Editorial

Further evidence supports the clinical utility of sperm DNA fragmentation testing in male infertility workup and assisted reproductive technology

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Drs. Johnson and Sandlow critically scrutinized the utility of sperm DNA fragmentation (SDF) testing in clinical scenarios as discussed by Agarwal *et al.* in their recently published guideline article (1,2). The authors were quite skeptical about the value of SDF testing in male infertility workup and assisted reproductive technology (ART). They ponder that there is insufficient evidence to support obtaining SDF results in specific clinical scenarios presented by Agarwal *et al.* (2). We appreciate their insightful remarks as they give us an invaluable opportunity not only to address their concerns but also to provide readers with further evidence to confirm the validity of Agarwal *et al.* guidelines (2), as we will see.

Clinical scenario #1: clinical varicocele

Guideline's recommendation: "*While further studies are required, current evidence suggests that SDF testing may allow clinicians to better select varicocelectomy candidates among those men with clinical varicocele and borderline to normal semen parameters. SDF is recommended in patients with grade 2/3 varicocele with normal conventional semen parameters and patients with grade 1 varicocele with borderline/abnormal conventional semen parameter results (grade C recommendation)*" (2).

In their commentary, Johnson and Sandlow refer to the American Urological Association (AUA) Best Practice Statements (BPS) on varicocele, which states that "varicocele repair should be reserved for patients complaining of infertility in the presence of both a palpable varicocele and abnormal semen parameters or sperm function tests. Varicocele treatment is not indicated in patients with normal semen quality" (3). The authors question the recommendation provided by Agarwal *et al.* for SDF testing in men with large varicoceles and normal semen parameters.

We reason that there is no incongruity whatsoever between the AUA statements and Agarwal *et al.* guidelines, which propose the use of SDF testing in challenging varicocele infertility scenarios, like (I) high grade varicocele in the presence of routine semen analysis results within reference limits; and (II) low grade varicocele associated with borderline semen analysis results. As clearly stated in the AUA BPS, varicocele repair should be considered in infertile men with clinical varicocele and abnormal semen analysis or abnormal sperm function tests (3). Notably, SDF measures an essential aspect of sperm function, namely, chromatin integrity, which is unquestionably associated with male infertility (4-10), embryo development, implantation, and pregnancy (11-13). SDF is, therefore, a classic example of a sperm function test (14).

Along these lines, we feel that it is also critical to place the results of conventional semen analysis in the right perspective. Classifying patients as having 'normal' or 'abnormal' semen analysis will depend upon the World Health Organization (WHO) edition utilized for the examination of human semen (15-17). The above-quoted AUA's BPS still use the outdated 1999 WHO reference values (18) to base their recommendations. In the manual

mentioned above, the reference values for sperm count, motility and morphology are markedly higher than those presented in the latest 2010 WHO manual (16). These differences have clinical implications, as a given infertile man with a low-grade clinical varicocele and sperm count of 17×10^6/mL will be deemed eligible for varicocele repair as per the AUA's BPS, since the cutoff point utilized to discriminate between normal versus abnormal sperm count is 20×10^6/mL (3). However, this very same patient would be deemed ineligible for varicocele repair according to the AUA criteria if the 2010 WHO reference values were utilized because 15×10^6/mL is the 'normalcy' cutoff point (19). In the example above, the patient in question has normal and borderline sperm count as per the 2010 and 1999 WHO manuals, respectively (18,19). SDF testing, in this case scenario, would allow the assessment of DNA integrity, which is shown to be negatively influenced by varicocele (20,21). In the face of abnormal SDF results, urologists could recommend varicocelectomy, a decision that would be endorsed by the AUA's BPS, as the criteria for varicocele repair were met, namely (I) infertility; (II) presence of clinical varicocele; and (III) abnormal sperm function test (3).

Drs. Johnson and Sandlow also pointed that if SDF testing was to be advocated in men with clinical varicocele and normal routine semen analysis, then evidence that improvements in SDF alone can affect pregnancy outcomes after surgical correction must be provided. The authors go further by arguing that in patients with borderline normal semen parameters and low-grade varicoceles, there is a need for evidence that either SDF was a predictive factor for improvement of semen parameters or pregnancy outcomes, but such evidence has not yet been established.

We disagree from their views as evidence does exist indicating that improvements in SDF after varicocelectomy are associated with improvement of semen parameters, and such effects translate into increased pregnancy outcomes, as presented below.

Smit *et al.* prospectively evaluated 49 infertile men with palpable varicocele and oligozoospermia who underwent varicocelectomy (22). While a significant improvement in DNA fragmentation index (DFI; SCSA) was noted 3 months after varicocelectomy (preoperative 35.2%±13.1%; postoperative 30.2%±14.7%, P=0.019), couples that conceived naturally or with ART had lower DFI (26.6%±13.7%) than those who did not (37.3%±13.9%, P=0.013). The decrease in DFI was more pronounced in patients showing amelioration of semen parameters after varicocelectomy, in particular sperm concentration, than those who did not. These results suggest that varicocele repair is useful for decreasing DFI in most patients. Moreover, the magnitude of change in DFI seems to be more distinct in patients exhibiting a concomitant increase in sperm quality overall (22).

Notably, Johnson and Sandlow commented that in a paper by Nasr-Esfahani *et al.*, the increase in chromatin compaction after varicocelectomy did not translate in higher pregnancy rates (23). We contemplate that the likely reason for the discrepancy between the results of Smit *et al.* (22) and Nasr-Esfahani *et al.* (23) is the method used for SDF assessment. In the former, SCSA was used whereas in the latter, chromomycin A3, a test of sperm nuclear decondensation (SND), was utilized. SND refers to defects in chromatin compaction (e.g., protamine mispackage via defective DNA-protein crosslinking), which is intrinsically associated with the later stage of spermatogenesis (6,14). Although defective chromatin compaction makes the DNA more vulnerable to damage by reactive oxygen species (ROS) and as a consequence SDF may ensue, the effect depends on the seminal redox properties and level of oxidative stress (OS) (24). In contrast, SCSA specifically assesses the presence of both existing DNA breaks as well as DNA more prone to exhibit single and/or double breaks after denaturation (25), such as those resulting from OS in the male reproductive tract. In varicocele, ROS and nitrogen species are released in endothelial cells of the dilated pampiniform plexus, testicular cells (germ cells, Leydig cells, macrophages, and peritubular cells), and principal cells of the epididymis (21,26,27). Excessive ROS affect the membranes by lipid peroxidation and chromatin by inducing DNA breaks (20,28,29). Given the ubiquity of OS in varicocele, we therefore argue that tests that measure the presence of DNA breaks are preferable over those that assess chromatin compaction in this patient population, as discussed elsewhere (24,30).

In another report, Ni *et al.* found that SDF results were associated with pregnancy rates after varicocele repair (31). In their study evaluating 42 infertile men with varicocele and 10 normozoospermic fertile controls, a marked improvement in sperm concentration, motility, morphology, and a decrease in DFI (preoperative: 28.4%; postoperative: 22.4%; P=0.018) was observed after varicocele repair. Notably, SDF results in patients who achieved pregnancy after varicocele repair (20.6%±3.5%) were not significantly different than controls (11.5%±3.9%), but were lower than

both preoperative values (27.4%±6.3%; P<0.01) and the results of non-pregnant patients (24.7%±6.5%; P<0.01).

As for the authors' question 'How many patients with varicoceles have an elevated SDF and how many of these elevated SDF levels resolve to normal levels after varicocelectomy?', the answer is provided below.

Smith *et al.* examined semen samples from 55 patients with clinical varicocele and 25 normozoospermic donors (32). In the group of patients with varicocele, increased SDF (defined as the mean of the control group plus 2 SD) was seen in 49% patients with normal semen profile and 58% of patients with abnormal semen parameters. In another report, Werthman *et al.* studied 11 men with clinical varicocele and observed that 90% of the patients showed a significant decrease in the rates of SDF 3 to 6 months after varicocelectomy (33). Lastly, Moskovtsev *et al.* reported improvements in SDF rates in 78% of the treated patients (34).

Collectively, these observations provide some evidence that improvement in sperm DNA integrity after varicocele repair translate into higher pregnancy rates. However, the authors' remark that increased pregnancy rates should be confirmed by improvements in SDF alone seems unrealistic, because amelioration in SDF is often associated with an overall improvement in sperm quality. Given the existing evidence, we assert that SDF should be obtained in men with clinical varicocele but otherwise normal range or borderline semen parameters as per conventional semen analysis.

Clinical scenario #2: unexplained infertility/ recurrent spontaneous abortion (RSA)/intrauterine insemination (IUI) failure

Guideline's recommendation: "*A high DNA fragmentation index in clinical scenario #2 patient would provide a possible explanation for RSA and IUI failure. Therefore, it is reasonable to offer SDF testing to infertile couples with RSA or prior to initiating IUI as these couples may be better served by IVF or ICSI sooner rather than later (grade C recommendation)*" (2).

Drs. Johnson and Sandlow contend that there is no published data looking at the usefulness of screening for elevated SDF in couples with unexplained infertility/RSA/IUI failure. And go further by raising a few questions "(I) what level SDF would be considered "elevated" in these couples? (II) How would you decide which couples should have SDF levels evaluated: after 2nd *vs.* 3rd pregnancy loss? (III) How would you counsel a couple with RSA and elevated SDF?" Lastly, they call for a cost-analysis evaluation as a means of improving the clinical role of SDF in these patients.

Despite the relative paucity of studies, there is data concerning the role of SDF in unexplained infertility, a term used to define couples with routine semen analysis within reference values and in whom definitive male and female infertility factors have not been identified (35). As a matter of fact, 25–40% of infertile men with conventional semen analysis within normal ranges present with SDF rates >20–30% (36). In a recent prospective study, Vandekerckhove *et al.* enrolled 25 couples with unexplained infertility applying strict criteria for diagnosis. The percentage of patients with SDF levels above 20% and 30% [evaluated by the sperm chromatin dispersion (SCD) test] was 43% and 29%, respectively. All couples were treated by ovarian stimulation and IUI. The proportion of couples who achieved a clinical pregnancy was significantly reduced when SDF rates were >20% (37).

Concerning IUI, a higher probability of successful pregnancy (7.0 to 8.7-fold) is observed in the general population of infertile couples when the male partners have specimens with low sperm DNA damage [reviewed by Agarwal *et al.* (12) and Evgeni *et al.* (38)]. Although the exact cutoff SDF values for IUI pregnancy prediction are still debated, most studies report that values greater than 25–30% are worrisome.

As for RSA, which is usually defined by two or more spontaneous miscarriages prior to 20 weeks gestation, Kumar *et al.* evaluated 45 RSA couples and found that SDF rates (by SCSA) were 1.2-fold higher than controls (28.1 ±4.9 *vs.* 21.7±4.7, respectively; P<0.05) (39). From a ROC curve analysis, SDF rate of 26% discriminated between RSA cases and controls with 73% sensitivity, 90% specificity, and accuracy of 83% [area under the curve (AUC): 0.830; 95% CI, 0.715–0.912; P<0.0001].

These findings were corroborated by Bareh *et al.* who compared SDF rates by TUNEL between couples with RSA and fertile controls (40). In their prospective study involving 26 RSA couples and 20 fertile controls, the mean SDF rates were higher in couples with RSA than fertile controls (36.8%±5.0% *vs.* 9.4%±2.7%; P<0.001). Furthermore, Zidi-Jrah *et al.* studying a small cohort of 22 couples with RSA and 20 fertile controls showed that SDF rates (using TUNEL) were higher in the RSA group than controls (17.1% *vs.* 10.2%; P=0.01) (41). In their study, the rates of spermatozoa with nuclear chromatin decondensation assessed by aniline blue staining (23.6% *vs.* 11.8%; P=0.001) and sperm aneuploidy by fluorescence *in*

situ hybridization (10.6% *vs.* 1.5%; P<0.001) also differed between RSA patients and controls.

Additional evidence of an association between SDF and RSA is provided by Carlini *et al.* (42). The authors examined SDF rates by TUNEL among male partners of 112 couples experiencing RSA and compared the results with those of (I) infertile men with abnormal semen parameters (CONTROL 1) and (II) fertile men with normal semen parameters according to the WHO criteria (CONTROL 2). Despite normal semen analysis results, SDF in the RSA group was higher than fertile controls (18.8%±7.0% *vs.* 12.8%±5.3%; P<0.001) and similar to infertile patients (20.8%±8.9%). The three groups were divided at the cutoff of 12.8%, which corresponded to the mean SDF value for the fertile men with normal semen parameters. A total of 81.3% of RSA patients, 81.6% of CONTROL 1, and 44.7% of CONTROL 2 had SDF ≥12.8% (P<0.001: RSA and CONTROL 1 *vs.* CONTROL 2). The authors also reported a significant positive correlation between the number of pregnancy losses events and elevated SDF (r=0.20, P<0.05).

In summary, the above observations indicate that SDF greater than 20–30% should be considered elevated. Couples with unexplained pregnancy loss should be offered SDF after the second event. Male partners of such couples should be counseled to take all measures to reduce SDF, including changing lifestyle, using oral antioxidants, and treating the underlying causative condition (if identifiable). ICSI with testicular sperm can also be offered in selected cases, as we will see below. Despite the need for further research, increasing evidence indicates that the clinical utility of SDF testing in the above-mentioned patient populations has been realized.

Clinical scenario #3: IVF and/or ICSI failure

Guideline's recommendation: "*While further research in this area is still warranted, DNA fragmentation testing in patients with recurrent ART failure is indicated as it can provide useful prognostic information on subsequent ART cycles. Several studies have shown some benefit in using testicular sperm rather than ejaculated sperm in men with oligozoospermia, high SDF and recurrent IVF failure (grade B–C recommendation)*" (2).

Drs. Johnson and Sandlow dispute that when comparing IVF to ICSI outcomes with regards to SDF, the only outcomes described are pregnancy rates, and not live birth rates (LBRs), and that this is an important shortcoming that significantly limits the utility of this data in recommending ICSI *vs.* IVF. The authors go further by asking which patients should be offered testicular sperm for ICSI (Testi-ICSI), "*those with elevated SDF/male factor alone or both male and female factor?*" They also inquired about the cutoff SDF level for recommending Testi-ICSI.

In this section, we rebut their observations by discussing the evidence with regards to the association between SDF and IVF/ICSI LBRs and the role of testicular sperm for ICSI in men with high SDF.

A recent meta-analysis looked at SDF in patients undergoing IVF and ICSI (43). In this study, Osman *et al.* included six studies and 998 couples and found that, overall, men with low SDF had a higher LBR than those with high SDF (RR 1.17, 95% CI, 1.07–1.28; P=0.0005). The cut-off level for high SDF in the selected studies was 27% (44) and 30% (45,46) for the SCSA test, 35% (47) and 10% (48) for the TUNEL assay, and 50% for the COMET assay (49). As for IVF, higher LBR was observed in men with low SDF than those with high SDF (4 studies, 553 couples; RR 1.27, 95% CI, 1.05–1.52; P=0.01). With regards to ICSI, a marginally significant difference was seen in LBR in men with low SDF compared to those with high SDF (5 studies, 445 couples; RR 1.11, 95% CI, 1.00–1.23, P=0.04) (44).

Additional evidence was recently provided by Jiang and Zhou evaluating 605 IVF cycles (50). In their study, high SDF rates were negatively correlated with the rates of fertilization (r=–0.32, P<0.01), cleavage (r=–0.19, P<0.01), high-quality embryos (r=–0.40, P<0.01), clinical pregnancy (r=–0.20, P<0.01), and live birth (r=–0.09, P=0.04), and positively correlated with miscarriage rates (r=0.23, P<0.01).

The issue of using testicular sperm in preference over ejaculated sperm for ICSI has been a matter of recent debate [reviewed by Esteves *et al.* (51) and Zini *et al.* (52)]. Testi-ICSI has been associated with higher LBR in men with confirmed high SDF in semen. In a prospective cohort study, Esteves *et al.* compared ICSI outcomes between ejaculated and testicular sperm in 172 infertile couples whose male partners had oligozoospermia and elevated SDF (>30% by SCD assay) (53). The comparison groups were similar concerning male and female demographic characteristics. The adjusted relative risk for live birth between testicular and ejaculated groups was 1.76 (95% CI, 1.15–2.70; P=0.008), favoring testicular sperm. In another study, Bradley *et al.* evaluated LBR between Testi-ICSI versus ICSI with ejaculated sperm among men with high SDF [≥29% by sperm chromatin integrity test (SCIT)—a variation of sperm chromatin structure assay] (54). In the ejaculated sperm group, the authors utilized interventions

[intracytoplasmic morphologically selected sperm injection (IMSI) and physiological intracytoplasmic sperm injection (PICSI)] to increase the likelihood of having specimens with low SDF for ICSI. They found that LBRs were higher with Testi-ICSI (49.8%) than IMSI (28.7%) and PICSI (38.3%) (P<0.05). The lowest LBRs (24.2%) were achieved when no intervention was applied to ejaculated sperm (P=0.020). The biological plausibility of these observations relates to obviating exposure of spermatozoa to oxidative DNA damage in the epididymis (51). Despite the lack of cost-analysis evaluations, the study of Esteves *et al.* showed that the number needed to treat (NNT) by Testi-ICSI compared to ICSI using ejaculated sperm was 4.9 (95% CI, 2.8–16.8) to obtain an additional live birth per fresh embryo transfer (53). From a different perspective, it can be said that one out of five oocyte pick-ups will be avoided by using testicular sperm in preference over ejaculated sperm in this particular population.

On the contrary, a recent meta-analysis comparing Testi-ICSI versus Ejac-ICSI among men with cryptozoospermia concluded that outcomes were not better with Testi-ICSI than ejaculated sperm (55). However, SDF was not assessed in any of the studies included in this meta-analysis probably due to the technical difficulties of performing the test in specimens with such low numbers. Moreover, high SDF is seen in only about 30% of the men from the general ICSI population (44), thus suggesting that the lack of a beneficial effect by Testi-ICSI in this particular study may be due to the inclusion of men with acceptable SDF levels.

To sum up, the currently existing literature supports Testi-ICSI for men with confirmed post-testicular SDF in the neat ejaculate. Despite the debate as to which sperm DNA assay should be adopted, the existing evidence shows that Testi-ICSI is beneficial when SDF values exceed 29%. The best candidates for Testi-ICSI seems to be couples with repeat ICSI failure and those with male factor alone (53,54,56,57). In view of the increasing evidence supporting the clinical utility of SDF testing in the clinical scenario of IVF and ICSI failure, we feel that screening SDF for couples undergoing ART is a sound and timely decision.

Clinical scenario #4: borderline (or normal) semen analysis and lifestyle risk factors for infertility

Guideline recommendations: "*Infertile men with evidence of exposure to pollutants or those found to have a modifiable lifestyle risk factor during evaluation should be offered SDF testing. The sperm DNA test can help reinforce the importance of lifestyle modification [e.g., cessation of cigarette smoking, antioxidant therapy (AOX)], predict fertility and monitor the patient's response to intervention* (2).

Drs. Johnson and Sandlow claim that there is insufficient evidence that interventions such as *AOX* or lifestyle modification will result in resolution of DNA fragmentation, or improve fertility outcomes. On one hand, we concede that information on the effects of smoking cessation, weight loss, and exposure avert to environmental/occupational chemicals on SDF is lacking in spite of the fact that the negative impact of such risk factors on SDF has been consistently reported by several studies. For instance, workers exposed to polycyclic aromatic hydrocarbon, ionizing radiation, and organophosphate and carbamate pesticides have decreased sperm DNA integrity (58-62). The reproductive toxicity of lead poisoning on SDF has been also documented (63). Furthermore, recent evidence is suggestive of an association between exposure to air pollutants, such as PM2.5, PM10, NO_x, SO_2, and O_3, and SDF (64,65). Tobacco users have increased ROS levels, 8-hydroxy-2'-deoxyguanosine (8-OHdG), and SDF in the semen (66).

On the other hand, recent data indicates that Prudent dietary pattern (high in fruits, vegetables, fish, and poultry) may decrease SDF (67). A decline in OS and an improvement in sperm DNA integrity following adoption of meditation and yoga-based lifestyle modification have been reported (68). Additionally, a recent Cochrane review suggests that AOX in the male might increase LBR (69). In this meta-analysis, two trials (n=100) reported on SDF and found that AOX reduced SDF rates compared to placebo (MD –13.8%; 95% CI, 10.4–17.7%, I^2 =0%; P<0.00001). In one trial, 64 men with unexplained infertility and SDF by TUNEL ≥15% in the neat ejaculate were randomized to AOX (1.0 g vitamin C and 1.0 g vitamin E daily for 2 months) and placebo (70). While no differences in conventional sperm parameters were found between AOX and placebo groups before and after treatment, SDF rates were reduced in the AOX group (pre: 22.1%±7.7%; post: 9.1%±7.2%; P<0.001). In another double-blind controlled study, 21 men received a supplement of 1,050 mg/day docosahexaenoic acid (DHA; an omega-3 fatty acid) for 10 weeks whereas the placebo group (n=15) was given 1,050 mg/day of sunflower oil for the same duration (71). The authors observed a decrease in SDF (measured by TUNEL) proportional to the number of weeks of treatment (0 weeks 26.0%±4.7%, 5 weeks 15.6%±2.5%, 10 weeks 8.8%±1.9%; P< 0.01). In controls, SDF was not changed

by placebo (0 weeks 17.8%±2.6%, 5 weeks 23.5%±4.5%, 10 weeks 29.0%±6.1%).

In summary, although further research is needed to confirm the role of lifestyle changes in sperm DNA integrity and how these changes translate into better reproductive outcomes, the clear association between SDF and the risk factors mentioned above makes SDF testing an ideal tool not only to identify individuals at risk but also to monitor response to intervention.

Lastly, it is true that Agarwal *et al.*'s recommendations (2) are primarily based on levels B and C evidence and that more clinical data should be attained to support their advice further. Based on this premise, Johnson and Sandlow (1) pondered that the recommendations by Agarwal *et al.* do not yet have sufficient evidence to justify the use of SDF testing as part of the male factor fertility evaluation. However, it is opportune to mention that Dr. Sandlow himself has contributed guidelines and recommendations with similar levels of evidence (72-74). The limitations on the quality of evidence did not refrain the author and his esteemed colleagues to make specific recommendations for the diagnosis and management of infertile males in the above mentioned guidelines. We feel that the driving force of Sandlow and colleagues is essentially the same of Agarwal and his peers; both groups contributed to translating the best evidence available into practice to serve as a framework for standardized care while maintaining physician autonomy (75). The existent shortcoming of SDF testing should not refrain physicians to take full advantage of its clinical benefits provided the data supporting that specific test is made clear to the patient.

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Footnote

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