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Commentary

A clinical assay for reactive oxygen species – ready for primetime?

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A B S T R A C T

Reactive oxygen species (ROS) have been demonstrated to have damaging effects on human sperm function. The measurement of ROS as an adjunct to traditional semen analysis has clinical relevance as part of the diagnosis of male infertility. The assay best suited to the clinical laboratory environment for detecting ROS generation remains somewhat controversial. A recent report on a multicenter study evaluating the reduction of nitroblue tetrazolium (NBT) to formazan precipitate as an indirect reporter of ROS-generating activity in spermatozoa, seminal plasma and semen has received a critique raising questions as to the sensitivity and specificity of the assay for detecting ROS. The authors of the report argue in response that the assay has validity and yields results that are potentially clinically significant. This dialogue serves to (re)direct readers to the original article and to consider carefully the intent and potential application of the assay, and whether there is sufficient scientific evidence to judiciously support its clinical diagnostic application.

The traditional semen analysis has, arguably, limited predictive value for pregnancy and, as a consequence, adjunct sperm tests have been developed to augment clinical decision making for infertility treatment. One putative adjunct test is the measurement of reactive oxygen species (ROS).

In a landmark publication, Aitken and Clarkson (1987) demonstrated that washed human spermatozoa generated ROS in response to treatment with a calcium ionophore, A23187. Further, they found variation amongst subject specimens in response to ionophore, with some specimens producing a dramatically higher release of ROS than others. Significantly, the greater the burst of ROS in response to ionophore treatment, the poorer the fusion of those sperm with zona-free hamster oocytes. Collectively, the data reveal that elevated concentrations of ROS negatively impact on critical aspects of human sperm function.

In a subsequent study, Aitken and Clarkson (1988) published data that transformed the way in which semen samples are processed in the clinical andrology laboratory. They found that techniques that selected motile from poorly motile sperm populations prior to centrifugation, such as density gradient centrifugation (DGC) or direct swim up, produced a highly functional suspension of sperm as demonstrated by high percentages of both motility and ionophore-induced sperm-oocyte fusion. In contrast, if semen samples were processed without such a preselection technique, then the functional ability of motile spermatozoa was diminished and reduced to that of the dysfunctional spermatozoa, as reflected by poor motility and sperm-egg fusion. A burst of ROS evolving from poorly motile sperm 'induced' dysfunction to the otherwise functional population of spermatozoa. This was further confirmed when upper layer DGC fractions, consisting primarily of poorly motile spermatozoa, were analyzed and found to mirror, in both functional attributes of motility and sperm-oocyte fusion, a sperm suspension isolated using no preselection technique.

Since these pioneering reports, the nature and impact of ROS on human sperm function and male fertility has been actively investigated. ROS are known to damage sperm, resulting in reduced human in-vitro fertilizing ability and increased DNA damage that correlates with miscarriage. A spectrum of conditions has been identified that contribute to seminal ROS, e.g., environmental, lifestyle, medical and others. Thus, a valid conclusion is that the measurement of ROS during

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the male (in)fertility evaluation may be of clinical significance. The question is what test best surveys ROS generation that has the critically damaging effects on spermatozoa?

Adjunct tests to semen analysis have been developed in the past, e.g., sperm DNA fragmentation, for which, like ROS, there are different techniques that assay different targets, e.g., single versus double DNA strand breaks. While a technique may be valid for its specific purpose, what remains controversial is whether the test been rigorously evaluated, with results that are clearly linked to clinical outcomes and with the added benefit of being able to be performed in the clinical laboratory environment.

Gosálvez et al (2017a) report on a multicenter study evaluating the reduction of nitroblue tetrazolium (NBT) to formazan precipitate as an indirect reporter of ROS-generating activity in spermatozoa, seminal plasma and semen. The NBT assay they used is commercially available as a kit, requires no special instrumentation, is amenable to the clinical laboratory environment and results from different laboratories can easily be compared. A second study objective they reported on was the degree of DNA fragmentation in low- and high-level NBT reactivity sperm populations over time. The results from experimentation are nicely presented, easy to understand and analyzed using proper statistical methods. Based on the procedures used, results generated and conclusions made, is there rationale to consider the NBT assay for use in the clinical laboratory?

Incontrovertibly, it is right and necessary to scrutinize any assay that has intention for clinical laboratory application. In recent correspondence in this journal (Aitken, in press), Professor John Aitken has provided a thoughtful critique regarding the NBT method for specifically assaying ROS source(s) and generation, and interpretation of results and conclusions drawn by the authors. In my opinion, there is no benefit to be gained by repeating or interpreting further his straightforward comments. In response to Professor Aitken's critique, Gosálvez, (in press) have provided appropriate rebuttal and substantiation for their approach and conclusions drawn. The discourse between these colleagues is not only respectful but also, collectively, meaningful, instructive and of equivalent merit. Importantly, the dialogue serves to (re)direct readers to the original article and to consider more carefully the intent and potential application of the assay, and whether there is sufficient scientific evidence to judiciously support its clinical diagnostic application. As for any new assay that is introduced into the clinical laboratory, rigorous testing is required across the breadth of a target patient population and, finally, the results weighed against meaningful clinical end points. The question of whether this assay has passed the litmus test falls to the discretion of the reader. One thing is certain, important questions were raised in the Gosalvez paper and by the correspondence and rebuttal pieces that merit aggressive investigation.

In the paper's Key Message section, Gosálvez et al. offer comment that extends on Aitken and Clarkson (1988): 'Semen samples with high levels of oxidative stress, as determined by a nitroblue tetrazolium assay, have diminished sperm DNA longevity after ejaculation. Given that seminal plasma was found to be the primary source of oxidative stress, rapid separation of this fraction may improve sperm DNA quality in these patients.' Thus, the conclusions from the present and historical study combine to reinforce the requirement for human spermatozoa to be quickly and selectively removed directly from seminal plasma in order to have optimal functional ability for capacitation, to fertilize the oocyte and, terminally, to contribute to a functional new genome. If a test, such as the NBT assay, can reliably and cost-effectively be used to discriminate between ROS-affected versus unaffected semen samples then effort towards possible remediation using, for example, antioxidant treatment can be applied to either the patient or specimen, the effectiveness of intervention validated by re-assay and then assisted or, preferably, unassisted fertility attempts can be safely initiated. Until that time, however, it seems there is a bit more work that needs to be done to address some of the questions raised before this assay can be adjuncted as part of the traditional semen analysis.

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