Clinical aspects of sperm DNA fragmentation
detection and male infertility

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Abstract

Over the past 25 years, various methods have been developed to measure sperm DNA strand breaks in situ. Currently, there are four major tests of sperm DNA fragmentation, including the Comet, Tunel, sperm chromatin structure assay (SCSA) and the acridine orange test (AOT). The Comet assay is a light microscope technique where the sperm cells are mixed with melted agarose and then placed on a glass slide. The cells are lysed and then subjected to horizontal electrophoresis. The Tunel assay, another light microscope technique, transfers labeled nucleotide to the 3'OH group of a broken DNA strand with the use of terminal deoxynucleotidyl transferase. The fluorescence intensity of each scored sperm is determined as a "yes" or "no" for sperm on a light microscope slide or by channels of fluorescent intensity in a flow cytometer. The light microscope-based AOT, uses the metachromatic properties of acridine orange to stain sperm cells. The SCSA treats sperm with low pH to denature DNA at the sites of DNA strand breaks, followed by acridine orange (AO) staining of green for native DNA and red for denatured DNA as measured by flow cytometry (FCM) as well as % sperm with high DNA stainability (HDS: immature sperm with intact DNA related to decreased fertilization rates). The SCSA method has defined a 27–30% DNA fragmentation index (DFI) as the point in which a man is placed into a statistical category of taking a longer time to in vivo pregnancy, intra uterine insemination (IUI) and more routine in vitro fertilization (IVF) cycles or no pregnancy. Current data suggest that intracytoplasmic sperm injection (ICSI) may help overcome the diminished pregnancy prognosis with high DFI over the other ART or natural methods.

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Keywords: Sperm DNA fragmentation techniques; SCSA; Clinical diagnosis and prognosis

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1. Introduction

Although the first manuscript on the in situ detection of sperm DNA fragmentation with automated instrumentation was published 25 years ago, only in the past 5 years or so a surge in papers on sperm DNA fragmentation have appeared from a variety of clinics and laboratories [1–5]. These data provide strong evidence that semen samples containing a statistical threshold of ≥30% sperm DNA fragmentation have a reduced level of pregnancy success.

The testis is characterized by very high rates of cell proliferation and both extensive and unique cell differentiation. Since developing testicular sperm need supportive Sertoli cells, an overabundance of cell proliferation requires that excess germ cells are eliminated by apoptosis. One characteristic of apoptosis in histone containing cells is endonuclease cutting of DNA into discrete sizes; a similar mechanism may be at play in early germ cells but not protamine containing late germ cells. Thus, even though a large amount of data exist to demonstrate the presence of fragmented DNA in mature sperm, there is a variety of thought on whether these DNA breaks represent residual apoptotic cells passing through the seminiferous tubules, or apoptosis occurring in late mature sperm, or DNA fragmentation occurring by another mechanism(s) [6–10].

Many of the reproductively toxic agents studied exhibit cell-type specificity resulting in increased DNA fragmentation in epididymal or ejaculated sperm. Thus, it appears that there are various mechanisms that result in DNA strand breaks in mature sperm. One of the primary DNA damaging agents receiving a lot of research attention is reactive oxygen species (ROS) [6,10].

Whatever the mechanism of sperm DNA strand breaks, the important point is to develop a strategy for clinical management of humans or animals with high levels of sperm DNA fragmentation. Depending on the percentage of sperm with damaged DNA in an ejaculate, a clinician can be guided as to the method for ART fertilization or obtaining sperm from the testis. This paper is a limited overview of techniques used to detect sperm DNA fragmentation with some insights into clinical interpretation of derived data.

2. Methods for assessment of sperm DNA fragmentation

2.1. Acridine orange test (AOT)

The pioneering article on in situ detection of sperm DNA damage using automated instrumentation (flow cytometers) was published in Science in 1980 [1]. In that study, sperm were heated to 100 °C for 5 min to potentially denature DNA followed by staining with the metachromatic dye, acridine orange (AO). AO intercalates into native DNA and fluoresces green when exposed to blue light and fluoresces red when associated with single stranded DNA. Red and green sperm obtained from subfertile humans and bulls could be seen under the fluorescent light microscope and roughly corresponded to the proportion of green and red sperm as measured by flow cytometry. The differential color of the sperm seen on the cover of Science prompted Tejada et al. [11] to develop the light microscope-based acridine orange test (AOT). Although papers continue to be published on clinical
samples using this AO test, we have extensively studied the technique and, at least in our hands, the data simply are not sufficiently reliable for clinical use. The primary reason is that the metachromatic staining by acridine orange requires a very strict equilibrium concentration of the dye, i.e., ~two AO molecules per/DNA phosphate group [12]. Since commercial glass slides and cover slips are not perfectly flat at a molecular level, there are some regions of the slide where flatness occurs, which then forces any ‘excess’ AO from that region and all sperm fluoresce green. Other regions where there are dips or pockets in the slide-cover slip interface, an increased effective AO concentration will cause all sperm to be red.

2.2. *Tunel*

The Tunel assay was first developed for somatic cells and then later adapted for sperm [13]. The essence is to transfer a labeled nucleotide to the 3’OH group of a broken DNA strand with the use of terminal deoxynucleotidyl transferase. The fluorescence intensity of each sperm examined is determined as a “yes” or “no” for sperm on a fluorescence light microscope slide or by channels of fluorescent intensity in a flow cytometer.

The Tunel assay is a very promising technique; however, it has been criticized as having as many variations of the methodology as investigators doing the method [14]. Incorporation of the Tunel assay in the clinical setting shows limited promise until strict quality control between labs is accomplished.

Henkel et al. [6] found routine IVF pregnancy rates significantly reduced with Tunel positive sperm. A cut-off value of 36.5% for the percentage of Tunel-positive spermatozoa was used for the distinction of groups. The mean pregnancy rate for Tunel-negative ejaculates was 34.7% while only 18.7% of the patients became pregnant if the Tunel-positive spermatozoa was >36.5%. In another study, Henkel et al. [7] found that with >36% Tunel-positive sperm the fertilization rate was the same but the pregnancy rate was decreased from 34.7 to 19.1%, \(P = 0.03\) by natural conception and intrauterine insemination (IUI) ART techniques of fertilization.

2.3. *Comet assay*

The Comet assay consists of DNA strand breaks detected in single cells on a microscope slide. The methodology consists of mixing the sperm with melted agarose, which is placed on a glass slide. The cells are lysed and then subjected to horizontal electrophoresis. The high molecular weight, unbroken DNA remains in the sperm head, while smaller broken pieces of DNA migrate out to take on the form of a comet, thus the name.

The original Comet studies on mouse sperm used a very high pH buffer (pH 13) that produced DNA strand breaks at “alkaline sensitive” sites. These sites are not understood and they are presumed not to be equivalent to toxicant induced DNA strand breaks.

IVF and ICSI patients were 1.5× more likely to become pregnant when DNA damage was low as assessed by Comet assay. A trend for increased early abortions was also seen. Three of the nine pregnancies in group 1 (high DNA damage; \(n = 31\)) spontaneously aborted in comparison to group 2 (low DNA damage; \(n = 22\)), where all six pregnancies ended in live births [15].
While the Comet assay may be useful for determining the percentage of sperm with DNA fragmentation, no clinically useful thresholds have been established. The method suffers by definition by being a fluorescent light microscope technique where only 200–300 sperm are analyzed and by the fact that different laboratories often use different methodologies. Yet, within a single laboratory following strict methodology with positive and negative controls, some laboratories successfully use this technique for patient diagnosis and prognosis.

3. Sperm chromatin structure assay (SCSA®)

In our initial flow cytometric study, it was not clear just what we were measuring, so we referred to the metachromatic shift from green to red fluorescence as alterations of chromatin structure which included the possibility of DNA fragmentation, proteins alterations allowing or causing DNA denaturation, or a combination of both. Unfortunately, even today, authors speak of the SCSA as only showing alterations in “chromatin structure”; however, SCSA data are now seen as much of an indicator of DNA strand breaks as the Tunel or Comet assay.

Later studies in 1983, showed that whole sperm and isolated and purified sperm nuclei provided the same data thus eliminating any thought that the red fluorescence was derived from cytoplasmic RNA. In that study, it was also shown that heating the sperm or treating the sperm with an acid detergent buffer resulted in the same data. This improvement constituted the low pH method as the basis for the current SCSA methodology [16]. The latest detailed methodology and terminology on the SCSA was described in Evenson et al. [17].

SCSA data resolve four different cell populations: % sperm without DNA fragmentation, % moderate DNA fragmentation index (DFI), % high DFI and % high DNA stainability index; percentage of immature sperm (HDS) (Fig. 1). Of great interest, the SCSA® soft software has another parameter that has shown a higher correlation with infertility than % DFI. This factor is the standard deviation (S.D.) of the DFI (red) + (red/ red + green) fluorescence), which measures the extent of sperm DNA fragmentation. Data from bull fertility trials and mouse toxicology studies show the S.D. of DFI was 20–30% more sensitive than the % DFI [18–20]. Preliminary results indicate that the S.D. of DFI is at least 10% more sensitive than % DFI alone in human infertility studies. This interesting parameter is now being studied for application into SCSA defined risk of human infertility.

3.1. SCSA for livestock sperm

Using the terminology of Saacke et al. [21], we had a great interest in what was termed compensable and uncompensable semen quality traits. For example, sperm numbers used for AI can be considered a compensable trait, i.e., increased sperm numbers can be added to produce a higher pregnancy rate. However, sperm DNA fragmentation is considered a non-compensable trait since it is the percent of sperm with fragmented DNA being considered and no matter how many sperm are added, the percent sperm with fragmented DNA
remains the same; thus, the probability is the same for decreased pregnancy outcome due to this factor alone.

Perhaps the best experiments to determine what semen quality traits are most important for pregnancy outcome are heterospermic matings. In such a comparison, equal numbers of motile sperm and seminal fluid are mixed from phenotypically different males and then the mixture is inseminated into a number of females. As an example, if a hundred cows were inseminated with mixed sperm from a black bull and a white bull and 80 black and 20 white calves were born, then the heterospermic data would show that the black bull had superior sperm.

3.2. Heterospermic examination of bovine sperm and DFI

Correlations with mean of % DFI (red/red + green fluorescence), S.D. of DFI and % DFI with competitive index for dairy bulls were $-0.81$, $-0.94$ ($P < 0.01$) and $-0.74$ ($P < 0.05$), respectively. The multiple correlation ($R^2$) of the competitive index with motility estimate and S.D. $\alpha_t$ (describes the extent of chromatin structure abnormality within the sperm cell population) was 0.96 ($P < 0.01$) [19].
3.3. Heterospermic examination of porcine sperm and DFI

In a heterospermic trial, the SCSA identified the three highest and three lowest boars for fertility. Semen from six boars was mixed in equal sperm numbers in six three-way combinations and inseminated in at least three gilts per combination. Four of the six combinations yielded two litters, while the remaining two combinations yielded three litters. Boars siring more piglets had significantly lower mean of DFI ($P < 0.05$), % DFI ($P < 0.05$) and S.D. of DFI ($P < 0.01$) than the less fertile boars [22]. An inverse relationship was noted in farrowing rate and number of pigs born/litter in regards to % DFI values [21].

3.4. Relationship of SCSA to the natural fertility of bulls and boars

Bull and boar sperm DNA integrity was evaluated by Rybar et al. [23] using a $k$-means cluster test. Boars and bulls were divided into two groups according to the levels of mean percentages of spermatozoa with DNA fragmentation index (DFI). Results showed five boars had a significantly higher DFI at 12.4% in comparison to the other 63 boars which had a 4.9% DFI. Bulls in group 1 ($n = 31$) had a DFI of 4% in comparison to group 2 ($n = 6$) which had a DFI of 14.2% ($P < 0.0001$). Bulls with a 43.3% pregnancy rate had significantly higher % high DFI and % HDS than those with a 60.0% pregnancy rate ($P < 0.01$). Note that the threshold for sub fertility is much lower than the 30% DFI for humans. For boars it is in the range of about 8% DFI and for bulls in the 10–20% range. It is interesting how such low numbers can make such a large influence in the pregnancy rate and litter size for pigs. We have described this before as the “tip of the iceberg” effect; i.e., DNA damage likely exists in the sperm that are below the threshold of detection. However, when the percent detected reaches a threshold of >30% DFI, this places that male into a statistical “fair to poor” category of reduced pregnancy outcome (see Figs. 2 and 3 for men with excellent and fair-to-poor sperm DNA integrity).

3.5. Relationship of SCSA and clinical fertility data of humans

The DFI threshold for humans was first established using data from 200 presumed fertile couples attempting to conceive naturally in the “Georgetown Male Factor Infertility Study”. Fertility data from this study were used to establish the statistical thresholds of >30% DFI for ‘significant lack of’, 15–30% DFI for ‘reasonable’ and <15% DFI for ‘high’ fertility status [2]. SCSA data obtained by Spano et al. [5] on 215 first pregnancy Danish couples showed an odds ratio of $10 \times$ greater probability of pregnancy via natural intercourse if the DFI values were <40%. Bungum et al. [3] found that IUI patients were $8.7 \times$ more likely to deliver a baby with an SCSA defined DFI $\leq 27\%$ ($P = 0.01$); these data have been recently confirmed by this same group with a larger data set [24].

While the predictivity of the SCSA for in vivo and IUI fertilizations are clear for reduced pregnancy odds, the results from IVF and ICSI are less so. Larson et al. [25] showed no ICSI pregnancies with % DFI >27% while Bungum et al. [3] found the results of ICSI were significantly better than those of routine IVF in the >27% group. Gandini et al. [26] found no differences in SCSA parameter values between patients initiating pregnancies or not
doing so in either ICSI or routine IVF. Possible reasons for the lack of clarity in these reports are the small number of patients in many studies, lack of distinction between routine IVF and ICSI, length of time between SCSA testing and pregnancy attempts and, importantly, the success rate of different ART clinics.

Fig. 2. Clinical results of the SCSA. Histogram shown is an example of the DNA Fragmentation Index for sperm from an individual human male for which the outcome resulted in a pregnancy. S.D. of DFI = standard deviation of DFI, high DNA stainability = HDS, DNA fragmentation index = DFI.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Date</th>
<th>Measurement</th>
<th>mean DFI</th>
<th>SD DFI</th>
<th>DFI (%)</th>
<th>HDS (%)</th>
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<tbody>
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<td>8.3</td>
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<td>5.4</td>
<td>4.6</td>
<td>1.1</td>
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</tr>
</tbody>
</table>

Fig. 3. Clinical results of the SCSA. Histogram shown is an example of the DNA Fragmentation Index for sperm from an individual human male for which the outcome did not result in a pregnancy. S.D. of DFI = standard deviation of DFI, high DNA stainability = HDS, DNA fragmentation index = DFI.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Date</th>
<th>Measurement</th>
<th>mean DFI</th>
<th>SD DFI</th>
<th>Total DFI (%)</th>
<th>HDS (%)</th>
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<td></td>
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<td>sd</td>
<td>1.2</td>
<td>1.1</td>
<td>0.0</td>
<td>0.4</td>
</tr>
</tbody>
</table>
Current research indicates a trend in increased human spontaneous abortions when the DFI is \( \geq 30\% \). A \( \geq 30\% \) DFI score was associated with increased miscarriage rates [26] and a higher rate of spontaneous abortions at 12 weeks of gestation (\( P < 0.01 \)) in comparison to the \(<30\% \) group (4). Data from Evenson et al. [2] suggested that 39\% of miscarriages were related to a DFI >30\%. Sanchez et al. [28] reported a 31\% abortion rate when spermatozoa from cryptozoospermia (\(<1 \text{ million sperm/cc} \)) or severe OAT (oligo-astheno-teratozoospermia) syndrome patients were used for ICSI. The high abortion rate in this study possibly reflects genetically compromised sperm.

3.6. Apoptosis and oxidative damage to sperm

DNA fragmentation can be attributed to various pathological conditions including cryptorchidism, cancer, varicocele, fever, age, infection, and leukocytospermia among others. Many environmental conditions can also affect DNA fragmentation such as chemotherapy, radiation, prescribed medicine, air pollution, smoking, pesticides, chemicals, heat and ART prep protocols. Current thoughts suggest that DNA fragmentation is due to an apoptotic events or to reactive oxygen species (ROS); however, Henkel et al. [7] showed no correlation with fertilization rates, pregnancy, DNA integrity or DNA fragmentation using early markers of apoptosis (Annexin V binding and Fas expression). Given, the clear relationship of high DNA fragmentation and lower pregnancy rates it seems plausible that DNA damage may be due to ROS.

Antioxidant therapy has shown promise in decreasing sperm DNA fragmentation. After supplementation with an antioxidant, male factor patients significantly decreased their \% DFI [29].

Seminal plasma and spermatozoa contain several antioxidant mechanisms that help counteract the harsh effects of ROS activity. Exposure to episodic air pollution in the Czech Republic has been associated with poor semen quality and sperm DNA damage [30]. The SCSA was the only measure to detect a correlation between air pollution and semen quality in 18-year-old army conscripts from Teplice, Czech Republic. Teplice had a history of infertility and spontaneous abortions hypothetically related to the fact that it was a valley town that burned soft brown coal for energy and suffered from winter smog inversions. One-fifth of the 18-year-old men had DFI values of more than 30\% [31]. Harmful metabolites from polycyclic aromatic hydrocarbons in polluted air are detoxified by glutathione s-transferase. In a follow-up study, the authors hypothesized that men who lacked the gene for the enzyme glutathione s-transferase would be more susceptible to the effects of air pollution resulting in sperm DNA damage. While DFI was associated with both genotype and exposure, the interaction between genotype and exposure was even more significant.

Sperm DNA fragmentation has been evaluated in men who were occupationally exposed to a mixture of organophosphorus pesticides. Nearly three-fourths of the men had DFI values \( >30\% \) [32]. Sperm DNA was also a sensitive target in smokers. Semen from 35 non-smokers and 35 smokers was analyzed by SCSA. The smoking group possessed higher levels of DNA strand breaks compared to non-smokers (\( P < 0.05 \)) [33].
4. Results and discussion

4.1. Meta-analysis of SCSA human infertility studies

To investigate the relationship between pregnancy rate and sperm DNA fragmentation, a meta-analysis on SCSA infertility studies has been performed. A meta-analysis of four studies \((n = 1962)\) was conducted to investigate the relationship of sperm DNA fragmentation on pregnancy outcome using in vivo and IUI procedures \([2,3,5,24]\). Using the Cochran–Mantel–Haenszel (CMH) statistic, the meta-analysis indicated that patients were \(7.1 \times \) (C.I. 3.37, 14.91) more likely to achieve a pregnancy/delivery if the DNA fragmentation index (DFI) <30\% \((P = 0.0001)\). The Breslow-Day test (BDT) was done to test the homogeneity of all the odds ratios for in vivo and IUI procedures used in the meta-analysis. The odds ratio for all studies tested was not significantly different \((P = 0.99)\) and showed similar trends.

When routine IVF was considered, couples were \(\sim 2.0 \times \) (C.I. 1.10, 2.96) more likely to become pregnant if their DFI was \(< 30\% \) \((n = 375, P = 0.02)\) \([3,7,34,35]\). The odds ratio for all studies tested was not significantly different \((\chi^2 = 5.11, P = 0.16)\) and showed similar trends.

A meta-analysis of six studies using ICSI and/or routine IVF fertilization was also done to investigate the relationship of sperm DNA fragmentation on pregnancy outcomes \([3,15,25,27,34,36]\). The meta-analysis (CMH) indicated a trend where patients were \(1.8 \times \) (C.I. 0.97, 3.14) more likely to achieve a pregnancy/delivery if the DFI was \(< 30\% \) \((n = 322, P = 0.06)\). The BDT showed that the odds ratio for all studies tested were not significantly different \((\chi^2 = 10.50, P = 0.06)\) and showed similar trends. The above meta-analysis shows that the SCSA infertility test is significantly predictive for reduced pregnancy success using in vivo, IUI, and routine IVF and to a lesser extent ICSI fertilization.

The above meta-analyses show that the SCSA infertility test is significantly predictive for reduced pregnancy success using in vivo, IUI and to a lesser extent routine IVF and ICSI. These data clearly indicate that the SCSA is an important component of the infertility workup and suggest that if a man has a DFI of \(> 30\% \) that IUI should probably not be considered and that the couple move to routine IVF or ICSI.

An alternative fertility treatment for infertility patients might be the use of testicular sperm. Greco et al. \([37]\) found successful treatment of infertility due to sperm DNA damage using testicular sperm. Couples \((n = 18)\) with sperm DNA fragmentation \(\geq 15\% \) Tunel positive in the ejaculate underwent an unsuccessful ICSI attempt. In all of these men a second ICSI attempt was performed using testicular sperm. The percentage of sperm DNA fragmentation in the testicular sample was \(< 6\% \) with the exception of one patient (18\%). PG rate using the testicular sperm was 44.4\% versus 5.6\% in the ejaculate \((P < 0.05)\). ICSI with ejaculated sperm resulted in one pregnancy, which was spontaneously aborted. Duran et al. \([38]\) found no pregnancies initiated with \(> 12\% \) Tunel positive of sperm with fragmented DNA was used for insemination. Host et al. \([39]\) found sperm DNA breaks to be more predictive of IVF than ICSI procedures. Furthermore, elevated sperm DNA strand breaks for the unexplained infertile couples were associated with a decreased pregnancy rate. Repeat pregnancy loss patients defined as \(\geq 3\) losses were \(2 \times \) and \(4 \times \) more likely to
miscarry in comparison to the general population (22% of sperm staining positive for sperm DNA fragmentation) and fertile donors (11% of sperm staining positive for sperm DNA fragmentation), respectively [40]. Sun et al. [41] found that men (n = 35) who smoked had a significant increase in sperm DNA fragmentation as compared to non-smokers (P < 0.01).

4.2. Overview on the use of the various DNA fragmentation tests

The SCSA can offer couples an effective, highly reproducible diagnostic and prognostic tool to evaluate male factor infertility. In contrast, interlab measurements using fluorescent light microscope assays such as the Comet do not appear to be consistent [42–44]. The same appears to be true for the Tunel assay [7,14,38]. Recent articles suggest the same, i.e., Duran et al. [38] suggest from their data that no pregnancies occur when more than 12% of sperm are Tunel positive while other manuscripts [6,7] suggest a threshold of about 36% for a 40% reduction rate in pregnancy rate compared to those samples with less than 36% positive sperm. More than a hundred thousand animal and human sperm samples have been measured by the SCSA. The 30% threshold for humans has been corroborated in recent research in regards to in vivo and IUI procedures. Studies from Evenson et al. [2], Bungum et al. [3] and Spano et al. [5] show patients were 6.5–10× more likely to achieve a pregnancy when their DFI was <30%. When routine IVF data were considered, patients were 2–9.5× more likely to achieve a pregnancy when their DFI was <30%.

In addition to the % DFI, the SCSA analysis uniquely measures HDS. The HDS population has less chromatin condensation, which leads to increased DNA stainability. Increased HDS values correlated with reduced fertilization rates and a longer time to pregnancy in in vivo, IUI and routine IVF data [2–4] whereas HDS in ICSI showed no correlations to fertilization or pregnancy [3,26].

5. Conclusions

The SCSA analysis is a precise, timely and cost-effective diagnostic tool to help manage a couple’s infertility treatment, whereas the Comet and Tunel assays currently lack the consistency and reliability needed in a clinical setting.

Patients presenting with the following should have a DNA fragmentation test:

- all idiopathic couples;
- men older than 40 years, even if prior fertility;
- men with known exposures to toxicants;
- men with above mentioned pathologies; and
- all couples presenting for infertility.

In the words of Dr. Larry Lipshultz, a well recognized urologist who is at the forefront of modern urological practice at Baylor Medical College, “I perform DNA fragmentation testing for all my patients who are undergoing IVF with ICSI so that I can give them a
general idea of how successful their cycles might be. If the tests show greater than 26% DNA fragmentation, chances for poor embryo development increase.” [45].

For those couples who are just beginning infertility investigations, the SCSA is a powerful diagnostic tool to better understand the male partner fertility potential, perhaps lessening the emotional and financial cost of potentially non-effective treatments.

References


