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Andrology Lab Corner

Sperm Chromatin Structure Assay: Its Clinical Use for Detecting Sperm DNA Fragmentation in Male Infertility and Comparisons With Other Techniques

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The Sperm Chromatin Structure Assay (SCSA) was first described in the December 5, 1980 issue of *Science* (Evenson et al, 1980a). The data described in that article showed a significant difference between proven fertile and subfertility or infertility in men and bulls and the susceptibility to denaturation of their sperm nuclear DNA. A subsequent study (Evenson et al, 1984) described SCSA data obtained from men with testicular cancer. The data were heterogeneous, and begged the question, what was SCSA measuring? Great effort has been spent over the past 15 years on animal model systems, mostly related to dose-response toxicology experiments and large animal fertility trials. The results from these studies showed that SCSA was highly dose-responsive to toxicants, highly repeatable, and provided meaningful biological information on sperm nuclear DNA defects. The extensive data from nonhumans (Evenson et al, 1985, 1986, 1989a,b, 1993a,b,c, 1994, 1995b; Ballachey et al, 1987, 1988; Evenson and Jost, 1993; Sailer et al, 1995a,b; Evenson, 1999a), and human toxicology and fertility studies (Evenson et al, 1978, 1980a,b, 1984, 1991, 1999, 2000, 2001; Evenson and Melamed, 1983; Evenson and Jost, 1994; Evenson, 1999b; Larson et al, 1999, 2000, 2001) provide compelling evidence that SCSA will be useful in clinical human semen analysis (Figure 1).

After reviewing current infertility center sites on the World Wide Web and approximately a dozen lay-oriented books on infertility and assisted reproductive technology (ART), it was amazing that not a single reference contained any information about the negative influence of fragmented sperm nuclear DNA on successful pregnancy outcome. Furthermore, few physicians are aware of scientific literature on this subject. Currently, if a spermatozoon has some motility as seen under a light microscope, and reasonable morphology, then many ART clinics assume that the sperm DNA must be fine. However, a number of cases have been documented in which couples have had multiple failed attempts at intracytoplasmic sperm injection (ICSI), even using donor eggs, and we found that the fragmentation of sperm DNA was above our threshold for less than 10% probability of achieving a successful pregnancy. Spermatozoa with defective DNA can fertilize an oocyte, produce high-quality early stage embryos, and then, in relationship to the extent of DNA damage, fail in producing a successful term pregnancy (Ahmadi and Ng, 1999).

Although a variety of methods have been developed to evaluate paternal DNA integrity, these assays are often labor intensive and lack statistical power for diagnosis and prognosis. Some of the leading professionals in the field of human reproductive medicine now view SCSA as the most efficient method for measuring thousands of single spermatoza in an ejaculate and the most successful assay for providing both diagnostic and prognostic evaluations of man's potential for infertility.

Details are provided in this article on the nature of sperm chromatin physiology and structure that may lead to DNA strand breaks and altered chromatin structure, including abnormal protein interactions with the sperm DNA. We emphasize that despite the promise of SCSA from previous measurements of thousands of sperm samples, we will be conducting large-scale, randomized trials to verify the statistical predictions of SCSA.

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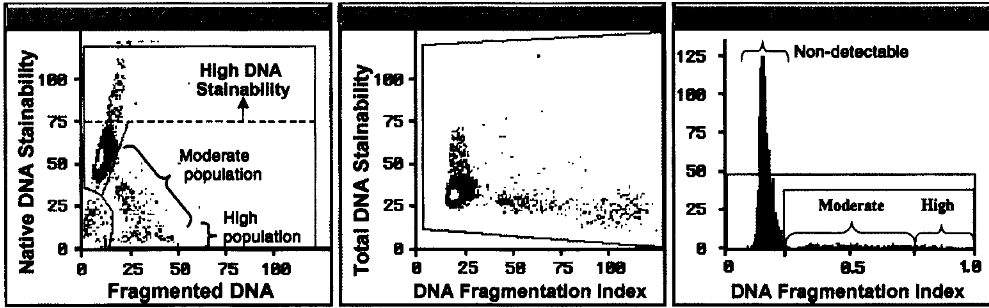
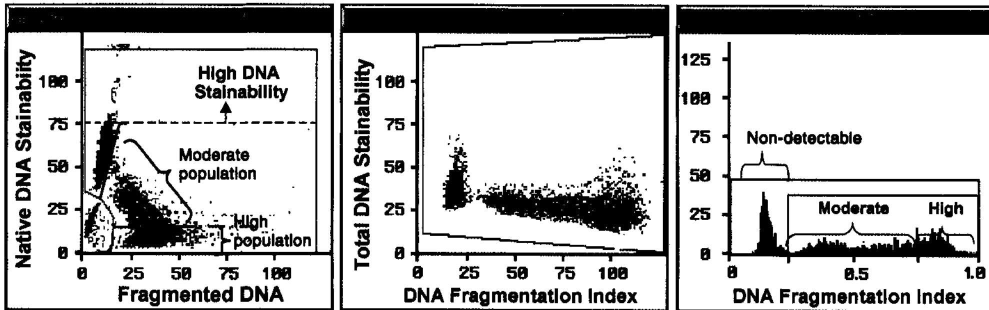
Pregnancy Outcome = Pregnant



Patient	Date	Measurement	mean DFI	SD DFI	DFI	HDS
7272-87	####	1	213.5	111.6	6.8	5.0
		2	221.2	118.1	8.3	5.4
		mean	217.4	114.8	7.5	5.2
		sd	5.4	4.6	1.1	0.2

Pregnancy Outcome = NOT Pregnant



Patient	Date	Measurement	mean DFI	SD DFI	DFI	HDS
7272-113	####	1	563.7	307.0	64.9	6.4
		2	561.4	304.8	64.9	7.2
		mean	562.6	305.9	64.9	6.8
		sd	1.2	1.1	0.0	0.4

Figure 1. Clinical results of SCSA. Typical SCSA cytograms obtained from 2 patients, 1 achieving a sustained term pregnancy and 1 who did not achieve pregnancy (Evenson et al, 1999). The cytograms and histogram show the source of each component of SCSA data. **(Left cytogram)** X axis, fragmented DNA (red fluorescence); Y axis, native DNA stainability (green fluorescence). The reader can visualize where each constituent part (non-detectable, moderate, high) of fragmented DNA is located and how the percentage of high DNA stainability (HDS) is calculated. **(Center cytogram)** X axis, DFI (a ratio of red to total fluorescence); Y axis, total DNA stainability (total fluorescence; ie, red + green). **(Right histogram)** DNA fragmentation index with each component (non-detectable, moderate, and high) clearly marked.

Diagnosing Male Infertility

Twenty percent of couples of childbearing age in the United States are considered infertile, defined as experiencing 1 year of unprotected intercourse without a pregnancy occurring. About 360 infertility clinics in North America treated 1 600 000 couples in 1998 (Centers for Disease Control and Prevention, 1998). These couples struggle to determine the basis of their problem, as well as attempt to circumvent it. The cause of this infertility is shared equally between male and female partners. At least 30% of cases of male infertility are still not diagnosed (Nieschlag, 2000) and are referred to as idiopathic infertility. Physicians and scientists who address the practical aspects of male infertility are driven toward identifying why the spermatozoa from a particular man are not

capable of fertilization (diagnostic) and whether spermatozoa from a particular man have the capacity to fertilize an egg (prognostic; De Jonge, 1999). One hopes that the information gathered will lead to an effective, therapeutic strategy for correcting or bypassing sperm defects (Burkman, 1996). In clinical practice, the traditional, manual/visual light microscopic methods for evaluating semen maintain their central role in the assessment of male fertility. However, a definitive diagnosis of male fertility often cannot be made as a result of a basic semen analysis (Centola and Ginsburg, 1996). Conventional semen analysis (World Health Organization, 1993) consists of measuring the classical semen parameters, including volume, pH, sperm concentration, vitality, morphology, and number of leukocytes. This analysis is used to determine whether all or some of the parameters of an ejaculate fall within a range characterized by fertile men. For a test to be useful, it must have a threshold above and below one that will provide discriminatory and predictive capabilities, with little overlap between fertile and infertile men. Conventional semen parameters often do not meet these standards.

To upgrade the prognostic and diagnostic ability of semen analysis beyond the conventional parameters, laboratories have included assays of sperm function, defined as measuring the ability of spermatozoa to complete the physiologic processes, which are classically viewed as necessary for natural fertilization in vivo (Burkman, 1996). De Geyter et al (2000) recently stated, "at present, none of the available sperm function tests can reliably predict the absence of fertilizing ability." It is important to understand that even when all conventional and functional semen parameters are normal, the sperm population in an ejaculate may be subfertile or infertile.

This failure to identify infertile men using conventional semen parameters and sperm function tests stems in part from the use of light microscopy, in which the number of spermatozoa analyzed for each parameter is typically only 100 to 200. Thus, the coefficient of variation, repeatability, and statistical soundness of these measures are weak. Also, systematic investigations have shown that the estimation of concentration, motility, and morphology of spermatozoa is strongly influenced by significant subjective factors (Neuwinger et al, 1990). Intensive efforts are being made to establish objective laboratory methods. The development of computer-assisted sperm assessment (CASA) is an example (Mortimer, 2000b). In contrast to light microscopic methods, the flow cytometric-based SCSA is the most objective and statistically robust measure of all tests in the infertility clinic. The terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay can also use flow cytometry; a standardized assay has not yet been validated.

Human Sperm Chromatin

Sperm chromatin is a highly organized, compact structure consisting of DNA and heterogeneous nucleoproteins. The condensed and insoluble nature of sperm chromatin protects the genetic integrity during transport of the paternal genome through the male and female reproductive tracts. However, the relatively homogeneous nucleoprotein packing of sperm chromatin seen in the light or electronic microscope belies the importance of the unique and complex structure in the proper expression of the paternal genome in early embryo and fetal development (Ward and Zalensky, 1996; De Jonge, 2000).

Relative to other species, human sperm chromatin is exceptionally variable within and between men. Although human spermatozoa can exhibit a dense, homogeneous chromatin structure, a considerable proportion may have a coarse, granular appearance with vacuoles of varying number and size (Bedford et al, 1973; Evenson et al, 1978; Jager, 1990). This coarse, granular appearance can be found within morphologically normal spermatozoa (Seligman et al, 1991) and is similar to that seen in late spermatids of other species (Fawcett, 1958). Therefore, the presence of this type of chromatin may be indicative of immaturity due to inadequate time in the epididymis (Seligman et al, 1994). This view is supported by evidence showing that the majority of disulfide bonds form when free thiol groups are oxidized during epididymal transit (Evenson et al, 1989a).

Variable sperm chromatin structure in a single human ejaculate also has been attributed to protamine content. The ratio of total protamine mass to DNA mass in human sperm chromatin is less than in the sperm nuclei of bull, stallion, hamster, and mouse (Bench et al, 1996). This observation is consistent with the retention of 15% histone nucleoproteins that leads to the formation or the retention of less-compact nucleosome structures (Bench et al, 1996). In contrast to the bull, rat, ram, boar, and guinea pig, whose spermatozoa contain only one type of protamine (P1), human sperm chromatin includes a second type of protamine, P2, which is deficient in cysteine residues. Therefore, human sperm chromatin has a lower level of free-sulfhydryl groups available for disulfide bonding and is therefore potentially less stable than the chromatin in species that contain P1 alone (Jager, 1990). Furthermore, P2 is synthesized as a precursor and modified after deposition into DNA. Abnormalities in proteolytic cleavage of the P2 precursor may act as an additional source of human sperm chromatin heterogeneity and potential infertility (de Yebra et al, 1998; Evenson et al, 2000).

Sperm Chromatin Structure in Embryonic Gene Expression

Variations in the highly defined nuclear architecture of sperm chromatin may influence the initiation and regulation of paternal gene activity in early embryo development (Haaf and Ward, 1995). In reference to the importance of chromatin structure, McCarthy and Ward (2000) described the sperm nucleus as an "ordered library of DNA organized into functional zones." Histone-associated DNA in the male pronucleus is preferentially acetylated and transcriptionally active during S/G_2 (Adenot et al, 1997) and early embryonic development (Gardiner-Garden et al, 1998). The association of histones with DNA sequences is highly controlled and specific with either protamine- or histone-containing chromatin changing within as few as 400 bp of DNA (Ward and Coffey, 1991; Gardiner-Garden et al, 1998). This high level of specificity indicates the fine control that histone-DNA associations play in gene expression (Gardiner-Garden et al, 1998). Abnormalities in the male genome have been shown to lead to postfertilization failure. (Sakkas et al, 1999a; Mortimer, 2000a; Sakkas and Tomlinson, 2000).

Oocyte Remodeling of Sperm Chromatin Structure

Oocytes and early embryos have been shown to repair sperm DNA damage (Matsuda and Tobari, 1988; Genesca et al, 1992). Consequently, the biological effect of abnormal sperm chromatin structure depends on the combined effects of the level of chromatin damage in the spermatozoa and the capacity of the oocyte to repair that preexisting damage. Therefore, if spermatozoa are selected from samples with extensively damaged DNA and used for in vitro fertilization, the oocyte's repair capacities may be inadequate, leading to a low rate of embryonic development and high early pregnancy loss (Ahmadi and Ng, 1999).

Sources of Sperm Chromatin Abnormalities

Physiological and environmental stress, as well as gene mutations and chromosomal abnormalities, can perturb the highly refined biochemical events that occur during spermatogenesis. This disruption can ultimately lead to abnormal chromatin structure that is incompatible with fertility. Stress can cause sperm chromatin abnormalities by inducing chromatin structural problems such as apoptosis or necrosis (Darzynkiewicz et al, 1997). Chromatin structural problems can arise during spermiogenesis if the DNA nicking and ligating activities of the endogenous nuclease, DNA topoisomerase II (topo II) are abnormal. High levels of both topo II and DNA nicks are present in elongating spermatids (Roca and Mezquita, 1989; McPherson and Longo, 1993). The presence of DNA nicks may reflect the need to relieve torsional strain resulting from negative supercoiling associated with the displacement of nucleosomal histones by protamines and modification of tertiary structure in elongating spermatids (Balhorn, 1982; Risley et al, 1986; McPherson and Longo, 1993). Therefore, in elongating spermatids, the presence of nicks is likely a physiological necessity. These nicks are not deleterious when they are ligated by topo II prior to the completion of spermiogenesis and ejaculation (McPherson and Longo, 1993). However, if topo II ligating activity is abnormal or blocked by exposure to topo II inhibitors (Morse-Gaudio and Risley, 1994), nicks may not be repaired properly, and they may remain in otherwise mature, morphologically normal, ejaculated spermatozoa. A recent paper by Caron et al (2001) indicates that the repair of these transient nicks can also be accomplished by an undefined activity of mammalian transitional proteins. These proteins are associated with in vivo repair of UV-induced lesions. Therefore, the role of transition proteins could extend beyond compaction and participate in the repair of DNA following various insults during spermatogenesis and/or spermiogenesis. If the abnormal DNA strand breaks remain, labeling by the TUNEL assay could lead to the erroneous conclusion that spermatozoa are apoptotic.

Sperm chromatin abnormalities associated with infertility can also result from abnormal protamine content; specifically, low levels of P2. An increased P1:P2 ratio was detected in some infertile men concomitant with an increased level of a putative P2 precursor (de Yebra et al, 1998). A fertile man with an influenza-induced fever (39.9°C; Figure 2) also expressed an unprocessed P2 precursor coincident with an increased histone:protamine ratio, decreased free -sulfhydryl groups, and increased DNA stainability (Evenson et al, 2000). The presence of an unprocessed P2 precursor (de Yebra et al, 1998) combined with evidence that genes encoding P1, P2, and transition proteins are normal (Schlicker et al, 1994) indicates that incomplete protein processing of P2 can lead to chromatin abnormalities. The role of abnormal P2 in infertility is substantiated by the importance of P2 in the formation of zinc-finger structures (Bianchi et al, 1994) and intermolecular and intramolecular disulfide bonds, which are necessary for chromatin stabilization (Calvin and Bedford, 1971; Bedford and Calvin, 1974; Evenson et al, 1980b).

Sperm chromatin abnormalities have also been attributed to abnormal apoptotic degeneration. Apoptosis plays a physiological role in germ cell output (Sinha Hikim et al, 1995) by regulating overproliferation (Rodriguez et al, 1997) and restricting normal proliferation levels during conditions unsuitable for sperm development (Pentikainen et al, 1999). The most characteristic feature of apoptosis is DNA double-strand breaks, which are formed by the activation of endogenous nucleases (Gorczyca et al, 1993). In somatic cells, apoptotic degeneration in-

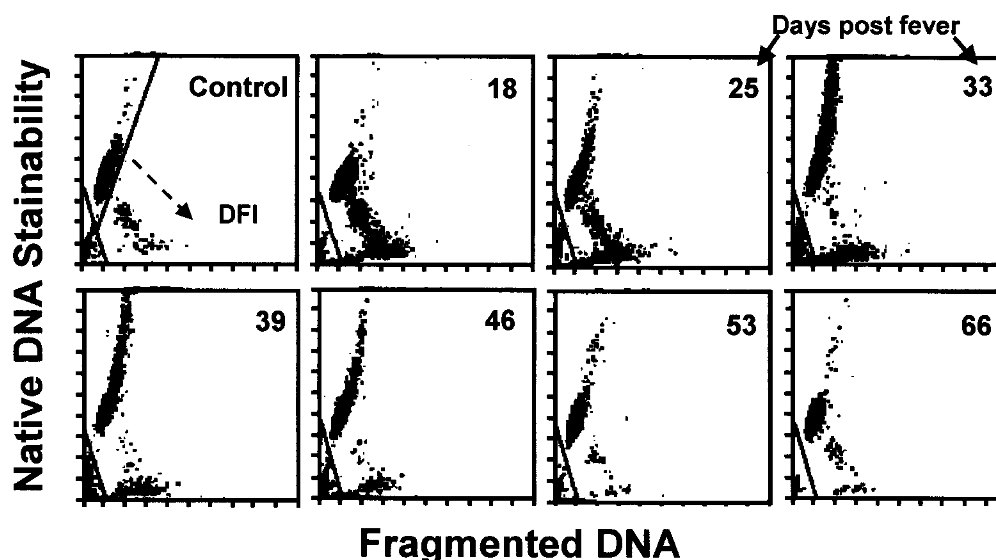


Figure 2. SCSA data showing the effects of influenza-related 39.9°C fever for 1 day on semen sample obtained on days 18, 25, 33, 39, 46, 53, and 66 days following the fever. Fever induced changes were different depending on the differentiation level of the developing spermatozoa. Note the change from a high percentage DFI at 18 days to one with mostly high DNA stainability at 33 days postfever. Of interest, the nuclear proteins at 33 days contained unprocessed P2 protamine. The data make a very strong case for physicians to postpone ART for nearly 2 months after a high fever (Evenson et al, 2000).

cludes a cascade of events, including the formation of apoptotic bodies seen by microscopy. However, in highly differentiated elongated spermatids or mature spermatozoa, these events may be modified (Sinha Hikim et al, 1995). For example, the highly condensed nature of the sperm nucleus may limit the formation of apoptotic bodies, although their presence has been reported in spermatozoa by Baccetti et al (1996). Furthermore, organellar and cytoplasmic apoptotic events may be modified in mature spermatozoa. Therefore, spermatozoa marked for apoptotic degradation may have mitochondrial activity as well as normal motility and morphology. Apoptotically labeled spermatozoa (TUNEL-positive or annexin V staining) were found in sperm fractions with both high and low motility (Barroso et al, 2000), and can be found in spermatozoa with normal morphology (Host et al, 2000a,b). Therefore, apoptotic spermatozoa can have normal kinematic and morphologic features, leaving the reproductive potential to be defined by the extent of endonuclease activity and resultant DNA degradation.

The work by Maione et al (1997) showed that in response to internalizing exogenous DNA, mature sperm cell nucleases were activated in a process similar to apoptosis. This indicates that even highly condensed, putatively inert mature spermatozoa may be able to respond to apoptotic triggers by dissociating suicidal machinery from inhibitors and beginning the cascade of molecular events that lead to apoptotic DNA degeneration (Vinatier et al, 1996). Oosterhuis et al (2000) reported that 20% of ejaculated spermatozoa showed DNA strand breaks and an apoptotic marker (annexin V). However, Sakkas et al (1999b) reported that DNA strand breaks and apoptotic markers did not exist together in the same mature spermatozoa. Ejaculated spermatozoa with apoptotic markers appeared to have escaped programmed cell death in a process called abortive apoptosis (Sakkas et al, 1999b). Therefore, it is critically important to distinguish between cells that show high levels of DNA strand breaks and cells that are positive for apoptotic markers. It is inappropriate to assume that strand breaks are synonymous with apoptotic degeneration.

Oxidative Stress Contributes to Abnormal Chromatin Structure

Reactive oxygen species (ROS) affect sperm function by contributing important physiologic functions at low levels and pathologic functions at high levels (Beckman and Ames, 1997). At physiological levels, ROS modulate gene and protein activities that are vital for sperm proliferation, differentiation, and function. However, at high levels, ROS are implicated in the 3 sources of abnormal chromatin structure discussed above—abnormal topo II activity, apoptosis, and necrosis.

In fertile men, the timing and amount of ROS generation is normal and properly controlled by antioxidants. Under these conditions, ROS play key roles in maintaining sperm kinematic and fusigenic functions. A specific ROS, H_2O_2, is involved in the hyperactivated movement necessary for spermatozoa to traverse the female genital tract and cumulus cells (Bize et al, 1991). ROS initiate lipid peroxidation, which increases sperm fusigenic func-

tions, and which is necessary for binding and traversing the zona pellucida (Aitken et al, 1989).

High levels of ROS that lead to pathogenicity can result when spermatozoa and contaminating leukocytes produce them in excess of the antioxidant capabilities of the male reproductive tract or seminal plasma (Aitken et al, 1992). Excess ROS production by spermatozoa is related to the retention of residual cytoplasm in the sperm midpiece (Gomez et al, 1996) and to sperm functional abnormalities (Aitken et al, 1995; Agarwal et al, 1996). In oligozoospermic samples, the spermatozoa themselves are the major source of excess ROS generation (Aitken et al, 1992). In fertile donors, leukocytes spontaneously generate the majority of ROS in the ejaculate (Aitken et al, 1992). Even at low concentrations, leukocytes are associated with abnormal sperm function due to their high-power ROS generation (Aitken et al, 1995). Therefore, spermatozoa, leukocytes, or both can contribute to excess ROS production and lead to abnormalities in sperm function and chromatin structure.

Spermatozoa are susceptible to oxidative damage due to the extrusion of antioxidant-rich cytoplasm during sperm maturation as well as the high concentration of polyunsaturated fatty acids in the plasma membrane (Griveau and LeLannou, 1994). Therefore, excess ROS produced in the testis and epididymis are especially harmful to spermatozoa due to the extended periods of exposure and the lack of antioxidant protection (Ochsendorf, 1999). After ejaculation, the seminal plasma, which is rich in the antioxidants catalase and glutathione peroxidase, aids in protecting spermatozoa from oxidative damage by catalyzing the degradation of H_2O_2 to H_2O and O_2.

Free radicals and H_2O_2 also are implicated in the induction of germ cell death via apoptosis and necrosis (Vinatier et al, 1996; Barroso et al, 2000). Physiological levels of apoptosis are necessary to regulate germ cell proliferation and can occur when ROS levels are relatively low or when spermatozoa are protected by the seminal plasma (Blanc-Layrac et al, 2000). However, elevated ROS production by spermatozoa in the male reproductive tract and ejaculate are associated, respectively, with oligozoospermia (Aitken et al, 1992) and the presence of chromatin degradation in mature spermatozoa (Barroso et al, 2000; Ollero et al, 2001). These abnormalities may be due to high levels of unregulated apoptosis, which result from exposure to excess sperm-derived ROS (Barroso et al, 2000). Conclusive evidence shows that centrifugal pelletting of unselected human sperm populations causes the production of ROS in the pellet and irreversible damage to spermatozoa (Aitken and Clarkson, 1988), which can impair the fertilization potential of motile sperm fractions subsequently prepared by swim-up (Mortimer, 1991). This indicates that when seminal plasma is removed, leukocyte-derived or sperm-derived ROS exposure can induce apoptotic or necrotic events and lead to impaired fertilization potential.

Elevated ROS levels are routinely reported in 25% of semen samples from men who consult infertility clinics (Griveau and LeLannou, 1997). Yet, even with the evidence that elevated ROS are associated with these potentially mutagenic changes in chromatin/DNA structure, these patients are still considered ideal candidates for ICSI. The potentially compromised spermatozoa from these patients can be further damaged during their preparation for ART procedures in which ROS can be enhanced and unregulated, which causes further iatrogeneic damage to sperm chromatin structure. ROS production may be enhanced 20- to 50-fold during repeated centrifugation steps, a technique still used in some clinics to prepare spermatozoa for ART (Aitken and Clarkson, 1988; Zalata et al, 1995). In addition, sperm preparation for ART could lead to unregulated ROS production when spermatozoa are separated from seminal plasma, thereby eliminating the seminal plasma's antioxidant protection afforded by superoxide dismutase, catalase, and glutathione peroxidase/reductase (Griveau and LeLannou, 1997).

Therefore, although some ART procedures such as ICSI, overcome fertilization-related sperm dysfunction associated with oxidative stress, these technical manipulations can also create and perpetuate oxidative damage in the genome of the next generation. By studying the impact of ROS on sperm nuclear chromatin integrity and resulting fertility, we may better understand their potential effect on the health of children conceived via ART.

Assisted Reproductive Techniques Require Sperm Chromatin Evaluations

In the vast majority of studies, sperm quality is defined by conventional parameters including concentration, motility, and morphology. The inclusion of these parameters is based on the assumption that spermatozoa "selected" by the female reproductive tract and oocyte vestments in vivo are also genetically adequate (Yanagimachi et al, 1995). This theory is not proven and is called into question by the heterogeneous chromatin condensation of spermatozoa that penetrate cervical mucus (Freundl et al, 1981), the high rate of spontaneous abortion in the general population, and by the absence of clinically significant correlations between conventional semen parameters and the presence of DNA strand breaks (Sun et al, 1997) or the susceptibility of sperm nuclear DNA to denaturation (Evenson et al, 1991; Evenson and Jost, 1994; Spano et al, 2000). Therefore, the fundamental definitions of sperm quality in andrology laboratories do not adequately assess the parameters required for optimum fertility success; namely, sperm DNA/chromatin integrity.

Mounting evidence for the presence of abnormal chromatin in morphologically normal spermatozoa and its ad-

verse effects on pregnancy outcomes indicates that it is now critically important to develop tools to assess chromatin damage. These tools will allow quantitative evaluation of sperm preparation techniques designed to select more spermatozoa with normal chromatin structure. Furthermore, these techniques may predict fertility potential prior to ART. The advancements in sperm preparation as well as the predictive power resulting from the quantitative assessment of sperm chromatin structure is seen as the next major advance in assisting couples with suspected fertility problems.

Assessment of Sperm Chromatin Structure in Fertility Evaluations

Normal sperm chromatin has an approximately fivefold decrease in binding capacity for DNA dyes and fluorochromes relative to the same DNA content in round spermatids. This decrease is due to the compact, insoluble structure of the molecule, which restricts access to DNA intercalators, minor groove ligands, and phosphate-binding dyes (Evenson et al, 1980b, 1986; Bianchi et al, 1993; Allen et al, 1996; Bench et al, 1996).

Feulgen, aniline blue, and chromomycin-A_3 (CMA_3) staining techniques have been used as measures of chromatin condensation anomalies. The potential value of adding these techniques to standard infertility evaluations lies in their ability to identify abnormal chromatin condensation in morphologically normal and abnormal spermatozoa (Dadoune et al, 1988; Hofmann and Hilscher, 1991). Aniline blue selectively stains lysine-rich histones (Terquem and Dadoune, 1983) and has been used as a clinical assessment of sperm chromatin condensation and associated infertility (Auger et al, 1990). High percentages of aniline blue–stained spermatozoa were found in patients affected by severe teratozoospermia (Franken et al, 1999), asthenozoospermia (Colleu et al, 1988), varicocele, idiopathic infertility, and with a history of unilateral cryptorchidism (Foresta et al, 1992). Furthermore, higher levels of aniline blue staining were found in subfertile men following cryopreservation than in fertile controls (Hammadeh et al, 1999b). Most importantly, aniline blue staining was found in some studies to be predictive of fertilization and pregnancy rates following IVF (Haidl and Schill, 1994; Hammadeh et al, 1998).

Aniline blue staining may not be related to IVF success solely on the basis of chromatin condensation assessment. Instead, the relationship to IVF success may be that aniline blue staining is indicative of abnormal epididymal maturation and associated sperm functional abnormalities. Functional abnormalities include failed induction of progressive motility and the acrosome reaction (Haidl and Schill, 1994), inability to bind to the zona pellucida, and abnormal morphology (Liu and Baker, 1992). These functional abnormalities, not chromatin structural abnormalities, may cause IVF failures in aniline blue–stained spermatozoa. ICSI obviates these sperm functional requirements by injecting these potentially immature spermatozoa directly into the oocyte where they can form a functional pronucleus and support a viable pregnancy. The potential viability of these immature spermatozoa is indicated by the inability of aniline blue staining to predict ICSI fertilization, embryo cleavage, or pregnancy rates with either ejaculated (Hammadeh et al, 1996) or testis biopsy–extracted spermatozoa (Hammadeh et al, 1999a). Therefore, aniline blue staining could be limited to assessing chromatin immaturity associated with functional abnormalities that lead to a decrease in fertilization rates following IVF.

CMA_3 is a fluorochrome specific for GC-rich sequences and is believed to compete with protamines for association with DNA, and therefore is related to the degree of protamination of mature spermatozoa (Bianchi et al, 1993). CMA_3 staining is related to low sperm counts, abnormal morphology, and low in vitro fertilization rates (Bianchi et al, 1996; Lolis et al, 1996; Esterhuizen et al, 2000; Iranpour et al, 2000). Neither Lolis et al (1996) nor Sakkas et al (2000) found a significant change in CMA_3 staining following swim-up preparation, confirming the absence of a relationship between CMA_3 staining and motility. However, a significant decrease in CMA_3 staining was reported following density gradient centrifugation by Sakkas et al (2000), although not by Lolis et al (1996). The decrease in CMA_3 staining is somewhat intuitive due to the selection of highly condensed spermatozoa by density gradient preparations.

Although CMA_3 staining is associated with abnormal morphology, 58% of sperm samples from patients treated by subzonal insemination (SUZI) showed both normal morphology and CMA_3 staining (Bianchi et al, 1996). These results indicate that a high percentage of morphologically normal spermatozoa may have hidden abnormalities in chromatin structure. However, no clear cutoff value for infertility has been demonstrated (Lolis et al, 1996). While one study reported an association between CMA_3 staining and fertilization ability following SUZI, this was not found in routine IVF (Bianchi et al, 1996) or ICSI (Sakkas et al, 1996). Furthermore, even in patients with SUZI treatment, there was no relationship between CMA_3 staining and the ability of fertilized oocytes to cleave. There was, however, a positive correlation between CMA_3 staining and the percentage of spermatozoa whose DNA remained condensed within unfertilized oocytes following ICSI (Sakkas et al, 1997). Therefore, although CMA_3 staining may be a useful method for screening patients for abnormal spermiogenesis that has resulted in intrinsic chromatin abnormalities, its utility in the ART laboratory remains limited due to the absence of a predictive threshold for fertility.

Overview of Assays for DNA/Chromatin Integrity

Assays using fluorochromes with more specific and complex chromatin/DNA interactions have been developed to identify more subtle defects in DNA and chromatin integrity. These assays, which might identify nonviable spermatozoa and provide predictive thresholds for male infertility, include the single cell gel electrophoresis assay (COMET), terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL), in situ nick translation (NT), sperm chromatin structure assay (SCSA), and acridine orange test (AOT). The methodologies of these assays and their application in male infertility investigations are outlined below.

COMET Assay

For the COMET assay, spermatozoa suspended in a thin agarose gel on a microscope slide are lysed, electrophoresed, and stained with a fluorescent DNA-binding dye. DNA strand breaks release supercoiling and allow migration of fragmented DNA to occur in the direction of the anode (Haines et al, 1998). Spermatozoa with high levels of DNA strand breaks have increased comet tail fluorescent intensity (Hughes et al, 1996) and comet tail length (Singh and Stephens, 1998).

The alkaline COMET assay (pH >10) denatures sperm DNA and therefore identifies both single- and double-stranded DNA breaks. A study using a pH 13 alkaline COMET assay showed that 20% of the total DNA fluorescence was in the comet tail of untreated spermatozoa, which is significantly higher than the 5% characteristic basal level of breaks in somatic cells (Hughes et al, 1996; Singh and Stephens, 1998). High basal levels of strand breaks were found in normozoospermic fertile men, indicating that normal human sperm chromatin has a high concentration of single-strand breaks that are alkali labile but not necessarily associated with reduced fertility (Singh et al, 1989). The presence of alkali labile DNA fragments in COMET assays could decrease the sensitivity of the assay to DNA fragments resulting from double-strand breaks that are indicative of DNA damage and infertility. This decrease in sensitivity may be why the alkaline COMET assay was unable to distinguish between sperm samples from normozoospermic fertile men, normozoospermic infertile men, and men with asthenozoospermia (Hughes et al, 1996). However, Irvine et al (2000) used a pH 12.3 alkaline COMET assay and found that spermatozoa from infertile men contained higher levels of DNA damage than did fertile controls. In addition, Hughes et al (1996) found that when DNA damage was increased by exposure to x-rays and H_2O_2, asthenozoospermic samples were more susceptible to damage than normozoospermic infertile samples, which in turn, were more susceptible to damage than normozoospermic fertile samples (McKelvey-Martin et al, 1997). The alkaline COMET assay also was used to show that ascorbic acid, α-tocopherol, and urate pretreatment protected DNA from x-ray-induced DNA damage (Hughes et al, 1998). However, pretreatment with acetyl cysteine alone or ascorbate and α-tocopherol in combination resulted in more DNA damage following x-ray irradiation.

The neutral COMET assay may be more sensitive to double DNA strand breaks and therefore better able to identify DNA damage related to infertility because the conditions of the assay (pH 9) do not denature DNA. The neutral assay measures only double-strand breaks and closely associated single-strand breaks, but does not measure strand breaks associated with alkali labile sites (Haines et al, 1988; Singh et al, 1988; Singh and Stephens, 1998). Singh and Stephens (1998) suggested that RNase A digestion in the neutral method allowed DNA to dissociate from other macromolecules and freely migrate through the gel. In this way, comet tail length increased in response to DNA damage rather than merely shifting the fluorescent intensity away from the nucleus. RNA association may have limited DNA migration and caused the lack of relationship between comet tail length and DNA damage reported by Hughes et al (1996).

Clinically useful thresholds have not been established for either the alkaline or neutral COMET assay. However, Irvine et al (2000) showed that the COMET assay was a more sensitive measure of DNA damage than in situ NT with prior chromatin decondensation. This level of sensitivity indicates that when methodologies are fully optimized, COMET parameters will likely be related to ART success.

TUNEL Assay

The TUNEL assay quantifies the incorporation of deoxyuridine triphosphate (dUTP) at single- and double-stranded DNA breaks in a reaction catalyzed by the template-independent enzyme, terminal deoxynucleotidyl transferase (TdT). Incorporated dUTP is labeled such that breaks can be quantified either by flow cytometry, fluorescent microscopy, or light microscopy as discussed below.

The flow cytometric TUNEL assay provides clinically significant results. Freezing raw or extended samples does not affect the results of the TUNEL assay (Sailer et al, 1995). After swim-up preparation, the majority of IVF samples have at most 4% damaged cells with 27% of the samples containing between 5% and 40% damaged cells (Sun et al, 1997). Sun et al also reported that TUNEL labeling was related to semen analysis parameters suggested by the World Health Organization, fertilization rate, and embryo cleavage in IVF samples. Yet, despite

highly significant χ^2 values, correlations were weak and do not provide adequate predictive power to be of clinical value.

Even with the weaknesses of the microscopic TUNEL assay, this method effectively quantifies the percentage of spermatozoa with DNA damage (Lopes et al, 1998a,b). ICSI samples had a higher mean percentage of damaged cells (14.5% ± 1.5%) with a much larger range (0.5% to 75%) than reported for IVF samples (Sun et al, 1997; Lopes et al, 1998b). DNA damage was also related to sperm motility and morphology of the unprocessed sample as well as fertilization following ICSI, but was not significantly correlated with embryo cleavage (ie, number of divided embryos after 48 hours compared to the number of zygotes after 24 hours) following ICSI (Lopes et al, 1998b). The microscopic method also shows that antioxidant treatment (reduced glutathione, hypotaurine, or *N*-acetylcysteine) before exposure to the ROS-generating xanthine-xanthine oxidase system significantly protects spermatozoa from DNA damage (Lopes et al, 1998a).

The microscopic TUNEL assay has been modified by some researchers to include a peroxidase enzyme labeling system that catalytically generates an intense signal from chromogenic substrates. This labeling technique eliminates the problems associated with fluorescence fading in the microscopic method, thereby giving technicians more time to analyze a greater number of cells (300 cells; Host et al, 1999a,b; 2000a,b). This technique has been used effectively to show that men with oligozoospermia have a greater level of sperm DNA damage than men with unexplained infertility (normal World Health Organization parameters), which in turn have more sperm DNA damage than normal fertile controls (Host et al, 2000a,b).

While some of the results reported using this nonfluorescent technique confirms the results based on the fluorescent TUNEL labeling techniques, other results seem to contradict previous findings. Host et al (2000a,b) showed that DNA damage was directly related to IVF rates in patients with unexplained infertility and oligozoospermia. These results are consistent with the work of Sun et al (1997) and Lopes et al (1998), but are in stark contrast to both heterologous human spermatozoa/zona-free hamster oocyte and homologous mouse experiments that showed fertilization was unaffected by sperm DNA damage (Ahmadi and Ng, 1999). Interestingly, in patients undergoing ICSI, Host et al (2000a,b) found that sperm DNA strand breaks were not related to fertilization. The difference in results between IVF and ICSI may indicate that the DNA strand breaks are associated with conventional parameters, including motility and morphology, which would influence fertilization rates in conventional IVF, but not in ICSI. To support this hypothesis, they reported finding a relationship between strict morphology and DNA damage in individual spermatozoa (Host et al, 1999a,b).

In Situ Nick Translation Assay

The in situ NT assay quantifies the incorporation of biotinylated dUTP at single-stranded DNA breaks in a reaction catalyzed by the template-dependent enzyme, DNA polymerase I. The NT assay identifies spermatozoa that contain appreciable and variable levels of endogenous DNA damage (Manicardi et al, 1995). These levels have been used as thresholds to predict the ability of sperm chromatin to decondense following ICSI. Sperm morphology parameters showed no such patterns (Sakkas et al, 1996), indicating that sperm morphology did not predict DNA integrity and could not be used to identify spermatozoa with adequate integrity for ART. Of particular concern was the finding that increased DNA damage (>10% nicks) does not appear to affect fertilization rates following ICSI. Therefore, adverse consequences of sperm DNA damage may be expressed during postimplantation development or during fetal development when children are conceived through ICSI (Sakkas et al, 1996).

Twigg et al (1998a,b,c) used NT to show 1) the impact of the source of ROS on sperm DNA damage and antioxidant effectiveness, 2) the vulnerability of sperm DNA to ROS resulting from protocols for ART, and 3) the normal levels of fertilization achieved by spermatozoa with high levels of ROS and DNA damage.

The ability of antioxidants to moderate sperm DNA damage depended on the source of ROS (Twigg et al, 1998a). Exposure to nicotinamide adenine dinucleotide phosphate (NADPH) increased intracellular ROS production, which increased sperm DNA damage and decreased sperm motility. Antioxidants that combat extracellular ROS (eg, superoxide dismutase, catalase, vitamin E, hypotaurine) did not decrease the effects of intracellular ROS on sperm nuclear DNA and motility. Albumin effectively neutralized lipid peroxide–mediated damage to the sperm plasma membrane but did not protect against DNA damage induced by NADPH. Albumin did protect sperm DNA from damage caused by the suppression of glutathione peroxidase activity with mercaptosuccinate. These results emphasize the extreme complexity of antioxidant supplementation in vivo and in vitro, similar to findings reported by Lopes et al (1998a).

Protocols for ART overcome some pathologies associated with male infertility. Yet many induce ROS, a fundamental cause of impaired sperm function and male infertility (Mortimer, 1991). The swim-up sperm preparation technique completed in the absence of seminal plasma caused significantly more ROS and sperm DNA damage than when the technique was completed in the presence of seminal plasma (Twigg et al, 1998c). This indicated not only the importance of seminal antioxidant

protection to sperm DNA in procedures for ART, but also the potential for these procedures to induce iatrogenic sperm DNA damage. Interestingly, sperm motility is not similarly affected. Therefore, without DNA assessment following this or other protocols, sperm DNA damage induced by ROS could remain hidden.

The effect of using oxidatively damaged spermatozoa for ICSI was studied by microinjecting human spermatozoa that had been exposed to oxidizing environments into golden hamster oocytes (Twigg et al, 1998b). Although exposure to oxidizing conditions can result in DNA and protein cross-linking, base modifications, and DNA strand breaks, the level of strand breaks identified by NT was not related to fertilization. These data confirm the results of Sakkas et al (1996) and show the potential for oxidatively induced sperm DNA damage to affect postfertilization events. Currently, NT thresholds for postfertilization embryo viability have not been established, which severely limits the clinical usefulness of this assay.

Sperm Chromatin Structure Assay

The SCSA, a flow cytometric assay developed by Evenson et al (1980a), has provided evidence for a relationship between sperm chromatin structure and function (Ward and Coffey, 1991). SCSA defines abnormal chromatin structure as an increased susceptibility of sperm DNA to acid-induced denaturation in situ. In samples from individual men, the SCSA parameters were more constant over 8 months than World Health Organization semen analysis parameters (Schrader et al, 1988; Evenson et al, 1991). Also, because SCSA was only weakly correlated with conventional semen parameters, it provided additional prognostic criteria for male infertility evaluations (Spano et al, 1998; Evenson et al, 1999; Larson et al, 2000). The ability to freeze raw or extended semen without affecting SCSA parameters (Evenson et al, 1994; Sailer et al, 1995; Ollero et al, 2001) allows effective collaborations between laboratories with SCSA capability and andrology/infertility laboratories around the world.

SCSA was first used approximately 20 years ago. It showed that sperm nuclear DNA was more resistant to heat-induced denaturation in fertile compared with infertile men and bulls (Evenson et al, 1980a). Heat-induced denaturation has since been replaced with acid-induced denaturation due to the similarity of results and the greater ease of the low pH technique (Darzynkiewicz et al, 1975; Evenson et al, 1985). A collaborative study showed that SCSA parameter values of 115 men seeking infertility counseling at the andrology laboratory affiliated with the National Hospital in Oslo, Norway, were twice those of men with proven fertility (Evenson et al, 1999).

Data from a study at Georgetown University in Washington, DC (Evenson et al, 1999) went beyond distinguishing fertile and infertile men and showed that SCSA parameters differentiated men with high in vivo fertility (pregnancy initiated in less than 3 months), moderate fertility (pregnancy initiated within 4–12 months), and no proven fertility (no pregnancy by 12 months). In addition, an SCSA parameter threshold was established that identified levels of DNA damage (30% of spermatozoa susceptible to the SCSA treatment conditions) that were incompatible with pregnancy (in the month of measurement) as well as a range (0%–15% damaged spermatozoa) that included 85% of fertile men. The 30% threshold was described as the "tip of the iceberg" effect; that is, chromatin damage identified in 30% of the spermatozoa indicated an abnormality in the entire population causing in vivo infertility. Spano et al (2000) also reported that in vivo fecundity decreased progressively when more than 20% of the spermatozoa were identified by SCSA as being damaged, and became essentially zero when at least 40% of the sperm population was damaged.

The relationship between SCSA parameters and in vivo infertility led to our study showing that sperm preparation protocols used in ART, including density gradient centrifugation (Larson et al, 1999; 2000) and glass wool filtration (Larson et al, 1999), isolated spermatozoa with more normal chromatin structure. Spano et al (1999) showed that swim-up also dramatically decreased the percentage of spermatozoa identified as having damaged chromatin structure (12% ± 8% damage in the unprocessed samples vs 4% ± 3% in the prepared samples). However, our results indicate that the improvement in sperm chromatin structure following ART was not associated with improvements in fertilization rates, embryo quality, or pregnancy outcomes. Instead, only the percentage of damaged spermatozoa in the unprocessed samples was predictive of negative pregnancy outcomes (Larson et al, 2000). Consistent with in vivo results reported by Evenson et al (1999), no IVF or ICSI patients achieved a pregnancy if more than 27% of the spermatozoa showed susceptibility to DNA denaturation via SCSA (Larson et al, 2000). To maintain consistency in SCSA data generated from the international collaborative team, raw semen aliquots will always be measured; measurements on other processed semen will be optional.

The case study of a patient with globozoospermia further illustrates the apparent independence of morphologic features and sperm chromatin structure (Larson et al, 2001). Transmission electron microscopy (TEM), the SCSA, and the COMET assay were used to determine if globozoospermia was associated with sperm chromatin structure abnormalities, DNA fragmentation, or both. It was unexpected but of great interest that a human sperm population with 100% sperm head morphology abnormalities (Tygerberg strict criteria) had molecular chromatin integrity equivalent to sperm populations shown in previous studies to be highly fertile. These data support

previous reports that ICSI in globozoospermia may result in pregnancy and that the lower success rates in some patients may be due to factors unrelated to chromatin integrity, such as oocyte-activating factor (Rybouchkin et al, 1996, 1997).

SCSA also has been used effectively in epidemiological studies of male infertility (Spano et al, 1998). SCSA values varied significantly by sexual abstinence time (88.8% of men less than 6 days; Spano et al, 1998), donor age, smoking history (18–55 years; Spano et al, 1998; Potts et al, 1999), and exposure to high levels of air pollution (Selevan et al, 2000; Evenson et al, 2001; Perreault et al, 2001). Cigarette smoke and air pollution share common genotoxins, polycyclic aromatic hydrocarbons, which may form protein or DNA adducts resulting in spermatozoa with increased amounts of fragmented DNA. This increase can result in DNA mutations that predispose offspring to greater risk of malformations, cancer, and genetic diseases (Potts et al, 1999). Specifically, the increase in chromatin abnormalities may also explain the reported effect of air pollution on the incidence of conception and congenital abnormalities in the Czech Republic (Selevan et al, 2000).

In clinical applications, SCSA showed that sperm chromatin structure was compromised in patients with leukocytospermia (Ollero et al, 2001), febrile illness (Evenson et al, 2000), and testicular cancer (Evenson et al, 1984; Fossa et al, 1997; Hiroshi et al, 2001). Ollero et al (2001) found a significant correlation between the concentration of leukocytes in semen and DNA damage in all the subsets of human spermatozoa isolated by a 3-step density gradient (immature germ cells and immature and mature spermatozoa). It was hypothesized that the DNA damage in spermatozoa from leukocytospermic samples could be related to alterations in the regulation of spermatogenesis. SCSA was also used to show that fever associated with disease resulted in a transient decrease in chromatin quality followed by an increase in chromatin stainability (see Figure 2). In further clinical studies, SCSA indicated that patients with untreated cancer express a variable but significantly higher incidence of sperm chromatin abnormalities than controls. Therefore, SCSA is able to quantify sperm chromatin abnormalities resulting from epidemiological factors and disease, and should be included in the modern semen analysis to predict infertility.

Acridine Orange Test

Tejada et al (1984) introduced the microscopic acridine orange test (AOT), a simplified microscopic method of the SCSA that does not require expensive flow cytometric equipment and an SCSA-trained technician. Both SCSA and AOT measure the susceptibility of sperm nuclear DNA to acid-induced denaturation in situ by quantifying the metachromatic shift of acridine orange fluorescence from green (native DNA) to red (denatured DNA). Although the AOT has been used by some laboratories in an attempt to improve male fertility evaluations (Tejada et al, 1984; Foresta et al, 1989; Peluso et al, 1992; Hoshi et al, 1996; Duran et al, 1998), any predictive value by AOT for human fertility is still controversial. AOT relies on visual interpretation of fluorescing spermatozoa and debris that fall into a broad range of colors under microscopic examination (Duran et al, 1998). Problems with interpretation are exacerbated by indistinct color (Claassens et al, 1992; Duran et al, 1998), rapidly fading fluorescence (Duran et al, 1998), and heterogeneous slide staining (Evenson et al, 1999). To determine the importance of these problems, AOT and SCSA were compared to determine whether AOT is adequately repeatable to allow its use in place of SCSA in predicting ART outcomes in the clinical laboratory. Our study (unpublished) showed that AOT introduced sources of variation that significantly affected our evaluation of relative levels of red and green fluorescence. It is obvious that if the majority of spermatozoa are green, then the statistical correlation is high between the two techniques. However, in samples with more than 20% red fluorescing cells, the correlations between slides ($r = 0.17$), areas within slides ($r = 0.67$) and scorers ($r = 0.53$) dropped dramatically. The range of 20%–30% red spermatozoa is the critical area and this is where the precision of the microscopic AOT drops off. The primary factor for the acridine orange metachromatic shift from green to red fluorescence is the critical equilibrium staining of acridine orange (Darzynkiewicz and Kapusinski, 1990). Because glass adsorbs acridine orange and, more importantly, glass microscope slides and coverslips are not perfectly flat, micro-pockets of higher and lower concentrations of acridine orange on the slides produce artifactual staining. This is most dramatically visualized on a single slide prepared for AOT on which all spermatozoa in one region are green and in an adjacent area of the same slide, all spermatozoa are red. Such conditions preclude use of AOT for critical clinical diagnosis and prognosis of a semen sample.

Sperm Chromatin Structure Assay

Advantages

The SCSA has several major advantages over existing clinical assays of sperm quality, including:

1. Twenty years of experience resulting in dozens of studies on animal and human spermatozoa that have been summarized in numerous symposia and more than 20 book chapters.
2. Aliquots of the same raw semen samples used for ART

can be easily flash frozen in liquid nitrogen or an ultracold freezer, stored, and sent for later analysis to a laboratory proficient in SCSA. Importantly, this provides a snapshot of the same semen sample used or, for prognostic purposes, a semen analysis performed within a few days prior to attempting fertilization by ART or in vivo methods.

3. The raw semen is thawed, diluted to a concentration of 1–2 million spermatozoa per mL, treated with a low pH buffer to potentially denature the sperm DNA, stained with acridine orange, and measured by flow cytometry. The unique metachromatic and equilibrium staining properties of acridine orange provide a very sensitive measurement and resolution between double- and single-stranded DNA by a shift from green to red fluorescence (Darzynkiewicz and Kapuscinski, 1990). This is the essence of the SCSA, and it requires strictly following the protocol for cell preparation, staining, and measuring.
4. Five thousand to 10 000 spermatozoa are measured by flow cytometry in less than 5 minutes, providing 1) objective, machine-defined criteria rather than biased human eye measures; 2) a higher level of repeatability (0.98–0.99) than any other currently measured semen parameter; and 3) data that are related to male factor infertility with reasonable sensitivity, high precision, and a statistical threshold for fertility prognosis.

Flow Cytometry

A variety of commercially available flow cytometers differ in their flow cell configuration (including sorting and nonsorting flow cells), optics, and data handling. Some flow cytometers do not adequately resolve sperm signal from debris signal, which is a crucial factor. SCSA data with the seminal debris-staining pattern overlapping the sperm fluorescence signal causes a serious and confounding artifact. Therefore, regardless of whether the problem originates with the brand of flow cytometer or the failure to set up the SCSA properly, it is simply intolerable for proper SCSA data. In samples in which the seminal debris to sperm ratio is very high, SCSA evaluation may have to be done only after purification of the spermatozoa from the debris though gradient centrifugation. A small percentage of samples are impossible to evaluate due to a very small sperm number (eg, $<$10 000/mL) and/or a massive level of cellular debris.

Using aliquots of the same semen samples, we have obtained equivalent SCSA data derived from our Ortho Diagnostics Cytofluorograf II, BD FACSCAN, Coulter Elite (Evenson et al, 1995a) and Coulter XL. Neither BD nor Coulter instruments have flow cytometry software that can convert the native DNA and fragmented DNA list mode data into a cytogram of total DNA stainability vs DNA fragmentation index (DFI; formerly termed alpha-t [α_t]). This conversion is vital for correctly calculating the percentage of spermatozoa with non-detectable DFI (formerly termed the Main or normal population of cells), moderate and high DFI (together, formerly termed COMP, for cells outside the main population) as well as the important standard deviation of DFI. We have recently developed a proprietary software program, SCSASoft, to handle list mode input from all flow cytometers for statistical analysis of SCSA parameters (refer to http://www.SCSAdiagnostics.com).

Protocols

Several techniques may qualify for use in analyzing mammalian sperm DNA fragmentation status (NT, COMET, and TUNEL assays). However, there are probably almost as many variant methods as there are investigator laboratories, rendering it impossible to rigorously compare data from one laboratory to another. Our laboratory has done the COMET assay several different ways; and efforts are in progress (Tritle et al, unpublished) to refine our earlier efforts (Aravindan et al, 1997). Even so, one study in our laboratory (Tritle et al, unpublished) indicates that the repeatability of the COMET assay is clinically inadequate. Likewise, we have used several methods for the flow cytometric TUNEL assay and found that in some cases, the data were poorly correlated (unpublished).

Over the last 2 decades a precise SCSA protocol has been developed and tested on thousands of animal and human spermatozoa/semen samples. It is important that other investigators who wish to use the assay conduct it in exactly the same way, not only so that data can be compared among clinics, but also in order for our threshold values to have clinical relevance. Therefore, we have now received trademark protection for the SCSA and our laboratory will serve as a quality control reference center. Those investigators who wish to deviate in any way from our protocol cannot call their test the SCSA, and their "new" technique will no longer have the benefit of all of our years of research and development.

The SCSA has been described by our laboratory numerous times in the literature (see introduction). Briefly, a frozen/thawed raw semen sample (usually 200 μL is flash frozen in a small cryovial) is diluted with TNE buffer (0.15 M NaCl, 0.01 M Tris HCl, 1 mM disodium EDTA pH 7.4, 4°C) to a sperm concentration of $1–2 \times 10^6$ mL and immediately mixed with 400 μL of 0.08 N HCl, 0.15 M NaCl, 0.1% Triton X 100 pH 1.2, 4°C. Exactly 30 seconds later, 1.20 mL of acridine orange staining solution (0.037 M citric acid, 0.126 M Na_2HPO_4, 0.0011 M disodium EDTA, 0.15 M NaCl pH 6.0, 4°C) containing 6 μg/mL electrophoretically purified AO (Polysciences, Inc, Warrington, Pa) is added, placed in the flow cytom-

eter, and the sample flow initiated. If the flow rate exceeds 300 spermatozoa/second, a new diluted sample is prepared. This low-pH solution potentially denatures DNA that is damaged. Acridine orange stains normal double-stranded DNA green, and denatured, single-stranded DNA is stained red. The flow cytometer can resolve 1024 X-channels vs. Y-channels of spermatozoa that emit green (515–530 nm band pass filter) and red (630 or 640 nm long pass filter) fluorescence to precisely characterize each of the 5000 cells measured. Three minutes after the start of the procedure, allowing time for hydrodynamic equilibration, signal acquisition to a computer list mode file is initiated. The raw data from the flow cytometer are collected on our interfaced Cicero computer (Cytomation, Ft Collins, Colo). Computer gates are used to determine the proportion of spermatozoa with increased levels of red fluorescence (fragmented DNA) and green fluorescence (immature spermatozoa). The DFI histogram profiles the spermatozoa with fragmented DNA within the entire population and mean and standard deviation values for DFI are derived from this flow cytometric data. Because SCSA data may play a significant role in how a couple will respond to ART and other options for obtaining a child, we have always independently measured every human semen sample twice, even though the repeatability is usually in the range of more than 0.98. This is especially important when sample values have detectable DFI values (moderate plus high) of 25%–35%, the statistical range of a greatly decreased probability of achieving a successful pregnancy.

Data Analysis—New Terminology

Raw data from SCSA measurements are stored in computer list mode files and transferred to an offline computer for final statistical analysis using our proprietary SCSA-Soft software. The report generated shows a native DNA vs. fragmented DNA cytogram, total DNA stainability vs. DFI scattergram, and a DFI frequency histogram. From these data the percentage of spermatozoa with an abnormally high DNA stainability and the level of DNA fragmentation (ie, DFI) are calculated. Although SCSA is an indirect measure of DNA strand breaks, the large amount of data showing a high level of correlation of percentage of DFI values and the proportions of cells positive by the TUNEL and COMET assays have made us conclude that the percentage of spermatozoa with denatured DNA is, in fact, the percentage of spermatozoa with significant amounts of DNA strand breaks. Further research will indicate whether these different tests will detect other factors that may be significant for pregnancy outcome. Other studies on rodents that have used the same sperm samples known to have DNA strand breaks have confirmed a high level of DNA denaturation (Estop et al, 1993; Evenson et al, 1993b). Currently, the previous single COMPα_t population has been replaced by percentage spermatozoa with non-detectable (formerly the main population) and detectable (moderate and high populations) DFI. Moderate plus high DFI equates to the previous COMPα_t value. Current clinical studies suggest that using both of these populations will improve the clinical diagnostic and prognostic values of SCSA. Statistical analysis of variance will be performed in the future to further test this hypothesis.

Data Interpretation

First, there is no doubt from the animal and human studies that DNA fragmentation and altered chromatin structure, as measured by SCSA, are detrimental for achieving and sustaining a pregnancy. Therefore, the primary question is, What is the SCSA threshold between subfertile or infertile men and fertile men? Note the use of the concept *infertility potential*, because spermatozoa with excellent chromatin structure but with other abnormalities and without motility will still be infertile. However, motile and otherwise normal spermatozoa with damaged DNA and chromatin can certainly be infertile.

At this point we have a relatively large accumulation of in vivo and in vitro data that provide a diagnostic and prognostic outlook on the effects of abnormal sperm DNA on male infertility. However, most of the data published and quoted are from the 200 couples not known to have a fertility problem in the comprehensive Georgetown male factor infertility study (Evenson et al, 1999). We still use that study's statistically derived threshold (using moderate plus high DFI) of more than 30% DFI, established for significant lack of fertility potential, 15%–30% DFI for reasonable, and less than 15% DFI for high fertility status. These 3 categories have been extended to 4 categories in our current clinical report: excellent, less than 15% DFI; good, 15%–24% DFI; fair, 25%–30% DFI; and poor, more than 30% DFI. These data show that SCSA has reasonable sensitivity. Specificity (ie, the proportion of normal individuals correctly predicted to have a fertility problem and who actually experienced a fertility problem), was 52% (27 out of 52). Had the SCSA data been based on a single measurement taken within several days of attempted conception over all 12 months rather than just during the first 3, the predictive power would likely have increased. Eighty-four percent of the male partners of couples who conceived in the first 3 months ($n = 73$) had a DFI of less than 15%. SCSA data from those couples demonstrating pregnancy during the first 3 months of the study were significantly different ($P < .01$) from the data on the 40 couples achieving pregnancy in months 4–12 and from the 31 couples not achieving pregnancy ($P < .001$). Based on logistic regression, the percentage of spermatozoa with fragmented DNA was the best predictor of all those measured for whether a couple would not achieve pregnancy. Using selected cutoff val-

ues for chromatin integrity, including spermatozoa with both DNA denaturation (DFI spermatozoa) and an abnormal increased level of DNA green stainability (high DNA stainability), the SCSA data predicted 7 of 18 miscarriages (39%) that could be considered likely to have been due to the male partner's genetic contribution.

Other as-yet-unpublished studies, in collaboration with large IVF clinics, have shown that if the DFI in raw semen is above 30%, there were rare sustained pregnancies (less than 10%) in more than 700 attempts at IVF, ICSI, or both. Currently, in these cases, the proportions of cases in the 0%–15%, 15.1%–25%, 25.1%–30%, and more than 30% DFI categories are 28%, 34%, 10%, and 27%, respectively. Spontaneous abortion is highest in the more than 30% DFI category. When more than 30% of the spermatozoa in a sample have fragmented DNA to the extent that the color of spermatozoa shifts from green to red under our specific conditions, then these samples have a level of sperm DNA strand breaks that are simply incompatible with proper paternal gene contributions to the embryo. An obvious question is, if 30% of spermatozoa in a sample are seen to be abnormal by the SCSA, why can't the other 70% be sufficient for fertility? Other data from our laboratory strongly suggest that the 70% of spermatozoa not showing DNA denaturation by our current protocol are the "remainder of the iceberg" (ie, they also contain the same kind of damage albeit to a lesser degree, but to an extent that significantly reduces paternal genome competence). If the sperm DNA damage is minor, the oocyte may be able to repair the damage, and this could become a major factor when a spermatozoon fertilizes an oocyte from an older woman. Age definitely has an effect on the level of DFI. Current collaborative studies in our laboratory show a significant increase in DFI after age 46. Thus, the male fertility potential appears to decrease at a later age than that of a woman and at a slower pace (Evenson et al, in preparation).

Much of the development and establishment of the SCSA as a meaningful biological assay for sperm nuclear integrity were conducted on rodent and livestock systems to study toxicology and fertility (Evenson et al, 1985, 1986, 1989a,b, 1993a,b,c, 1994, 1995b; Ballachey et al, 1987, 1988; Evenson and Jost, 1993; Sailer et al, 1995a,b; Evenson, 1999a). One study with our laboratory showed that SCSA-defined chromatin damage in bovine spermatozoa negatively affects early embryo formation from what appear to be normally fertilized eggs (Ellington et al, unpublished). The success of these animal model studies and preliminary studies on human systems led to the hypothesis that SCSA is the only practical means for diagnosing the integrity of sperm DNA for the couple with idiopathic infertility.

In previous SCSA studies, the total percent of spermatozoa showing DNA fragmentation was calculated as a single parameter (COMPα_t, now DFI). Yet SCSA data from patients often show that the DFI population consists of two resolvable populations, moderate and high DFI. These two populations likely have distinct biochemical properties that differentially influence male fertility potential. Furthermore, as pertains to the discussion of the "tip of the iceberg" effect, it is obvious that the non-detectable population must contain some spermatozoa with fragmented DNA, but not to the point that the flow cytometry signal was pushed from this population into the moderate DFI population by the chemical/physical forces imposed on the spermatozoa to reveal the DNA defects. Our laboratory has done a number of experiments to determine whether other spermatozoa in this non-detectable population could be pushed over the threshold to be counted as a "red" cell with fragmented DNA. Whereas this was found to be possible in dose-response studies, the SCSA fragmentation index curves were parallel and therefore, no useful information is added. By collecting SCSA data on a linear scale and keeping the SCSA protocol exactly the same, the flow cytometer is able to identify the important sperm parameters available.

High DNA-stainable (HDS) spermatozoa is another distinct population in semen that characterizes immature spermatozoa. Recent data support the idea that high DNA stainability can significantly influence male fertility potential and that this biomarker is currently underutilized in infertility investigations (Evenson et al, 1999). These cell types cannot be resolved and detected by the TUNEL, COMET, NT, or AOT methods. This provides a huge advantage to SCSA in being able to identify spermatozoa with at least 3 major levels of DNA fragmentation (low, moderate, and high) as well as the proportion with high DNA stainability plus an evaluation of the amount of seminal debris.

Development of the SCSA Clinical Report

The continued development of a comprehensive clinical SCSA report for enhanced diagnosis and prognosis of male idiopathic infertility is a major goal as additional samples are evaluated. At this time, a semen sample is measured twice and the mean and standard deviation of each SCSA variable are included on the report. Clinical reports are prepared that include native vs. fragmented DNA cytograms. Physicians use these data to offer diagnosis and prognosis. At present, we are using the statistically significant criteria generated from our Georgetown fertility study (Evenson et al, 1999) as well as taking into account the data from more than 700 IVF cases (unpublished). This includes check-off boxes for high, good, fair, and poor fertility potential, taking into account both the percentage of spermatozoa with fragmented DNA and the percentage with high DNA stainability. The report will evolve over time as more data are accumulated and

interactions with more fertility clinics increase. In particular, we hypothesize that the two resolvable sperm populations with high and moderate fragmentation will add to the prognostic and diagnostic potential of the clinical report. In addition, we hypothesize that the high DNA stainability parameter will provide an additional threshold for ART success as already implied by the in vivo study (Evenson et al, 1999).

Conclusion

Some personnel in infertility clinics may not be aware of the research completed over the past decade that has strongly implicated DNA damage in otherwise normal appearing spermatozoa as a major problem for achieving a successful pregnancy. Such information does not exist on infertility clinic Web sites or in the lay press. Internet chat groups between couples experiencing infertility problems are likewise unaware of the problem.

This is not surprising because the tests being worked on in the research laboratory using semen from patients attending infertility clinics are rather new, and TUNEL, COMET, and NT tests are being performed differently in different laboratories. There is essentially very little standardization and without it, there is no proven test to offer the infertility clinics.

The SCSA test is different because other laboratories and researchers currently using this test have been trained by our laboratory to adhere strictly to the protocol as developed over the past 20 years. The international SCSA network that is being established will maintain a very high level of quality control. No data will be accepted into the consensus report that do not meet the highest criteria set out for both an embryology laboratory and the SCSA measurement center. At this juncture, we believe that SCSA will make a solid contribution to the semen analysis profile that will be used for both diagnostic and prognostic purposes. Rapidly accumulating data strongly suggest a significant threshold above which there is a very small likelihood of a sustained pregnancy.

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