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Cost-effective diagnosis of male oxidative stress using the nitroblue tetrazolium test: useful application for the developing world

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Summary

Seminal oxidative stress plays an important role in male factor infertility (MFI), worldwide. A study was thus undertaken for the first time to establish seminal reactive oxygen species (ROS) as a clinical marker of MFI in a cohort of Sri Lankan males. The nitro blue tetrazolium (NBT) assay for ROS estimation and modified Endtz test for detecting leucocytes were carried out on semen samples ($N = 102$) of sub-fertile males. Age-matched individuals ($N = 30$) with proven past paternity served as controls. Significantly higher ROS production was evident in individuals with asthenozoospermia and unexplained infertility (Mann–Whitney U-test, $P = 0.000$), than of the fertile and the other sub-fertile groups tested. Receiver operating characteristic plot analysis established cut-off points of 40.57 and 42.02 μg formazan/10^7 spermatozoa for ROS to distinguish fertile males from asthenozoospermics (71.4% sensitivity: 70% specificity; AUC = 0.82), and from unexplained infertile males (74.1 % sensitivity: 73.3% specificity; AUC = 0.85) respectively. As ROS appear to be a potential marker of male infertility, it is imperative to validate this test as a simple, cost-effective hence a widely accessible diagnostic tool to be included in MFI investigations in the developing world.

Introduction

Infertility is defined as the inability of a couple to conceive after adequate regular, unprotected intercourse for a period of twelve months. If a man has never impregnated or initiated a pregnancy in a woman independent of the outcome of that pregnancy, or when a man who impregnated a woman in the past irrespective of the outcome of that pregnancy presents with inability do so again, is considered as male factor infertility (MFI) (Rowe *et al*., 2000).

Male factor infertility is a significant global health issue that contributes 50% to the cause amongst infertile couples seeking treatment (Seshagiri, 2001; Sharlip *et al*., 2002). In Sri Lanka, the prevalence of MFI exceeds 60% (Fernando,2002; Wijeratne, 2003), and according to available data, of the major contributory aetiological factors, medical causes recorded the highest prevalence (54%) where mumps in the pre-pubertal period was the commonest (43.5%), while the rest were attributed to diabetes, hypertension, endocrine problems and tuberculosis. Defects in the male genital tract such as varicocoele, hydrocele and other conditions contributed to around 14% of the male factor defects, while occupational hazards (7%) and substance abuse (2%) were among other recorded factors. Of the MFI problems, 23% were of unknown aetiology (Fernando, 2001).

Previous studies have proven that reactive oxygen species (ROS) play an important role in MFI (De Lamirande & Gagnon, 1995; Pardon *et al*., 1997). ROS, which comprise both radicals and nonradicals, are oxidising agents generated as a result of metabolism of oxygen and have at least one unpaired electron that make them very reactive (Cocuzza *et al*., 2007). Semen peroxidase positive leucocytes and abnormal spermatozoa produce ROS continuously (Aitken & West, 1990; Kessopoulou *et al*., 1992). Small amounts of ROS produced play a significant role in sperm physiological processes such as capacitation, hyper activation and sperm–oocyte fusion (de Lamirande

et al., 1997; Agarwal *et al.*, 2004). However, ROS are continuously inactivated by antioxidants to maintain the balance for normal sperm functioning. Excessive generation of ROS in semen can cause DNA, sperm cell membrane and protein damage (Alvarez & Storey, 1995; Aitken, 1999; Saleh & Agarwal, 2002).

Although currently more than 30 methods are available for the detection of ROS levels in semen globally (Tremellen, 2008), this semen quality parameter is not tested for in Sri Lanka. As such, there is an unmet need for the introduction of a suitable, cost-effective method to diagnose ROS in semen. Amongst these nitro blue tetrazolium (NBT) test is a readily available, easily performed, inexpensive and highly sensitive test for assessing the contribution of spermatozoa and leucocytes to ROS production in semen (Esfandiari *et al.*, 2003). NBT is a yellow, water-soluble nitro-substituted aromatic tetrazolium compound that reacts with cellular superoxide ions to form a purple coloured formazan derivative that can be monitored spectrophotometrically after solubilising in an appropriate solvent (Rook *et al.*,1985; Choi *et al.*, 2006).

Although excessive production of ROS is detrimental to spermatozoa and thereby fertility of the male, it is not included in the evaluation of the infertile male in the Sri Lankan setting. Adding ROS analysis to the MFI investigation profile will widen the information available for the diagnosis of the infertile male. As diagnostic tests in developing countries need to be widely accessible, inexpensive and simple to use, this study was undertaken to seek the possibility of adding value to local MFI investigations, using the cost-effective NBT assay.

Materials and methods

Ethical approval

This study received the approval of the Ethics Review Committee (ERC) of the Faculty of Medicine, University of Colombo, Sri Lanka (ERC/09/006; 26/03/2009). Written informed consent of all participants was obtained for their voluntary involvement in this study.

Study populations

The study populations were recruited between April and August 2009 from men undergoing infertility assessment, both at the Reproductive Biology Laboratory (RBL), Faculty of Medicine, University of Colombo ($N = 54$), and at a private infertility clinic, the *Vindana* Reproductive Health Centre, Colombo ($N = 48$), Sri Lanka. Care was taken to ensure that the inter- and intra observer variability was negligible if not minimal with regards to collection and processing of semen samples from the two laboratories. Identical standard operation procedures are being used in both these andrology laboratories and both the laboratories participate in an inter laboratory comparison programme.

Men above 45 years, those with any chronic medical disorders and those on medication within the last 6 months, were excluded from this study. Facilities for infertility management are not readily available in many parts of the country. Thus, this study cohort included sub-fertile males from most parts of the island, other than from the North and the Eastern provinces due to the ethno-political environment prevailing in the country during the period of sample collection.

An age-matched group of men ($N = 30$) with proven paternity within the past two years with normal semen parameters according to World Health Organization guidelines was selected and recruited as the control group (WHO, 1999).

Seminal fluid analysis (SFA)

Collection, analysis and interpretation of results of all semen samples were performed according to the guidelines of the WHO (1999).

NBT test for assessing semen ROS production

All test ($N = 102$) and control ($N = 30$) semen samples were subjected to the modified NBT test to assess the total amount of ROS produced by both spermatozoa and leucocytes (Tunc *et al.*, 2008).

NBT assay procedure

Spermatozoon was washed prior to the NBT test by centrifuging 200 μl of semen in 1000 μl of phosphate buffered saline (PBS, pH = 7.4) at 300 g for 5 min. One millilitre of the supernatant was replaced by PBS, and the pellet was washed again as before. Washed spermatozoon was re-suspended in 200 μl of PBS, divided into 100-μl aliquots, and each incubated with an equal volume of 0.1% nitro blue tetrazolium (NBT) (Sigma-Aldrich, MO, USA) at 37°C for 45 min. Following incubation, sperm cells containing the formazan product were washed twice in PBS for 10 min at 500 g to remove all residual NBT leaving only a sperm pellet. The intracellular formazan product was solubilised in 60 μl each of 2 M KOH and dimethyl sulphoxide (DMSO) (Sigma-Aldrich, MO, USA). The reaction mixture after 5 min was dispensed into an ELISA plate (Immulon 2HB, Thermolab Systems, Inc., Beverly, MA, USA) and the resulting colour was measured using a microplate reader (Model 680; Bio-Rad Instruments Inc., CA, USA) at 655 nm. ROS production was expressed as micro-

gram of formazan per 10^7 spermatozoa, derived from a standard curve plotted with the absorbance values vs known concentrations of formazan substrate solubilised in DMSO.

Endtz test for quantification of seminal leucocytes

The presence of neutrophils and macrophages in semen was assessed by the Endtz test, a modified myeloperoxidase staining method (Shekarriz *et al.*, 1995).

Assay procedure

Liquefied semen (20 μl) was placed in a 2-ml cryogenic vial (Corning Costar Corp., Cambridge, MA, USA), and 20 μl of PBS and 40 μl of benzidine solution were dispensed in to semen samples. The solutions were mixed and allowed to incubate at room temperature for 5 min. Peroxidase positive leucocytes (PPL) that stained brown were counted using a haemocytometer under light microscopy (Hay & Westwood, 2003) (×40 objectives; Nikon, Japan).

Statistical analyses

Data management and statistical analyses were performed using computer software package SPSS 16 for windows (SPSS Inc, Chicago, USA). Values were represented as mean ± standard deviation and median values. Correlation analysis was carried out using Spearman rank correlation test. Mann–Whitney *U*-test was used for pair wise analysis. Comparisons of means in more than two groups were carried out using the Kruskal–Wallis test. The significance level was set at $P < 0.05$.

Cut-off points in terms of sensitivity and specificity of ROS values were established by constructing Receiver operating characteristic (ROC) curves, where the area under the curve (AUC) provided the accuracy of the NBT assay (Obuchowsk, 2005).

Results

Seminal fluid analysis (SFA)

Routine semen parameters with the exception of volume were significantly different (Mann–Whitney *U*-test; $P < 0.05$) between the fertile and infertile study groups (Table 1).

Table 1 Summary statistics of routine semen parameters of fertile and infertile study groups

Sperm parameter	Fertile group Mean ± SD	Infertile group Mean ± SD	*P*-value
Sperm Concentration (10^6 cell ml^{-1})	77 ± 33.20	40.26 ± 31.61	<0.000*
Motility %	51.57 ± 14.38	36.06 ± 24.18	0.005*
Morphology %	48.17 ± 11.40	38.74 ± 16.80	0.008*
Volume (ml)	2.65 ± 1.42	2.59 ± 1.17	0.990
Vitality %	68.54 ± 1.80	61.72 ± 1.65	0.020*

Values are represented as mean ± SD.
*Significant difference between fertile and infertile groups (Mann–Whitney *U*-test).

ROS and infertility

The production of formazan by infertile men on average was 73.40 μg of formazan/10^7 spermatozoa, which was significantly higher than that of healthy fertile donors (29.88 μg of formazan/10^7 spermatozoa) (Mann–Whitney *U*-test; $P = 0.001$), implying that oxidative stress is prominent in the infertile male population (Fig. 1b).

For further analysis, the infertile group was subdivided into two: Group 1 included men with sperm count defects (azoospermia and oligozoospermia), while Group 2, the 'other infertile group', included all other sub-fertile men with the conditions of asthenozoospermia, teratozoospermia, asthenoteratozoospermia, oligoasthenozoospermia, oligoteratozoospermia, oligoasthenoteratozoospermia and also men with unexplained infertility.

A significant difference in ROS production was evident among infertile groups 1 and 2, and the fertile group (Kruskal–Wallis test; $P < 0.000$), while pairwise analysis revealed that the significant difference was between the fertile group and the other infertile group. Sequential comparison of all the groups clustered under the other infertile group (Group 2) (asthenozoospermia, teratozoospermia, asthenoteratozoospermia, oligoasthenozoospermia, oligoteratozoospermia, oligoasthenoteratozoospermia and unexplained) with the fertile group revealed a significant difference in ROS production of both the asthenozoospermic group (Mann–Whitney *U*-test, $P = 0.000$), and of the unexplained infertile group (Mann–Whitney *U*-test, $P = 0.000$). Thus, males with asthenozoospermia (sperm motility defects only) and unexplained infertility were detected to have high ROS levels in semen compared to their fertile counterparts (Fig. 1a).

ROS and semen parameters

Highly significant correlations were established between the production of formazan with sperm motility (Spearman's correlation; $P = 0.009$, $r = 0.272$), sperm morphology (Spearman's correlation; $P = 0.001$, $r = 0.398$) and sperm concentration (Spearman's correlation; $P = 0.000$, $r = 0.510$) exclusively in the infertile group compared to those of the fertile control group.

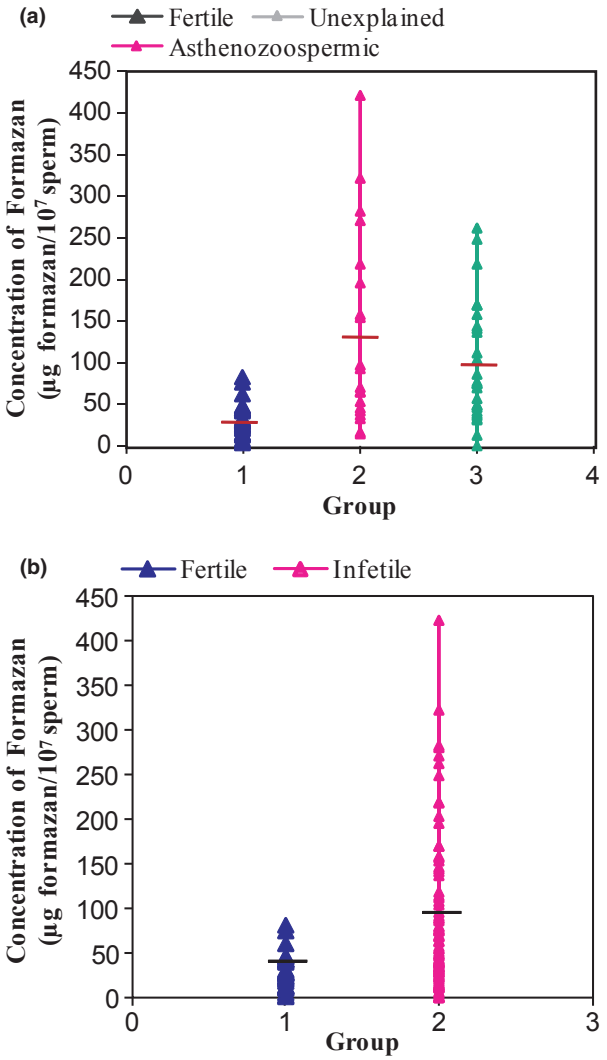

Fig. 1 Comparison of ROS production in (a) fertile, asthenozoospermic and unexplained infertility groups (b) fertile and infertile groups and (Horizontal bars represent the mean of each group).

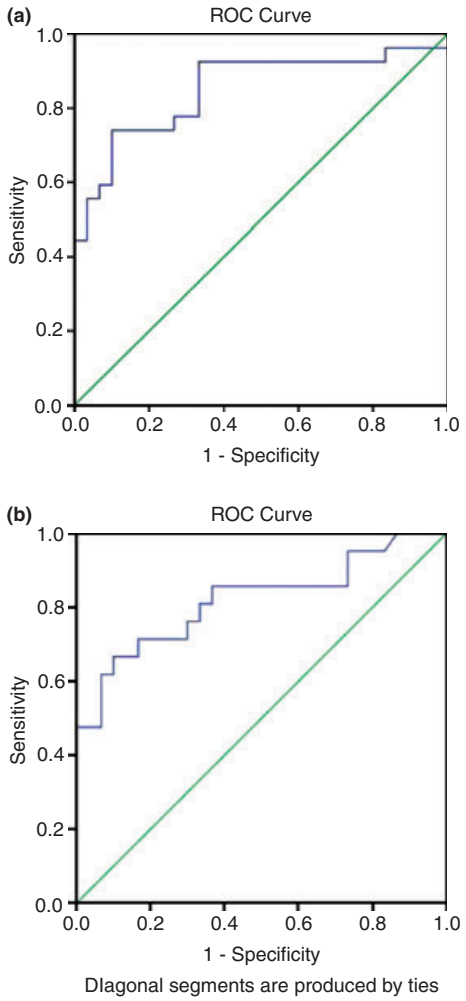

Fig. 2 ROC curves to differentiate (a) fertile and asthenozoospermic groups and (b) fertile and unexplained infertile groups. Area under curves for these assays were 0.820 and 0.852, respectively, indicating that the assay was accurate at distinguishing fertile and asthenozoospermic & fertile and unexplained infertile groups.

Normal reference values

Receiver operating characteristic plot analysis established cut-off points of 40.57 µg formazan/10^7 spermatozoa for ROS (71.4% sensitivity: 70% specificity) to distinguish fertile and asthenozoospermics, and 42.02 µg formazan/10^7 spermatozoa for ROS (74.1% sensitivity: 73.3% specificity) to differentiate fertile from unexplained infertile males. Estimated area under the curve denoted a very good ability to distinguish between fertile males from that of asthenozoospermics (0.82), and unexplained infertiles (0.85) respectively (Fig. 2).

ROS and leucocytes

The mean number of neutrophils and macrophages present in the semen of infertile individuals was significantly higher than that of the fertile donors (Mann–Whitney *U*-test; $P < 0.001$). Interestingly, contrary to expectations, none of the infertile individuals were detected in the leucocytospermic level (>10^6 leucocytes ml^{-1} semen). No significant correlation was evident between the PML concentration and ROS production in both the fertile control (Spearman's correlation; $r = 0.054$, $P = 0.778$) and infertile groups (Spearman's correlation; $r = 0.188$, $P = 0.073$).

Discussion

There is considerable debate concerning the diagnostic and prognostic significance of semen quality parameters in the evaluation of MFI (Ombelet, 1997; Tomlinson

et al., 1999). A basic semen analysis does not adequately reflect all the parameters of semen quality and function that are required for predicting optimum fertility status, more so in unexplained MFI. It is evident that seminal oxidative stress is involved in many cases of unexplained MFI, resulting in abnormal semen parameters and sperm damage (Ramadan *et al.*, 2002; Venkatesh *et al.*, 2009; Yumura *et al.*, 2009).

This is the first study carried out in Sri Lanka to assess the seminal ROS levels in infertile males. The results of this study using the simple, low-cost NBT test revealed that ROS levels in semen could be developed as a potential marker in the diagnosis of asthenospermia-based MFI and of unexplained infertility. Even though the gold standard recommended by the WHO for ROS in semen is the expensive, chemiluminiscence test as a developing nation sophisticated and lavish techniques are beyond our access. Therefore, introducing a cost-effective, easy to perform, sensitive technique such as the NBT test for ROS assay in semen would be much more practical for local laboratories.

The existence of highly significant correlations between sperm concentration, sperm motility and sperm morphology with ROS production in terms of formazan concentration in the NBT assay exclusively in the infertile group of this study substantiated previous studies (Agarwal *et al.*, 1994; Pasqualotto, 2000; Tunc *et al.*, 2008), but opposed findings by Venkatesh *et al.* (2009).

Sperm count and sperm motility are the most important predictors of fertility potential (Saalu, 2010). ROS can directly damage spermatozoa by inducing peroxidation of the lipid-containing sperm plasma membrane, which decreases its integrity, and may also affect sperm motility by damaging the axonemal structure (Saleh & Agarwal, 2002). This study indicated that high levels of ROS may be a causative factor in the impairment of sperm motility and hence the occurrence of asthenozoospermia.

Several researchers have correlated leucocyte numbers with seminal ROS production, as leucocytes are potent producers of ROS (Shekarriz *et al.*, 1995; Esfandiari *et al.*, 2003). The present investigation having taken into consideration ROS production by both seminal macrophages and neutrophils, however, found no correlation between leucocyte numbers and seminal ROS production. The most likely reason for this may be that none of the infertile participants had evidence of infection and hence were nonleucocytospermic, and thereby weakening our ability to correlate leucocyte activity with the ROS output.

The Endtz test is restricted to staining of semen leucocytes (specifically PMN granulocytes and macrophages) and hence to indicate the ROS production by semen leucocytes. The current study assayed the total ROS production by both spermatozoa and leucocytes in semen by the NBT assay, while the contribution of leucocytes to ROS production was detected by the Endtz test. Thus, the current study revealed that at least in nonleucocytospermic patients, sperm cells are by far the dominant producer of ROS and not seminal leucocytes.

The present study suggests that ROS have major effects on sperm function. Antioxidant treatment may therefore be helpful to maintain the balance between pro-oxidant and antioxidant, which may prevent damage of spermatozoa by oxidative stress and help in improvement of the sperm function ability (Nicoles *et al.*, 2004). SFA being the backbone of clinical evaluation of male infertility does not reflect all the semen quality parameters that are required for optimum infertility evaluation, for example ROS levels. This study for the first time established that individuals with asthenozoospermia and unexplained infertility have high ROS levels in semen compared with fertile individuals in Sri Lanka. ROS levels in semen could therefore be developed as a potential independent marker in the diagnosis of asthenozoospermia and unexplained infertility-based MFI, locally.

Diagnostic tests in developing countries need to be widely accessible, inexpensive in terms of reagents and equipment used, and simple to use. Although governments of Sri Lanka, over many decades, have shown greater commitment to strengthen public health by providing free health services, yet, areas of high priority such as reproductive health require more focused attention. Tests to determine the levels and sources of excessive ROS generation in semen are currently not included in routine evaluation of sub-fertile men, locally. For countries like ours with free health services, WHO recommended diagnosis of ROS using expensive tests such as chemiluminescence is unaffordable. The NBT test that can be performed spending less than US$ 1 per test on reagents, with access to an ELISA plate reader, will make it promising to diagnose MFI and affordable for the government in serving the public. Then again, it would be undoubtedly affordable for the public who are serviced by the private healthcare sector as well. Eventually studies on types and doses of antioxidants that can be used to treat asthenozoospermics and unexplained infertile men with elevated ROS levels will help to improve natural and assisted reproduction success rates in Sri Lanka.

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