Oxidative stress in viable spermatozoa: detection in native semen samples

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INTRODUCTION **It is of great importance to have methods able to detect oxidative stress in native semen samples to be used in male infertility work-up.**

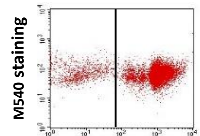
OBJECTIVES **We challenged three fluorescent probes, CM-H2DCFDA, CellROX Green and MitoSOX Red, each coupled to a suitable staining able to exclude both non-viable spermatozoa and non-sperm elements from flow cytometric analysis: Merocyanine 540 (M540) for CM-H2DCFDA and CellROX Green Reagent and LIVE/DEAD green fixable stain (LD-G) for MitoSOX Red**

PATIENTS AND METHODS **To assess probe specificity, we treated semen samples (n=5) with H2O2 (1 mM, 1 h) and Menadione (50 µM, 30 min) and then double stained with: i) CM-H2DCFDA/M540, ii) CellROX Green/M540 or MitoSOX Red/LD-G and acquired by Flow cytometry.**

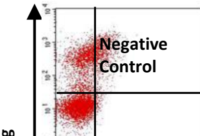
To assess whether the probes were able to detect spontaneously produced oxidative stress, spermatozoa were incubated in vitro for 1 h at RT and then were stained with the three above procedures. Probe localization was studied by Fluorescence Microscopy. Sperm Sorting was performed by a FACSMelody ™ Cell Sorter. Sperm DNA damage was detected by both COMET and SCD test.

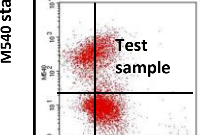
RESULTS

Double staining with CM-H2DCFDA and M540



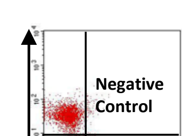
M540/Yo-Pro1 staining after induction of cell death: M540 stains all dead spermatozoa and apoptotic bodies.

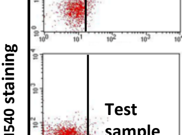




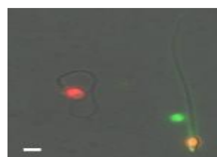
CMH2DCFDA

Double staining with CellROX Green and M540

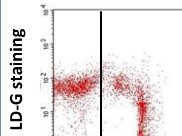




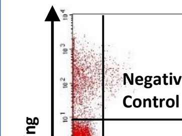
this probe does not label non-viable spermatozoa, but stains all viable spermatozoa

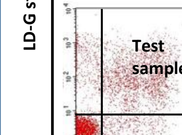


Double staining with MitoSOX Red and LD-G

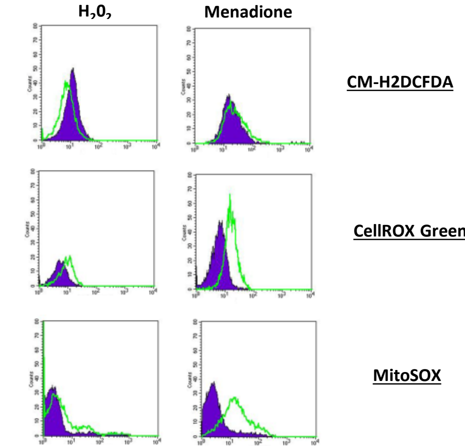


LD-G/PI after membrane permiabilisation: PI stains all dead spermatozoa and apoptotic bodies

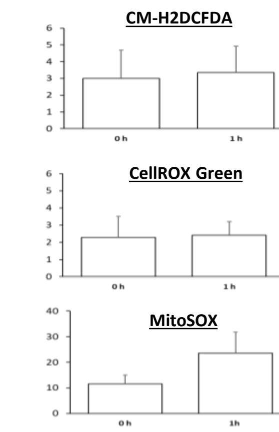




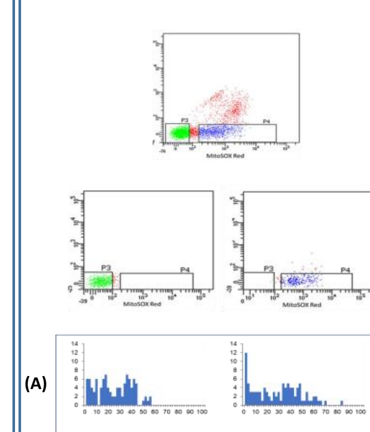
Mitosox red

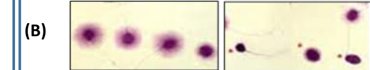


Overlapping of fluorescence of negative control (solid) and test sample (open) after gating viable sperm. Only CellROX Green and MitoSox Red detect H2O2 and superoxide production induced by menadione.



MitoSOX Red revealed an increase of ROS production during 1h incubation, at variance with CM-H2DCFDA and CellROX Green





Sperm Sorting was used to separate MitoSOX-positive from MitoSOX-negative viable spermatozoa

As expected spermatozoa with oxidation showed higher levels of oxidative stress, also exhibited higher percentage of sDF, as assessed by both Comet (A) and SCD (B).

We revealed sDF in the two fractions with Comet and SCD assay.

DISCUSSION **All the three methods presented here are able to focus on semen viable spermatozoa, thus providing measurements on the most relevant sperm fraction for seminal OS determination. At variance with MitoSOX Red, well discriminating between negative and positive viable spermatozoa, CM-H2DCFDA and CellROX Green labelling was present in all viable spermatozoa.**

MitoSOX Red and CellROX Green, but not CM-H2DCFDA increased fluorescence upon H2O2 and menadione treatment. MitoSOX Red fluorescence, but not CM-H2DCFDA or CellROX Green labelling, increased during sperm incubation, suggesting that only the first probe detects spontaneous sperm ROS production. As expected, the viable sperm fraction with ROS production also exhibited higher percentages of sDF.

CONCLUSION **The three probes showed different localization and specificity for ROS in human spermatozoa. MitoSOX Red appears a probe able to detect both spontaneous and H2O2- and menadione-induced ROS production. Labelling with this probe also identifies a viable sperm fraction susceptible to sDF, a clinically important semen parameter.**