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Asian J Androl 2008; 10 (3): 455–459
DOI: 10.1111/j.1745-7262.2008.00399.x

AJA

·*Original Article*·

Stability of fluorochrome based assays to measure subcellular sperm functions

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Abstract

Aim: To evaluate the long-term stability of the fluorescence signals of new fluorescence-based semen analysis assays for clinical application. **Methods:** Semen samples from 87 unselected infertile patients were used to perform the following assays: (i) detection of active caspase-3 (n = 17); (ii) integrity of the mitochondrial membrane potential (MMP) (n = 17); (iii) externalization of phosphatidylserine (EPS) (n = 16); and (iv) detection of intact acrosomes via CD46 (n = 37). After the assays, 4% paraformaldehyde was added to all aliquots. The fluorescence intensity of each sample was evaluated by flow cytometry on days 0, 3, 7, 10 and 14. **Results:** Differences of up to ± 5% positive spermatozoa from the value measured at day 0 were estimated as acceptable deviation. The Caspase-3 FLICA™ showed mean differences < 5% at day 3, 7 and 10. At day 14 the mean difference was 7.6%. In contrast, the disrupted MMP and the EPS detection showed differences > 5% at day 3. The CD46-FITC labeling displayed absolute differences < 5% CD46-positive spermatozoa at days 3, 7, 10 and 14. **Conclusion:** Although immediate analysis of the fluorescence signals is recommended, it is possible to evaluate caspase-3 activation up to 10 days and CD46 up to 14 days after staining of sperm. The FACS evaluation of MMP and EPS detection should be conducted on the same day. *(Asian J Androl 2008 May; 10: 455–459)*

Keywords: human spermatozoa; apoptosis assays; caspase-3; mitochondrial membrane potential integrity; CD46; semen analysis

1 Introduction

Applications of assisted reproductive techniques (ART) to treat infertile couples have expanded rapidly within the past decade. However, the pathophysiological diagnosis of male infertility is often missed and the current pregnancy and live-births success rates remain unsatisfactory [1]. Standard semen analysis does not provide information about impaired sub-cellular processes in human sperm [2]. Several fluorescence-based techniques have been applied to evaluate functional ultrastructure of sperm defects: the integrity of the plasma membrane, the mitochondrial transmembrane potential (MMP) and caspase-3 activation [3–5]. These variables, which represent components of the apoptosis signaling cascade from somatic cells [6] correlate well with sperm motility [7–9] and morphology [10] as well as with the fertilizing ability of human sperm [11, 12]. Sperm preparation techniques used before ART procedures, like cryopreservation and thawing or centrifugation, induce sub-cellular damage in various segments of sperm [13]. An increase in

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Received 2007-10-11 Accepted 2008-02-20

the numbers of sperm having externalized phosphatidylserine in the outer plasma membrane, showing a disrupted MMP, or possessing activated caspase-3 (cytosolic aspartate-specific protease 3) might play a role in male infertility [14], and their depletion might improve the outcome of ART.

The measurement of the percentage of acrosome-reacted sperm before and after induction of the acrosome reaction gives insight as to the functional ultrastructure of sperm. Decreased proportions of acrosome-reacted sperm after induction of the acrosome reaction are found significantly more often in ejaculates from subfertile patients [15]. Labeling with anti-CD46 antibodies is an established method to measure the amount of acrosome-reacted sperm [16, 17]. The integrity of the MMP and the activation of caspase-3 can be evaluated using commercially available kits. FITC-labeled antibodies raised against phosphatidylserine and CD46 are used to detect membrane changes and the proportion of acrosome-reacted sperm, respectively.

All of the previously mentioned assays are fluorescence-based. The evaluation by fluorescence microscopy reveals much more imprecise results compared to flow cytometric analysis. Fluorescence microscopy allows the measurement of only a limited number of cells because of fading under the fluorescence light and due to time limitations. For example, a recent study demonstrated in comparison to flow cytometric analysis consistently lower levels of caspase-activation when the evaluation was performed by fluorescence microscopy. However, the intra-observer and inter-observer reliability of both methods were comparable [18]. Immediate access to a flow cytometer is not possible in all laboratories. The present study was conducted to evaluate the stability of the fluorescence signals of these recently developed tests for clinical application.

2 Materials and methods

2.1 Samples and experimental design

This study was approved by the Institution Review Board of the Faculty of Medicine, University of Leipzig (Leipzig, Germany). Aliquots were collected from semen samples of 87 unselected male partners of infertile couples after given informed consent. Following liquefaction and density gradient centrifugation at 500 × *g* for 20 min (PureCeption, SAGE BioPharma, Bedminster, NJ, USA), the resulting 80% pellet was re-suspended in phosphate buffered saline (PBS: pH 7.4, Na_2HPO_4 8.0 mmol/L, K_2HPO_4 1.5 mmol/L, KCl 2.7 mmol/L, NaCl 140.0 mmol/L) for the various assays.

2.2 Monitoring of externalization of phosphatidylserine (EPS)

EPS was examined in 16 semen samples using a monoclonal mouse anti-human phosphatidylserine antibody, clone 1H6 (Upstate cell signaling solutions, Lake Placid, NY, USA). Spermatozoa were incubated with the phosphatidylserine-antibody at a final concentration of 0.5 μg/mL in PBSB (PBS, pH 7.4 containing 2% bovine serum albumin [BSA]) for 20 min on ice, followed by addition of 150 μL PBSB and centrifugation at 400 × *g* for 5 min at 20ºC. After discarding the supernatant each sperm pellet was incubated protected from light with 50 μL of secondary antibody (goat anti-mouse IgG [H + L], fluorescein conjugate, Upstate Cell Signalling Solutions) on ice for 20 min. A second washing step in PBSB (400 × *g* for 5 min at 20ºC) was performed to remove excess antibody that was not bound to the spermatozoal surface. For assessment by flow cytometry, sperm pellets were diluted in 400 μL PBSB. Human neutrophils (5×10^6 cells) treated with 1 mmol/L cycloheximide for 6 h served as positive controls for induction of apoptosis. The negative controls were processed identically for each fraction, except that the primary antibody was replaced with 200 μL PBS.

2.3 Detection of activated caspase-3

In 17 aliquots, the levels of activated caspase-3 were detected using a fluorescein-labeled inhibitor of caspase-3, which is cell permeable, non-cytotoxic, and binds covalently to activated caspase-3 [19]. The inhibitor was used with the appropriate controls according to the kit instructions provided by the manufacturers (Fluorescence Labeled Inhibitor of Caspase-3, Caspase-3 FLICA™; Immunochemistry Technologies, Bloomington, MN, USA). A 150-fold stock solution of the inhibitor was prepared by dissolving the lyophilized caspase-inhibitor in 50 μL dimethyl sulfoxide (DMSO) and was further diluted 1:5 in PBS to yield a 30-fold working solution (per aliquot: 2 μL of the stock solution plus 8 μL PBS). All test aliquots and controls (with 100 μL PBS) were incubated at 37ºC for 1 h with 10 μL of the working solution and subsequently washed with the rinse buffer. In concordance with the monitoring of MMP, human neutrophils (5×10^6 cells) treated with 1 mmol/L cycloheximide for

6 h were used as positive controls for induction of apoptosis. The negative controls were processed identically for each fraction, except that the stain was replaced with 10 μL PBS.

2.4 Monitoring of MMP

In further 17 aliquots a lipophilic cationic dye was applied. Sperm with intact mitochondria emit an intense red fluorescence as a result of the formation of dye aggregates. The monomer dye fluoresces green, indicating a disrupted MMP. Mitosensor™ was used according to the instructions of the manufacturer (Apoalert Mitochondrial Membrane Sensor Kit; Clontec Laboratories, Palo Alto, CA, USA). Briefly, all aliquots were incubated at 37ºC for 20 min in 1 μg of the lipophilic cation diluted in 1 mL PBS. Human neutrophils (5×10^6 cells) treated with 1 mmol/L cycloheximide for 6 h were used as positive controls for induction of apoptosis. Negative controls were processed identically for each fraction except that the stain was replaced with 1 mL PBS.

2.5 Evaluation of the acrosomal status

In 37 aliquots, the acrosome reaction was monitored with and without induction. Sperm were capacitated for 3 h at 37ºC with 5% CO_2 in human tubar fluid (HTF) medium containing 3 mg/mL BSA (Purity = 98%, Merck; Darmstadt, Germany). Induction of acrosome reaction was performed using the calcium ionophore A23187 (Sigma; St. Louis, MO, USA). On the day of use, a frozen aliquot of stock solution of calcium ionophore A23187 in DMSO (Sigma) was diluted 1:5 with HTF medium and 20 μL of the solution was added to 100 μL of the sperm suspension. As a control, an aliquot of the same sperm suspension was left untreated. Both tubes were incubated for 1 h at 37ºC in 5% CO_2 in air before the acrosomes were assessed.

The amount of spontaneous and ionophore-induced acrosome reaction was detected using a monoclonal FITC-labeled mouse anti-human CD46 antibody (IgG2a; Biomeda, Foster City, CA, USA). All aliquots were washed in 900 μL PBS for 4 min at 400 × *g*. The resulting sperm pellet was resuspended in 100 μL PBS containing 4 μL of CD46-FITC and incubated for 30 min under light protection. A second washing step in PBS (4 min at 400 × *g*) was performed before the pellet was resuspended in 0.4 mL PBS.

2.6 Flow cytometry analysis

The extent of CD46 and EPS on the sperm surface as well as the percentage of sperm with activated caspase-3 and a disrupted MMP was evaluated by flow cytometry in neat aliquots at day 0 and after the addition of 4% paraformaldehyde 1:1 to the remaining portion of all samples on days 0, 3, 7, 10 and 14. A minimum of 10 000 spermatozoa was examined for each assay at a flow rate of < 100 cells/s. The sperm population was gated using 90 degree and forward-angle light scatter to exclude debris and aggregates. The excitation wavelength was 488 nm, supplied by an argon laser at 15 mW. Green fluorescence (480–530 nm) was measured in the FHL-1 channel and red fluorescence (580–630nm) in the FHL-2 channel. The percentage of positive cells and the mean fluorescence was calculated on a 1023 channel scale using software Expo32ADC (Coulter, Krefeld, Germany).

2.7 Statistical analysis

Data were analyzed using inbuilt functions within the Statistica 6.0 software (StatSoft; Tulsa, OK, USA). Study variables were not normally distributed. Summary statistics are presented as mean absolute difference. Univariate comparison of sperm variables at different time points was performed with Wilcoxon's signed rank test. All hypothesis tests were two-tailed. $P < 0.05$ was considered statistically significant.

3 Results

All applied assays can be performed in a reasonable timeframe: for measurement of the integrity of the MMP only 30 min is necessary, for detection of the amount of caspase-3 positive sperm 70 min is required, and for the evaluation of EPS 60 min is needed. To label sperm with the anti-CD46-FITC antibody, 40 min is required, but prior capacitation takes 3 h. Depending on the number of samples it is possible to run the assays simultaneously.

The percentage of sperm containing activated caspase-3, an intact or disrupted MMP, EPS and CD46 was detected by FACS analysis on days 0, 3, 7, 10 and 14. The fluorescence signals of all assays diminished with time, but there were both positive and negative differences when compared to day 0. The differences ($\triangle x$) between the values measured at day 0 (x_0) and day 3 (x_1), day 7 (x_2), day 10 (x_3) and day 14 (x_4) were evaluated as absolute values of differences: $\triangle x = |x_i - x_j|$ (i, j = 0–4). Absolute differences of up to 5% of positive sperm from the value

measured at day 0 were estimated as an acceptable deviation.

Caspase-3 activation was detected in 55% ± 18% of spermatozoa on day 0 (mean ± SD). The FACS analysis showed mean differences < 5% on day 3 (3.7%), day 7 (3.5%) and day 10 (3.2%). The mean absolute difference on day 14 was 7.6% (Figure 1A). The Mitosensor™ revealed that 41% ± 24% of the sperm contained a disrupted MMP on day 0. In contrast to the Caspase-3 FLICA™ the fluorescence signal was not stable, showing mean differences of 6% (day 3), 31% (day 7), 17% (day 10) and 34% (day 14) of sperm with a disrupted MMP (Figure 1B). Similar results were derived from stability analyses of EPS detection: 42% ± 22% of sperm were EPS positive at day 0. Flow cytometry of the same aliquots demonstrated mean differences of 7.8% (day 3), 13% (day 7), 12% (day 10) and 17% on day 14 (Figure 1C). CD46-FITC labeling (before induction of acrosome reaction: 6.9% ± 12%, after induction of acrosome reaction: 44% ± 16% CD46 positive sperm) displayed absolute differences < 5% of CD46 positive sperm at days 3 (1.5%), 7 (2.9%), 10 (3.8%) and 14 (4.5%, Figure 1D).

4 Discussion

Ejaculates of infertility patients often demonstrate normal sperm variables as determined by standard semen analysis [2]. Results derived from fluorescence-based assays like Caspase-3 FLIC™, Mitosensor™ or the FITC-based EPS and CD46 detection will provide a better diagnosis in the future. There is significant increase in caspase activation in various cases of "unexplained" infertility [20]. In addition, measurement of subcellular markers like apoptosis signaling allows further optimization of sperm preparation (e.g. during cryopreservation and thawing) [21]. The novel assays will increase our knowledge of the pathophysiology of known conditions like varicocele associated with an increase in caspase activation [22]. Methods widely used in ART like centrifugation, cryopreservation and thaw-

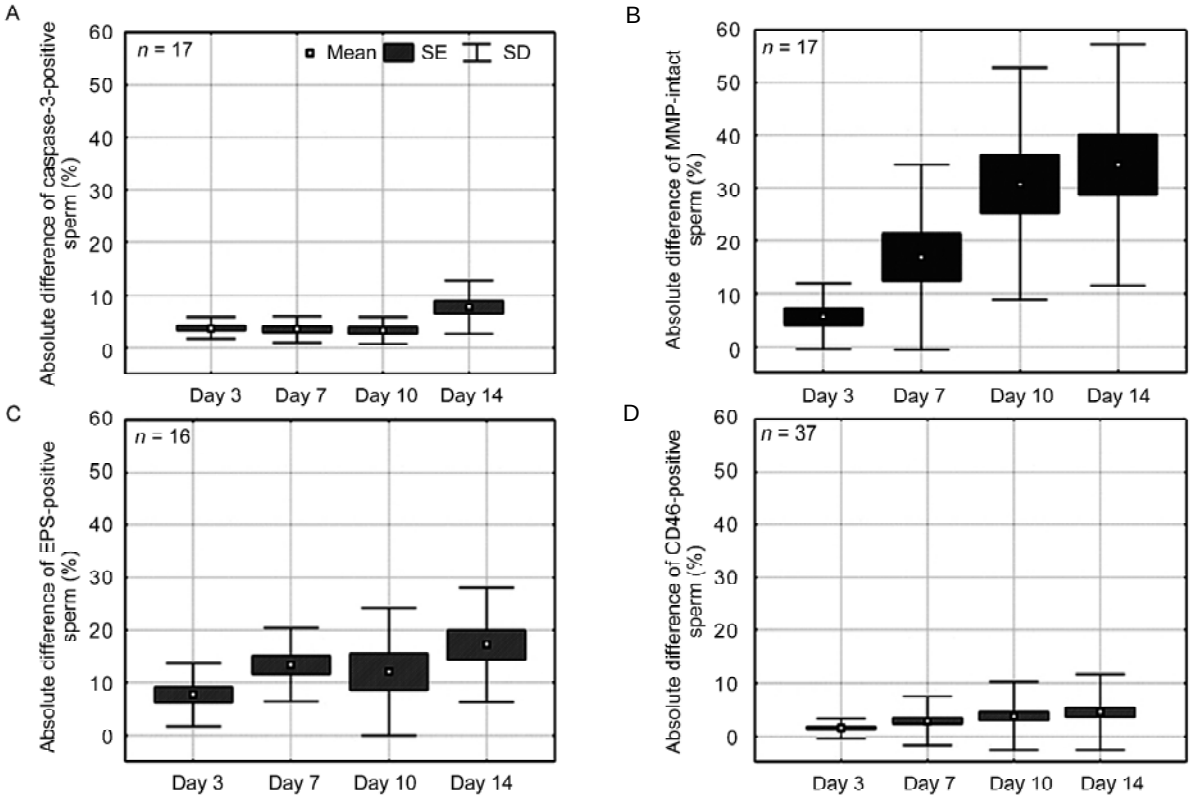

Figure 1. Absolute difference of the percentage of caspase-3 positive sperm (A), sperm with disrupted mitochondrial transmembrane potential (MMP, B), with externalized phosphatidylserine (EPS, C) and CD46 positive sperm (D) at days 3, 7, 10 and 14 compared to day 0. While the fluorescence signal of caspase-3 positive sperm remained stable for 10 days and of CD46 positive sperm for 14 days, analysis of MMP and EPS should be performed at the same day to avoid differences > 5%.

ing might be subjected to further optimization according to cellular changes on a molecular level [23]. The commercially available assays as well as the detection by FITC-coupled antibodies are easy to perform in a reasonable timeframe. The Caspase-3 FLICA™ showed mean differences of < 5% until day 10. In contrast, the Mitosensor™ and the EPS detection showed differences of > 5% as early as day 3. The CD46-FITC labeling displayed absolute differences of < 5% of CD46 positive sperm on days 3, 7, 10 and 14. Although immediate analysis of the fluorescence signals is recommended, it is possible to evaluate activation of caspase-3 up to 10 days after staining of human sperm. Labeling with anti-CD46-FITC on the sperm surface gives very stable fluorescence signals and flow cytometric analysis can be performed within at least the following 2 weeks. The FACS evaluation of MMP integrity and EPS detection should be conducted on the same day.

Acknowledgment

We would be grateful to funds from the German Research Council (Deutsche Forschungsgemeinschaft, DFG GL 199/4-4).

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