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CLINICAL APPLICATION OF SPERM CHROMATIN STRUCTURE ASSAY IN DIAGNOSIS AND TREATMENT OF INFERTILITY

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Abstract

The diagnosis of male infertility is traditionally based on microscopic evaluating of sperm concentration, motility and morphology. Nowadays, when the assisted reproductive technology has gained a major role in the treatment of infertility, this method is not efficient in assessment of male fertility potential. Among a large number of new diagnostic methods Sperm Chromatin Structure Assay (SCSA), expressed in DNA fragmentation index (DFI), is the most scrutinized technique and seems to be most promising from a clinical point of view. The overall aim of this thesis was to evaluate the clinical value of Sperm Chromatin Structure Assay in diagnosis and therapy of infertility.

The intra-individual variation of DFI, evaluated in the first study, was 30.1% and considered as high compared to previous studies. Nevertheless, 85% of the tested men, when repeating the analysis, were still in the same side of the 30% cut-off value. Moreover, the next study revealed, that 26.1% of men from couples previously diagnosed as unexplained infertile had DFI>20%, compared to 10.5% of men with proven fertility. These findings illustrate that cases with the diagnosis ”unexplained infertility” can, to a certain extent, be explained by impairment of sperm DNA and that SCSA is a good supplement to the traditional semen analysis. The third study illustrates that SCSA can contribute to a choice of the optimal fertilization method in couples undergoing *in vitro* fertilization. The chance of live birth in standard *in vitro* fertilization (IVF) when DFI was above 20% was significantly lower than for those with lower DFI. Moreover, for the high DFI subgroup, live birth rates were significantly higher for intracytoplasmic sperm injection (ICSI) as compared to IVF. The results corresponded with negative association between DFI and fertilization rate as well as the chance of obtaining at least one good quality embryo (GQE), in standard IVF but not in ICSI. This suggests that ICSI might be a preferred method in cases with high DFI. Increased risk of miscarriage was seen in combined calculation for both IVF and ICSI when DFI exceeded 40%. The last study demonstrates that increased DFI makes some early embryo morphokinetics longer within IVF group and shorter in ICSI group which suggests that sperm DNA integrity plays an important role in early embryo development. In addition to clinical conclusions, the finding contributes to the theoretic knowledge about the potential impact of sperm DNA integrity on embryo during the first days after fertilization.

In conclusion, SCSA is recommended as a complement to traditional semen test in assessment of male fertility potential.

Key words: infertility, sperm DNA damage, SCSA, DFI, sperm chromatin, intra-individual variation, IVF, ICSI, fertilization, GQE, embryo development, pregnancy, miscarriage, live birth

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Abbreviations

AIH	artificial insemination by husband
AO	acridine orange
ART	assisted reproductive technology
BMI	body mass index
CI	confidence interval
CMA3	chromomycin A3
CSP	conventional semen parameters
CV	coefficient of variation
DFI	DNA fragmentation index
DGC	density gradient centrifugation
DNA	deoxyribonucleic acid
DSB	double-strand breaks
dUTP	deoxyuridine triphosphate
EDTA	ethylenediaminetetra acetic acid
ELISA	enzyme-linked immunosorbent assay
ET	embryo transfer
FACS	fluorescence-activated cell sorting
FAS	first apoptosis signal
FISH	fluorescent in situ hybridization
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
GQE	good quality embryo
HDS	high DNA stainability
ICSI	intracytoplasmic sperm injection
IUI	intrauterine insemination
IVF	*in vitro* fertilization
MAR	matrix attached region
NT	in situ nick translation
LBR	live birth rates
LH	luteinizing hormone
OPU	oocyte pick-up
OR	odds ratio
OS	oxidative stress
PCR	polymerase chain reaction

PRM1	protamine-1
PRM2	protamine-2
ROS	reactive oxygen species
SCD	sperm chromatin dispersion
SCGE	single cell gel electrophoresis assay (Comet assay)
SCSA	sperm chromatin structure assay
SD	standard deviation
SPSS	Statistical Package for the Social Sciences
SSB	single-strand breaks
TdT	terminal deoxy nucleotidyl transferase
tPBe	time of polar body extrusion
tPNa	time of pronuclei appearance
tPNf	time of pronuclei fading
tSB	time of start of blastulation
TNE	tris + NaCl + EDTA solution
TUNEL	terminal deoxy nucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labelling
t2	early cleavage
WHO	World Health Organizationyte

Preface

In recent decades, intensified depopulation processes in high developed countries can be observed. In Europe birth rates continue to decline and have dropped to below 1.5 children per couple. This phenomenon has its source from several factors such as lifestyle and migration, as well as infertility/subfertility problems. Infertility is recognized by the World Health Organization (WHO) as a public health issue and the European Parliament acknowledged in 2008 that falling birth rates were a major reason for the decline of the European population.

List of original papers

This thesis is based on the following original papers, which are referred to in the text by their Roman numerals:

I. **Oleszczuk K**, Giwercman A and Bungum M. Intra-individual variation of the sperm chromatin structure assay DNA fragmentation index in men from infertile couples. *Hum Reprod* 2011,26:3244-3248.

II. **Oleszczuk K**, Augustinsson L, Bayat N, Giwercman A and Bungum M. Prevalence of high DNA fragmentation index in male partners of unexplained infertile couples. *Andrology* 2013,1:357-360.

III. **Oleszczuk K**, Giwercman A and Bungum M. Sperm chromatin structure assay in prediction of in vitro fertilization outcome. *Andrology* 2016,4(2):290-6.

IV. **Oleszczuk K**, Lundberg T, Hambiliki F, Sundström T, Jerre E, Sjöberg A, Dimberg A, and Bungum M. Sperm DNA integrity is associated with time-lapse parameters of early embryonic development. *Submitted.*

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Background

Infertility

Infertility, defined as an inability to obtain pregnancy after at least one year of unprotected intercourse, affects approximately 15-20% of couples of reproductive age (Templeton *et al.*, 1990). Up to 50% of all infertility cases have a male factor as a sole or additional reason (Comhaire, 1987). The standard diagnostic tool which plays the central role in the assessment of male fertility in clinical practice is the conventional semen parameters (CSP). This traditional light microscopic method evaluates semen volume, sperm concentration, motility and morphology (WHO, 2010). The method is unfortunately subjective (Auger *et al.*, 2000), poorly standardized (Jorgensen *et al.*, 1997) and not powerful as a predictor of male infertility (Guzick *et al.*, 2001).

In the last decades assisted reproductive technology (ART) has gained a major role in the treatment of infertility. During this time ART has recorded considerable progress including pharmacological capability of woman's hormonal stimulation, technological equipment and the advent of new laboratory techniques. The development of these new techniques, especially intracytoplasmic sperm injection (ICSI) have created a request for a more effective investigation tools of male fertility potential. A large number of these new diagnostic methods focuses on the genomic integrity of the male gamete (reviewed in (Erenpreiss *et al.*, 2006)). This attention has been increased by the growing anxiety about imaginable transmission of genetic diseases through ICSI where natural control mechanisms during sperm-oocyte interaction are bypassed with potential subsequent chromosomal abnormalities, congenital malformations and developmental abnormalities in ICSI-born offspring (Hansen *et al.*, 2012; Okun *et al.*, 2014). At the same time ART has met the maximum limit of effectivity, the results assessed by "take-home baby" rate remains unchanged for the last several years (Kupka *et al.*, 2014; Sunderam *et al.*, 2014). The deficiency in the male diagnosis and therapy can be one of the reasons.

Sperm DNA and chromatin structure

The vast majority of sperm DNA is accumulated in the nucleus. This DNA will be further discussed and referred to as “sperm DNA” or “sperm chromatin”. The mitochondrial DNA which represents a small part of the whole DNA volume and is responsible for sperm motility is not an object of interest in this thesis.

The mature spermatozoon is formed during the hormonally regulated process of spermatogenesis. Two gonadotropins: follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are synthesized and released by adenohypophysis which represents the anterior lobe of the pituitary gland. The hypophysis itself is under the control of the hypothalamus which, via the hypophyseal portal system, affects it by gonadotropin-releasing hormone (GnRH). The hypothalamus is highly interconnected with other parts of the central nervous system and works under the stimulating and inhibiting influence of brain neurotransmitters. GnRH and gonadotropins, together with testosterone and other androgens, form a hypothalamic-pituitary-gonadal axis, which is a strictly controlled feedback system. LH stimulates testosterone production in the Leydig cells of the testes. Testosterone inhibits LH secretion through a negative feedback system. FSH acts on Sertoli cells by stimulating spermatogenesis and secretion of inhibin, which in its turn applies a negative feedback suppressing hypophyseal release of FSH. Testosterone has a large biological effect and is also required for normal spermatogenesis.

Diploid spermatogonia, which are initial cells in the spermatogenesis pathway, proliferate by mitosis to generate the primary spermatocytes. These cells are characterized by the ability to enter meiosis. Each primary spermatocyte converts during the first meiotic division into two secondary spermatocytes. DNA duplicates by replication in the beginning of the division (prophase I) and then cleaves into two cells, thus secondary spermatocyte contains the same amount of DNA. It continues dividing into the next two cells during the second meiotic division. DNA halves again forming the haploid spermatid. The spermatids undergo vast morphological changes during the spermiogenesis. They transform from round to elongated spermatids and then to spermatozoa. This involves nuclear condensation, reduction of cytoplasmic volume, transformation of the Golgi apparatus into the acrosomal cap and tail development. In mature sperm cell chromatin occupies almost the entire nuclear volume (Agarwal and Said, 2003). Completely formed spermatozoa are released into the lumen of seminiferous tubule and transported with the testicular fluid to the epididymis where they continue maturation and gain the motility and fertilizing capabilities.

The mature spermatozoa are morphologically and functionally specialized to transport the paternal genome through the male and female genital tracts and protect genetic material. They ensure that the paternal DNA is delivered in the form that allows the correct fusion of parental genomes and, thanks to the chromatin's decondensing properties at an appropriate time in the fertilization process (Amann, 1989), enables the developing embryo to properly express the genetic potential (De Jonge, 2000; Ward and Zalensky, 1996). To fulfill the uniquely high degree of condensation, sperm DNA must be arranged in a particular mode, which diverges fundamentally from that of somatic cells (Ward and Coffey, 1991).

Somatic cell's chromatin is organized into nucleosomes (Pienta and Coffey, 1984). These basic structure units consist of two laps of DNA wrapped around a protein core formed by an octamer of histones. The nucleosomes are then additionally wound into solenoids (Finch and Klug, 1976). This type of DNA packaging increases the volume of the chromatin (Ward and Coffey, 1991). Thus, an entirely different type of DNA structure, with large reduction of the volume, is present in mammalian sperm nuclei.

The chromatin structure transforms from the loose nucleosomal organization characteristic for somatic cells, via transition proteins to highly packed protamine bound chromatin in the sperm cell (Poccia, 1986). The condense structure and insolubility are the features which make sperm nucleus stable and have a protective role on the genetic material. Figure 1 presents a graphic illustration of this process. The replacement of the histones by protamines happens during the spermiogenesis and covers the vast majority of sperm DNA (Hud *et al.*, 1995). Protamine binding silences gene expression and has a protective role (Carrell *et al.*, 2007; Martins *et al.*, 2004). The smaller parts of the sperm chromatin remain bound to histones (Churikov *et al.*, 2004; Hammoud *et al.*, 2009; Ward and Coffey, 1991) or are attached to the sperm nuclear matrix at MARs (matrix attached regions) (Martins *et al.*, 2004; Nadel *et al.*, 1995). The mature sperm nucleus is characterized by very compressed bundling of the primary sperm DNA which allows for retaining a much smaller volume than that of normal somatic nuclei, containing yet half as much DNA (Ward and Coffey, 1991). The essential packaging unit of sperm chromatin is a doughnut-shaped toroid representing the DNA loop-domains, highly condensed by protamines and fixed at their bases to the nuclear matrix. Toroids are stacked side by side and crosslinked by disulfide bonds, formed by oxidation of sulfhydryl groups of cysteine present in the protamines (Fuentes-Mascorro *et al.*, 2000, Ward, 1993). The bonds are essential for the high order of chromatin packaging necessary for normal sperm function (Courtens and Loir, 1981). A large garland of toroids forms the chromosome.

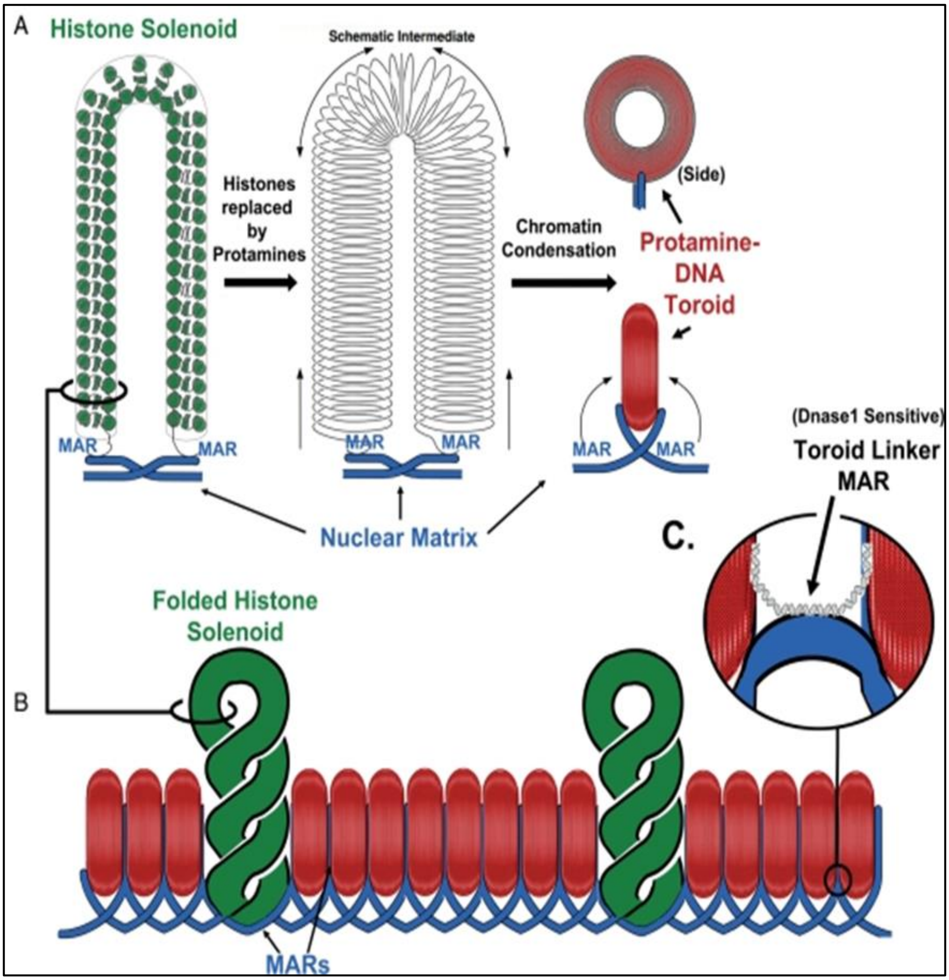

Figure 1. Three major structural elements of sperm chromatin.
During spermiogenesis, histones are replaced by protamines (A), condensing the DNA into tightly packaged toroids (B). Protamine toroids may be organized by stacking side by side (C) The DNA strands that link the protamine toroids may be bound to histones, as well.
Function of sperm chromatin structural elements in fertilization and development. Ward 2010.

Mammalian spermatogenesis usually results in highly homogenous spermatozoa in terms of nucleoprotein contents. The histone, solenoid formed component varies between 2% and 15% depending on the species and method used to quantify it (Ward, 2010). Whilst many mammal sperm nuclei are characterized by very high amounts of protamine-bound sperm chromatin i.e., over 90% (Bench *et al.*, 1996) or even over 95% (van der Heijden *et al.*, 2005), human sperm nuclei contain considerably fewer protamines (approximately 85%-90%) (Bench *et al.*, 1996; Brykczynska *et al.*, 2010; Gatewood *et al.*, 1987). Human sperm chromatin is therefore less regularly compacted, frequently contains DNA strand breaks (Irvine *et al.*, 2000; Sakkas *et al.*, 1999) and demonstrates considerable inter- and intra-individual variability related mostly to its protein element. The retention of 15% histones, which are less basic than protamines, leads to the formation of a less compact chromatin structure (Bench *et al.*, 1996). Two types of protamine have been identified: protamine-1 (PRM1) which is found in almost all mammals (Queralt *et al.*, 1995), and protamine-2 (PRM2) which is limited to relatively few species including humans (McKay *et al.*, 1986; Oliva, 2006). PRM2 is characterized by a deficiency in cysteine residues (Corzett *et al.*, 2002). Consequently, the disulfide crosslinking responsible for more stable packaging is diminished in human sperm as compared to species containing PRM1 alone (Jager, 1990). Altered PRM1/PRM2 ratio, absence of PRM2 and occurrence of protamine abnormalities results in deregulated protamine expression are associated with human male fertility problems (Aoki *et al.*, 2005; Mengual *et al.*, 2003).

Sperm DNA damage

The integrity of sperm DNA plays a considerable role in the proper processing of transfer of paternal genetic material into the oocyte during fertilization. DNA fragmentation is defined as both single (SSB) and double DNA strand breaks (DSB). The etiology of the sperm DNA damage is multifactorial and can be divided into intrinsic and extrinsic reasons (Zini and Sigman, 2009). The first appear on the molecular level. There are a number of pathophysiological phenomena which can occur during the spermatogenesis and lead directly to the DNA breaks. The latter ones are the external reasons which can cause or accelerate these phenomena.

Intrinsic reasons of sperm DNA damage

Deficiencies in recombination during spermatogenesis

Meiotic crossing-over is the exchange of chromatid fragments between homologous chromosomes. It is one of the phases of genetic recombination which occurs during prophase I of meiosis. It is associated with the physiologically programmed introduction of DNA double strand breaks by specific nucleases (Bannister and Schimenti, 2004). Then the DNA DSB are joined together by the enzyme ligase. Finally DNA damage checkpoint is activated and, depending on whether the DNA is entirely repaired or not, proceeding to meiosis is approved or disapproved (Page and OrrWeaver, 1997). An incorrect checkpoint may be the reason for occurrence of fragmented DNA in ejaculated spermatozoa. However, the conclusion that deficiencies in DNA recombination result in decreased chromatin integrity in mature spermatozoa is based on theoretical speculation and there is no evidence-based data which directly confirms this hypothesis in humans.

Abnormal chromatin remodeling

Spermiogenesis and histone-to-protamine replacement running during this phase is another moment when the physiologically programmed DNA breaks occur. Most of the sperm chromatin structure is established during spermiogenesis, therefore this process is crucial to the genetic integrity of the developing spermatids (Laberge and Boissonneault, 2005). The structural changing and formation of toroids create stage specific, transient torsional stress that is relieved by DNA breaks (Marcon and Boissonneault, 2004). These breaks favor the replacement of the nucleosome histone cores by transitional proteins and final protamination during spermiogenesis. They are created and ligated by the endogenous nuclease (topoisomerase II) (McPherson and Longo, 1993). They have been found in round and elongating spermatids. Thus, DNA strand breaks during chromatin remodeling are part of the normal differentiation program of these cells. Any alteration in the protein exchange process leading to chromatin remodeling may therefore lead to considerable consequences on the integrity of the sperm chromatin (Laberge and Boissonneault, 2005). Thus, the presence of endogenous DNA breaks in spermatozoa may indicate anomalies during spermiogenesis and an incomplete maturation process (Manicardi *et al.*, 1995).

Abortive apoptosis

Apoptosis is defined as a programmed and highly-controlled cell death. It is common in every kind of cell in multicellular organisms. It is a physiological process and its role is to remove abnormal cells and control their overproliferation. Thanks to apoptosis the balance between cell production and cell death is established. Apoptosis of testicular germ cells occurs normally throughout life and is necessary to limit the quantity of the germ cell population to a number that is adjusted to the Sertoli cell capacity (Rodriguez *et al.*, 1997). Deficiencies in this process may lead to sperm DNA damage (Sakkas *et al.*, 1999). The early apoptotic pathway, initiated in spermatogonia and spermatocytes, is related to the FAS (First Apoptosis Signal) pathway. Sertoli cells express FAS ligand, which by binding to FAS receptor begins a cascade reaction leading to activation of caspase enzymes and elimination of appropriately marked sperm cells by phagocytosis (Said *et al.*, 2004; Suda *et al.*, 1993). However, if this mechanism works inefficiently a number of defective germ cells may escape apoptosis and enter the process of sperm remodeling appearing later on in the ejaculate (Sakkas *et al.*, 1999). Abortive apoptosis initiated at the early stage of spermatogenesis is unlikely to be seen in semen. This is because apoptosis is an irreversible process at the stage of spermatogonia and these cells are usually digested by Sertoli cells (Zhivotovsky and Kroemer, 2004). Contrary, if the apoptotic cascade is initiated at the round spermatid phase, abortive apoptosis might be an origin of the DNA breaks.

Oxidative stress (OS)

Oxidative stress is recognized as a factor in generating sperm DNA damages (Aitken and Krausz, 2001). It reflects the disrupted balance between activity of reactive oxygen species (ROS) and endogenous defense agents of antioxidants (Sikka, 2001). Cells living under aerobic conditions constantly face the oxygen paradox: O_2 is required to support life, but its metabolites such as ROS can endanger cell survival (de Lamirande *et al.*, 1997). Hence, ROS must be continuously inactivated to keep only a small amount necessary to maintain normal cell function (Agarwal *et al.*, 2003). The determinants of oxidative stress are regulated by an individual's unique hereditary factors, as well as environment and lifestyle characteristic. In spermatogenesis ROS modulate gene and protein activities vital for sperm proliferation, differentiation and function. They induce sperm hyperactivation, capacitation, acrosome reaction and oocyte fusion *in vitro* (de Lamirande and Gagnon, 1993). Low levels of ROS are necessary to enhance the spermatozoa's ability to bind with the *zona pellucida* and facilitate sperm-oocyte adhesion (Kodama *et al.*, 1996). On the other hand, there are reports with solid evidence that high levels of ROS induce various forms of DNA damage including SSB and DSB frequently observed in spermatozoa of infertile men (Agarwal *et al.*, 2003; Aitken and Krausz, 2001; Kodama *et al.*, 1997). Consequently, antioxidant treatment significantly protects spermatozoa from DNA damage (Lopes *et al.*, 1998). The fine balance between ROS production and scavenging enzymes is of high importance for the acquisition of fertilizing ability (de Lamirande *et al.*, 1997). The pathogenic effects of ROS occur with increased generation and/or decreased antioxidant capabilities of the male reproductive tract or seminal plasma. Morphologically abnormal spermatozoa (with residual cytoplasm, in particular) and leukocytes are the main source of excess ROS generation in semen (Aitken *et al.*, 1992).

The origin and interaction of different sources of sperm DNA damage is shown in Figure 2.

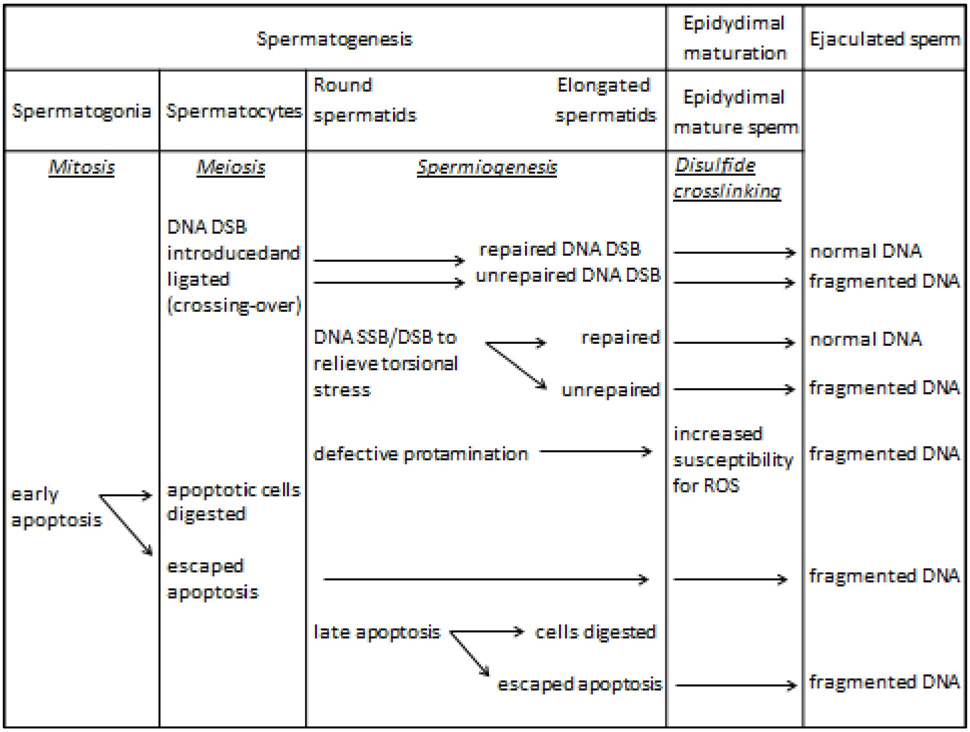

Figure 2. Sperm chromatin damage (modified from Erenpreiss 2006).

Extrinsic reasons of sperm DNA damage

It is worth noting that all four intrinsic causes of DNA damage are derived from processes which occur physiologically during spermatogenesis. The fragmented chromatin is a result of described disorders, insufficiencies, disturbed balances and other anomalies in these processes. There are numerous extrinsic reasons which can induce, strengthen and accelerate these anomalies and in this way impact the formation of DNA breaks. This is a broad spectrum of external factors. They are of interest of clinical praxis and are items of diagnostic examination of patient.

The most common extrinsic reasons of sperm DNA damage can be enumerated as following:

Cancer

Cancer can affect sperm in various ways, including disruption the spermatogenesis and in this way impact sperm chromatin integrity. Thus, patients with some types of cancer manifest a significantly higher range of sperm chromatin abnormalities before beginning therapy (Kobayashi *et al.*, 2001). Generally neoplasmatic transformation implies increased oxidative stress and apoptosis (Engel and Evens, 2006; Waris and Ahsan, 2006) and these two intrinsic reasons cam explain increased formation of sperm DNA breaks in men with cancer diagnosis (Agarwal and Said, 2005; Gandini *et al.*, 2000). Data about sperm DNA integrity in pre-treatment cancer men depend on type of cancer. A large study was performed on testicular germ cell cancer, lymphomas- either Hodgkin’s or non-Hodgkin’s and leukemia. These three groups make up the majority of men having their semen cryopreserved due to the sickness. In this group of patients an intra-testicular alteration in the system of apoptotic control as a reaction to the neoplastic cell proliferation can be observed. This deregulation is responsible for parallel sperm damage (Gandini *et al.*, 2000). Further studies, in vast majority, confirm a higher risk for poor semen quality and increased sperm DNA damage prior to cancer-specific therapy (Kobayashi *et al.*, 2001; O'Donovan, 2005; O'Flaherty *et al.*, 2008). However contradicting researches do not observe this rule or only confirm it partly (Smit *et al.*, 2010).

Iatrogen reasons

Regarding cancer therapy there is a considerable consensus that all three therapeutic methods i.e., chemotherapy, radiotherapy and surgery may have a negative and permanent impact on the individual's fertility potential (Romerius *et al.*, 2010). The two first methods may contribute to a disturbance of DNA integrity, but data on to which extent the antineoplasmatic treatment affects DNA integrity is more conflicting. It can be anticipated that treatment which effectively annihilates cancer cells because of their intensive proliferation, can also affect cells with a fast multiplication rate, such as germ cells (Paoli *et al.*, 2015). Due to constant sperm production and development, they are a prime target for chemotherapy. Diagnosis, type and number of therapeutic cycles, cumulative dose received as well as the pre-treatment status influence the extent of damage to gonadal cells and their subsequent recovery (O'Donovan, 2005). Increased sperm DNA damage has been shown in many studies (Paoli *et al.*, 2015; Spermon *et al.*, 2006; Stahl *et al.*, 2004). However, there is still a minority of studies with contradicting results (Smit *et al.*, 2010; Stahl *et al.*, 2009), where no significant differences were found in pre- and post-treatment chromatin integrity status. Prior to puberty there is no sperm production, but germ cells are present and are the target of chemotherapy as well. Germ cells and sperm can also be damaged by radiation, even in low doses (Singh and Stephens, 1998).

Varicocele

Varicocele is found in about a quarter of men who undergo infertility investigations, compared with 12% in the population. It is accompanied with impaired sperm quality assessed by CSP (Said *et al.*, 1992). It is strongly associated with OS by increased levels of ROS and diminished seminal plasma antioxidant capacity (Hendin *et al.*, 1999). The exact pathways by which a varicocele damages spermatogenesis and sperm quality remain elusive. Scrotal hyperthermia, hormonal disturbances, testicular hypoperfusion and hypoxia as well as backflow of toxic metabolites are described as potential mediators of varicocele related infertility (Agarwal *et al.*, 2012). Levels of ROS positively correlate with the degree of varicocele and infertile patients with varicocele had a significantly increased DNA damage than healthy controls (Allamaneni *et al.*, 2004). It has been shown that varicocele is also associated with the abnormal retention of sperm cytoplasmic droplets (a morphologic feature associated with high levels of semen ROS) and that these retained droplets are correlated with sperm DNA damage (Fischer *et al.*, 2003; Zini *et al.*, 2000). Another potential cause of sperm DNA damage in patients with varicocele is apoptosis. Sperm DNA integrity has been shown to improve after varicocele repair (Smit *et al.*, 2010; Zini *et al.*, 2005).

Nicotine

The majority of reports have shown that smoking is associated with lower values in CSP (Zenzes, 2000) as a result of impaired spermatogenesis (Saleh *et al.*, 2002) and also with an increase in sperm chromatin damage (Potts *et al.*, 1999). The common tobacco toxins may cause increased amounts of fragmented DNA (Evenson *et al.*, 2002). Smoking is linked to significantly increased levels of seminal ROS and as a consequence to the oxidative stress (Saleh *et al.*, 2002). Metabolites of cigarette smoke components may induce an inflammatory reaction in the male genital tract, with subsequent release of chemical mediators of inflammation (Agarwal and Said, 2003). ROS in the seminal plasma of smokers may have three main origins: a leukocytospermia induced by a chronic inflammation of the genital tract, an imbalance between the antioxidant capacity of the spermatozoa and the amount of ROS and the presence of ROS in the cigarette itself (Sepaniak *et al.*, 2006). This increased ROS activity results in apoptosis (Sakkas *et al.*, 2002) and then consequently in elevated DNA fragmentation.

ART

Assisted reproductive technology carries a potential risk of inducing DNA breaks. OS is a common phenomenon in the context of ART. The ICSI procedure itself induces both oocyte and spermatozoa membrane damage to allow sperm-oocyte interaction. This makes the sperm nucleus more accessible for ROS released from plasma membrane, and potentially can induce DNA breaks (Agarwal *et al.*, 2003). Media and their components used in ART vary widely in their ability to protect DNA from the ROS (Cummins *et al.*, 1994). Cryopreservation, which is a generally accepted and available option for fertility preservation is another technique that might lead to DNA damage (Donnelly *et al.*, 2001; Thomson *et al.*, 2010). Repeated gradient centrifugation during sperm preparation before ART stimulates production of ROS (Agarwal *et al.*, 1994). However single centrifugation selects spermatozoa with low DNA damage (Bungum *et al.*, 2008; Lopes *et al.*, 1998; Malvezzi *et al.*, 2014).

Heat stress

The prospective studies have shown that mild induced testicular and epididymal hyperthermia impairs sperm chromatin integrity (Ahmad *et al.*, 2012). Moreover, an association between testis overheating and reduced male fertility potential has also been demonstrated (Thonneau *et al.*, 1998). Although studies are limited it can be assumed that certain behaviors and occupations which are associated with increased scrotal temperatures might impair sperm DNA integrity. Consequently patients with febrile status show compromised sperm DNA integrity too (Evenson *et al.*, 2000).

Air pollution, xenobiotics, drugs

Even exposure to air pollution can have deleterious effects on sperm chromatin integrity (Evenson and Wixon, 2005; Rubes *et al.*, 2005). Various occupational hazards involving industrial chemicals like toluene, xylene, herbicides, pesticides and organochlorines are also known to significantly stimulate DNA damage in spermatozoa (Bian *et al.*, 2004; Sanchez-Pena *et al.*, 2004; Spano *et al.*, 2005). Use of cocaine or marijuana might reduce the number and quality of sperm as well. Cocaine has also been proven to affect sperm DNA. The exposure leads to a rise in sperm DNA strand breaks, attributed to an increase in apoptosis (Li *et al.*, 1999).

Infections

Leukocytes in general are present in ejaculate and play an important role in immunosurveillance and phagocytic clearance of abnormal sperms (Tomlinson *et al.*, 1992). ROS is attached to inflammation status in genital tract by leukocytospermia and its association with increased DNA damage. If leukocytes enter the male reproductive tract at the level of the secondary sexual glands, the first contact that the spermatozoa have with these cells is at the moment of ejaculation. At this point, the spermatozoa are shielded from leukocyte attack by the protective properties of seminal plasma (Aitken and De Iuliis, 2007; Zini *et al.*, 2002). However, if significant numbers of leukocytes enter the male tract at the level of the testes or epididymides, or if the number of leukocytes is so high as to overwhelm the antioxidant protection offered by seminal plasma, then a state of oxidative stress can generate DNA damage (Aitken *et al.*, 1995). Thus, the inflammation dependent DNA leisure is even valid in posttesticular genital tract infections and inflammations like epididymidis or prostatitis (Erenpreiss *et al.*, 2002).

Age

Men produce gametes generally their entire adult life. However, the quality of spermatozoa deteriorates and their fertility declines (Moskovtsev *et al.*, 2006; Wyrobek *et al.*, 2006). This also pertains to the sperm DNA integrity. An increased amount of sperm double-stranded DNA breaks appears. Simultaneously, a decrease in sperm apoptosis can be observed which may indicate worsening of healthy sperm cell selection process with age (Singh *et al.*, 2003).

Other factors

There are additionally a number of medical, environmental and lifestyle factors which can theoretically influence sperm DNA integrity. The following have been reported in literature: sexual abstinence time (Spano *et al.*, 1998), electromagnetic radiation (Aitken *et al.*, 2005) including mobile telephones (Fejes *et al.*, 2005), hormone imbalance (Meeker *et al.*, 2008), cryptorchidism (Smith G *et al.*, 2007). In contrast, according to some studies, Body Mass Index (BMI) is not associated with sperm DNA integrity (Bandel *et al.*, 2015). Other factors like nutritional status (Vujkovic *et al.*, 2009) and folate in seminal plasma (Boxmeer *et al.*, 2009) have not provided definitive results.

Assays for evaluating sperm DNA structure

Several techniques have been developed in the last decades to assess sperm DNA damage. They differ from each other according to the physical and chemical phenomena they utilize and the aspect of DNA damage they detect. Three of them seem to give the best hope for practical use and have been studied more extensively. They can be specified as follows:

Sperm chromatin structure assay (SCSA)

Sperm Chromatin Structure Assay is a diagnostic method based on the flow cytometric technology. SCSA adopts fluorescence-activated cell sorting (FACS) which is a specialized type of flow cytometry suitable for sorting a heterogeneous mixture of biological cells according to fluorescent characteristics of each cell. SCSA was invented by Evenson and co-workers (Evenson *et al.*, 1980).

The assay detects and measures the susceptibility of sperm chromatin to acid-induced DNA in situ denaturation and is relied on the fact that spermatozoa with abnormal chromatin structure are much more susceptible to this denaturation (Darzynkiewicz *et al.*, 1975). Both fresh and frozen samples can be used. When used in the assay, frozen samples should be thawed in a 37°C water bath and diluted to a concentration of 1-2 x 106 sperm cells per ml with 1x TNE buffer, thus sperm samples with very low concentration cannot be used. The diluted samples are exposed to acid solution for 30 seconds when DNA denatures in situ. Immediately following acidic treatment, cells are stained with acridine orange (AO) in a phosphate-citrate buffer. AO is a fluorescent dye and its metachromatic properties are utilized (Evenson and Jost, 2000). AO binds to the DNA helix as an intercalator and emits green fluorescence when bound to intact, double strand DNA or red fluorescence when bound to the fragmented DNA (Darzynkiewicz *et al.*, 1975). The stained sample is placed on the flow cytometer where a fluidics system transports spermatozoa in a stream to the laser beam for interrogation. The optics system illuminates the particles and directs the resulting light signals to the appropriate detector. The device is equipped with an electronic system which converts the light signals into electronic signals that can be processed by the dedicated software. The extent of DNA denaturation is expressed in terms of the DNA fragmentation index (DFI), which is the percentage ratio of red to total (red plus green) fluorescence intensity, i.e., the level of denatured DNA over the total DNA (Evenson *et al.*, 2002). It represents the population of sperm with DNA damage as a percentage of the total number of spermatozoa. The principles of flow cytometry and SCSA are depicted in figure 3.

Apart from DFI, SCSA measures another index which characterizes sperm chromatin: immature sperm nuclei with abnormal proteins and/or altered protamine/histone ratios -high DNA stainability (HDS) (Evenson *et al.*, 1999). The index reflects the presence of immature spermatozoa. However, predictive value of this parameter for the outcome of ART is doubtful (Bungum *et al.*, 2007).

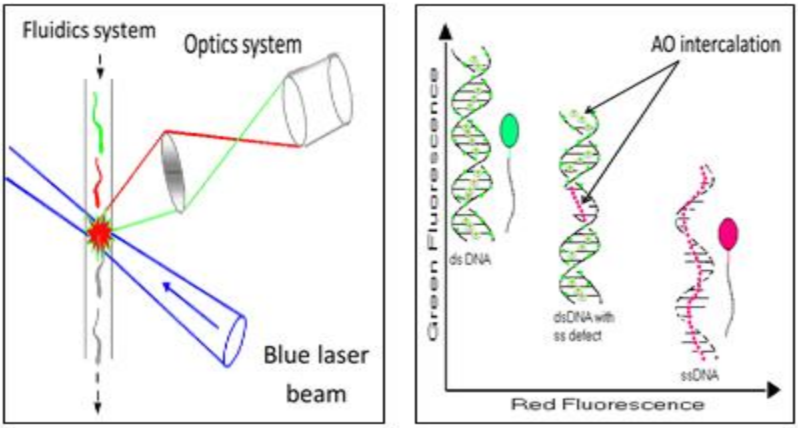

Figure3. Principles of flow cytometry and SCSA.

Single Cell Gel Electrophoresis assay (SCGE or Comet assay)

This method quantifies the SSB and DSB in individual cells using electrophoretic technology (Ostling and Johanson, 1984). Two versions of the technique exist: alkaline and neutral. In the first one, spermatozoa are lysed by the alkaline detergent of pH 10-13 which makes that disulfide bonds break down and DNA decondenses. Sperm cells are then stained with a fluorescent DNA-binding dye and placed in an electromagnetic field between two electrodes. The migrating spermatozoa emit the fluorescent light which forms the shape of comet therefore the name of the assay. The intact, high molecular unbroken DNA migrates slowly and tends to stay in the comet head, while short fragments of damaged double- and single-stranded DNA migrate faster into the tail area (Klaude *et al.*, 1996). These characteristics (diameter of the nucleus and the comet length) in resulting images are measured to determine the extent of DNA damage (Hughes *et al.*, 1996). Comet assay characterization is highly sensitivity. The neutral version of Comet serves pH 9. If the DNA is not denatured then the assay is more sensitive to double DNA strand breaks and therefore better able to identify DNA damage related to infertility (Singh and Stephens, 1998).

TUNEL assay- TdT (terminal deoxynucleotidyl transferase)-mediated dUTP nick-end labelling

This technique utilizes fluorochromes to determine real, “actual” DNA damage. The principle of this assay is to quantify the incorporation of deoxyuridine triphosphate (dUTP) at SSB and DSB (Gorczyca *et al.*, 1993). Incorporated dUTP is labelled such that breaks can be assessed by flow cytometry as well as microscopic methods with application of fluorometric or colorimetric labelling. The method is especially suitable in cases with severely reduced sperm amount or when spermatozoa are extracted by the biopsy of epididymis alternatively testis (Lewis *et al.*, 2013). The method is recognized as precise and reproducible, however low sensitivity is its big disadvantage (Mitchell *et al.*, 2011). It can be used in the same extent in the frozen and thawed semen samples (Sailer *et al.*, 1995). The flow cytometric TUNEL provides clinically significant results; nevertheless the assay cannot be employed for routine clinical use due to a lack of standardization of the thresholds (Evenson *et al.*, 2002).

These three, most commonly used techniques have principle differences. SCSA quantifies DNA strand breaks by measuring in situ susceptibility to acid denaturation, contrary to TUNEL which assesses the actual strand breaks in the individual spermatozoa as fragmentation positive or negative. Comet examines degrees of DNA damages in an individual spermatozoon but valuates it from 0 to 100% (Lewis *et al.*, 2013). This means that SCSA is attributed as an indirect measurement of sperm DNA damage. The TUNEL and SCSA assays correlate well (Evenson *et al.*, 2007), although they determine chromatin breaks in a different way (Mitchell *et al.*, 2011). In contrast to SCSA, TUNEL and Comet detect DNA damage induced by alkaline conditions. All three tests show correlation with infertility diagnosis according to CSP (Larson-Cook *et al.*, 2003; Sharma *et al.*, 2010; Simon and Lewis, 2011), however this correlation is not strong. Comet test is relatively inexpensive but requires special equipment and experienced staff. SCSA and TUNEL implicate purchasing of expensive equipment. Furthermore both protocols are very demanding and require good quality laboratory routines. Testing with SCSA has several advantages. It analyzes a high number of spermatozoa in a short period of time; 5000-10 000 cells compared to the classic microscopic tests where 100-300 cells normally are examined. Spermatozoa are measured by flow cytometry in a few minutes providing objective, machine-defined criteria rather than subjective eye measures (Evenson *et al.*, 2002). However the solution of sperm concentration minimum $1x10^6$/ml is required, thus sperm samples with very low concentration cannot be routinely analyzed which can be attributed to the technique's disadvantages. In this aspect both TUNEL and Comet distinguish themselves positively since they can utilize very low sperm count, even testicular samples. SCSA is suitable for both frozen and fresh samples, so the analysis can be done in a clinic's routine schedule (Ollero *et al.*, 2001). All three tests damage spermatozoa irreversibly during the process. The biggest advantage of the SCSA is that it is standardized and performed according to a strict protocol (Evenson *et al.*, 2002). This makes the technique universal and high repeatable. TUNEL assay has also proved its clinical value (Henkel *et al.*, 2004) and even reference intervals for DNA damage have been established (Aitken *et al.*, 2010; Sharma *et al.*, 2010) but its lack of standardization is an obstacle to widespread use. Thus, both TUNEL and Comet, despite their advantages, need more efforts on their standardization to make them useful in clinical practice.

The summary of the other common techniques that have been developed to assess sperm DNA damage are specified in Table 1.

Table 1. Sperm chromatin integrity assays.

Assay	Principles	Advantages	Disadvantages	Clin value
AO-test	Simplified modification of the SCSA, based on visual microscopic examination of fluorescing spermatozoa after AO chromatin staining	Cheap, simple, flow cytometry equipment and trained technician not required, a strong positive correlation with TUNEL, negative correlation with sperm motility	Indistinct, rapidly fading colors, heterogeneous staining, test not repeatable	No
SCD (Halo)	Ability of sperms with intact DNA, deprived of chromatin proteins to loop around the sperm nucleus, measures the absence of damage. Sperm cells are counted manually with bright-field or fluorescence microscopy	Simple, cheap	Not validated	No
NT-test	Quantification of dUTP incorporation at SSB in reaction catalyzed by DNA polymerase I, quantified using FISH or blotting techniques, detected by fluorescence microscopy	Simple, high correlation with CSP	No relation between the level of strand breaks identified by NT and fertilization during *in vivo* studies, low sensitivity	No
Sperm nuclear matrix stability assay	Similar to Halo test, based on fluorescence microscopy, determines the DNA organization and sperm nuclear matrix ability to organize DNA into loop domain structure (fluorescence microscopy)	Relatively simple, inexpensive	Not extensively validated	No
Chromomycin- A3	CMA3 competes with protamines for association with DNA and detects protamine deficiency in mature spermatozoa (fluorescence microscopy)	Simple, inexpensive	Absence of a predictive threshold for fertility	No
Aniline blue	DNA protein stained of Aniline blue, detected by bright field microscopy	In some studies predictive of fertilization and pregnancy rates following IVF	Heterogeneous slide staining, inter-lab variability not tested	No
Toluidine blue	Spermatozoa dyed with toluidine blue which stains nucleic acids are evaluated by bright field microscope or image cytometry. The results are analyzed by a special computer program	Inexpensive, simple and correlates well with SCSA and TUNEL assays	The scrutiny of assay's clinical relevance is not completed which limits its introduction to the clinical practice	No

As seen from this summary SCSA is currently the most scrutinized technique of all which assess sperm chromatin integrity. So far it seems to be most promising from a clinical point of view. Nevertheless SCSA, despite of clear favors, still has detailed fields concerned accuracy, discriminating power, predictive value which are less investigated what hampers its common use in the clinical practice. The completion of these shortages determines a step forward to establish SCSA as an essential platform of knowledge about sperm chromatin integrity.

Clinical value of SCSA

The value of SCSA can be considered as a diagnostic tool during investigation of male infertility and as a predictor of chance of pregnancy in the various scenarios.

The technique has an acceptably low intra- and inter-laboratory variation (described in detail in section “Methods”). To gain a complete view on the variability of DFI a detailed study concerned intra-individual variation is needed. The data from previous research shows large differences in the results, examples presented in Table 2. However most of them are based on a low number of men from different cohorts.

Table 2. Intra-individual variations of SCSA/DFI (CV-coefficient of variation).

	Remarks	Number of men	CV(%)
Evenson *et al.*, 1991	not diagnosed men	45	10
Zini *et al.*, 2001	infertile men	21	21
Erenpreiss *et al.*, 2006	infertile couples	282	29

It is still unclear as to what extent the intra-individual variation of SCSA/DFI disturbs the method’s accuracy. Since it is an important limitation for clinical application, the problem requires further investigation on a larger study group.

An association has been observed between sperm DNA integrity and time to achieve a spontaneous pregnancy and a chance of achieving it in general (Evenson *et al.*, 1999; Spano *et al.*, 2000) as well as increased miscarriage rate (Evenson *et al.*, 1999).

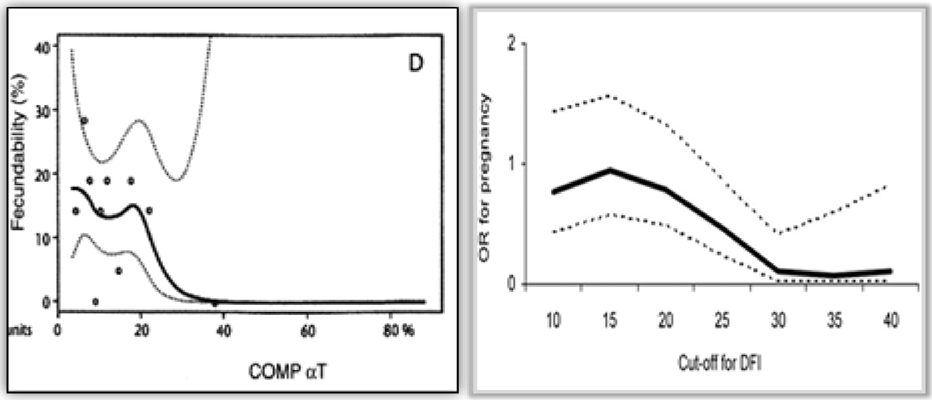

Figure 4(a)
Spano. Sperm chromatin damage impairs human fertility. The Danish First Pregnancy Planner Study Team.
Fertil Steril 2000.

(b)
Bungum. Sperm DNA integrity assessment in prediction of assisted reproduction technology outcome.
Hum Rep 2007.

In two separately lead studies concerning the probability of conception *in vivo*, one achieved by intercourse (Spano *et al.*, 2000) and the other one by intra-uterine insemination (IUI) (Bungum *et al.*, 2007) a similar pattern could be observed. In the interval of DFI 0–20%, the chance of the pregnancy was constant. When DFI was above 20% the chance of obtaining a pregnancy was decreased and approach zero when the DFI level passed 30%. Graphic illustration of these two studies is presented on the Figure 4a and b.

Men with decreased CSP manifest elevated levels of sperm DNA damage detected by various methods (Sun *et al.*, 1997; Virro *et al.*, 2004). Men with normal CSP demonstrate decreased fertility for DFI above 20%, men with one aberrant conventional semen parameter had a statistically significant decline in fertility already when DFI exceeded 10% (Giwercman *et al.*, 2010). However, the direct relation between CSP and sperm chromatin integrity has not been found which suggests that exploring sperm DNA contributes the new data not accessible through a conventional semen analysis (Simon *et al.*, 2010). DFI assessed by SCSA has been shown as a relatively independent predictor of male subfertility *in vivo* (Giwercman *et al.*, 2010). This finding was confirmed by an analysis of a systematic review by Castilla (Castilla *et al.*, 2010). Thus SCSA is a clinically useful technique with good predictive value of DFI for *in vivo* pregnancy (spontaneous and IUI). Table 3 presents a list of example studies.

Table 3. Impact of sperm DNA integrity assessed by SCSA on decrease in vivo pregnancy rates.

Reference	Method	Patients	Result significant(s)/not significant(ns)	DFI cut-off value (%)
Evenson, *et al.*, 1999	spontaneous	280	s	30
Spano *et al.*, 2000	spontaneous	215	s	40
Saleh *et al.*, 2003	IUI	19	s	30
Bungum *et al.*, 2004	IUI	131	s	27

However, a study revealing prevalence of increased DFI in a cohort of men from couples diagnosed as "unexplained infertile" has not been performed. Such knowledge would give the perception as to what extent assessment of sperm chromatin integrity can complement CSP.

The predictive value of sperm chromatin damage on IVF/ICSI outcome is still enigmatic. Already in 1980, inventors of the technique observed the association between decreased sperm DNA integrity and impaired fertilization (Evenson *et al.*, 1980). A similar relationship was later observed according to negative pregnancy outcome (Larson *et al.*, 2000), poor quality embryo (Virro *et al.*, 2004) low implantation rate (Speyer *et al.*, 2010) and non-significant increased spontaneous abortion (Check *et al.*, 2005; Virro *et al.*, 2004), confirmed by meta-analyses together with other methods (Robinson *et al.*, 2012; Zini *et al.*, 2008). The suggestion of some studies that ICSI can improve clinical pregnancy outcome in case of high DFI (Bungum *et al.*, 2007; Gandini *et al.*, 2004) was not confirmed by others (Larson *et al.*, 2000; Niu *et al.*, 2011). Recently made systematic reviews and meta-analyses which combine different methods of sperm DNA integrity assessment, mainly detect the impact of high DFI on outcomes of IVF or ICSI (Collins *et al.*, 2008; Ozmen *et al.*, 2007) including deleterious effects on live birth rates (Osman *et al.*, 2015). A meta-analysis carried out by Zhang didn't confirm predictive values specifically for SCSA, but instead generally indicated an impact of high DFI on the pregnancy outcome after IVF/ICSI (Zhang *et al.*, 2015).

Table 4 presents a list of example studies.

Table 4. Impact of sperm DNA integrity assessed by SCSA on decrease *in vitro* pregnancy rates.

Reference	Method	Patients	Result Significant (s) /not significant (ns)	DFI cut-off value (%)
Chohan *et al.*, 2004	IVF/ICSI	52	ns	30
Larson, *et al.*, 2000	ICSI	21	ns	27
Check, *et al.*, 2005	ICSI	106	ns	30
Bungum, *et al.*, 2004	IVF/ICSI	109/66	ns	27
Larson-Cook, *et al.*, 2003	IVF/ICSI	55/26	ns	27
Virro, *et al.*, 2004	IVF	249	s	30

As seen from the Table 4 the study which shows statistically significant impact of DFI on *in vitro* pregnancy rate involved standard IVF (Virro *et al.*, 2004) and a relatively high sample size compared to the other studies. This suggests that previous publications which question the SCSA prognostic value could depend on low numbers of patients and suboptimal study design. The larger study concerning only SCSA is of a great value.

Lack of evidence-based data concerning the impact of sperm chromatin integrity on IVF/ICSI outcome prompts to look for new tools for used during IVF/ICSI. The time-lapse technique enables continuous monitoring of early embryo development during the first days after fertilization (Armstrong *et al.*, 2015). It can be profitable to examine the potential impact of paternal genome on this development. Observing embryo development in relation to DFI level opens new possibilities for studying the impact on its development and general biological background of the problem. Because time-lapse is a link between IVF/ICSI therapy and clinical outcomes, the results of this observation can have a predictive value for future, potential pregnancy.

Aims of the thesis

The overall aim of these studies was to evaluate the clinical value of Sperm Chromatin Structure Assay in diagnosis and therapy of infertility.

The specific aims were:

1. To assess the intra-individual variation in DFI, as measured by SCSA, in order to evaluate the clinical utilization of this parameter (Paper I).

2. To assess the prevalence of high DFI in men from couples diagnosed with “unexplained infertility” (Paper II).

3. To estimate the impact of high DNA on the outcome of standard IVF and of ICSI, in order to develop tools for optimizing *in vitro* fertilization methods (Paper III).

4. Using “time lapse” technology to get deeper insight in association between high DFI and early embryo development (Paper IV).

Materials and methods

Subjects

All the papers were retrospective in design. The data is based on the internal register of the Center of Reproductive Medicine (RMC), Skåne University Hospital, of infertility examination (Paper I and Paper II) and IVF/ICSI treatments (Paper III and Paper IV) between 2007 and 2015.

Inclusion criteria for male partners for ART were below 56 years of age at start of the treatment and semen sample with sperm concentration at least 1 x 10^6 /ml when SCSA analysis was possible to perform. For infertility examination no age limitations for men were imposed.

For female partners, the criteria for being included were: age below 39 years at start of the treatment and body mass index (BMI) preferably below 30 kg/m^2. It was mandatory for both partners to be non-smokers. Ovarian reserve assessment in the form of FSH control and count of the number of antral follicles was mandatory during the diagnostic process. However, apart from the cases with extreme poor ovarian reserve, it wasn’t a hindrance for being accepted for ART and included to the study sample.

The present thesis was divided into four papers. The summary of methodological considerations is presented in Table 5.

Table 5. Subjects and study settings.

	Paper I	Paper II	Paper III	Paper IV
Setting	Patient's database in RMC Malmö			
Study design	Retrospective design			
Study period	May 2007-November 2009	June 2008-April 2011	May 2007-March 2013	May 2013- March 2015
Subjects	616 men aged 18-66 years who performed at least two SCSA analyses	119 men aged 22-55 years with diagnosis "unexplained infertility"	1633 couples who performed IVF/ICSI treatment	639 couples: 256 IVF and 383 ICSI treatments
Statistical methods	Coefficient of variation, Spearman's rho test	Proportion calculation, Fisher's exact test	Logistic regression, Univariate analysis of variance	Univariate analysis of variance
Outcomes	Intra-individual variation for DFI, correlation between the intra-individual variation and time interval between samples	Distribution of DFI value in relation to analogical value in a cohort of fertile men	IVF/ICSI results: fertilization, good quality embryo, pregnancy, miscarriage, live birth	Embryo morphokinetic characteristics: tPNa; tPNf; t2; tSB

Paper I.

The study sample was selected from 2409 consecutive men under infertility investigation. Repeated SCSA (2-7) analyses were performed on 616 samples from men between 18 and 66 years of age.

Paper II.

The cohort of 122 men from couples diagnosed as "unexplained infertile" was identified among 212 consecutive men under infertility investigation. Calculations were performed on 119 of 122, where SCSA data were available.

Paper III.

The study is based on 6660 consecutive, routinely driven IVF/ICSI treatments. The patients' recruitment took place according to the following flowchart (Figure 5).

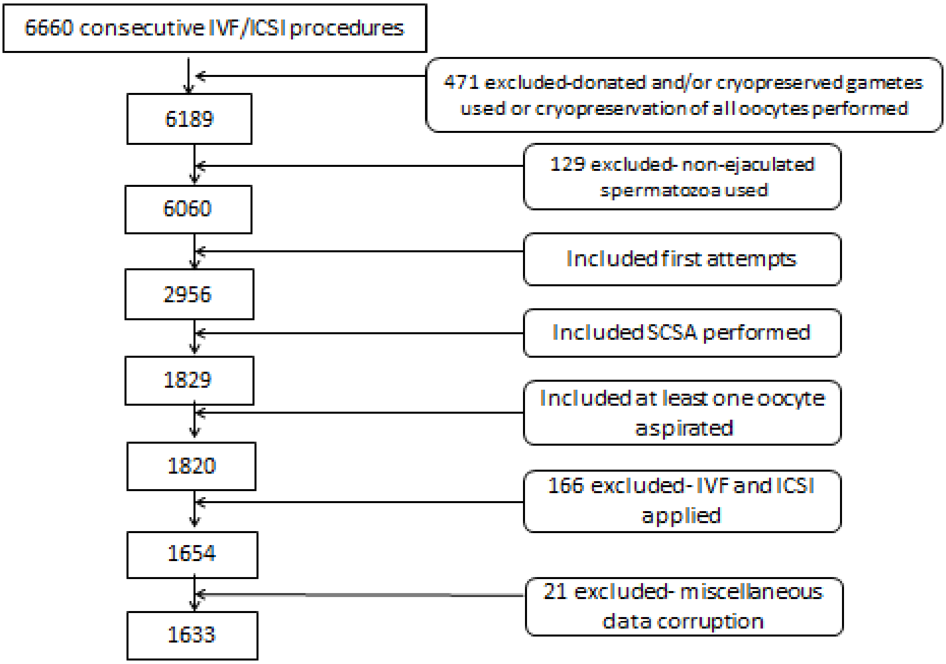

Figure 5. Patients' recruitment in Paper III.

Paper IV.

The study is based on 639 consecutive, routinely driven IVF/ICSI treatments: 256 IVF and 383 ICSI. Ninety-four couples were included in the study twice, 17 couples three times and 1 couple four times. Cycles where both standard IVF and ICSI were applied in the same cycle and cycles with donated sperm were excluded from the study. Thirty-three couples included in the study had donated oocytes, among them 7 with standard IVF. Oocyte distribution in the study is presented in Figure 6.

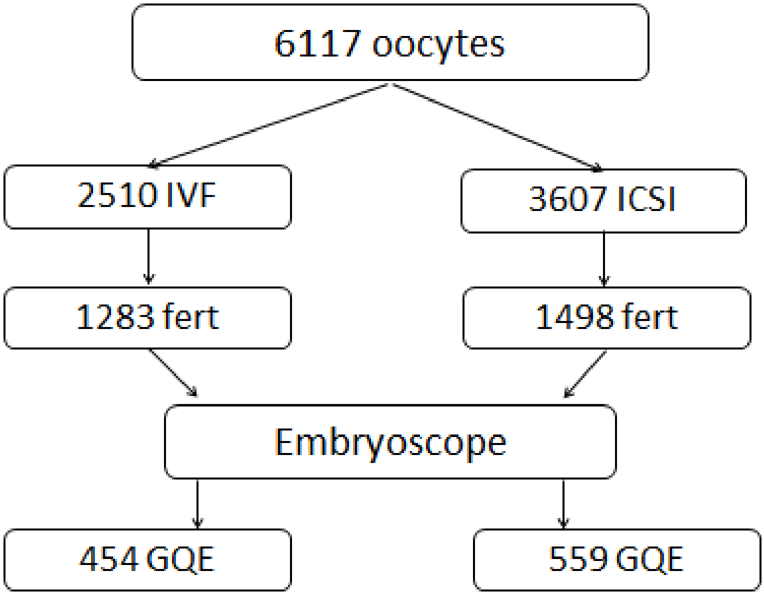

Figure 6. Oocyte distribution in Paper IV.

Methods

Collection and handling of semen samples

Semen samples were collected by masturbation. A sexual abstinence time of 2-7 days was recommended for Paper I-III and 2-4 days respectively for Paper IV. One hundred microliters of the ejaculate was frozen in Eppendorf snap-cap tubes in ultra-cold freezer at -80°C, for subsequent analysis.

Conventional semen parameters

All semen samples were examined in the laboratory within 30 minutes after collection. Five µl of well liquefied semen was placed on a Neubauer –chamber. All measurements were performed on a phase contrast microscope on a heating stage (37°C) at a total magnification of x40. Sperm concentration was assessed by using undiluted semen. The number of spermatozoa counted in any strip of 10 squares of the grid of the Neubauer-chamber indicated their concentration in million/ml. A mean of 10 x 2 squares was calculated. Motility was scored according to the WHO guidelines (WHO, 1999) for Paper I, II and III or WHO guidelines (WHO, 2010) respectively for Paper IV.

Sperm chromatin structure assay

SCSA was carried out in all papers following the procedure described by Evenson (Evenson *et al.*, 2002). A total number of 5000-10000 cells were accumulated for each measurement at a flow rate 200-300 cells/s. Analysis was performed by FACSort (Becton Dickinson, San Jose, CA, USA). Analysis of the flow cytometric data was carried out using dedicated software (SCSASoft; SCSA Diagnostics, Brookings, SD, USA) which implies that the DFI histogram is used to precisely determine the percentage DFI. All SCSA measurements were performed on raw semen, which on the day of analysis was quickly thawed and analyzed immediately. For the flow cytometer setup and calibration, a reference sample was used from a normal donor ejaculate retrieved from the laboratory repository (Evenson and Jost, 2000). The same reference sample was used for the whole study period. A reference was run for every fifth sample. A single SCSA measurement was made for each reference sample. The intra-laboratory variation is very limited, the coefficient of variation for DFI used to be about 4.5% (Giwercman *et al.*, 1999). Furthermore the SCSA analysis has demonstrated very low inter-laboratory variations. There is not only a high level of correlation between the results reported by two independent laboratories that strictly followed the SCSA protocol, but the absolute DFI values obtained at two different places, using different equipment, did not on average differ by >1% (Giwercman *et al.*, 2003). This makes the technique universal and highly repeatable.

In this thesis DFI values of 20% and respectively 30% are recognized as “cut-off” or delimit DFI intervals. This choice is based on well-documented studies and described before knowledge that chances for pregnancy *in vivo* begin to decrease in DFI 20% and are nearly zero if DFI>30% (Bungum *et al.*, 2007; Spano *et al.*, 2000). For the calculation of miscarriage risk in Paper III an extra interval for DFI>40 was established. DFI value of 10% delimits reference interval in Paper III and IV.

ART procedures

Paper III and IV are based on a cohort of consecutive IVF/ICSI procedures. All the patients underwent controlled ovarian stimulation with either “down regulation” GnRH-agonist long protocol or alternatively GnRH-antagonist short protocol. Ovarian stimulation was achieved with recombinant FSH or alternatively urine derived gonadotrophin. Detail procedure regimes were followed as described in respective paper.

Selection of spermatozoa with good motility and normal morphology was made with density gradient centrifugation (DGC). PureSperm, (Nidacon Ltd, Sweden) a standard colloidal silica suspension diluted with culture medium, G-Sperm^{TM} (Vitrolife, Sweden), to 45% and 90% was used. Semen sample was then placed on the top of two layer gradient dilution and centrifuged at 300xg for 15 min. The pellet which contains the functionally normal spermatozoa is washed twice in IVF-100^{TM} (Vitrolife, Sweden) and centrifuged at 200xg between each washing. Finally the sample was diluted and incubated in 5% CO2 in ambient air 37°C before use for fertilization. Non-motile and abnormal spermatozoa, leukocytes, bacteria etc. are preserved in the separating layer.

In Paper III clinical outcomes of IVF/ICSI was examined. The study patients received embryo transfer with one or alternatively two embryos on day two, three or alternatively on day five after oocyte pick up (OPU). Positive pregnancy test was defined by plasma concentration of β-hCG>15 IU/L.

Time-lapse embryo monitoring

Time-lapse embryo monitoring system is new technology for observing early embryo development. The technique is non-invasive and is used in reproductive medicine to select effectively good quality embryo with optimal implantation potential. The system consists of an IVF incubator with a built-in camera. The embryo is captured repeatedly with defined time intervals without removing it from the incubator. The continuous culture medium, especially designed for time-lapse, makes the pH, osmolality and supporting compounds unchanged during the entire culture period and the intracellular stresses are minimized. Thus, the system serves the possibility of very detailed monitoring of the embryo without disturbance. The images are compiled and create a time-lapse sequence of embryo development which is analyzed subsequently. Many details can be assessed like timing of cell divisions, intervals between cell cycles and other important events including multinucleation, equality of blastomeres and dynamic pronuclei patterns.

Vitrolife “Embryoscope® Time-Lapse System” was used for Paper IV. Embryos were cultured for 2-6 days at 37°C with a gas concentration of 6% CO2, 5%O2 and 89% N_2. All embryos were cultured individually in 25 µL droplets in G-TL^{TM}, (Vitrolife, Sweden), a single step time-lapse medium. For the IVF patients the Embryoslides were prepared with 25 µL droplets covered with 1.4 ml mineral oil, G-Oil^{TM} (Vitrolife, Sweden) the day before oocyte pick-up to equilibrate overnight. For the ICSI patients the slides were prepared the same morning with pre-equilibrated media. The images were taken every 20 minutes in 7 focal planes.

Ethical considerations

Ethical approval was obtained from ethical committee of Lund University and, following written information, the couples were given an option to be excluded from the study.

Statistical methods

The statistical analyses in Papers I, III and IV were done using the Statistical Package for the Social Sciences for Windows (SPSS Inc., Chicago, IL, USA), version 14 and 22. In Paper II calculations were performed with Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA) and graphpad online calculator (www.graphpad.com) for Fisher exact test.

In Paper I coefficient of variation (CV) was adopted using the formula (SD/mean) x 100%. The correlation between the length of the interval between semen sampling and CV of DFI was calculated using Spearman's rho test. Binomial distribution was assumed in calculation of men changing DFI category in two SCSA tests. Proportion was calculated, with 95% confidence interval (CI).

In Paper II percentage of men with $20\% < \text{DFI} \leq 30\%$ and $\text{DFI} > 30\%$, respectively, in relation to the total number of included men was calculated. Cut-off values 20% and respectively 30% for DFI for chances for achieving pregnancy *in vivo* was established according to previous reports (Bungum *et al.*, 2007; Spano *et al.*, 2000). Fisher's exact test was used to examine the significance of the association between two variables in 2x2 contingency table.

In Paper III logistic regression was applied to calculate the chances for IVF/ICSI outcomes expressed as odds ratio with 95 % confidence interval (CI).

Univariate analysis of variance was used for calculations differences in rates for IVF/ICSI outcomes.

Detailed calculations:

- Fertilization rate is expressed as the number of fertilized oocytes as percentage of the number IVF/ICSI procedures. Univariate analysis of variance was applied.
- Embryo quality rate is calculated as the number of good quality embryos (GQE) as a percentage of the number of successful fertilizations. To do this calculation an additional 158 cases where no oocyte were fertilized were excluded. Univariate analysis of variance was performed on 1475 residual procedures.
- Odds ratio (OR) for at least one GQE in those having done OPU were calculated using binary logistic regression.
- Odds ratio for pregnancy for 1107 couples who have undergone embryo transfer with GQE. Binary logistic regression was applied for calculation of OR.
- Odds ratio for miscarriage for 471 women that got pregnant was calculated using binary logistic regression. For this end point, additional calculations were performed for DFI > 40%.
- Successful pregnancy outcome is defined as OR for live births in those who have had OPU. In order to obtain higher statistical power, for this calculation the two highest DFI groups were merged. Moreover the OR for live birth by ICSI was calculated with standard IVF as reference.

In Paper IV following early embryo time-lapse morphokinetic characteristics are defined as study outcomes:

- time of pronuclei appearance (tPNa) - the first observed time point when two separate pronuclei are visible,
- time fading of pronuclei (tPNf) - the first observed time point when pronuclei disappear,
- time early cleavage (t2) - the first observed time point when the newly formed blastomeres are completely separated by confluent cell membranes,
- time starting blastulation (tSB) - the first observed time point when blastocoele is visible.

Univariate analysis of variance was used for calculating differences in morphokinetic mean times for IVF/ICSI outcomes.

Detailed calculations:

- The potential presence of interaction between DFI category (≤10%; 10%-20%; >20%), type of fertilization (IVF or ICSI) and time lapse outcomes (tPNa, tPNf, t2 or tSB). The interaction parameter was defined as “DFI category x fertilization type” and included as independent variable.
- Differences in meantime of morphokinetics in the DFI groups: 10-20% and above 20% with the reference group (≤10%) separately for standard IVF and ICSI.
- Differences in meantime of morphokinetics for each of the three DFI groups mentioned above, by comparing ICSI to standard IVF as reference.

Results in Paper III and IV were adjusted for female age as a covariate.

All the tests were conducted at a significance level of two-sided $p < 0.05$.

Results

Paper I

The coefficient of variation (CV) for DFI in the study group was 30.1% (SD: 21.5%; median 26.9%).

Cut-off value 30% for DFI was established for achieving pregnancy in ART according to previous reports (Bungum, Humaidan, *et al.*, 2007; Spano, *et al.*, 2000). Dichotomization of patients was done according to whether the DFI was ≤ 30% (Category I) or >30% (Category II). The proportion of men switching from one category in the first test to the other category at the second examination was calculated, with 95% confidence interval (CI), based on the assumption of binomial distribution. Results are presented in Figure 7.

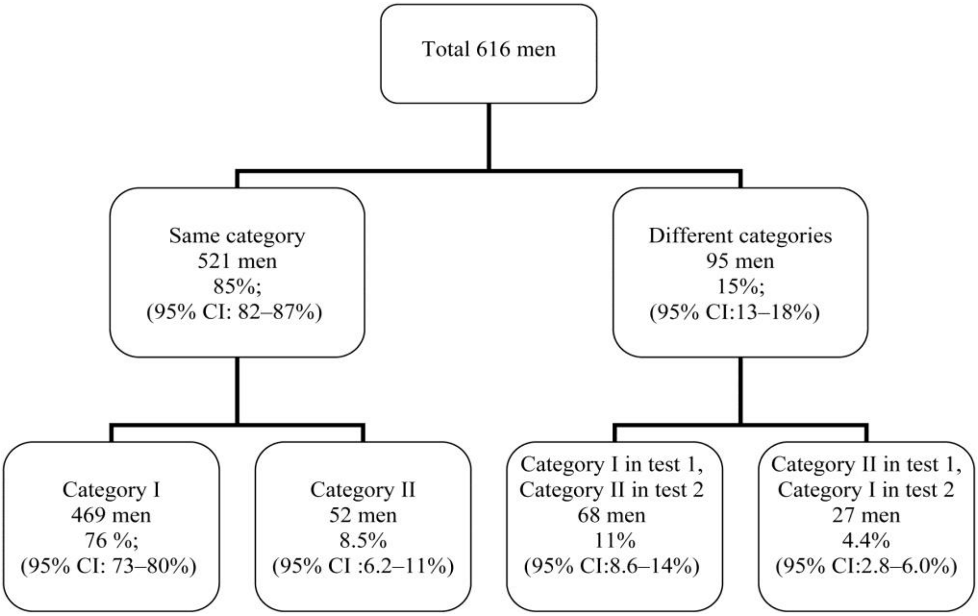

Figure 7. Variation in DFI between tests 1 and 2, in relation to the two categories of DFI (Category I: DFI ≤ 30% and Category II: DFI> 30%), which are used in the assessment of male fertility.

There was no significant correlation between the intra-individual CV and time interval between samples (Spearman's ρ-test; ρ = 019; p = 0.82).

Paper II

In the cohort of couples with diagnosis "unexplained infertility" 17.7% of men (95% CI 10.8%–24.5%) presented with 20% <DFI <30% and 8.4% (95% CI 3.40%–13.4%) had DFI >30%. Previously calculated corresponding figure for men with proven fertility was 10.5% for DFI>20%.

Paper III

The main finding of the study was significantly decreased OR for live birth in standard IVF treatments performed with spermatozoa with DFI above 20% as seen in Figure 8. For this DFI group OR for live birth was significantly higher for ICSI as compared to IVF (OR 1.7; 95% CI: 1.0–2.9; p = 0.05).

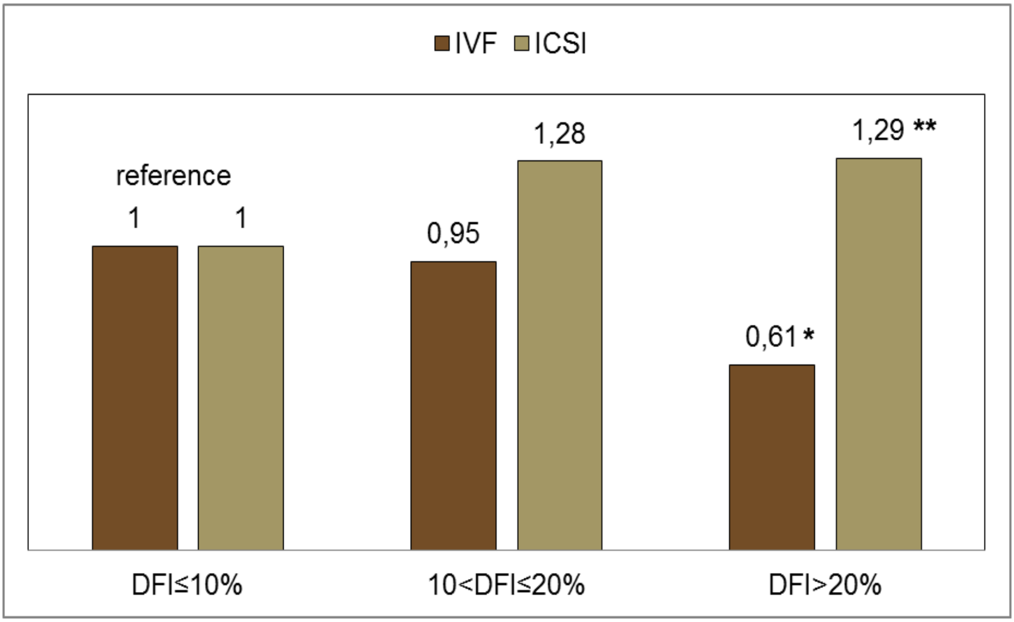

Figure 8. Odds ratio for live birth following OPU according to DFI.
* significant in relation to reference DFI within IVF group (p=0.04)
** significant for ICSI in relation to IVF as reference, for DFI>20% (p=0.05).

Fertilization rate and OR for obtaining at least one GQE were significantly negative associated with DFI in IVF group as depicted in Figure 9 and 10.

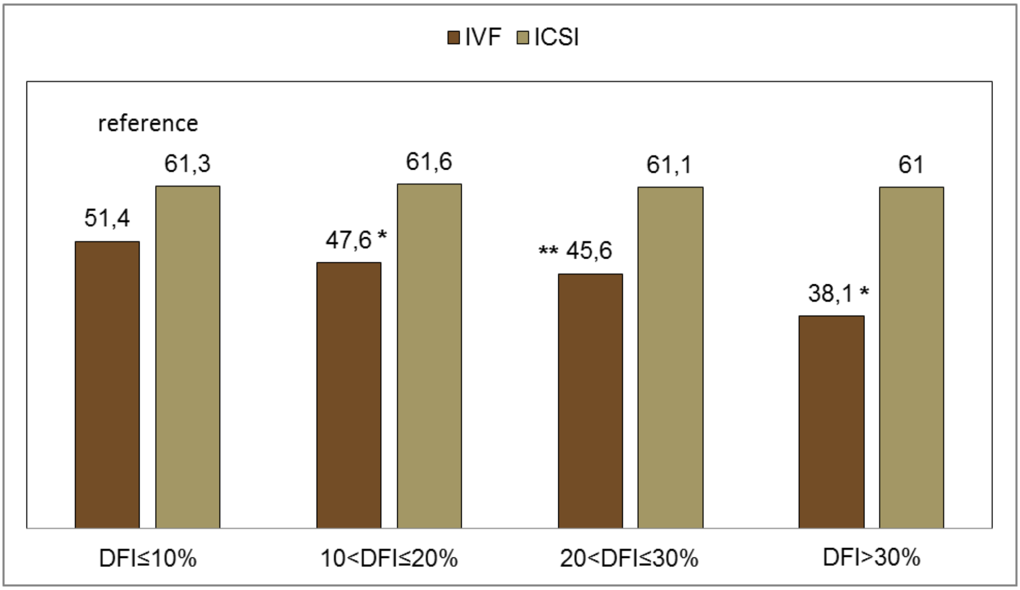

Figure 9. Fertilization rate according to DFI (* p≤0.05; ** p=0.056).

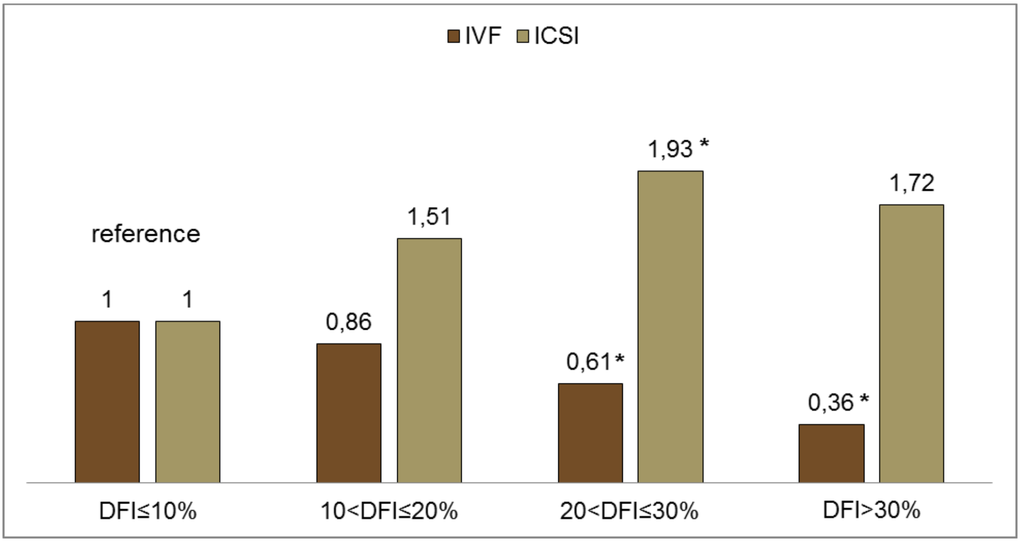

Figure 10. Odds ratio for at least one good quality embryo following OPU, according to DFI (* p≤0,05).

OR for obtaining at least one GQE were significantly increased in ICSI group for 20<DFI≤30 (Fig. 10). OR for miscarriage was significantly increased when IVF and ICSI were merged together and an extra interval for DFI>40 was created (OR 3.8; 95%CI: 1.2–12; p = 0.02). No more significant results for merged IVF and ICSI were observed. No significant results were seen for GQE rate and for OR for achieving pregnancy.

Paper IV

Meantime tPNa was statistically significantly shorter for DFI>10%within ICSI group and independently of the DFI level in the ICSI compared to IVF.

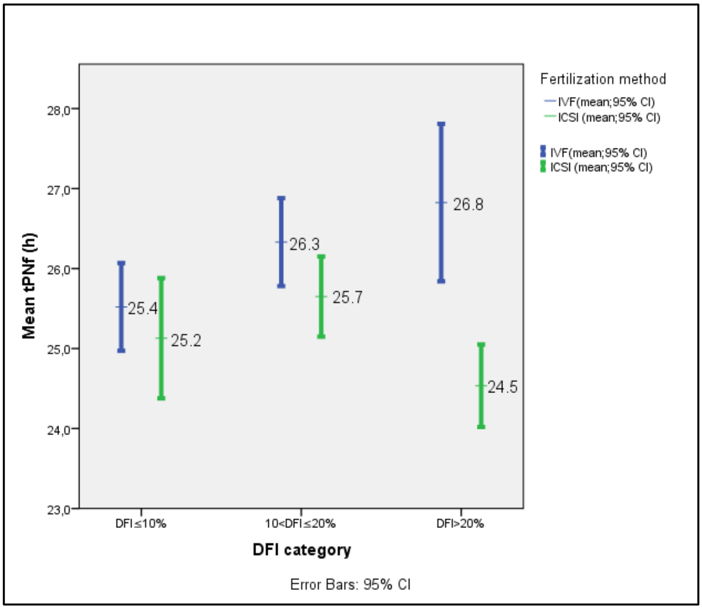

Figure 11. Mean time of PN fading according to IVF or ICSI.

Interaction was observed between DFI category and type of fertilization in relation to the meantime of tPNf. Meantime of tPNf was statistically significantly increased in the standard IVF group for 10%<DFI≤20% and DFI>20%. Statistically significantly shorter mean time tPNf for ICSI compared to IVF for

DFI>20% was observed as shown in figure 11. Similar observation was noted also for t2 as depicted in Figure 12.

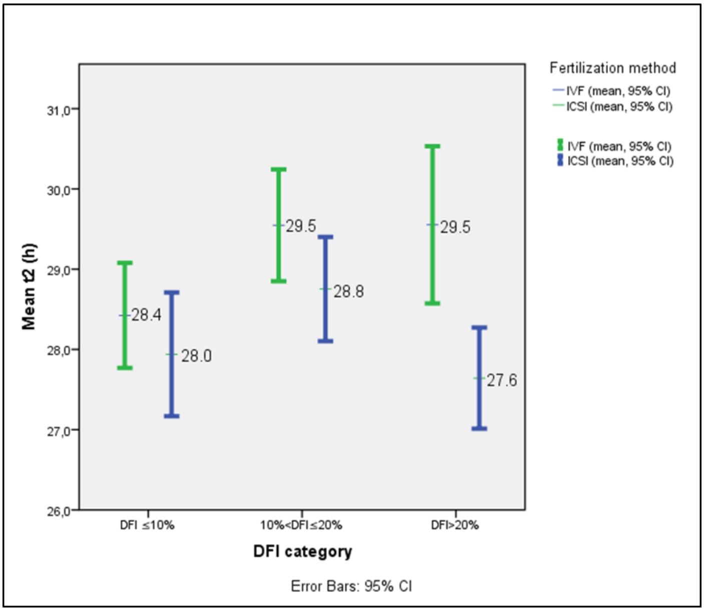

Figure 12. Mean time for early cleavage (t2) according to IVF or ICSI.

Mean time of t2 for standard IVF was statistically significantly increased in the DFI group 10%-20% as compared to the reference group. The meantime of starting (tSB) was longer for ICSI as compared to IVF in DFI≤20.

Discussion

The testing of sperm chromatin integrity is essential for assessment of fertilizing ability *in vivo*. Although clinical value of SCSA in IVF/ICSI therapy is more controversial, the technique is already now often recommended as a standard test to complement CSP in assessment of male fertility potential. This thesis strengthens this recommendation.

The intra-individual variation of DFI in male partners of infertile couples demonstrated in Paper I was 30.1% which is considerable compared to previous studies. The result of SCSA testing was first described as very homogeneous as the CV was 10% (Evenson *et al.*, 1991). However this study was made on a low number of healthy men. The outcome was 29% when the study was derived on men from infertile couples (Erenpreiss *et al.*, 2006). Findings from most of the other studies are located between these two values (Evenson *et al.*, 2000; Zini *et al.*, 2001).The corresponding studies made on CSP show that their intra-individual variation range from 28% to 34% (Leushuis *et al.*, 2010). It illustrates that DFI oscillates to the same extent as sperm concentration, motility and morphology. In Paper I, any lifestyle or medical factors which can affect sperm DNA integrity has been neglected. There are several extrinsic reasons of sperm chromatin damage with proven impact on DFI (described in details in “Background”). Most of them change DFI value temporarily and can be regarded as confounders in the calculation of intra-individual variation. By considering only a few of them calculations seems to be more biased. The pure cohort of men better reflects the real clinical situation. Nevertheless, 85% of the tested men, when repeating the analysis, were still in the same DFI category. Moreover, the CV of DFI is not dependent on the time period between semen samples. These observations give the SCSA a good clinical value. Additionally, according to previous studies, SCSA is a relatively independent measurement of semen quality and its correlation with CSP is weak (Giwercman *et al.*, 2010; Spano *et al.*, 1999). Other studies, using different methods of assessment of sperm DNA damage, confirm this association (Lopes *et al.*, 1998) and show that CSP is not a good predictor of disturbed sperm chromatin integrity (Sakkas *et al.*, 1998). All these findings indicate that CSP and SCSA can work complementarily to each other.

Due to the low accuracy of male fertility testing, the diagnosis ”unexplained infertility” is placed in excess, although abnormalities are likely to be present but are not detected by current methods. Paper II examines the distribution of DFI value in male partners in couples who primarily received the diagnosis “unexplained infertility”. By including only patients with this diagnosis, severe confounders of other infertility factors are eliminated. To the author’s knowledge similarly designed research has not been performed before. The study reveals that 26.1% of men in couples diagnosed as ”unexplained infertile” according CSP have a DFI >20%, previously found to be associated with a decreased fertility *in vivo* (Giwercman *et al.*, 2010). This result was confronted with a previous, retrospective study (Giwercman *et al.*, 2010) which shows that 10.5% of men with proven fertility had a DFI level of 20% or higher. We observed that a statistically significantly higher percentage of men from couples previously diagnosed with traditional diagnostic methods as unexplained infertile had remarkably high degrees of fragmented sperm DNA. These results suggest that cases with diagnosis ”unexplained infertility” can to a certain extent be explained by impairment of sperm DNA. Sperm chromatin integrity assessment may support to differentiate men with fertility problems. The complementary characteristic of CSP and DFI is the potential way to optimize the diagnostic process of infertile couple.

The advantage of the SCSA technique is that it can be exploited in every stage of infertility diagnosis and therapy. Paper I and Paper II have shown its utility in the beginning of the process i.e., to select more effectively men with the presence of male factor of infertility. Papers III and IV are focused on the later stage of the process, when infertile couples already participate in *in vitro* fertilization. The dilemma which fertilization method is most adequate is often still present. The Scandinavian principle to promote standard IVF is based on the anxiety of transmission of genetic diseases through ICSI (Hansen *et al.*, 2002; Hansen *et al.*, 2013). This technology eliminates natural processes of gametes selection during oocyte-sperm interaction which is still active in conventional IVF. On the other hand the frequent adaptation of a standard IVF is connected with some risks and inconveniences like decreased fertilization rate and, complete fertilization failure in extreme cases (Neri *et al.*, 2014; Palermo *et al.*, 2009). The main conclusions coming from Paper III are of particular importance to reduce this decision dilemma. The paper shows that the chance of live birth in standard IVF treatments performed with sperms with DFI above 20% is significantly lower than if sperms with lower DFI are used. Moreover, for the high DFI subgroup the live birth rates were significantly higher for ICSI as compared to IVF. The results corresponded with negative association between DFI and fertilization rate as well as the chance of obtaining at least one GQE- a prerequisite for performing embryo transfer- in standard IVF treatments but not in ICSI. These findings indicate that the control of sperm chromatin integrity assessed by SCSA technique can be helpful to more effectively differentiate men whose sperms have reduced fertilizing ability and in this way optimize the decision about fertilizing method. The effect of high miscarriage risk in *in vitro* pregnancies (both IVF and ICSI) in case of high DFI reported from systematic reviews and meta-analyses (Robinson *et al.*, 2012; Zini *et al.*, 2008) is also seen in our study in combined calculation for both fertilization methods when additional interval with DFI>40 was created (Paper III).

Taking into account the existing knowledge and the conclusions of this thesis we suggest the following formula to utilize SCSA clinically (Figure 13). These guidelines can be customized based on details according to both man's and woman's fertility status.

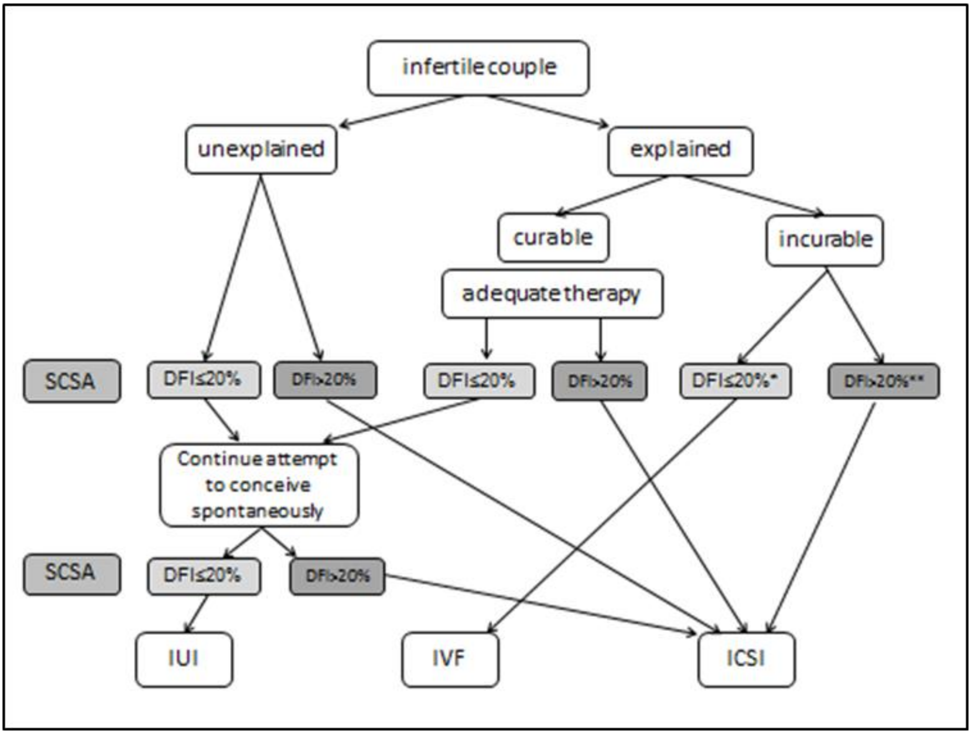

Figure 13. SCSA in clinical practice. * - SCSA preferably done on sample used for ART, other sperm parameters adequate for IVF; ** - SCSA preferably done on sample used for ART, DFI>20% and/or other sperm parameters not adequate for IVF.

Summarizing, the theses in Paper I-III state that sperm chromatin integrity testing with SCSA technique can be an effective tool which can reduce all suboptimal scenarios during the entire procedure of infertility investigation and therapy. It's clearly beneficial for all parties of the process. Infertile couple can get more apt diagnosis and can react more accurately, either continue with the most proper treatment method, or contrary- depending on other biological, economic or social factors continue attempts to conceive spontaneously. The infertility clinics can improve their results and optimize resource management. Since many clinics operate on the basis of public financing, all these advantages have a positive impact on public economy. Furthermore, the question of possibilities of pharmacological (Hamada *et al.*, 2012) and surgical (Smit *et al.*, 2010; Zini *et al.*, 2005) therapy in order to improve sperm chromatin integrity is still open. If the reports of the clinical effects of respective therapies (Showell *et al.*, 2014) are definitely proven, it will open a new crucial demand for an effective method for monitoring the effects of this treatment.

In addition to clinical conclusions arising from the thesis, it also contributes to the theoretic knowledge about the potential impact of sperm DNA integrity on the development of early embryo. It is worthwhile to emphasize that all the observations of significant impact of sperm DNA damage on the pregnancy outcomes concern spontaneous fertilizations. This is obviously the truth for pregnancy *in vivo* both by intercourse (Spano *et al.*, 2000) and IUI (Bungum *et al.*, 2007). But even concerning *in vitro* results the significant finding is only relevant to standard IVF, i.e., the situation where fertilization itself happens spontaneously, even in the IVF laboratory (Paper III) (Evenson and Wixon, 2006; Osman *et al.*, 2015). This rule doesn't apply in cases of ICSI. Thus, it's not IVF but ICSI, with its totally artificial fertilization, overcomes the detrimental effect of impaired sperm chromatin integrity on fertilization process. The concept is illustrated in Figure 14.

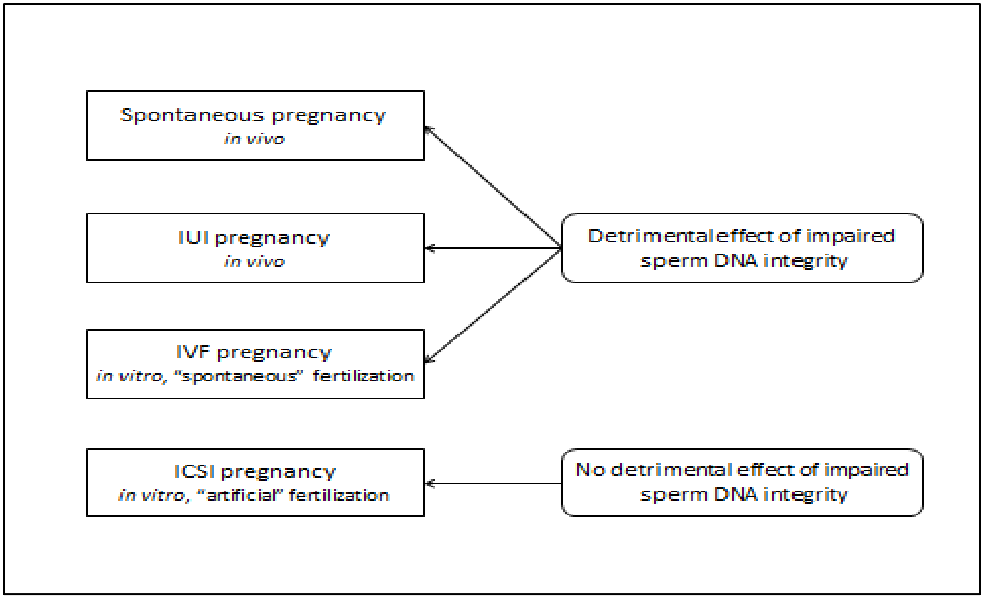

Figure 14. Effect of impaired sperm DNA integrity on fertilization.

Moreover, the processing of statistical material concerning standard IVF performed in Paper III have shown a characteristic rule. All the calculations made in relation to the base data i.e., number of inseminated oocytes have shown significantly decreased outcome results. Contrary, none of calculations made in relation to the later "checkpoints" was significant. This observation allows the author to hypothesize that the potential negative influence of paternal genome deriving from sperms with highly damaged chromatin is placed on the early stage of embryo development. The observation is depicted on the Figure 15. Paper IV examines this hypothesis. It is a continuation of the study made in Paper III although it concerns the laboratory outcomes instead of clinical ones.

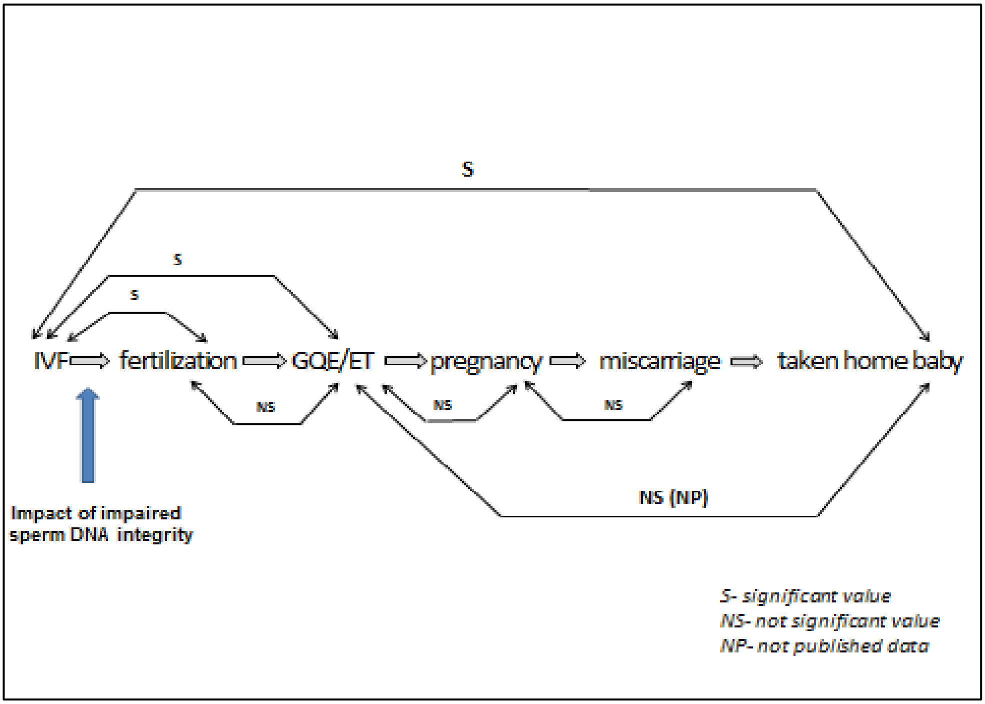

Figure 15. SCSA and standard IVF fertilization outcome.

It has been shown on animal studies that oocytes and early embryos, to a certain limited extent, have the capability to repair sperm DNA damage (Genesca *et al.*, 1992). According to previous reports embryonic genome significantly expresses first in the third cleavage i.e., between the four- and eight-cell stage (Braude *et al.*, 1988). The current question is to what extent the amending ability depends on oocyte capabilities and how much on efficiency of paternal genome's internal repair mechanisms. Many authors point out the considerable role of oocyte in this process (Adenot *et al.*, 1997). McLay shows in his study that the ability to remodel sperm chromatin from protamine-associated DNA of the sperm into functional somatic-like chromatin with the removal of sperm protamines followed by the addition of oocyte histones develops in oocytes during meiotic maturation (McLay and Clarke, 1997) (see relevant section in “Background”). These results reveal that the maturing oocyte has a full panel of mRNAs coding for nucleotide repair and thereby have the capacity to modify the structure of the paternal chromatin (Menezo *et al.*, 2007; Osman *et al.*, 2015). However, the influence of paternal genome seems to be substantial as well (Ioannou *et al.*, 2015; Tesarik *et al.*, 2002). Unrepaired DNA damage that remains above a crucial limit has been considered to result in the arrest of the embryo development (Dumoulin *et al.*, 2000; Seli *et al.*, 2004). All these findings are paralleled by several previous reports noting high predictive value of the early cleavage parameters on embryo quality and its implantation potential (Lundin *et al.*, 2001; Salumets *et al.*, 2003; Van Montfoort *et al.*, 2004). Paper IV examines how much early embryo morphokinetic characteristics depend on sperm chromatin integrity. The study was focused on very early embryonic developmental stage described by Simon as peri-fertilization effects of sperm DNA damage (Simon *et al.*, 2014) i.e., its main outcomes were embryo morphokinetics within the first cell cycle: tPNa, tPNf ,t2 and additionally tSB as the last outcome before ET. Few studies that addressed this, initial period in embryo development note a significantly faster development of ICSI embryos compared with standard IVF. This general observation, regardless DFI value, is also seen in our study. However, all of them note a characteristic pattern that this discrepancy diminishes and disappears after the first division (Kirkegaard *et al.*, 2012), 3-cell stage (Dal Canto *et al.*, 2012), in day two (Lemmen *et al.*, 2008) or after t4 stage (Bodri *et al.*, 2015). Cruz hypothesizes that the observed time difference could reflect in standard IVF unknown variability in sperm penetration of *corona radiata* and *zona pellucida* as well as fertilization timing and suggests that when PNf, rather than time of insemination, was established as start time, the differences between the two procedures disappeared. This time difference could actually be constant but just not significant at the later stages of development due to the larger variability of the late-stage parameters (Cruz *et al.*, 2013). Since none of these studies was related to sperm DNA integrity testing, it can be a point for future research. So far only one report has been focusing on the association between time-lapse embryo development parameters and sperm DNA fragmentation (Wdowiak *et al.*, 2015). After analyzing 165 couples who underwent ICSI which led to ET, they found that embryos developed from sperms with low DFI reached the blastocyst stage faster than embryos from a high DFI. In our study DFI doesn’t change significantly tSB neither within IVF nor ICSI group. However, our two studies differ in many aspects i.e., the technique for assessing sperm DNA, sample characteristic and sample size, finally, the time-lapse parameters. But yet, significant impact of DFI is seen during peri-fertilization stage. The results are significant only for some morphokinetic characteristics. In general, a high DFI results in longer mean times for the morphokinetic characteristics assessed within the IVF group and shorter or neutral in ICSI group. The influence of sperm DNA integrity on early embryo development was examined before, utilizing Comet assay and standard embryo assessment (Simon *et al.*, 2014). It confirmed the impact of sperm DNA damage on embryo development. These findings together with previous observations (Paper III) favoring ICSI instead of IVF in cases of high DFI indirectly suggest that sperm DNA integrity plays an important role not only in fertilization moment but also in early embryo development. ICSI procedure which omits processes of natural selection during sperm-oocyte interaction permits fertilization with sperm with high DFI which changes morphokinetic characteristics of embryo development. Successful ICSI fertilization with sperm with high DFI gives a higher chance to achieve at least one good quality embryo, a higher chance for live birth, however, at the same time a higher risk for miscarriage. This rule is partly valid also for standard IVF which is exemplified in higher miscarriage risk in cases of high DFI which concerns both fertilization methods (Paper III), which is in agreement with previous review reports and meta-analyses (Robinson *et al.*, 2012; Zini *et al.*, 2008).

The biological explanation of superiority of ICSI over the IVF technique in case of increased DFI is not directly documented. There are several possible explanations. IVF and ICSI contrast considerably according to culture environments. IVF oocytes are exposed to spermatozoa for longer time, contrary to ICSI, when the spermatozoon are injected directly into the oocyte and therefore probably less exposed to ROS than in IVF. Oxidative stress originates from different sources during the IVF process. The major source of ROS is an estrogenic compound of the oocyte (Bennetts *et al.*, 2008). In the IVF environment, the whole cumulus oophorus i.e., oocyte and surrounding it *corona radiata* is placed together with sperms. Corona cells are a considerable source of estradiol. Contrary in the ICSI environment, all corona cells are removed. It is observed that sperms with high DFI are more sensitive to the harmful effects of ROS (Kattera and Chen, 2003), which has also been proven to have a straightforward unfavorable effect on the embryo (Valbuena *et al.*, 2001). Also culture media itself represent suboptimal conditions for sperm cells. The ICSI procedure provides spermatozoa within a short time into the optimal circumstances in the oocyte (Dumoulin *et al.*, 2010). A huge amount of sperm placed together with one oocyte during the standard IVF process may release a lot of ROS as well (Lewis *et al.*, 2013). Generally a standard IVF procedure is associated with a high exposure of both gametes on oxidative stress. This can have a negative influence on sperm chromatin integrity and paternal genome after fertilization. Another explanation is that in the ICSI group, infertility is mainly caused by male factor which means that women in this group might be more fertile, e.g., due to a younger age, and possibly produce oocytes with a better DNA repair capacity (Bungum *et al.*, 2007), which was confirmed by the observation that donors' high-quality oocytes atone the negative effect of sperm chromatin damages in early embryo development (Meseguer *et al.*, 2011). Evidence that the method of fertilization can improve the repair of paternal DNA is lacking (Osman *et al.*, 2015). Therefore, the claim that ICSI takes advantage over IVF is based on empirical observation. Paper IV is long from any definitive conclusion. It's more the beginning of discussion about the impact of sperm chromatin integrity on early embryo morphokinetics and its clinical consequences and stimulus to further research.

All the studies in this thesis are based on the relatively large and complete cohorts of study sample compared to corresponding publications on the respective topics. SCSA was performed on the semen sample used for fertilization. Woman's age, as the most important confounder was included to the calculations as a covariate in Paper III and IV. Other potential confounders like woman's BMI and ovarian reserve were taken into account already during the patient's recruitment however, the latter one, apart from the cases with extreme poor ovarian reserve, wasn't a hindrance for being accepted for ART and included to the study sample. Socioeconomic and ethnical factors were not adjusted. The retrospective design of the studies represents their major weakness. Especially the results of Paper III and IV warrant the continuation with well-designed, prospective trials. Technological development in both sperm genomic diagnosis and observation of early embryo by time-lapse provide opportunities to considerable progress. Thus, ideally, the patients with high DFI fulfilling the criteria for standard IVF should be randomized to this treatment or to ICSI. Having in mind high heterogeneity of both CSP and DFI (Paper I), collecting enough number of men with repeatable high DFI and at the same time repeatable normal CSP to achieve satisfactory trial's power seems to be the biggest logistic problem. Such studies are not yet available but our results that indicate impairment of the outcome of standard IVF for DFI exceeding the level of 20% facilitates a design for future studies.

Concluding remarks

The conclusions of this thesis implicate further progress in assessing the clinical value of SCSA technique and the next step in the application of the method to the clinical praxis. The technique can be an effective device both during the infertility investigation and therapy. This can reduce risk for potential suboptimal scenario during the whole way of ART procedure.

The detailed conclusions from the studies are:

- intra-individual variation of the SCSA/DFI in men from infertile couples is 30.1% and this value is classified as high,
- the test performed once has an 85% chance that repeated test remains on the same side of cut-off value of 30% which gives the test acceptable clinical value,
- SCSA is useful as a complement to CSP, to effectively select more men with present “male factor” of infertility, who according to previous studies have low chance to conceive spontaneously,
- SCSA is useful for differentiation of couples for whom ICSI or alternatively standard IVF is the appropriate fertilization method,
- sperm DNA damages may lead to some early embryo morphokinetics changes, which suggest that sperm chromatin integrity plays an important role not only in the fertilization act but also in early embryo development. SCSA and time-lapse technologies seem to be useful in future research, which examines this observation in more detail.

Future perspectives

To verify the results of this thesis by randomized prospective trials seems to be the hottest challenge in the nearest future. A theoretically optimal design of such a trial should include patients with high DFI fulfilling the criteria for standard IVF randomized to this treatment or to ICSI. Collecting enough number of men with repeatable high DFI and at the same time repeatable normal CSP to achieve a satisfactory trial's power seems to be the biggest logistic problem in this situation when both DFI and CSP are characterized with high heterogeneity.

The research on the relationship between sperm DNA integrity and early embryo development using time-lapse technique should be continued. The technological progress opens new possibilities in this area and research carried out so far seems to be only the beginning.

In order to achieve higher accuracy an idea to combine SCSA technique with other modern method for assessment of male factor can be an interesting idea to investigate, e.g., high magnification optical technology (Bartoov *et al.*, 2001) or sperm hyaluronic acid binding assay (Huszar *et al.*, 2003). Even combination with ROS analysis in order to detail examination its different impact on paternal genome during IVF and alternatively ICSI procedure should be considered.

Svensk sammanfattning

Bakgrund och syfte

Utredningen av manlig infertilitetsfaktor baseras sedan flera decennier på standard spermieparametrar. Denna traditionella, ljusmikroskopiska metod utvärderar spermavolym, spermiekoncentration, rörlighet och morfologi. Metoden är emellertid subjektiv, dåligt standardiserad och har ett lågt värde i bedömningen av manlig fertilitetsförmåga.

Samtidigt, har assisterad reproduktionsteknologi (ART) genom de senaste decennierna utvecklats som en av de viktigaste åtgärderna mot infertilitet. Enorma framsteg har gjorts vad gäller möjligheter för hormonstimulering, teknisk utrustning och nya laboratorietekniker. Trots denna utveckling är resultaten av ART behandlingarna under de senaste decennierna oförändrade. En av orsakerna kan vara bristen i metoderna för diagnos och terapi av manliga infertilitetsfaktorer. Man har därför sökt efter nya metoder för bedömning av spermakvalitet. En stor del av dessa fokuserar på spermie kromatinintegritet, det vill säga om huruvida det finns brott i spermiens kromosom eller inte. Det finns et flertal analyser, bland annat ”sperm chromatin structure assay” (SCSA) som har visat sig vara kliniskt användbar. Efter tillsättning av ett fluorescerande färgämne färgas spermier med kromosombrott röda och normala spermier gröna. Andelen av spermier som färgas röd kallas för DNA-fragmenteringsindex (DFI) och visar andel spermier med kromosombrott. Kliniska studier har visat att SCSA/DFI har ett högt prediktivt värde när det gäller graviditet *in vivo* d.v.s. när befruktningen sker i kvinnans kropp. Man har också visat chansen för att uppnå spontan graviditet eller vid intrauterin insemination sjunker när DFI överstiger 20 % och blir obefintlig vid DFI 30 % eller högre. Andra studier har visat att SCSA och standardparametrar är relativt oberoende av varandra. När det gäller *in vitro*-fertilisering (IVF) och mikroinjektionsbehandling (ICSI) resultaten är det mer oklart om huruvida DFI spelar en roll för chanserna till att uppnå graviditet.

Syftet med denna avhandling var att undersöka det kliniska värdet av SCSA i diagnostik och behandling av manlig infertilitet.

Metoder och resultat

Delarbete I

Här studerats den intra-individuella variationen av DFI hos 616 män från par som genomgick infertilitetsutredning. Männen lämnade 2-7 prov. Variationskoefficienten (CV) för DFI i studiegruppen var 30,1%. Patienter delades i grupper beroende på om DFI var ≤ 30 % (kategori I) eller> 30 % (kategori II). Vid upprepade SCSA är det emellertid 85% chans att DFI förblir på samma sida av cut-off värdet på 30% vilket ger testet ett acceptabelt kliniskt värde.

Resultaten visade ingen signifikant korrelation mellan den intraindividuella CV och tidsintervallen mellan proverna.

Delarbete II

Bland 212 par under infertilitetsutredning blev 119 med diagnosen oförklarad infertilitet identifierats. Procentandelen män med DFI> 20 % eller DFI> 30 % beräknades. I gruppen med diagnosen oförklarad infertilitet hade 17.7% av männen 20 <DFI <30 medan 8.4 % hade DFI> 30 %. I grupp av män med beprövad fertilitet, 10.5% av dem hade DFI>20%. Konklusionen är att en signifikant andel av män med diagnosen oförklarad infertilitet har anmärkningsvärt hög andel av spermier med kromatinbrott.

Delarbete III

I en grupp av 1633 IVF/ICSI cykler undersöktes sambandet mellan DFI och resultatet av behandling. DFI värden blev indelat i fyra intervaller: DFI ≤ 10 % (referensgrupp) 10 % <DFI ≤ 20 %, 20 % <DFI ≤ 30 % och DFI> 30 %. För de tre sistnämnda intervallen analyseras resultaten av IVF/ICSI i förhållande till referensgruppen: befruktning, embryo av god kvalité (GQE), chans för graviditet och levande födda barn samt risk för missfall. Resultaten visade att för de par som genomgick standard IVF var där en negativ sammanhäng mellan DFI och fertiliseringsgrad. Chansen för att ha ett embryo av god kvalité och få ett levande född barn var också mindre vid stigande DFI. Inga sådana associationer sågs i ICSI gruppen. Resultaten tyder på att i fall med hög DFI bör ICSI vara föredragen behandlingsmetod.

Delarbete IV

I en grupp av 256 IVF och 383 ICSI-behandlingar (6117 ägg) undersöktes sambandet mellan DFI och tidig embryo utveckling med hjälp av et time-lapse system, det vill säga en fortlöpande bildtagning av embryon under utveckling. DFI värden indelats i 3 intervaller: DFI ≤10% (referensgrupp), 10 % <DFI≤20% och DFI> 20 %. Resultaten visade att ett högt DFI förlänger tiden för embryoutveckling för IVF medan den i ICSI gruppen är kortare eller neutral.

Slutsatser

Den intraindividuella variationen av SCSA/DFI hos män från infertila par är hög. Vid upprepade SCSA är det emellertid 85% chans att DFI förblir på samma sida av cut-off värdet på 30% vilket ger testet ett bra kliniskt värde. I diagnosen av infertilitet är SCSA ett gott komplement till standard spermaanalysen. SCSA är också användbart i valet av den optimala behandlingen för ett givet par.

Spermie kromatinintegritet spelar en viktig roll i befruktningen och den tidiga embryoutvecklingen.

Sammanfattningsvis implicerar denna avhandling ytterligare framsteg vad gäller det kliniska värdet av SCSA och tillämpningen av metoden till den kliniska praxisen. SCSA kan vara till stor hjälp både under infertilitetsutredning och behandling.

Streszczenie w języku polskim

Wprowadzenie i cel pracy:

Diagnostyka męskiej niepłodności oparta jest od dziesięcioleci na klasycznym badaniu standardowych parametrów nasienia. Ta tradycyjna metoda mikroskopowa ocenia objętość nasienia, stężenie plemników, ich ruchliwość i morfologię. Metoda ta ma niestety niską wartość predykcyjną dla oceny niepłodności męskiej.

W ostatnich dziesięcioleciach, dominującą role w leczeniu niepłodności zajęła tzw technologia wspomaganego rozrodu (assisted reproductive technology, ART). Ogromne postępy poczyniono w zakresie możliwości farmakologicznej stymulacji hormonalnej, wyposażenia technicznego oraz powstawania nowych technik laboratoryjnych. Pomimo tak znaczącego postępu, skuteczność lecznia niepłodności natrafiła na pewną barierę i nie rośnie znacząco w ostatnich kilkunastu latach. Brak postępu w diagnostyce i terapii czynnika męskiego niepłodności może być jedną z przyczyn tego stanu. Z tego powodu, na całym świecie trwają badania nad nowymi, nowoczesnymi metodami oceny jakości nasienia. Znaczna część z tych badań koncentruje się na ocenie struktury chromatyny płciowej plemnika. Wśród nich test SCSA (sperm chromatin structure assay) wydaje się być najbliższy wdrożeniu do praktyki klinicznej. Po dodaniu oranżu akrydyny (AO) oraz zastosowaniu cytometru przepływowego fragmenty prawidłowego DNA emitują zielone światło fluorescencyjne natomiast fragmenty uszkodzone emitują światło czerwone. Frakcja plemników wybarwionych na czerwono zwana jest DFI (DNA fragmentation index) i pokazuje odsetek plemników z uszkodzonym DNA. Badania kliniczne wykazały, że SCSA/DFI ma dużą wartość predykcyjną zakresie określenia szans na uzyskanie ciąży w warunkach *in vivo*, tzn.gdy zapłodnienie następuje w organizmie kobiety. Zarówno jeśli chodzi o ciążę całkowicie naturalną jak i tę uzyskaną przy pomocy wewnątrzmacicznej inseminacji, szanse na jej uzyskanie zaczynają maleć gdy DFI przekracza 20%, a balansuje w okolicach zera dla DFI 30% lub wyższego. Ponadto okazało się, że SCSA i standardowe parametry nasienia są testami od siebie niezależnymi. Jeśli chodzi o technologię *in vitro* (standardowe zapłodnienie *in vitro*- IVF oraz mikroiniekcję- ICSI) to wyniki są bardziej rozbieżne.

Celem tej pracy jest zbadanie wartości klinicznej SCSA w diagnozowaniu i leczeniu czynnika męskiego bezpłodności.

Material, metody i wyniki:

Praca I:

Dokonano analizy zmienności wewnątrzosobniczej testu SCSA na próbie 616 mężczyzn wśród par, poddanych diagnostyce niepłodności, u których co najmniej dwa razy wykonano badanie. Współczynnik zmienności (CV) dla DFI w grupie badanej wynosił 30.1%. Pacjentów podzielono na dwie grupy: kategoria I gdy DFI ≤ 30% oraz kategoria II gdy DFI> 30%. Następnie zbadano odsetek mężczyzn, którzy zmienili kategorię w drugim badaniu. W rezultacie stwierdzono, iż z prawdopodobieństwem 85% powtórzona próba SCSA da wynik znajdujący się w tej samej kategorii. Obserwacja ta daje badaniu SCSA akceptowalną wartość kliniczną.

Praca nie wykazała znaczącej korelacji między współczynnikiem zmienności CV a odstępem czasu między dwoma badaniami.

Praca II:

Wśród 212 par poddanych diagnostyce niepłodności, 119 otrzymało rozpoznanie niepłodności o nieustalonej przyczynie (idiopatycznej). W grupie tej, u 17,7% mężczyzn badanie nasienia wykazało 20 <DFI <30 a 8,4% mężczyzn miało DFI> 30%. Analogiczne badanie w grupie mężczyzn płodnych wykazało, że 10.5% z nich ma DFI>20%. Statystycznie stotny odsetek mężczyzn z rozpoznaniem "niepłodności idiopatycznej" zgodnie z klasyczną metodą badania nasienia miało podwyższony współczynnik DFI.

Praca III:

W grupie 1633 procedur IVF lub ICSI zbadano związek między DFI a wynikami leczenia przy pomocy technik *in vitro*. Wartości DFI podzielono na cztery zakresy: DFI ≤ 10% (referencyjny) 10% <DFI ≤ 20%, 20% <DFI ≤ 30%,> 30% DFI. Dla trzech ostatnich przedziałów zanalizowano następujące wyniki IVF / ICSI w odniesieniu do grupy referencyjnej: odsetek zapłodnionych oocytów, zarodki o wysokiej jakości (GQE), ciąże zakończone poronieniem i żywe urodzenia. W grupie ze standardowym IVF stwierdzono statystycznie istotną ujemną zależność pomiędzy DFI i odsetkiem zapłodnionych oocytów. Szansa uzyskania co najmniej jednego zarodka dobrej jakości oraz szansa urodzenia dziecka było znacząco niższe w grupie standardowego IVF gdy DFI> 20%. Takich zależności nie stwierdzono w grupie ICSI. Wyniki sugerują, że ICSI może być optymalną metodą leczenia *in vitro,* w przypadku wysokiego DFI.

Praca IV:

W badaniu retrospektywnym opierającym się na obserwacji 6117 oocytów poddanych zapłodnieniu IVF (256) oraz ICSI (383) zanalizowano ewentualny związek pomiędzy DFI a wczesnym rozwojem embrionów z wykorzystaniem technologii ”time-lapse” polegającej na wykonywaniu zdjęć w regularnych odstępach czasu a następnie wyświetlanie ich w przyśpieszonym tempie. Praca wykazała, iż podwyższone DFI wydłuża czas rozwoju embrionu w pierwszej dobie w grupie standardowego IVF, natomiast skraca ten czas lub jest całkowicie neutralne w grupie ICSI.

Podsumowanie:

Zmienność wewnątrzosobnicza SCSA/DFI u mężczyzn z niepłodnych par jest wysoka i wynosi 30,1%. Jednak 85% szans, że powtórzony test pozostaje na tej samej stronie wartości referencyjnej 30% daje badaniu kliniczne akceptowalną wartość kliniczną. SCSA może stanowić uzupełnienie do klasycznego badania nasienia, bardziej efektywnie selekcjonuje mężczyzn z obecnością czynnika męskiego niepłodności. SCSA jest użyteczny do różnicowania pacjentów, dla których ICSI lub IVF jest odpowiednią metodą zapłodnienia. Integralność DNA plemników odgrywa ważną rolę nie tylko w momencie zapłodnienia, ale również we wczesnym rozwoju zarodka. SCSA i technika ”time-lapse” wydają się być przydatne w przyszłych badaniach dotyczących tego zagadnienia.

Podsumowując, rozprawa ta stanowi dalszy postęp w zakresie oceny wartości klinicznej technologii SCSA i jest kolejnym krokiem ku wdrożeniu metody do praktyki klinicznej. Metoda ta może być skuteczna zarówno w diagnostyce i leczeniu niepłodności i może zmniejszyć ryzyko ewentualnych negatywnych scenariuszy dla wszystkich procedur wspomaganego rozrodu.

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Original publications

Paper I

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human reproduction ORIGINAL ARTICLE ***Andrology***

Intra-individual variation of the sperm chromatin structure assay DNA fragmentation index in men from infertile couples

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BACKGROUND: The sperm chromatin structure assay (SCSA) is a valuable tool for prediction of fertility *in vivo*, with DNA fragmentation index (DFI) of 30% as a clinically useful cut-off level. Previous studies on fertile men have shown a high level of repeatability, with an intra-individual variability in DFI of ~10%. However, conflicting data on how much the DFI fluctuates within individuals exist. The aim of the present study was to investigate the intra-individual variation of DFI in order to further evaluate the clinical use of SCSA.

METHODS: Among 2409 consecutive men under infertility investigation, repeated SCSA analyses were performed on 616 samples from men between 18 and 66 years of age. The coefficient of variation (CV) for DFI was calculated. For each patient, we also analyzed whether the DFI value in tests I and II switched the category from <30 to >30%, or *vice versa*.

RESULTS: Mean CV for DFI for men with at least two SCSA analyses within a 30-month period was 30.1% (SD 21.5). Compared with the first test, 85% (95% confidence interval: 82–87%) of the men remained on the same side of the cut-off point of 30%.

CONCLUSIONS: Despite showing a high intra-individual CV for DFI, 85% of the men from infertile couples did not change category between tests, with respect to the cut-off level of 30%. Thus, using the previously established DFI cut-off value of 30%, a single SCSA analysis has a high predictive value for assessing fertility *in vivo*.

Key words: sperm DNA / DNA fragmentation index / infertility / intra-individual variation

Introduction

Conventional semen analysis, including assessment of sperm counts, morphology and motility, is a standard laboratory test of male fertility, according to the World Health Organization (2010). However, these parameters are not sufficient to interpret the fertility status or chance of pregnancy in a couple (Bonde *et al.,* 1998; Auger *et al.,* 2000; Guzick *et al.,* 2001; Jequier, 2004), regarding neither natural nor assisted conception. A search for better predictors of fertility has brought the genomic integrity of the male gametes in focus (Reviewed in Agarwal and Said, 2003; Erenpreiss *et al.*, 2006b) and during the last decades several methods to assess sperm DNA damage have been developed. The sperm chromatin structure assay (SCSA®), a flow cytometric technique first described by Evenson *et al.* (1980), is one such test that provides additional information about the fertility capacity of the sperm. With SCSA the proportion of spermatozoa with impaired DNA integrity, expressed numerically as the DNA fragmentation index (DFI), is measured. SCSA was shown to be an independent marker of fertility *in vivo*, defined as the capability to get pregnant by either intercourse (in unstimulated cycle or after ovulation stimulation) or by intrauterine insemination (Evenson *et al.*, 1999; Spanò *et al.*, 2000; Bungum *et al.*, 2004; Evenson and Wixon, 2006b; Giwercman *et al.*, 2010). The SCSA has also a potential to contribute to more efficient use of *in vitro* assisted reproduction techniques (ARTs) in the future (Evenson and Wixon, 2006a; Bungum *et al.*, 2007).

A well-known problem with using conventional semen analysis as a diagnostic tool is the high intra-individual variation reported for sperm concentration, motility and morphology (Mallidis *et al.*, 1991; Amann and Hammerstedt, 1993; Alvarez *et al.*, 2003; Keel, 2006). In contrast, previous studies on men who had a DFI of ~10% have shown a high level of repeatability (Evenson *et al.*, 1991, 2002). A comprehensive study on the variation of multiple SCSA measures for non-infertility patients showed SCSA measures which were significantly lower than those derived using common semen measures. In a study by Evenson *et al.* (1991), semen samples collected once per month for

8 months from 45 men (recruited by newspaper advertisement) were assessed using the common semen parameters and SCSA. The green versus red fluorescence cytogram patterns were strikingly homogeneous within a donor overtime, with a mean coefficient of variation (CV) for within donor green fluorescence of 3%, and for red fluorescence of 7%. Furthermore, average intra-individual CV for DFI expressed as a percentage of any given individual's mean was around 10%, which is significantly lower than that derived from measures of common semen parameters. From these observations the authors concluded that 'the SCSA is an objective, technically sound, biologically stable, sensitive and feasible measure of sperm quality'.

However, in another study, the intra-individual CV for DFI was found to be between 18 and 25% (Spano *et al.*, 1998). In addition, more recently a single study has reported a significant intra-individual variation in infertile men with a CV of ~30% (Erenpreiss *et al.*, 2006a), corresponding to the magnitude of intra-individual variation reported for other standard sperm parameters (Erenpreiss *et al.*, 2008; Castilla *et al.*, 2010).

However, unlike other sperm parameters, DFI has a distinct cut-off value for infertility *in vivo* (when exceeding 30%) and is, therefore, a clinically applicable fertility marker (Evenson *et al.,* 1991, 1999; Spanò *et al.*, 2000; Bungum *et al.,* 2007; Giwercman *et al.,* 2010). Thus, from a clinical point of view, the proportion of subjects who are switching between levels above and below 30% is more important than the magnitude of the intra-individual CV. In order to further elucidate this issue, we aimed to investigate the variation of DFI in repeated tests from the same patient, both in fertility work-up and during ART treatment. In particular, the study was aimed at assessing the feasibility of using SCSA in a clinical environment where the control of patient behavior, access to patient information and opportunity to maintain the highest levels of assay control and standardization may not be possible.

Materials and Methods

Patients

The study is based on a database of 2409 men aged between 18 and 66 years (mean 34.3 ± SD 6.3) who underwent infertility investigation and/or ART treatment at the Reproductive Medicine Centre, Skåne University Hospital, Malmö, Sweden, during the period May 2007 to November 2009. Six hundred and sixteen men with at least two SCSA (2–7) analyses were included in this retrospective observational descriptive study.

In order to obtain sufficient numbers of sperm for SCSA analysis, only men having a sperm concentration of at least 1×10^6/ml in neat semen were included in the study.

Semen collection and standard sperm analysis

Semen samples were collected by masturbation after the recommended abstinence period of 2–7 days. Standard semen analysis was performed according to the WHO guidelines (WHO, 1999).

Sperm chromatin structure assay

The principles and procedure to measure sperm DNA damage by flow cytometry SCSA are described in detail elsewhere (Evenson and Jost, 2000; Spanò *et al.*, 2000; Bungum *et al.*, 2004). In brief, the SCSA is based on the phenomenon that a 30 s treatment with a pH 1.2 buffer denatures the DNA at the sites of single- or double-strand breaks, whereas normal double-stranded DNA remains intact. Thereafter, the sperm cells are stained with the fluorescent DNA dye Acridine orange, which differentially stains double- and single-stranded DNA. After blue light excitation in a flow cytometer, the intact (double-stranded) DNA emits green fluorescence, whereas denaturated (single-stranded) DNA emits red fluorescence. Sperm chromatin damage is quantified by the flow cytometry measurements of the metachromatic shift from green (native, double-stranded DNA) to red (denatured, single-stranded DNA) fluorescence and displayed as red versus green fluorescence intensity cytogram patterns. The extent of DNA denaturation is expressed as the DFI, which is the ratio of red to total fluorescence intensity i.e. the level of denatured DNA over the total DNA. The frequency histogram of DFI provides a more precise calculation of percentage DFI than the use of computer gating on the green versus red cytogram.

Five thousand cells were analyzed by FACSort (Becton Dickinson, San Jose, CA, USA). Analysis of the flow cytometric data was carried out using dedicated software (SCSASoft; SCSA Diagnostics, Brookings, SD, USA) which implies that the DFI histogram is used to precisely determine the percentage DFI. All SCSA measurements were performed on raw semen, which on the day of analysis was quickly thawed and analyzed immediately. For the flow cytometer setup and calibration, a reference sample was used from a normal donor ejaculate retrieved from the laboratory repository (Evenson and Jost, 2000). The same reference sample was used for the whole study period. A reference was run for every fifth sample. The intra-laboratory CV for DFI analysis was found to be 4.5%. A single SCSA measurement was made for each reference sample.

Statistical analysis

Results were expressed as mean (±SD). The CV for DFI in each man was calculated using the formula (SD/mean) × 100%.

According to previous reports suggesting 30% DFI as a cut-off value for achieving a pregnancy in IVF, the patients were dichotomized according to whether the DFI in raw semen was $\leq 30\%$ (Category I) or $>30\%$ (Category II). Subsequently, the proportion of men switching from one category in the first test to the other category at the second examination was calculated, with 95% confidence interval (CI), based on the assumption of binomial distribution. Subsequently, the same calculation was performed using an interval of 29–31% instead of the 30% cut-off value (switch from <29 to $>31\%$ or *vice versa*). For the subjects for whom the date of the delivery of the first and the second ejaculate were computed in the database, the correlation between the length of the interval between sampling and CV of DFI was calculated using Spearman's ρ-test.

Statistical analysis was performed using the Statistical Package for the Social Sciences 14.0 for Windows (SPSS Inc., Chicago, IL, USA). Statistical significance was regarded as a two-sided $P < 0.05$.

Results

Mean CV for DFI of all repeated SCSA measurements in the study group was 30.1% (SD: 21.5%); median 26.9% (range: 0–130%).

Of the 616 patients included, 521 (85%; 95% CI: 82–87%) did not change DFI category from first to second sample (Category I: DFI $\leq$ 30% versus Category II: DFI $>$ 30%). Ninety percents (95% CI: 87–93%) of these men had DFI $\leq$ 30% and the remaining 10% of the men had a DFI $>$ 30% (95% CI: 7.4–13%).

Sixty-eight patients (11%; 95% CI: 8.6–14%) had belonged to Category I in test 1 and to Category II in test 2. The DFI in those subjects was 31–71% (mean 40%, SD 10%). Twenty-seven patients

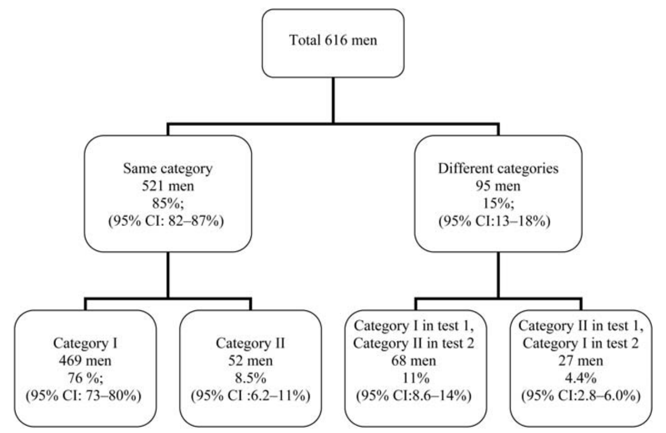

Figure 1 Variation in DFI between tests 1 and 2, in relation to the two categories of DFI (Category I: DFI $\leq$ 30% and Category II: DFI $>$ 30%), which are used in the assessment of male fertility. Based on 616 patients. CI, confidence interval.

(4.4%; 95% CI: 2.8–6.0%) switched from Category II in test 1 to Category I in test 2. Of these, 19 had a DFI between 20 and 30% and 8 had a DFI between 15 and 20% (mean for DFI in test 2 was 22%, SD 4.4%). These results are summarized in Fig. 1.

When the DFI interval 29–31% was used instead of the 30% cut-off level, 12% of the subjects (95% CI: 9.2–14.2) switched from a value $<$29 to $>$31%, or *vice versa*.

For 141 of the 616 men (23%), the date of both measurements was registered in the database. The mean for CV for the two DFI assessments in this group was 29.5%, whereas the mean CV for the remaining 475 subjects was 25.7%. The mean time interval between the two samples for this subgroup of men was 134 days. There was no significant correlation between the intra-individual CV and time interval between samples (Spearman's ρ-test; $\rho = 019$; $P = 0.82$).

Discussion

The present study demonstrated that in men from infertile couples the variation of DFI in repeated samples is approximately of the same magnitude as for standard sperm parameters, previously being estimated as ~30% for concentration, motility and morphology (Leushuis *et al.*, 2010).

However, using the DFI of 30% as a clinical cut-off level, the result of the SCSA analysis is relatively robust, since 85% of the men, when repeating the analysis, were still in the same DFI category. This figure is similar to the previously reported 82% in another cohort of men under infertility assessment (Erenpreiss *et al.*, 2006a). Furthermore, there was no correlation between the length of the time period between the delivery of the two semen samples and the intra-individual CV, indicating that a single SCSA analysis is equally predictive for the DFI level some days, as well as several months, after the first sampling.

A strength of this study is the high number of subjects included. Furthermore, it is based on men coming for investigation owing to infertility problems, thereby representing the target group for which the prediction of chances of fertility *in vivo* is of the greatest interest. Previous studies have shown that variation might be lower for non-infertile men in contrast to the men from infertile couples studied here (Evenson *et al.*, 1991).

Although data from the present study demonstrated a high intra-individual DFI variation, this does not invalidate the use of the test in clinical practice. The reason is robustness of the estimation based on one analysis in relation to whether the patient belongs to the DFI category below or above 30%, the clinically significant cut-off for predicting *in vivo* infertility (Evenson *et al.*, 1999; Spanò *et al.*, 2000; Bungum *et al.*, 2007; Evenson and Wixon, 2008).

A major weakness of the study is lack of information about changes in life style and health during the follow-up of the men included in the study. Factors such as smoking, medication and fever were previously suggested to have a possible influence on sperm DNA integrity (Evenson *et al.*, 1991, 2000; Niu *et al.*, 2010; Elshal *et al.*, 2009; Rubes *et al.*, 2010). However, use of medication is not that common in men belonging to the age group seeking help for infertility. Change of smoking habits during infertility investigation, if occurring, most often implies that the patient stops smoking, which might explain the observed lowering of DFI between tests I and II. However, Spano *et al.* (1998) found no significant impact of smoking, alcohol consumption, fever or genital viral infection on DFI, which recently was confirmed by Smit *et al.* (2007), who demonstrated that neither life style nor occupation had any influence on the intra-individual variation of chromatin fragmentation.

All the patients were asked to keep an abstinence period of 2–7 days. In principle, the DFI outcome can be compromised by the presence of older spermatozoa that still remain after previous ejaculations. Although we did not correct for the actual length of the abstinence period, the DFI was found to increase by 0.45% per day of increase of the abstinence period (Richthoff *et al.*, 2002). Furthermore, our set up reflects the daily situation where one analysis of semen quality is supposed to predict the chance of the couple to achieve pregnancy during the following months, and for each subject a day-to-day variation in abstinence period can be expected.

Apart from the clinical implications of our finding, the high intra-individual variation in the DFI raises some questions related to biological aspects of regulation of semen quality. As the intra-laboratory CV for determination of DFI was as low as 4.5%, the variation in the results of the SCSA analysis can hardly be explained by technical aspects of the analysis, although we only measured each reference sample once with no repeat measurement to verify that sample debris caused no artifact in the measurement. As for other sperm parameters, our knowledge of biological factors which may have a major impact on the intra-individual variation in DFI is limited. Sperm, during its development, transport and storage, can be negatively affected by different mechanisms (Sakkas *et al.*, 2010); abortive apoptosis during spermatogenesis, DNA strand breaks during the remodeling of sperm chromatin under the spermiogenesis process, and oxidative stress caused by reactive oxygen species, which may lead to post-testicular DNA fragmentation (Aitken *et al.*, 1998). Moreover, DNA fragmentation can be induced by endogenous caspases and endonucleases, or external factors, such as radiotherapy, chemotherapy and environmental toxicants. Although the highly organized, compact and insoluble nature of the sperm chromatin with its protective system of histones and protamines (Erenpreiss *et al.*, 2006b; Shamsi *et al.*, 2008), make the spermatozoa exposed for conspicuous disintegration. It appears plausible that the factors which cause an increase in DFI are more pronounced in subfertile men, thereby also leading to higher intra-individual variation in infertile subjects as compared with men without fertility problems.

In conclusion, this study describes a considerable intra-individual variability in sperm DNA damage within a large group of infertile men. However, in the vast majority of the subjects, repeated SCSA testing does not result in a switch in DFI category, in relation to the clinical cut-off level of 30%. This finding adds to the utility of SCSA DFI as a valuable tool in the investigation of men from infertile couples.

Authors' roles

K.O., A.G. and M.B. have all given substantial contributions to conception and design of the present study. All authors have contributed to acquisition of data, analysis as well as interpretation of data. K.O. has drafted the manuscript and A.G. and M.B. have revised the content critically. All three authors have made final approval of the version to be published.

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Paper II

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ORIGINAL ARTICLE

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Prevalence of high DNA fragmentation index in male partners of unexplained infertile couples

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SUMMARY

The sperm chromatin structure assay (SCSA) parameter DNA fragmentation Index (DFI) is a valuable tool for prediction of fertility in vivo. Clinical data show that a DFI above 30% is associated with very low chance for achieving pregnancy by natural conception or by insemination. Already when DFI is above 20% the chance of natural pregnancy is reduced, this despite normal conventional semen parameters. The aim of the present study was to investigate the prevalence of high DFI in male partners of unexplained infertile couples to further identification of male factors contributing to subfertility. Among 212 consecutive men under infertility investigation, 122 cases with the diagnosis 'unexplained infertility' were identified. For all but three, SCSA data were available. The percentage of couples with diagnosis 'unexplained infertility' in which the male partner has DFI >20% or DFI >30% was calculated. In the group diagnosed with 'unexplained infertility' 17.7% of the men (95% CI 10.8–24.5) presented with $20 \leq$ DFI <30 and 8.4% (95% CI 3.40–13.4) had DFI $\geq$30%. A significant part of men diagnosed as unexplained infertile according to traditional diagnostic methods has remarkably high degrees of fragmented sperm DNA. Apart from adding to our understanding of biology of infertility our finding has clinical implications. Couples in which the DFI of the male partner is high can avoid prolonged attempts to become spontaneously pregnant or referral for intrauterine insemination, both having low chances of leading to conception.

INTRODUCTION

Infertility is a common problem that affects up to 25% of couples in societies in various parts of the world (Schmidt *et al.* 1995; Bushnik *et al.* 2012; Cai *et al.* 2011; Dunson *et al.* 2004). The exact prevalence of male factor infertility is difficult to define referable to the lack of sufficient diagnostic tools Jequier 2004). Although the World Health Organization (WHO, 1987) has estimated that up to 50% of the infertility cases are predominantly or partly caused by male factors, the incidence of infertile couples diagnosed as unexplained infertile is around 10–20% (Isaksson & Tiitinen 2004).

Investigation of the male partner in infertile couple is mainly based on the conventional semen analysis, which includes assessment of sperm concentration, motility and morphology. These parameters have, however, a limited power in regard to prediction of chance of conception (Bonde *et al.* 1998) and can only in selected cases point to options for specific therapeutic measures. To overcome these limitations, a number of new sperm tests have been developed (Erenpreiss *et al.* 2006). The sperm chromatin structure assay (SCSA), first described by Evenson (Evenson *et al.* 1980) evaluates sperm chromatin integrity and provides additional information about the fertilizing capacity of the sperm. Studies have shown that the SCSA parameter DNA fragmentation index (DFI) is an independent predictor of male sub-fertility in vivo (Bungum *et al.* 2007), Giwercman *et al.* (2010). Recently we demonstrated that men having normal standard semen parameters and an increased DFI above 20% had a higher odds ratio for infertility compared with fertile controls (Giwercman *et al.* 2010). If one of the standard semen parameters according to World Health Organization criteria was abnormal (WHO 1999), the odds ratio for infertility increased already at DFI above 10%. Thus, chances of conception achieved by intercourse or by intra-uterine insemination decreased already at DFI levels above 20% and are being close to zero when DFI exceeds the level of 30% (Giwercman *et al.* 2010). These findings indicate that DFI is a potentially, clinically useful marker of male fertility as it can add to explaining, at least some cases of 'unexplained infertility'. Clinically, DFI can be of help in selecting couples who, referable to low in vivo fertility potential, should be referred directly for in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI). Furthermore, it has been suggested that high DFI is a potentially curable condition and causal treatment may become an option for cases of infertility associated with impairment of sperm DNA integrity (Agarwal *et al.* 2009; Li *et al.* 2012).

So far, there is only limited information regarding the prevalence of high DFI in couples diagnosed with 'unexplained infertility'. The purpose of the study was, therefore, to find out the percentage of couples with diagnosis 'unexplained infertility' in which the male partner has a DFI >20% or a DFI >30%. Furthermore, we wished to compare this proportion with the corresponding figure in a cohort of proven fertile men with normal standard sperm parameters (Giwercman *et al.*, 2010).

MATERIALS AND METHODS

Study design and patient population

This is a case series study based on data from files of 212 consecutive couples who underwent infertility investigation at the Reproductive Medicine Centre (RMC), Skåne University Hospital, Malmö, Sweden between June 2008 and April 2011. Reproductive Medicine Centre is a tertiary referral centre; however, the couples can refer themselves after more than 1 year of unprotected intercourse not leading to pregnancy. As cases with obvious male or female factor are usually referred directly to RMCs andrological or gynaecological outpatient clinic from secondary referral level, couples with 'unexplained infertility' are over-represented in this group.

The diagnosis of 'unexplained infertility' was based on the following

- At least 1 year of unprotected intercourse without pregnancy;
- Normal sperm concentration, motility and morphology according to WHO, 1999;
- Unremarkable andrological history (no cryptorchidism, drug abuse, cancer treatment or other iatrogenic factors), no genetic abnormalities such as Klinefelter's syndrome or Y-chromosome microdeletion and no hypogonadotropic hypogonadism;
- No female factors (anovulation, hormonal infertility, tubal factor or endometriosis).

Among the 212 couples included, 27 couples had a female related infertility diagnosis (anovulation, hormonal infertility, tubal factor or endometriosis) and were excluded from the study. The same was true for additional 63 couples with 'male factor infertility', defined as one or more abnormal standard sperm parameters. All, except three men, who only had one ejaculate investigated, delivered at least two semen samples for analysis according to WHO criteria (WHO 1999). The SCSA analysis is a routine test for all male patients in our clinic. However, among the 122 'unexplained infertile' couples only 119 (97%) had a SCSA analysis and could thus be included in the data analysis.

For comparison, retrieving data from a previous publication (Giwercman *et al.*, 2010) we included a cohort of 95 proven fertile men with normal standard sperm parameters. Among 95 of these men, 10 presented with DFI $\geq 20\%$.

Semen samples and standard semen analysis

Semen samples were collected by masturbation after the recommended abstinence period of 2–7 days. Semen parameters were scored according to the WHO guidelines (WHO 1999). For assigning semen quality as normal, following cut-off levels, which were valid at the time of the collection of our material, were used:

- Volume ≥ 2.0 mL;
- Sperm concentration $\geq 20 \times 10^6$/mL or total number $\geq 40 \times 10^6$;
- Sperm motility: $\geq 25\%$ rapidly progressive motile or $\geq 50\%$ progressively motile sperm;
- Sperm morphology: $\geq 5\%$ normal forms.

Sperm chromatin structure assay

The principles and procedure to measure sperm DNA damage using flow cytometry SCSA are described in detail elsewhere (Bungum *et al.* 2004; Evenson & Jost 2000; Spano *et al.* 2000). In brief, the SCSA is based on the phenomenon that a 30 sec treatment with pH 1.2 buffers denatures the DNA at the sites of single- or double-strand breaks, whereas normal double-stranded DNA remains intact. Thereafter, the sperm cells are stained with the fluorescent DNA dye acridine orange, which differentially stains double- and single-stranded DNA. After blue light excitation in a flow cytometer, the intact (double-stranded) DNA emits green fluorescence, whereas denatured (single-stranded) DNA emits red fluorescence. Sperm chromatin damage is quantified using the flow cytometry measurements of the metachromatic shift from green (native, double-stranded DNA) to red (denatured, single-stranded DNA) fluorescence and displayed as red vs. green fluorescence intensity cytogram patterns. The extent of DNA denaturation is expressed as DFI, which is the ratio of red to total fluorescence intensity that is, the level of denatured DNA over the total DNA.

A total of 5–10 000 cells were analysed by FACSort (Becton Dickinson, San Jose, CA, USA). Analysis of the flow cytometric data was carried out using dedicated software (SCSASoft; SCSA Diagnostics, Brookings, SD, USA), which imply that the DFI histogram is used to precisely determine the percentage of DFI. All SCSA measurements were performed on raw semen, which on the day of analysis was quickly thawed and analysed immediately. For the flow cytometer setup and calibration, a reference sample was used from a normal donor ejaculate retrieved from the laboratory repository (Evenson & Jost 2000). The same reference sample was used for the whole study period. A reference was run for every fifth sample. The intra-laboratory CV for DFI analysis was found to be 4.5%. A single SCSA measurement was made for each reference sample.

Statistical analysis

Results were expressed as percentage of men with $20 \leq \text{DFI} < 30\%$ and DFI $\geq 30\%$, respectively, in relation to the total number of couples diagnosed with 'unexplained infertility'. The rationale for using these DFI thresholds was based on previous reports in which the SCSA was performed (Bungum *et al.* 2007; Evenson & Jost 2000; Giwercman *et al.* 2010). A 95% confidence interval (CI) was estimated for each group. The data analysis was performed on the first semen analysis in which SCSA was performed.

The additional parameters: age of man/woman and woman's body mass index (BMI) were expressed as mean/median (range) separately for each group. These values were also calculated for conventional semen parameters (sperm concentration, motility

A + B) for those 119 semen samples with DFI included in the analysis.

Using Fisher's exact test (www.graphpad.com), the percentages of men with DFI $\geq 20\%$, was compared to a corresponding figure in the previously reported cohort of proven fertile men with normal standard sperm parameters (Giwercman *et al.* 2010). All other statistical analyses were performed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA).

RESULTS

In Table 1 the demographic characteristics of the 119 included couples with 'unexplained infertility' are given.

The mean DFI was 16.2% (median 15%, range 4–50%). Twenty one of these men (17.7%) (95% CI 10.8–24.5%) presented with $20 \leq$ DFI $< 30\%$ and 10 men [8.4%, (95% CI 3.40–13.4%)] had a DFI $\geq 30\%$. In total, 31 men [26.1%, (95%CI 18.2–33.9%)] had a DFI $\geq 20\%$.

The percentage of men with DFI $\geq 20\%$, in the cohort of fertile men with normal standard sperm parameters was 10.5% (95% CI 6.29–17.0%), this value being significantly lower than those found in men from 'unexplained infertility couples' ($p = 0.005$).

DISCUSSION

The present study shows that one quarter of men in couples diagnosed as 'unexplained infertile' according to traditional diagnostic methods have a DFI level $\geq 20\%$, previously found to be associated with a decreased fertility in vivo. This figure was statistically significantly higher than in proven fertile men. In a retrospective study (Giwercman *et al.* 2010) found that 10.5% of men with proven fertility had a DFI level of 20% or higher. Thus, in a significant proportion of so called 'unexplained' cases impairment of sperm DNA integrity can at least partly explain the subfertility problem of the couple. In line with previous accumulated data (Bungum *et al.* 2011) our results suggest that sperm DNA integrity assessment may help to differentiate men with fertility problems and can therefore be of help in counselling of infertile couples.

Recent research has indicated that sperm chromatin integrity testing as assessed with SCSA may contribute to the evaluation of men in infertile couples, however, none of these previous studies have been specifically related to the diagnosis 'unexplained infertility' (Giwercman *et al.* 2010). Previously we reported that if sperm concentration, motility and morphology were normal, fertility impairment is seen at DFI levels exceeding 20% (Spano *et al.* 2000). It has also been shown in studies based on pregnancy planners (Spano *et al.* 2000) and on couples referred for intrauterine insemination (Bungum *et al.* 2007) that the probability of conception in vivo decreases when the DFI, as determined by SCSA, exceeds 20% and is almost zero if this value is more than 30%. This was the reason for selecting 'cut off' values of 20% and 30% respectively. Numerous studies have demonstrated that the association between SCSA and other semen parameters is only weak to moderate (Giwercman *et al.* 2003; Spano *et al.* 1998). This indicates that impairment of sperm DNA integrity is an independent predictor of male fertility (Bungum *et al.* 2007; Giwercman *et al.* 2010).

Table 1 Background characteristics of the 119 couples with 'unexplained infertility'. Semen parameters based on the first sample delivered by the patient

	'unexplained infertility'
Age man (years), mean/median (range)	34/33 (22;55)
Age woman (years), mean/median (range)	31/31.5 (21;39)
BMI woman (kg/m^2), mean/median (range)	25/24 (18.5;43.4)
Sperm concentration ($\times 10^6$/mL), mean/median (range)	93/73 (15;640)
Sperm motility (A + B) (%), mean/median (range)	60/61 (25;81)
DFI (%), mean/median (range)	16/15 (4.0;50)

Motility A – rapid progressive motility. Motility B – slow progressive motility.

Sperm DNA integrity assessment has been suggested as being useful in the clinical guidance in choice of assisted reproduction technique (Boe-Hansen *et al.* 2006; Bungum *et al.* 2004; Jiang *et al.* 2011; Zini *et al.* 2001), although some disagreement regarding this matter exists (Lin *et al.* 2008). Data indicate that in cases with DFI above 30% the 'baby take home rate' is higher when using ICSI instead of standard IVF (Bungum *et al.* 2011).

One limitation of this study is the possibility to exclude female sub-fertility as a factor contributing to the infertility of the couple. Today, the work up of the female partner in an infertile couple is rather sparse (Crosignani & Rubin 2000), often limited to hormonal evaluation only. Even though we have excluded female factors such as endometriosis, tubal occlusion or ovulatory disturbances, other causes of female subfertility, as for example poor oocyte quality cannot be excluded. However, as infertility, in many cases, is believed to be ascribable to accumulation of several adverse factors, even in case of presence of some 'female factor', the contribution of impairment of sperm DNA integrity may play an important role.

The calculations are based on one SCSA analysis only. However, despite some intra-individual variation in the DFI, we have shown (Oleszczuk *et al.* 2011) that in 85% of cases when repeating SCSA- analysis the DFI value remained at the same side of the 30% cut-off level. Thus, multiple SCSA testing only rarely impels a change of DFI category from normal to abnormal, or *vice versa*.

Our study has biological and clinical implications. From a biological point of view, it is interesting that sperm DNA impairment can, at least partly, explain as many as 25% of previously unexplained cases. Clinically, our data indicate that SCSA testing may help in management of couples with unexplained infertility. It has been suggested that some cases of impairment of sperm DNA are potentially curable (Agarwal *et al.* 2009; Li *et al.* 2012). Furthermore, finding of high DFI will in incurable cases point to direct referral to IVF or ICSI, instead of continuing attempts to achieve spontaneous pregnancy or using intrauterine insemination.

AUTHORS' CONTRIBUTIONS

K. O, A. G and M. B have all given substantial contributions to conception and design of the present study. All authors have contributed to acquisition of data, analysis as well as interpretation of data. K. O has drafted the manuscript and A. G and M. B have revised the content critically. All authors have made final approval of the version to be published.

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CONFLICT OF INTERESTS

The authors declare that they have no competing interests.

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Paper III

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ORIGINAL ARTICLE

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Sperm chromatin structure assay in prediction of in vitro fertilization outcome

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SUMMARY

Sperm DNA fragmentation index (DFI) assessed by sperm chromatin structure assay is a valuable tool for prediction of fertility in vivo. Previous studies on DFI as predictor of in vitro fertilization (IVF) outcome, based on relatively small materials, gave contradictory results. The present study examines, in a large cohort, the association between sperm DFI and the outcome of IVF/ICSI procedure. The study is based on 1633 IVF or ICSI cycles performed at the Reproductive Medicine Centre, Skåne University Hospital, Malmö, Sweden, between May 2007 and March 2013. DFI values were categorized into four intervals: DFI $\leq$ 10% (reference group), 10% $<$ DFI $\leq$ 20%, 20% $<$ DFI $\leq$ 30%, DFI $>$ 30%. For the three latter intervals, the following outcomes of IVF/ICSI procedures were analyzed in relation to the reference group: fertilization, good quality embryo, pregnancy, miscarriage, and live births. In the standard IVF group, a significant negative association between DFI and fertilization rate was found. When calculated per ovum pick-up (OPU) Odds Ratios (ORs) for at least one good quality embryo (GQE) were significantly lower in the standard IVF group if DFI $>$ 20%. OR for live birth calculated per OPU was significantly lower in standard IVF group if DFI $>$ 20% (OR 0.61; 95% CI: 0.38–0.97; $p = 0.04$). No such associations were seen in the ICSI group. OR for live birth by ICSI compared to IVF were statistically significantly higher for DFI $>$ 20% (OR 1.7; 95% CI: 1.0–2.9; $p = 0.05$). OR for miscarriage was significantly increased for DFI $>$ 40% (OR 3.8; 95% CI: 1.2–12; $p = 0.02$). The results suggest that ICSI might be a preferred method of in vitro treatment in cases with high DFI. Efforts should be made to find options for pharmacologically induced reduction of DFI. The study was based on retrospectively collected data and prospective studies confirming the superiority of ICSI in cases with high DFI are warranted.

INTRODUCTION

Infertility is a profound medical and social problem affecting one in six couples trying to become pregnant (Templeton *et al.*, 1990). Up to 50% of the infertility problems are described to be related to a male factor (Comhaire, 1987). Investigation of the male partner in the infertile couple is traditionally based on the conventional semen analysis, which includes assessment of sperm concentration, motility, and morphology. The analysis has, however, a limited value both as a diagnostic tool and as a guide to selection of the therapeutic procedure (Bonde *et al.*, 1998; Jequier, 2004). To overcome these limitations, a number of new sperm tests have been developed, perhaps most promising being those assessing sperm DNA integrity (reviewed in (Erenpreiss *et al.*, 2006)). Among them, sperm chromatin structure assay (SCSA), introduced by Evenson (Evenson *et al.*, 1980), is based on a rather standardized methodology and has been shown to be of clinical value (Spano *et al.*, 2000; Bungum *et al.*, 2007). It has been shown that DNA fragmentation index (DFI) as measured by SCSA is a relatively independent predictor of male sub-fertility in vivo (Giwercman *et al.*, 2010). The chance of conception achieved by intercourse or by intra-uterine insemination decreases already at DFI levels above 20% and approaches zero when DFI exceeds the level of 30% (Spano *et al.*, 2000; Bungum *et al.*, 2007). These findings indicate that DFI is a clinically useful marker of male infertility. A further question is to what degree sperm chromatin integrity affects the outcome of in vitro-assisted reproductive technology (ART) (Evenson *et al.*, 1999; Larson *et al.*, 2000; Larson-Cook *et al.*, 2003). It is agreed that even spermatozoa with high DFI can be used to achieve pregnancy with help of in vitro techniques i.e., in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) (Gandini *et al.*, 2004; Bungum *et al.*, 2007), but it is still unclear whether the chance of pregnancy is related to the level of DFI.

In addition to pregnancy and the implantation rate, the outcome of IVF and ICSI can be assessed by fertilization rate and embryo quality. Whilst the impact of sperm DNA integrity on embryo development and implantation was confirmed by animal studies (Ahmadi & Ng, 1999; Penfold *et al.*, 2003), the findings in human studies are conflicting. While some of studies do not reveal any value of assessment of sperm chromatin damage in prediction of fertilization failure or pregnancy rate (Niu *et al.*, 2011), this association is clearly seen by others (Saleh *et al.*, 2003; Bungum *et al.*, 2007). A recent meta-analysis indicated an impact of high DFI on the pregnancy outcome after IVF or ICSI but no statistical significance was seen when SCSA specifically was evaluated as a method of assessment of DFI. Thus, the predictive value of SCSA was not confirmed for IVF or ICSI (Zhang *et al.*, 2015). These ambiguous results can, at least partly, be the effect of the small study sizes, lack of distinction between various types of ART and the use of different techniques for assessment of DFI. The present study evaluates in a larger sample the predictive value of DFI assessed by SCSA in relation to fertilization rate, embryo quality, pregnancy rate, the risk of miscarriage as well as probability of live birth following IVF and ICSI.

Table 1 The characteristics of participants and those excluded because of lack of sperm chromatin structure assay (SCSA) data

	Participants *n* = 1633	Non-participants because of lack of SCSA data *n* = 1127
Age (years), median/range	35/21-55	34/21-55
Sperm concentration ($\times 10^6$/mL), median/range	45/0.1-480	35/0.1-290
Sperm motility (%), median/range	67/0-100	70/0-100

Table 2 Background characteristics for participants

	DFI ≤ 10%	10% <DFI ≤ 20%	20% < DFI ≤ 30%	DFI > 30%
Male age (years), mean/SD	33.5/5.3	33.9/5.3	34.7/5.9	35.9/6.3
Female age (years), mean/SD	32.4/4.1	32.4/4.1	32.7/4.1	32.4/4.4
Female BMI (kg/m^2), mean/SD	23.5/3.1	23.5/3.3	23.3/3.2	23.6/3.6
Agonist/Antagonist/ Other (%)	69/31/0.3	66/34/-	64/36/-	72.4/27.6/-
FSH total dose (IU), mean/SD	1896/825	1951/885	1868/832	1954/868
Asp oocytes (*n*), mean/SD	9.9/6.8	10.2/6.1	10.1/5.7	10.1/5.7
IVF/ICSI (%)	85/15	68/32	48/52	37.2/62.8

DFI, DNA fragmentation index; IVF/ICSI, in vitro fertilization/intracytoplasmic sperm injection.

MATERIALS AND METHODS

Study design and patient population

This is a cohort study where data were analyzed retrospectively. The outcome of IVF treatments in regard to fertilization, embryo quality, pregnancy, miscarriage, and live birth were analyzed in relation to the level of DFI. The study is based on a database of 6660 consecutive IVF procedures performed at the Reproductive Medicine Centre (RMC), Skåne University Hospital, Malmö, Sweden, between May 2007 and March 2013. Generally, the criteria for being treated at this public university clinic included female age below 39 years at start of the treatment, female BMI preferably below 30 kg/m^2 as well as both partners being non-smokers. Four hundred and seventy-one cycles were excluded because donated and/or cryopreserved gametes were used or cryopreservation of all oocytes was performed. Furthermore, one hundred and twenty-nine cycles were excluded because non-ejaculated spermatozoa were used for fertilization. Among residual 6060 cycles, only the 2956 first attempts were included in order to avoid multiple involvement of the same couple. Finally 1829 of them (62%) had SCSA analysis performed. In 1820 of female partners at least one oocyte was aspirated. One hundred and sixty-six cycles where both standard IVF and ICSI were applied in the same cycle were also excluded. Additional 21 cycles were excluded because of miscellaneous data corruption. Finally, data on 1633 cycles were included in the statistical analysis.

During the sample collection period, saving aliquots of ejaculate used for IVF/ICSI for subsequent SCSA analysis was, gradually, introduced as clinical routine, which is the reason for 38% of treatments with no SCSA data. However, the selection of samples for SCSA was random, apart from those ICSI treatments where sperm concentration was below 1×10^6/mL or which no SCSA was done. This is, probably, the explanation as to why the participants and those excluded because of lack of SCSA data did not differ as considers age and sperm motility whereas sperm concentration is slightly higher in the former group. The data are presented in Table 1. Background characteristics for participants considering male and female age, female BMI, type of stimulation, follicle-stimulating hormone (FSH) total dose, and number of aspirated oocytes are given in Table 2. Among the 1107 embryo transfers, the 22 were performed as double embryo transfers (DET) and in the remaining 1085 cases, a single embryo was transferred (SET). Mean DFI value was 15.7% in the SET group and 15.4% in the DET group.

The study was approved by the ethical committee of Lund University and, following written information, the couples were given an option to be excluded from the study.

Semen collection and analysis

Semen samples were collected by masturbation. Conventional semen analysis including sperm concentration, motility, and morphology was performed according to the World Health Organization guidelines (WHO, 1999). Two hundred microliter of the raw semen was stored in Eppendorf snap-cap tubes in −80 °C ultra-cold freezer following the procedure described by Evenson (Evenson *et al.*, 2002) for subsequent SCSA analysis.

Sperm chromatin structure assay

The principles and procedure of SCSA are described in detail elsewhere (Evenson & Jost, 2000; Bungum, 2012). The technique is based on the phenomenon that a 30-sec treatment with pH 1.2-buffer denatures the fragments of DNA with single- or double-strand breaks, whereas normal double-stranded DNA remains intact. The sperm cells are then stained with the fluorescent DNA dye acridine orange, which stains differently intact and fragmented DNA. After blue light excitation in a flow cytometer, the intact DNA emits green fluorescence, whereas denatured DNA emits red fluorescence. Sperm chromatin damage is quantified using the flow cytometry measurements of the metachromatic shift from green (native, double-stranded DNA) to red (denatured DNA) fluorescence and displayed as red vs. green fluorescence intensity cytogram patterns. The extent of DNA denaturation is expressed as DFI, which is the ratio of red to total fluorescence intensity i. e. the level of denatured DNA over the total DNA. A total of 5–10,000 cells were analyzed by FACSort (Becton Dickinson, San Jose, CA, USA). Analysis of the flow cytometric data was carried out using dedicated software (SCSASoft; SCSA Diagnostics, Brookings, SD, USA), which imply that the DFI histogram is used to precisely determine the percentage of DFI. All SCSA measurements were performed on raw semen, which on the day of analysis was quickly thawed and analyzed immediately. For the flow cytometer setup and calibration, a reference sample was used from a normal donor ejaculate retrieved from the laboratory repository (Evenson & Jost, 2000). The same reference sample was used for the whole study period. A reference was run for every fifth sample. The intra-laboratory CV for DFI analysis was found to be 4.5%.

IVF and ICSI procedures

Controlled ovarian stimulation was achieved using a GnRH antagonist short protocol or a GnRH-agonist down-regulation long protocol. Ovarian stimulation was performed with recombinant FSH alternatively urine derived gonadotrophin. Patients were monitored with transvaginal ultrasound for a count and size of follicles and serum-estradiol level if necessary. Human chorionic gonadotropin (hCG) injection was administered with the presence of at least two >17 mm follicles. Oocyte retrieval was conducted 35 h later under conscious sedation.

Gamete handling as well as IVF/ICSI procedures, culturing and embryo transfer (ET) were performed as previously described (Bungum *et al.*, 2004).

Assessment of fertilization, embryo morphology classification, cryopreservation and embryo transfer

Fertilization was determined 18 ± 20 h after the IVF/ICSI procedure. The oocytes were considered as fertilized when two distinct pronuclei were visible.

Cleavage and classification of morphology was assessed on day 2 or 3 (Bungum *et al.*, 2006). On day 5, embryos were assessed according to scoring criteria for blastocysts (Gardner & Schoolcraft, 1999).

The term good quality embryo included embryos selected for embryo transfer in which on day 2 were 4–6 cells, grade 1 or 2, on day three 8–10 cells, grade 1 or 2, or on day 5 blastocysts with good expansion, inner cell mass and trophectoderm (A or B according to Gardner criteria).

One embryo with the best morphology was selected for embryo transfer on day 2, 3 or 5 after oocyte retrieval. In the 22 cases two embryos were transferred. All not transferred good quality embryos, were cryopreserved.

All embryo transfers were performed with a Cook Soft 5000 catheter (Cook, Brisbane, Qld, Australia).

Luteal phase support, pregnancy test and miscarriage

All the patients received luteal phase support in the form of daily vaginal administration of micronized progesterone, 90 mg once a day starting on the day following oocyte retrieval and continuing until the day of the pregnancy test (i.e., day 12 after embryo transfer). A positive pregnancy test was defined by a plasma βHCG concentration >15 IU/L. A clinical pregnancy was defined as ultrasound detected intrauterine gestational sac with a heart activity 3 weeks after a positive HCG test. Miscarriage was defined as spontaneous expulsion of gestational sac up to 18th week of gestation which is verified by gynecological examination/ultrasound.

Statistical analysis

Statistical analysis was performed using the IBM SPSS Statistics 22 software (SPSS Inc., Chicago, IL, USA). The couples were categorized into four groups, according to the DFI value: DFI ≤ 10% (reference group), 10% < DFI ≤ 20%, 20% < DFI ≤ 30%, DFI > 30%. All the calculations were done separately for standard IVF and ICSI and after merging both procedures. All the results were adjusted for female age as a covariate. Following calculations were performed:

- Fertilization rate expressed as number of fertilized oocytes as percentage of the number used for IVF/ICSI procedures [(100 × Fertilized eggs/total number of injected oocytes) and (100 × Fertilized eggs/total number of oocytes inseminated)]. Univariate analysis of variance was applied.
- Embryo quality rate, calculated as number of good quality embryos (GQE) as a percentage of the number of successful fertilizations. To do this calculation additional 158 cases where none oocyte was fertilized were excluded. Univariate analysis of variance was done on 1475 residual procedures.
- Since GQE is a pre-requisition for performing ET, the cases with at least one GQE were identified and odds ratio (OR) for at least one GQE in each DFI group were calculated using binary logistic regression.
- Pregnancy rate defined as the number of pregnancies as a percentage of the number of ET with GQE. Pregnancy was defined as serum hCG ≥ 15 IU/L on day 12 post ET. For this analysis the cases with no GQE as well as those in which ET was not performed for other reasons (e. g. ovarian hyperstimulation syndrome) were excluded. Totally 526 cases were excluded and 1107 used for analysis. Binary logistic regression was applied for calculation of OR.
- Miscarriage rate defined as a number of spontaneous abortions as a percentage of all pregnancies. Only the 471 cases where the pregnancy was achieved were included in this calculation. Odds ratio was calculated using binary logistic regression. For this end point, additional calculations were done for DFI > 40%.
- Successful pregnancy outcome defined as OR for live births in those having done ovum pick-up (OPU). In order to obtain higher statistical power, for this calculation the two highest DFI groups were merged. Apart from comparing the groups with DFI higher than 10% with the reference group (≤10%) for each DFI group the OR for live birth by ICSI was calculated with standard IVF as reference.

RESULTS

Fertilization rate

Mean fertilization rate according to DFI group is shown in Table 3. No significant statistical difference in fertilization rate in respective DFI groups were seen when results of IVF and ICSI

Table 3 Fertilization rate according to DNA fragmentation index (DFI)

DFI (%)	IVF			ICSI			Total (IVF/ICSI)		
	n	Mean % (SE)	*p*-value	*n*	Mean % (SE)	*p*-value	*n*	Mean % (SE)	*p*-value
0-10	501	51.4 (1.32)	–	89	61.3 (2.71)	–	590	52.8 (1.2)	–
>10-20	445	47.6 (1.4)	0.05	208	61.6 (1.78)	0.94	653	52.1 (1.14)	0.68
>20-30	117	45.6 (2.73)	0.056	128	61.1 (2.27)	0.95	245	53.8 (1.86)	0.66
>30	54	38.1 (4.0)	0.02	91	61 (2.68)	0.92	145	52.6 (2.41)	0.94

IVF/ICSI, in vitro fertilization/intracytoplasmic sperm injection. Fertilization rate = fertilized oocytes/total number of injections or inseminations. Univariate analysis of variance. Reference = the '0–10%' DFI category. Results adjusted for female age.

Table 4 Good quality embryo rate according to DNA fragmentation index (DFI)

DFI (%)	IVF			ICSI			Total (IVF/ICSI)		
	n	Mean% (SE)	*p*-value	*n*	Mean% (SE)	*p*-value	*n*	Mean% (SE)	*p*-value
0-10	453	46.6 (1.73)	–	85	42.9 (4.36)	–	538	45.9 (1.64)	–
>10-20	386	46.3 (1.87)	0.91	201	44.2 (2.84)	0.80	587	45.7 (1.57)	0.91
>20-30	98	43.5 (3.72)	0.45	124	40.7 (3.63)	0.69	222	41.8 (2.55)	0.17
>30	40	37.2 (5.81)	0.12	88	43.6 (4.29)	0.91	128	41.6 (3.36)	0.25

IVF/ICSI, in vitro fertilization/intracytoplasmic sperm injection. Good quality embryo rate = number of good quality embryo/number of successful fertilizations. Univariate analysis of variance. Reference = the '0–10%' DFI category. Results adjusted for female age.

were merged. However, when standard IVF and ICSI were calculated separately, in the standard IVF group fertilization rate, as compared to the reference group, was lower for all DFI groups, this difference reaching statistical significance for DFI > 10–20% and DFI > 30% and borderline statistical significance for those with DFI > 20–30%. No such differences were seen in the ICSI group.

Good quality embryo

Good quality embryo rate according to DFI group is shown in Table 4. When expressed in relation to successful fertilizations, no statistically significant association between DFI level and the GQE was observed. The results in the standard IVF group show a trend toward a decreasing GQE rate with increasing DFI.

The data regarding OR for achieving at least one GQE are shown in Table 5. Whilst the groups with DFI above 10% did not differ from the reference group when IVF and ICSI were merged, in standard IVF group the ORs for GQE were significantly lower for 20% < DFI ≤ 30% and for DFI > 30%. In ICSI group, ORs for GQE were higher in all DFI intervals reaching the significance for 20% < DFI ≤ 30%.

Pregnancy and risk of miscarriage

Table 6 presents the OR for pregnancy rate in those receiving ET with GQE according to DFI intervals. No statistically significant differences between the DFI groups were seen, neither when IVF and ICSI were treated separately nor for the merged group.

Odds ratios for miscarriage are presented in Table 7. No statistically significant differences between the DFI groups were seen, when IVF and ICSI were treated separately. If the additional group with DFI > 40% was extracted the OR for miscarriage was significantly increased for the merged group (OR 3.8; 95% CI: 1.2–12; $p = 0.02$).

Live births

Table 8 presents OR for live birth for couples who underwent OPU. For DFI > 20%, statistically significantly lower OR was seen for IVF but not ICSI. When comparing ICSI to IVF the OR for live birth by ICSI were statistically significantly higher for DFI > 20% (OR 1.7; 95% CI: 1.0–2.9; $p = 0.05$), whereas for DFI ≤ 10% and 10% < DFI ≤ 20%, no such difference was seen.

DISCUSSION

The main clinically applicable finding of this study was significantly decreased chance of live birth in standard IVF treatments performed with ejaculates with DFI above 20%. For this DFI subgroup the live birth rates were also significantly higher for ICSI as compared to IVF. These findings were paralleled by negative association between DFI and fertilization rate as well as the chance of obtaining at least one GQE- a prerequisite for performing embryo transfer- in standard IVF treatments but not in ICSI. Our results are in agreement with some previous studies reporting negative association between DFI level, fertilization rate and embryo quality after IVF/ICSI procedure (Virro *et al.*,

Table 5 Odds ratio for at least one good quality embryo following oocyte pick-up, according to DNA fragmentation index (DFI)

DFI (%)	IVF			ICSI			Total (IVF/ICSI)		
	n	OR (95% CI)	*p*-value	*n*	OR (95% CI)	*p*-value	*n*	OR (95% CI)	*p*-value
0-10	501	Ref	–	89	Ref	–	590	Ref	–
>10-20	445	0.86 (0.64-1.15)	0.32	208	1.51 (0.87-2.61)	0.14	653	0.97 (0.75-1.25)	0.83
>20-30	117	0.61 (0.40-0.94)	0.025	128	1.93 (1.04-3.59)	0.04	245	0.94 (0.67-1.31)	0.69
>30	54	0.36 (0.2-0.63)	0.000	91	1.72 (0.88-3.35)	0.12	145	0.75 (0.51-1.12)	0.16

IVF/ICSI, in vitro fertilization/intracytoplasmic sperm injection. Logistic regression. Reference = the '0–10%' DFI category. Results adjusted for female age.

Table 6 Odds ratio for pregnancy for couples who have undergone embryo transfer, according to DNA fragmentation index (DFI)

DFI (%)	IVF			ICSI			Total (IVF/ICSI)		
	n	OR (95% CI)	*p*-value	*n*	OR (95% CI)	*p*-value	*n*	OR (95% CI)	*p*-value
0-10	345	Ref	–	52	Ref	–	397	Ref	–
>10-20	302	0.98 (0.71-1.34)	0.89	149	0.92 (0.49-1.73)	0.79	451	1.02 (0.77-1.33)	0.92
>20-30	71	0.79 (0.46-1.34)	0.37	95	0.78 (0.4-1.54)	0.48	166	0.90 (0.62-1.3)	0.58
>30	26	1.04 (0.47-2.34)	0.92	67	0.79 (0.38-1.65)	0.54	93	1.02 (0.64-1.61)	0.95

IVF/ICSI, in vitro fertilization/intracytoplasmic sperm injection. Logistic regression. Reference = the '0–10%' DFI category. Results adjusted for female age.

Table 7 Odds ratio for spontaneous abortion according to DNA fragmentation index (DFI)

DFI (%)	IVF			ICSI			Total (IVF/ICSI)		
	n	OR (95% CI)	*p*-value	*n*	OR (95% CI)	*p*-value	*n*	OR (95% CI)	*p*-value
0-10	144	Ref	–	26	Ref	–	170	Ref	–
>10-20	122	1.04 (0.6-1.81)	0.9	72	1.4 (0.53-3.71)	0.5	194	1.27 (0.8-2.01)	0.31
>20-30	25	0.95 (0.36-2.51)	0.91	41	0.78 (0.26-2.33)	0.66	66	0.99 (0.52-1.89)	0.97
>30-40	9	1.99 (0.49-8.0)	0.34	18	0.92 (0.24-3.53)	0.09	27	1.45 (0.6-3.51)	0.42
>40	2	2.32 (0.14-38.2)	0.56	12	3.08 (0.72-13.1)	0.12	14	3.75 (1.2-11.7)	0.02

IVF/ICSI, in vitro fertilization/intracytoplasmic sperm injection. Spontaneous abortions/total number pregnancies. Logistic regression. Reference = the '0–10%' DFI category. Results adjusted for female age.

Table 8 Odds ratio for live birth following ovum pick-up, according to DNA fragmentation index (DFI)

DFI (%)	IVF			ICSI			Total (IVF/ICSI)		
	n	OR (95% CI)	*p*-value	*n*	OR (95% CI)	*p*-value	*n*	OR (95% CI)	*p*-value
0-10	501	Ref	–	89	Ref	–	590	Ref	–
>10-20	445	0.95 (0.70-1.29)	0.76	208	1.28 (0.69-2.36)	0.43	653	1.01 (0.77-1.31)	0.97
>20	171	0.61 (0.38-0.97)	0.04	219	1.29 (0.70-2.37)	0.42	390	0.85 (0.62-1.16)	0.30

IVF/ICSI, in vitro fertilization/intracytoplasmic sperm injection. Logistic regression. Reference = the '0–10%' DFI category. Results adjusted for female age.

2004; Check *et al.*, 2005; Zini *et al.*, 2005; Jiang *et al.*, 2011), also confirmed by some meta-analysis data (Evenson & Wixon, 2006). In contrast, some other studies have not been able to show such association (Larson-Cook *et al.*, 2003; Bungum *et al.*, 2007; Lin *et al.*, 2008; Speyer *et al.*, 2010; Dar *et al.*, 2013). This study represents, so far, the largest IVF/ICSI single center study in which the outcome of the treatment was related to the level of DFI.

A meta-analysis made by Collins (Collins *et al.*, 2008) has shown a statistically significant negative association between DFI and pregnancy in IVF and ICSI cycles. However, it was concluded that the magnitude of the effect of high DFI was not sufficiently high to provide a clinical indication for routine use of these tests in male infertility evaluation.

Our data show that both as considers the OR for live birth as well as for obtaining a GQE, the alteration in OR for the high DFI group, as compared to the reference group (DFI $\leq$ 10%), was of a magnitude which may have profound implications for the clinical outcome of ART. Our results do also indicate that the decreased fertilization rate was the major biological mechanism leading to the negative association between DFI and the lower birth rate as well as chance of obtaining a GQE.

Numerous of studies demonstrate that a significant part of men in infertile couples has remarkably high degrees of fragmented sperm DNA (Erenpreiss *et al.*, 2008; Oleszczuk *et al.*, 2013) and also men with high DFI have lower chance to cause pregnancy (Giwercman *et al.*, 2003; Sakkas & Alvarez, 2010). This problem can be overcome by using ART, especially by the use of ICSI (Bungum *et al.*, 2007). The results of our study which shows a significant difference of fertilization rate in the standard IVF group and does not show this difference in ICSI group are in agreement with previous observation regarding pregnancy in vivo (Spano *et al.*, 2000; Bungum *et al.*, 2007). Thus, our findings suggest that the cause and effect link between fertilization rate and sperm chromatin integrity is placed on the early stage of fertilization process based on a fusion between an oocyte and a spermatozoon. This can theoretically be bypassed by ICSI which can be confirmed by our observation that the fertilization rate is generally higher in the ICSI group. However, it must be noted that results in standard IVF and ICSI group are not entirely comparable, because in the ICSI but not the IVF group, the immature oocytes are excluded prior to assessment of the fertilization rate. On the other hand, significantly higher live birth rates in the ICSI group as compared to IVF for DFI > 20% might indicate that the former method is more efficient in this group of patients.

The biological explanation of superiority of ICSI over the IVF technique in case of increased DFI is not directly documented. Two possible explanations were suggested by Bungum (Bungum *et al.*, 2007). In the ICSI group, infertility is mainly caused by male factor which means that women in this group might be more fertile, e.g., as a result of younger age, and possibly produce oocytes with a better DNA repair capacity. In our material there was 1 year difference in the mean age of ICSI and IVF women. Another possible explanation was based on two completely different culture environments used for IVF and ICSI. While IVF oocytes were exposed to spermatozoa for 90 min, in ICSI, the spermatozoon are injected directly into the oocyte and therefore probably less exposed to reactive oxygen species (ROS) than in IVF. The general knowledge about the negative influence or ROS and oxidative stress on sperm chromatin integrity can also support our observation of the difference in success rates between ICSI and IVF. It is observed that the high level of estrogenic compounds causes oxidative stress, which leads to DNA damage in human spermatozoa (Bennetts *et al.*, 2008). In the IVF environment, not only the oocyte and the sperm are present, but also the cumulus complex consisting of a high number of corona cells is a natural part of the culture. In contrast, in the ICSI environment, all corona cells are chemically and mechanically removed. It may be speculated that sperms with high DFI are more vulnerable to the adverse effects of ROS because of release of estradiol from corona cells surrounding the oocyte during standard IVF procedure (Kattera & Chen, 2003), which has also been shown to have a direct toxic effect on the embryo (Valbuena *et al.*, 2001).

Our study has several strengths one of them being the large sample size, giving the study sufficient statistical power and making it possible to defining multiple DFI subgroups and, thereby, defining a DFI-threshold for impairment of fertilization and higher miscarriage risk. We have also been able to perform a separate analysis for standard IVF and ICSI treatments and were, thereby, able to conclude that the impact of DFI on ART outcome differs in those two scenarios. Furthermore, we have been able to focus on one method for assessment of DFI, some of the previous studies mixing both different types of ART and methods of DFI analysis (Zini *et al.*, 2008). Also, by collecting large numbers of treatments from a single center and having almost 100% SET, we excluded the potentially confounding effect of heterogeneous patient cohorts, diverging treatment protocols and differences in methodology used for assessment of DFI (Collins *et al.*, 2008). This may be the reason why we, in contrast to a recent published study (Simon *et al.*, 2014) found even SCSA to be predictive for the outcome of IVF treatment. Although the SCSA data were available for only 62% of eligible couples, apart from exclusion of those with sperm concentration below 1×10^6/mL no selection bias is expected. For those cases excluded because of very low sperm counts ICSI is, anyhow, the only feasible method of treatment and a comparison with IVF is not relevant.

Although it is common for infertility studies that a distinction between presences of male and/or female factor is made, we have omitted to include this classification in this study. The reason is that we find such categorization as quite inaccurate and highly dependent on the number of investigations included in the work up of the couple. Thus, in a recent paper (Oleszczuk *et al.*, 2013) we have shown, that in 25% of cases of ‘unexplained infertility’ the DFI is above the level of 20%, which indicates that impairment of sperm DNA integrity might be one of the explanations of the couple’s infertility problem. The fact that DFI seems to have a predictive value in relation to the IVF outcome, without discriminating between possible causes of infertility, makes this marker even more valuable in the daily clinical practice.

The retrospective design of the study represents its major weakness. Thus, ideally, the patients with high DFI fulfilling the criteria for standard IVF should be randomized to this treatment or to ICSI. Such a study are not yet available but our results indicating impairment of the outcome of standard IVF for DFI exceeding the level of 20%, facilitates a design of such study. Our study has profound clinical implications. Thus, the DFI as measured by SCSA above the level of 20–30% may be an indication for switching from standard IVF treatment to ICSI, in order to increase the chance of embryo transfer. Owing to a certain level of intra-individual variation in DFI (Oleszczuk *et al.*, 2011), the analysis should, ideally, be performed on the semen sample to be used for IVF or ICSI. Furthermore, since a recent Cochrane analysis (Showell *et al.*, 2014) has indicated increased pregnancy rates following antioxidant treatment of males in couples seeking fertility assistance, there is an urgent need of clarifying the effects of this treatment in management of men with high DFI.

In conclusion, we found that DFI-SCSA levels of 20% or higher, as seen in almost 25% of men entering IVF or ICSI treatment are associated with significant lowering of live birth rate when, using standard IVF but not ICSI treatment. Furthermore, the miscarriage rate was significantly increased for those having DFI of 40% or higher. These results point to sperm DNA testing as a useful tool in selection of the most effective ART-method in a given couple and also encourage to testing new treatments modalities which might improve sperm DNA integrity.

AUTHOR CONTRIBUTIONS

K. O, A. G, and M. B have all given substantial contributions to conception and design of the present study. All authors have contributed to acquisition of data, analysis as well as interpretation of data. K. O has drafted the manuscript and A. G and M. B have revised the content critically. All authors have made final approval of the version to be published.

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COMPETING INTERESTS

The authors declare that they have no competing interests.

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