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Associate Editor

Sperm DNA fragmentation: mechanisms of origin, impact on reproductive outcome, and analysis

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Objective: To review the mechanisms responsible for DNA fragmentation in human sperm, including those occurring during spermatogenesis and transport through the reproductive tract. The mechanisms examined include: apoptosis in the seminiferous tubule epithelium, defects in chromatin remodeling during the process of spermiogenesis, oxygen radical-induced DNA damage during sperm migration from the seminiferous tubules to the epididymis, the activation of sperm caspases and endonucleases, damage induced by chemotherapy and radiotherapy, and the effect of environmental toxicants. The different tests currently used for sperm DNA fragmentation analysis and the factors that determine the predictive value of sperm DNA fragmentation testing and their implications in the diagnosis and treatment of infertility are also discussed. Finally, we also scrutinize how the presence in the embryonic genome of DNA strand breaks or modifications of DNA nucleotides inherited from the paternal genome could impact the embryo and offspring. In particular we discuss how abnormal sperm could be dealt with by the oocyte and how sperm DNA abnormalities, which have not been satisfactorily repaired by the oocyte after fertilization, may interfere with normal embryo and fetal development.

Conclusion(s): Sperm DNA can be modified through various mechanisms. The integrity of the paternal genome is therefore of paramount importance in the initiation and maintenance of a viable pregnancy both in a natural conception and in assisted reproduction. The need to diagnose sperm at a nuclear level is an area that needs further understanding so that we can improve treatment of the infertile couple. (Fertil Steril® 2010; ■: ■–■. ©2010 by American Society for Reproductive Medicine.)

Key Words: DNA fragmentation, in vitro fertilization, oxygen radicals, oxidative stress, caspases, endonucleases, chemotherapy, ionizing radiation

Spermatozoa with DNA damage are able to efficiently fertilize an egg (1–3). However, the question remains as to what the most likely effects are to normal embryo and fetal development when the paternal genome introduces nucleotide or DNA damage that has not been repaired by the oocyte after fertilization (4–7). A relatively high number of women fail to have a pregnancy despite the apparent absence of a male or female factor of infertility. It is likely that many of these couples could have a genomic male factor of infertility, which may include sperm DNA damage, meiotic alterations, or sperm aneuploidy. The ability of the human oocyte and embryo to repair DNA damage is, however, poorly understood and our knowledge is currently limited to a number of gene expression studies showing that both the oocyte and the embryo are equipped with mechanisms to possibly cope with some paternal DNA anomalies (8, 9). First, the ability of the oocyte to initiate repair is, to a large extent, going to

depend on the cytoplasmic and genomic quality of the oocyte, which is impacted dramatically by increasing age. Second, the quality of sperm DNA present in a spermatozoon is increasingly being linked to paternal age (10) and this may further exacerbate the decrease in pregnancy rate (PR) observed in women of advanced age (11).

The presence of unrepaired DNA damage above a critical threshold in embryos generated in vivo and in vitro has been postulated to explain the block in embryo development observed after embryo implantation in embryos with a normal karyotype. Recent studies suggest that this type of damage is expressed during/after implantation and has been characterized as *late paternal effect* (12, 13). There are also indications that the high levels of DNA damage in a sperm sample with failure to obtain blastocysts (14) and it is believed that some loss of preimplantation embryos occurs between postembryonic genome activation and the blastocyst stage (15, 16).

It should be pointed out that the DNA damage found in the embryo will not always be related to DNA damage in the spermatozoon that fertilized the oocyte. A significant level of DNA abnormalities arise from the oocyte. If aneuploidy studies are taken as an example the problems in the oocyte remain as the major contributory factor (17, 18). Future analytical techniques may concentrate on the combined use of chromosomal probes and DNA fragmentation testing in

Received May 12, 2009; revised ■■, 2010; accepted October 27, 2009. D.S. is chief scientific officer for Molecular Biometrics. J.G.A. has nothing to disclose.

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embryo biopsies that could allow a more robust analysis, including not only diagnosis of monogenic diseases and aneuploidy, but also the degree of DNA damage in the embryo, thus allowing to select embryos of the highest genomic quality. Another potential strategy to test whether sperm DNA damage has or has not been repaired by the oocyte or the embryo would be by analyzing DNA fragmentation in trophoblast cells obtained by blastocyst biopsy.

MECHANISMS OF SPERM DNA FRAGMENTATION

How does sperm DNA damage take place?

DNA damage in spermatozoa can affect both mitochondrial as well as nuclear DNA and can be induced by six main mechanisms. These can occur during either the production or the transport of the sperm cells and include (Fig. 1): [1] apoptosis during the process of spermatogenesis; [2] DNA strand breaks produced during the remodeling of sperm chromatin during the process of spermiogenesis; [3] post-testicular DNA fragmentation induced mainly by oxygen radicals, including the hydroxyl radical and nitric oxide, during sperm transport through the seminiferous tubules and the epididymis; [4] DNA fragmentation induced by endogenous caspases and endonucleases; [5] DNA damage induced by radiotherapy and chemotherapy; and [6] DNA damage induced by environmental toxicants.

Of these six mechanisms, one that can play a major role in causing sperm DNA fragmentation is post-testicular damage during sperm transport through the epididymis. This is supported by previous reports demonstrating that DNA fragmentation is higher in the caudal epididymal and ejaculated (19, 20) sperm compared with testicular spermatozoa. Recent reports have confirmed this hypothesis (21). The question, however, still remains whether failures during spermatogenesis have made sperm more susceptible to post-testicular damage.

Induction of Apoptosis During the Process of Spermatogenesis

During the process of spermatogenesis the germ cell screening mechanism governed by the Sertoli cell is responsible for the induction of apoptosis in 50%–60% of all germ cells that enter meiosis I. These cells are earmarked with apoptotic markers of the *Fas* type and should be phagocytosed and eliminated by the Sertoli cell to which these germ cells are associated (22–24). However, this mechanism may not always operate efficiently and a variable percentage of these defective germ cells enter the process of sperm remodeling during spermiogenesis, appearing later on in the ejaculate. With regard to the failure of this screening mechanism, the results of a recent study by Burrello et al. (25), suggest that there is dissociation between genomic quality in the germ cell and the sperm remodeling that takes place during the process of spermiogenesis. That is, a germ cell may have its nucleus “disrupted” by apoptosis or be aneuploid and, still, the resulting spermatozoon will have normal morphology. Therefore, when a spermatozoon with normal morphology is microinjected it does not necessarily mean that the genomic quality is also normal. Interestingly, we have recently proposed that the mechanism of apoptosis in spermiogenesis may be less related to that of cell death but more responsible for the process of stripping the cytoplasm in the final stages of sperm maturation (26).

It has been shown that in men with oligospermia, the probability that a spermatozoon with normal morphology to be aneuploid is much higher than when the man is normozoospermic (25). This is probably related to the partial maturational arrest associated with meiotic alterations. The fact that a variable percentage of spermato-

zoa in the ejaculate express apoptotic markers, for example, *Fas*, *phosphatidylserine*, *Bcl-X_L*, *p53* (27–30), indicates that this phenomenon could be used to select nonapoptotic spermatozoa from semen samples (31). A method recently introduced for this purpose is the use of Annexin-V-conjugated microbeads (ANMB Microbead Kit; Miltenyl Biotec, Germany). The principle in which these columns are based is that apoptotic spermatozoa express *phosphatidylserine* in the outer leaflet of the sperm membrane and bind to Annexin-V. When a magnetic field is applied to the columns, those sperm bound to Annexin-V conjugated to the magnetic microbeads are retained in the column, whereas nonapoptotic sperm go through the column (32).

DNA Breaks During the Process of Spermiogenesis

Alterations in chromatin remodeling during the process of spermiogenesis could result in DNA fragmentation. McPherson and Longo (33–35) postulated that the presence of DNA nicks in ejaculated sperm may be indicative of incomplete maturation during spermiogenesis. They postulated that chromatin packing may necessitate endogenous nuclease activity to create and ligate nicks that facilitate protamination. These nicks are thought to provide relief of torsional stress to aid chromatin arrangement during the displacement of histones by the protamines and the same procedure is thought to occur in humans (36). Alterations in the control of this process could result in the presence of chromatin packaging anomalies or unrepaired DNA nicks. These DNA breaks would occur before spermiation and are likely to make them more susceptible to post-testicular assault.

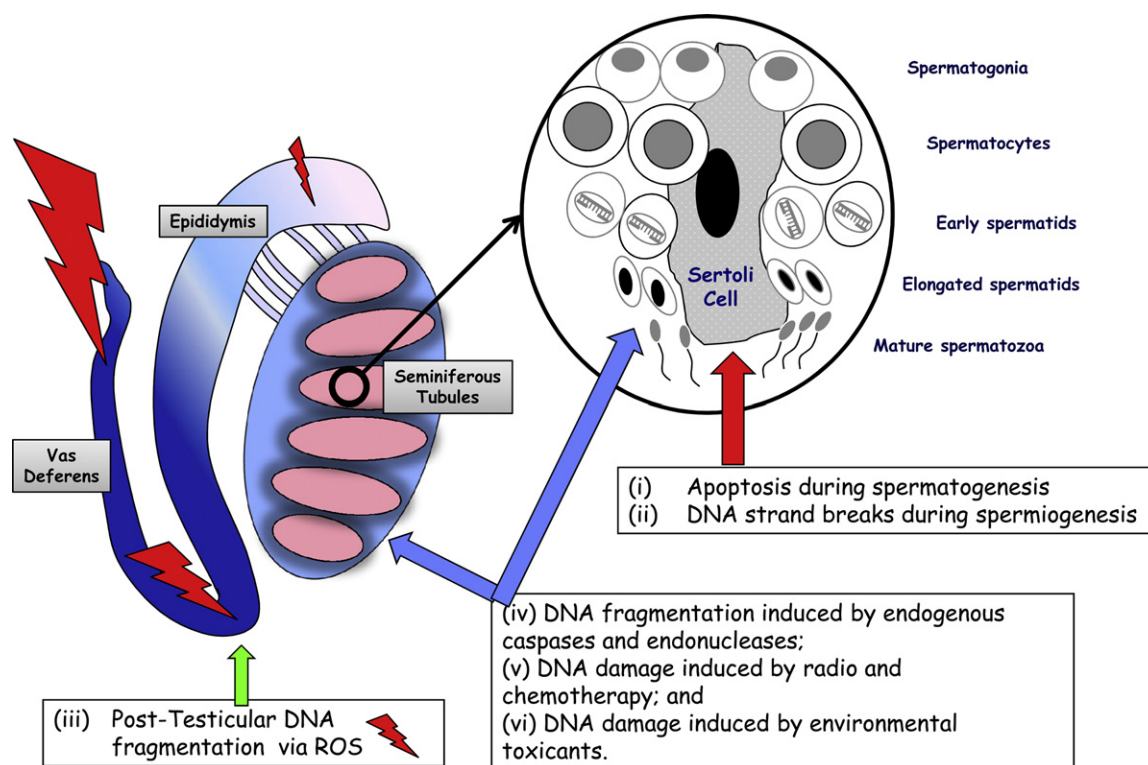
Post-Testicular Sperm DNA Fragmentation

Recent studies show that immature sperm, which produce high levels of ROS, can induce DNA damage in mature sperm. This damage would be produced after spermiation during comigration of mature and immature sperm from the seminiferous tubules to the cauda epididymis (20). Although the half-life of ROS is of the order of nanoseconds to microseconds, as sperm are highly packed in the epididymis, this would facilitate ROS-induced DNA damage. The ROS can damage sperm DNA directly or indirectly through the activation of sperm caspases and endonucleases. This is consistent with the fact that cocentrifugation of immature sperm (that produce high levels of ROS) with mature sperm results in the induction of sperm DNA fragmentation in mature sperm, because under these conditions, mature and immature sperm are in close contact (37). This is also consistent with the fact that the in vitro exposure of mature sperm to ROS results in significant DNA damage (3, 38). On the other hand, the epithelial cells from the epididymis could also play an active role in ROS-induced DNA damage, [1] through ROS such as the hydroxyl radical or nitric oxide (39) or [2] through the activation of sperm caspases and endonucleases by physicochemical factors such as high temperature (40) and environmental factors (41). In the first case, this damage could theoretically be prevented with the use of antioxidants, whereas in the second case this treatment would not be effective. This is supported by the study by Greco et al. (19) in which the use of antioxidants resulted in a significant reduction in the levels of sperm DNA fragmentation. However, care must be exercised when using antioxidant therapy. It has been previously reported that some antioxidants, like vitamin C, may increase chromatin compaction by reducing disulfide cross-linking in sperm protamines (42).

Most likely, the sperm that have higher levels of DNA damage would be those that acquire lower levels of disulfide cross-linking

FIGURE 1

Major mechanisms of inducing DNA damage in spermatozoa during either the production or the transport of sperm cells: (i) apoptosis during the process of spermatogenesis; (ii) DNA strand breaks produced during the remodelling of sperm chromatin during the process of spermiogenesis; (iii) post-testicular DNA fragmentation induced, mainly by oxygen radicals, during sperm transport through the seminiferous tubules and the epididymis (increasing DNA damage is indicated by size of red flashes and gradient darkening in tract); (iv) DNA fragmentation induced by endogenous caspases and endonucleases; (v) DNA damage induced by radiotherapy and chemotherapy; and (vi) DNA damage induced by environmental toxicants.



Sakkas. Sperm DNA fragmentation. Fertil Steril 2010.

in their sperm chromatin during the process of sperm maturation in the epididymis. An interesting feature that is arising from recent studies is that, in general, the degree of sperm DNA fragmentation in ejaculated spermatozoa is higher than in testicular sperm (19, 43) and in sperm from the *corpus* and *caput* epididymis, which is precisely where the process of disulfide cross-linking takes place, and that the induction of sperm DNA fragmentation in the epididymis could be related to their genomic quality. That is, in addition to the screening mechanism exerted by the Sertoli cell during the process of spermatogenesis, there would be another screening mechanism at the level of the epididymis directed to eliminate genomically defective sperm (21).

The potential damage that sperm may experience during passage through the epididymis could be limited by removing them before that passage. This has greater clinical relevance as in cases of high levels of DNA fragmentation in semen and repeated IVF failure where one could resort to the use of testicular sperm obtained by testicular sperm extraction (TESA or TESE) (preferably TESA, as it is less invasive and would enjoy greater acceptance by the gynecologist and the patient). This is supported by the results reported by Greco et al. (19), where microinjection of testicular sperm in patients who previously failed with their ejaculated sperm, with DNA fragmentation levels in semen >15%, as measured by TdT-mediated-dUTP nick-end labeling (TUNEL), resulted in a clinical

PR of 44.4% compared with a clinical PR of 0% with ejaculated spermatozoa.

It should be pointed out that sperm DNA fragmentation induced by the hydroxyl radical and ionizing radiation results in the formation of 8-OH-guanine and 8-OH-2'-deoxyguanosine (8-OHdG) in a first stage and single-stranded DNA fragmentation thereafter (44). In addition, hydroxyl radical formation may result in the induction of double-stranded sperm DNA damage through the activation of sperm caspases and endonucleases. Although DNA damage of the first type may be repaired by the oocyte or the embryo, fertilization of an oocyte by a spermatozoon with extensive double-stranded DNA fragmentation is virtually not repairable and incompatible with normal embryo and fetal development.

Activation of Caspases and Endonucleases

The activation of sperm caspases and endonucleases by oxygen radicals and physicochemical factors can also induce sperm DNA fragmentation. Previous studies indicate that exposure of mouse sperm to 40°C results in a significant increase in sperm DNA fragmentation (45). More recently, Banks et al. (40) have shown the induction of sperm DNA fragmentation after *in vivo* exposure of mouse testis at 42°C. Because the increase in sperm DNA fragmentation observed in these mice occurred within 1 hour after heat exposure

the investigators concluded that the observed damage must have occurred in the epididymis and could have been caused either by ROS or the activation of sperm caspases and endonucleases. Considering that mouse sperm nuclei are more homogeneously packaged the likelihood that human sperm would be susceptible to heat is much greater, because of lower levels of protamines in the nuclei and more heterogeneity in nuclear packaging (46).

DNA Fragmentation Induced by Chemotherapy and Radiotherapy

It has been previously reported that exposure to chemotherapy and radiotherapy may also result in the induction of sperm DNA fragmentation. It is generally believed that cancer treatments adversely affect male fertility and that reduction of sperm output arises from the cytotoxic effects of chemotherapy or radiotherapy on the spermatogenic epithelium (47). Although specific examination of sperm DNA has been limited in cancer patients, a recent study by O'Flaherty et al. (48) found that sperm DNA integrity and compaction were affected in patients with testicular cancer and Hodgkin's Lymphoma before chemotherapy. When examining different techniques of DNA damage assessment the investigators concluded that although sperm chromatin structure assay (SCSA), TUNEL, and comet assays all detected DNA damage, the latter was optimal for use in cancer patients. A combination of the comet assay with tests that evaluate sperm DNA compaction, such as flow cytometry-based CMA3 and mBBR assays, is thought to be a reliable strategy to characterize sperm chromatin quality in cancer patients at the time of sperm banking.

DNA Damage Induced by Environmental Toxicants

Previous studies have provided evidence of an association between exposure to high levels of air pollution and increased DNA damage in human sperm. In a recent study, Rubes et al. (41) extended these observations and addressed the hypothesis that men who are homozygous null for glutathione-S-transferase M1 are less able to detoxify reactive metabolites of carcinogenic polycyclic aromatic hydrocarbons found in air pollution. Consequently, these men are more susceptible to the effects of air pollution on sperm chromatin. Using a longitudinal study design in which men provided semen samples during periods of both low (baseline) and episodically high air pollution, the investigators found a statistically significant association between glutathione-S-transferase M1 null genotype and increased sperm DNA fragmentation. Furthermore, men with the null genotype for glutathione-S-transferase M1 also showed higher levels of sperm DNA fragmentation in response to exposure to intermittent air pollution (41).

DNA FRAGMENTATION TESTS

During the past two decades a number of tests have been introduced for the analysis of sperm DNA fragmentation. These tests include TUNEL (49), comet (50), CMA3 (51), in-situ nick translation (46, 52), DBD-FISH (DNA breakage detection fluorescence in situ hybridization) (53), sperm chromatin dispersion test (SCD) (54), and the SCSA (55–58).

An important aspect in sperm DNA fragmentation analysis is the question related to the type of DNA breaks produced in the DNA strands, i.e., whether the breaks are single- or double-stranded and whether they require an initial step of denaturation in order to detect DNA breaks, such as the SCSA, SCD (54), or comet, with an acid or alkaline pH (59). In fact, when DNA damage is observed under acid or alkaline conditions and not under neutral pH conditions we

should be talking about acid/alkali labile DNA sites (10). On the other hand, the TUNEL (49), in situ-nick translation (ISNT) (46), and comet at neutral pH (60) tests do not require an initial denaturation step and, therefore, measure single-stranded (ISNT, TUNEL, and comet) or double-stranded (TUNEL and comet) DNA breaks directly. Because the intracellular pH in the oocyte is around 7.0 (61, 62) the presence of single-stranded DNA breaks or acid/alkali labile sites should not be of significant consequences in the formation of the male pronucleus, as at neutral pH DNA strands would not dissociate and, therefore, this type of damage would be easier to repair than double-stranded DNA damage.

Therefore, as a first examination, we could consider two types of tests: [1] tests that measure DNA damage directly, such as TUNEL, ISNT, or comet at neutral pH, and [2] tests that measure DNA damage after denaturation, such as the SCSA, SCD, and comet, at acid or alkaline pH (59). A recent study reported by Borini et al. (13) showed that sperm DNA fragmentation values in aliquots of the same spermatozoa used for IVF, measured by TUNEL, were significantly correlated with pregnancy outcome. This is in sharp contrast to the results reported by Bungum et al. (63) where no correlation was found between sperm DNA fragmentation values in the samples used for IVF, as measured by the SCSA test, and pregnancy outcome.

It is important to note that although the TUNEL test is frequently used for the determination of cell apoptosis, TUNEL positivity is not synonymous with apoptosis, as DNA damage induced by the hydroxyl radical or ionizing radiation also causes DNA fragmentation that can be detected as a positive test result by the TUNEL test (64). The concept reported in many sperm DNA assessment articles stating that the TUNEL test is associated to apoptosis is a vast overestimation of this test's capability.

The test that has been more extensively studied to date from the clinical point of view is the SCSA test, developed by Evenson et al. (55, 58, 65–68). The SCSA test, as claimed by Evenson, measures DNA susceptibility to DNA denaturation after exposure to mild acid. DNA of sperm with a normal chromatin structure do not denature, whereas if the DNA is somewhat damaged and contains breaks in the DNA strands it can reach different degrees of denaturation. To determine the degree of sperm DNA denaturation, after mild acid treatment, the sperm cells are stained with acridine orange (AO). Because AO is a metachromatic fluorochrome, it fluoresces in green when intercalated between intact double-stranded DNA and fluoresces in red in single-stranded DNA. The degree of DNA damage is measured by flow cytometry and is expressed as a DNA fragmentation index or DFI. Previous studies indicate that a DFI value >27% is associated with pregnancy failure in assisted reproductive technology (ART) (57, 69). However, as indicated previously, recent reports challenge the predictive value of the SCSA test (2, 70, 71). It must be cautioned, however, that the SCSA test has undergone more scrutiny than other tests and further clinical studies may unveil limitations in other tests such as TUNEL and the SCD/Halo test.

PREDICTIVE VALUE OF SPERM DNA FRAGMENTATION TESTING

The predictive value of sperm DNA fragmentation testing is going to depend on a number of factors. These factors include:

1. *Type of DNA damage: single-stranded versus double-stranded DNA damage.* As a general approximation, single-stranded DNA damage is of better prognosis and easier to repair than double-stranded DNA damage. As indicated previously, single-stranded DNA fragmentation may be caused by unrepaired

DNA nicks generated during the process of chromatin remodeling. It might be also caused by oxygen radical-induced damage. Double-stranded DNA damage is usually caused by apoptosis, hydrolysis by caspases and endonucleases, and by oxygen radical-induced DNA damage through the activation of caspases and endonucleases. The extent of double-stranded DNA damage induced by oxygen radicals during passage of sperm through the epididymis may be determined not only by the levels of oxygen radicals produced by immature sperm, epididymal epithelial cells, or activated leukocytes, but also by the levels of antioxidant enzymes present in the lumen of the epididymis (39).

2. *Percent of sperm with DNA damage.* To date most studies have analyzed this aspect of the relationship between fertility and sperm DNA damage. In general the results point to a greater utility of sperm DNA tests in relation to natural conception and intrauterine insemination rather than ART treatments such as normal IVF and intracytoplasmic sperm injection (ICSI) (72). It is important to note that the quality of the sperm DNA in the fertilizing sperm is the only important factor. In techniques, such as IVF or ICSI, although a high percentage of sperm in a sample may have damaged DNA, a “non-DNA damaged” sperm can still be selected. Both the extent of DNA damage in individual spermatozoa and how different sperm processing techniques can remove DNA-damaged sperm need to be better understood.
3. *Extent of DNA damage per spermatozoon.* The main untested questions in humans are: [1] What is the degree of sperm DNA damage in a single sperm? and [2] How much sperm DNA damage can an oocyte deal with? In mouse studies, a correlation has been shown between increasing amounts of DNA damage inducers (heat and radiation) and reproductive outcome (73–75). For example, the studies by Ahmadi and Ng (74, 75) showed that fertilization rates of around 60% were achieved when sperm were subjected to 0, 5, 10, 50, and 100 GY. Blastocyst development, however, decreased from 49.8% in the control group to 2.3% with sperm exposed to doses of 5–100 GY. Of the transferred blastocyst in the control group, 33.9% developed into live fetuses, whereas these rates were 20% and 0% when sperm were exposed to doses of 5 and 10 GY. The investigators concluded that embryonic and fetal development are very much related to the degree of DNA damage and that the oocyte has the capacity to repair DNA damage of sperm when it is damaged less than 8%.
4. *Whether there is combined nucleotide damage and DNA fragmentation.* Post-testicular sperm DNA damage induced by the hydroxyl radical or after exposure to ionizing radiation is associated with nucleotide damage of the 8-OHdG type. As indicated previously, with this type of damage, in a first stage the damage produced is of the 8-OHdG type, followed by double-stranded DNA fragmentation that may be mediated by caspases and endonucleases. The implications of combined nucleotide damage and DNA strand fragmentation is that, in addition to its diagnostic value (oxygen radical-induced damage is amenable to antioxidant treatment), it may have a worse prognosis as the majority of the sperm cells will be affected by one type of damage or the other.
5. *Whether DNA damage affects introns or exons.* More than 90% of the DNA is comprised of introns. Therefore, the probability that DNA damage affects exons, which are the sequences that encode for proteins, is relatively low. There is therefore the question of whether the majority of DNA damage is architectural in nature and whether this may have any long-term implications.
6. *Ability of the oocyte to repair sperm DNA damage in the fertilizing spermatozoon.* Of the different factors that impact on the pre-

dictive value of sperm DNA fragmentation tests, the DNA repair ability of the oocyte is perhaps the most important. The mouse oocyte has the capacity to repair sperm DNA damage (76), although this capacity is limited and may vary from oocyte to oocyte and even between different cohorts of oocytes from the same patient or from different patients. It may also depend on the woman's age. On the other hand, the ability of the oocyte to repair sperm DNA damage in the fertilizing spermatozoon is also going to depend on the type of sperm DNA damage. As indicated previously, sperm DNA damage can be classified as single-stranded and double-stranded. In general, single-stranded DNA damage is easier to repair than double-stranded DNA damage, although there is evidence that polymerases can also repair double-stranded DNA damage (77). Therefore, even if the fertilizing spermatozoon carries DNA damage in its genome, the oocyte could repair this damage and, therefore, it would be of no consequence for embryo and fetal development. However, we cannot determine whether the oocyte would be capable of repairing this damage. In addition, DNA fragmentation tests currently available cannot provide information concerning the “repairability” of DNA damage. Therefore, there are two levels of uncertainty that cannot be resolved with currently available diagnostic tests. However, if we postulate that unreparable sperm DNA damage is not compatible with normal embryo or fetal development, those couples in whom the man has sperm DNA fragmentation of the unreparable type, a pregnancy should be not be achieved and the couple would present to infertility clinics with long-standing infertility or repeated pregnancy failure in ART without an apparent cause. If sperm DNA fragmentation is the limiting factor, strategies directed to decrease sperm DNA fragmentation levels should improve pregnancy outcome in couples with no apparent cause for their infertility problem.

As previously stated reports have shown that sperm DNA damage is significantly lower in the seminiferous tubules compared with the cauda epididymis (21, 78) or ejaculated sperm (19), and that use of testicular sperm in couples with repeated pregnancy failure in ART and high sperm DNA fragmentation in semen resulted in a significant increase in PRs in these couples (19, 79). In addition, a recent report shows that PRs in the first cycles of TESA–ICSI are relatively high (Table 1). These results are consistent with the notion that in couples with long-standing infertility or repeated pregnancy failure with ART without an apparent cause, sperm DNA fragmentation could be the limiting factor responsible for their pregnancy failure, and that use of testicular sperm with very low levels of sperm DNA fragmentation eliminates the burden of DNA repair of the fertilizing spermatozoon by the oocyte. However, the use of testicular sperm may not always solve the problem as sperm DNA damage may also occur in the seminiferous tubule epithelium by apoptosis or be due to defects in chromatin remodeling during the process of spermiogenesis. Nevertheless, in the majority of cases, sperm DNA damage occurs or is increased in the epididymis and, therefore, DNA fragmentation levels in testicular sperm should be lower (19).

One issue that ought to be considered when using testicular sperm is the possibility of microinjecting testicular sperm with a higher probability of carrying chromosomal abnormalities. It is well known that the rate of aneuploidy in testicular sperm is higher than in ejaculated sperm. This could be due to the selective elimination of aneuploid sperm during passage through the epididymis (80). However, the conclusion that the rate of aneuploidy in testicular sperm is significantly higher than in ejaculated sperm has been drawn, for the most part, from studies using sperm from severe oligospermic or

TABLE 1

Outcome results of TESA–ICSI in couples with repeated IVF failure and high levels of sperm DNA fragmentation in semen.

Characteristics	Values
Semen and case parameters	
Cases evaluated	68
Cases of TESA–ICSI performed	31
Mean DFI in semen	39.4%
Mean sperm concentration (million/mL)	44.7
Mean male age	41.9
Outcome results	
Fertilization rate	58% (20%–100%)
Number of embryos transferred	2.3
Clinical pregnancy rate	40.0%
Pregnancy rate in the first TESA–ICSI cycle	93%

DFI = DNA fragmentation index; TESA–ICSI = testicular sperm extraction–intracytoplasmic sperm injection.

Sakkas. *Sperm DNA fragmentation. Fertil Steril* 2010.

azoospermic men. In contrast, in most patients with high DNA fragmentation levels in ejaculated sperm in which the use of testicular sperm is indicated, sperm concentration in semen is normal and, therefore, we are not dealing with severe oligospermic or azoospermic men where the risk of sperm aneuploidy is higher. In a recent report it has been shown that PRs, using testicular sperm in couples with high levels of sperm DNA fragmentation, were significantly higher than when using ejaculated sperm and that miscarriage rates were significantly lower (Table 2). If the use of testicular sperm in couples with high levels of sperm DNA fragmentation were a significant risk, PRs should be lower and miscarriage rates higher. However, the results indicate the contrary and that this is a relatively safe technique when applied to these couples (13).

- Type of sperm DNA fragmentation test used.* As indicated previously, tests that measure DNA damage directly without a prior denaturation step, such as TUNEL, should be preferentially recommended, especially when coupled with flow cytometry. This increases the reproducibility of the test result and the reliability of the test.
- Sperm processing in ART.* DNA damage may occur during sperm processing. Previous studies have shown that incubation of

semen at room temperature (81) or at 37°C (82) after their isolation by density gradient centrifugation may lead to an increase in the levels of sperm DNA fragmentation. Also, sperm cryopreservation may lead to an increase in DNA fragmentation (83). In addition, centrifugation of raw semen may result in an increase in DNA fragmentation. Therefore, sperm processing in ART should be governed by the principle of *primum non nocere*. That is, [1] unnecessary incubation of semen and sperm suspensions should be avoided; [2] density gradient centrifugation appears to be the best way of processing semen (84–89), and [3] cryopreservation methods that minimize sperm DNA fragmentation should be used. A DFI value may be normal in liquefied semen and abnormal in the sperm used for ART. This is going to give false-negative test results and alter the predictive value of sperm DNA fragmentation testing. One way of minimizing the effect of semen incubation on sperm DNA fragmentation would be by shortening the time between semen processing and insemination. In addition, in the case of sperm cryopreservation, the use of media supplemented with antioxidants should be recommended. An important point to be made concerning the relationship between sperm processing and DNA fragmentation is that, should a critical number of the sperm in the semen sample have intact chromatin, sperm processing may actually result in the enrichment of these spermatozoa and, therefore, improve the chances of achieving a viable pregnancy.

- Oocyte number.* In couples in whom the level of sperm DNA fragmentation is relatively high, the probability that normal embryos or a viable pregnancy be produced is going to depend, at least in part, on the number of metaphase II oocytes obtained and their ability to repair DNA damage in the fertilizing spermatozoon. For example, if we obtain 10 metaphase II oocytes, the probability of having a pregnancy is going to be higher than if we only obtain 1 oocyte. In the second case (only 1 oocyte obtained), the probability of obtaining at least one embryo derived from a spermatozoon with intact DNA that fertilized a normal oocyte or from a spermatozoon with damaged DNA that fertilized an oocyte with high DNA repair capacity is going to be much lower than in the first case (10 oocytes obtained). In the first case, embryo selection and the transfer of two embryos significantly increases the probability of obtaining at least one embryo with intact DNA from which a viable pregnancy can derive. However, this does not negate the value of sperm DNA fragmentation testing in these couples. Therefore, the number of metaphase II oocytes available is an important factor impacting on the predictive value of sperm DNA fragmentation testing. The

TABLE 2

Pregnancy outcome in patients with high levels of sperm DNA fragmentation when treated with ejaculated compared with testicular sperm.

	Biochemical pregnancy rate	Clinical pregnancy rate	Implantation rate	Miscarriage rate
Ejaculated sperm (n = 42)	6.90%	13.79%	6.56%	75.0%
Testicular sperm (n = 30)	2.5%	40.0%	28.09%	6.25
P value	NS	.035	.0021	.017

Note: All patients included in the study had a DNA fragmentation index value in semen by TUNEL >20%; statistical analysis by χ^2 test.

Sakkas. *Sperm DNA fragmentation. Fertil Steril* 2010.

same would apply to natural or IUI cycles in which the number of oocytes obtained is usually one. In this scenario, PRs would be more dependent on the DNA fragmentation test value, as the probability of obtaining one embryo with intact DNA is lower and, in addition, we do not have the advantage of transferring two or three embryos as in the case IVF. Therefore, the negative predictive value of DNA fragmentation testing should be higher in natural and IUI cycles. This hypothesis is consistent with the results of the study of Duran et al. (90) in which they found that sperm DNA fragmentation values, as measured by the TUNEL test, were highly correlated with PRs in IUI cycles.

To conclude, we propose that the predictive value of DNA fragmentation testing, Π_{test} , is the sum of several factors: $\Pi_{\text{test}} = \Pi_{(1)} + \Pi_{(2)} + \Pi_{(3)} \dots + \Pi_{(n)}$, whereby $\Pi_{(n)}$, represents many of the factors 1–9 discussed previously, and each factor may be given a different weight of importance.

APPLICATIONS TO THE DIAGNOSIS AND TREATMENT OF INFERTILITY

What are the implications of sperm DNA testing in the diagnosis and treatment of infertility? The fertilization of metaphase II oocytes by spermatozoa with DNA damage could lead to defects in embryo development, implantation failure, or to an increase in miscarriage rate (13, 14, 91–95). Mature oocytes with functional DNA repair mechanisms have the ability to repair moderate sperm DNA damage (96, 97). However, oocytes whose DNA repair mechanisms are not functional or that have been damaged by endogenous (e.g., free radicals) or exogenous (e.g., radiation, environmental toxicants) factors would not be able to repair this damage.

One important aspect that is currently emerging related to sperm DNA fragmentation testing is the presence of subgroups of infertile couples who present to infertility clinics with a history of longstanding infertility or repeated IVF failure without an apparent cause in which “unrepairable” sperm DNA damage may be the limiting factor responsible for their infertility problem. This may help to explain, at least in part, why PRs in first cycles of TESA–ICSI are relatively high in these couples (79). These data suggest that if pregnancy does not take place in the first TESA–ICSI cycle, the cause of infertility in these couples may lie elsewhere. Currently, there is some controversy concerning the predictive value of sperm DNA fragmentation testing in ART. Even when using tests with a high predictive value, such as TUNEL by flow cytometry, given their low specificity, mainly due to the sperm DNA repair ability of the oocyte, sperm DNA fragmentation test values do not always correlate with pregnancy outcome. Some investigators report such a correlation and others do not. In a recent report, Collins et al. (98) conclude that “the small but statistically significant association between sperm DNA integrity test results and pregnancy in IVF and ICSI cycles is not strong enough to provide a clinical indication for routine use of these tests in infertility evaluation of men. It is possible that yet to be determined subgroups of infertile couples may benefit from sperm DNA integrity testing.” This assessment implies that sperm DNA fragmentation testing may be selectively applied to couples with a poor prognosis in ART. As indicated previously, there are a number of factors that impact on the predictive value of DNA fragmentation testing, which may help answer this question. However, perhaps the most likely explanation to this apparent paradox is related to the type of DNA damage—repairable versus nonrepairable. For example, two patients may have the same DNA fragmentation test value by TUNEL (e.g., 50%) and yet the prognosis may be completely different, as in one case the DNA damage may be repair-

able and in the other it is not. However, this, in itself, does not explain why PRs would be so low in the second case. For this to apply, all spermatozoa must have some type of DNA damage and this damage cannot be repaired by the oocyte or the embryo. An “iceberg effect” type of scenario should apply. Given the fact that one of the most important mechanisms of sperm DNA fragmentation is oxidative damage during passage through the epididymis and that, as mentioned previously, a combination of nucleotide damage (i.e., 8-OHdG and double-stranded DNA damage), it is possible that, in these couples, DNA damage would affect the majority of the sperm cells, although current tests only allow to measure a fraction of this DNA damage. Therefore, the value of 50% could be misleading. The concept that some patients with a more severe type of DNA damage (e.g., DNA damage that is not repairable by the oocyte or the embryo), who may benefit from DNA fragmentation testing and the use of sperm with low DNA fragmentation values (e.g., testicular sperm), is worthwhile considering. Unfortunately, no tests are currently available, at least in a clinical setting, that would allow for the differentiation of these types of DNA damage. However, recently, the 2D-COMET has been introduced as a novel test that can distinguish between these two types of damage (99). This test may help evaluate the impact of double-stranded sperm DNA damage on ART pregnancy outcome and improve the specificity of DNA fragmentation testing. In addition to the DNA damage measured by tests, such as TUNEL, comet, SCD, or SCSA, with the exception of the 8-OHdG test, no tests are currently available that measure other types of DNA damage (oxidative and nonoxidative) that may be present in sperm DNA. Until such tests are developed the specificity of sperm DNA fragmentation testing will continue to be low.

Therefore, based on the factors that determine the predictive value of sperm DNA fragmentation testing, the clinical value of sperm DNA fragmentation testing in predicting pregnancy outcome in ART should be necessarily based on two main assumptions: [1] DNA damage should affect the entire sperm population in the sample, and [2] the DNA damage present in these spermatozoa cannot be repaired by the oocyte. Should any one of these assumptions fail to apply, no significant correlation whatsoever would be expected between an abnormal DFI value and pregnancy outcome in ART.

Another important aspect of the clinical applications of DNA fragmentation testing is the use of testicular sperm in patients with high levels of sperm DNA fragmentation in semen, as previously indicated. In these cases, the use of testicular sperm obtained by TESE or TESA should be recommended.

In addition to the use of testicular sperm, another strategy that could be used in patients with high levels of sperm DNA fragmentation is the selection of spermatozoa with low levels of DNA damage. Although this strategy would be mainly applied to ejaculated spermatozoa, it could also be applied to testicular sperm, since, as indicated previously, not all sperm DNA damage is post-testicular. Currently a number of techniques have been proposed to select sperm with lower levels of DNA fragmentation. These include the use of Annexin-V columns (100, 101), which have been shown to significantly reduce the percentage of spermatozoa with DNA fragmentation, as measured by the TUNEL test and a sperm selection method based on sperm hyaluronic acid binding (102). Other techniques include the selection of spermatozoa devoid of surface vacuoles by high-magnification ICSI (103) and the recently introduced confocal light absorption scattering spectroscopy (CLASS) technology, which allows for the noninvasive visualization of subcellular structures (103). These subcellular structures can be identified by two main modes: direct visualization by confocal microscopy or by their specific spectrum. With

regard to the applications of the spectral mode in ART, a spectroscopy would be attached to the inverted microscope and the spectrum of subcellular structures, such as the sperm chromatin, can be collected in less than a second. Therefore, the CLASS technology may allow, in the near future, selecting spermatozoa with the intact chromatin to be microinjected by ICSI. However, caution must be exercised concerning the potential deleterious effects of using these noninvasive technologies, as iatrogenic damage may be induced under some unanticipated conditions. Because time of sperm selection and visible light exposure in the case of the CLASS technology would be limited to about 1 second, this is more likely to apply to the IMSI technology where sperm selection time is significantly higher. With regard to the utility of IMSI in ART, a recent study indicates that DNA fragmentation levels, as assessed by the SCD test, in individual sperm selected by IMSI, is negligible in sperm with no vacuoles (Gosalvez et al., unpublished results).

In conclusion, the presence of DNA fragmentation in sperm nuclei has generated a plethora of articles during the past decade. The distinction between all sperm being affected versus just the “DNA damaged” sperm is critically relevant to clinical management of these patients. In relation to ART, the onus must now be shifted to identification of the DNA damaged sperm and how to select individual or populations of “normal” sperm. Finally, the presence of DNA fragmented sperm is irrefutable whatever the mechanisms of its origin, effect on the embryo and offspring, and the need to diagnose it are critical areas that need further understanding.

Acknowledgments: The authors thank Dr. Esther Velilla and Dr. Estefania Toro for technical assistance in the performance of the TUNEL test and to Dr. Ferran García for the retrieval of testicular sperm in the TESA-ICSI studies. We also thank Dr. López-Teijón for her valuable comments and her unconditional support of this research.

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