSI cycle which can cause selection bias. The previously mentioned factors all potentially negatively influence the predictive value of the pre-

treatment sperm DNA integrity testing on the outcome of IVF/ICSI. In the present study, we aimed to evaluate the prognostic value of SCSA testing in infertile couples with documented severe OAT in predicting the outcome of their first IVF/ICSI cycle. Despite the notably lower sperm concentration and greater prevalence of higher levels of DFI in our study population, our results confirm the observations of other authors that sperm DNA integrity expressed as DFI is not associated with IVF/ICSI outcome, even in a well documented population with severe male factor infertility.

Zini et al previously demonstrated the potential detrimental effect of DGC on sperm DNA integrity in 10% of infertile patients studied (Zini et al. 1999). In patients with severe OAT we found that DFI increased following DGC in 40% of patients. The initial poor semen quality in our cohort may account for the high percentage of increase of DFI induced by centrifugation, washing, the removal of protective seminal plasma and potential induction of damage by reactive oxygen species (ROS) since it was previously shown that SCSA results after DGC were related to the initial sperm quality (Zini et al. 2000). Despite the increase in DFI found in 40% of patients in our study after DGC, we could confirm previous reports that DFI assessed in the processed sperm sample used for IVF/ICSI cannot predict the outcome of the subsequent treatment (Bungum et al. 2008; Gandini et al. 2004; Larson et al. 2000).

Recent studies suggested that impaired sperm DNA integrity as measured by SCSA may have the greatest effect on pregnancy loss in IVF/ICSI treatment (Zini et al. 2008). We could not confirm an association between the DFI levels and miscarriage rates in our study population, indicating that either such a relationship is not present or the number of participants was too small to detect a statistically significant difference.

Several explanations for the apparent lack of prognostic value of sperm DNA integrity assessment for the outcome of IVF/ICSI can be put forward. First, the oocyte may be capable of repairing a substantial proportion of the DNA damage present in the spermatozoa, thereby preventing a negative effect on IVF/ICSI outcome (Ahmadi et al. 1999). A second reason for the observed discrepancy might be that since sperm DNA damage correlates moderately with sperm motility and morphology it may be justified to conclude that selection of spermatozoa for injection based on these sperm characteristics warrants the selection of sperm with the least chromatin abnormalities in a sample (Giwercman et al. 2003). Finally, the assay used in the present study estimates a DNA damage profile in a sperm suspension which is not necessarily evenly representative for all spermatozoa present in this sample.

Recently, Avendano et al. showed that even morphologically normal sperm in the purified sperm population used for IVF/ICSI can contain a high level of DNA fragmentation as measured by deoxynucleotidyl transferase dUTP end labelling (TUNEL). Sperm DNA fragmentation in morphologically normal sperm correlated with embryo quality and may be a useful predictor of pregnancy probability (Avendano et al. 2009). In this

view, the lack of prognostic value of SCSA parameters for IVF/ICSI outcome may be the result of the inability of SCSA to detect chromatin abnormalities that are associated with a reduced fertilizing capacity of spermatozoa and subsequent pregnancy following IVF/ICSI.

In general, pregnancy as an outcome measurement is difficult to interpret altogether because of the multifactorial male as well as female factors which are involved. One of the dominating factors that influence the outcome of ART is female age. Hence, the relatively young age of our subgroup of couples with severe male factor infertility may have masked this association and may explain the lack of association between female age and outcome of IVF/ICSI in our study.

Some authors have suggested that infertile couples with high DFI should be counselled for IVF/ICSI since DFI has prognostic value for in vivo pregnancy rates, both among healthy first pregnancy planners and following IUI treatment (Bungum et al. 2007; Evenson et al. 1999; Spano et al. 2000). Because pregnancies can be achieved with IVF/ICSI, irrespective of the DFI, this ART modality may be the most appropriate therapy and save couples with high DFI the disappointment of embarking on unsuccessful IUI or IVF treatment. Reports on congenital malformations, increased prevalence of sex chromosome abnormalities and loss of imprinted genes in IVF/ICSI offspring raises the question as to whether IVF/ICSI is a safe treatment option for patients with high percentage of DNA damage (Kurinczuk 2003). On one hand, SCSA may not be able to elucidate the safety issues for IVF/ICSI offspring but one could argue that IVF/ICSI in severe male factor infertility may be rendered a hazardous undertaking based on our observation that high DFI levels were found in 45% of patients with severe OAT and DFI can be increased by sperm processing prior to IVF/ICSI in as much as 40% of these patients. On the other hand, we must take into account that the current visual selection of spermatozoa for injection based on morphology and motility result in selecting sperm with the least amounts of chromatin damage from an abnormal sperm fraction. Future studies selectively assessing sperm DNA damage in spermatozoa to be used in IVF/ICSI and follow-up studies among children conceived via IVF/ICSI with documented paternal sperm DNA damage are needed to clear these current uncertainties.

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CHAPTER 8

Sperm DNA integrity in cancer patients before and after treatment

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ABSTRACT

Background: We assessed sperm DNA fragmentation index (DFI) in cancer patients before and after treatment to evaluate if sperm DNA integrity is compromised by cancer itself or its treatment.

Methods: In a prospective study, DFI was assessed in 127 patients diagnosed with testicular germ cell tumours (TGCT), Hodgkin's lymphoma (HL), Non Hodgkin Lymphoma (NHL) and various malignancies. The severity of cancer and tumour markers at diagnosis was recorded. Follow-up DFI after treatment was available in 52 patients who were mostly less severely affected.

Results: In patients diagnosed with TGCT, HL, and various malignancies, pretreatment DFI levels were not significantly different from that of proven fertile controls, but in patients with NHL an increased DFI was found. An overall significant decrease in post-treatment DFI (13.2% range 5.0-70.5) compared with pretreatment values (17.1% range 5.1-66.6) was found (p=0.040). In TGCT patients, post-treatment DFI was significantly higher in patients who were treated with radiotherapy (16.9% range 11.5-39.9) compared with that in patients treated with chemotherapy (CT) alone (10.9% range 5.5-39.9) (p=0.037). In HL patients, type of treatment or number of CT cycles was not associated with DFI. Overall, post-treatment DFI in cancer patients was not significantly different from that of proven fertile controls.

Conclusions: In this study, the presence of cancer does not seem to negatively affect the sperm DNA integrity in TGCT and HL patients; only NHL patients showed increased DFI at the time of diagnosis compared with healthy controls. Our results confirm previous reports that DFI decreases significantly following various anti-cancer treatments. In contrast, radiotherapy in TGCT patients is associated with an increase in DFI compared with CT treatment alone.

INTRODUCTION

Fertility preservation has become an important issue in the counselling and therapy of cancer patients of reproductive age, particularly for cancer types with a high cure rate, such as testicular germ cell tumour (TCGT) and Hodgkin's (HL) or non-Hodgkin's lymphomas (NHL). More than 90% of all TGCT patients are considered cured in a 5-year period following treatment (Kopp et al. 2006). In HL and NHL patients, more than 80% of all patients are successfully treated (Bernard et al. 2005; Diehl et al. 2003).

Impaired spermatogenesis is commonly observed at the time of cancer diagnosis, both for TGCT and for lymphomas (Gandini et al. 2003; Howell et al. 2005; van Casteren et al. 2009). TGCT is associated with testicular malfunction and it is postulated that the disease is part of the testicular dysgenesis syndrome also including defective spermatogenesis and cryptorchidism (Skakkebaek et al. 2001). The gonadal dysfunction observed in lymphoma is believed to be caused by a systemic effect of the disease, possibly because of cytokine activity. Increased levels of interleukins have been associated with more severe dysfunction of spermatogenesis (Rueffer et al. 2001).

Cancer treatment might further affect gonadal function, though these effects may be (partly) reversible, depending on the agents and cumulative dosage used. Current chemotherapy (CT) agents and radiotherapy treatment protocols aim at cure in combination with minimal long-term morbidities such as treatment related infertility. TGCT patients treated with at least 3 cycles of bleomycin, etoposide and cisplatin (BEP) initially all develop azoospermia with recovery of spermatogenesis in 48% after 2 years and 80% after 5 years (Lampe et al. 1997). Alkylating agents such as mustine, vincristine, procarbazine and cyclofosfamide, formerly used to treat lymphomas, cause permanent azoospermia in up to 90% of patients. Current therapy of HL with a combination of non-alkylating agents like adriamycin, bleomycin, vinblastine and dacarbazine (ABVD) result in post-treatment spermatogenesis recovery in 90% of patients (Fossa et al. 2004). Chemotherapeutic treatment of NHL with cyclophosphamide, doxorubicin, vincristine and prednisolone (CHOP) results in permanent azoospermia in 30% of patients (Howell et al. 2005).

Cryopreservation of spermatozoa prior to cancer treatment is a generally accepted and available option for fertility preservation (van Casteren et al. 2008). Nowadays, in vitro fertilization with intracytoplasmatic sperm injection (IVF/ICSI) enables men with severe oligoasthenoteratozoospermia to father children. Paternity has become an option for cancer survivors who were able to cryopreserve even a few spermatozoa prior to therapy and for those with only partial recovery of spermatogenesis after discontinuation of cancer treatment (van Casteren et al. 2008). However, the safety of the use of sperm cryopreserved at the time of cancer diagnosis and the use of recovered sperm from males exposed to cancer treatment has recently been questioned (Spermon et al.

2006). Assessment of sperm DNA integrity has been proposed as a means to investigate the impact of cancer and its treatment on the functional quality of spermatozoa. In animal studies sperm DNA damage induced by gonadotoxic treatment has been associated with increased number of pathological pregnancies (Dobrzynska et al. 2005; Trasler et al. 1985). In humans, however, there is no epidemiological proof of increased malformations in children born to parents who were formerly treated for cancer (Morris 2002).

In the present study, we determined sperm DNA damage in cancer patients before and following treatment to evaluate whether the cancer itself or its treatment induced changes in the genomic integrity of sperm. We evaluated the association between sperm DNA damage and disease stage, pretreatment tumour markers and type of treatment, to establish the impact of tumour characteristics and intensity of treatment on sperm DNA integrity.

MATERIAL AND METHODS

Study population

Between September 2003 and September 2005, study participants were recruited among all men who visited our Andrology outpatient clinic for an attempt to cryopreserve their semen before oncological treatment. One hundred and twenty-seven non-azoospermic men were included in the study and provided written informed consent to have the Sperm Chromatin Structure Assay (SCSA) performed in addition to classic semen analysis, prior to cryopreservation and in future semen samples. All patients were categorised according to oncological diagnosis comprising TGCT, HL, NHL, leukemia, sarcoma and various malignancies requiring CT like astrocytoma, rectal cancer and so on (other). Information regarding cancer stage, prognosis group, tumour markers at the time of diagnosis, histological tumour type and oncological treatment was obtained from patient records. TGCT patients were categorized as good prognosis if, at the time of diagnosis, the primary tumour was found in the testis or retroperitoneum, beta-human chorionic gonadotrophin (ßHCG) was <1.9 IU/l (normal value 0-1.9 IU/l) and lactate dehydrogenase (LDH) was <1.5 x 449 U/l (normal value 0-449 U/l). Intermediate prognosis comprised extrapulmonary visceral metastasis in seminomas or alfafoetoprotein (AFP) 1-10 ug/l (normal value 0-9 ug/l) or LDH <1.5-10x 449 U/l or ßHCG 5-50 IU/L in non-seminomas. Tumour markers above these thresholds or the presence of extrapulmonary visceral metastases in non-seminomas resulted in classification as poor prognosis (Mead et al. 1997). For lymphomas, the Ann Arbor staging system was used, defined as cancer in a single lymph node region (stage I), two separate lymph node regions either above or below the diaphragm (stage II), cancer on both sides of the diaphragm (stage III) or disseminated involvement (stage IV) (Lister et al. 1989).

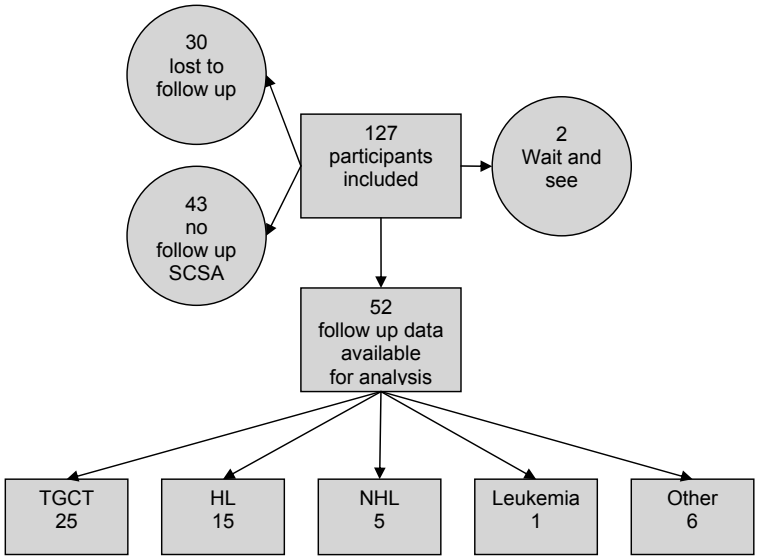

Figure 1 Flowchart of patient inclusion and followup
TGCT=testicular germ cell tumours, HL=Hodgkin's lymphoma and NHL=Non-Hodgkin's lymphoma, other= divers malignancies like astrocytoma, rectal cancer etc.

Patients were advised to repeat semen analysis at least 6 months after the last oncological treatment to assess fertility and decide whether cryostorage of the pretreatment semen sample should be prolonged. When multiple follow-up samples were available, the most recent sample was used for the analysis. A total of 30 patients were lost to follow-up. In 43 patients follow-up semen analysis and SCSA results could not be obtained because of progression and death, persistent azoospermia due to treatment, anorchia or retrograde ejaculation. Two patients were managed in a wait and see protocol and did not receive treatment. In 52 patients, follow-up data were available for analysis. A study flowchart is depicted in Figure 1. For fertile controls, 22 proven fertile healthy men donated a semen sample prior to vasectomy.

Semen analysis

Semen samples were collected by masturbation prior to oncological treatment. Following liquefaction, a droplet of semen was used for gross examination of the sample. If motile, viable sperm was found, the sample was considered adequate for cryopreservation. After cryopreservation, semen analysis in the droplet of semen was carried out according to WHO guidelines (WHO 1999). Remnant material was stored at -80°C for SCSA analysis at a later stage. Whenever patients were able to have multiple samples cryopreserved prior to therapy, results from the first semen sample were used in this study. During follow-up semen samples were collected by masturbation after a period of abstinence of 3-5 days. Semen analysis was performed according to WHO guidelines

(WHO 1999), and material was stored at -80°C for sperm DNA integrity assessment at a later stage.

Sperm DNA integrity assessment

The SCSA was used to assess sperm DNA integrity. O'Flaherty et al. recently concluded that SCSA and deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) are significantly correlated and suggested that any of these assays can be used to determine DNA damage in cancer patients (O'Flaherty et al. 2008). SCSA was performed essentially as described by Evenson and Jost (Evenson et al. 2000a), using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). In brief, frozen samples were quickly thawed, diluted to a concentration of 1-2 x 10^6 sperm cells per ml, exposed to acid detergent solution and stained with acridine orange. A reference sample, treated similarly, was run prior to the actual sample measurements and used to adjust the voltage gains of the flow cytometer FL3 and FL1 photomultipliers that analyse red and green fluorescence, respectively. The voltage gains were adjusted to obtain stable mean red (X) and mean green (Y) values of the reference sample at 110 and 370 channels, respectively, with a maximum deviation of five channels. An aliquot of reference sample was run after every 5-10 samples. The voltage gains were re-adjusted whenever the fluorescent signal of the reference sample drifted. Data collection of the fluorescent pattern in 5000 cells was performed at 3 min after acid treatment. Debris, bacteria and leukocytes were gated out during acquisition as recommended by Evenson and Jost (Evenson et al. 2000a). The extent of DNA damage is expressed as the DNA fragmentation index (DFI), reflecting the ratio of red fluorescence to total fluorescence. Cell Quest Pro and WinList software were used to calculate the DFI of each sample. Each sperm sample was measured in duplicate and the mean values of the results were used in the analysis.

Statistics

All variables were tested for normal distribution using the one sample Kolmogorov-Smirnov test. Because the majority of semen analysis and SCSA variables were not normally distributed, non-parametric tests were used for all variables. We aimed to assess whether pre- and post-treatment semen analysis and DFI values were statistically different using the Wilcoxons-paired test. Possible associations between cancer characteristics, timing of semen sampling after therapy and sperm quality parameters were evaluated using Spearmans-signed rank coefficient of correlation. Differences in classic semen analysis and SCSA variables between patients groups and in comparison with proven fertile controls were assessed with the Mann-Whitney U-test. All results are expressed as median with range. A p-value of <0.05 was considered statistically significant.

RESULTS

Pre-treatment

Pre-treatment semen analysis results and sperm DFI for patients with various types of cancer are depicted in Table 1.

In TGCT patients, 41 non-seminomas and 11 seminomas were diagnosed. No significant differences were found in semen variables or DFI between the two histological TGCT types. In the TGCT patients with a poor or intermediate prognosis (n=7), total sperm count and sperm concentration were significantly lower compared with TGCT patients with a good prognosis, n=45, (8.2 x 10^6 (2.2-105.6) versus 50 x 10^6 (1.9-898.0) p=0.040 and 4.9 x 10^6/ml (0.5-33.0) versus 16.0 x 10^6/ml (1.0-749.0) p=0.049 respectively). Higher DFI values were found in the intermediate/poor prognosis patients (25.4% (12.1-42.0)) compared with patients with good prognosis (13.6% (4.0-65.3)), but the difference was not statistically significant (p=0.086). Serum AFP, ßHCG and LDH at diagnosis were 9.0 ug/l (1.0-12264.0), 4.7 IU/l (0.2-88915.0) and 342.0 U/l (146.0-3698.0), respectively. Both total sperm count and sperm concentration were significantly negatively correlated with serum ßHCG at diagnosis (r=-.286, p=0.049 and r=-.306, p=0.034, respectively). Serum LDH level at diagnosis was significantly correlated with DFI (r=0.354, p=0.017).

In HL patients stage I/II disease was diagnosed in 24 patients, whereas 7 men were diagnosed with stage III/IV disease. In the NHL patient group, stage I/II was found in 10

Table 1. Semen parameters and sperm DNA fragmentation index (DFI) at time of cancer diagnosis, before treatment.

	TGCT	HL	NHL	Leukemia	Sarcoma	Other
N=	52	31	15	8	2	19
Sperm count (x10^6)	41.2 (1.9-898.0)	49.4 (0.6-915.0)	110.4 (18.5-430.0)	96.0 (6.0-302.4)	12.6 (5.4-19.7)	83.2 (8.6-1282.6)
Sperm concentration (x10^6/ml)	13.5 (0.5-749.0)	26.0 (0.1-327.0)	41.0 (6.6-237.0)	40.0 (1.5-176.0)	5.5 (2.7-8.2)	42.0 (4.3-283.0)
Progressive motility (%)	34.0 (8.0-68.0)	42.0 (13.0-68.0)	32.0 (10.0-70.0)	29.5 (21.0-60.0)	44.5 (39.0-50.0)	42.0 (12.0-84.0)
Normal morphology (%)	5.0 (0-21.0)	6.0 (1.0-16.0)	4.0 (0-10.0)	5.5 (4.0-21.0)	5.0 (5.0-5.0)	6.0 (1.0-12.0)
DFI (%)	14.0 (4.8-65.3)	13.5 (3.1-52.6)	25.1 (8.7-66.7)	17.7 (5.6-61.1)	15.7 (9.1-22.3)	11.1 (3.8-58.7)

All values are median (range). DFI in NHL patients was significantly higher compared to all other cancer diagnosis groups. In TGCT patients sperm concentration was significantly lower compared to other diagnosis groups, except sarcoma patients. Higher percentage progressive sperm motility seen in HD patients compared to NHL (p=0.041) and TGCT patients (p=0.028). TGCT=testicular germ cell tumours, HL=Hodgkin's lymphoma and NHL=Non-Hodgkin's lymphoma, Other= divers malignancies like astrocytoma, rectal cancer etc.

patients and 5 patients were diagnosed with stage III/IV disease. For both HL and NHL patients, no association between disease stage and pretreatment semen parameters or DFI was observed.

Post-treatment

In 73 patients, no follow-up data were available because no spermatozoa were available due to death, azoospermia, anorchia or retrograde ejaculation (n=43) or semen analysis could not be obtained (n=30). When compared with the 52 patients in whom follow-up data were available for analysis, NHL, leukaemia and high-dose treatment were over-represented in the former group. Median follow-up time was 1.1 years (0.5-3.3) since semen cryopreservation and 0.8 years (0.2-3.1) since the last oncological treatment. DFI levels were not significantly correlated with the time since the last treatment to semen sampling. Overall, total sperm count and sperm concentration decreased significantly at follow-up (from 80.1 x 10^6 (2.2-915.6) to 22.9 x 10^6 (0.1-612.0) $p<0.001$ and from 30.0 x 10^6/ml (0.5-327.0) to 9.9 x 10^6/ml (0.1-102.0) $p<0.001$, respectively). Progressive motility and normal morphology were not significantly different after treatment. A significant decrease in DFI following treatment in the population with available follow up was found (from 17.1% (5.1-66.7) to 13.2% (5.0-70.5) $p=0.040$).

The three largest patient categories, TGCT, HL and NHL, were analysed separately.

Of the 25 TGCT patients with available follow-up data, 17 patients were treated with 3-4x BEP alone, whereas eight patients were treated with either a combination of 3-4x BEP and 13-25 fractions of 2.0 Gy radiotherapy (RT) (n=3) or RT alone (n=5) on the retroperitoneum. DFI at follow-up was significantly higher in patients who were treated with RT or a combination of CT and RT (16.9% (11.5-39.9)) compared with patients treated with BEP CT alone (10.9% (5.5-37.1) $p=0.037$). In the whole TGCT group, DFI decreased from 15.1% (6.7-52.0) to 12.0% (5.5-39.9) upon treatment but the difference was not statistically significant ($p=0.061$).

In the 15 patients with HL in whom follow-up semen analysis was available, two patients were treated with ABVD only, and 13 patients were treated with 15-20 fractions of 2.0 Gy RT in addition to ABVD. RT was administered to the groin in one patient, whereas all other RT was localised above the diaphragm. Only sperm concentration was shown to decrease significantly following therapy from 77.0 x 10^6/ml (11.0-327) to 40.0 x 10^6/ml (5.9-102.0) ($p=0.041$). The other sperm characteristics and the DFI did not change significantly following treatment. Post-treatment DFI was not significantly different in patients who were treated with CT alone or a combination of CT and RT. The number of CT cycles was not correlated with the DFI at follow-up.

In the NHL patient category, three patients were treated with a combination of 6x CHOP CT and 20 fractions 2 Gy RT and 2 patients received only high dose CT (either 8x

Table 2. Pre- and post-treatment sperm DNA fragmentation index (DFI) in patients in whom follow-up data were available for analysis and fertile controls.

diagnosis	N	DFI pre-treatment <30%	DFI pre-treatment >30%	N	DFI post-treatment <30%	DFI post-treatment >30%
TCGT	52	41 (79%)	11 (21%)	25	22 (88%)	3 (12%)
HD	31	22 (71%)	9 (29%)	15	14 (93%)	1 (7%)
NHL	15	10 (67%)	5 (33%)	5	5 (100%)	0 (0%)

TGCT=testicular germ cell tumours, HL=Hodgkin's lymphoma and NHL=Non-Hodgkin's lymphoma.

CHOP or 6x CHOP followed by third line CT). Because of the small number of patients, no statistical analysis was performed.

In TGCT, HL and NHL groups, the number of patients with pre- and post-treatment DFI levels above 30% were calculated and depicted in Table 2.

DFI in cancer patients versus controls

Overall, both pre- (17.1% 5.1-66.7) and post-treatment (13.2% 5.0-70.5) DFI levels in cancer patients were not significantly different from that of proven fertile controls (15.3% 6.4-25.7) (Figure 2). No statistical differences in DFI between proven fertile controls and TGCT patients, before or after treatment, were found. The same holds true for HL patients. In NHL patients, however, pretreatment DFI was significantly higher compared with proven fertile controls (p=0.008 (Figure 2)).

DISCUSSION

We determined pretreatment DFI levels in 127 cancer patients and compared this with the results obtained for proven fertile controls. Sperm DNA integrity was found to be compromised in NHL patients but was not significantly changed in patients diagnosed with TGCT or HL. This finding is in agreement with a recent study by Ribeiro et al who found that DNA fragmentation was not significantly increased in 48 TGCT patients compared with proven fertile controls (Ribeiro et al. 2008). In contrast, several other studies have reported increased sperm DNA damage in pretreatment sperm samples of patients with TGCT and HL compared with controls (Meseguer et al. 2008; O'Donovan 2005; O'Flaherty et al. 2008; Spermon et al. 2006; Stahl et al. 2008). This discrepancy may have been caused by low numbers of patients studied, selection bias in patients who were offered semen cryopreservation or selection bias of controls. To our knowledge, our study cohort contains the largest number of patients in whom pretreatment DFI was assessed. All patients who were offered semen cryopreservation were eligible for study inclusion. Consequently, our cohort consisted of patients with normal prevalence and variation in severity of cancer stage and prognosis. It should be emphasized that at clini-

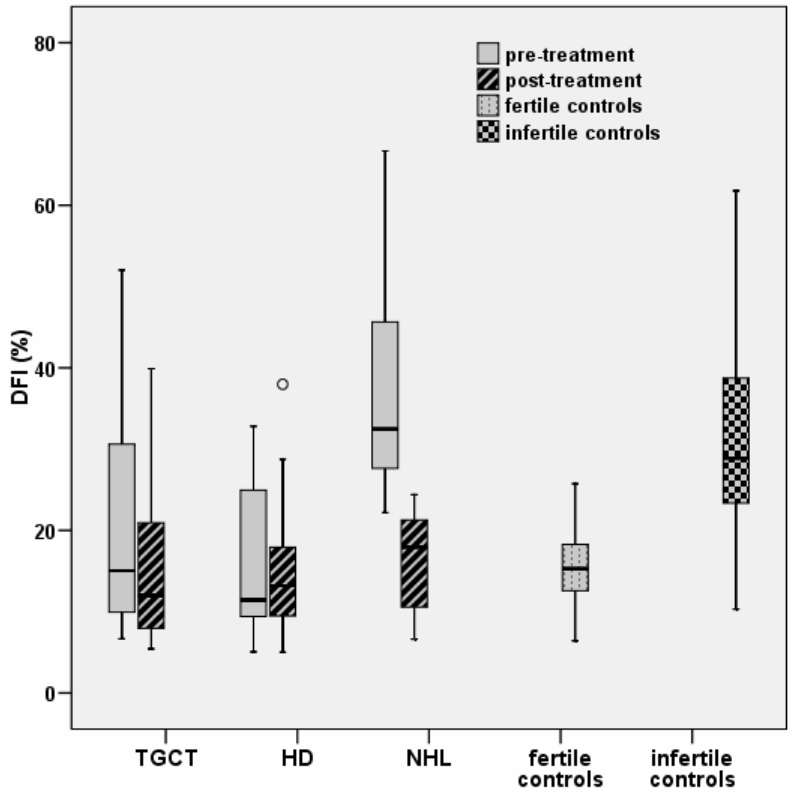

Figure 2 Pre- and post-treatment DFI values in the 3 most prevalent cancer diagnosis groups and 22 fertile and 22 infertile controls.
Boxes show the mean DNA fragmentation index (DFI) with standard deviation, whiskers show the range of non-outlier DFI values, ○ represents an outlier.

cal presentation, patients with poor prognosis and high-stage disease are the minority. We used proven fertile controls but acknowledge that population-based controls are the most appropriate controls because proven fertile controls may have lower DFI levels than men on average.

Our results are in agreement with previous findings that pretreatment sperm concentration in TGCT patients is significantly lower compared with that of HL and NHL patients and that there is no interrelationship between the TGCT histological type and pretreatment semen quality (Gandini et al. 2003; van Casteren et al. 2009). More severe testicular cancer, indicated as intermediate or poor prognosis, is associated with decreased sperm quality and slightly increased DFI values, although the latter did not reach statistical significance. Our data confirm the previous observation by Gandini et al that pathological ßHCG is correlated with abnormalities in sperm quality in TGCT patients (Gandini et al. 2003). In addition, we found a significant correlation between serum LDH at the time of TGCT diagnosis and pretreatment DFI that may reflect a positive association between

the severity of the TGCT and sperm DNA damage. For HL and NHL, we did not find an association between disease stage and sperm quality or sperm DNA integrity.

Literature reports describing sperm DNA integrity of patients at the time of cancer diagnosis and following treatment are scarce (O'Donovan 2005; Spermon et al. 2006; Stahl et al. 2008; Stahl et al. 2004, 2006). Several studies report improvement of the chromatin structure following CT, although some authors report abnormal post-treatment sperm DNA damage levels compared with healthy controls (O'Donovan 2005; Spermon et al. 2006; Stahl et al. 2006). It has been suggested that spermatogonia with abnormal chromatin might be more susceptible to CT and are thus eliminated by the treatment. Remaining normal spermatogonia are responsible for the restoration of spermatogenesis after a recovery time (Spermon et al. 2006). Our observation that the time since the last oncological treatment was not associated with post-treatment DFI levels in our study, may further indicate that normal spermatogonia with normal chromatin structure are responsible for the recovery of spermatogenesis. Studies among childhood cancer survivors indicate that the sperm DNA integrity in men with recovered spermatogenesis is not different compared with healthy, fertile controls (Thomson et al. 2002; van Beek et al. 2007). Fertility status follow-up after cancer treatment can be troublesome due to variable recovery rates of spermatogenesis and anorchia or retrograde ejaculation due to oncological treatment. Our study results were further influenced by selection bias because a significant number of patients were lost to follow-up and the majority of patients included were categorized as good prognosis or low grade. Despite these shortcomings, we were able to analyse the impact of cancer and its treatment on the sperm DNA integrity in as many as 52 patients. In the present study, we observed a significant decrease of DFI levels following oncological therapy. As shown before, irradiation therapy of TGCT patients resulted in more sperm DNA damage than did CT of TGCT patients (Stahl et al. 2006). It is presumed that sperm DNA damage can be induced by direct gonadal irradiation or by radiation scatter during treatment despite shielding of the gonads. In HL patients, however, irradiation therapy was not associated with increased post-treatment DFI in our study.

It is common practice to recommend patients to postpone conception for 6-12 months after therapy to bypass possible negative effects of radiation and chemotherapy such as single gene mutations and chromosomal translocations in spermatogonia (Meistrich 1993). A longer period up to 24 months has been suggested by others because of the persistence of increased aneuploidy after CT (De Mas et al. 2001). Our results support previous reports that sperm DNA damage is significantly reduced in post-treatment semen samples compared with pretreatment samples after a median follow-up time of 1.1 year. Moreover, we found that the presence of cancer does not seem to negatively affect the sperm DNA integrity in TGCT and HL patients, whereas NHL patients showed increased DFI at the time of diagnosis and compared with proven fertile controls. On

the basis of our results and a review of the literature, RT in TGCT patients may have a more profound negative impact on the sperm DNA integrity resulting in increased DFI levels, persisting 1-2 years following radiation treatment, than CT alone (Stahl et al. 2006). Finally, we have shown that about one-third of TGCT, HL and NHL patients have increased DFI levels above 30% at the time of diagnosis. Following treatment only 0-12% of these patients have increased DFI levels.

DFI levels above 30% are associated with significantly lower pregnancy chances in vivo (Evenson et al. 1999), which can be overcome by use of IVF/ICSI (Bungum et al. 2007). Moreover, apart from diminished in vivo fertilizing potential in patients with high DFI levels, it is unclear if high DFI's in patients who achieve pregnancies have a negative impact on the offspring. One could argue that SCSA may be unable to assess the subtle changes associated with the cancer- or therapy-induced damage of the paternal genome and that it is therefore unjustified to conclude that the use of spermatozoa of cancer survivors with low DFI in IVF/ICSI is safe. Further, larger studies that evaluate aneuploidy rate, genomic stability and IVF/ICSI offspring follow-up will be needed to further address the safety and timing issues of the use of spermatozoa in IVF/ICSI in cancer survivors formerly exposed to cancer treatment.

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CHAPTER 9

General discussion and Summary

This thesis deals with the validation of the Sperm Chromatin Structure Assay (SCSA), the evaluation of the additional value of SCSA compared to conventional (surrogate) sperm quality parameters, the discriminative value of SCSA for the diagnosis of the infertile male and the association between SCSA and outcome parameters. Various possible clinical applications of the SCSA as a prognostic tool in the management and treatment of Andrology patients are investigated in the context of prospective controlled clinical trials. In this general discussion we will summarize and discuss our results in view of the literature.

1. ASSAY VALIDATION OF SCSA

1.1 Evaluation of SCSA as a laboratory test

A diagnostic test can be designated to be a valuable clinical tool if the results are reproducible and consistent over time. The SCSA is a robust test by which sperm DNA damage can be determined objectively (Evenson et al. 2000) and with minimal interassay variability (Giwercman et al. 2003). In our laboratory, the interassay coefficient of variation is acceptably low at less then 5%. Although the biological variation of SCSA parameters is markedly less than in classic semen parameters, we have shown that SCSA parameters are indeed subject to biological variation over time (**chapter 2**). We found that substantial biological variation of the SCSA parameters DNA fragmentation index (DFI) and high DNA stainability (HDS) occurred in about half of the Andrology patients examined. Smoking, the presence of varicocele and exposure to gonadotoxins were not associated with the biological variation of the DFI and HDS.

Our findings suggest that unknown, perhaps predominantly post-testicular, factors influence the biological variation of sperm DNA fragmentation as measured by SCSA (**chapter 2**).

1.2. Correlation with conventional semen parameters

SCSA has been advanced as an independent predictor of fertilizing potential because only weak to moderate correlations between SCSA and classic semen parameters were previously demonstrated (Giwercman et al. 2003; Spano et al. 1998). In the couples attending our Andrology clinic, midlevel correlations between the DFI and sperm count, concentration, progressive motility and normal morphology were found (**chapter 3**). The observation by Moskovstev et al (Moskovtsev et al. 2009b) that the degree of DNA damage increases with the number of abnormal parameters in a sample, coincides with our observation that the TZI (that is: the number of morphological abnormalities per abnormal spermatozoa) is significantly correlated with the DFI (**chapter 3**).

We conclude that sperm DNA damage assessed by the SCSA provides additional information to WHO semen parameters.

2. CLINICAL VALIDATION OF SCSA

2.1. Sperm DNA damage in normozoospermic men

Increased DFI in patients with normozoospermia may reveal the reason for "unexplained" infertility in these couples. In **chapter 3**, we have demonstrated that 10% of normozoospermic men attending our Andrology clinic have DFI levels above 30%. These results are consistent with previous studies that reported high DFI levels in normozoospermic patients in 5-8% of cases (Erenpreiss et al. 2008; Zini et al. 2002), but in contrast to a large study where DFI >30% was found in only 0.5% of patients with otherwise normal semen parameters (Moskovtsev et al. 2009b). Selection bias and differences in severity of infertility in patients attending different Andrology clinics may have caused these contradictory results.

We further explored the complimentary value of SCSA by evaluating DFI in proven fertile men. In several of the studies reported in this thesis, we used 22 proven fertile men who donated semen prior to vasectomy as controls. Although the range of DFI in these men was high (median 15.3%, range 6.4-25.7%), DFI levels did not exceed the 30% fertility threshold. In our study that evaluated the association of B-vitamins in seminal plasma and DFI we defined a proven fertile subpopulation of men who had fathered children spontaneously or were previously treated successfully with ART (**chapter 4**). In this subgroup of patients, the median DFI was 19.6% with a range of 3.7-74.8. It should be noted that this selection was biased, as it was taken from couples attending our infertility clinic who were treated with in vitro fertilization/ intracytoplasmatic sperm injection (IVF/ICSI).

Based on our results, we conclude that routine application of SCSA testing with the purpose to diagnose unexplained infertility in men with normozoospermia remains controversial and may not be efficient because of the low prevalence of abnormal DFI in these men and the high range of DFI in proven fertile males. On the other hand, SCSA testing in normozoospermic sperm samples can be justified in clinical setting in cases of infertility in the absence of female factors because it may provide complimentary information in addition to classic semen analysis.

2.2. SCSA as a diagnostic tool for the evaluation of infertile males

It is generally accepted that male infertility is linked to decreased sperm DNA integrity. Common entities diagnosed in infertile males, such as varicocele and cryptorchidism, are associated with sperm DNA damage (Saleh et al. 2003; Sousa et al. 2009). We have shown that infertile men categorized by diagnosis display a wide range of DFI. Moreover, DFI levels overlap the different male infertility diagnoses. We therefore conclude that SCSA parameters provide additional information to classic semen analysis but are not sufficient to differentiate in male infertility diagnoses as a sole test (**chapter 3**). Our findings are confirmed in recent reports (Cohen-Bacrie et al. 2009; Moskovtsev et al. 2009b).

We have shown that the quality of spermatogenesis, based on the complete andrological work up of patients, rather than the diagnostic classification seems to be associated with the DFI (**chapter 3**). This finding may explain the substantial overlap of DFI in Andrology patients with various diagnoses and may account for the low discriminative power of SCSA in the diagnosis of infertile patients.

Recently Aitken et al. proposed a two step hypothesis for the origin of sperm DNA damage. The first step has its origin in defective spermiogenesis where decreased sperm production and impaired chromatin remodelling create a state of vulnerability, whereby the spermatozoa become more susceptible to oxidative damage. In the second step, the chromatin is attacked by reactive oxygen species (ROS) that is mainly originated from the mitochondria of the poorly remodelled, dysmature spermatozoa themselves. This theory might explain why sperm concentration, motility, morphology and DNA damage are so often correlated (Aitken et al. 2009). A different perspective is discussed in a review by Oliva. Oxidative stress may negatively influence spermatogenesis itself through targeting proteins responsible for chromatin remodelling. Oxidative stress may either negatively affect the structure of protamines or their binding to DNA (Oliva 2006).

In conclusion, all three plausible theories about the origin of sperm DNA damage like compromised chromatin remodelling, oxidative stress and abortive apoptosis (**chapter 1**) are linked, but their exact relationship remains unclear. The study described in **chapter 3** provides clinical evidence for the hypothesis that defective spermatogenesis in general, irrespective of its aetiology, increases sperm susceptibility to chromatin abnormalities and fragmentation.

2.3. Effect of possible confounders

Every human sperm sample contains leukocytes. ROS generation in semen depleted of its anti-oxidant components is significantly correlated with seminal leukocyte concentration (Aitken et al. 1995). However, leukocytospermia alone may be an insufficient parameter to evaluate the association between ROS and sperm DNA fragmentation. The capability of leukocytes to induce oxidative stress with consequent sperm DNA damage probably depends on their origin and the antioxidant capacity of an individual. Henkel et al suggested that oxidative stress caused by testicular/epididymal inflammation may trigger protamination or maturation problems in underprotaminated sperm and consequent lead to sperm DNA fragmentation (Henkel et al. 2010). In a clinical setting, treatment of male accessory gland infection is recommended as it may increase sperm quality and enhance a couple's chance of natural conception (Dohle et al. 2005). In our experience, the effect of such treatment is often limited. So far, only one study reported a decrease in DFI in patients with bacteriospermia in response to antibiotic treatment (Moskovtsev et al. 2009a).

We have shown that in an Andrology population, the correlation between stressors that may lead to ROS mediated sperm DNA damage (such as smoking and leukocytospermia) and DFI is not significant (**chapter 3**). One can argue that in an infertile population the additional negative effect of ROS mediated risk factors on the sperm DNA integrity may not be profound because other causes of sperm DNA damage that lead to defective spermatogenesis are predominant.

It is widely acknowledged that oxidative stress is associated with sperm DNA damage seen in male infertility, making the assumption that antioxidant treatment can improve male fertilizing potential plausible. Several studies aimed to investigate if antioxidant suppletion may reduce the levels of sperm DNA damage in infertile men (Greco et al. 2005a; Moskovtsev et al. 2009a), but to date, no large randomized controlled trials have been conducted to test this hypothesis.

We observed a significant positive correlation between male age, body mass index (BMI) and DFI in infertile populations (**chapter 3**). It is generally accepted that sperm DNA damage increases with advancing male age (Moskovtsev et al. 2006; Schmid et al. 2007). We confirm other studies that the significant positive correlation between male age and DFI also applies for infertile study populations (Wyrobek et al. 2006). In an era where the age of first time fathers is well beyond 30 in Western societies, this finding may have implication for the pre-conceptional counselling in first pregnancy planners.

Our results are in agreement with a study by Kort el al who demonstrated a positive correlation between sperm DNA fragmentation and BMI (Kort et al. 2006). Excess body fat, genetic and endocrinologic factors may contribute to the observed correlation. To our knowledge, no studies have been published evaluating if weight reduction improves male fertility and leads to improvement of the sperm chromatin structure.

In conclusion, in men attending an Andrology outpatient clinic, sperm DNA damage assessed by SCSA is associated with male age and BMI. No significant correlation was found between DFI and smoking or leukocytospermia. Future studies are needed to evaluate intervention related changes.

2.4. SCSA associated with the outcome parameter pregnancy

In the recently published 5th edition of the World Health Organization (WHO) manual, reference values for classic semen parameters are reported, based on data from 1800 recent fathers in 15 countries on 3 continents (WHO 2010). Semen analysis data are indicative of a man's fecundity, but the discriminative power of classic semen parameters is low, with a substantial overlap among men who do or do not achieve pregnancy (Guzick et al. 2001). The opposed major clinical advantage of SCSA is the reported decreased chance of spontaneous pregnancy when the DFI exceeds 20-30% in the general population that is further reduced to nil if the DFI is more than 30% (Evenson et al. 1999; Spano et al. 2000). Similar results were demonstrated in intra uterine insemination (IUI)

programmes (Bungum et al. 2007). Giwercman et al recently reported increased odds ratio for infertility in patients with one or no abnormal standard sperm parameter when the DFI exceeded 20% in a case-control study among 127 infertile and 137 proven fertile men (Giwercman et al. 2010). They suggested that SCSA adds to the value of classic semen parameters in prediction of the chance of natural conception especially in men with normozoospermia. They propose that optimizing female fertility might be considered if DFI <10%, even in the presence of one abnormal semen parameter. Referral for assisted reproduction technique (ART) should be considered when the DFI is high (Giwercman et al. 2010).

The clinical value of sperm DNA testing in unselected or infertile populations remains controversial. In response to the most frequently cited study with first pregnancy planners by Evenson (Evenson et al. 1999), it was recently opposed that the sensitivity of the SCSA is low. In 165 first pregnancy planners who did not achieve pregnancy within one year, only 6.2% had DFI levels above 30%. Out of all patients examined with abnormal SCSA results (e.g. a DFI >30%), pregnancy still occurred in 40% (Zini et al. 2009).

One of the objectives of our studies described in **chapter 5 and 6** was to evaluate the association between postoperative DFI following surgical intervention and spontaneous pregnancy. The mean postoperative DFI in varicocelectomy and vasectomy reversal patients who achieved spontaneous pregnancy was 30% and 33% respectively. In both studies the DFI was high in couples who achieved spontaneous pregnancy, considering the reported nil change of spontaneous pregnancy when DFI exceeds 30% (Evenson et al. 1999; Spano et al. 2000). Both our study among varicocele patients (**chapter 5**) and vasectomy reversal patients (**chapter 6**) indirectly indicate that the 30% DFI fertility threshold for spontaneous pregnancies may be questionable, at least in couples with females with normal fertility.

These conclusions and the considerations discussed above may implicate that the clinical usefulness of SCSA as a prognostic tool for in vivo pregnancy may be less than initially reported. In the following paragraphs we will discuss two possible explanations.

As we discussed in the general introduction, all currently available assays measure single and or double DNA strand breaks at random location in the genome. The biological consequences of randomly induced DNA damage can be highly variable, depending on the location. It seems reasonable to assume that DNA damage in those areas relevant for conception and embryo development has clinical significance. On the other hand, damage of untranscribed sequences such as intron DNA (comprising more than 90% of total DNA) may only be architectural in nature and will probably have little if any implication for sperm function. Moreover, in the general introduction we discussed that sperm DNA nicking occurs as a physiological process during the winding and unwinding of DNA in preparation of protamination. Current sperm DNA fragmentation assays do not differentiate physiological from pathological nicking.

Several methods are currently used to detect sperm DNA fragmentation (**chapter 1**). It can be questioned whether (additional) DNA damage is induced as a result of the sample preparation procedures. The treatments used to prepare semen samples may induce DNA damage. For the SCSA, the red emitted fluorescence may not be limited to pre-existing DNA damage. It has been stated that the susceptibility to denaturation by acid treatment correlated with sperm DNA fragmentation, but it is unclear whether all chromatin is equally accessible to acid denaturation. During ejaculation normal spermatozoa are suspended in zinc-rich prostate fluid and the zinc-chelating seminal vesicular fluid. It is known that this environment has a marked influence on chromatin stability (Bjorndahl et al. 2003).

In conclusion, sperm DNA fragmentation was shown to provide prognostic information for spontaneous pregnancy rate in initial studies. We have demonstrated that in couples attending an Andrology clinic the proposed 30% DFI threshold for spontaneous pregnancy may be questionable. High levels of sperm DNA damage are certainly a negative trait, but an absolute threshold for failure to achieve a spontaneous pregnancy is not established.

3. INTERVENTION RELATED EVALUATION OF SCSA

3.1. The influence of nutritional status on sperm DNA integrity

The folate/homocysteine pathway is responsible for the generation of methyl donors and is therefore central to the process of DNA methylation for all cells. In general, hypomethylation of DNA is associated with gene transcriptional activity, while hypermethylation is associated with gene silencing. DNA methylation is considered as a major mechanism in epigenetic processes involved in reproduction to control gene expression and imprinting. Several studies indicate that DNA methylation is altered in imprinted genes, oligozoospermic men and men with improper histone to protamine replacement (Carrell et al. 2010). Folate shortage increases DNA fragility and may lead to increased susceptibility to DNA damage since the protective mechanism of DNA methylation is impaired (Blount et al. 1997; Koury et al. 1997). Tavalaee et al recently observed a significant negative correlation between DFI and sperm DNA methylation (Tavalaee et al. 2009). A recent study by Tunc et al suggested that oxidative damage to sperm DNA integrity inhibits methylation, rather than abnormalities in the methyl donor pathway, because sperm DNA methylation was significantly related to seminal ROS production but not to serum homocysteine concentrations. Interestingly, they observed a significant decrease in ROS and sperm DNA fragmentation after 3 months of antioxidant treatment, while sperm DNA methylation increased (Tunc et al. 2009).

In **chapter 4** we describe the results of the FOLFO study designed to prospectively evaluate the influence of pre-conceptional nutrition and lifestyle on fertility and pregnancy outcome in subfertile couples undergoing IVF with or without ICSI. Here we report the novel finding that low folate concentration in seminal plasma is associated with increased sperm DNA damage in fertile men.

The discussed observations and our study results require confirmation in larger series. Basic studies are needed to establish the role of methylation defects in the development and growth of ART offspring (Carrell et al. 2010).

In the same study population, Vujkovic et al showed that a health conscious diet, consisting of high intakes of fruit, vegetables, fish and whole grains, is inversely associated with DFI after adjustment for male age, BMI, smoking, vitamin use and the presence of a varicocele (Vujkovic et al. 2009).

These important findings are the first evidence that nutritional status and folate concentration in seminal plasma are associated with sperm DNA fragmentation assessed by SCSA. It seems logical to encourage patients to amend lifestyle factors such as smoking, obesity and malnutrition, but so far no studies have been published to show that a decrease in sperm DNA damage can indeed be achieved. SCSA may be a useful tool to evaluate the effect of nutrition intervention on the sperm chromatin structure in future studies. It would be an important step forward if future research can show that correction of lifestyle factors can improve a couple's chance of spontaneous or ART based pregnancy.

3.2. DFI assessment following surgical intervention

Varicocele is a common abnormality found in 25% of infertile patients. Varicoceles may present heterogeneously and coincide with other fertility related effects (Marmar 2001; WHO 1992). Varicocelectomy remains a controversial treatment for infertility because surgery may not increase spontaneous pregnancy rate and not all patients show improvement in semen parameters following surgery. Some authors suggest that the postoperatively increased sperm concentration and motility could also have occurred without treatment and may be the result of the biological variation of semen parameters over time (Evers et al. 2003). Although randomized controlled trials are scarce, two recent meta-analyses demonstrated that surgical varicocele repair in infertile men with palpable varicocele and abnormal pre-operative semen parameters increases the odds ratio of spontaneous pregnancies compared with infertile men with a varicocele who were not treated (Ficarra et al. 2006; Marmar et al. 2007). Although the pathophysiology of male infertility due to varicocele is still not clear, it has been reported that sperm DNA damage is associated with varicoceles (Saleh et al. 2003). Patients with a varicocele express more apoptotic markers than fertile controls (El-Domyati et al. 2008) and have

significantly higher ROS concentrations compared to controls (Agarwal et al. 2006) which can be significantly reduced following varicocelectomy (Chen et al. 2008).

We previously showed that the presence of a varicocele is not associated with the biological variation of DFI, the biological variation is still markedly less than conventional semen parameters and is not correlated with clinical features of infertile men (**chapter 2**). As discussed previously, earlier studies indicated that SCSA parameters are prognostic for spontaneous pregnancy. These considerations led us to the conclusion that SCSA may be used to evaluate the effect of interventions like varicocelectomy or chemotherapy on the sperm DNA integrity in Andrology patients.

In **chapter 5** we confirm that sperm DNA damage can be significantly decreased following varicocelectomy. This finding provides evidence that varicocele induced changes to the chromatin structure can be reversed by varicocelectomy. The significant decrease in DFI following varicocele repair indicates that surgery can restore spermatogenesis in most patients. However, DFI could not predict positive response to varicocele repair. The contribution of unknown underlying genetic or epigenetic causes of infertility may explain the heterogeneous response to treatment of patients with a varicocele. It can also be suggested that in some patients the negative effect of the varicocele on spermatogenesis is too profound to be reversed by varicocele repair.

Furthermore, our results provide evidence for the therapeutic effect of varicocelectomy because lower postoperative DFI was associated with higher pregnancy rate, both spontaneous and following ART.

In our study among vasectomy reversal patients DFI was not associated with pregnancy rate and high DFI levels in postoperative semen samples did not exclude spontaneous pregnancies (**chapter 6**). This may be explained by the large numbers of dead cells in the semen samples of these patients, which is possibly due to remnant cell debris from the formerly obstructed epididymis or to an inflammatory component after obstruction caused by vasectomy. Non-vital spermatozoa contain fragmented DNA that translates into high DFI levels. The predictive value for spontaneous pregnancy in vasectomy reversal patients of DFI in one postoperative semen sample may therefore be biased as dead cells and debris are washed out once vas deferens continuity is repaired. On the other hand, we demonstrated the lack of association between DFI and vasectomy reversal to semen analysis interval.

We conclude that high DFI may be characteristic for vasectomy reversal patients. It is unlikely that high DNA damage following vasectomy reversal is solely caused by nonviable sperm that need to be washed out in the months following vasectomy. High DFI levels in these patients may be caused by an ongoing ROS mediated inflammation reaction in the genital tract that may also account for the relatively lower pregnancy rate after reversal surgery compared to the general population (Kolettis et al. 1999).

3.3. Considering the use of SCSA in ICSI

The initial enthusiasm about the existence of an upper DNA fragmentation threshold for the outcome of ART (Larson et al. 2000; Lopes et al. 1998) cooled down as more investigations were published. Several of these studies showed that healthy pregnancies could be obtained with IVF/ICSI in men with high levels of damaged sperm DNA (Gandini et al. 2004; Lin et al. 2008; Payne et al. 2005). Bungum et al published the largest series thus far and concluded that the chance of pregnancy after IUI was significantly higher in patients with a DFI <27%, but no statistical difference in the outcome of IVF/ICSI was shown if the same threshold was applied (Bungum et al. 2007). Recent meta-analyses suggest that sperm DNA damage has only a modest impact on IVF pregnancy rates and the predictive power of SCSA seems to lose its strength in the order, natural conception, IUI, IVF to ICSI (Collins et al. 2008; Zini et al. 2009).

In a well documented study population with severe male infertility we confirm that DFI is not associated with ICSI outcome (**chapter 7**).

The absence of an association between DFI and ICSI outcome lies primarily in the use of one single sperm per injected oocyte and the fact that SCSA refers to a DNA status profile in a whole ejaculate. A major drawback of all currently available assays is that the sperm is destroyed during the process of sperm DNA assessment. A sperm DNA quality profile of a native sample or processed sample can be made, but an individual cell with optimal sperm chromatin structure that can be used in IVF/ICSI cannot be assessed. In future studies methods that can assess the chromatin structure in a single cell, such as micro-Raman spectroscopy, may provide essential information about the efficiency of DNA packaging in morphologically normal spermatozoa (Huser et al. 2009).

In spontaneous pregnancies and IUI usually one oocyte is available for conception. In these cases, the level of sperm DNA fragmentation is more likely to have predictive value, compared with IVF/ICSI in which the probability of the formation of a normal embryo is in part dependent on the number of metaphase II oocytes obtained after stimulation. In addition, the quality of these oocytes and their ability to repair DNA damage in the fertilizing spermatozoon further impacts on the predictive value of sperm DNA fragmentation in IVF/ICSI. The extent of sperm DNA damage per sperm cell and the amount that can be dealt with by the oocyte is unknown. The mouse oocyte has the capacity to repair sperm DNA damage (Derijck et al. 2008). In humans, the same applies, but this capacity is thought to be limited and may vary between oocytes and is dependent upon women's age. In general, single strand DNA breaks are easier to repair than double strand breaks. Current available tests cannot provide information concerning the "repair ability" of DNA damage.

A matter of concern is the detrimental effect of density gradient centrifugation on the sperm DNA integrity in patients with severe oligoasthenoteratozoospermia (**chapter 7**). In 40% of patients an increase in the DFI was found following sperm preparation.

Our finding is in contrast with earlier studies that concluded that sperm processing techniques like gradient centrifugation and swim up enhance the sperm DNA integrity (Donnelly et al. 2000; Gandini et al. 2004; Spano et al. 1999), although Zini et al previously demonstrated that sperm preparation techniques can increase sperm DNA damage in poor quality semen samples (Zini et al. 1999). The recent observation that incubation of processed semen at room temperature after isolation by density gradient centrifugation may lead to an increase in the levels of sperm DNA fragmentation (Gosalvez et al. 2009) may explain our result. New sperm selection procedures that aim to recover spermatozoa with normal DNA integrity like the zeta potential sperm processing method and the hyaluronic acid sperm selection procedure are under development and may proof to enhance sperm selection for ART (Kheirollahi-Kouhestani et al. 2009).

It has been suggested that new markers for infertility, such as DFI, may facilitate the choice of treatment and help decide whether optimizing female fertility may give a couple hope of achieving a spontaneous pregnancy or whether referral for ART is necessary (Giwercman et al. 2010). Results from sperm DNA fragmentation analysis may help to direct towards the most suitable ART to use and save couples with high DFI the disappointment of embarking on an unsuccessful IUI or IVF treatment. Concerns have been raised that successful fertilization with damaged DNA may cause iatrogenic transmission of genetic and epigenetic abnormalities in ICSI offspring (Bonduelle et al. 2002). Moreover, damaged DNA, for instance caused by DNA oxidation, may cause misreading errors that may lead to de novo mutations. This theory is subscribed by a study by Ji et al who that found children of heavily smoking fathers have a higher risk of developing childhood cancer (Ji et al. 1997).

Injection of abnormal sperm DNA remains a matter of concern, and there is need for new tools for sperm selection. Selection of morphologically normal sperm using high magnification before injection may significantly improve pregnancy outcome (Bartoov et al. 2003). Because previous reports have shown that sperm DNA damage is significantly lower in the seminiferous tubules compared with the cauda epididymis (Steele et al. 1999) or ejaculated sperm (Greco et al. 2005b), the use of testicular sperm in couples with repeated pregnancy failure in ART with high sperm DNA fragmentation has been suggested. In a recent report pregnancy rates where significantly higher when using testicular sperm than ejaculated sperm with high sperm DNA damage and miscarriage rates were significantly lower in patients with repeated IVF failure without an apparent cause (Sakkas et al. 2010).

As a conclusion sperm DNA fragmentation testing may be beneficial in patients with repeated IVF/ICSI failure with apparent normal classic semen parameters. In these couples DFI assessment may have consequence for management decisions in the choice of ART and may give insight into the reason for previous failures.

3.4. Sperm DNA integrity in cancer patients

In patients diagnosed with cancer, cryopreservation of spermatozoa prior to cancer treatment enables patients to father a child in the future, even if fertility is destroyed by the treatment and not restored later in life. Little is known, however, about the reprotoxic safety of cryopreserved sperm.

We evaluated the DFI in cancer patients before treatment and found that the presence of cancer does not seem to negatively affect the sperm DNA integrity in testicular germ cell tumours (TGCT) and Hodgkin's Lymphoma (HL) patients; only non-Hodgkin's Lymphoma (NHL) patients showed increased DFI at the time of diagnosis compared with fertile controls. Following cancer treatment, the DFI significantly decreased. In contrast, radiotherapy in TGCT patients is associated with an increase in DFI compared with chemotherapy treatment alone. In HL patients, irradiation therapy was not associated with increased post-treatment DFI in our study (**chapter 8**).

Our results are in accordance with several studies that reported improvement of the chromatin structure following chemotherapy in cancer patients, although some authors report abnormal post-treatment sperm DNA damage levels compared with healthy controls (O'Donovan 2005; Spermon et al. 2006; Stahl et al. 2006). It has been suggested that spermatogonia with abnormal chromatin may be more susceptible to chemotherapy and are eliminated by the treatment. Remaining normal spermatogonia may be responsible for the restoration of spermatogenesis after a recovery time (Spermon et al. 2006).

As we discussed previously, our results should be interpreted with caution because SCSA may not be able to detect changes to the chromatin structure associated with cancer or treatment-induced damage. It is unjustified to conclude that the use of spermatozoa at the time of cancer diagnosis or following cancer treatment with low DFI is safe for use in IVF/ICSI. Future studies should determine the location of impact by chemotherapy and radiotherapy on the male genome and establish whether recovered spermatogenesis is free of transmittable induced effects.

CONCLUSIONS

Our studies show that SCSA is a validated test with a low inter/intraobserver variability, and with lower biological variation than classic semen analysis, that provides complimentary information about sperm quality. However, the extensive overlap between the DFI values found in fertile and infertile men, the low prevalence of abnormal DFI values in normozoospermic males and the non-discriminative value for andrological diagnosis prevent routine implementation of SCSA as a standard diagnostic tool at this moment. For selected patients, such as couples with unexplained infertility with normozoospermic males, SCSA may be indicative of the cause of decreased infertility.

In infertile men attending an Andrology clinic, sperm DNA fragmentation is not correlated to smoking habits or leukocytospermia. On the contrary, BMI and age are positively correlated with sperm DNA damage. There is evidence for an association between nutritional status, folate concentration in seminal plasma and DFI. In general, sperm DNA fragmentation seems to be a reflection of impaired spermatogenesis, independent of its aetiology. The origin of sperm DNA damage is most likely multi-factorial. Future studies will need to elucidate the role of confounders and contributing factors of abnormal sperm chromatin structure. Hopefully, this will lead to new treatment options for male infertility.

We have shown that sperm DNA fragmentation can be reduced following varicocelectomy and that lower DFI is associated with pregnancy rate in these couples. DFI was not associated with pregnancy rate in vasectomy reversal patients. In agreement with the present literature, sperm DNA fragmentation is not associated with ICSI outcome. We have shown that sperm DNA damage may be increased by sperm processing in severe OAT samples. Finally, following cancer treatment the sperm DNA integrity assessed by SCSA improved.

The clinical consequence of these observed decreases in sperm DNA damage remain unclear because high levels of DFI did not exclude pregnancies and the actual damage to the genome cannot be assessed by SCSA. From the data presented in this thesis and published elsewhere, we can conclude that sperm DNA damage is a negative trait that reduces the chance to father a child. However, an absolute upper threshold of DFI not compatible with pregnancy has not been established yet. DFI testing may not contribute to the initial management of couples attempting ART, as long as the association between DFI and the risk of iatrogenic transmission of genetic and epigenetic abnormalities in ICSI offspring remains to be elucidated.

We have shown that SCSA is a useful tool to investigate the association between possible confounders, etiological factors and sperm DNA damage. In clinical studies, SCSA can aid to evaluate the effect of surgical or chemical intervention on the sperm chromatin structure although the SCSA has some limitations, mainly due to the indirect method of analysing generalized sperm DNA damage.

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CHAPTER 10

Samenvatting

Infertiliteit, gedefinieerd als het uitblijven van spontane zwangerschap binnen 1 jaar, is een algemeen voorkomend probleem. Andrologie is het aandachtsgebied binnen de urologie waarbinnen de diagnose en therapie van mannen met vruchtbaarheidsstoornissen centraal staat.

Van oudsher richt onderzoek naar de mannelijke vruchtbaarheid zich op evaluatie van de sperma kwaliteit door middel van semen analyse. Klassieke semen parameters als zaadcel concentratie, motiliteit en morfologie hebben echter beperkte voorspellende waarde voor de kans op zwangerschap. Bovendien is semen analyse onderhevig aan hoge variabiliteit; zowel inter observer variatie als ten gevolge van biologische variatie tussen verschillende monsters van een patiënt. Er is behoefte aan nieuwe sperma functie markers. De mate van DNA schade in zaadcellen zou mogelijk van waarde kunnen zijn als diagnostische en prognostische maat voor mannelijke vruchtbaarheid. Het complex van DNA en DNA bindende eiwitten in zaadcellen wordt chromatine genoemd. Met de Sperm Chromatin Structure Assay (SCSA) kan door middel van een DNA bindende fluorescente stof schade aan het chromatine gemeten worden. De uitkomst maat van de test in een semen monster is de DNA fragmentatie index (DFI). Eerder onderzoek liet zien dat een DFI boven de 30% geassocieerd is met verminderde kans op spontane zwangerschap bij gezonde paren die voor het eerst zwanger probeerden te worden.

In dit proefschrift wordt de SCSA gevalideerd, zowel als test als in klinische studies. Om te beoordelen of de SCSA klinisch toepasbaar is in de dagelijkse Andrologie praktijk, evalueerden wij factoren die de test kunnen beïnvloeden, de associatie van de DFI met zwangerschap en de waarde van de test om het effect van vruchtbaarheidsbevorderende interventies te beoordelen.

Hoofdstuk 1 is een algemene introductie waarin oorzaken van DNA schade in zaadcellen worden besproken. We introduceren en vergelijken de verschillende testen waarmee afwijkingen aan de chromatine structuur kunnen worden bepaald.

In **hoofdstuk 2** toonden wij aan dat de technische variatie van de SCSA verwaarloosbaar is. De biologische variatie van de DFI is significant lager dan de biologische variatie van klassieke semen parameters als zaadcel concentratie, motiliteit en morfologie. Toch kent de SCSA onverklaarde klinisch relevante biologische variatie in bijna de helft van de 100 patiënten die wij onderzochten.

Wij constateerden een matige correlatie tussen klassieke semen parameters en de DFI. Hieruit kan geconcludeerd worden dat SCSA additionele informatie over de zaadcel kwaliteit geeft (**hoofdstuk 3**). In 10% van de mannen met normale klassieke semen parameters vonden wij een DFI boven de 30%. SCSA kan in deze mannen een verklaring bieden voor het uitblijven van zwangerschap. Het routinematig toepassen van SCSA bij mannen met normale zaadcel concentratie, motiliteit en morfologie is wellicht niet efficiënt gezien de lage prevalentie van verhoogde DFI.

In **hoofdstuk 3** evalueerden wij de diagnostische waarde van DFI bepaling in Andrologie patiënten. In bijna 280 patiënten werd een compleet Andrologisch onderzoek uitgevoerd, aangevuld door SCSA. Op basis van lichamelijk onderzoek, laboratorium waarden, echografie van het scrotum en semen analyse werd per patiënt een diagnose gesteld. Wij concluderen dat de kwaliteit van de spermatogenese (de aanmaak van zaadcellen) geassocieerd is met de DFI, in tegenstelling tot de Andrologische diagnose. Eerder onderzoek toonde aan dat DNA schade in zaadcellen veroorzaakt kan worden door oxidatieve stress, veroorzaakt door Reactive Oxygen Species (ROS). In diverse studies wordt een associatie beschreven tussen roken, het voorkomen van witte bloedcellen in het ejaculaat, ROS en afwijkende chromatine structuur. Wij constateerden geen relatie tussen roken, leukocyten in het semen en DFI in onze studie onder 279 patiënten met vruchtbaarheidsstoornissen. Leeftijd van de man is echter wel geassocieerd met DNA schade in zaadcellen in Andrologie patiënten.

De oorzaak van vruchtbaarheidsstoornissen is multifactorieel, maar voor therapie is het identificeren van behandelbare oorzaken van afwijkende chromatine structuur van belang. De voedingsstatus en in het bijzonder de concentratie van B vitaminen en homocysteine in bloed en seminaal plasma hebben wellicht invloed op het bevruchtend vermogen van zaadcellen. In **hoofdstuk 4** evalueerden we mogelijke associaties tussen B vitaminen en homocysteine met zowel klassieke semen analyse parameters als de DFI. In een subgroep van fertiele mannen constateerden wij een negatieve correlatie tussen foliumzuur in seminaal plasma en de DFI. Toekomstig onderzoek moet uitwijzen of het aanpassen van levensstijl en verbeteren van de voedingstoestand leidt tot betere kansen op spontane zwangerschap of zwangerschap na geassisteerde voortplanting.

Een veel voorkomende bevinding bij infertiele mannen is een varicocele (spatader van het scrotum). Ook bij vruchtbare mannen komen varicoceles voor, hetzij in mindere mate (10% versus 25%). Chirurgische correctie van de varicocele is nog steeds controversieel vooral omdat de spontane postoperatieve zwangerschapskans wellicht niet toeneemt. Sceptici stellen dat de toename van zaadcel concentratie en motiliteit na de operatie berust op biologische variatie van de semen parameters. In **hoofdstuk 2** toonden wij aan dat het voorkomen van een varicocele niet geassocieerd is met de biologische variatie van DFI. Onze bevinding dat na varicocele correctie zowel klassieke semen parameters significant verbeteren als de DFI significant afneemt (**hoofdstuk 5**) levert bewijs dat varicocele correctie spermatogenese in geselecteerde patiënten kan herstellen. In dezelfde studie constateerden wij dat lagere postoperatieve DFI geassocieerd is hogere zwangerschapskans, zowel spontaan als na geassisteerde voortplanting.

Hoofdstuk 6 beschrijft de waarde van SCSA als voorspeller voor spontane zwangerschap na sterilisatie hersteloperaties. Ondanks succesvolle rekanalisatie is de spontane zwangerschapskans verminderd ten opzichte van mannen die niet eerder een vasectomie (sterilisatie) ondergingen. De tijd tussen vasectomie en hersteloperatie en de

leeftijd van de vrouw zijn belangrijke voorspellers voor zwangerschapskansen. In onze studie was DFI niet geassocieerd met zwangerschap. Mogelijke verklaringen voor de afwezige associatie zijn een voortdurende ROS gemedieerde ontstekingsreactie of het voorkomen van debris in het ejaculaat na hersteloperatie.

Over het voorspellend vermogen van SCSA voor de uitkomst van geassisteerde voortplanting wordt niet eensluidend gepubliceerd. De meest recente studies tonen aan dat een DFI onder 30% geassocieerd is met betere zwangerschapskansen na intra uteriene inseminatie maar beperkte voorspellende waarde heeft voor de uitkomst na in vitro fertilisatie (IVF). In **hoofdstuk 7** bevestigen wij eerdere studies dat SCSA geen voorspellende waarde heeft voor de uitkomst van intracytoplasmatische sperma injectie (ICSI) waarbij een zaadcel per eicel wordt geïnjecteerd. Wij onderzochten een goed gedocumenteerde populatie mannen met ernstige semen analyse afwijkingen en evalueerden het voorspellend vermogen van zowel het gehele semen monster, als het bewerkte monster waaruit zaadcellen voor ICSI worden geselecteerd. Selectie vindt plaats op uiterlijke kenmerken van de zaadcel. Wellicht worden voornamelijk zaadcellen met intact DNA geselecteerd voor ICSI. Anderzijds beschikt de eicel over DNA herstellend vermogen waardoor de voorspellende waarde van SCSA beperkt wordt. Een andere mogelijke verklaring is dat de SCSA niet in staat is om DNA schade relevant voor embryo ontwikkeling te objectiveren. Wij beschrijven de opvallende observatie dat DNA schade, bepaald door de SCSA, toeneemt bij 40% van de patiënten met ernstige Andrologische factor infertiliteit.

De behandeling van kanker kan invloed hebben op de vruchtbaarheid. Om die reden wordt het invriezen van zaadcellen aangeboden aan kanker patiënten met kinderwens voor aanvang van chemotherapie en/of bestraling. Bij aandoeningen als zaadbalkanker en lymfklierkanker kan de vruchtbaarheid al voor oncologische behandeling aangetast zijn. In **hoofdstuk 8** evalueren wij de DFI in patiënten met zaadbalkanker (TGCT), de ziekte van Hodgkin (HD) en non-Hodgkin lymfoom (NHL) voor en na behandeling. Alleen in NHL patiënten constateerden wij een verhoogde DFI ten opzichte van vruchtbare mannen voor aanvang van therapie. Na oncologische behandeling nam de DFI voor de hele studie populatie significant af. Een mogelijke verklaring is dat alleen gezonde voorloper cellen, die verantwoordelijk zijn voor de aanmaak van nieuwe zaadcellen, chemo- en radiotherapie kunnen overleven. Deze resultaten moeten echter voorzichtig worden geïnterpreteerd en bieden geen uitsluitsel over de veiligheid van gebruik van zaadcellen voor geassisteerde voortplanting voor en na kanker therapie.

Hoofdstuk 9 geeft een overzicht van de hierboven samengevatte hoofdstukken en bediscussieerd bevindingen en conclusies. Samenvattend concluderen wij dat de SCSA een gevalideerde test is met lagere biologische variatie dan klassieke semen analyse die aanvullende informatie geeft over zaadcel kwaliteit. Er is grote overlap in DFI tussen vruchtbare en onvruchtbare mannen en tussen verschillende Andrologische diagnose

groepen. De prevalentie van abnormale DFI in mannen met ogenschijnlijk ongestoorde vruchtbaarheid is laag. Hieruit concluderen wij dat het routinematig implementeren van SCSA in de Andrologie praktijk niet zinvol is. In geselecteerde patiënten kan SCSA aanvullende informatie over de oorzaak van infertiliteit geven. SCSA is wel een bruikbaar middel om de associatie tussen patiënt en levensstijl karakteristieken te bestuderen en het effect van chirurgische of chemische interventies te evalueren.

CHAPTER 11

Dankwoord

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Marij Dinkelman-Smit
Januari 2011

CHAPTER 12

List of publications

PhD portfolio

LIST OF PUBLICATIONS

Surveillance with microsatellite analysis of urine in bladder cancer patients treated by radiotherapy
European Urology 2003;43(4):369-373
B.W.G. van Rhijn, M. Smit, D. van Geenen, A.J. Wijnmaalen, W.J. Kirkels, Th.H. van der Kwast, V. Kuenen-Bouwmeester, E.C. Zwarthoff

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M. Smit, P.C. Verhagen

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Fertil Steril, 2009;92(2):548-56
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J Urol, 2010;183(jan):270-4
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Human Reprod, 2010;25(8):1877-83
M. Smit, N.J. van Casteren, M.F. Wildhagen, J.C. Romijn, G.R. Dohle

Reply of the Authors: Acridine orange binding to RNA interferes with DNA fragmentation index calculation in sperm chromatin structure assay.
Fertil Steril, 2010 94:1
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J Urol, 2010;184(4):1577-78
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PHD PORTFOLIO

Name PhD student: Marij Smit
Erasmus MC Department: Urology, section Andrology
PhD period: July 2003 – March 2011
Promotor: Prof.dr. C.H. Bangma
Supervisor(s): Dr. G.R. Dohle, Dr. J.C. Romijn

PhD training	Year	ECTS
Research skills		
In-depth courses		
European Society Human Reproduction & Embryology (ESHRE) course for semen analysis	2003	2
Basic microsurgery course, Skills Lab, Rotterdam	2005	2
European Andrology Academy. Two year training and exam clinical Andrology, Kopenhagen, Denmark	2003-5	10
National conferences		
Externe refereeravond Urologie Erasmus MC Spreker: Introducing measurement of sperm DNA fragmentation in the standard Andrology practice	2004	1
Colloquim vergadering Josphine Nefkens Instituut Spreker: Voorspellende waarde van DNA fragmentatie in zaadcellen voor de uitkomst van geassisteerde voortplanting	2004	1
Wetenschapsdag voorplantingscentrum Erasmus MC Spreker: DNA schade in zaadcellen Spreker: Sperm DNA fragmentatie: klinische toepassing in Andrologie Spreker: Sperm DNA fragmentatie: klinische toepassing in Andrologie	 2004 2005 2007	 1 1 1
Interne refereeravond Urologie Erasmus MC Spreker: Sperm DNA fragmentatie vs morfologie	2007	1
Voorjaarsvergadering NVU Spreker: Kan Inhibine-B de uitkomst van MESA voorspellen in patiënten met obstructieve azoöspermie?	2005	1
Voorjaarsvergadering NVU Spreker: Afname van DNA fragmentatie in zaadcellen na varicocelectomie is geassocieerd met zwangerschap	2010	1
International conferences		
European Association of Urology, Istanbul, Turkey. Podium session: Sperm Chromatin Structure Assay (SCSA): A new technique to predict the outcome of ART Poster presentation: Determinants of ejaculatory dysfunction in a community-based longitudinal study	2005	2 2
European Society of Andrological Urology, Amsterdam Podium session: Sperm DNA fragmentation in patients with testicular germ cell tumours	2006	2

American Society for Andrology, Seattle, USA Poster presentation: Sperm DNA fragmentation in patients attending an Andrology outpatient clinic	2006	2
Society for Gynecologic Investigation, Reno, Nevada, USA Poster presentation: Seminal plasma cobalamin significantly correlates with sperm concentration	2006	2
Seminars and workshops		
Didactic skills		
Flowcytometric Sperm Chromatin Structure Assay	2003	2
Other		
Workgroup Guideline semen cryopreservation in cancer patients, IKR	2004-5	2
Reviewer Asian J Androl	2007-present	1
Reviewer Int J Androl	2010-present	1
Reviewer SRMB	2010-present	1
Teaching activities		
Lecturing		
Speaker European Society Human Reproduction & Embryology (ESHRE) course for semen analysis. Presentation sperm antibodies Presentation sperm motility Presentation sperm chromatin structure assay	2004-5	2 2 2
Keuze onderwijs: Echografie in de Andrologie	2004	1
Medical students: Subfertiliteit en Spermaanalyse	2005	1
Medical students: Het scrotum	2008	1
Supervising practicals		
Microsurgical vasovasostomia course (teacher)	2005-7	3
Supervising Interns	2004-5	2

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