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·*Review*·

Sperm chromatin structure and male fertility: biological and clinical aspects

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Abstract

Aim: Sperm chromatin/DNA integrity is essential for the accurate transmission of paternal genetic information, and normal sperm chromatin structure is important for sperm fertilizing ability. The routine examination of semen, which includes sperm concentration, motility and morphology, does not identify defects in sperm chromatin structure. The origin of sperm DNA damage and a variety of methods for its assessment are described. Evaluation of sperm DNA damage appears to be a useful tool for assessing male fertility potential both *in vivo* and *in vitro*. The possible impact of sperm DNA defects on the offspring is also discussed. *(Asian J Androl 2006 Jan; 8: 11–29)*

Keywords: infertility; sperm; DNA damage; human

1 Introduction

Infertility affects approximately 15 % of couples trying to conceive and a male cause is believed to be a sole or contributing factor in approximately half of these cases [1]. In clinical practice, the traditional, manual-visual light microscopic methods for evaluating semen quality maintain their central role in assessment of male fertility potential. However, often a definitive diagnosis of male fertility cannot be made as a result of basic semen analysis. This consists of measuring seminal volume, pH, sperm concentration, motility, morphology and vitality [2]. Abnormalities in the male genome characterized by damaged sperm DNA may be indicative for male subfertility regardless of routine semen parameters [3, 4], and these parameters do not reveal sperm DNA defects.

Focus on the genomic integrity of the male, gamete has been intensified by the growing concern about transmission of genetic diseases through intracytoplasmic sperm injection (ICSI). This technique bypasses processes of natural selection during sperm-oocyte interaction, which are still present in conventional *in vitro* fertilization (IVF). There are concerns relating to potential chromosomal abnormalities, congenital malformations and developmental abnormalities in ICSI-born progeny [5–8].

2 Human sperm chromatin structure

In many mammals, spermatogenesis leads to the production of highly homogenous spermatozoa. For example,

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mouse sperm nuclei contain more than 95 % protamines in their nucleoprotein component [9]. This allows the mature sperm nuclei to adopt a volume 40 times less than that of normal somatic nuclei [10]. The final, very compact packaging of the primary sperm DNA filament is produced by DNA-protamine complexes, which, contrary to nucleosomal organization in somatic cells provided by histones, approach the physical limits of molecular compaction [11]. Human sperm nuclei, however, contain considerably fewer protamines (approximately 85 %) than those of bull, stallion, hamster and mouse [12, 13]. Human sperm chromatin, therefore, is less regularly compacted and frequently contains DNA strand breaks [14, 15].

To achieve this uniquely condensed state, sperm DNA must be organized in a specific manner, which differs substantially from that of somatic cells [10]. The fundamental packaging unit of mammalian sperm chromatin is a toroid containing 50–60 kb of DNA. Individual toroids represent the DNA loop-domains highly condensed by protamins and fixed at the nuclear matrix; toroids are cross-linked by disulfide bonds, formed by oxidation of sulfhydryl groups of cysteine present in the protamins [11, 16]. Thus, each chromosome represents a garland of toroids, and all 23 chromosomes are clustered by centromeres into a compact chromocenter positioned well inside the nucleus with telomere ends united into dimers exposed to the nuclear periphery [17, 18]. This condensed, insoluble and highly organized nature of sperm chromatin acts to protect genetic integrity during transport of the paternal genome through the male and female reproductive tracts. It also ensures that the paternal DNA is delivered in the form that sterically allows the proper fusion of two gametic genomes and enables the developing embryo to correctly express the genetic information [18–20].

In comparison with other species [21], human sperm chromatin packaging is exceptionally variable, both within and between men. This variability has been mostly attributed to its basic protein component. The retention of 15 % histones, which are less basic than protamines, leads to the formation of a less compact chromatin structure [13]. Moreover, in contrast to the bull, cat, boar and ram, whose spermatozoa contain only one type of protamine (P1), human and mouse spermatozoa contain a second type of protamine (P2), which is deficient in cysteine residues [22]. Consequently, the disulfide cross-linking responsible for more stable packaging is diminished in human sperm as compared to species containing P1 alone [23]. It is noteworthy that altered P1/P2 ratio and the absence of P2 are associated with human male fertility problems [24–31].

3 Origin of sperm DNA damage

DNA fragmentation is characterized by both single and double DNA strand breaks, and is particularly frequent in the ejaculates of subfertile men [15]. Oocytes and early embryos have been shown to repair sperm DNA damage [32, 33]. Consequently, the biological effect of abnormal sperm chromatin structure depends on the combined effects of sperm chromatin damage and the capacity of the oocyte to repair it.

Abnormal sperm chromatin/DNA structure is thought to arise from four potential sources: 1) deficiencies in recombination during spermatogenesis, which usually lead to cell abortion; 2) abnormal spermatid maturation (protamination disturbances); 3) abortive apoptosis; and 4) oxidative stress [14, 34].

3.1 Deficiencies in recombination

Meiotic crossing-over is associated with the genetically programmed introduction of DNA double strand breaks (DSBs) by specific nucleases of the *SPO11* family [35]. These DNA DSBs should be ligated until the end of meiosis I. Normally the recombination checkpoint in the meiotic prophase does not allow meiotic division I to proceed until the DNA is fully repaired or ablates defective spermatocytes [35, 36]. A defective checkpoint may lead to persistent sperm DNA fragmentation in ejaculated spermatozoa. However, direct data for this hypothesis in humans is lacking.

3.2 Abnormal spermatid maturation

Stage-specific introduction of transient DNA strand breaks during spermiogenesis has been also described [37–39]. DNA breaks have been found in round and elongating spermatids. DNA breaks are necessary for transient relief of torsional stress, favoring casting off of the nucleosome histone cores, and aiding their replacement with transitional proteins and protamines during maturation in elongating spermatids [37, 39–41]. DNase I-hypersensitive sites were found to be localized throughout the maturing spermatid nuclei or in a graduated manner, increasing from the anterior to posterior pole of the spermatid nucleus, mirroring the pattern of chromatin re-packaging and condensation [40]. Subsequently, their native DNA superhelicity was found to decrease

from the anterior to posterior pole as detected by the acridine orange test (AOT) [42]. Thus, chromatin re-packaging includes a sensitive step necessitating endogenous nuclease activity, which is evidently fulfilled by coordinated loosening of the chromatin by histone hyper-acetylation and introduction of breaks by topoisomerase II (topo II), capable of both creating and ligating breaks [40, 41]. Chromatin packaging around the new protamine cores is completed and DNA integrity restored during epididymal transit [42]. Although there is little evidence that spermatid maturation-associated DNA breaks are fully ligated, biologically broken DNA ends should not be allowed [43]. Ligation of DNA breaks is necessary not only for preserving the integrity of the primary DNA structure but also for reassembly of the important unit of genome expression, the DNA loop-domain. However, if these temporary breaks are not repaired, DNA fragmentation in ejaculated spermatozoa may occur.

In practice, in sperm DNA, contrary to somatic cell DNA, it is nearly impossible to distinguish single strand breaks from DSBs [44]. A huge radiation dosage of 30 Gy or more is necessary to produce detectable levels of X-ray-induced damage in elongated spermatids [45]. This is probably due to the uniquely tight chromatin packaging produced by protamines [38, 44]. The link between disturbances in chromatin packaging and the consequent occurrence of DNA strand breaks is also confirmed in knock-out mouse models defective in the expression of transition proteins and protamines [46–52].

It should be noted that elongated spermatids are enriched in both alkali-labile [53] and DNase I-hypersensitive sites [40], which evidently represent the same sensitive chromatin conformation. DNase I-sensitive sites are formed in pachytene in the chromatin domain containing protamine 1 (P1) and protamine 1 (P2) and the transition protein *Tnp2* genes, in the histone-enriched region. This configuration is necessary to induce transcription of these genes, however, it is also preserved in mature sperm [54].

The other methodical approach showed that human sperm DNA, compared to leukocytes, is enriched in segments of partially denatured DNA, which can also be considered alkali-sensitive sites [55]. These sites represent potential DNA breaks if induced by any factors. Although protected by proper chromatin packaging [53], the relative spermatid DNA/chromatin conformational fragility may be responsible for the presence of higher levels of spontaneous DNA damage in sperm than in somatic cells [45]. In addition, elongating chromatids have a lower repair capacity for strand breaks [56].

Enzymatic activity involved in the creation of DNA breaks in spermatids has only been proven (by decatenating activity and specific inhibition) for topo II generating and ligating DSBs [37, 41, 57]. Re-modelling of chromatin by histone H4 hyperacetylation weakens the ionic interactions between the DNA and histone cores and is needed for topo II activity to be introduced in spermatids [57]. The presence of DNase I in acrosome vesicles, from their initial formation in early spermatids to their presence in mature sperm, was shown in rats [58]. The ability of spermatozoa to use it and to digest their own DNA, if exposed to stressful conditions, has been suggested [59].

3.3 Abortive apoptosis

An alternative etiology for the DNA DSBs in the spermatozoa of infertile patients can arise through an abortive apoptotic pathway. Apoptosis of testicular germ cells occurs normally throughout life, controlling their overproliferation [60, 61]. It has been suggested that an early apoptotic pathway, initiated in spermatogonia and spermatocytes, is mediated by Fas protein. Fas is a type I membrane protein that belongs to the tumour necrosis factor–nerve growth factor receptor family [62, 63]. It has been shown that Sertoli cells express Fas ligand, which by binding to Fas leads to cell death through apoptosis [62], limiting the size of the germ cell population to numbers Sertoli cells can support [61]. Ligation of Fas ligand to Fas in the cellular membrane triggers the activation of caspases, therefore this pathway is also characterized as a caspase-induced apoptosis [64]. Men exhibiting deficiencies in their semen profile often possess a large number of spermatozoa bearing Fas. This fact prompts the suggestion that these dysfunctional cells are the product of an incomplete apoptotic cascade [14]. However, the contribution of aborted apoptosis in the DNA damage seen in the ejaculated spermatozoa is doubtful in cases where this process is initiated at the early stages of spermatogenesis. This is because that at the stage of DNA fragmentation apoptosis is an irreversible process [65] and these cells should be digested by Sertoli cells and removed from the pool of ejaculated sperm. Some studies have not found correlations between DNA damage and Fas expression [66], or, in contrast, have not revealed ultrastructural evidence for the association of apoptosis with DNA damage in sperm [67].

Alternatively, if the apoptotic cascade is initiated at

the round spermatid phase, when transcription (and mitochondria) are still active, abortive apoptosis might be an origin of the DNA breaks. *Bcl2* anti-apoptotic family gene member *Bclw* has been shown to be suppressing apoptosis in elongating spermatids [68].

Although many apoptotic biomarkers have been found in the mature male gamete, particularly in infertile men, their definitive association with DNA fragmentation remains elusive [69–78].

3.4 Oxidative stress

Reactive oxygen species (ROS) play an important physiological role, modulating gene and protein activities vital for sperm proliferation, differentiation and function. In the semen of fertile men the amount of ROS generation is properly controlled by seminal antioxidants. The pathogenic effects of ROS occur when they are produced in excess of the antioxidant capabilities of the male reproductive tract or seminal plasma [79]. Morphologically abnormal spermatozoa (with residual cytoplasm, in particular) and leukocytes are the main source of excess ROS generation in semen [79]. It seems that sperm DNA is more prone to leukocyte-induced ROS damage in infertile men with abnormal semen parameters likely possessing "masked" DNA damage and/or more fragile chromatin structure which are under the sensitivity threshold of the assays used for the sperm DNA damage assessment [80]. Such samples from infertile men frequently show depressed fertilization rates *in vitro* associated with the DNA damage [81].

Processes leading to DNA damage in ejaculated sperm are inter-related. For example, defective spermatid protamination and disulphide bridge formation because of inadequate oxidation of thiols during epididymal transit, resulting in diminished sperm chromatin packaging, makes sperm cells more vulnerable to ROS-induced DNA fragmentation. The origin and interaction of different sources of sperm DNA damage is shown schematically in Figure 1.

4 Assessment of sperm chromatin structure

Several assays have been developed to evaluate sperm chromatin/DNA integrity, and their capability to assess male fertility potential has been under active scrutiny [34, 82–86]. In general, all assays can be divided into three groups: 1) sperm chromatin structural probes, 2) tests for direct assessment of sperm DNA fragmentation, and 3) sperm nuclear matrix assays (see Table 1).

4.1 Chromatin structural probes using nuclear dyes

Chromatin structural probes using nuclear dyes are both sensitive and simple to use and therefore attractive for clinical use. Their cytochemical bases, however, are rather complex. Several factors influence the staining of the chromatin by planar ionic dyes: 1) secondary structure of DNA, 2) regularity and density of chromatin packaging, and 3) binding of DNA to chromatin proteins.

4.1.1 DNA secondary structure and conformation

Fragmented DNA is easily denatured [87]. However, even a single DNA strand break causes conformational transition of the DNA loop-domain from a supercoiled state to a relaxed state. Supercoiled DNA avidly takes up intercalating dyes (like acridine orange [AO]) because this reduces the free energy of torsion stress. In contrast, the affinity for intercalation is low in relaxed DNA and is lost in fragmented DNA. In this case, an external mechanism of dye binding to DNA phosphate residues and dye polymerization (metachromasy) is favored [88, 89]. Nevertheless, fragmentation of DNA is not the only factor affecting the determination between metachromatic versus orthochromatic staining. Chromatin packaging density also influences this balance.

4.1.2 Chromatin packaging density

If the chromatin is regularly arranged and sufficiently densely packed, dye co-planar polymerization providing metachromatic shift (change of color) is favored [90, 91]. However, if the chromatin is packaged even more densely (as in normal sperm), the polymerization of the dye is hindered [92] and may even prevent dye binding, especially by large, bulky dyes at an unfavorable pH. The latter case is seen with aniline blue (AB) at low pH where it stains basic proteins loosely associated with DNA and is unable to bind to the chromatin of normal sperm, which is very densely packaged. Explanations of how protamine molecules interact to facilitate DNA condensation and toroid formation have only been published recently [93–95]. Substitution of histones to more cationic protamines occurring during spermiogenesis neutralizes DNA charge and decreases the accessibility of DNA-specific dyes. Thus, the fluorescence staining intensity of a haploid sperm is much lower than the fluorescence intensity of a haploid round spermatid. However, after removal of nuclear proteins (e.g., by acid extraction), the net gain of stainability of sperm DNA can vary depending on the chemical structure of the fluorescent probe and from the

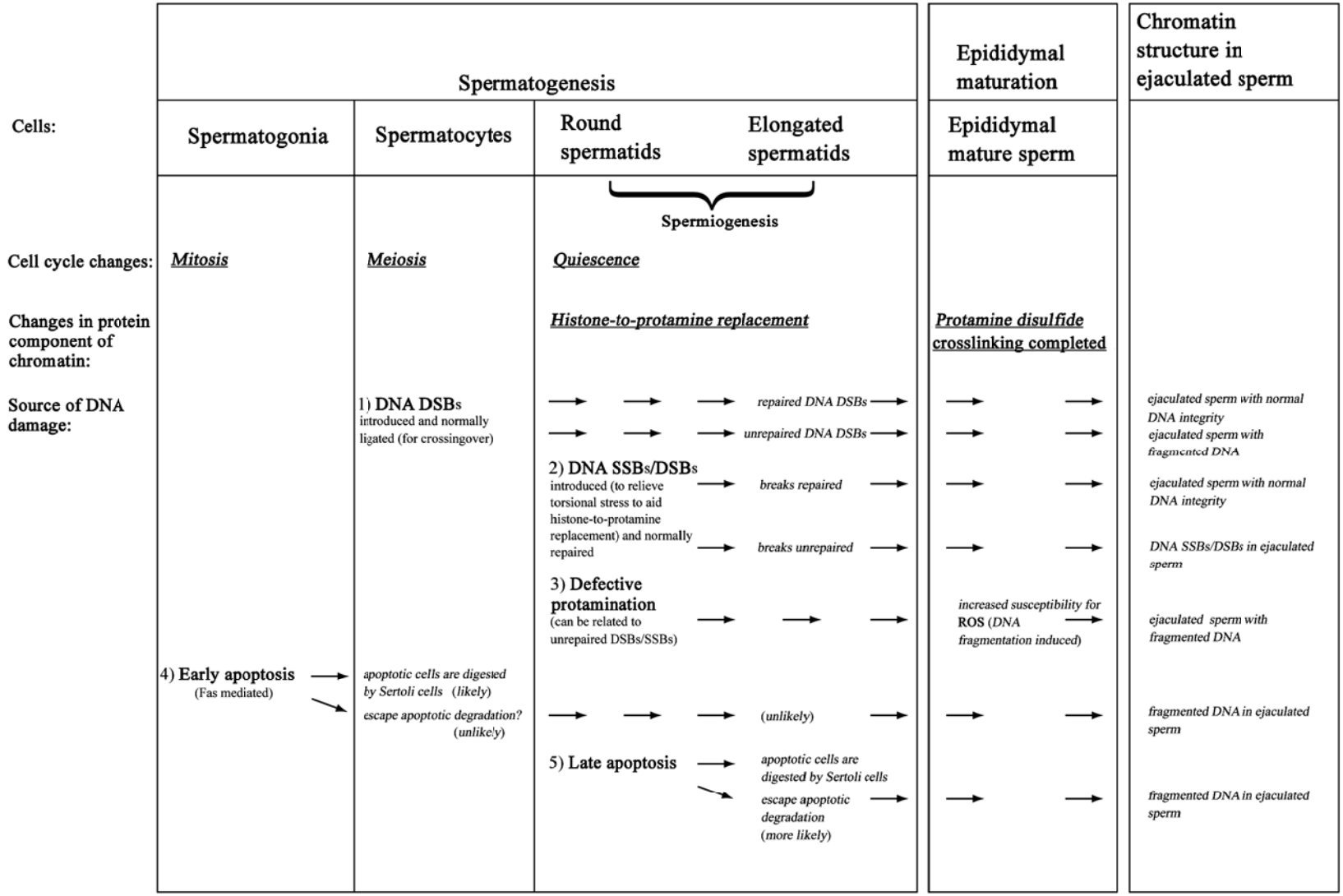

Figure1. Scheme of possible origin of DNA damage in ejaculated sperm. DSB, double strand break; ROS, reactive oxygen species; SSB, single strand break.

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Table 1. Methods to assess sperm chromatin/DNA integrity. AO, acridine orange; COMET, single-cell gel electrophoresis assay; DSB, double strand break; ICSI, intracytoplasmic sperm injection; PCR, polymerase chain reaction; SCSA, sperm chromatin structural assay; SSB, single strand break; TB, toluidine blue; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay.

Technique	Assay principle	Detection method	Advantages	Disadvantages	Clear clinical levels demonstrated
Chromatin structural probes					
AO test [98, 99, 105, 113–115]	Measures *in situ* DNA susceptibility to the acid-induced conformational helix-coil transition	Fluorescence microscopy	Inexpensive simple	Indistinct colour Heterogeneous slide staining Necessity to evaluate slides shortly after staining (fading) Inter-labvariability not tested	No
Aniline blue [42, 106]	Stains proteins in loosely condensed chromatin	Bright field microscopy	Inexpensive simple	Heterogeneous slide staining Inter-lab variability not tested	No
Chromomycin–A_3 [109, 110]	Compete with protamines for association with DNA, related to the degree of protamination of mature spermatozoa	Fluorescence microscopy	Inexpensive simple	Inter-lab variability not tested	No
Toluidine blue [42, 116–121]	Stains phosphate residues of the DNA of sperm nuclei with both loosely packed chromatin and fragmented	Bright field microscopy Image cytometry	Inexpensive simple Correlates well with SCSA and TUNEL assays	Inter-lab variability not tested	No
	DNA				
SCSA [100-104]	Measures *in situ* DNA susceptibility to the acid-induced conformational helix-coil transition by AO fluorescence staining	Flow cytometry	Quantitative detection of sperm with DNA breaks and sperm with nuclear immaturity Extensively standardized High statistical robustness High intra- and inter-lab repeatability	Needs flow cytometer and dedicated software	Yes
Direct methods for assessment of fragmented sperm DNA					
In situ nick translation assay [15, 109, 123, 124]	Quantifies the incorporation of biotinylated dUTP at single-stranded DNA breaks in a reaction catalyzed by the template-dependent enzyme, DNA polymerase I	Fluorescence microscopy	Relatively simple	Lack of sensitivity compared with other sperm assays	No

(to be continued)

Table 1 (continued)

Technique	Assay principle	Detection method	Advantages	Disadvantages	Clear clinical levels demonstrated
TUNEL assay [121, 122, 127–128]	Quantifies the incorporation of dUTP at breaks in double-stranded DNA in a reaction catalyzed by terminal deoxynucleotidyl transferase	Bright field microscopy Fluorescence microscopy Flow cytometry	Sensitive exclusively for DNA DSBs and SSBs Correlates well with other assays like SCSA, TB and COMET	Relatively expensive and labour consuming High intra-assay variability, inter-lab variability	No
COMET assay [15, 129-135]	Quantifies DNA SSBs and DSBs, using electrophoresis of DNA-fluorochrome-stained single sperm cells	Fluorescence microscopy	High level of sensitivity	Time consuming Requires computer-assisted image analysis High inter-assay variability, different inter-lab protocol options and high variability in data report format Difficult to standardize	No
a) Alkaline COMET assay (pH ≥ 12)	Denatures sperm DNA and therefore identifies both DNA SSBs and DSBs		High sensitivity as identifies both DNA SSBs and DSBs	Possible overestimation of DNA breaks due to induced conversion of alkali-labile sites into breaks	
b) Neutral COMET assay (pH < 9)			Specific for the detection of DNA DSBs	Low sensitivity	
Sperm nuclear matrix stability assay [134, 135]	Determines the high level DNA organization or aberrations in the sperm nuclear matrix's ability to organize the DNA into loop-domains	Fluorescence microscopy	Relatively simple and inexpensive	Preliminary stage, not extensively validated	No
Sperm chromatin dispersion test [136]	Sperm with fragmented DNA fails to produce the characteristic halo when mixed with aqueous agarose following acid/salt treatment removing nuclear proteins and, possibly, fragmented DNA	Bright field microscopy Fluorescence microscopy	Relatively simple and inexpensive	Preliminary, not extensively validated	No
Combinations of tests					
TUNEL and COMET [132]		Fluorescence microscopy	Improved assessment of male fertility	Applicablility in routine andrology laboratory appears quite problematic	No
COMET and long PCR [133]	Detects both DNA strand breaks (COMET) and mitochondrial DNA deletions (PCR)		Associated with pregnancy in ICSI	Applicability in routine andrology laboratory appears quite problematic	No

type binding the dye forms with the DNA substrate [96].

4.1.3 Chromatin proteins

Chromatin proteins affect the binding of DNA dyes in the way that they themselves bind differently to relaxed/fragmented or supercoiled DNA. DNA supercoiling requires covalent binding of some nuclear matrix proteins and tighter ionic interactions between DNA and chromatin proteins to support negative supercoils [97]. Relaxed/fragmented DNA has looser ionic interactions with chromatin proteins, which can be more easily displaced from the DNA, thus favoring external metachromatic binding of the dye to DNA phosphate groups. Both mechanisms of dye binding, external and intercalating, compete within each constraint loop-domain (toroid) depending on its conformational state.

Since the 1960s it has been known that DNA is more prone to denaturation by heat or low pH in sperm nuclei with abnormal chromatin structure [98, 99], as shown by AO. This test has been applied using flow cytometry as the sperm chromatin structural assay (SCSA) [100], which has been shown to have a predictive value for both *in vivo* and *in vitro* fertilization [101–104]. Tejada *et al*. [105] introduced the microscopic AOT, a simplified fluorescent microscopic method using acid fixative that does not require flow cytometry equipment. Both SCSA and AOT measure the susceptibility of sperm nuclear DNA to acid-induced conformational transition *in situ* by quantifying the metachromatic shift of AO fluorescence from green (native DNA) to red (denatured or relaxed DNA).

Chromatin proteins in sperm nuclei with impaired DNA appear to be more accessible to binding with the acidic dye, as found by the AB test [42, 106]. An increase in the ability to stain sperm by acid AB indicates a looser chromatin packaging and increased accessibility of the basic groups of the nucleoprotein. This is due to the presence of residual histones [107] and correlates well with the AOT [42, 108]. Chromomycin–A_3 (CMA_3) is another staining technique, which has been used as a measure of sperm chromatin condensation anomalies. CMA_3 is a fluorochrome specific for GC-rich sequences and is believed to compete with protamines for association with DNA. The extent of staining is therefore related to the degree of protamination of mature spermatozoa [109, 110].

In turn, it can be inferred that the phosphate residues of sperm DNA in nuclei with loosely packed chromatin and/or impaired DNA will be more liable to binding with basic dyes. Such conclusions were also deduced from the results of staining with basic dyes, such as toluidine blue (TB), methyl green and Giemsa stain [42, 110–112].

The most widely used techniques for sperm chromatin structure assessment are the SCSA [100–104], AO [105, 113–115] and TB tests [42, 116–122].

4.2 Tests for direct assessment of sperm DNA fragmentation

The most widely used of these tests are *in situ* nick translation assays, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay (TUNEL) and single-cell gel electrophoresis assay (COMET). Their basic principles are well described elsewhere [15, 109, 121–133] and are summarized in Table 1. Nick translation is a relatively simple assay for fluorescence microscopy that quantifies the incorporation of biotinylated dUTP at single-stranded DNA breaks in a reaction catalyzed by the template-dependent enzyme, DNA polymerase I. The TUNEL assay quantifies the same incorporation at breaks in double-stranded DNA using a reaction catalyzed by terminal deoxynucleotidyl transferase. TUNEL can be applied in both bright field and fluorescence microscopy, and also using flow cytometry. The COMET assay quantifies single- and/or double-stranded DNA breaks (dependant on the pH conditions, see Table 1), using single-cell electrophoresis of spermatozoa stained with a fluorescent DNA-binding dye. It is therefore suggested as a very sensitive assay for DNA damage evaluation.

4.3 Sperm nuclear matrix assays

Two similar assays have been described that can be allocated to this group. The sperm nuclear matrix stability assay and the sperm chromatin dispersion test are based on the ability of intact DNA deprived of chromatin proteins to loop around the sperm nucleus carcass [134–136]. These two recently described assays are at the developmental stage and no studies verifying their usefulness in routine clinical practice have been reported to date. However, published data show that germ-line mutations in the nuclear matrix protein may lead to deficient DNA repair and chromatin organisation [137], so matrix pathologies can impair fertility and should be considered in future.

The assays' principles, as well as the advantages and disadvantages of assays from all three groups, are described in Table 1.

5 Clinical significance of sperm DNA damage

5.1 Relationship of DNA damage to other semen parameters

Relationships between sperm chromatin/DNA damage and conventional semen analysis parameters are summarized in Table 2.

Although some studies have reported either only a weak or no correlation between conventional semen parameters and sperm DNA damage, most of them do indicate that spermatozoa from patients with abnormal sperm count, morphology and motility have increased levels of DNA damage. It can be hypothesized that both testicular and extratesticular factors (see also Figure 1) contribute to the final load of sperm DNA damage in ejaculated sperm, therefore it is not surprising that different studies have found various correlation levels with other parameters of sperm quality. If we assume that DNA damage in a particular patient arises solely from the failure to repair DNA breaks introduced during spermatogenesis, one could logically expect that it would also correlate well with other indices of spermatogenic failure, like oligozoospermia and teratozoospermia. Alternatively, if sperm DNA damage is mostly as a result of the adverse effects of ROS, then a relationship to sperm motility could be expected. This factor is also affected by ROS, due to the lipid peroxidation of sperm membranes rich with unsaturated fatty acids. In fact some studies report a correlation solely between sperm DNA damage and motility [140, 145]. However, it should be remembered that these processes are inter-related. Unrepaired DNA DSBs can lead to defective sperm packaging which, in turn, as a consequence of persistent DNA fragmentation or due to the other reasons, can cause increased

Table 2. Relationships between sperm chromatin/DNA damage and conventional semen analysis parameters. † expressed as *r* = correlation coefficient. —, not reported; AOT, acridine orange test; COMET, single-cell gel electrophoresis assay; NS, not significant; NT, *in situ* nick translation assay; SCSA, sperm chromatin structural assay; TB, toluidine blue test; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay.

Technique	Study population		Results †			Reference
	Donors (*n*)	Patients (*n*)	Concentration	Normal morphology	Motility	
TUNEL	0	18	– 0.57	– 0.71	– 0.71	[121]
TUNEL	0	25	—	—	NS	[125]
TUNEL	0	262	NS	– 0.44	– 0.60	[126]
TUNEL	20	113	—	NS	NS	[139]
TUNEL	23	0	NS	NS	– 0.49	[140]
TUNEL	0	29	NS	– 0.56	NS	[140]
TUNEL	7	33	—	– 0.40	– 0.65	[141]
TUNEL	0	140	NS	– 0.31	– 0.28	[66]
TUNEL	0	65	– 0.33	–0.39	NS	[69]
TUNEL	0	34	– 0.63	—	—	[142]
TUNEL	0	104	– 0.44	–0.36	–0.28	[143]
SCSA	0	115	– 0.27	– 0.21	– 0.30	[101]
SCSA	0	306	NS	—	– 0.25	[104]
SCSA	0	35	– 0.64	– 0.71	– 0.77	[42]
SCSA	0	25	—	—	NS	[125]
SCSA	7	33	—	–0.38	–0.53	[139]
SCSA	16	92	– 0.31	– 0.40	– 0.47	[144]
SCSA	171		—	—	– 0.53	[145]
SCSA	0	201	—	– 0.46	– 0.56	[146]
NT	0	140	– 0.24	NS	– 0.20	[79]
COMET	12	29	– 0.54	– 0.37	– 0.37	[15]
AOT	0	119	– 0.58	– 0.48	– 0.42	[80]
TB	0	35	– 0.70	– 0.72	– 0.74	[42]

access to ROS attack. It is therefore understandable that clear unimodel patterns are not evident among the various published reports when different study populations with varying causes of sperm DNA damage were investigated.

5.2 Natural conception

Available studies clearly indicate a significant impact on *in vivo* fertilization from sperm DNA damage. Many studies, using a variety of techniques, have shown significant differences in sperm DNA damage levels between fertile and infertile men [102, 103, 139–141, 147]. The probability of fertilization *in vivo* seems to be close to zero if the proportion of sperm cells with DNA damage exceeds 30 % as detected by SCSA [101, 102].

5.3 Intrauterine insemination (IUI)

The probability of fertilization by IUI also seems to be close to zero if the proportion of sperm cells with DNA damage exceeds 30 % by means of SCSA [104, 144]. In addition, it has been shown that when semen samples containing > 12 % sperm with fragmented DNA (as detected by TUNEL assay) were used for insemination, no pregnancies were achieved [148].

Therefore, sperm DNA damage assessment has a high predictive value for the outcome of both natural conception and IUI.

5.4 In vitro fertilization (IVF)

The results from assisted reproductive techniques (ART) (IVF and intracytoplasmic sperm injection [ICSI]) in connection to sperm DNA damage are more controversial.

Sperm DNA damage was reported to show a significant negative correlation with embryo quality in IVF cycles [149]. Several authors have also reported significant correlations between sperm DNA damage and blastocyst development following IVF [150], and sperm DNA damage and fertilization rates following IVF [151] and ICSI [128], even though sperm DNA damage may not necessarily preclude fertilization and pronucleus formation during ICSI [124]. It has also been reported that a sperm DNA fragmentation index (DFI) predictive threshold of 27 %, detected by SCSA, is necessary to obtain a successful pregnancy both by IVF and ICSI [152, 153]. However, these results could not be repeated either by the same authors [138] or by other research groups [104, 154, 155], demonstrating that successful pregnancies in IVF/ICSI cycles can even be obtained using semen samples with a high proportion of DNA damage. Nevertheless, a study by Virro *et al*. [138] showed that men with DFI ≥ 30 % were at risk for low blastocyst rates and no ongoing pregnancies when IVF/ICSI were performed. The study by Bungum *et al*. [104] did not find such a difference between groups of men with low and high DFI proportions, however, it demonstrated that significantly higher clinical pregnancy rates (52.9 % *vs*. 22.2 %) and delivery rates (47.1 % *vs*. 22.2 %) were seen after ICSI compared with IVF when semen samples with high levels of sperm DNA damage were used. In this study, when DFI exceeded 27 % the odds ratio for a positive reproductive outcome after ICSI compared with standard IVF was 8 for biochemical pregnancy, 4 for clinical pregnancy and 3 for delivery. This data is in agreement with other reports showing that sperm DNA damage is more predictive in IVF and much less so in ICSI [151, 153, 156]. Apparently, sperm chromatin integrity, evaluated on neat semen, becomes particularly relevant when contact between the two gametes occurs in a more natural way when selective pressures operate to avoid the development of an embryo derived from sperm with a high load of genetic damage [157]. On the other hand, it is not surprising that the ICSI procedure, which bypasses normal egg-sperm interactions, and was initially developed for men with very seriously impaired semen parameters [158], allows even very low quality sperm to initiate a successful pregnancy. Pregnancies by ICSI using testicular spermatids have been reported [159–164], which stresses the fact that ICSI can lead to pregnancy regardless of traditional sperm quality parameters and sperm chromatin structural integrity. As it is likely that sperm with high DNA damage levels contributes to successful fertilization and *in vitro* development, the potential adverse effects when sperm with high loads of DNA damage are used still remain to be clarified.

5.5 Embryonal loss

Adverse male-mediated developmental outcomes can occur if the fertilizing sperm has a defective genome with, for example, DNA strand breaks. Depending on the severity of the genetic damage and the ability of the oocyte to repair it, the embryo may fail at any stages of pregnancy or might develop to term with abnormalities. Studies of miscarriages may be a feasible and sensitive approach to increase knowledge on male-mediated developmental toxicity. However, data on miscarriages as a possible consequence of sperm DNA damage is rather scarce. Whether conventional measures of semen qual-

ity are related to embryonic loss or not, sporadic but suggestive clues have been offered [165, 166]. It has been shown that the proportion of sperm with DNA damage (as detected by TUNEL) is significantly higher in men from couples with recurrent pregnancy loss(38.0 ± 4.2 %), compared with the general population (22.0 ± 2.0 %) or fertile donors (11.9 ± 1.0 %) [167]. It has also been reported that 39 % of miscarriages could be predicted using a combination of selected cut-off values for percentage spermatozoa with denaturated (likely fragmented) DNA and/or abnormal chromatin packaging as assessed by SCSA [101]. In this study, 7 of 18 men from couples that had experienced miscarriages had an increased sperm DNA fragmentation index or percentage of immature sperm cells as detected by SCSA. The study by Virro *et al.* [138] also showed an increased trend of spontaneous abortions following IVF/ICSI when sperms from men with high loads of damaged DNA, as detected by SCSA, were used. Recently, the SCSA test was performed on 106 male partners from couples failing to have a successful pregnancy despite at least two previous IVF attempts. Authors found that DFI ≥ 30 % was associated with a trend for lower ongoing pregnancy rates especially related to a high miscarriage rate [155]. The activation of embryonic genome expression occurs at the four- to eight-cell stage in human embryos [168], suggesting that the paternal genome may not be effective until that stage, therefore we can speculate that an elevated level of sperm DNA strand breaks seems to be of importance in the later stages of embryonic development [169]. In conclusion, it is possible that sperm DNA damage assessment could be a good predictor of possible miscarriages, which are dependent on the male factor. However, the findings mentioned above should be supported by more extended studies.

5.6 Effect of sperm DNA quality on offspring

Sperm DNA damage can affect the health of the embryo, fetus, and offspring [165, 166, 170, 171]. A possible consequence of sperm DNA damage is infertility in the offspring [172–174].

One concern raised from studies of smokers is the increased risk of childhood cancer in the offspring of men with a high proportion of sperm DNA fragmentation in their semen. It was shown that the offspring of these men, whose ejaculates are under oxidative stress [109] and whose semen is characterized by high chromatin fragmentation, are four to five times more likely to develop childhood cancer than the children of non-smoking fathers [175]. Another study has demonstrated that 15 % of all childhood cancers are directly attributable to paternal smoking [176]. However, the linkage between sperm DNA damage and abnormalities in offspring is not confined to smokers. For example, powerful associations exist between childhood disease and paternal occupation [177].

Of particular concern is recent data showing that ICSI is able to overcome the normal barrier of high loads of sperm DNA damage and initiate a successful pregnancy when this would hardly be possible through natural conception, IUI, or even to some extent IVF. The safety of the ICSI procedure has been questioned [178], and findings from the latest studies [104, 140] provide further reason for concern. Aitken and Krausz [174] proposed that sperm DNA damage is promutagenic and can give rise to mutations after fertilization, as the oocyte attempts to repair DNA damage prior to the initiation of the first cleavage. Mutations occurring at this point will be fixed in the germline and may be responsible for the induction of not only such pathologies as described above (infertility and childhood cancer in the offspring), but also for a higher risk of imprinting diseases [179, 180]. So far, however, follow-up studies of children born after ICSI compared with children born after conventional IVF have not been conclusive regarding the risks of congenital malformations, imprinting diseases and health problems in general [5, 181–189]. The recent meta-analysis of 25 studies addressing the prevalence of birth defects in infants conceived following IVF and/or ICSI compared with spontaneously conceived infants demonstrated that two-thirds of these studies show a 25 % or greater increased risk of birth defects in infants conceived through ART [190].

6 Suggestions for a clinical approach

Without doubt the existing data justify the necessity to introduce sperm DNA damage assessment into the routine infertility investigation. Some cases of unexplained or idiopathic infertility, when a traditional semen analysis falls into normal range and no evident female reproductive system pathologies can be revealed, will probably meet an explanation. In addition, the ART method of choice can be recommended based on sperm DNA damage assessment. It is clear that the chance of conception using IUI is negligible if the sperm DFI as de-

tected by SCSA exceeds 30 %, and these couples should be transferred to either IVF or ICSI. DFI can therefore be used as an independent predictor of pregnancy and birth in couples undergoing IUI [104]. In addition, an extended study by Bungum *et al.* (personal communication), including a large study population from ART cycles, presents preliminary data [104] that exceeding the 30 % DFI threshold as detected by SCSA is not compatible with *in vivo* fertilization by means of IUI. They also report that even though high DFI does not exclude successful treatment by means of IVF, ICSI is far more successful compared with IVF in these cases.

Therefore, a considerable number of patients can benefit from improved male infertility diagnosis and prognosis by means of sperm DNA damage assessment, enabling them to avoid unnecessary medical interventions with a very low chance of success (IUI when DFI > 30 %), and giving them the opportunity to choose a method with the highest chance of success (ICSI when DFI > 30%). However, it should be kept in mind that IVF, and especially ICSI, are able to overcome the natural barriers of sperm DNA damage levels not compatible with fertilization under natural circumstances, and the consequences of this for the progeny are still not clear. Further studies are needed in order to investigate whether treatment modalities as administration of antioxidants (Greco *et al.*, 2005[191]) to men with high DFI, can play a role in infertility treatment.

A suggestive clinical approach flow chart for infertile couples is shown in Figure 2.

It has to be mentioned that at the moment SCSA is the only method which has demonstrated clear clinically useful cut-off levels between fertile and infertile men [101, 102], and its prognostic value for ART has also been shown [104, 138]. The undisputed advantages of this technique are its robustness and small intra- and inter-assay variations [122, 145, Spano and Giwercman, unpublished data].

SCSA is not yet very common in andrological laboratories worldwide. However, alternative and cheaper

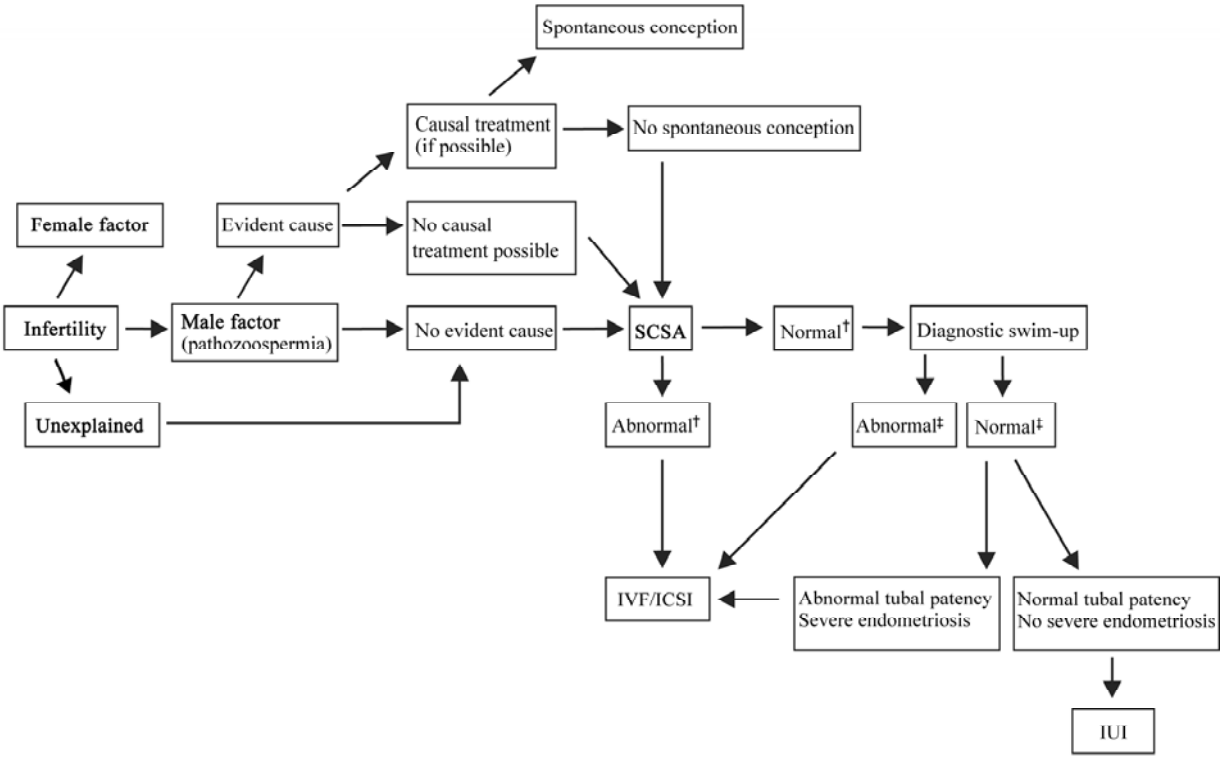

Figure 2. Suggestive clinical algorithm for use of sperm DNA damage assessment in men from infertile couples. †Normal sperm chromatin structural assay (SCSA): DNA fragmentation index (DFI) ≤ 30 %, abnormal SCSA: DFI > 30 %; ‡Normal diagnostic swim-up: ≥ 1 million sperms/mL, abnormal swim-up: < 1 million sperms/mL (can differ between laboratories).

tests of the same clinical value for measuring sperm DNA damage are not yet available. Our studies show that the TB test [42, 121, 122] has potential to become a robust assay and the search for clinically valuable predictive thresholds both *in vivo* and *in vitro* is currently under investigation.

Whether sperm DNA damage can be decreased by some treatment modalities, allowing these couples to switch from ICSI to IVF/IUI or even achieve a pregnancy in a natural way, remains to be elucidated.

7 Conclusion

Normal structure of sperm chromatin is essential for the fertilizing ability of spermatozoa *in vivo*. It is a relatively independent measure of semen quality that yields diagnostic and prognostic information complementary to, but distinct from, that obtained from standard sperm parameters (concentration, motility and morphology). Accumulated data allows sperm DNA damage assessment to be recommended among routine tests for infertility investigations. Several methods are used to assess sperm chromatin/DNA status. SCSA is currently the only method that has provided clear clinical cut-off levels and that can be recommended for a robust sperm DNA damage evaluation. The normality ranges and predictive thresholds for male fertility potential of the other assays discussed still need to be established or clarified. It seems that ART, especially ICSI, are able to overcome the natural barriers of sperm DNA damage levels not compatible with fertilization under natural circumstances. The consequences of this for the progeny are still not clear.

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