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ANDROLOGY

Sperm DNA damage in male infertility: etiologies, assays, and outcomes

Ryan T. Schulte · Dana A. Ohl · Mark Sigman · Gary D. Smith

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Abstract Male factor infertility is the sole cause of infertility in approximately 20% of infertile couples, with an additional 30% to 40% secondary to both male and female factors. Current means of evaluation of male factor infertility remains routine semen analysis including seminal volume, pH, sperm concentration, motility, and morphology. However, approximately 15% of patients with male factor infertility have a normal semen analysis and a definitive diagnosis of male infertility often cannot be made as a result of routine semen analysis. Attention has focused on the role of sperm nuclear DNA integrity in male factor infertility. Here we review the structure of human sperm chromatin, the etiology and mechanisms of sperm DNA damage, current tests available to assess sperm DNA integrity, and effect of sperm DNA integrity on reproductive outcomes.

Keywords Etiologies · Assays · Sperm, DNA Damage

Capsule Integrity of sperm DNA is essential for normal fertilization, embryo development and reproduction. Numerous factors can impact sperm chromatin structure and currently multiple diagnostic test are available to ascertain sperm DNA integrity.

R. T. Schulte · D. A. Ohl · G. D. Smith
Department of Urology, University of Michigan,
Ann Arbor, MI 48109-0617, USA

M. Sigman
Department of Urology, Brown Medical School,
Providence, RI 02912, USA

G. D. Smith
Department of Obstetrics and Gynecology,
University of Michigan,
Ann Arbor, MI 48109-0617, USA

G. D. Smith
Department of Integrated and Molecular Physiology,
University of Michigan,
Ann Arbor, MI 48109-0617, USA

G. D. Smith
Department of Reproductive Sciences Program,
University of Michigan,
Ann Arbor, MI 48109-0617, USA

G. D. Smith (✉)
6428 Medical Sciences I, 1301 E. Catherine St,
Ann Arbor, MI 48109-0617, USA
e-mail: smithgd@umich.edu

Introduction

Male factor infertility is the sole cause of infertility in approximately 20% of infertile couples, with an additional 30% to 40% secondary to both male and female factors [90, 120]. Thus, male factor infertility is present in approximately half of all infertile couples. Current means of evaluation of male factor infertility remains routine semen analysis including seminal volume, pH, sperm concentration, motility, and morphology [125]. However, approximately 15% of patients with male factor infertility have a normal semen analysis [1] and a definitive diagnosis of male infertility often cannot be made as a result of routine semen analysis [19].

Over the past decade, there has been a growing body of research investigating the role of sperm nuclear DNA integrity in male factor infertility. It has been suggested that sperm DNA integrity may be a better predictor of male fertility than routine semen analysis. There is evidence that sperm of infertile men contain more DNA damage than fertile men and that this sperm DNA damage may have a negative effect on fertility potential of these patients [40, 55, 71, 114, 127, 130]. While high levels of sperm DNA damage often correlate with poor seminal parameters such as reduced count and motility or abnormal morphology [65,

80, 92], sperm DNA damage is also found in 8% of men with normal seminal parameters [127, 130]. Additionally, there have been concerns about potential consequences of the use of DNA damaged sperm in intracytoplasmic sperm injection (ICSI) as this technique overrides the process of natural selection [109].

Although there has been a significant amount of research on human sperm DNA integrity over the last decade, our understandings of mechanisms of sperm DNA damage and their effects on reproductive outcomes are far from complete. Additionally, there continues to be questions and controversies as to how to best apply this knowledge to clinical practice. What exactly do these tests measure? What are the normal and abnormal thresholds? What do abnormal results mean in terms of clinical recommendations to the patients? Are there effective treatments to improve sperm DNA integrity?

In this review article, we will review the structure of human sperm chromatin, the etiology and mechanisms of sperm DNA damage, current tests available to assess sperm DNA integrity, and effect of sperm DNA integrity on reproductive outcomes.

Sperm chromatin structure

Chromatin of mammalian sperm has a unique structure that is highly organized, condensed, and compacted. This allows protection of the paternal genome during transport through the male and female reproductive tracts and its subsequent delivery to the ova in good condition. Mammalian sperm DNA is the most tightly compacted eukaryotic DNA [124]. This feature is in contrast DNA structure in somatic cell nuclei. Somatic cell nuclear DNA is wrapped around an octamer of histones and packaged into nucleosomes and then further coiled into a solenoid [84]. This type of packaging adds histones, which increase chromatin volume. Sperm cell nuclei simply do not have the volume for this type of packaging and thus must undergo a different type of packaging [124].

During spermiogenesis, sperm chromatin undergo a series of modifications in which histones are lost and replaced with transition proteins and subsequently with protamines [27, 68, 77]. Protamines are approximately half the size of histones [44]. The DNA strands are highly condensed by these protamines and form the basic packaging unit of sperm chromatin, a toroid. The toroids are further compacted by the intramolecular and intermolecular disulfide cross-links between cysteine residues present in protamines [72]. All of these levels of compaction and organization help to protect sperm chromatin during transport through the male and female reproductive tract and also ensures the paternal genome is delivered in a form that allows developing embryo to accurately express genetic information [103].

Although human sperm chromatin contains this highly organized and compact structure, it is less compact than in other mammals. Approximately 15% of histones are retained in human sperm chromatin subsequently making chromatin less tightly compacted [11, 47]. Infertile men have been reported to have a higher histone to protamine ratio in their sperm chromatin [94, 116]. Human sperm also contain two types of protamines, P1 and P2. P2 protamines contain fewer cysteine groups and thus contain less disulfide crosslinks [25]. This theoretically leaves the DNA more susceptible to damage. It has been reported that altered P2 expression is common in men with infertility [18].

Etiologies and mechanisms of sperm DNA damage

There are several different levels of sperm chromatin abnormalities that are important to consider: 1) damage to the actual DNA physical integrity in the form of single-stranded or double-stranded DNA strand breaks, 2) nuclear protein defects that may interfere with histone to protamine conversion and subsequent DNA compaction, and 3) chromatin structural abnormalities causing altered tertiary chromatin configuration. Environmental stress, gene mutations, and chromosomal abnormalities can all disturb biochemical events that occur during spermatogenesis, which can ultimately lead to abnormal chromatin structure incompatible with fertility [41]. Ova are able to repair sperm DNA damage to a certain extent [48]. However, when sperm DNA damage is extensive, ovum may not have repair capacities to allow normal development.

Etiologic factors

There are a variety of etiologic factors that have been associated with sperm DNA fragmentation and/or impaired chromatin integrity. These causes are many and range from environmental conditions such as cigarette smoking [99], irradiation [8], and chemotherapy [20, 88] to pathophysiologic conditions such as leukocytospermia [5, 35], varicoceles [105, 107], and cancer [70]. Even iatrogenic causes such as sperm cryopreservation [32, 73] have been associated with sperm DNA damage. Exact molecular mechanisms by which these conditions lead to sperm DNA damage and/or chromatin abnormalities are not fully understood, but there are currently three main theories which we will review: 1) chromatin packaging abnormalities, 2) reactive oxygen species, and 3) apoptosis.

Chromatin packaging abnormalities

As discussed previously, during spermiogenesis, sperm chromatin undergoes an important step in remodeling in

which histones are replaced by protamines. This chromatin remodeling is facilitated by the coordinated loosening of chromatin by histone hyper-acetylation as well as the enzyme DNA topoisomerase II (topo II) which produces temporary nicks in the sperm DNA to relieve torsional stress resulting from supercoiling [74, 83, 85]. These temporary nicks are then normally repaired by this same enzyme, topo II, prior to completion of spermiogenesis and ejaculation. However, if these nicks are not repaired, DNA fragmented sperm may be present in the ejaculate [91].

Reactive oxygen species

Sperm DNA damage has also been associated high levels of reactive oxygen species (ROS; [10]). At low levels, ROS play an important role in sperm maturation and functions such as capacitation and the acrosome reaction [30]. Seminal plasma contains antioxidants which help protect sperm DNA [122]. However, when an excessive amount of ROS is produced beyond the antioxidant capacity of seminal plasma and male reproductive tract, the pathogenic result is often cellular and DNA damage [3, 30]. Increased levels of ROS have been reported in the semen of approximately 25% of infertile men [128]. Additionally, a positive correlation was reported between sperm DNA fragmentation and ROS [10]. Major sources of ROS in semen are leukocytes and the sperm themselves, particularly immature sperm with cytoplasmic retention and abnormal head morphology characterized by retention of residual cytoplasm [2, 51, 95]. Both leukocytospermia and retention of residual cytoplasm within sperm have been associated with increased sperm DNA damage, likely secondary to increased level of ROS produced by these cells [5, 35, 43].

Apoptosis

Another theory of sperm DNA damage is through abortive apoptosis. Apoptosis is a process of programmed cell death that occurs in many cells throughout the body. In the testes, apoptosis normally occurs to prevent the overproduction of germ cells and to selectively destroy injured germ cells [113]. Sertoli cells are only able to support a limited number of germ cells in the testis. Clonal expansion of germ cells is in excess and thus apoptosis is necessary to limit the size of the germ cell population to one which Sertoli cells are able to support [101]. It has been suggested that this apoptotic pathway is triggered by the interaction of Fas Ligand (FasL) secreted by Sertoli cells with the Fas protein located in the germ cell surface [78]. However, there has been more recent evidence that this may not always be the case as FasL defective mice still show evidence of germ cell apoptosis [61]. Men with poor seminal parameters often display a large percentage of Fas expressing sperm in the ejaculate [103]. This has led to the suggestion that some of these sperm with DNA damage and Fas expression have undergone “abortive apoptosis”, in which they started but subsequently escaped the apoptotic pathway [104]. There has been controversy regarding this theory as some studies have found no correlation between DNA damage and Fas expression and other markers of apoptosis [86, 92]. Interestingly, recent loss-of-function studies indicate that DNA damage checkpoints occur during spermatogenesis and may involve excision repair genes, mismatch repair genes, and p53 [97].

Tests of sperm DNA integrity

Over the years, there have been an increasing number of tests developed to assess sperm DNA integrity. Mechanisms by which sperm DNA integrity is assessed in these assays varies, with some measuring abnormalities in sperm chromatin structure while other directly measure DNA strand breaks.

Sperm Chromatin Structure Assay (SCSA)

The SCSA was first described over 25 years ago [38]. This assay is based on the premise that DNA in sperm with abnormal chromatin structure is more prone to acid or heat denaturation [29, 100]. Using the metachromatic properties of acridine orange (AO), SCSA measures susceptibility of sperm DNA to acid-induced denaturation in situ. By quantifying this metachromatic shift of AO from green to red after acid treatment using flow cytometry, the extent of DNA denaturation is determined [29, 38]. The parameter obtained by SCSA most commonly referred to in the literature is DNA fragmentation index (DFI), a measure of DNA denaturation.

Acridine orange test

The acridine orange test (AOT) is based on similar principles as the SCSA in which the metachromatic shift of AO from green to red is used to determine extent of DNA denaturation. The AOT is simpler and less expensive than the SCSA since it can be done by visual interpretation under fluorescent microscopy without the need for flow cytometry or a SCSA trained technician [118]. However, issues of indistinct colors, rapid fading, and the heterogeneous staining can cause difficulties during visual interpretation [22].

Toluidine blue

Toluidine blue (TB) is a basic dye used to evaluate sperm chromatin integrity. Phosphate residues of sperm DNA in

nuclei with loosely packed chromatin and/or impaired DNA are more liable to binding with basic dyes such as TB [87]. Thus, using light microscopy, damaged sperm will be stained blue while normal sperm will remain colorless.

Aniline blue

Aniline blue is an acidic dye which is used to evaluate sperm chromatin integrity. Sperm with impaired DNA often display the presence of residual histones. These residual histones lead to looser chromatin packaging allowing increased accessibility of basic groups of the nucleoprotein and subsequently liable to bind acidic dyes such as aniline blue [9, 28].

TUNEL

The terminal deoxynucleotidyl transferase-mediated (TdT) deoxyuridine triphosphate (dUTP) nick end labeling assay (TUNEL) is a direct quantification of sperm DNA breaks [52]. dUTP is incorporated at single-stranded and double stranded DNA breaks in a reaction catalyzed by the enzyme TdT. The DNA breaks based on the incorporated dUTP are then labeled and can be measured using bright field or fluorescent microscopy as well as flow cytometry [52]. Sperm are then classified as TUNEL positive or negative and expressed as a percentage of the total sperm in the population. Typical results of the TUNEL assay are shown in Fig. 1A.

In situ nick translation assay

The in situ nick translation (NT) assay is similar to the TUNEL assay in that it quantifies incorporation of dUTP into DNA breaks. However, in contrast to TUNEL which identifies both single-stranded and double-stranded DNA breaks, the in situ NT assay only identifies single-stranded DNA breaks in a reaction catalyzed by the template-dependent enzyme, DNA polymerase I. Although this can be a relatively simple test to perform, it lacks sensitivity when compared to other assays [122].

COMET

The single-cell gel electrophoresis (Comet) assay is another test for direct assessment of sperm DNA breaks [56]. Decondensed sperm are suspended in an agarose gel, subjected to an electrophoretic gradient, stained with fluorescent DNA-binding dye, and then imaged with imaging software. Low-molecular weight DNA, short fragments of both single-stranded and double-stranded DNA, will migrate during electrophoresis giving the characteristic comet tail [69]. High-molecular weight intact segments of DNA will not migrate and remain in the head of the "comet." Imaging software is then use to measure comet tail length and tail fluorescent intensity, which are increased in sperm with high levels of DNA strand breaks [64, 112].

Sperm chromatin dispersion test

The sperm chromatin dispersion (SCD) test is based on induced condensation which is directly linked with sperm DNA fragmentation [93]. Intact sperm are immersed in an agarose matrix on a slide, treated with an acid solution to denature, and then treated with a lysis buffer to remove sperm membranes and proteins giving rise to nucleoids with a central core and a peripheral halo of dispersed DNA loops. Sperm with non-fragmented DNA release their DNA loops forming large halos (Fig. 1B). However, sperm which produce a very small halo or no halo at all contain DNA fragmentation [42]. Sperm can be stained with Wright's stain for visualization under bright field microscopy or an appropriate fluorescent dye for visualization under fluorescent microscopy.

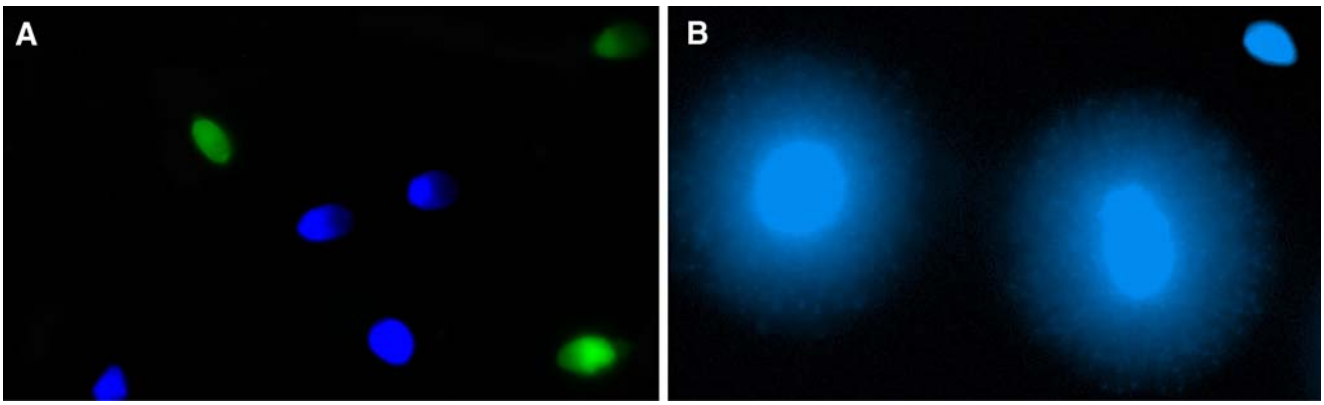

Fig. 1 Sperm DNA fragmentation assays. **A**. TUNEL assay. Blue sperm are TUNEL negative while green sperm are TUNEL positive indicating DNA fragmentation. **B**. Sperm Chromatin Dispersion Test. The two sperm in the center with non-fragmented DNA form large halos, while the sperm in the upper right hand corner has no halo indicating DNA fragmentation. Results from both assays are typically expressed as percentage of sperm demonstrating DNA fragmentation

Correlations with other tests

As displayed above, there are many tests to measure sperm DNA integrity. Although the mechanisms by which sperm DNA integrity is assessed in these assays varies, most tests of sperm DNA integrity generally correlate well with each other. The SCSA has been shown to have a significant positive correlation with multiple other assays including COMET [7], TUNEL [22, 102], toluidine blue staining [36], and SCD test [22]. TUNEL has also been shown to have a strong positive correlation with toluidine blue staining [36] and the AOT [127, 130]. However, more recently, AOT was shown to display extreme variations for sperm DNA fragmentation [22]. While SCSA, TUNEL, and the SCD test predicted similar levels of DNA fragmentation, AOT consistently showed higher levels of DNA fragmentation and interestingly had no significant correlation with SCSA [22].

Standard seminal parameters of sperm concentration, motility, and morphology have been consistently shown to have a negative correlation with levels of sperm DNA fragmentation whether measured by COMET [65], TUNEL [11, 127, 130], or SCSA [40, 105, 107]. While degree of correlation is somewhat variable between studies, they consistently indicate that sperm from patients with abnormal sperm concentration, motility, and morphology have increased levels of DNA damage.

Another important aspect to consider in evaluating these tests is their repeatability over time in the same patient. Standard seminal parameters of sperm concentration, motility, and normal morphology can be highly variable over time within individuals [4, 6, 82]. In comparison, measures of sperm DNA damage has been shown to have good stability over time within individual with TUNEL [110, 111] and SCSA assays [39, 127, 130]. Earlier studies have reported low coefficients of variation (CV) of 10% [39] and 21 % [127, 130] of DFI with the SCSA. However, a recent study with a larger study population reported a significant intra-individual variation with a CV of 29% and recommended that SCSA be repeated when the DFI in the first measurement is >20% [34].

Sperm DNA damage and reproductive outcomes

In vivo fertilization

Increasing evidence suggests that sperm DNA damage has a negative impact on male fertility potential [40, 45, 62, 105–107, 114, 129]. These studies utilized a variety of different assays to analyze sperm DNA integrity but consistently showed that infertile men have a significantly higher level of sperm DNA damage when compared with fertile men. A threshold value of 20% sperm DNA fragmentation with TUNEL assay has been suggested to distinguish between fertile controls and infertile men [110, 111]. Additionally, ≥30% or >40% DFI as measured by the SCSA has also been suggested as specific threshold levels, above which the chance of pregnancy approaches zero [40, 114]. However, these proposed thresholds are based on few patients that actually exceed the threshold. The study by Evenson and coworkers included only 10 patients with a DFI greater than the proposed threshold of 30% and the study by Spano and colleagues included only 4.5% of the cycles with a DFI above the proposed threshold of 40%. A recent meta-analysis by Evenson and Wixon of these two studies stated that couples with no known infertility problems were 7.0 time more likely to achieve a pregnancy/delivery via in vivo fertilization if the DFI <30 [37]. As a general trend in these studies, lower pregnancy rates are associated with increasing % DFI, but a high % DFI does not preclude pregnancy. Although evidence suggests that sperm DNA damage is associated with male infertility, more large scale studies are needed before suggested threshold levels are to be validated.

There is also evidence that sperm DNA integrity may have a high predictive value for in vivo fertilization when intrauterine insemination (IUI) is performed. In multiple studies, sperm DNA damage has been shown to be significantly higher in couples who failed to obtain a pregnancy after IUI [16, 17, 33, 105, 107]. One study found that no samples with >12% DNA fragmentation as measured by TUNEL resulted in pregnancy using IUI [33]. In the largest series to date involving 387 IUI cycles the odds ratio for biochemical pregnancy, clinical pregnancy, and delivery in IUI were all significantly lower in patients with a DFI >30% [16]. Additionally, infertile couples using IUI were 7.3 times more likely to achieve pregnancy delivery if the DFI was <30% in a metanalysis by [37]. Given these findings, measures of sperm DNA integrity appear to have a high predictive value for IUI outcomes.

In vitro fertilization

In the last five years, there has been an increase in number of clinical studies evaluating the relationship between sperm DNA integrity and reproductive outcomes of IVF and IVF with ICSI. While tests of sperm DNA integrity appear to have high predictive value for outcomes of in vivo fertilization, the relationship with IVF and ICSI are more controversial. Fertilization, embryo quality, and pregnancy rates are all important reproductive parameters that could potentially be affected by sperm DNA damage and thus are often examined in the literature. Several clinical studies have found no significant correlation between sperm DNA damage and fertilization rates in vitro [46, 59, 60, 75, 76, 89, 119, 121, 123, 131]. In a recent

meta-analysis by Li and colleagues found no relationship between sperm DNA damage and fertilization rates during IVF or ICSI using either SCSA or TUNEL assays [79]. This is not completely unexpected since the embryonic genome is not expressed until the four to eight-cell stage and thus fertilization may not be dependent on sperm DNA integrity [15]. Despite this, other studies have found a negative correlation between sperm DNA damage and fertilization rates [12, 63, 80, 98]. Additionally, there has been no consistent relationship between sperm DNA damage and embryo quality during IVF cycles. Several studies did not identify any adverse effects of sperm DNA damage on embryo quality [12, 63, 75, 80, 121], while others reported a negative correlation [89, 108, 123, 131].

Perhaps the most important reproductive parameter to consider in relation to sperm DNA damage is pregnancy rates. Unfortunately, there is also controversy as to the influence of sperm DNA damage on pregnancy rates with IVF or ICSI. Early studies reported a significant reduction of pregnancy rates following IVF or ICSI for patients with increased levels of sperm DNA damage [12, 59, 60, 75, 76, 121, 123]. It was reported in multiple studies that no clinical pregnancy could be obtained after IVF or ICSI when the DFI as measured by the SCSA was >27% [75, 76]. Additionally, Benchaib and coworkers reported that DNA fragmentation as measured by TUNEL was significantly lower when a pregnancy was obtained using ICSI and that no pregnancy was obtained when DNA fragmentation was >20% [12]. Although in a more recent study there was no significant differences in pregnancy rates after IVF or ICSI between patients with high and low levels of DNA fragmentation using a TUNEL value of 15% as a cutoff [13]. Several studies have failed to confirm these earlier findings by demonstrating no significant differences in pregnancy rates following IVF or ICSI between patients with high and low levels of sperm DNA damage [13, 16, 17, 46, 63, 108, 131]. Successful pregnancies have also been obtained using IVF or ICSI cycles despite high levels of sperm chromatin damage (DFI > 27%) [16, 17, 21, 46]. A recent study found no significant differences in IVF and ICSI pregnancy outcomes between low and high DFI groups. However, when there were high levels of sperm DNA damage (DFI > 30%), they reported pregnancy rates with ICSI were significantly better than IVF [16]. Given that couples undergoing conventional IVF in this study did not also undergo ICSI, making comparisons between these two different groups precludes definitive conclusions. With this increasing number of studies in recent years, meta-analyses have been published evaluating effects of sperm DNA damage on reproductive outcomes. In the meta-analysis by Evenson and Wixon, infertile couples were approximately 2.0 times more likely to become pregnant after routine IVF if their DFI was <30%. For ICSI and/or routine IVF, the results showed a non-significant trend where infertile couples were 1.6 times more likely to achieve pregnancy if the DFI was <30% [37]. In contrast, another meta-analysis showed no significant effects of sperm DNA damage on the clinical pregnancy rate after IVF or ICSI when using the SCSA. However, when using the TUNEL assay, clinical pregnancy rates following IVF, but not ICSI, decreased significantly for patients with a high degree of sperm DNA damage [79]. A recent meta-analysis demonstrated a small but statistically significant association between sperm DNA integrity test results and pregnancy in IVF and ICSI cycles, yet questioned its clinical utility [23].

Despite the growing body of literature on these issues, there continues to be a degree of uncertainty. It has been proposed that sperm DNA integrity becomes particularly relevant when fertilization occurs in a more natural way, such as in normal circumstances or conventional IVF [46]. Subsequently, the SCSA and other tests of sperm DNA integrity seem to lose their predictive power in respect to reproductive outcomes from natural conception and IUI to IVF to ICSI [115]. We speculate that men with very high levels of sperm DNA damage will have a lower chance of producing a pregnancy. However, based on the available evidence, there is currently no established absolute upper threshold that would preclude a successful pregnancy.

It has been proposed that sperm DNA damage is promutagenic and can give rise to mutations after fertilization as oocytes attempts to repair the DNA before initiation of the first cleavage division [3]. Any mutations occurring at this point may be fixed in the germ line and may be responsible for induction of pathology [3]. Children conceived by ICSI have been found to have an increased incidence of genetic aberrations [14]. Whether this is a treatment or patient population association is currently unknown. Additionally, epigenetic abnormalities such as rare human imprinting disorders, Angelman's syndrome and Beckwith-Wiedemann syndrome, have been associated with IVF and ICSI [26, 31, 50, 53, 81]. An increased risk of birth defects has been associated with IVF and ICSI when compared to natural conception [57, 58, 96], but this has not been observed by several other studies [66, 126]. While the consequences of using sperm with DNA damage in assisted reproductive technologies are far from being fully understood, these current reports raise concern and warrant further investigation into this subject.

Evolving treatment strategies: role of antioxidants

Because ROS have been associated with sperm DNA damage, investigators have studied possible protective roles of antioxidants in preventing or treating sperm DNA

damage. Several previous studies have reported improved sperm DNA integrity with use of oral antioxidants [24, 49, 54, 67, 71, 117]. However, few of these studies report on how these improvements affect pregnancy rates. In a recent study 38 men with an elevated percentage of DNA fragmented sperm were treated with antioxidants, vitamin C and vitamin E, for 2 months after one failed ICSI attempt. A second ICSI cycle was then performed which demonstrated a marked improvement of clinical pregnancy rates (48.2% vs. 6.9%) when compared with pretreatment ICSI outcomes [54], although there was no placebo control for comparison. Additionally, 76% of these cases led to a decrease in the percentage of DNA fragmented sperm after treatment with antioxidants. However, another study reported that even without treatment, 37% of patients with an abnormal result on first SCSA (DFI >30%) were subsequently found to have normal result (DFI < 30%) on a second SCSA test [34]. Larger scale, prospective, randomized studies will be necessary before reaching any definitive conclusions on the clinical usefulness of antioxidants in treatment of male infertility.

Summary

Sperm chromatin has a highly specialized and compact structure that is essential for protection and subsequent transmission of the paternal genome. A variety of etiologies have been associated with increased levels of sperm DNA damage, but the exact pathophysiologic mechanisms by which sperm DNA damage occurs are not completely understood. A large number of tests are available to assess different aspects of sperm DNA integrity, but there remains no consensus on the optimal technique or appropriate clinical cut-off levels. High levels of sperm DNA damage are found in infertile men and have a negative correlation with reproductive outcomes by natural conception or IUI. However, the impact of sperm DNA damage on IVF and ICSI reproductive outcomes remain more controversial. Our understanding of the potential consequences on the offspring of using DNA damaged sperm in ICSI remain very basic and warrants further investigation. While the testing of sperm DNA integrity has the potential for great impact on the field of male infertility, additional studies and large scale trials are needed to further elucidate and define the mechanisms of sperm DNA damage and their clinical significance in reproductive outcomes.

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