Editorial

An evidence-based perspective on the role of sperm chromatin integrity and sperm DNA fragmentation testing in male infertility

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Response to: Pandiyan N, Pandiyan R, Raja DR. A perspective on sperm DNA fragmentation. Transl Androl Urol 2017;6:S661-4.

Submitted May 23, 2017. Accepted for publication May 23, 2017.
doi: 10.21037/tau.2017.05.39
View this article at: http://dx.doi.org/10.21037/tau.2017.05.39

We read with interest the commentary by Dr. Pandiyan and colleagues (1) about the merit of our recently published clinical practice guidelines for sperm DNA fragmentation (SDF) testing based on clinical scenarios (2).

We noted that these authors were quite skeptic about the integration of SDF testing into the clinical workup of male infertility and management algorithms for couples embarking on assisted reproductive technology (ART). Their opinion is mainly based on the argument that most (or all) sperm function tests introduced in the last decades failed to provide useful information for clinical management of patients facing infertility. The authors argued that the role of molecular biology testing in infertility remains elusive, as does the association between SDF and infertility. In addition, the authors present their views about the futility of treating infertile men with varicocele. They go further by questioning varicocele as causative of male subfertility and argue that reactive oxygen species (ROS), which have been implicated in varicocele pathophysiology, bring more good than harm. Lastly, Dr. Pandiyan and colleagues advise against the use of sperm function tests, including SDF, given the effectiveness of ART. And they conclude with a quote from Edward Wallach—a Professor Emeritus of Gynecology at Johns Hopkins School of Medicine—that says "*It is easy to fall prey to accepting an unproven therapy as dogma, while overlooking the basic principles responsible for infertility, especially when the overall climate encourages aggressiveness in the use of high-tech measures*".

In our reply to the authors, we address their remarks and discuss the principles of why SDF is associated with infertility. While being respectful to their viewpoints and opinions, we provide readers with overwhelming proof embedded in science and medicine to scrutinize the commentary by Dr. Pandiyan and colleagues' with a grain of salt.

ICSI (over)use and its implication to the health of generating offspring

The striking evolution of ART in the past few decades has undoubtedly impacted the urological practice. Given the success of ICSI, sperm function tests, including post-coital test, antisperm antibodies, hypoosmotic swelling test, hemizona assay, and hamster egg penetration test have rarely been utilized nowadays (3). Indeed, ICSI has become the most common fertilization method used for ART. In the United States, ICSI represents about 65% of all fresh IVF cycles performed (4). Worldwide, fertilization by ICSI remains relatively constant over the past years, being utilized in approximately 67% of all ART cycles (5). However, there is a considerable variation by region; ICSI is performed in around 55% of ART procedures in Asia, 65% in Europe, 85% in Latin America, and close to 100% in the Middle East (5).

It is also true that in the era of ICSI the value of proper male evaluation and treatment is overlooked since ICSI may give the couple a baby without the need of explaining the

nature or cause of underlying male infertility. In contrast, the workup of female partner remains relevant because the woman is subjected to ovarian stimulation, oocyte collection and embryo transfer, and ultimately holds gestation (6). Also, remarkable attention is invested in improving embryo quality and pregnancy outcome after ART. Despite its obvious success to overcome male factor infertility, ICSI pregnancy rates (PRs) and delivery rates (DRs) remain suboptimal worldwide, being 28.7% and 18.9% for the year 2008; 27.7% and 19.9% for 2009, and 26.8% and 20.0% for 2010, respectively (5). Notably, when compared to conventional IVF among couples undergoing ART for non-male factor infertility, ICSI is associated with lower implantation rates [23.0% *vs.* 25.2%; adjusted relative risk (RR) 0.93; 95% CI: 0.91, 0.95] and live birth rates (36.5% *vs.* 39.2%; adjusted RR 0.95; 95% CI: 0.93, 0.97) (4).

Furthermore, the (over)use of ICSI has come with a price tag. The introduction of ICSI has raised concerns about the health and wellbeing of resulting offspring because of its invasive nature that circumvents natural selection mechanisms and related infertility conditions. The sperm injection technique *per se* may compromise sperm nuclear decondensation, possibly leading to embryo aneuploidy (7). Also, the microinjection pipette used to inject the spermatozoon into the oocyte cytoplasm may accidentally disrupt the oocyte meiotic spindle, possibly leading to abnormal chromosomal segregation (8). Besides, handling oocytes outside the incubator for prolonged periods of time, as in ICSI, can alter, even slightly, the temperature and pH, which may increase the rates of stress-induced aneuploidy (9).

Equally important are the studies reporting methylation defects in embryos originated from ART. The altered hormonal milieu associated with ovarian stimulation by exogenous gonadotropins and the retrieval of epigenetically immature oocytes can result in increased epigenetic/imprinting defects in children conceived through ART (10). Moreover, sperm manipulation and embryo culture conditions *in vitro* may alter the methylation processes thus increasing the risk of imprinting disorders (11). Embryos with altered methylation patterns may inherit these modifications paternally, as it has been demonstrated that aberrant methylation of promoters of specific genes (e.g., DAZL and MTHFR) and general gene classes, such as imprinted loci, is associated with oligozoospermia and azoospermia (12,13).

As a result, children conceived through ICSI, in general, have an increased risk of chromosomal abnormalities, in particular, sexual chromosome aneuploidy, when compared to naturally conceived children (9). Some evidence also indicates that certain cancer types are more common in ICSI children than naturally conceived counterparts (14,15). Lastly, there are reports suggesting that children born with the use of testicular sperm extracted from men with non-obstructive azoospermia have a slightly higher risk of autistic disorders and mental retardation than children born through ART using ejaculated sperm (16).

Association between the quality of sperm genome and epigenome and health of resulting ART offspring

The effects of ICSI on the health of offspring have been related not only to the technique *per se* but also to the quality of the male gamete. Indeed, the integrity of the sperm genome and epigenome is essential for the birth of healthy children (17). The sperm nuclear genome includes a central compact toroid comprised of protamine-bound DNA that is both transcriptionally and translationally inert. The peripheral compartment, composed of histone-bound DNA, retains the nucleosomal structure and contains promoters for developmentally critical genes, microRNAs, and signaling factors [reviewed by Kumar *et al.*, 2013 (17)]. The histone-bound DNA is highly susceptible to environmental insults, especially oxidative damage. The sperm epigenome is maintained through the retention of histones, the compaction of significant portions of the genome by protamines, DNA methylation, and covalent histone modifications. Since the male gamete loses the majority of cytosolic antioxidants during spermiogenesis, the cell is highly vulnerable to free radical-induced DNA damage. Low levels of key DNA repair enzymes and poor oocyte quality to fix sperm DNA damage may explain the persistence of DNA damage in sperm, particularly in subfertile men and sperm exposed to *in vitro* conditions, like those undergoing ART (18,19). The fertilization of oocytes by such sperm, either naturally or through IVF and ICSI, may pose an increased risk for fertilization failure, embryo arrest, miscarriage, congenital malformations, childhood cancers and perinatal morbidity (20).

Association between SDF and infertility

There is overwhelming evidence indicating that sperm chromatin integrity is essential for effective transmission of genetic information to subsequent generations and that abnormal sperm chromatin adversely affects both natural

fertility and ART outcomes [reviewed by Agarwal *et al.* (21)] (17,20,22). For instance, high SDF determined by SCSA was shown to be associated with failure to achieve natural pregnancy with an unequivocal odds ratio of 7.01 (95% CI: 3.68–13.36) (23). Time-to-pregnancy, which is a marker of fecundability, is increased in first pregnancy planners (without any infertility history) if the male partner has high SDF in the semen, as shown by both the prospective LIFE study (24) and the Danish first pregnancy planner study (25). Recent data also demonstrates the clinical value of SDF testing in the prediction of natural pregnancy, with sensitivity and specificity of over 80% with the use of sperm chromatin dispersion (SCD) test and terminal deoxynucleotidyl transferase mediated dUTP-biotin nick end labeling (TUNEL) assay (26,27). Notably, normozoospermic partners of infertile couples may exhibit elevated SDF (28). Furthermore, poor IUI outcomes have also been reported in association with elevated levels of SDF in the semen (29,30). These observations support the utilization of SDF testing in the clinical scenarios of unexplained infertility and IUI failure, as proposed in our guidelines, as poor sperm DNA integrity is a possible sound causative factor for infertility and IUI failure (31).

As for ART, a number of studies have shown the deleterious effect of SDF on IVF and ICSI outcomes (32-34). An analysis of a total of 8,068 treatment cycles revealed a significant adverse effect of SDF on clinical pregnancy in both IVF and ICSI (32). In a meta-analysis evaluating 2,969 couples, the risk of miscarriage was increased by 2.2 fold when semen specimens with an abnormally high proportion of DNA damage were used for ICSI (95% CI: 1.54–3.03; P<0.00001) (33). In another meta-analysis pooling data from 14 studies, elevated SDF was associated with higher miscarriage rates in ICSI cycles (OR: 2.68; 95% CI: 1.40–5.14; P=0.003) (34). Moreover, among couples experiencing recurrent pregnancy loss (RPL), SDF was shown to be higher in the RPL group than fertile controls (18.8%±7.0% *vs.* 12.8%±5.3%, P<0.001) and similar to the levels of infertile patients (20.8%±8.9%) (35).

To sum up, fair evidence indicates SDF plays a critical role in IVF/ICSI outcomes and RPL, thus supporting the clinical utility of SDF testing in the scenarios of repeat ART failure. In the face of abnormal results, patients should be counseled about the increased risk of pregnancy loss and decreased effectiveness of conventional IVF. Finally, in the face of persistent elevated SDF—after all measures had been attempted to ameliorate SDF—ICSI with testicular sperm is recommended (36,37).

Association between varicocele, ROS, SDF and infertility

Several etiological factors have been implicated in the impairment of sperm DNA content, including environmental and lifestyle factors, varicocele, male accessory gland infections, advanced paternal age, and systemic diseases (38-43). In this context, varicocele has been an often-debated issue (44). However, there is overwhelming evidence confirming the adverse effect of varicocele on several sperm markers, including SDF, and the benefit of varicocelectomy in selected men [reviewed by Tiseo *et al.* (45)] (46).

A meta-analysis of seven studies assessed SDF rates in men with varicocele. Higher sperm DNA damage was found in patients with varicocele than controls. The overall estimate showed a mean difference of 9.84% (95% CI: 9.19 to 10.49; P<0.00001) in SDF rates between patients and controls (47). In another article involving a total of sixteen case-control studies that measured SDF in fertile and infertile men with varicocele, SDF rates were higher in infertile men with varicocele than infertile men without varicocele in four studies (48). The remaining seven studies specifically included fertile men with varicocele. In six of them, SDF rates were higher in men with varicocele (and no history of infertility) than fertile men or sperm donors without varicocele (48). In a multicenter study, we evaluated SDF by SCD test in 593 men with various etiologies attending infertility clinics. A total of 98 men with varicocele and 80 fertile controls were included (38). Both men with varicocele and those with leukocytospermia exhibited the highest SDF rates among the studied men, with 35.7% (±18.3%) and 41.7% (±17.6%) damaged sperm, respectively. Notably, we identified two distinctive sperm subpopulations within fragmented DNA in the varicocele subgroup, namely, standard fragmented sperm and degraded sperm (DDS). Spermatozoa with standard fragmented DNA exhibited either the absence of a halo or the presence of a small halo of chromatin dispersion around a compact nucleoid (49). On the contrary, spermatozoa with degraded DNA exhibited a ghost-like morphology owing to massive single- and double-strand DNA breaks as well as nuclear protein damage. The rates of degraded sperm (DDSi), determined by the proportion of degraded sperm in the whole population of spermatozoa with fragmented DNA, were 8 fold higher in varicocele than donors. Although DDS is not pathognomonic of varicocele, it was possible to identify varicocele based solely on SCD results with 94% accuracy,

thus making DDSi an attractive marker for the presence of a varicocele (38).

As for ROS, it is important to distinguish between physiological ROS—which are essential for sperm function—and excessive ROS (18). The latter have been associated with several infertility conditions, including varicocele (18,42). ROS in excess may overcome the body's antioxidant protection and result in oxidative stress (OS). In men with clinical varicocele, ROS and nitrogen species are released in endothelial cells of the dilated pampiniform plexus, testicular cells (developing germ cells, Leydig cells, macrophages, and peritubular cells), and principal cells of the epididymis (50,51). As human spermatozoa contain high concentrations of unsaturated fatty acids, lipid peroxidation ensues in the presence of excessive ROS (52). As a result, damage to sperm membrane occurs, affecting both sperm motility and sperm-oocyte fusion. Furthermore, OS may negatively affect the sperm chromatin by inducing breaks in the DNA strands (18,53,54). Therefore, in men with varicocele SDF may result from excessive ROS production by spermatozoa themselves and the surrounding environment.

Varicocele repair has been shown to improve or normalize total antioxidant capacity (TAC) levels both in the seminal plasma and peripheral blood, as well as retinol, selenium and zinc levels (54,55). These studies indicate that varicocelectomy is beneficial not only for alleviating OS and its negative effect on fertility but also for protecting against the progressive nature of varicocele and its consequent upregulation of systemic OS (18,56). Along the same lines, a number of studies have shown that repair of clinical varicocele lowers SDF and increases the chances for achieving a natural pregnancy [reviewed by Tiseo *et al.* (45)]. A meta-analysis including 6 studies evaluated the effect of varicocelectomy on SDF rates. The authors found that SDF rates were reduced overall, with a mean difference of –3.37% (95% CI: –4.09 to –2.65; P<0.00001) (47). Kadioglu *et al.* retrospectively analyzed 92 consecutive infertile men presenting with clinical varicocele and who were subjected to subinguinal microsurgical varicocele repair. SDF was evaluated using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. In addition to the improvements in conventional semen parameters, there was a significant decrease in DNA fragmentation index (DFI) from a preoperative mean of 42.6% to a postoperative mean of 20.5% (P<0.001) (57). Ni *et al.* evaluated 42 subfertile patients with clinical varicocele and altered seminal parameters subjected to microsurgical varicocelectomy. SDF was measured by sperm chromatin structure assay (SCSA), and the protamine-1/2 mRNA ratio was also assessed. The preoperative results were compared to a control group of semen donors. The protamine-1/2 ratio and SDF indexes were significantly higher in the patient group than in the control group. After varicocelectomy, the mean P1/P2 ratio was markedly improved after a mean time of 3 to 6 months in men who were able to impregnate their wives, and postoperative results did not differ from the control group. Overall, SDF was also significantly lower 3 to 6 months after surgery when compared to the preoperative levels, although still higher than in controls. However, in the group of patients unable to impregnate their wives naturally after a follow-up of 6 months, postoperative P1/P2 mRNA and SDF rates remained unchanged in comparison to the preoperative results (58). Lastly, Smit *et al.* prospectively evaluated 49 men with clinical varicocele, oligozoospermia, and at least one year of infertility. These authors also observed postoperative improvements in sperm parameters and decreases in SDF indexes. Lower postoperative SDF results were associated with a higher chance of pregnancy, both naturally and with ART (59).

Not surprisingly, the latest Cochrane review on varicocele and infertility confirmed that there might be a benefit to performing varicocelectomy in subfertile men (60). Indeed, the beneficial role of performing varicocele repair using microsurgery techniques has been advocated for a long time by eminent urological microsurgeons (61-64).

To summarize, given the massive evidence implicating oxidative stress in the pathophysiology of varicocele, and its association with SDF, it seems there is robust proof to assess SDF status in men with varicocele as test results may provide valuable information to guide therapeutic interventions. Determining which patients are affected by SDF could enable clinicians to better select varicocele candidates for early surgical interventions. Moreover, SDF testing can be used to monitor the effectiveness of interventions.

Importance of male infertility diagnosis and treatment: a plea for less invasive treatments and the exercise of devoted medical principles in male infertility

We advocate the incorporation of clinical and surgical andrology as means to overcome male infertility. A comprehensive male evaluation, including history and

physical examination, semen analysis, sperm function testing, such as SDF, ultrasound, and measurement of hormones, as appropriate, is paramount. We also prescribe microsurgical techniques for varicocele repair and all measures for counteracting risk factors, like smoking cessation, weight loss, and refraining from using medication with gonadotoxic effects (65-73).

Several conditions associated with SDF are correctable, including varicocele, lifestyle factors, and genital infections (19,22,38,42,56,74,75). Besides varicocele repair, oral antioxidant therapy may also alleviate SDF in infertile men (76,77) and improve the chances of natural pregnancy (65). Modifiable lifestyle factors such as smoking, obesity, and occupational exposure have been associated with high rates of SDF making them potential targets for interventions (66-68,75,78,79). Taken together, these observations indicate that correction of underlying factors can alleviate SDF and potentially enable natural conception or allow the use of less complex ART methods. If ICSI is still to be used, lower miscarriage rates are anticipated after treatment of the conditions causing SDF. Clinical evaluation of the infertile male is, therefore, essential to identify the causes of infertility mentioned above and allow treatment of these men to reduce SDF.

To conclude, the role of sperm chromatin integrity to both natural and assisted conception is unquestionable. SDF testing has emerged as a simple tool complementary to the conventional semen analysis that may enable clinicians to better manage infertile couples. While there is a need for further refinement of existing assays, SDF testing—provided it is properly conducted and standardized- reflect the quality of the entire semen specimen, not just the damaged sperm detected in the test result. In the presence of abnormal values, several strategies can be undertaken to alleviate SDF aiming to increase both natural fertility and ART outcomes. Quoting Robert Heinlein, an American science-fiction writer "*We should not handicap ourselves making our lives easier by neglecting what is in front of our eyes*".

Acknowledgements

None.

Footnote

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

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Cite this article as: Esteves SC, Agarwal A, Majzoub A. An evidence-based perspective on the role of sperm chromatin integrity and sperm DNA fragmentation testing in male infertility. Transl Androl Urol 2017;6(Suppl 4):S665-S672. doi: 10.21037/tau.2017.05.39

