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Comparison of four fluorochromes for the detection of the inner mitochondrial membrane potential in human spermatozoa and their correlation with sperm motility

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BACKGROUND: Sperm motility evaluation is associated with fertility in IVF programmes. The visual estimation of sperm motility is extremely subjective. Hence, alternative methods are required. Among them, determination of mitochondrial membrane potential ($\Delta\psi_m$) changes of spermatozoa using potentiometric dyes may be a reliable test to determine sperm quality. However, the use of the potentiometric dyes in sperm samples has not been compared. METHODS: We have studied sperm samples from 28 infertile patients enrolled in an IVF programme in flow cytometry after staining of spermatozoa with four commonly used potentiometric dyes. Sperm motility was evaluated visually. RESULTS: As expected, JC-1 seems to detect specifically $\Delta\psi_m$ changes, CMX-Ros, $DiOC_6(3)$ and TMRE fluorescence is easily analysed and the latter three fluorochromes are particularly suitable for multiparametric staining. Irrespective of the $\Delta\psi_m$-dependent fluorochromes used to stain spermatozoa, a positive correlation was found between the percentage of $\Delta\psi_m^{high}$ cells and forward motility and also with high fertilization rates after IVF. CONCLUSION: The four fluorochromes may be useful for evaluation of sperm samples from infertile patients. The choice of the potentiometric dyes will depend on their fluorescence characteristics in order to use them in combination with other fluorescent markers.

Key words: flow cytometry/IVF/mitochondria/spermatozoa

Introduction

Failed fertilization happens in 5–10% of IVF cycles and may result from defective spermatozoa and/or oocytes. In the IVF setting, most cases result from male factor deficiencies (Liu and Baker, 2000). Consequently, a variety of tests assessing sperm quality may be useful in determining the likelihood of successful IVF (for a review see Mahutte and Arici, 2003). Among them, standard semen analysis including sperm count, motility and morphology is the most commonly used as a fundamental indicator of male fertility. However, semen analysis has limited clinical value for predicting IVF since 50% of couples with failed fertilization have a normal pre-IVF semen analysis (Liu and Baker, 2000). Due to extreme variability in estimating sperm motility, it is important to develop objective measurements. Thus, other sperm function tests are needed to improve the clinical management of patients and to allocate them to the best assisted reproductive technology programme (IVF or ICSI).

Analysing mitochondrial function may offer a means of assessing the motility of sperm. This can be achieved by determining the inner mitochondrial membrane potential ($\Delta\psi_m$) in sperm cells. The $\Delta\psi_m$ is a sensitive indicator for the energetic state of the mitochondria and the cell, and can be used to assess the activity of the mitochondrial respiratory chain, electrogenic transport systems and the activation of the mitochondrial permeability transition (for a review see Ly *et al.*, 2003). Thus, determination of $\Delta\psi_m$ is widely used for characterization of cellular metabolism, viability and apoptosis in various cellular models. In human, a correlation exists between poor sperm mitochondrial function detected by reduction of $\Delta\psi_m$, and diminished motility and reduced fertility (Troiano *et al.*, 1998; Donnelly *et al.*, 2000; Marchetti *et al.*, 2002; Piasecka and Kawiak, 2003; Wang *et al.*, 2003).

Several fluorimetric methods using cationic lipophilic dyes have been utilized to measure the $\Delta\psi_m$. The cationic lipophilic dyes accumulate in mitochondria depending on $\Delta\psi_m$, and the fluorescence of the accumulated fluorochromes corresponds to this potential. During the past decades, rhodamine 123 (Rh123) has been widely used as a fluorescent probe to assess $\Delta\psi_m$. However, contradictory data indicated that this probe was not fully satisfactory to measure $\Delta\psi_m$ because of the existence of several energy-independent Rh123-binding sites (Lopez-Mediavilla *et al.*, 1989).

To overcome these drawbacks, several potential sensitive dyes were developed including rosamines, rhodamine and carbocyanine derivatives: (i) Chloromethyl-X-rosamine (CMX-Ros) dye, that contains a mildly thiol-reactive chloromethyl moiety, is much more photostable than Rh123 and constitutes a valuable dye to analyse mitochondrial morphology and function (Poot *et al.*, 1996); (ii) The rhodamine derivative tetramethylrhodamine ethyl ester (TMRE) which has reduced hydrophobic character, also exhibits less potential-independent binding to cells than other rhodamines and has been described as one of the best fluorescent dyes for $\Delta\psi_m$ measurements in living cells (Loew *et al.*, 1993). However, like other rhodamines, TMRE used at high concentration induces fluorescence quenching so that an increase in mitochondrial fluorescence corresponds to depolarization (O'Reilly *et al.*, 2003); (iii) The carbocyanine derivative 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] offers the important advantage of not causing quenching effects (Metivier *et al.*, 1998) but $DiOC_6(3)$ uptake depends on both mitochondrial membrane and plasma membrane potentials (Salvioli *et al.*, 1997); (iv) The carbocyanine fluorescent probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) has been proposed to evaluate changes in $\Delta\psi_m$ accurately (Salvioli *et al.*, 1997).

Thus, interpreting and evaluating changes in $\Delta\psi_m$ may be somewhat confusing because there are substantial variations between these dyes depending on various susceptibilities to the surrounding environment. Therefore, comparing results obtained with different potentiometric dyes is useful to select the most accurate probe for a particular application.

In recent years, many investigators have used lipophilic cationic fluorochromes including Rh123 (Troiano *et al.*, 1998), JC-1 (Donnelly et al,. 2000; Piasecka and Kawiak, 2003) and $DiOC_6(3)$ (Marchetti *et al.*, 2002; Wang *et al.*, 2003) for $\Delta\psi_m$ determinations in sperm samples. A comparison of the ability of these potentiometric dyes to evaluate $\Delta\psi_m$ in sperm samples has not been made. To our knowledge, fluorescence from CMX-Ros or TMRE has never been analysed in human spermatozoa. Flow cytometry, when used in conjunction with $\Delta\psi_m$-dependent fluorochromes, could be an ideal method to study the mitochondrial potential in sperm samples. Indeed, flow cytometry provides a rapid, accurate and reliable estimation of the $\Delta\psi_m$ in a large number of cells and is of considerable relevance for laboratory practice.

Therefore, we have studied sperm samples from infertile patients enrolled in an IVF programme in flow cytometry after concomitantly staining with CMX-Ros, $DiOC_6(3)$, TMRE and JC-1.

We have compared the results obtained with these dyes and have established the correlation with the quality of sperm evaluated by conventional light microscopic analysis in spermatozoa prepared for IVF. This approach allowed us to discuss the advantages and limitations of $\Delta\psi_m$-dependent cytofluorometric assays for assessment of sperm quality in the reproductive biology laboratory.

Materials and methods

Materials

Ferticult medium was purchased from Fertipro NV (Beernem, Belgium) and PureSperm gradient from NidaCon International AB (Gothenburg, Sweden). $DiOC_6(3)$, JC-1, TMRE, CMX-Ros, propidium iodide (PI) and YOPRO-1 were obtained from Molecular Probes Inc. (Eugene, OR). All other reagents were purchased from Sigma Chemical Co. (St Louis, MO).

Collection of semen samples

We studied 28 male subjects who underwent seminal fluid evaluation at the Laboratory of Reproductive Biology (CHRU, Lille). All subjects were the partners of women who had failed to conceive after 2 years of unprotected intercourse. Patient information remained confidential and within the institution. This study was conducted according to guidelines established for research on human subjects (Ethical committee, CHRU Lille). The samples were collected by masturbation into sterile plastic jars, after 3–5 days of sexual abstinence.

Preparation of semen samples

To isolate spermatozoa, an aliquot of semen was purified using a three-step discontinuous Pure Sperm gradient (90–70–50%) diluted in Ferticult medium. After centrifugation at 300 *g* for 20 min, purified populations of highly motile spermatozoa (from the 90% layer) were recovered, washed in Ferticult medium,

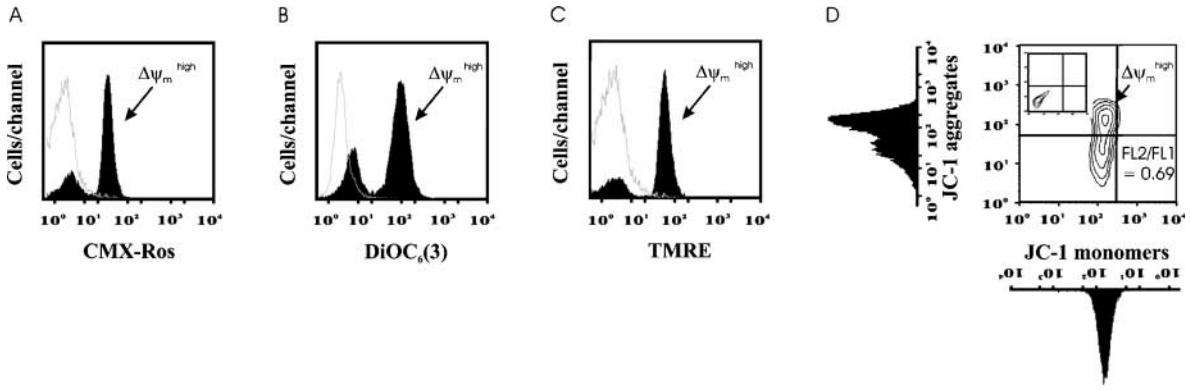

Figure 1. Representative examples of stained spermatozoa. Cytofluorometric analysis of histograms of spermatozoa from one sample stained with the following potentiometric dyes: (**A**) CMX-Ros 50 nmol/l for 20 min, (**B**) $DiOC_6(3)$ 20 nmol/l for 20 min and (**C**) TMRE 300 nmol/l for 20 min (black profiles). Unlabelled sample is represented by white profiles. (**D**) Example of a contour plot (and corresponding histograms) of JC-1-stained spermatozoa (JC-1 1.5 μmol/l for 20 min). The insert shows a contour plot of unlabelled spermatozoa.

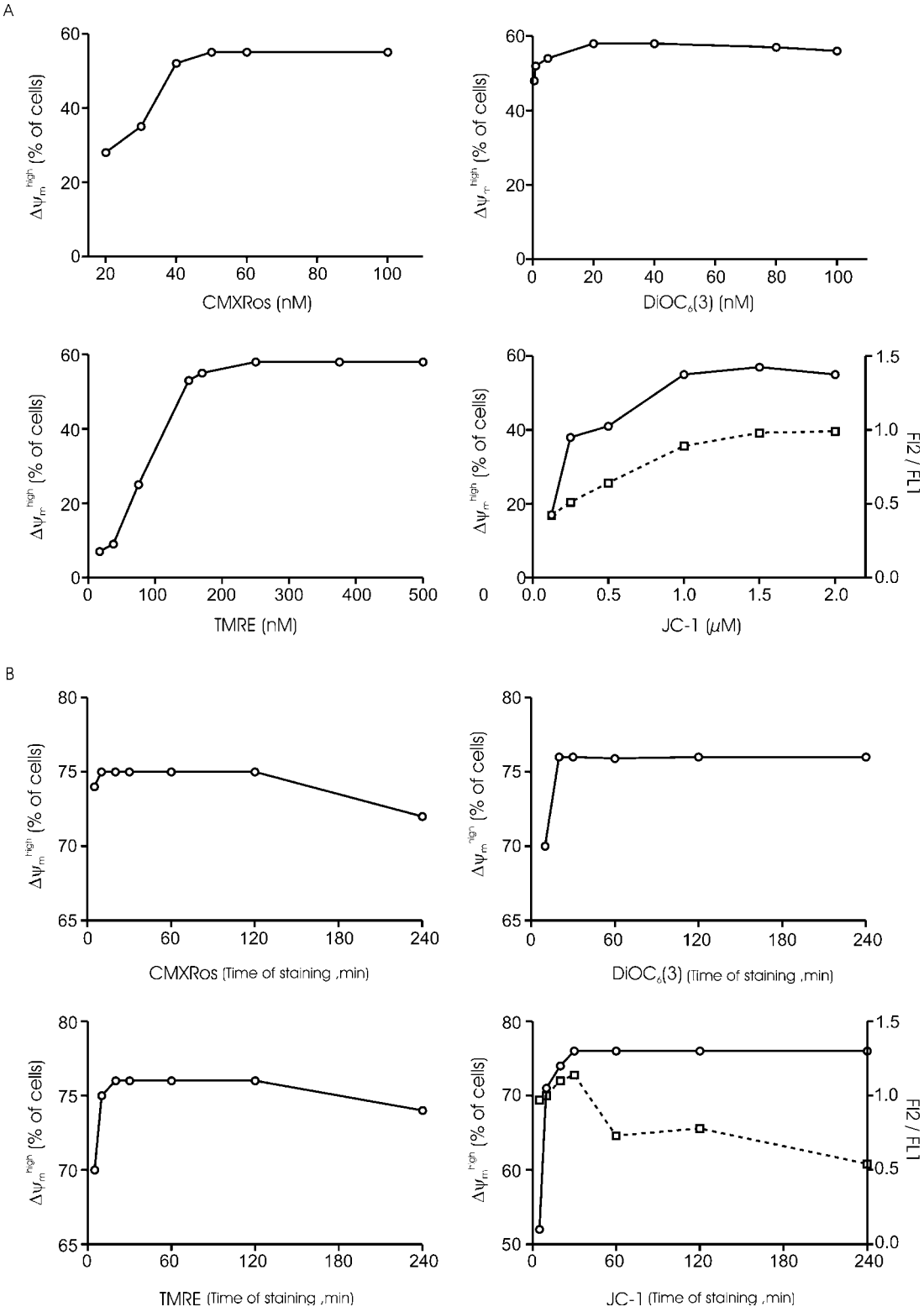

Figure 2. Variations of the percentage of $\Delta\psi_m^{high}$ spermatozoa depending on experimental conditions. (**A**) Representative dose response of the loading concentration of fluorochromes. Spermatozoa were incubated in medium for 30 min with different doses of CMX-Ros (upper left panel), $DiOC_6(3)$ (upper right panel), TMRE (lower left panel) or JC-1 (lower right panel) and analysed by flow cytometry. The results are representative of three samples, each from a different patient. (**B**) Kinetics of fluorochrome uptake. Spermatozoa were incubated with 50 nmol/l CMX-Ros (upper left panel), 20 nmol/l $DiOC_6(3)$ (upper right panel), 250 nmol/l TMRE (lower left panel) or 1 μmol/l JC-1 (lower right panel). At the indicated times, cells were analysed by flow cytometry. The results are representative of five samples, each from a different patient. The percentage of $\Delta\psi_m^{high}$ cells was calculated as previously described. When cells are stained with JC-1 (lower right panel in A and B), the open circles indicate the values of the percentage of $\Delta\psi_m^{high}$ spermatozoa and the open squares represent the values of the FL2/FL1 ratio. (A) and (B) refer to different samples.

and resuspended in 1 ml of the same medium. Prepared spermatozoa were counted and the percentage of forward motile spermatozoa was calculated. Prepared sperm was used for IVF and aliquots taken for cytofluorometric experiments.

Conservation of spermatozoa before $\Delta\psi_m$ labelling

Typically, purified spermatozoa were subjected to flow cytometry within 1 h. In some experiments, we set up conditions of spermatozoa conservation that allow for the retention of the $\Delta\psi_m$. For this experiment, samples were stored at 4°C, room temperature or 37°C in medium until the times (0, 30, 60 and 120 min) that the staining with the potentiometric dyes were performed (see below).

Cytofluorometric assessment of mitochondrial membrane potential

Stock solutions of JC-1 (2.5 mmol/l), TMRE (1 mmol/l) and CMX-Ros (1 mmol/l) were made in dimethylsulphoxide (DMSO). $DiOC_6(3)$ (4 mmol/l) was dissolved in ethanol. Stock solutions were stored in small aliquots at −20°C, and subsequent working solutions [dilutions 1:250 for JC-1, 1:40 for TMRE; 1:200 for CMX-Ros; 1:2000 for $DiOC_6(3)$] were made in experimental medium immediately before use. A total of 5×10^5 spermatozoa were incubated in the Ferticult medium with the fluorochromes at 37°C, followed by analysis on a cytofluorometer. Except when indicated for Figures 1 and 2, we used optimal conditions of staining defined as 50 nmol/l CMX-Ros for 20 min, 20 nmol/l $DiOC_6(3)$ for 20 min, 250 nmol/l TMRE for 20 min, and 1 μmol/l JC-1 for 30 min. All flow cytometry experiments were performed on a Coulter XL cytofluorometer (Coulter Corp., Hialeah, FL). Data were acquired using Expo 32 software (Coulter). The analyser threshold was adjusted on the forward scatter channel to exclude subcellular debris. Forward and side scatters were gated on the major population of normal size cells and a minimum of 10 000 cells was analysed. The fluorescent probes $DiOC_6(3)$, JC-1, CMX-Ros and TMRE were excited with the 488 nm argon laser. Signals from the $DiOC_6(3)$ fluorescence were collected through the FL1 detector (525 ± 5 nm band pass filter), CMX-Ros through the FL3 detector (620 ± 5n m band pass filter) and TMRE through the FL2 channel (575 ± 5 nm band pass filter). The fluorescence signals of JC-1 monomers and aggregates were detected through the FL1 (525 ± 5 nm band pass filter) and FL2 channels (575 ± 5 nm band pass filter), respectively. Control experiments were performed in the presence of carbamoylcyanide *m*-chlorophenylhydrazone (ClCCP) or the K^+ ionophore valinomycin. ClCCP is a protonophore that uncouples oxidation from phosphorylation by dissipating the chemiosmotic gradient and induces dissipation of $\Delta\psi_m$. Spermatozoa were incubated with 0.5 μmol/l ClCCP or 100 nmol/l valinomycin for 30 min at 37°C then stained with the potentiometric dyes as described above.

Double staining of $\Delta\psi_m$ and cell viability

YOPRO-1 and PI were used as supravital fluorescent stains. Both YOPRO-1 and PI are membrane-impermeant nuclear fluorescent dyes. When plasma membrane integrity is altered, these dyes enter cells (non-viable cells), bind to nucleic acids and exhibit fluorescence. YOPRO-1 and PI were excited at 488 nm. YOPRO-1 fluorescence was monitored in FL1 (525 ± 5 nm band pass filter) and PI in FL3 (620 ± 5 nm band pass filter). These emission wavelengths allow us to define double staining with potentiometric dyes. We used the following probe combinations: CMX-Ros and YOPRO-1; $DiOC_6(3)$ and PI; and TMRE and YOPRO-1. Preliminary results shown that $\Delta\psi_m$ fluorescence did not change with YOPRO-1 or PI addition.

Spermatozoa were exposed for 20 min at 37°C to CMX-Ros (50 nmol/l) and YOPRO-1 [200 nmol/l in phosphate-buffered saline (PBS)], to $DiOC_6(3)$ (20 nmol/l) and PI (5 μg/ml) or to TMRE (250 nmol/l) and YOPRO-1 (200 nmol/l in PBS). Immediately after staining, spermatozoa were analysed by flow cytometry.

Fluorescence microscopy

Immediately after the $\Delta\psi_m$ staining procedure, counterstaining of nuclei was performed with Hoescht 33342 1 μg/ml for 10 min in the dark. Then, cells were washed once in PBS and resuspended in Vectashield H-100 mounting medium (Vector Laboratories, Burlingame, CA), coverslipped and analysed with a Leica DMLR epifluorescence microscope, using a Leica 63 × /1.32 HCX PL APO objective (Leica S.A., Rueil Malmaison, France). Images were captured using Leica software.

Statistical analysis

Results were analysed using GraphPad Prism version 3.00 (GraphPad Software, San Diego CA). The Pearson rank correlation test was used to calculate the correlation coefficient between flow cytometric analyses. The Spearman rank correlation test was employed to evaluate the relationship between semen analysis parameters and cytofluorometric results. Statistical significance was set at $P < 0.05$. To test the reproducibility of the assays, 10 replicates from a single sample were processed and acquired by the same operator for each staining, then typical intra-assay precision tests were performed.

Results

Conventional semen analysis

Table I shows patients' age and contains results of classical semen analysis performed by light microscopy with samples from a total of 28 men consulting for sterility.

Typical representation of cytofluorometric profiles after spermatozoa staining

Figure 1 represents an example of cytofluorometric profiles of spermatozoa stained with the four putative $\Delta\psi_m$-sensitive fluorescence probes used in this study. All these potentiometric probes are lipophilic cationic fluorochrome able to detect $\Delta\psi_m$ in viable cells. They distribute passively between the cytosol and mitochondria according to the Nernst

Table I. Patient age, and sperm characteristics of semen and prepared spermatozoa from infertile patients

	No. of samples analysed	Mean ± SEM	Minimum	Median	Maximum
Patient age	28	35 ± 1	20	34.5	55
Sperm concentration (× 10^6/ml)	28	101 ± 17	25	74	232
Progressive motility (a + b) in neat semen (%)	28	30 ± 3	5	30	50
Forward motility (a) in prepared spermatozoa (%)	28	79 ± 3	50	80	98
Normal morphology by David's criteria (%)	28	43 ± 3	10	44	72

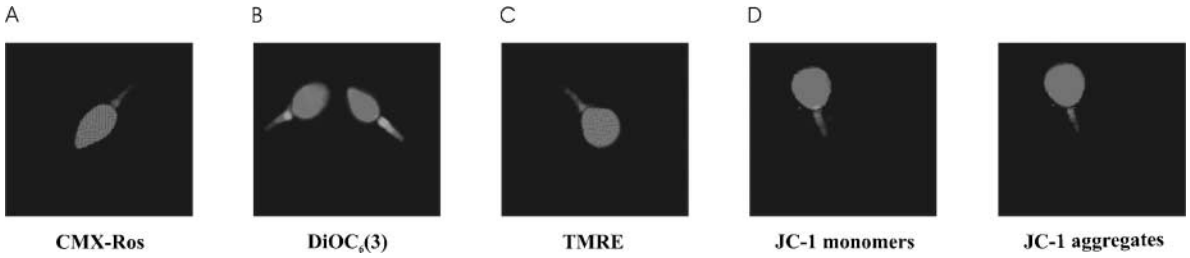

Figure 3. Photomicrographs of spermatozoa labelled with fluorochromes. The sample was stained with either 50 nmol/l CMX-Ros for 20 min (**A**), 20 nmol/l $DiOC_6(3)$ for 20 min (**B**), 250 nmol/l TMRE for 20 min (**C**) or 1 μmol/l JC-1 for 30 min (**D**), then counterstained with the nuclear dye, Hoechst 33342. Spermatozoa that were stained with CMX-Ros or TMRE displayed midpieces (where mitochondria are located) that fluoresced bright red, and $DiOC_6(3)$-stained midpieces appeared green. After JC-1 staining (D), the left and right panels show the monomer (green) and aggregate (red) fluorescence, respectively. In this case, a perfect co-localization of green and red fluorescence signals was observed. Original magnification ×630.

equation, where transmembrane distribution depends on the mitochondrial membrane potential. Flow cytometric analysis of spermatozoa stained with either CMX-Ros (Figure 1A), $DiOC_6(3)$ (Figure 1B) or TMRE (Figure 1C) revealed in each case two cellular subsets displaying different levels of fluorescence. These two subpopulations had distinct incorporation of fluorochromes into spermatozoa. One subpopulation that incorporated more (1–1.5 log more) fluorochrome which corresponded to spermatozoa with high fluorescence signals [CMX-Ros^{high}, $DiOC_6(3)$ or TMRE^{high}] were called $\Delta\psi_m{}^{high}$ cells (Figure 1A, B and C, respectively). For each fluorochrome, the percentage of cells with $\Delta\psi_m{}^{high}$ was determined in sperm samples and used for the study (see below).

To monitor the $\Delta\psi_m$, we have also chosen the carbocyanine dye JC-1 which has been shown to be more reliable than other fluorescent dyes for detecting changes in $\Delta\psi_m$ due to its dual emission characteristics (Cossarizza *et al.*, 1996). Mitochondria with $\Delta\psi_m{}^{high}$ concentrate JC-1 into aggregates (red-orange fluorescence in the FL2 channel), while in depolarized mitochondria JC-1 forms mainly monomers (green fluorescence in the FL1 channel). A two-parameter fluorescence display of JC-1-stained spermatozoa reveals that most of the cells emitted relatively high levels of both green and orange-red fluorescence, whereas a subpopulation exhibited a reduced JC-1 aggregation and a decrease in the orange-red fluorescence emission, a finding that indicates a drop in $\Delta\psi_m$ (Figure 1D). By flow cytometry, a high correlation has been found previously between mitochondrial membrane potential values in isolated mitochondria and fluorescence ratio (mean red-orange fluorescence intensity/mean green fluorescence intensity corresponding to the FL2/FL1 ratio) (Cossarizza et al,. 1996). Therefore, in order to determine the population of cells with $\Delta\psi_m{}^{high}$ in sperm samples after JC-1 staining, we evaluated both the percentage of cells which concentrate JC-1 into aggregates (high fluorescence of JC-1 red-orange in the upper left quadrant) called $\Delta\psi_m{}^{high}$

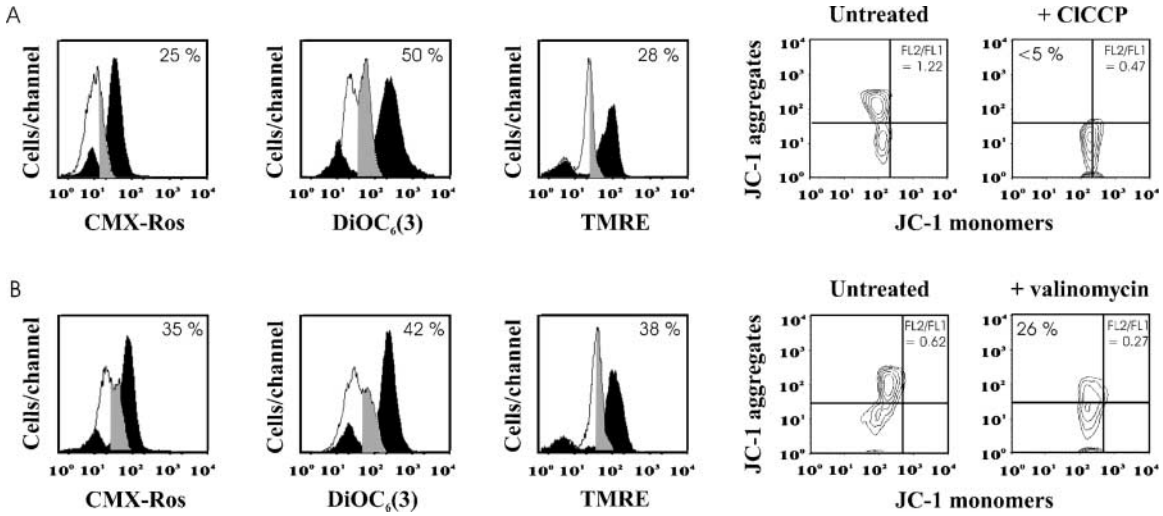

Figure 4. Cytofluorometric analysis of $\Delta\psi_m$ in spermatozoa treated with depolarizing agents. Fluorescence pattern of spermatozoa treated with ClCCP (**A**) or valinomycin (**B**) then stained with either CMX-Ros, $DiOC_6(3)$, TMRE or JC-1 according to staining procedures defined in Figure 3. Fluorescence histograms (left) of spermatozoa kept untreated (black profiles) or treated with depolarizing agents (white profiles) are represented when spermatozoa are stained with either CMX-Ros, $DiOC_6(3)$ or TMRE. When cells are stained with JC-1, contour plots are indicated (right). Quadrant boundaries were set with reference to untreated spermatozoa. Grey profiles correspond to the ionophore-treated spermatozoa which maintain a high $\Delta\psi_m$. Numbers indicate the percentage of ionophore-treated spermatozoa with $\Delta\psi_m{}^{high}$ (grey profiles). The results are representative of five samples, each from a different patient. (A) and (B) refer to different samples.

Table II. Correlations (Pearson correlation test) among flow cytometric methods for the detection of $\Delta\psi_m$ changes in spermatozoa

r	$DiOC_6(3)$[a]	CMX-Ros[b]	TMRE[c]	JC-1[d]	JC-1 ratio[e]
$DiOC_6(3)$[a]		0.94*	0.97*	0.78*	0.66*
CMX-Ros[b]			0.96*	0.70*	0.65*
TMRE[c]				0.74*	0.64*
JC-1[d]					0.60*

[a] Percentage of $DiOC_6(3)^{high}$.
[b] Percentage of CMX-Ros^{high}.
[c] Percentageof $TMRE^{high}$.
[d] Percentage of JC-1 $aggregates^{high}$.
[e] Ratio of JC-1 aggregates/JC-1monomers.
*$P < 0.05$.

cells and the values of the fluorescence ratio (JC-1 red-orange/JC-1 green or FL2/FL1 ratio) (Figure 1D).

Optimization of conditions for spermatozoa labelling

Incubation of spermatozoa with different concentrations of fluorochromes revealed that the percentage of cells emitting high fluorescence was influenced by the concentration of dye used (Figure 2A). This study indicated that the minimal dose of dye required to achieve an effective spermatozoa loading was 50 nmol/l of CMX-Ros, 20 nmol/l of $DiOC_6(3)$, 250 nmol/l of TMRE and 1 μmol/l of JC-1.

Figure 2B represents the time course for the uptake of dyes used at optimal concentrations. Under these conditions, a 20 min period of incubation was enough for $DiOC_6(3)$, CMX-Ros and TMRE to equilibrate into the cells. For JC-1 staining, a 30 min period of incubation was needed and allowed a better separation of the high fluorescence peak of JC-1 red-orange (data not shown). These patterns of cell fluorescence were stable and remained unchanged for at least 120 min of incubation in the medium at 37°C, except for the FL2/FL1 ratio.

In the optimal conditions of staining based on the above results, fluorescence microscopy (Figure 3) was used to verify that each dye accurately measured the correct sperm compartment. The spermatozoa stained with the $\Delta\psi_m$-sensitive fluorescence probes in conditions defined above were counterstained with the DNA marker Hoechst 33342. In each case, a high level of fluorescence was associated with the sperm midpiece where mitochondria are located. No other portion of spermatozoa displayed fluorescence that could be detected microscopically, indicating a characteristic mitochondrial uptake of all fluorochromes.

Sensitivity of potentiometric dyes to $\Delta\psi_m$ changes

Under the optimal conditions of staining defined above, we used mitochondria-targeted drugs to ascertain that the fluorochromes were able to measure $\Delta\psi_m$ variations in spermatozoa. Exposure of spermatozoa to ClCCP significantly reduced fluorochrome incorporation into cells by 0.5–1 logs for all fluorochromes used (Figure 4A).

To confirm the ability of fluorochromes to detect changes in $\Delta\psi_m$, we also used valinomycin, which is able to collapse $\Delta\psi_m$ (Figue 4B). After 30 min of incubation with valinomycin, spermatozoa stained with CMX-Ros, $DiOC_6(3)$, TMRE or JC-1 changed their fluorescence pattern, as observed after ClCCP incubation.

These results indicate that under our experimental conditions, the four potentiometric fluorochromes reveal a $\Delta\psi_m$ variation in spermatozoa. However, when we displayed the percentage of ionophore-treated spermatozoa which retain a high $\Delta\psi_m$ (grey profiles in Figure 4A and B), the remaining fluorescence was much lower in JC-1-stained spermatozoa than in cells labelled with the other fluorochromes.

We also found a highly significant relationship between all four cytofluorometric methods in samples (Table II), confirming that any potential-sensitive fluorochromes detect $\Delta\psi_m$ changes of spermatozoa.

To test the reproducibility of the assays, 10 replicates of a sperm sample were analysed by each staining. The mean percentage of $\Delta\psi_m{}^{high}$ was 95 ± 0.4 by CMX-Ros staining, 91.8 ± 1.4 by $DiOC_6(3)$ staining, 92.6 ± 1 by TMRE staining and 88.5 ± 1 by JC-1 staining. We found that the coefficients of variation (CVs) of these flow cytometric assays were 0.5% for the CMX-Ros staining, 1.6% for the $DiOC_6(3)$ staining, 1.1% for the TMRE staining and 1.2% for the JC-1 staining.

Conservation of spermatozoa before $\Delta\psi_m$ labelling

Results showed that spermatozoa stored at 4°C undergo a decrease in the percentage of $\Delta\psi_m{}^{high}$ cells irrespective of the $\Delta\psi_m$-dependent dye used to stain spermatozoa (Figure 5). In any case, prepared spermatozoa stored at room temperature or at 37°C maintained a constant proportion of $\Delta\psi_m{}^{high}$ cells at least for the first 60 min.

Simultaneous determination of $\Delta\psi_m$ and cell viability

A double staining procedure was developed to assess $\Delta\psi_m$ and cell viability simultaneously in spermatozoa (Figure 6). We used the following probe combinations: CMX-Ros and YOPRO-1 (Figure 6A), $DiOC_6(3)$ and PI (Figure 6B) and TMRE and YOPRO-1 (Figure 6C), in order to have compatible emission wavelengths. All non-viable spermatozoa (YOPRO-1- or PI-marked spermatozoa) had low $\Delta\psi_m$ [CMX-Ros^{low}, $DiOC_6(3)^{low}$ or $TMRE^{low}$], whereas viable spermatozoa (YOPRO-1- or PI-negative) contained two distinct subpopulations, one that exhibited a reduction in $\Delta\psi_m$ comparable with non-viable spermatozoa and the other that displayed high $\Delta\psi_m$ (Figure 6). This suggest that the $\Delta\psi_m$ collapse occurs at an early stage before the loss of viability.

Table III. Correlations of cytofluorometric $\Delta\psi_m$ markers in spermatozoa with fertilization rate

Percentage spermataozoa	*n*	Spearman coefficient *r*	*P*-value
CMXRos^{high}	28	0.40	0.02
$DiOC_6(3)^{high}$	28	0.44	0.01
$TMRE^{high}$	28	0.36	0.03
JC-1^{high}	28	0.36	0.03
JC-1 ratio	28	0.34	0.03

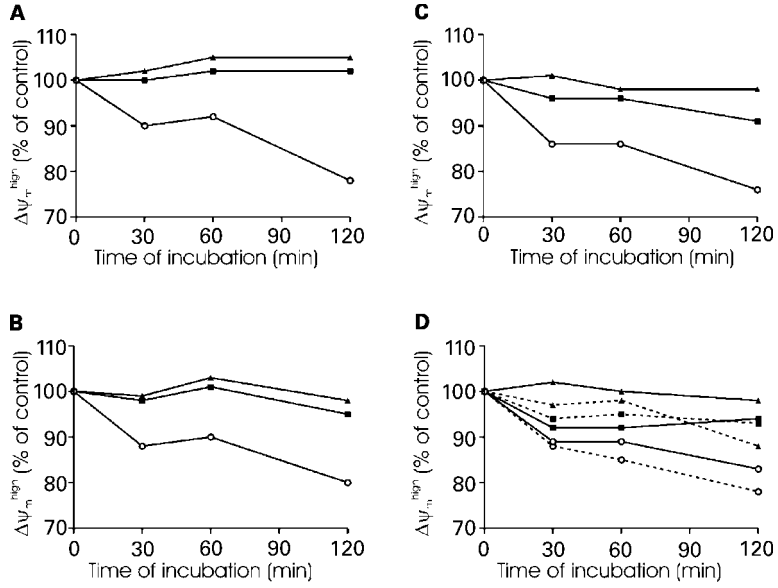

Figure 5. Effect of sample storage on $\Delta\psi_m$. Spermatozoa were kept at 4°C (○), room temperature (■) or 37°C (▲) over time (120 min). At the indicated times, cells were stained with either CMX-Ros (**A**), $DiOC_6(3)$ (**B**), TMRE (**C**) or JC-1 (**D**) according to staining procedures defined in Figure 3. Results are expressed as a percentage of $\Delta\psi_m^{high}$ cells at time zero (control). When cells are stained with JC-1 (D), the results are presented both as a percentage of $\Delta\psi_m^{high}$ cells at time zero (solid lines) and as a percentage of the FL2/FL1 ratio at time zero (dotted lines). Data are representative of three samples, each from a different patient.

Correlation between $\Delta\psi_m^{high}$ cells and sperm motility

Regarding the relationship between the percentage of spermatozoa that were highly motile and the percentage of $\Delta\psi_m^{high}$ spermatozoa measured by the four fluorochromes, a positive correlation was found to be significant for all $\Delta\psi_m$-dependent dyes (Figure 7).

Correlation between $\Delta\psi_m^{high}$ cells and the fertilization rate

We also determined the correlation between the percentage of $\Delta\psi_m^{high}$ spermatozoa determined by the four potentiometric dyes and fertilization rates (Table III). Fertilization rate correlated positively with $\Delta\psi_m^{high}$ cells for all $\Delta\psi_m$-dependent dyes.

Discussion

The assessment of $\Delta\psi_m$ in intact spermatozoa is attracting growing interest since functional mitochondria have been related mainly to sperm motility. Thus, the assessment of $\Delta\psi_m$ could represent an important test to determine the quality of sperm of infertile men enrolled in an assisted reproductive programme. Cationic lipophilic fluorochromes have been widely used to assess the functionality of mitochondria in numerous cells including spermatozoa (Garner *et al.*, 1997; Troiano *et al.*, 1998; Gravance *et al.*, 2000, 2001; Marchetti *et al.*, 2002; Piasecka and Kawiak, 2003; Wang *et al.*, 2003). These fluorochromes are permeable to the plasma membrane and therefore can be easily used on living cells to assess $\Delta\psi_m$ changes. However, the behaviour of the dyes may depend on environmental events independent of the $\Delta\psi_m$. It has been observed that some of these fluorochromes (i) are highly toxic and interfere with the bioenergetic function of mitochondria (Modica-Napolitano *et al.*, 1996); (ii) may undergo self-quenching upon accumulation in the mitochondrial matrix which is responsible for

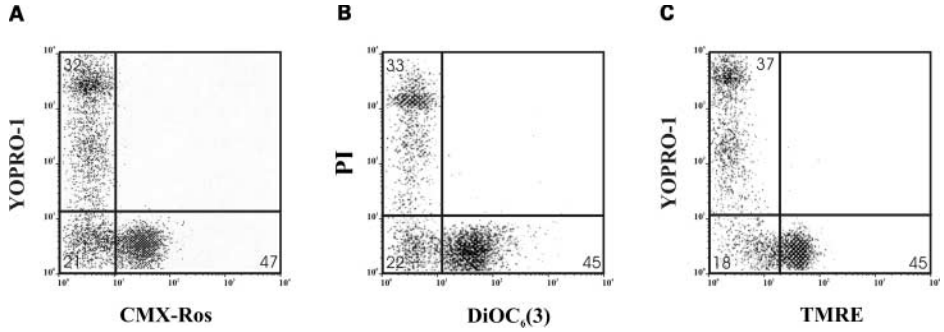

Figure 6. Representative examples for simultaneous $\Delta\psi_m$ measurement and cell viability in spermatozoa. Spermatozoa were stained with either CMX-Ros and YOPRO-1 (**A**), $DiOC_6(3)$ and PI (**B**), or TMRE and YOPRO-1 (**C**) as described in Materials and methods. The abscissa indicates the fluorescence intensity of spermatozoa stained with potentiometric dyes [CMX-Ros, $DiOC_6(3)$ or TMRE] and the ordinate indicates the fluorescence intensity of spermatozoa stained with impermeant nuclear dyes (YOPRO-1 or PI). Numbers indicate the percentage of spermatozoa in each quadrant. Results are representative of two samples, each from a different patient.

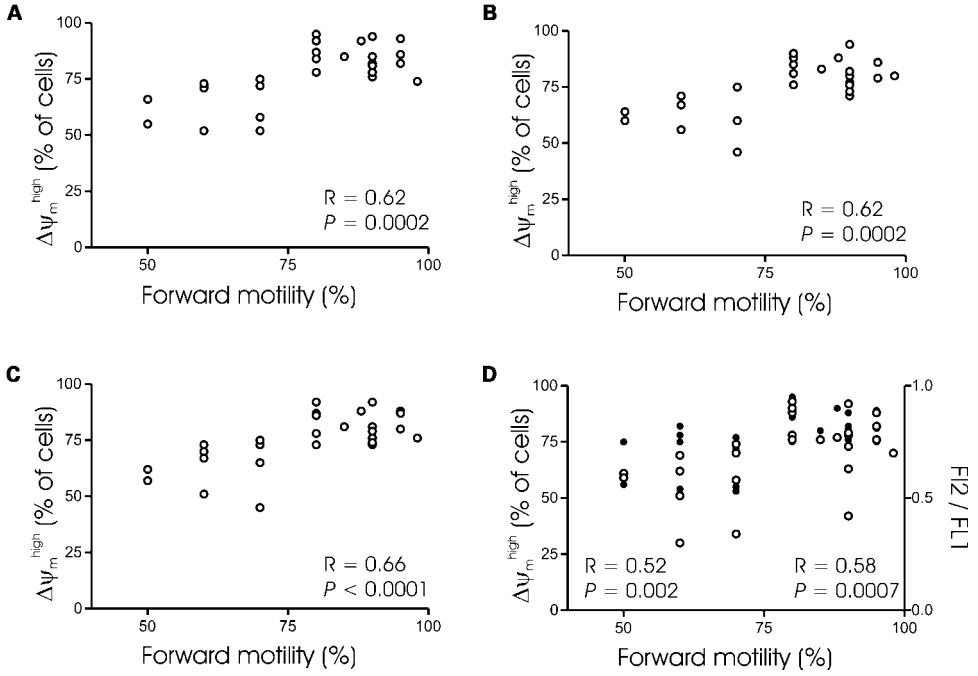

Figure 7. Relationship between forward motility and the percentage of $\Delta\psi_m^{high}$ spermatozoa. Spermatozoa from 28 subjects were stained with either CMX-Ros (**A**), $DiOC_6(3)$ (**B**), TMRE (**C**) or JC-1 (**D**) according to staining procedures defined in Figure 3. Results are expressed as a percentage of $\Delta\psi_m^{high}$ cells. R indicates the Spearman correlation factor. When cells are stained with JC-1 (D), open circles indicate the values of the percentage of $\Delta\psi_m^{high}$ spermatozoa and closed circles represent the values of the FL2/FL1 ratio. R and P indicate the statistical values when results are expressed as a percentage of $\Delta\psi_m^{high}$ cells (left) or as a FL2/FL1 ratio (right).

a paradoxical relationship between fluorochrome concentration and fluorescence (Metivier *et al.*, 1998); (iii) may show non-specific interaction with lipids or thiols (Ferlini *et al.*, 1998); (iv) may be influenced by the multidrug resistance-associated proteins (Kuhnel *et al.*, 1997); and (v) may depend on the magnitude of plasma membrane potential ($\Delta\psi_p$) (Rottenberg and Wu, 1998).

Thus, determining advantages and limitations of different $\Delta\psi_m$-sensitive fluorescence probes in spermatozoa is an essential step to develop efficient test(s) to evaluate sperm quality.

We first optimized the conditions of dye labelling. To increase the actual contribution of $\Delta\psi_m$ to cell fluorescence, it has been suggested to reduce dye concentrations, i.e. the dye/cell ratio. Indeed, lowering the dye/cell ratio reduces the toxicity, the quenching effect and the importance of the $\Delta\psi_p$ in cell fluorescence (Rottenberg and Wu, 1998). Thus, we have chosen the lowest concentration of fluorochromes (and the shortest incubation time) that do not reduce the percentage of $\Delta\psi_m^{high}$ spermatozoa and that allow us to discriminate unambiguously between spermatozoa with high and low $\Delta\psi_m$. However, even in these optimal conditions, we cannot exclude that $\Delta\psi_m$-independent processes could contribute at least in part to the fluorescence observed.

For an ideal $\Delta\psi_m$-sensitive fluorescence probe, specific alterations of the $\Delta\psi_m$ should result in decreased uptake of the fluorescence probe in the mitochondrial matrix. To test the capacity of the dyes to detect $\Delta\psi_m$ variations, we pre-incubated spermatozoa with uncouplers (Figure 4). We used the protonophore ClCCP and the K^+ ionophore valinomycin because it has been demonstrated that ClCCP may change the $\Delta\psi_p$ in human spermatozoa (Guzman-Grenfell *et al.*, 2000). Valinomycin was used at concentrations that modulate the $\Delta\psi_m$ without collapsing the $\Delta\psi_p$ (Rottenberg and Wu, 1998). In these experimental conditions, we confirmed that the four classical dyes, $DiOC_6(3)$, CMX-Ros, TMRE and JC-1, are sensitive enough to detect changes in $\Delta\psi_m$ induced by mitochondrial uncouplers. ClCCP and valinomycin caused a complete modification of JC-1 fluorescence, suggesting that JC-1 had an exclusive distribution to mitochondria. This observation is consistent with the fact that JC-1 is considered as a fluorochrome which can measure $\Delta\psi_m$ with great accuracy in intact cells (Salvioli *et al.*, 1997) including cardiomyocytes (Mathur *et al.*, 2000) and spermatozoa (Troiano *et al.*, 1998). In contrast, the uncoupler-induced decrease in CMX-Ros, $DiOC_6(3)$ and TMRE fluorescence was much smaller as the peak was in an intermediate position between those of spermatozoa with high and low fluorescence (Figure 4), indicating that CMX-Ros, $DiOC_6(3)$ and TMRE staining respond not only to $\Delta\psi_m$ changes but also to other non-specific ($\Delta\psi_m$-independent) processes. Thus, JC-1 appears to be the best probe to detect specifically $\Delta\psi_m$ changes in spermatozoa.

For an ideal probe suitable for explicit determination of $\Delta\psi_m$ in clinical samples, the resolution between the $\Delta\psi_m^{high}$ and $\Delta\psi_m^{low}$ fluorescence peaks should be maximal. As shown here, cytofluorometric profiles of spermatozoa stained with either CMX-Ros, $DiOC_6(3)$ or TMRE unambiguously revealed two distinct populations with $\Delta\psi_m^{high}$ and $\Delta\psi_m^{low}$. In any case, the determination of the percentage of $\Delta\psi_m^{high}$ spermatozoa was easily done by setting markers on histograms and using statistical functions of the software. JC-1

differs from rhodamines and other carbocyanines because it produces two fluorescence emission peaks that reflect the existence of two forms of the dye. The JC-1 monomers which emit green fluorescence are predominant at low $\Delta\psi_m$, while the JC-1 aggregates (red-orange fluorescence) are predominant at high $\Delta\psi_m$. Typically, it is described that upon lowering the $\Delta\psi_m$, the JC-1 aggregates dissipate into monomers and lead to a shift from red to green fluorescence. In fact, the intensity of the green fluorescence from the JC-1 monomer form seems to be insensitive to $\Delta\psi_m$ changes (Cossarizza *et al.*, 1996) and was instead used to monitor changes in mitochondrial mass (Mancini *et al.*, 1997). In spermatozoa, we found that disruption of $\Delta\psi_m$ does not lead to a significant increase in green fluorescence even after incubation with valinomycin (Figure 4B). Consequently, we have relied on the red-orange fluorescence emission of JC-1 aggregates to monitor changes in $\Delta\psi_m$. Nevertheless, as shown in Figure 1, the discrimination of spermatozoa with reduced and high $\Delta\psi_m$ was rather difficult since the resolution between the two fluorescence peaks of JC-1 aggregates was weak in many samples (see Figure 1D). Thus, it is more subjective to set markers on the JC-1 aggregate histograms. In our study, the use of JC-1 would lead to variations in the determination of the percentage of $\Delta\psi_m$ and could explain why the correlation factor among $\Delta\psi_m$-dependent fluorochromes is lower with JC-1 (Table II). One other possibility is to employ JC-1 as a ratiometric probe since a strict correlation has been found between the FL2/FL1 ratio and the $\Delta\psi_m$ in isolated mitochondria (Cossarizza *et al.*, 1996). Consistent with previous results (Gravance *et al.*, 2000; Mathur *et al.*, 2000), assessment of JC-1 staining by a ratiometric analysis did not provide better results. This is not surprising because variations in the mitochondrial mass, which can influence the FL2/FL1 ratio independently of changes in $\Delta\psi_m$, have been demonstrated in some cases of asthenospermia (Piasecka and Kawiak, 2003). Thus, the analysis of the percentage of $\Delta\psi_m{}^{high}$ stained with JC-1 seems to be more subjective than after staining with other potential-sensitive dyes.

To develop objective measurements of $\Delta\psi_m$ in semen samples, it is fundamental to achieve standardized protocols. First, we demonstrated acceptable reproducibility. Secondly, it is important to establish what, if any, is the influence on the $\Delta\psi_m$ of storage conditions of spermatozoa before flow cytometric analysis. Indeed, $\Delta\psi_m$-dependent fluorochromes are used on living cells and inadequate storage could seriously alter the $\Delta\psi_m$ of spermatozoa. The results of our study indicate that a conservation period at 4°C of spermatozoa in culture medium decreases the percentage of $\Delta\psi_m{}^{high}$ spermatozoa irrespective of the fluorochromes used. The recommendations from our study are that spermatozoa should be stored in culture medium at room temperature or 37°C for a maximum period of 60–120 min before flow cytometric analysis if they cannot be used immediately.

We compared the evaluation of $\Delta\psi_m{}^{high}$ by four fluorochromes with respect to their ability to correlate with forward motility. Prepared sperm with high $\Delta\psi_m$ correlated with forward motility, thus confirming the strong link between the functional status of mitochondria and sperm cell quality (Marchetti *et al.*, 2002; Wang *et al.*, 2003). Whereas JC-1 and $DiOC_6(3)$ have been used previously for sperm sample evaluation (Donnelly *et al.*, 2000; Marchetti *et al.*, 2002; Piasecka and Kawiak, 2003; Wang *et al.*, 2003), it is, to our knowledge, the first report describing CMX-Ros and TMRE as valuable probes to measure $\Delta\psi_m$ in sperm samples. Importantly, we found that all $\Delta\psi_m$-dependent fluorochromes were able to predict successful IVF, providing additional evidence supporting the importance of a flow cytometric $\Delta\psi_m$-based test in evaluation of spermatozoa for clinical studies.

Spermatozoa need to possess many attributes including a high motility in order to fertilize an oocyte, and sperm may be infertile for numerous reasons. Therefore, measuring multiple sperm parameters simultaneously on individual spermatozoa should provide a better indication of fertilizating capacity than one single parameter. One advantage of the flow cytometry is the possibility of evaluating, in combination, multiple fluorescent markers associated with individual spermatozoa in a population (Graham, 2001). Thus, flow cytometry should be used to analyse multiple sperm parameters (including the determination of $\Delta\psi_m$ and cell viability) to enhance the capacity to estimate the fertilizing potential of semen samples. For this reason, it is important to have several reliable $\Delta\psi_m$-dependent probes emitting in different fluorescence channels, which can be used in combination with other probes evaluating different sperm attributes. In order to evaluate $\Delta\psi_m$ and cell viability simultaneously, we developed double staining protocols. We used CMX-Ros, $DiOC_6(3)$ and TMRE as potential-sensitive dyes because they produce a single fluorescent emission peak allowing the combination with supravital fluorochromes. In contrast, JC-1 emits two fluorescence peaks (green and orange-red) which preclude simultaneous assessment of cell viability by commonly used supravital probes, because of fluorescence overlap. However, it should be noted that JC-1 has been used recently in combination with the impermeant dye TOTO-3 to investigate cell death (Zuliani *et al.*, 2003). Nevertheless, the major limitation of this method is the need to use a cytofluorometer equipped with multiple lasers to excite both TOTO-3 and JC-1. Thus, in contrast to JC-1, the fluorochromes CMX-Ros, $DiOC_6(3)$ or TMRE permit the development of a simple method in combination with other probes for multiparametric evaluation of sperm quality.

In conclusion, our results indicate that four classical $\Delta\psi_m$-dependent fluorochromes provide valuable tests assessing changes in mitochondrial membrane potential of human spermatozoa and may be usable for evaluation of sperm sample quality from infertile patients. These $\Delta\psi_m$ changes can be easily detected using cytoflurometric analysis of spermatozoa. Whereas JC-1 detects $\Delta\psi_m$ changes in spermatozoa more specifically than other dyes tested, CMX-Ros, $DiOC_6(3)$ and TMRE fluorescence is easily analysed and these fluorochromes are particularly suitable for multiparametric staining. The choice of the $\Delta\psi_m$-dependent fluorochromes will depend on their fluorescence characteristics in order to use them in combination with other sperm attribute-dependent fluorescent markers.

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