# Human Sperm Nuclear DNA Fragmentation Assays and Their Values in Assisted Conception

(Values of Sperm Nuclear DNA Testing in Assisted Reproduction)

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Slide 2

In this presentation, I will briefly discuss the structure of DNA as it related to the tests developed to assess its structural integrity. Having a refreshed memory about this topic will provide a better understanding of the tests of DNA status developed for spermatozoa. Emphasizing on sperm, I also briefly discuss the changes in DNA responsible for facilitating its attachment to the nuclear matrix and inclusion within the nucleus. "Each of us has enough DNA to reach from here to the sun and back, more than 300 times. How is all of that DNA packaged so tightly into chromosomes and squeezed into a tiny nucleus?"
**Annunziato, A. (2008) DNA packaging: Nucleosomes and chromatin. *Nature Education* 1(1):26**

Next, I'll discuss the nature of sperm nuclear damage and the possible causes or reasons for this damage. Later, a discussion of the tests currently utilized to assess the integrity of sperm nuclear DNA followed by a brief discussion about the influence of sperm nuclear damage on reproduction.

Slide 3

I'll later discuss our experience with evaluations carried out to assess sperm nuclear damage and its impact on reproduction. Undoubtedly, any study that explores and finds an association between sperm nuclear DNA and any semen parameter will be considered an important investigation. I'll briefly touch on this subject as well followed by brief case presentations of the sperm nuclear DNA fragmentation results from 2 men who were IVF patients at our clinic. We conclude with some thoughts about the utility of DNA fragmentation assays and the current ASRM recommendation about these evaluations.

Slide 4

I briefly refresh your memory about the structure of DNA as it is important to remember the principles behind the assays developed to assess the status of nuclear DNA in human sperm as it related to reproduction. Accordingly, having a basic knowledge about these assays will help us putting into perspective the significance or shortcomings of the published studies.

The DNA molecule is made of 2 strands (double helix) kept together by in the interaction between corresponding bases of the 2 strands. The backbone (a strand) is made of pentose sugar molecules, deoxyribose, attached to the phosphate groups at their carbon 3 and 5. Also attached to the pentose are purine and pyrimidine bases which help linkage of the two strands forming a double helix. While we are discussing the bases, it is appropriate to note the interaction of certain compounds with these bases has been used in some of the diagnostic tests developed to assess the structural integrity of DNA. Certain dyes such as acridine orange can bind to these bases under different circumstances. If the molecule of DNA and the bases

are intact and binding to each other normally, the acridine orange can only intercalate as a monomer between the bases of DNA (meaning insert itself between the bases without any strong bonding between the dye and the bases). This intercalation in the form of a monomer, provides a special optic characteristic different from when the dye binds to the bases which have been separated from each other due to the damage to the DNA molecule/strands. With intercalation, the dye will emit green fluorescence (sperm look green) at 530 nM, when the DNA and the dye mixture are exposed to the light. However, if the linkages between the bases are broken/damaged and the DNA is not an intact double stranded structure, the acridine orange binds to the bases (and to the damaged DNA) as aggregates rather than intercalating as a monomer between the bases. In this case, the exposure of the mixture of DNA and acridine orange to light results in emission of red fluorescence (occasionally orange-red) at higher wavelength of 630-670 nM. This principle has been utilized in sperm chromatin structure stability assay (SCSA) developed by Evenson.

While we are discussing the DNA structure, we also need to note that links between the sugar and the phosphate groups often are damaged by various factors leading to the fragmentation of DNA. Damage can be on one of the strands (single-stranded) or on both strands (double-stranded). This type of damage often occurs at the phosphodiester linkage between carbon 3 of the deoxyribose and the phosphate group attached to carbon 5 of the adjacent deoxyribose (the 5 carbons of deoxyribose are numbered beginning from where the deoxyribose binds the bases, carbon 1, and ends with the –CH2- as carbon 5). When carbon 3 is unattached due to the damage to the phosphodiester bond(s) or when it is at the end of the DNA molecular, it is called the 3' end of the DNA molecule. An intact DNA molecule has two 3' and two 5' ends. However, damaged DNA molecules may have many 3' and corresponding 5' ends depending on the extent of the damage. Determination of these 3' ends is the principle behind another test for DNA fragmentation called the TUNEL assay which will be discussed shortly.

As is, the DNA molecule is huge in length. The nucleus of each cell holds nearly 1.8 meters (or 6 feet) of DNA. Without any further changes in the DNA molecule with the high number of negative charges by the phosphate how DNA can fir in the nucleus? The minor grooves in DNA play a role in this fitting. There are highly positive proteins called protamines which bind to the minor grooves neutralizing these charges allowing the DNA to become compact. We will briefly discuss this as well as the process may have something with how easily DNA can be denatured under certain conditions, a basis for several DNA fragmentation tests such as SCSA performed under acidic conditions and the Comet assay conducted at basic pH.

Determination of these breaking points have been utilized by some tests to determine the status of DNA and the degree of the damage. There are some dyes such as acridine orange that can bind to the DNA. If DNA is intact, the dye can intercalate between the bases and in this

case it emits light at a specific wavelength (530, green). However, if the strands of DNA have been separated (denatured DNA), the same dye can bind to the unpaired bases and emit light at a higher wavelength (600 nm) and emit red light. Therefore, this dye can be used to distinguish between an intact DNA molecule (intercalation of the dye and emission of green light) and a damaged.

Slide 5

This slide compares the DNA packaging in somatic cells versus what is believed to be the packaging in sperm. Remember that the DNA packaging in somatic cells and in germ cells up to spermatids are the same (drawings to the left). In somatic and early germ cells, DNA molecules wrap around octameric cores of proteins called histones (bases) to form nucleosomes with a diameter of approximately 11 nm. These wrappings (nucleosome formations) are repeated every 200 nucleotides. Because of these wrappings and configuration, DNA can fit within the nucleus as an integral part of chromosomes.

The main types of histones in somatic cells are named H2A, H2B, H3 and H4. Treatment of DNA formed as such with a high salt medium dissociates the bases (histones) from the DNA double helix. During late stages of germ cell development, (spermtids) nearly 85% of histones are replaced with more basic protamines. Protamines which are rich in arginine and cysteine provide two important properties to the DNA to which they are bound. Arginine molecules with abundant –NH2+ groups provided much needed positive charge to the DNA molecules to enable them to become more compact (neutralize the negative charges of the phosphate groups on the DNA molecules enabling the molecules to come closer to each other and become more compact). Cysteine molecules with their sulfhydryl groups (-SH) enable the DNA molecules to bridge (disulfide bond, -S-S-) rendering them more stable.

Please note that the DNA forms loops and the loops extend beyond the nuclear core/matrix. For this reason, when sperm chromatin dispersion test is performed (see later for the description of the test), if we see the halos are formed (meaning that strands of DNA are intact and expand beyond the borders of nucleus), it means that the nuclear DNA is intact as well. If the nucleus appears less fuzzy with more defined dimensions, then the DNA loops are not present (broken) and the cells is considered to have damaged nuclear DNA (see end of the cartoon/slide showing the loops). The results of this test look completely opposite to what we see in the comet assay where cells with damaged nuclear DNA form a dispersed (comet) type of pattern. Make sure to be able to distinguish cells with intact or with damaged nuclear DNA in “sperm chromatin dispersion assay” versus the “comet assay.” As noted, in one assay (sperm chromatin dispersion, SCD) the cells with fuzzy boundaries have intact nuclear DNA whereas in the other assay (the comet assay) cells with fuzzy boundaries have damaged nuclear DNA.

No notes for slides 6-10

Slide 11

During spermiogenesis, spermatid chromatin undergoes substantial compaction. Testis-specific nuclear proteins, the transition proteins and protamines, are responsible for this chromatin condensation [1-5]. The first step in this process occurs in haploid round spermatids and involves replacement of somatic histones with the transition proteins (TP1 and TP2). Subsequently, in elongating spermatids, the protamines (P1 and P2) replace TP1 and TP2. The resulting chromatin is highly condensed and transcriptionally silent. There is evidence that the P1:P2 ratio is more important for male fertility than the absolute amount of protamines [58, 76, 78]. A recent report found P2 precursors to be present in the sperm of infertile males who had reduction in P2 levels [79]. Presumably, this indicates incomplete processing of the P2 protein (V Aoki, et al, Asian J Androl, 2003). The importance of the P1/P2 ratio in spermatogenesis has recently been emphasized by a study, which showed that haplo-insufficiency of P1/P2 causes severe infertility in mice [80]. In addition, the haplo-insufficiency of P2 has recently been reported to lead to sperm DNA damage and embryo death in mice [81]. Consistent with this data, Yebra *et al* reported the complete loss of P2 protein in a small proportion of infertile males [82]. Carrell and Liu later found that P2 was undetectable in 13 of 75 severely infertile patients analyzed prior to *in vitro* fertilization. Conversely, P2 was seen in all 50 donors of known fertility analyzed [27]. That study also showed that low P2 levels were generally associated with low sperm counts, motility and morphology. Additionally, Mengual *et al* recently reported marked increases in the P1/P2 ratios of oligozoospermic and asthenozoospermic patients as compared with fertile control patients [83]. Taken together these studies may indicate that the abnormal protamine content in sperm is a reflection of abnormal transcriptional or translational regulation of P1/P2 expression (V Aoki, et al, Asian J Androl, 2003). Functionally, it appears as if the protamines are required to impart zona pelucida binding and penetration abilities. This was exemplified by a study that showed destruction of the protamines inhibited sperm binding and penetration in the hamster egg penetration test [84]. Does this mean that low protamines may lead to DNA fragmentation and inability to fertilize the egg under natural conditions (natural conception?). Recent reports have shown that abnormal P2 is associated with diminished fertilization ability [85]. However, it does not appear as if normal protamine replacement is required for pronuclear formation, because ICSI with round spermatids has been shown to produce chromatin decondensation and pronucleus formation [84]. Additionally, patients without P2 have good success with ICSI during IVF cycles [85]. Furthermore, it has been demonstrated that destruction of the protamines actually increases sperm decondensation [84-85]. Taken together, these studies demonstrate that protamines are important components of spermatid differentiation and aberrations in the

protamines are related to infertility and may reflect defects in spermiogenesis (V Aoki, et al, Asian J Androl, 2003).

This slide shows nuclear and cytoplasmic characteristics of various germ cells of spermatogenic lineage. Take a look at the volume of nucleus in early germ cells compared to spermatids. Although the volume of nucleus to the cell volume is high in spermatids and in sperm, the overall volume of nucleus in early germ cells is much larger. This is due to the DNA packaging that is more bulky than it is in spermatids/sperm. Note that the largest germ cells are of various types of primary spermatocyte.

No notes for slides 12-17

Slide 18

DNA damage can occur in various parts of the molecule and by various mechanisms. However, the most easily recognizable damage is when there is a breakage at the phosphodiester bond (i.e., hydroloytic attack) either on a single strand or across the molecule in the form of double-strand breaks.

No notes for slide 19

Slide 20

A variety of methods have been developed to assess the integrity of human sperm nuclear DNA. Each method has its own advantages as well as some technical and interpretive disadvantages. Sperm chromatin structure assay, one of the most widely utilized methods developed by Ronald Evenson, has the advantage of being rapid and able to assess the DNA status in a large number of sperm using flow cytometry. The assay appears to have a good reproducibility although a high number of sperm are needed to achieve a good level of precision. The sensitivity of the assay (the percentage of affected people who are correctly identified as having significantly high level of sperm nuclear DNA problems in their semen) also appears to be acceptable. The DNA fragmentation index and DNA high stainability parameters reported by the assay are unique. The assay has been shown to correlate with other frequently performed assays such as TUNEL, Comet and Sperm Chromatic Dispersion (Halosperm). It has also been utilized in environmental studies. However, the routine use of the assay has been hampered by the high cost equipment (flow cytometer) and the technical issues related to the flow cytometry techniques (i.e., gate settings and the requirement to have a reference sample to calibrate the system). SCSA does not represent a distinct and unique physiological process The assay requires a high number of sperm in order to achieve an acceptable degree of precision. As a result, the values reported for samples with very few sperm should be interpreted with caution. The technique may lead to formation of partially stained sperm

(orange in color rather than red) and inclusion or exclusion of these sperm from consideration introduces uncertainty and reduces the objectivity of the assay results. The interpretation of the results reported by SCSA needs a good level of expertise. In addition, the fragmentation index reported by the assay is an "indirect evaluation of the actual fragmentation of the DNA" Evengi E et al, 2014.

The TUNEL assay has extensively been used simply because the technique utilizes fluorescence microscopy and is useful for assessing a small number of spermatozoa particularly in samples such as testicular biopsies and does not require reference samples for parallel running. The assay detects both single- and double-stranded DNA fragmentations. Despite these advantages, the assay requires 4-6 hours to complete and can have a high level of intra-assay, intra-laboratory and inter-testing personnel variabilities. These have lead to variable thresholds suggested for the TUNEL assay. The TUNEL assay may not be suitable for detection of immature spermatozoa detectable by SCSA via its high DNA stainability reporting. In addition, it has been reported that TUNEL may underestimate the degree of DNA fragmentation because the technique may not expose the inner core of DNA (DNA compaction), thus, not revealing the extent of damage (L. A. Mitchell et al, Int J Anrol, 2012). The authors proposed a revised version of the TUNEL assay by exposing the samples to 2 mm dithiothreitol (DTT) for 45 min prior to the fixation step. DTT is a reducing agent and can open up the chromatin structure, thus, allowing the terminal transferase to reach the inner core damages and react with the DNA nicks. This revision resulted in enhanced detection capability of the TUNEL assay.

The comet assay is carried out in either neutral or alkaline pHs. At neutral pH, it mainly detects double stranded DNA damage while the test carried out at alkaline pH detects both single- and double stranded DNA damages, thus making it a more sensitive assay. Using special software, attempts have been made to make the assay quantify the DNA damage. However, the results have not been consistent. Some studies have shown that it correlates with the TUNEL and SCSA results. The test, however, is time consuming, difficult to standardize, requires extensive experience, and special equipment and software. The assay conducted under neutral condition may underestimate the DNA damage as it mainly detects the double-stranded breaks. On the other hand, the assay conducted under alkaline pH may overestimate the DNA breaks simply due to the treatment of DNA under harsh alkaline conditions which may induce breaks.

The sperm chromatin dispersion assay has been marketed in the form of a kit (i.e., Halosperm) and the technique is fairly simple and quick to complete. Similar to SCSA, the test can utilize neat semen, motile sperm fraction or cryopreserved sperm. However, the assay requires extensive training in order to avoid inter-technician subjectivity.

Acridine orange has been used either alone or as a part of other methods (i.e., SCSA) to assess the integrity of DNA (degree of denaturation). One of the issues with the use of acridine orange

is the presence of so called moribund DNA (sperm appearing in orange/yellow color) making it difficult to assess whether they have intact DNA (green) or are DNA damaged (red).

Acidic aniline blue (ABB), is a dye that stains the lysines of histones and can be sued to assess the residual histones attached to nuclear DNA of sperm.

Iodoacetamide in the form of fluorescein fluorescence (IAF) covalently binds to the sulfhydryl groups of protamines. It therefore, can assess the degree of DNA maturity. DNA molecules with a significant level of free –SH groups are prone to denaturation compared to the ones with –S-S- bonds.

Slide 21

In Toluidine staining, the integrity of sperm chromatin is assessed. The assay determines the interaction between DNA and proteins and is suitable for cases when there are only a small number of sperm available. Bright field microscopy can be utilized to read the results. However, similar to other assays the efficacy of the staining and the inter-laboratory variability can limit the utility of the assay.

Chromomycin A3 has also been used to assess the maturity of the chromatin by determining the level and the ratio of protamine 1 and 2 associated with the DNA.

No notes for slides 22-34

Slide 35

It must be noted that SCSA has been performed by many investigators with limited knowledge of the intricacies of the technique. This has certainly contributed to the variations reported in the literature. Performed by a competent and knowledgeable laboratory, the technique can provide valuable information about the status of human sperm nuclear DNA.

No notes for slides 36

Slide 37

Double-stranded DNA damage: Breakage in the sugar-phosphate backbone of DNA across both strands adjacently

Single-stranded DNA damage: Breakage in the sugar-phosphate backbone of a single strand of DNA

The comet assay can be performed at neutral pH or in an highly alkaline pH (sperm treated with a strong base).

To conform with the normal physiological pH, comet assays performed at neutral pH are better than those performed at highly basic pH conditions. However, at neutral pH, comet reveals double stranded (ds) DNA damage only (pieces of double stranded DNA). This is because nothing is used to destabilize the DNA to convert it to single strands.

At alkaline pH, comet detects ds and ss DNA. Why? Because not only you are detecting already existing ds DNA breaks, but with harsh treatments you'll also be able to detect unstable DNA molecules converted (denatured) to ss DNA. With neutral pH, there is no harsh treatment, therefore, whatever exist, which are mainly ds DNA breaks (DNA fragments), are determined.

Summary: Neutral pH: ds DNA damage; Basic pH: ds and ss DNA damage. The assay also is capable of measuring the magnitude of DNA damage. However, the assay has not been standardized so techniques are different from lab to lab and the results cannot be compared due to this lack of standardization.

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Sperm chromatin dispersion assay is based on the removal of the protamines in order to expose the DNA loops. If the DNA is intact, the exposed loops given an appearance of fuzziness (halo) to the sperm head. However, if the DNA has been damaged/fragmented the loops are not present and the nucleus (sperm heads) do not appear fuzzy.

No Notes for slides 39-47

Slide 45

A technique with good specificity and sensitivity is an excellent method for assessing the status of human sperm nuclear DNA. However, methods often have a better sensitivity or specificity at the expense of the other. In general, methods with good sensitivity (above 80%, and better if they are above 90%) have more reliable abnormal results. This means that if the results are abnormal, they have a better predictive value compared to the time when the results are reported to be normal utilizing the technique with high sensitivity. The opposite is true for the methods with high specificity. Looking at the table 2, in the hands of these investigators, the best method for normal and abnormal results is alkaline Comet assay. The neutral Comet is predictive when it gives abnormal results. Look at the other tests and make a judgment about their positive and negative predictive values (recognizing that we need to know the prevalence in order to be able to find truly about positive and negative predictive values).

No notes for slides 46 and 47

Slide 48

A good number of studies on sperm nuclear DNA damage and the outcome of various assisted reproductive techniques have been published. The conclusions of the studies, however, have not been consistent. A review of these studies reveal several issues:

1. Different methods of assessing DNA damage were used
2. Even if the same method (i.e., TUNEL) was used, the methodologies were not exactly the same
3. Some used neat semen, some cryopreserved semen or sperm and some utilized motile sperm, although for some assays the difference between neat semen, and motile sperm has been shown to be insignificant.
4. Different methods of ART were implemented
5. Importantly, the assay was carried out investigators not completely familiar with the technique utilized (i.e., SCSA)

Most importantly, the contribution (or lack of contribution) by the oocyte in repairing the sperm nuclear DNA damage was not investigated or taken into consideration. In many of these studies, the quality of oocyte and the age of the woman in each individual case were not investigated. To highlight this issue, Tesarik (2004) reported that the oocytes from young donors enabled the establishment of a term pregnancy with higher percentages of DNA-fragmented spermatozoa (TUNEL) as compared with attempts performed with oocytes coming from the significantly older patient wives population. Tesarik also reported that sperm DNA fragmentation may have different clinical significance in couples with a history of previous failures of assisted reproduction treatment as compared with couples without such a history.

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Bungum et al, 2004, evaluated a total of 306 consecutive couples undergoing various methods of assisted conception. One hundred thirty one (131) patients underwent IUIs, 109 used IVF and 66 utilized ICSI. “For IUI, the chance of pregnancy/delivery was significantly higher in the group with DFI <or=27% and HDS <or=10% than in patients with DFI >27% or HDS >10%. The odds ratios (ORs) (95% confidence intervals) were 20 (2.3-117), 16 (1.9-137) and 14 (1.6-110) for biochemical and clinical pregnancies and deliveries, respectively. No statistical difference between the outcomes of IVF versus ICSI was observed in the group with DFI <or=27%. In the DFI >27% group, however, the results of ICSI were significantly better than those of IVF. Comparing ICSI with IVF, the OR (95% CI) for biochemical was 26 (1.9-350).” The authors

conclude that SCSA was a useful method for prediction of the outcome of assisted reproduction.

Gosalvez et al study (Asian J Androl, 2013:

This study compared sperm DNA fragmentation (SDF) in neat semen samples and the swim-up (SU) from the same samples. The aim was to predict the pregnancy outcome following ICSI of swim up sperm from these samples using donor oocytes. A total of 81 infertile women were included in this study. The women (24-35 years of age) were transferred embryos resulting from intracytoplasmic sperm injection (ICSI) of spermatozoa from their partner (age 26-52 years) and proven fertile donor oocytes. The authors state that "This model normalized the impact of female factor in putative sperm DNA repair. Semen was blindly assessed for SDF using Halosperm immediately following ejaculation (NS) and after swim-up at the time of ICSI fertilization. There was a decrease in SDF values of the ejaculated semen sample following the swim-up protocol (P=000). Interestingly, pregnancy could be equally predicted from SDF values derived from either neat or swim-up semen samples. Receiver operator curves and the derived Youden's indices determined SDF cutoff values for NS and SU of 24.8% and 17.5%, respectively. Prediction of pregnancy from NS SDF had a sensitivity of 75% and a specificity of 69%, whereas for SU SDF was 78% and 73%, respectively. While increased levels of SDF negatively impact reproductive outcome, we have shown that a reduction in SDF following sperm selection using ICSI with proven donor oocytes is not mandatory for achieving pregnancy. This suggests that a certain level of DNA damage that is not detectable using current technologies could be impacting on the relative success of assisted reproductive technology (ART) procedures. Consequently, we propose a modification of the so called 'iceberg model' as a possible rationale for understanding the role of SDF in reproductive outcome."

Duran et al, 2002, evaluated the same semen samples used for 154 cycles of IUIs in 119 patients and noted that no pregnancies occurred when sperm had a TUNEL positive rate of more than 12%. Alkhayal et al, 2013 also evaluated 102 semen samples from patients undergoing IUIs (102 consecutive cycles) in their clinic and compared the results with the readings from 15 fertile donors. They use SCSA, acidic aniline blue (ABB) and iodoacetamide fluorescein fluorescence (IAF) staining of the same semen samples used for IUIs and noted that "The mean (±SD) percentage of spermatozoa with positive IAF fluorescence was significantly higher in the IUI population compared to fertile controls (17 %±10 % vs. 8 %±6 %, P =0.0011) and also in the normozoospermic subset (n =78) compared to controls (16 %±9 % vs. 8 %±6 %, P <0.0001, ANOVA). They also observed a trend toward lower %progressive motility and higher %AAB staining and %DFI in the IUI group compared to controls. It was also observed the existence of a significant relationships between sperm %DFI (15%) and progressive motility (r =−0.40, P

<0.0001) and between positive AAB staining and IAF fluorescence (r =0.58, P <0.0001). It must be noted that the r values are not high although the p values show a high significance.

No note for slide 51

Slide 52

Sperm from neat semen as well as the motile sperm fraction (swim up) from our fertile donors were evaluated for the DNA fragmentation using TUNEL assay. The results are shown in this graph. Similar to neat semen, DNA fragmentation in the motile fraction of sperm varied among donors. The mean TUNEL positive for neat semen was found to be 26% (mean $\pm$ SD of 27 $\pm$ 13) while the swim up fraction had only 5% TUNEL positive sperm. Interestingly, the IUI pregnancy rate was the best for the donors with the TUNEL positive of <= 5% in the motile sperm fraction compared to the donors with higher TUNEL positive levels. Donors with the lowest levels of DNA fragmentation in their swim up samples had the best pregnancy rates.

Slide 55

Our Small Study: Some patients who resumed sperm production post chemotherapy had low TUNEL and some had high in their motile sperm fractions. The wife (29 years old) of the one with the low TUNEL, SY, had positive clinical pregnancy.

Slide 56

With our current capabilities, we are unable to use the same sperm evaluated for DNA integrity for any type of assisted conception. It is, therefore, tempting to find if there is any association between the DNA status of spermatozoa and their characteristics determined following routine semen analyses. There have been published reports of the association between DNA fragmentation and various semen parameters. Carrell et al, 2003, noted an elevation of sperm chromosome aneuploidy, apoptosis and high levels of morphologically tapered sperm with unexplained recurrent miscarriages. Alkhayal et al, 2013, reported of a significant relationships between sperm %DFI (SCSA) and sperm progressive motility (r =−0.40, P <0.0001) in semen samples used for intrauterine inseminations. The %DFIs (>15%) were significantly higher in the asthenozoospermic samples (n =15) compared to the fertile controls. It is also known that the motile fraction of sperm separated by means of density gradient centrifugation or the swim-up technique has lower levels of DNA fragmentation compared to the native semen. There also have been reports of the association between normal sperm morphology and the DNA status and the negative impact on various methods of assisted conception (Younglai et al,2001; Tang et al, 2010 and Avendano 2009, 2010, 2013). In a recent communication (Avendaño et al, 2010), it was reported that when the percentage of normal sperm with fragmented DNA was 17.6% or lower, the likelihood of pregnancy was 3.5 times higher than when above this

level. Menkveld et al, 2011 also reported that sperm with abnormally small heads have poor IVF outcome and have high degree of DNA fragmentation. On the other hand, there have published studies failing to report any association between major semen parameters and DNA fragmentation indices (Lin et al, 2008; Xia et al, 2005; Karydis et al, 2005) The reasons for the reported discrepancies may be due to the variability in methods of semen analysis and DNA assessment and the differences in the populations studied and the assisted conception methods applied. It is generally believed that conventional semen parameters may not always be associated with the nuclear DNA status.

No notes for slides 57-61

Slide 62

In one of the studies carried out at our center, we looked at the sperm in swim-up samples from patients who have been undergoing ICSI. Individual sperm which appeared to be normal for ICSI purposes were evaluated for DNA fragmentation using TUNEL. Samples with TUNEL positive normal sperm of <17.6% had 3.5 times more pregnancies than the samples with higher TUNEL. High negative predictive value indicates that when the TUNEL results indicate that the values are normal (equal or below 17.6%), there is 79% chance that the value is correct and a higher pregnancy rate is possible. On the other hand, having a positive predictive value of 66.7% indicates that if the results are more than 17.6% there is a 66.7% chance that the pregnancy rate will adversely be affected. Higher specificity of 82.6% correlate with higher negative predictive value. If the area under the curve was higher (i.e., 0.8 or higher), the findings would have been more convincing.

Slide 63

Small unpublished study: overall, the percent TUNEL positive in the motile fraction of sperm from fertile men (4 donors) was near 4%. In these men, all normal sperm (wet prep) were TUNEL negative. The subfertile group (5, normal samples but attending fertility clinic) had an average of 9% TUNEL positive sperm. One of the 5 cases had normal sperm with DNA fragmentation. The infertile men (severely oligoasthenoteratozoospermic with partners having no female factor infertility) had an average of 17% TUNEL positive sperm and all normal looking sperm were TUNEL positive.

No notes for slide 64

Slide 65

Our report for the TUNEL assay includes the basic semen parameters as well as the DNA fragmentation in morphologically normal (wet prep) sperm and morphologically abnormal

sperm. This particular patient had been evaluated for basic semen parameters several times and his sperm morphology (Strict Criteria) ranged from 0-1.5%. The report provides information about the sperm nuclear DNA status in neat semen as well as that of the motile fraction of sperm retrieved from the semen sample. In addition, information about the DNA status in spermatozoa appear to be normal under ICSI conditions. Under ICSI conditions, the most "normal" looking sperm are selected for the injection into an oocyte. The report also provides an estimate of the chances of picking up a normal appearing sperm with intact DNA often selected for use in an ICSI. However, in considering this risk factor, three important issues must be kept in mind when a patient is advised about the chances. The DNA fragmentation assessed by TUNEL does not provide the information about the extent of the DNA damage. The "normal" in a wet prep can be different than the normal morphology based on the staining of a smear prepared from semen. The DNA repair (or lack of) by the oocyte is an important (and often ignored or unknown) factor which should be included in the equation.

No notes for slide 66

Slide 67

This is the TUNEL report for the 38 year old male partner of a 37 year old woman who had undergone several unsuccessful ICSI attempts. As you see he has a very high level of DNA fragmentation both in his neat semen and in the motile sperm fraction recovered from his semen (93% and 87%, respectively). Sixty seven (67%) of his motile sperm considered morphologically normal for ICSI (wet prep) were TUNEL positive and 33% were TUNEL negative. The TUNEL assay was repeated several times at different occasions with similar results. She did get pregnant at least 3 times following ICSI but miscarried every time during the first trimester. With the reports that these men may indeed have testicular sperm minimally affected by DNA fragmentation (Tesarik, 04, Greco, 05), a TESE was performed on him about 7 months later. The results are shown in next slide.

Slide 68

This is the TUNEL result for the TESE sample obtained from the same individual whose ejaculated sperm showed a high level of DNA fragmentation (see previous slide). The results clearly demonstrate that the testicular sperm in this individual show a significantly lower level of DNA fragmentation (only 29% TUNEL positive and 71% TUNEL negative compared to 87% TUNEL positive and 13% TUNEL negative for the motile fraction of his ejaculated sperm). Of the TUNEL negative TESE sperm, 7% had morphologies considered normal under ICSI conditions. She did not conceive using TESE sperm and the couple finally ceased their efforts to conceive. For this case, the following questions should have been asked:

1. With the fact that she was 37, what would have been her contribution to the failure?

2. Could the quality of oocytes (i.e., inability to repair DNA damage and/or to contribute to the development of the embryo) have been playing a major factor in the failure?

3. If the question to #2 above is yes, was TESE necessary?

4. Occasionally, we have noted that DNA fragmentation level in the ejaculated semen/sperm may change. Would running the TUNEL assay for the ejaculated sperm during the time of ICSI (i.e., just prior to ICSI) have been useful?

5. What would have been the best suggestion to this couple? Would you have suggested accepting a donor egg? With TESE or without?

No notes for slides 69 and 70

Slide 71

This is the TUNEL results for a 31 year old man whose 32 year old wife underwent several unsuccessful ICSI attempts. His semen analyses were normal with the exception of morphology which was 0% (Strict Criteria). Similar to the first case, this patient's sperm in neat semen, and following swim-up, showed high levels of DNA fragmentation using TUNEL under the conditions of testing at our center. She was a very poor responder although she was not very old. The quality of her oocytes, 1-2 each time, was also poor. Following several unsuccessful ICSI attempts, the couple accepted an donor egg. About 4 months later at the time of donor oocyte retrieval, he underwent another TUNEL assessment with the results shown in next slide. She successfully conceived and recently delivered.

Slide 72

This is the result of repeat TUNEL for the male partner of the couple described in case #2. His TUNEL results for the neat semen showed 67% DNA fragmentation. About 4 months prior, his neat semen had 92.5% DNA fragmentation. DNA fragmentation in his motile fraction of sperm was only 7.5% whereas the level was 74% about 4 months prior. This is a clear indication of the existence of an intraindividual variation since the change in DNA fragmentation result was not due to the techniques or the testing personnel given that they remained the same in both instances. Oleszczuk et al, 2011, investigated the intraindividual variability in SCSA results (DFI) for 616 semen samples with repeated SCSA testing. They concluded that compared to the first SCSA test, 85% of men (their semen samples) remained at the same level of DFI either higher or less than 30%. The intraindividual variability was reported to be about 10%. With the results obtained from this particular patient, several questions arise. We can ask whether or not the couple would have been successful following ICSI using his sperm with low DNA fragmentation and the oocyte from his partner. Another question is whether the oocyte from the donor egg

would have been able to repair the DNA had his level of DNA fragmentation remained the same as was about 4 months prior (74%). All these questions highlight the very important and often ignored factor of the contribution of oocyte to the success or failure of assisted conception methods and undoubtedly to the success of all methods of conception whether assisted or natural.

No slide notes for the rest of presentation.