

Reactive oxygen species measurement in human spermatozoa by flow cytometry using the fluorescent probe, 2',7'-dichlorofluorescein-diacetate (DCFH-DA).

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We declare that this manuscript has not been presented nor published elsewhere

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Abstract

Semen analysis constitutes the first biological step in the evaluation of male factor infertility (MFI) in an infertile couple. It is known that human spermatozoa generate reactive oxygen species in physiologic amounts, which play a role in sperm functions during sperm capacitation, acrosome reaction, and oocyte fusion. However, uncontrolled and excessive production of ROS, when it overwhelms the limited antioxidant defenses in semen, results in seminal oxidative stress. Recently, a substantial body of growing evidence suggests that such seminal oxidative stress is involved in many cases of idiopathic MFI. We have demonstrated that intracellular reactive oxygen species can be detected and measured in human spermatozoa by flow cytometry using the fluorescent probe 2',7'-dichlorofluorescein-diacetate (DCFH-DA). This method will help in assessing oxidative stress during basic semen analyses in assisted reproductive technology.

INTRODUCTION

Male fertility markers have been scrutinized in order to comprehend the molecular events that can lead to subfertility and permit an accurate diagnosis and design of therapeutic protocols. Among these markers, the study of oxidative stress (OS) status in semen has emerged as a promising field.^{1,2} OS can be defined as an imbalance between pro-oxidative and anti-oxidative molecules in a biological system which arises as a consequence of excessive production of free radicals and impaired antioxidant defense mechanisms.³

Those free radicals that are derived from oxygen are known as reactive oxygen species (ROS). ROS are produced primarily by the physiological metabolism of oxygen in cells under aerobic conditions. Examples of ROS include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), peroxy radicals (ROO^\cdot) and hydroxyl radicals (OH^\cdot).^{4,5,6,7}

Human spermatozoa are extremely susceptible to ROS induced damage due to large quantities of polyunsaturated fatty acids (PUFA) present in their plasma membranes.⁸ This is exacerbated by low concentrations of scavenging enzymes in their cytoplasm.⁹ Some studies have shown that almost 40% of infertile males display abnormally increased ROS levels in their semen.¹ There are many methods used to measure ROS in cells. One of the most commonly used methods in human spermatozoa is the chemiluminescence assay.¹⁰ This assay measures the oxidative end-products produced by an *in vitro* reaction between ROS and certain reagents. The reaction causes light to be emitted, which is measured with a luminometer, thus the results can be affected by many variables. Fluorescence-activated cell sorting is an accurate technique that can also be used to detect ROS even though no current studies are available on the standardization of the assay in human spermatozoa.¹¹ The advantages of flow cytometry over other fluorescence-detection techniques are that it rapidly measures and analyzes thousands of cells, distinguishes between cell sub-populations and analyzes intracellular events on a single-cell level.¹²

The aim of this study was to develop a method to measure ROS in human spermatozoa by flow cytometry using the probe, 2',7'-dichlorofluorescein-diacetate (DCFH-DA).

MATERIALS AND METHODS

Semen collection

Semen samples were obtained from normozoospermic donors according to World Health Organization¹³ guidelines. Institutional Review Board approval was obtained for this study.

Sperm preparation

Motile sperm fractions were retrieved from the samples using a double wash in synthetic human tubal fluid (400 x g, 5 min) swim-up technique (3% HTF-bovine serum albumin, 37°C, 5% CO_2). After 1 hour, the supernatant containing motile sperm was collected and the concentration adjusted to 2×10^6 cells/ml before it was divided into aliquots.

Flow cytometry

The detection technique used in this study was a modification of previous studies.^{14,15} Samples were loaded with DCFH-DA (5 μ M; Sigma Chemicals Co., St. Louis, MO, USA) and incubated (15 min, 37°C) in the dark. The cells were then washed twice in distilled water (5 min) and further incubat-

ed in probe free medium (30 min, 37°C) before analysis by flow cytometry. A Becton Dickinson FACSCalibur™ analyzer was used to quantify fluorescence (excitation wavelength 488nm and emission wavelength 530 nm) at the single-cell level, and data were analysed using Cellquest™ version 3.3 (Becton Dickinson, Sanjose, CA, USA) software. The mean fluorescence intensity of the analysed sperm cells was determined after gating the cell population by forward and side scatter signals. In total, 20 000 events were acquired, but non-sperm particles and debris were excluded by prior gating, thereby limiting undesired effects on overall fluorescence. The final gated cell populations usually consisted of 12 000-15 000 sperm cells. Fluorescence in these cells was recorded on a frequency histogram by logarithmic amplifiers (Fig. 1a).

Specificity of DCFH-DA was validated by administration of the non-specific ROS scavenger N-(2-mercaptopropionyl)glycine (MPG, 50 μ M) (Sigma-Aldrich Co. Ltd, St Louis, MO, USA) to the cells to see if it would attenuate the fluorescence. Subsequently, the ability of this technique to detect changes in ROS production was assessed. Aliquots were centrifuged for 10 min (400 x g) in the presence or absence of a ROS scavenger, MPG (50 μ M) which was added 15 min prior to centrifugation.

STATISTICAL ANALYSIS

In all the experiments the sample size was $n = 12$. All data are expressed as mean \pm SEM. Student's *t*-test or one-way analysis of variance (ANOVA) tests (with Bonferroni post hoc test if $p < 0.05$) were used for statistical analyses. Fluorescence data are expressed as mean fluorescence intensity (percentage of control, control adjusted to 100%). Differences were regarded statistically significant if $p < 0.05$.

RESULTS

Decreases or increases in DCFH-DA fluorescence were depicted as a shift in the histogram to either the left or right (Fig. 1b) as compared to baseline DCFH-DA fluorescence (Fig.1a). A significant decrease in mean DCFH-DA fluorescence was detected in cells treated with 50 μ M MPG ($60.41 \pm 3.36\%$ vs. control; $p < 0.05$; Fig. 1c). Cells subjected to centrifugation for 10 min showed a significant increase in DCFH-DA fluorescence ($144.50 \pm 10.73\%$ vs. control; $p < 0.05$) which was reversed by MPG treatment prior to centrifugation ($74.38 \pm 4.86\%$ vs. $144.50 \pm 10.73\%$; Fig. 1c).

DISCUSSION

It has previously been shown with the chemiluminescence technique that centrifugation of spermatozoa resulted in increased ROS generation.¹⁶ Our findings show that this newly employed DCFH-DA-flow cytometry detection method was also able to measure endogenous production of ROS induced by centrifugation. To further validate the specificity of DCFH-DA, the administration of the ROS scavenger, MPG, was able to attenuate DCFH-DA fluorescence. These findings demonstrate that the use of this detection method is effective and comparable to chemiluminescence as validated by detection of centrifugation induced ROS production which was reversed by MPG.

As far as we are aware, this is the first study that demonstrates, standardizes and establishes the utility of the DCFH-DA-flow cytometry technique as a tool for measuring intracellular ROS in human spermatozoa. Also, the findings of this study demonstrate that flow cytometry can be used as an accu-

rate and relatively time-saving technique to detect and measure intracellular ROS production in human spermatozoa, which is of particular importance as ROS appears to be implicated as one of the major culprits in male factor infertility.

ACKNOWLEDGEMENTS

We thank Dr Christo Muller for his technical assistance, as well as the NRF, University of Stellenbosch and Malawi College of Medicine NORAD project for funding.

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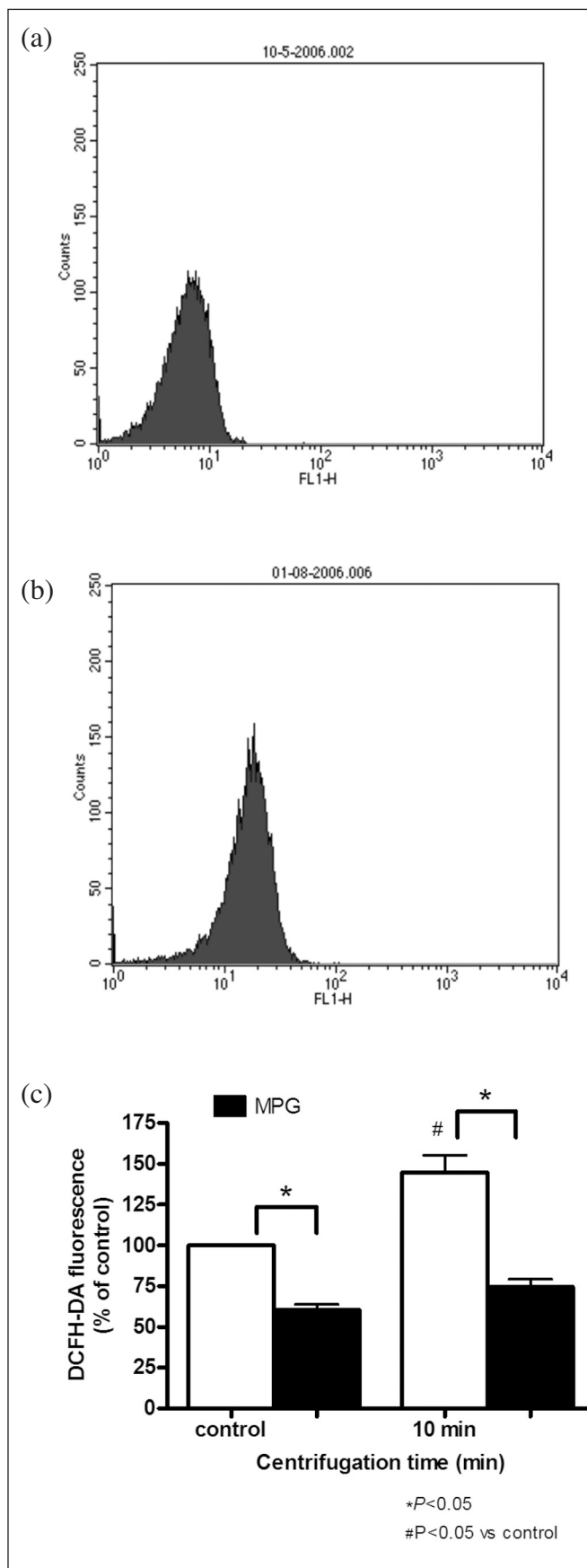


Figure 1
 (a) Histogram of baseline 2',7'-dichlorofluorescein-diacetate (DCFH-DA) fluorescence. (b) Histogram showing an increase in DCFH-DA fluorescence (shift to the right). (c) Effects of MPG on DCFH-DA fluorescence in centrifuged and uncentrifuged cells.