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Lewis, S. E. M. (2013). The place of sperm DNA fragmentation testing in current day fertility management. *Middle East Fertility Society Journal*, 18(2), 78-82. <https://doi.org/10.1016/j.mefs.2013.01.010>

Published in:
Middle East Fertility Society Journal

Document Version:
Publisher's PDF, also known as Version of record

Queen's University Belfast - Research Portal:
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DEBATE

Sperm DNA fragmentation testing: To do or not to do?

Comment by: **Khaldoun Sharif**

Reproductively speaking, the package carried in the sperm is the paternal DNA, which upon union with the oocyte will potentially lead to the creation of an embryo and consequently a baby. Therefore, it is both biologically plausible and clinically tempting to assume that testing sperm DNA damage (the so-called fragmentation) will increase our understanding of fertility problems and help us decide the most suitable treatment for them.

A lot of work has been done in this field in recent years, and a search in Google Scholar for “sperm DNA fragmentation” between the years 2001 and 2012 showed 2390 publications, in contrast to only 20 such publications between 1991 and 2000.

So given this amount of published work, the biological plausibility and the clinical appeal of the idea, one would think that incorporating sperm DNA fragmentation testing in clinical practice is a given. Well, the experts disagree, and in this debate we have asked two world-renowned experts in the field to debate the ‘for’ and ‘against’ of sperm DNA fragmentation testing.

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The place of sperm DNA fragmentation testing in current day fertility management

Comment by: **Sheena E.M. Lewis**

1. We know traditional semen analysis tells us little-why do we keep using an inadequate batch of tests?

Male infertility is the commonest cause of infertility yet we keep sidetracking it. It contributes to nearly 50% of the ~15% of couples of reproductive age. As a result of population aging and adverse changes in our lifestyle, infertility increases (with 4% more couples seeking ART per year) while we are only making a marginal improvement in pregnancy and birth rates after 30 years of trying. ART year on year (average live birth rate 21%, (6)). Why do we accept this as good enough for our patients? Why can we not improve? I believe that one major reason is that we do not understand the causes of male infertility at the molecular level. Furthermore, since the advent of intracytoplasmic sperm injection (ICSI), there has been no incentive to develop pharmaceutical therapies for male infertility. We believe we can get around the problem rather than solving it. But let us remember, in 30% of cases, women are subjected to invasive procedures for ICSI although they are fertile. Conventional semen analysis remains the only routine test to diagnose this condition although it cannot discriminate between the sperm of fertile and infertile men (10). This belief is reflected in the shifting values for normality (all ‘normal’ values are now lower) in the 5th edition of the WHO manual, (18) compared to previous WHO guidelines. Further, only 1% of sperm even reach the oocyte *in vivo*, so why would we expect an analysis of the widely ranging gross parameters of the whole ejaculate to give strong discriminatory information? That approach is simply not scientific. All that a semen analysis can do is identify men whose chance of achieving a natural pregnancy is very low i.e. they have few or no sperm. . .!

1.1. Why test sperm DNA?

Over the last decade, a plethora of studies (11) have confirmed that sperm DNA damage testing has strong associations with every early fertility check point. These include impaired fertiliza-

Peer review under responsibility of Middle East Fertility Society.



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tion, slow early embryo development, reduced implantation, miscarriage and, in animal studies, birth defects in the offspring. Childhood cancers have also been associated with oxidative damage to sperm DNA as a consequence of paternal smoking.

What couples would benefit from a sperm DNA test? To answer this question, we must first ask another. Why are we testing sperm DNA? The answer is so that we may guide couples with low damage to intrauterine insemination (IUI) or in vitro fertilization (IVF) and guide those couples with high DNA damage to ICSI (reasons why these sperm are successful after ICSI will be explained later). So, the only group who would not benefit from testing is that of couples with oligoastheno-terato-zoospermia as there is no treatment other than ICSI for them.

As for all other categories of couples, sperm DNA testing will provide essential information on which clinics can guide couples to bespoke treatment for their particular needs. These include couples with unexplained infertility, men with normal semen by semen analysis prior to embarking on IVF, couples who have had unsuccessful IVF and couples who have had miscarriages.

1.2. What tests are currently available for sperm DNA damage?

For a sperm DNA test to be clinically useful it must have strong predictive capacity for pregnancy with little overlap between fertile and infertile samples. The four tests most often used today are the Comet assay, SCSA, the terminal transferase dUTP nick end labeling (TUNEL) assay, and the Sperm Chromatin Dispersion (SCD or Halo) test. Sperm DNA tests are all different. Just as apples are NOT oranges! Both are fruits but they are very different types of fruits. The current range of sperm DNA tests measure different aspects of DNA damage and have different sensitivities. That is why combining them in a meta-analysis (19) must be viewed with a little caution.

2. The Sperm chromatin structure assay

The SCSA is a fluorescence cell sorter test which measures the susceptibility of sperm DNA to denaturation after exposure to heat or acid conditions. A strength of SCSA is its ability to measure large numbers of cells rapidly. This gives it robust statistical power. It measures only single stranded fragments, and has demonstrated repeatedly strong associations between native, although not DGC, sperm and ART outcomes. In terms of sensitivity, it can detect sperm DNA damage in ~20% of unexplained couples. However, the SCSA test has been tried and tested over many years and has a standardized protocol for all users. This has reduced inter-laboratory variation and allowed comparison of studies from different groups globally. The clinical threshold is a DNA fragmentation index (DFI) of 30%- that means 30% of the sperm have damage (with quantification into moderate or high damage) and 70% have no detectable damage. Couples with >30% damage are more likely to have success with ICSI than IVF.

3. The TUNEL assay

The TUNEL assay detects 'nicks' (free ends of DNA) by incorporating fluorescently stained nucleotides. This allows the detection of single and double stranded damage. The cells can be assessed

either microscopically or by flow cytometric (FCM) analysis. A disadvantage of the assay is its many protocols, which makes comparison between laboratories almost impossible and explains its many clinical thresholds. Recently, Aitken's group (2011) (14) has improved the TUNEL assay by including a preliminary step of DDT to relax the whole chromatin structure and allow access to all 'nicks'. They have also added a viability stain so that DNA damage is measured only in live sperm. This has eliminated a previous inaccuracy of measuring damage (often at high levels) in dead cells. The TUNEL has major potential but robust clinical thresholds have yet to be established.

4. The sperm chromatin dispersion (Halo) test

The Halo test is a 'cheap and convenient' kit form of sperm DNA testing. It is a simple and inexpensive assay, available in fertility labs for in house use. Unlike all the other tests, it measures the absence of damage rather than the damaged DNA in sperm. One limitation of the assay is that its low-density nucleoids are relatively faint, with less contrasting images. To date, correlations have been observed between DNA and other sperm parameters, although few correlations between sperm DNA damage and ART outcomes have been established with the Halo test, even in large studies.

5. The Comet assay

The comet assay is a second generation sperm DNA test. Unlike the other three tests, it quantifies the actual amount of DNA damage per sperm. As the mass of DNA fragments stream out from the head of unbroken DNA, they resemble a 'heavenly comet' tail, hence the name of the assay. One major advantage of this assay is that it uses only 5000 sperm, so is suitable for the assessment of small samples left over from clinical use, or for samples where only a few sperm are available. The Comet assay can measure both single and double strand breaks, and with an additional step can measure even altered bases. This is useful, because we do not yet know which types of DNA damage are most deleterious to male fertility. The Comet is sensitive, repeatable and capable of detecting damage in every sperm (even those of fertile donors). Since 2010, clinical thresholds for the diagnosis of male infertility and the prediction of successful IVF have been established.

5.1. Unexplained infertility is unexplained no more

As we all know, unexplained infertility is a very unsatisfactory diagnosis for couple and clinic alike. In our latest study we have shown that 80% of couples diagnosed with idiopathic infertility have sperm DNA damage (>25% damage per sperm). This suggests that sperm DNA damage is the cause of infertility in a substantial number of men if we compare these levels of damage we reported in sperm of donor men with proven fertility (15). We also reported that 40% of these couples had such high sperm DNA damage (>50%) that IVF had very poor success for them. Couples with idiopathic infertility had lower live birth rates following IVF (15%) compared with couples undergoing IVF (20%) following the detection of a female problem. This finding is also reflected in disappointedly low effectiveness of the IVF treatment when measured as the

cumulative incidence of live delivery after commencing IVF treatment. Previously we would have expected these couples to have high success rates since we could detect no anomalies but we now know this is a mis-diagnosis. Offering them IVF or in some cases (IUI) without DNA testing can lead to treatments with very low chances of success. From the clinic's viewpoint, using IVF for these couples is a poor choice too as it reduces their overall IVF success rates.

5.2. *The benefits of sperm DNA testing*

For all couples having IVF, after diagnosis with a female factor, sperm DNA fragmentation also has a close inverse relationship with live birth rates. Our latest results (15) were based on dividing couples into groups depending on the severity of their sperm DNA damage. Those with sperm DNA fragmentation (< 25%) had live births of 33% following IVF treatment. In contrast, couples with sperm DNA fragmentation (> 50%) had a much lower live birth rates of 13% following IVF treatment.

If we were to incorporate this new information into routine clinical care, we could direct these patients straight to ICSI treatment thus avoiding loss of valuable biological time, cost of failed cycles and heartache after repeatedly unsuccessful cycles of IVF treatment.

Further compelling reasons for testing sperm DNA come from its strong associations with miscarriage. A systematic review and meta-analysis (16) of 16 cohort studies (2969 couples), 14 of which were prospective studies which examined the effect of sperm DNA damage on miscarriage rates was performed. We used the terms 'DNA damage' or 'DNA fragmentation' combined with 'miscarriage', 'abortion' or 'pregnancy' to generate a set of relevant citations. Sub-group analyses were performed by the type of DNA damage test, whether the sperm examined were prepared or raw ejaculate and IVF or ICSI treatment. The meta-analysis showed a significant increase in miscarriage in patients with high DNA damage compared with those with low DNA damage (Risk Ratio (RR) = 2.16 [1.54, 3.03], $P < 0.00001$).

5.3. *A fresh look at the 'evidence' against Sperm DNA damage testing*

The meta- analyses of Zini and Sigman (19) and of Collins et al. (5) were the first to bring all the sperm DNA data together and highlight the substantial body of work. However, we must view their conclusions with caution due to the significant heterogeneity of different assays, different female ages, some sperm from raw semen, some from prepared sperm, different ART endpoints and different thresholds for clinical significance. The primary objective of the ESHRE position paper in 2009 was not to support or refute clinical DNA testing. It was written as an overview of the maturational vulnerabilities of sperm DNA and a mechanistic look at DNA damage and repair. The section on clinical testing was less than 10% of the content. In addition, as with the meta- analysis by Zini and Sigman (19), it did not include Comet data. The objective of the 'Cochrane' review by Van Rumste et al. (17) was to investigate if live birth rates were better with ICSI than IVF in couples with non male sub-fertility. The review began with ten randomized controlled trials but all but one study were excluded for design reasons. The one study as we know was

from Bhattacharya et al., and published in *The Lancet* in 2001 (4). However, the numbers of couples included in this one study are relatively small ($n = 224$ in IVF group, $n = 211$ in ICSI group). Even this study did not give live birth rates or miscarriage rates. The couples were identified as having non-male sub-fertility at time of study (2001) by semen parameters that are now viewed as neither predictive of diagnosis of male infertility nor useful in predicting ART outcome. As with all Cochrane reviews it concluded that further studies are needed.

The American Society of Reproductive Medicine (ASRM) (2) has recently released a position paper for consultation (2013) (3). In my opinion, this paper presents a negatively biased and unbalanced overview. It dismisses the findings of around 100 papers in high impact journals over the last 30 years (11) as 'insufficient evidence' and turns the range of different sperm DNA tests measuring different aspects of DNA damage into a weakness rather than accepting it as the strength it is. My specific objections include the ASRM requirement of Level 1 evidence of quality from randomized controlled trials prior to clinical use. This is in stark contrast to the approach adopted to ICSI in 1995, when it became a routine publically funded fertility treatment on a global scale without any human trials at all. Further, I would direct you to the paper by (9) followed by the letter by Richard Legro in *Fertility and Sterility* in December 2012 (12) commenting on the quality of RCTs over 2006–2011 in five leading human reproduction journals and pointing out that incomplete outcome data and inadequate allocation concealment led to bias in almost 50% of them.

In terms of ASRM Level 11 requirements, the sperm DNA test designed in our lab; the SpermComet; conforms to all of them. These data result from studies performed on large numbers of over 500 couples. These couples were consecutive cases and were controlled for female factors. The primary endpoints were live birth rates. However, despite numerous reviews showing its serious limitations of semen analysis and evidence of the stronger repeatability, sensitivity and specificity of sperm DNA damage testing, ASRM still supports the continued use of semen analysis alone for diagnosis. Further, the level of precision that ASRM requires, can never come from one test. Fertilization is a multi-factorial process and a successful ART outcome depends on many other traits of sperm quality and function, as well as the influences of the oocyte, uterine receptivity and maternal immune system competence. Finally, the ASRM document, having begun by criticizing DNA testing as measuring different aspects of DNA damage and therefore being non-uniform, then criticized DNA testing for being non specific 'for not providing an indication of specific DNA sequences that may be affected'. As no one yet knows what aspects of DNA damage at large, nor any specific sequences that are responsible for 'male infertility', neither of these opposing criticisms are based on sound science.

5.4. *Reasons why sperm with poor DNA are successful in ICSI*

One question that has exercised my mind much upon analyzing the data is 'Why do sperm with DNA damage not reduce success following ICSI?'

I have four hypotheses (all based on sound studies) to explain this:

Firstly, up to 30% of women having ICSI have no detectable problems. They may be fertile and their oocytes may have more capacity to repair DNA damage even if the injected sperm is of poor quality. This is supported by the findings of Meseguer et al. (13), where high quality oocytes from donors offset the negative impact of sperm DNA damage on pregnancy. Further, the study of Devroey et al. (7), shows that significantly fewer good quality embryos were available for transfer from women more than 40 years of age compared to the younger group, reflecting better DNA repair capacity of oocytes from young women. This is relevant in that women undergoing ICSI are often younger than those undergoing IVF as their cause of infertility has been diagnosed earlier and they have not spent time undergoing failed intrauterine insemination or IVF before embarking on a cycle of ICSI. Secondly, in ICSI, the gametes are not subjected to prolonged culture so the sperm may have less damage than those exposed to culture media overnight, as in IVF procedures. The recent major study from Dumoulin et al. (8) shows that even the birth weight of IVF babies can be markedly influenced by minor differences in culture conditions. In contrast to IVF, ICSI sperm are injected into the optimal environment of the oocyte within a few hours of ejaculation. This may protect them from laboratory induced damage. Thirdly, much sperm DNA damage is caused by oxidative stress (1) and so these sperm are producing reactive oxygen species. If they are used in IVF, the oocyte may be exposed to oxidative assault during the overnight incubation from these ~0.5 million sperm. In ICSI, the oocyte is protected from this attack and can use its energies to repair the damage in the sperm immediately following fertilization. Finally, there is now evidence that embryos created from sperm with high DNA damage are associated with early pregnancy loss (reviewed by Robinson et al. (16) so ICSI success rates are sometimes affected adversely by sperm DNA damage, but at a later stage.

5.5. Limitations of sperm DNA testing

The major limitation of testing for sperm DNA damage is that the assay renders the tested sperm unsuitable for clinical purpose. In an effort to overcome this problem, a number of non-invasive tests have been developed and their correlation with DNA damage assessed. These novel test include Birefringence, Intracytoplasmic morphologically selected sperm injection (IMSI) and Hyaluronic acid-selection of sperm for ICSI. If these tests can help embryologists choose sperm with low DNA damage for ART, a major step forward in sperm selection will be achieved, but presently this is not the case.

5.6. Why wait? The benefits for clinics and couples

Couples will avoid the loss of valuable biological time, the cost of failed cycles and the heartache of repeatedly unsuccessful cycles of IVF treatment. Unexplained couples will get a diagnosis and thus directs to the best treatment for them. Happier, better informed couples will spread the word of their clinic's success.

Clinics will have improved IVF success rates, a scientific rationale for using ICSI and additional revenue from appropriate male fertility testing. Their greater success rates will help

them retain their patient bases (with couples attending for their next child!).

Alas, although some of these novel biomarkers (particularly sperm DNA testing) have increasingly robust data to support them, there is still a reluctance to incorporate them into routine clinical care. While this inertia continues and our traditional tests prevail, it seems unlikely that success rates in the treatment of male infertility will improve and success rates will be doomed to mediocrity.

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Andrology is desperate for a new assay – Let us make sure we get it right this time . . .

**Comment by: Christopher L.R. Barratt,
Steven A. Mansell**

Although the diagnostic and predictive value of traditional semen parameters is limited, two facts are clear: (1) at the lower ends of the spectrum, e.g. low concentrations of motile spermatozoa, there are significantly higher chances of sub fertility (9,11) (2) except in rare cases values above these limits of semen analysis provide minimal diagnostic clarity (14). Consequently, semen analysis is very helpful, but andrology requires more robust sophisticated functional assays to be placed in the patient's pathway to assist/change management decisions. Unfortunately, the search for this Holy Grail has been littered with false dawns (2,13) continually blighted by two key problems (1) poor technical and methodological control of purported assays (2) low quality clinical trial information exemplified by poor design often accompanied by low numbers. Not surprisingly, the resultant data often produce conflicting results.

Two key questions arise: will the testing of DNA and its packaging in the human spermatozoon be an important tool in the armamentarium? Is it not already (3)? [We suggest the answers here are yes and no, respectively.](#)

The assessment of DNA integrity in the spermatozoon is not new. In 1980, a landmark publication by Don Evenson suggested that DNA integrity may be a useful and potentially independent marker of fertility for both animals and men (8). Significant data are now available to suggest that higher levels

of DNA damage is present in men with severe sperm defects and is an indication of a potentially negative impact on both natural and assisted conception outcomes (4). So why do we answer no to the second question? Simply, aside from a relatively low (but significant negative) influence of high levels of DNA on miscarriage (15), three comprehensive reviews of the clinical data concluded that the significance of sperm DNA integrity assessment for natural and ART remains unclear (7,4,16). Routine testing is not supported. This has been reinforced recently by the clinical practice guidelines produced by the British Fertility Society (19). The BFS concludes that 'there is evidence of a relationship between sperm DNA damage and either semen parameters and/or outcome of assisted conception. However, reports conflict and depend largely on the laboratory test utilized. Results are unlikely to alter patient management'. The draft document presented by the American Society for Reproductive Medicine (1) on 'The clinical utility of sperm DNA integrity testing: a guideline' concurs with these conclusions. Its final recommendation is: 'there is insufficient evidence to recommend the routine use of sperm DNA integrity tests in the evaluation and treatment of the infertile couple (Evidence Level B)'.

These are powerful, consistent and persuasive conclusions. But why is this so? Unfortunately assessment of DNA is affected by the three key problems identified for sperm function testing. Firstly clear technical difficulties of performing the three key assays used to test DNA integrity in sperm – SCSA, TUNEL and Comet have affected their clinical usefulness. However, rigorous attention to detail and defined methods now exists for all these methods, e.g. see (12) for TUNEL. Application of robust protocols will, hopefully, minimize future methodological challenges. Secondly, to date the clinical evidence is based on relatively low numbers and poorly designed trials. Surprisingly, in this area there are a large percentage of uncritical reviews compared to primary data which distort the field (3). In 2012 for example there was only one high quality clinical study examining outcomes for IVF/ICSI using relatively large numbers (18). This is very disappointing and unacceptable. No progress will be made if this is not urgently addressed. Thirdly, it has been unclear where the assays (when validated) fit within the patient pathway. For example, diagnostic tests can be used as replacement, triage or add-on with their usefulness being dependent on a large number of factors (6). A critical factor will be cost effectiveness. Two examples suffice here. Mitchell and colleagues showed that the TUNEL assay was highly correlated to sperm viability (12) demonstrating that a simple viability assay could effectively replace an expensive estimate of DNA damage. Improved methodology in the TUNEL assay now allows assessment of DNA damage in live cells – a substantial improvement. A second example is provided by a study comparing progressive motility and DNA assessment for IVF success where it was concluded that DNA assessment was more significant. However, close examination of the data shows this to be a relatively small difference and it is questionable if this is actually cost effective (17). Future studies in cost effectiveness clearly need to establish the additional role of DNA integrity assessment alongside a high quality semen assessment (See (19)).

For the field to progress we must address the above points. These are not new (10) or specific to reproductive medicine (5). However, to date, DNA damage assays have not been evaluated in a critical manner and, quite simply, they need to be. We are very hopeful that this will occur and DNA assessment