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Letter

Response: Nitroblue tetrazolium (NBT) assay

To the Editor

We are grateful to Professor Aitken (2017) for his comments on our recent study (Gosálvez et al., 2017), for he has highlighted critical issues in understanding the mechanism of action of the nitroblue tetrazolium (NBT) reaction test and its association with seminal oxidative stress. We are pleased to have the opportunity to expand on the discussion of this important topic.

Firstly, we emphasize that the NBT reduction test has been used extensively for the assessment of oxidative stress (OS) in several cell lines, including spermatozoa, for over four decades (Park et al., 1968; Urban and Jarstrand, 1979; Mercardo-Pichardo et al., 1981; Whittington and Ford 1999; Esfandiari et al., 2003; Choi et al., 2006; Tunc et al., 2010; Amarasekara et al., 2014; Habas et al., 2017). Our paper adds to the existing literature by showing the broad range and diverse response of NBT reactivity when applied to neat human semen samples and various semen fractions.

Secondly, we recognize that other molecules present in the seminal plasma may interfere with NBT reactivity as correctly pointed out by Professor Aitken. It is also plausible that given their high reactivity and short half-life superoxide anions are not the primary candidate for explaining the NBT reactivity in isolated seminal plasma fractions. However, it still remains to be determined whether intracellular enzymes, such as cytochrome P450 reductase and cytochrome b5 reductase, as suggested by Prof Aitken, can be found free in seminal plasma or in concentrations detectable by the assay (Baker et al., 2005). Notably, reduction of NBT by cytochrome P450 reductase and cytochrome b5 reductase requires external in-vitro addition of NADH, which was not a component of our research method. Furthermore, infiltrating leukocytes, a potential source of NAD(P)H, were unlikely to represent a major problem in our study, as the vast majority of specimens analyzed with positive seminal plasma reactivity had few, or entirely lacked, contaminating leukocytes. We, therefore, ponder that the origin of NBT reactivity in P3 specimens (neat ejaculate positive, seminal plasma positive, and spermatozoa negative) may have arisen from different sources which are still to be determined. Nevertheless, our findings of a strong NBT reactivity in the seminal plasma and the observation of accelerated dynamics of sperm DNA fragmentation are suggestive of the presence of oxidative stress, a hypothesis that needs to be investigated further.

Thirdly, at the level of the spermatozoon, we also acknowledge the possible contribution of other electron donors participating in NBT reactivity. However, we argue that it is presumptuous to fully disregard the contribution of mitochondrial activity as a cause of an unbalanced redox environment. Our results, as depicted in Figure 5a and 5b of the original article by Gosálvez et al (2017), clearly indicate that the midpiece of human spermatozoa exhibits low and moderate positive NBT reaction, respectively; furthermore, a massively reacted sperm mid piece region is noted in Figure 5c. Although further study is required, it is not unreasonable to assume that ROS generation, as depicted in Figure 5c of the original article by Gosálvez et al (2017), would have an adverse consequence on sperm fertilizing potential. As for the possible association between NBT results and sperm death, it is essential to understand that increased ROS levels and cell apoptosis are interrelated events (Aitken et al., 2014), thus negating the need to assess cell viability when applying the NBT assay.

Lastly, there are both direct and indirect techniques to assess for the presence of free radicals. While direct assessment can be performed using electron spin resonance (Rice-Evans et al., 1991) or spin trapping methods (Mason, 1996), these approaches are not commonly used in standard andrology laboratories due to technical complexity. Consequently, indirect methods have been used to assess redox cell status. While we concede that molecules such as -2',7'-dichlorofluorescin diacetate (DCFDA) and malondialdehyde (MDA) have the capacity to produce fluorescent signals, and are therefore useful to assess ROS at the cellular level by means of flow cytometry or epifluorescence microscopy, we would also point out that the same level of scrutiny must be applied to these molecules regarding their efficacy for discerning relevant and specific information, as that demanded by Professor Aitken concerning the NBT assay. This level of confidence seems to be the only way of overcoming any possible biased result derived from unexpected energy transmission from biomolecules to the reporter molecule necessary to produce fluorescence emission or a color conversion.

Despite being semi-quantitative in nature, we reason that the NBT assay, as used in our paper, can be utilized routinely by andrologists and reproductive endocrinologists alike as a surrogate for seminal oxidative stress assessment. In fact, we have already demonstrated that a high level of NBT reactivity is negatively associated with sperm

https://doi.org/10.1016/j.rbmo.2017.09.007

DNA longevity. The latter represents a prime practical finding from our study because prolonged in-vitro incubation of sperm in seminal plasma may result in a time-dependent DNA damage. Ejaculates with elevated levels of NBT reactivity are more prone to producing a faster rate of iatrogenic DNA damage than those with low NBT reactivity.

Despite the particular characteristics of NBT, we argue that the assay is useful for making rapid assessments of overall ROS in the human ejaculate. It also has a potential role in the evaluation of in-vivo anti-oxidant treatments designed to reduce ROS in the ejaculate.

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