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Letter

Nitroblue tetrazolium (NBT) assay

To the Editor

It was with great interest that I read the paper by Gosálvez et al. (2017) entitled 'Multi-centre assessment of nitroblue tetrazolium reactivity in human semen as a potential marker of oxidative stress'. This paper has been well written, is based on a sizeable patient cohort and has been carefully analysed using appropriate statistical methods.

Unfortunately, however, the interpretation of the results and the validity of the conclusions are undermined by a failure to understand the fundamental chemistry of the nitroblue tetrazolium (NBT) reaction that lies at the heart of this analysis. NBT will undergo reduction to form an insoluble blue-black product, formazan. While it is true that superoxide anion will give up an electron to reduce NBT, in truth, any enzyme capable of effecting NBT reduction using, for example, NADH or NADPH, as an electron donor, will generate a response that masquerades as a reactive oxygen species (ROS) signal. So, while $O_{2^{-\cdot}}$ is theoretically capable of reducing NBT, the same response can be generated by a number of oxidoreductases using alternative electron donors, including some that have already been identified as being involved in the reduction of redox-sensitive probes, such as the cytochrome P450- and cytochrome b5- reductases (Baker et al., 2004, 2005).

The result is that NBT exhibits high background levels of reduction in the presence of seminal plasma because this biological fluid is well endowed with reductases capable of reducing NBT to formazan. Because no evidence was presented by Gosálvez et al., (2017) to confirm that superoxide is the reductant in this reaction, the fundamental conclusions drawn in this paper such as 'Overall, 80.1% of the studied population showed semen reactivity to the NBT test, indicating the presence of superoxide anion in the ejaculate' are untrue. Similarly, apparent conundrums such as 'the reason why those individuals characterized as P3 (neat ejaculate positive, seminal plasma positive, and spermatozoa negative) showed a positive signal is difficult to ascertain' are not really conundrums at all. In such cases the positivity of the neat ejaculate was not due to the generation of superoxide by the spermatozoa but due to reductases in seminal plasma, possibly augmented by the copious amounts of ROS generated by contaminating leukocytes. Similarly, the discussion addressing the relationship between DNA damage and oxidative stress is not helpful because, fundamentally, the assay is not measuring oxidative stress. This is not the first time that this problem has arisen. An earlier study by Pujol et al. (2016) using the NBT assay erroneously concluded that oxidative stress is not related to seminal parameters, fertilization rate or pregnancy outcomes. In truth, this study only demonstrated that none of these clinical parameters are correlated with NBT reduction – a completely different outcome from the one stipulated.

Whether or not this assay can measure ROS generation by spermatozoa, which, because of their limited cytoplasmic volume are not richly endowed with reductases, is an interesting question that has been addressed previously. (Aitken et al., 2013). In the presence of potent ROS generation stimulators such as menadione, NBT was capable of generating a dose-dependent response, however it could not detect the mitochondrial ROS generated in response to arachidonic acid or 4-hydroxynonenal (Aitken et al., 2013). It was therefore concluded that NBT has neither the specificity nor the sensitivity to measure ROS generation by human sperm suspensions and should not be used for this purpose.

At a microscopic level, the variation in intracellular formazan deposition visualized by the authors is controversial because previous investigations have failed to find any evidence for NBT reduction by human spermatozoa, although contaminating leukocytes were effective in this regard (Armstrong et al., 2002). Very similar compounds such as MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) can certainly be reduced to formazan by spermatozoa (van den Berg, 2015; RJ Aitken unpublished observations) and serve as a valuable means of assessing cell viability. In the absence of a vitality marker, Gosálvez et al. (2017) can draw no conclusions about ROS generation by spermatozoa on the basis of their NBT assay since, in reality, they are only measuring cell death.

I commend the authors on the selection of this important topic for analysis and on the quality of the written text and illustrations. However the paper is flawed by a lack of understanding in relation to the mechanisms by which NBT reduction is effected. Overall, I would encourage andrologists to use flow cytometry methods to measure ROS generation by spermatozoa, avoid any notion of a 'dipstick' test on crude semen samples and, particularly, avoid the use of non-specific, unhelpful probes such as NBT.

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