Editorial

Sperm DNA fragmentation: overcoming standardization obstacles

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Response to: Jarvi KA. Is sperm DNA fragmentation testing ready for prime time? Transl Androl Urol 2017;6:S419-21.

Submitted Jun 27, 2017. Accepted for publication Jun 28, 2017.
doi: 10.21037/tau.2017.07.10
View this article at: http://dx.doi.org/10.21037/tau.2017.07.10

In his commentary on the "Clinical utility of sperm DNA fragmentation testing: practice recommendations based on clinical scenarios" by Agarwal *et al.* (1), Dr. Jarvi acknowledged the utility of sperm DNA fragmentation (SDF) testing in the workup of male factor infertility. He recognized that there is a growing interest in this diagnostic modality among urologists and reproductive endocrinologists. This interest, as he considered, is attributed to the increasing number of publications exploring the utility of SDF in various infertility related circumstances, which necessitates need for clinical guideline articles that would serve to identify the precise indications for such testing.

Nonetheless, Dr. Jarvi explored the current drawbacks that still hinder widespread use of SDF testing in clinical practice, particularly the lack of test standardization and the presence of poor correlations between different SDF testing methods. We would like to further elaborate on these particular issues.

Test standardization is necessary in order for any medical diagnostic test used clinically. Achieving such a characteristic is mainly related to the nature of the test being performed, its complexity and degree of subjectivity required for result interpretation. There are several SDF testing methods in practice. While some still suffer from high inter-laboratory [aniline blue (AB) staining, acridine orange (AO) assay] and inter-observer [toluidine blue (TB) staining, Chromomycin A3 (CMA3) staining] variability [reviewed by Agarwal *et al.* (1)], extensive effort has been made to standardize other tests such as the sperm chromatin structure assay (SCSA), terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and sperm chromatin dispersion test (SCD). The SCSA measures the vulnerability of sperm DNA to denaturation when exposed to heat or acid. It uses flow cytometry which, despite its cost and relative complexity, offers the advantage of assessing large numbers of sperm cells efficiently. AO, a nucleic acid-selective cationic fluorescent dye, is allowed to interact with single stranded (ss) or double stranded (ds) DNA to produce green or red fluorescence, respectively. The degree of metachromatic shift from green to red fluorescence, measured with a flow cytometer, represents the number of sperm with DNA damage. The SCSA has been standardized for users limiting inter-laboratory variation (2-4).

Evenson *et al.* typically recommend making two independent measurements of the same sample to ensure the absence of laser drift artifacts or channel flow blockage. They have confirmed the presence of extremely low standard deviations (SD) of the percentage DNA fragmentation index (DFI) between repeat samples (SD =0.0) indicating superior precision and repeatability of the SCSA test (5). In another study, Giwercman *et al.* (6) investigated the relationship between SCSA and sperm motility in samples obtained from 171 Danish first pregnancy planner males (group 1) and 278 Swedish military conscripts (group 2). In addition to finding a statistically significant negative correlation between DFI and percentage of motile sperm (group 1: r^2=–0.53; group 2: r^2=–0.38), the authors compared the DFI measurements that were performed in two laboratories revealing a high level of correlation (r^2=0.90; P<0.0005).

TUNEL utilizes flow cytometry or fluorescent microscopy to quantify the incorporation of dUTP into ss- or dsDNA breaks through an enzymatic reaction that increases with the number of DNA breaks (7). Using a

benchtop flow cytometer, the TUNEL assay has been recently standardized and validated on semen samples obtained from 95 fertile controls and 261 infertile men (8). A SDF cutoff value of 16.8% was found to have a specificity of 91.6 % and a positive predictive value of 91.4% in distinguishing infertile men from controls. Test validation was assessed in a blind fashion by two experienced observers with results showing absolute inter- and intraobserver differences of 1.73% and 6.68% and percent inter- and intraobserver differences of 3% and 9.68% in >80% of cases, respectively (8). The same authors further investigated test reliability and reproducibility between two laboratories (Basel, Switzerland and Cleveland, USA), where SDF measurements were done on 31 samples by two experienced operators using the same standardized approach (9). The average SDF level measured was similar with a strong correlation seen between results from both laboratories (r^2=0.94).

The SCD test (also Halo test), performed using a bright field or fluorescent microscope, measures the degree of dispersion of DNA loops that occur following acid denaturation. Sperm with fragmented DNA fail to produce the characteristic halos seen with non-fragmented DNA after denaturation (10). Although the test is easy to perform and does not require complex instrumentation, it was previously criticized for having some inter-observer variability based on the subjective nature of result interpretation. Recent efforts were made by the manufacturer of the Halo test® to provide easy-to-follow information on how to implement and conduct SDF analyses in andrology laboratories.

McEvoy *et al.* (11) assessed the clinical utility of the SCD test, using the Halosperm G2 test kit, and found significant associations between SDF and sperm concentration, normal sperm morphology and sperm motility. Moreover, intraobserver variability assessment depicted an absolute average difference in SDF values between replicate tests to be 1.02%±0.55% with an average percentage difference of 4.16%. Interobserver variability of SDF values between two technicians showed an absolute difference of 0.21%±0.57% and an average percentage difference of 9.56%.

Difficulties in establishing sound correlations between various SDF testing methods are attributed to the variable outcome measures these tests assess. While some tests measure the degree of sperm chromatin decondensation (AB staining and TB staining), others look for the presence of nicks, ss- or ds DNA breaks either directly or after denaturation [AO staining, SCSA, TUNEL, SCD and single cell gel electrophoresis (Comet)] [reviewed by Agarwal *et al.* (1)]. Despite that, several recent studies have investigated this particular issue finding different SDF testing methods to be significantly correlated, albeit the evidence was not unequivocal (12,13).

LeSaint *et al.* (14) examined SDF levels from semen samples of 38 infertile men and observed a strong correlation between the results of SCSA and TUNEL (r^2=0.7137, P<0.0001). Another insightful study by Ribas-Maynou *et al.* (15) investigated correlations between the five most commonly used SDF testing methods (TUNEL, SCSA, SCD, alkaline Comet and neutral Comet) performed on semen samples from 250 men. Strong correlations were found between SCSA and TUNEL (r^2=0.79; P<0.001), between SCD and SCSA (r^2=0.71; P<0.001) and between SCD and TUNEL (r^2=0.70; P<0.001). Moderate correlations were also found between alkaline Comet and SCD (r^2=0.61; P<0.001), between alkaline Comet and SCSA (r^2=0.59; P<0.001) and between alkaline Comet and TUNEL (r^2=0.72; P<0.001). Finally, no correlation was found between neutral Comet and all the four other testing methods.

To conclude, while international reproductive societies (16) have disfavored the routine use of SDF testing based on concerns with its accuracy and the presence of drawbacks such as those discussed above, it did not influence the extensive research this fertility test is currently partaking as implied by Dr. Jarvi. We believe that the continued use of SDF testing for the right indications, such as those set by Agarwal *et al.*'s clinical guideline article (1), would result in protocol refinements, applied experience and superior diagnostic results.

Acknowledgements

None.

Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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Cite this article as: Majzoub A, Agarwal A, Esteves SC. Sperm DNA fragmentation: overcoming standardization obstacles. Transl Androl Urol 2017;6(Suppl 4):S422-S424. doi: 10.21037/tau.2017.07.10

