Study of mitochondrial membrane potential, reactive oxygen species, DNA fragmentation and cell viability by flow cytometry in human sperm

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BACKGROUND: Sperm cell death appears to be a cause of male infertility. The objective of this study was to determine the most reliable method for the evaluation of sperm quality in semen samples during sperm preparation for IVF. METHODS: Conventional analysis of semen samples was compared with several cytofluorometric methods detecting death-associated changes. Neat semen from infertile patients and sperm prepared by PureSperm® gradient were studied by conventional microscopy and analysed for mitochondrial membrane potential ($\Delta\psi_m$), generation of reactive oxygen species, DNA fragmentation and cell viability. RESULTS: In neat semen, a positive correlation was found between the percentage of $\Delta\psi_m^{high}$ sperm cells and standard semen parameters (concentration/motility). Sperm cells depicting $\Delta\psi_m^{high}$ and cells with low DNA fragmentation displayed high fertilization rate after IVF. The only changes that could be detected in prepared sperm were changes in $\Delta\psi_m$, with $\Delta\psi_m^{high}$ sperm positively correlated with forward motility and also with high fertilization rates after IVF. CONCLUSION: Analysis of mitochondrial membrane potential is the most sensitive test by which to determine sperm quality. These findings promise development of a test that may help to predict successful IVF.

Key words: cell death/DNA fragmentation/infertility/mitochondria/sperm

Introduction

Apoptosis is the mechanism by which superfluous, unwanted or damaged cells are removed to maintain tissue homeostasis in most organ systems during organogenesis and in the adult. The physiological presence of apoptosis was demonstrated in the human spermiogenetic epithelium (Baccetti *et al.*, 1996), indicating that this process is also necessary for the normal function of the adult reproductive tissues. Moreover, selective apoptosis of germ cells occurs to counteract the excess of clonal expansion during spermatogenesis. Germ cell apoptosis is required for functional spermatogenesis and development of normal mature sperm (Rodriguez *et al.*, 1997). It has been postulated that some forms of male infertility may result from an abortive programme of germ cell death (Sakkas *et al.*, 1999). In this case, the apoptotic process is incomplete and fails to eliminate germ cells even though the apoptotic programme has been initiated. These rescued cells undergo spermatogenesis and spermiogenesis resulting in mature sperm with an apoptotic phenotype often found in human semen from infertile patients (Baccetti *et al.*, 1996).

Recently, growing attention has been paid to the studies on determination of cell death pathways. Nuclear morphology and presence of chromosomal DNA strand breaks, easily detectable by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) method, are typical nuclear changes occurring during the final degradation phase of cell death. In numerous experimental models, the advanced DNA fragmentation is preceded by severe perturbations in mitochondrial function detected as a decrease in mitochondrial membrane potential ($\Delta\psi_m$). This reduction in $\Delta\psi_m$ is accompanied by the production of reactive oxygen species (ROS) contributing to cell death (Kroemer *et al.*, 1997).

The quality of sperm samples is one of the factors determining successful IVF. Currently sperm quality is evaluated by conventional semen analysis, determining sperm concentration, motility and morphology using light microscopy. These parameters comply with the World Health Organization (World Health Organization, 1999) criteria recommended for sperm classification. Although the conventional analysis of semen gives considerable information, new methods are still needed to make the evaluation of sperm fertilizing capacity *in vitro* easier and more reliable.

Numerous reports indicate that human sperm from infertile patients contain fragmented DNA and are less viable than that

of fertile men (Garner *et al.*, 1986; Host *et al.*, 1999). Mitochondrial dysfunction (Troiano *et al*., 1998) and increased ROS production have also been reported (Sharma and Agarwal, 1996).

In the present paper, we performed concomitantly four well-defined cytofluorometric assays to assess $\Delta\psi_m$, ROS generation, DNA fragmentation and sperm cell viability in semen samples from infertile patients enrolled in an IVF programme. The aim of this study was to establish correlation, if any, between these death-associated changes and the quality of sperm evaluated using conventional light microscopy analysis during the preparation of sperm for IVF.

Materials and methods

Materials

Ferticult® medium was purchased from Fertipro NV (Beernem, Belgium) and PureSperm® gradient from <GenX> International Inc. (Guilford, CT, USA). 3,3′-dihexyloxacarbocyanine iodide [$DiOC_6(3)$] and hydroethidine (HE) were obtained from Molecular Probes Inc. (Eugene, OR, USA). The TUNEL kit was from Promega (Madison, WI, USA) and DNAase I from Boehringer Mannheim/Roche, (Basel, Switzerland). Carbamoylcyanide m-chlorophenylhydrazone (mClCCP), menadione, and all other reagents were from Sigma Chemical Co. (St Louis, MO, USA). All flow cytometry experiments were performed on a Coulter XL® cytofluorometer (Coulter Corp., Hialeah, FL, USA).

Collection of semen samples

We studied 111 subjects who underwent seminal fluid evaluation at the Biology of Reproduction Laboratory (CHU, Lille, France). 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, CHU Lille). The samples were collected by masturbation into sterile plastic jars, after 3–5 days of sexual abstinence. Within 1 h of collection, a routine semen analysis was performed using a light microscope to determine sperm quality. Semen profiles were classified into normal ($n = 39$) or abnormal sperm parameters ($n = 72$). According to the World Health Organization criteria (World Health Organization, 1999), normal sperm parameters were defined as (a + b) type motility or progressive motility $\geq$50% and 'a' type motility (forward motility) $\geq$25%; sperm cell concentration $\geq$20$\times$10^6 cells/ml; and sperm cells with altered morphology $\leq$70%.

All samples fulfilled the criteria to be selected for IVF rather than ICSI.

Preparation of semen samples

To isolate sperm, an aliquot of semen was purified using a three-step discontinuous PureSperm® gradient (90–70–50%) diluted in Ferticult® medium containing 0.4% HSA. After centrifugation at 400 *g* for 20 min, purified population of motile sperm (from the 90% layer) were recovered, washed in Ferticult® medium, and resuspended in 1 ml of the same medium. Prepared sperm were counted and the percentage of forward motile sperm ('a' type motility, WHO, 1999) was estimated. Prepared sperm were then used for IVF and an aliquot taken for cytofluormetric analyses. Both neat semen and purified sperm from the same semen samples were subjected to flow cytometry within 2 h.

Assessment of mitochondrial membrane potential

Mitochondrial membrane potentials ($\Delta\psi_m$) were measured by means of $DiOC_6(3)$ staining as previously described (Marchetti *et al*., 1996a,b). Briefly, 5×10^5 cells were incubated for 15 min at 37°C in 500 µl of 40 nmol/l $DiOC_6(3)$, immediately followed by analysis on a cytofluorometer with excitation and emission settings of 488 nm and 525 nm (FL1 channel) respectively. Cells were kept on ice before analysis. Control experiments were performed in the presence of 50 µmol/l mClCCP (15 min, 37°C), an uncoupling agent that abolishes the $\Delta\psi_m$.

Determination of reactive oxygen species by flow cytometry

It is possible to determine the production of ROS using HE, a substance that is oxidized by superoxide anion to become ethidium bromide, emitting red fluorescence (Rothe and Valet, 1990). Cells were exposed for 15 min at 37°C to 2 µmol/l HE before cytofluorometric analysis (excitation: 488nm; emission: 625nm in the FL3 channel). Control cells were incubated in the presence of 1 mmol/l menadione, a superoxide anion generator, for 1 h at 37°C, washed twice and labelled with HE.

Cytofluorometric analysis of nuclear apoptosis by TUNEL assay and determination of cell viability

Nuclear apoptosis was assessed by TUNEL assay. This assay was performed with the TUNEL kit according to the manufacturer protocol with minor modifications. Briefly, 1×10^6 washed sperm cells were fixed with 4% paraformaldehyde (Sigma Chemical Co.) for 30 min at room temperature. Cells were washed twice in phosphate buffered saline (PBS) followed by permeabilization with 0.2 % Triton X-100 for 2 min on ice. After washing with equilibration buffer, cells were incubated with 50 µl of TUNEL mix (dUTP-FITC and TdT enzyme in equilibration buffer) for 1 h at 37°C. Positive control was obtained by incubating one sample with 10 mg/ml DNAse I for 10 min at room temperature. To the negative control, TdT enzyme was not added. Cells were washed twice in PBS before analysis.

To determine cell viability, cells were incubated for 10 min in propidium iodide (PI) (10 µg/ml in PBS) at 37°C and analysed by cytofluorometry as described previously (Marchetti *et al.*, 1999a). For all cytofluorometric experiments, forward and side scatters were gated on the major population of normal-size cells and a minimum of 50 000 cells was analysed.

Statistical analysis

Data are presented as mean values $\pm$ SEM. Results were analysed using GraphPad Prism® version 3.00 (GraphPad Software, San Diego, CA, USA). For comparison of percentage of positive cells in neat semen and prepared sperm from the same ejaculate, a Wilcoxon matched rank test was employed. For comparison of two groups (normal and abnormal sperm parameters), a two-tailed, Mann–Whitney *U*-test was performed. The Pearson rank correlation test was used to calculate the correlation coefficient between cytofluorometric analysis. The Spearman rank correlation test was employed to evaluate the relationship between semen analysis parameters and cytofluorometric examination. Statistical significance was set at $P < 0.05$.

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 111 men consulting for infertility. According

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

	Number of samples analysed	Mean ± SEM	Minimum	Median	Maximum
Patient age	111	36 ± 1	26	34	64
Semen concentration ($\times 10^6$/ml)	111	65 ± 7	1	45	408
Progressive motility (grades a + b)[a] in neat semen (%)	111	40 ± 2	1	40	70
Forward motility (grade a)[a] in prepared sperm (%)	51	62 ± 3	1	70	90

[a]According to World Health Organization (1999).

to the World Health Organization criteria (World Health Organization, 1999), 39 subjects had normal, and 72 abnormal sperm characteristics. In particular, they exhibited asthenozoospermia (33 men), oligoasthenozoospermia (20 men), oligozoospermia (five men), teratoasthenozoospermia (two men) and oligoasthenoteratozoospermia (12 men).

Validation of cytofluorometric analyses of the sperm cell population in semen

Figure 1 shows an example of cytofluorometric profiles representing the distribution of sperm cells after staining with different probes used in this study. For each sample, percentage of cells with high $\Delta\psi_m$ (DiOC$_6$(3) ^{high} cells) (Figure 1A), producing ROS (HE$^+$ cells) (Figure 1B), presenting DNA fragmentation (TUNEL$^+$ cells) (Figure 1C) and viable cells (PI$^-$ cells) (Figure 1D) were determined. Regarding mitochondrial membrane potential of sperm cells, pilot experiments in which protonophore mClCCP was used to depolarize mitochondria membranes, allowed us to ascertain that the cationic lipophilic fluorochrome DiOC$_6$(3) was able to measure $\Delta\psi_m$ variations in sperm cells. Indeed, semen incorporated 1 to 1.5 log more DiOC$_6$(3) than semen incubated with the protonophore mClCCP, indicating that most of the dye incorporation is driven by the inner mitochondrial transmembrane proton gradient (Figure 1A). As shown in Figure 1A, staining with DiOC$_6$(3) revealed the presence of a major cell population with a high mitochondrial membrane potential ($\Delta\psi_m^{high}$ cells).

The determination of ROS production was performed after staining of sperm cells with HE. HE is a non-fluorescent lipophilic compound which is converted into the fluorescent hydrophilic ethidium in presence of superoxide anion. Menadione, a substance that undergoes redox cycles in mitochondria, enhanced the rate of HE to ethidium conversion in sperm cells and was used as positive control (Figure 1B). Consequently, HE$^+$ cells exhibit high ROS production (Figure 1B). In contrast to the positive control, semen sample in Figure 1B was mostly HE negative, indicative that these sperm cells do not produce ROS.

DNA strand breaks, indicative of advanced apoptosis, were studied by TUNEL staining which was negative for a major subpopulation of sperm cells (Figure 1C). Positive controls were performed using DNase I.

Finally sperm viability was assessed as cells excluding propidium iodide staining (PI$^-$ cells). Results in Figure 1D

show that sperm cell population is heterogenous. It contains viable cells (PI negative) and dead cells (PI positive).

We identified significant relationships between all four cytofluorometric methods detecting death-associated changes in semen samples (Table II).

Correlation between sperm characteristics of semen samples and cytofluorometric results

In sperm samples defined as normal by the World Health Organization (1999) criteria, the percentage of $\Delta\psi_m^{high}$ cells was significantly higher than in sperm samples defined as abnormal by the same criteria (Figure 2A; mean: 45% ± 3 versus 30% ± 2; $P = 0.0003$). In addition, sperm with abnormal parameters had more DNA fragmentation than sperm with normal parameters (Figure 2C; mean: 30% ± 2 versus 23% ± 2; $P = 0.04$). No significant difference was found between normal and abnormal sperm samples in the percentage of viable cells (Figure 2C) and cells generating ROS (Figure 2B).

Regarding the relationship between sperm concentration in the semen samples and the four cytofluorometric parameters (Figure 3), a significant positive correlation was found only with the percentage of cells with $\Delta\psi_m^{high}$ (Figure 3A), whereas ROS production (Figure 3B), DNA fragmentation (Figure 3C) and cell viability (Figure 3D) failed to give statistically significant values. Also, the relationship between the progressive motility of sperm [percentage of cells showing (a + b) motility] and the four cytofluorometric parameters was evaluated (Figure 4). The percentage of cells with $\Delta\psi_m^{high}$ correlated significantly with the percentage of sperm with progressive motility (Figure 4A). A statistically significant negative correlation was found, however, between progressive motility and DNA fragmentation (Figure 4C). There was no correlation between progressive motility and ROS production (Figure 4B) or cell viability (Figure 4D).

Cytofluorometric analysis in semen and in prepared sperm after PureSperm® gradient

We compared flow cytometry data from semen with the motile sperm prepared by PureSperm® gradient for each sample and expressed as a percentage of positively or negatively stained cells (Figure 5). This comparative study revealed that prepared
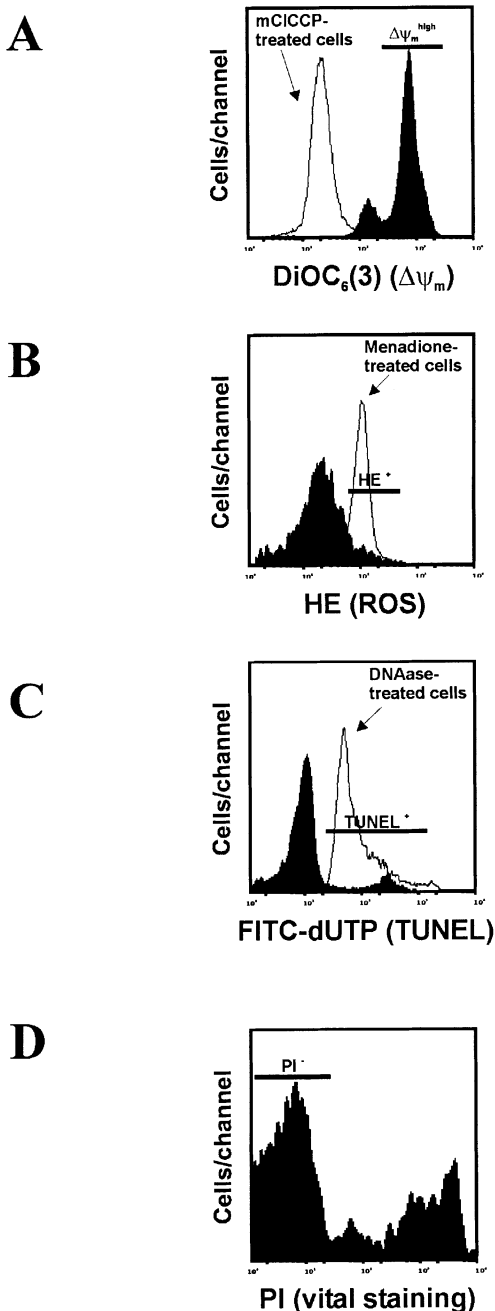
A

DiOC₆(3) (Δψₘ)

B

HE (ROS)

C

FITC-dUTP (TUNEL)

D

PI (vital staining)

Figure 1. Cytofluorometric analysis of the frequency histograms of mitochondrial membrane potential (**A**), reactive oxygen species (ROS) generation (**B**), DNA fragmentation (**C**) and cell vitality (**D**) in semen samples (black profiles). Sperm cells were labelled either with the $\Delta\psi_m$-sensitive dye 3,3′-dihexyloxacarbocyanine iodide [DiOC$_6$(3)] (**A**), or the superoxide anion oxidizable probe hydroethidine (HE) (**B**), or dUTP-FITC to detect DNA fragmentation (**C**) or propidium iodide (PI) (**D**) as described in Materials and methods. For controls (white profiles), sperm cells were treated with the uncoupling agent mClCCP (**A**), or with the superoxide anion-generating agent menadione (**B**), or with the DNAase I (**C**) followed by the staining. Similar results were obtained with prepared sperm (data not shown).

sperm had higher $\Delta\psi_m$ (Figure 5A) but lower DNA fragmentation (Figure 5C), generated lower ROS (Figure 5B) and were more viable (Figure 5D) than semen samples.

Table II. Correlation (Pearson correlation test) among flow cytometric methods for detection of cell death in semen samples

r	DiOC$_6$(3)[a]	HE[b]	TUNEL[c]	PI[d]
DiOC$_6$(3)[a]		−0.27[e]	−0.32[e]	0.48[e]
HE[b]			0.22[e]	−0.66[e]
TUNEL[c]				−0.47[e]

[a]% of DiOC$_6$(3)^{high}.
[b]% of HE$^+$.
[c]% of TUNEL$^+$.
[d]% of PI$^-$.
[e]$P < 0.005$.
PI = propidium iodide; HE = hydroethidine; TUNEL = terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling; DiOC$_6$(3) = 3,3′-dihexyloxacarbocyanine iodide.

Correlation between forward motility of prepared sperm, cytofluorometric results and fertilization rate

The detection of cells with high $\Delta\psi_m$ was the only cytofluorometric assay significantly correlating with the forward motility (Figure 6) and sperm concentration (data not shown) of prepared sperm.

In neat semen, the percentage of sperm cells with fragmented DNA correlated negatively with the fertilization rate after IVF, whereas the percentage of $\Delta\psi_m^{high}$ cells correlated positively (Table III). In neat semen, no correlation was found between standard semen parameters and fertilization rate (data not shown). In prepared sperm, fertilization rate correlated significantly only with $\Delta\psi_m^{high}$ cells and not with DNA fragmentation, ROS production or cell viability (Table III). A positive correlation between the forward motility of prepared sperm and fertilization rate was also found (correlation test $r = 0.35$; $P = 0.03$).

Discussion

During the last decade, a variety of biological assays detecting cell death have been proposed to evaluate sperm quality in human subjects. Assays based on flow cytometry give some advantages over the fluorescence microscopy assays. They are more accurate, particularly more discriminative, allow for automatic, therefore rapid, analysis of high number of cells and provide less subjective and statistically more reliable results than microscopic examination. In the present paper, we assessed four different cytofluorometric methods detecting cell death in sperm from infertile men including mitochondrial dysfunction, ROS production, DNA fragmentation and sperm cell viability. The methods used detect not only apoptosis (programmed cell death) of sperm cells but also changes associated with non programmed cell death. The aim of this study was to compare the presence of these cellular alterations detectable by flow cytometry with the conventional sperm analysis during sperm preparation for IVF. We evaluated sperm alterations in neat semen and in prepared sperm for each sample. After separation on discontinuous PureSperm® gradient, motile sperm were selected in the 90% fraction which contained sperm cells used in assisted reproduction techniques.

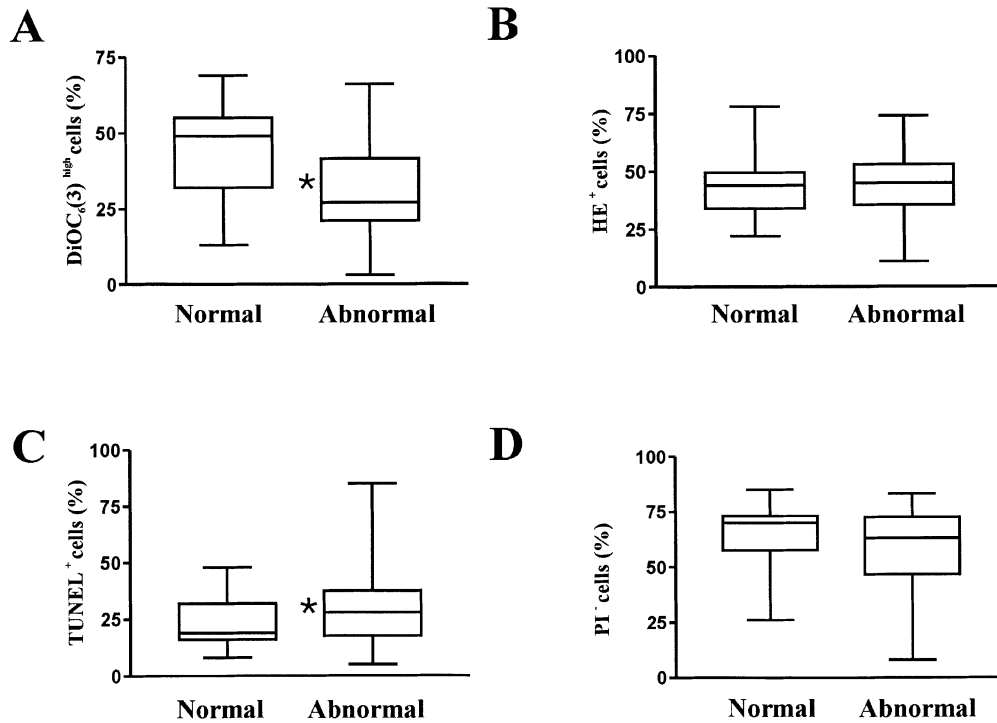The correlation found among the methods assessing sperm

Figure 2. Distribution of semen samples stained with $DiOC_6(3)$ (**A**), HE (**B**), terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL) (**C**) and PI (**D**) between patients with normal or abnormal sperm parameters. (**A**) Distribution of the percentage of $DiOC_6(3)$ high cells in semen from patients with normal ($n = 29$) and abnormal ($n = 55$) sperm parameters. *$P = 0.0003$. (**B**) Distribution of the percentage of HE^+ cells in semen from patients with normal ($n = 29$) and abnormal ($n = 55$) sperm parameters $P = NS$. (**C**) Distribution of the percentage of $TUNEL^+$ cells in semen from patients with normal ($n = 39$) and abnormal ($n = 59$) sperm parameters *$P = 0.04$. (**D**) Distribution of the percentage of PI^- cells in semen from patients with normal ($n = 23$) and abnormal ($n = 37$) sperm parameters $P = NS$. Values are medians (horizontal bars) with 25–75% interquartile ranges (boxes) and minimum–maximum values (I).
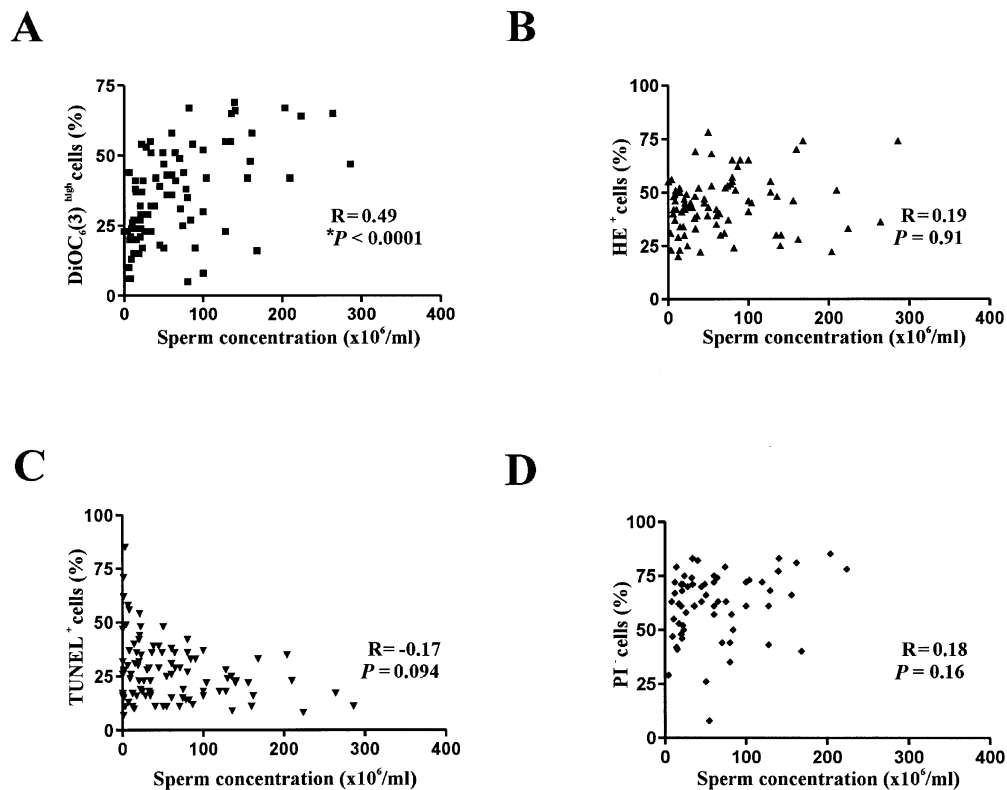

Figure 3. Relationship between the sperm concentration and percentage of $DiOC_6(3)$ high cells (**A**), HE^+ cells (**B**), $TUNEL^+$ cells (**C**), PI^- cells (**D**) in semen samples. R indicates the Spearman correlation factor.

A

B

C

D
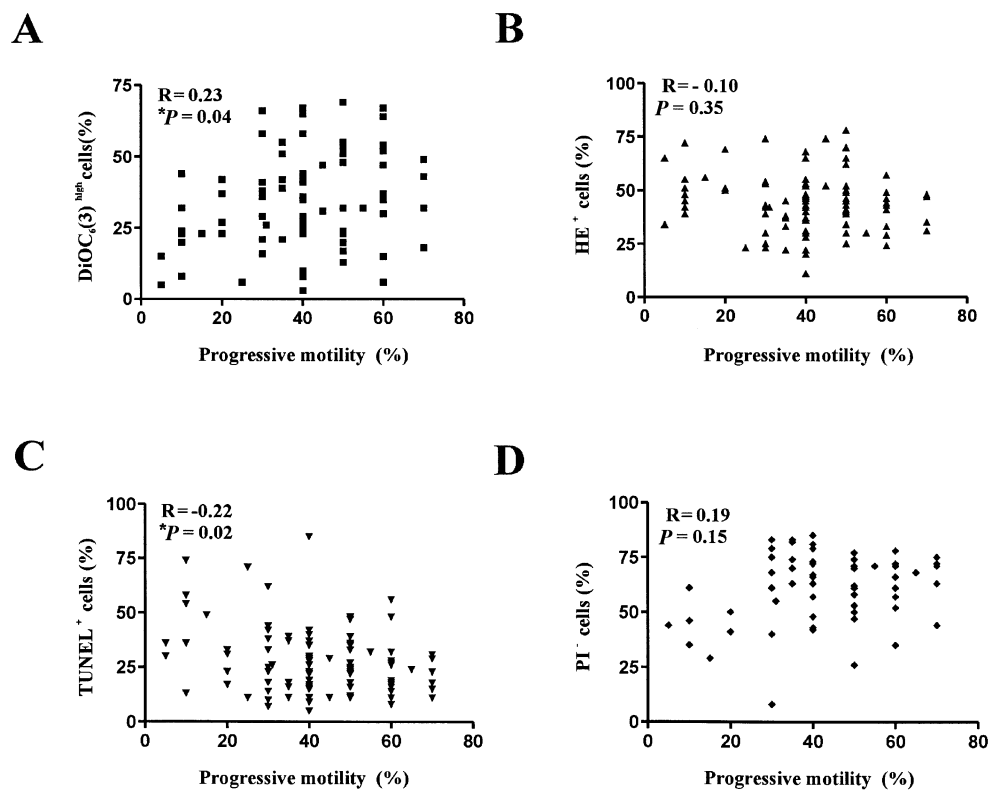
Figure 4. Relationship between progressive motility and percentage of $DiOC_6(3)$ [high] cells (**A**), HE^+ cells (**B**), $TUNEL^+$ cells (**C**), PI^- cells (**D**) in semen. R indicates the Spearman correlation factor.

cell damages (Table II) indicated that mitochondrial dysfunction, ROS generation, DNA fragmentation and loss of viability are likely to represent the interrelated aspects of the overall dead status of sperm. However, these assays assess the sperm quality with a different sensitivity. Indeed, we showed that determination of the mitochondrial membrane potential represents the most sensitive test by which to evaluate sperm quality. To determine mitochondrial membrane potential ($\Delta\psi_m$), several fluorochromes have been previously used for sperm samples evaluation including Rhodamine 123, J-C 1 or MitoSensor (Troiano *et al.*, 1998; Donnelly *et al.*, 2000; Gravance *et al.*, 2000). All of these fluorochromes stained mitochondria of live cells and validated usefulness of the cytofluorometric analysis. Rhodamine 123 is the most commonly used fluorochrome in sperm sample evaluation, however it appears to assess $\Delta\psi m$ incorrectly (Troiano *et al.*, 1998). In this study, we used the carbocyanine dye $DiOC_6(3)$, which is cell permeable and stains mitochondria at low concentration (Zamzami *et al.*, 2000). The mitochondrial uptake of $DiOC_6(3)$ is dependent on the mitochondrial membrane potential. Compared to Rhodamine 123, the $DiOC_6(3)$ offers the important advantage of not causing major quenching effects (Zamzami *et al.*, 2000). Consequently, $DiOC_6(3)$ fluorochrome is one of the most commonly used to monitor mitochondrial changes preceding nuclear apoptosis in other cell types, including various tumour cells, neurones, hepatocytes, thymocytes (Kroemer *et al.*, 1997) as well as lymphocytes from patients infected by HIV-1 (Macho *et al.*, 1995). Our results suggest that $DiOC_6(3)$ is also a valuable probe to measure $\Delta\psi_m$ in sperm samples. The analysis of sperm cells after $DiOC_6(3)$ staining indicated that

there is a significant correlation between the proportion of $\Delta\psi_m$ [high] cells and the motility of sperm in both a native sample and after PureSperm® gradient separation. These results confirmed those obtained after JC-1 staining of sperm from infertile men (Troiano *et al.*, 1998; Donnelly *et al.*, 2000) and rats (Gravance *et al.*, 2001) and suggested that sperm motility is associated with the functional status of mitochondria. Indeed, mitochondrial alterations can result in the reduction of sperm motility since this motility is an ATP-dependent process, thus dependent on the functional mitochondria producing energy powering the flagellar motion.

The $\Delta\psi_m$ reduction is a general feature of cell death. Decrease in the $\Delta\psi_m$ defines an early stage of apoptosis preceding other manifestations of this process such as DNA fragmentation, ROS production and the late increase in membrane permeability (Kroemer *et al.*, 1997). The significance of the $\Delta\psi_m$ reduction encountered in infertile sperm remains unknown but assumption that it manifests an early stage of cell death could explain why detection of mitochondrial changes represents the most sensitive test in our study. Several arguments indicating that mitochondria may be involved in the cell death pathways in germ cells support our interpretation. For example, it was found that partial oxygen pressure induced mitochondrial permeability transition and apoptosis in human testis, and that mitochondria from apoptotic germ cells were swollen (Erkkila *et al.*, 1999). This and other findings strongly imply association of male infertility with mitochondrial alterations observed during apoptosis. However, mitochondrial alterations have been associated with male infertility irrespective of apoptosis (Bourgeron, 2000).
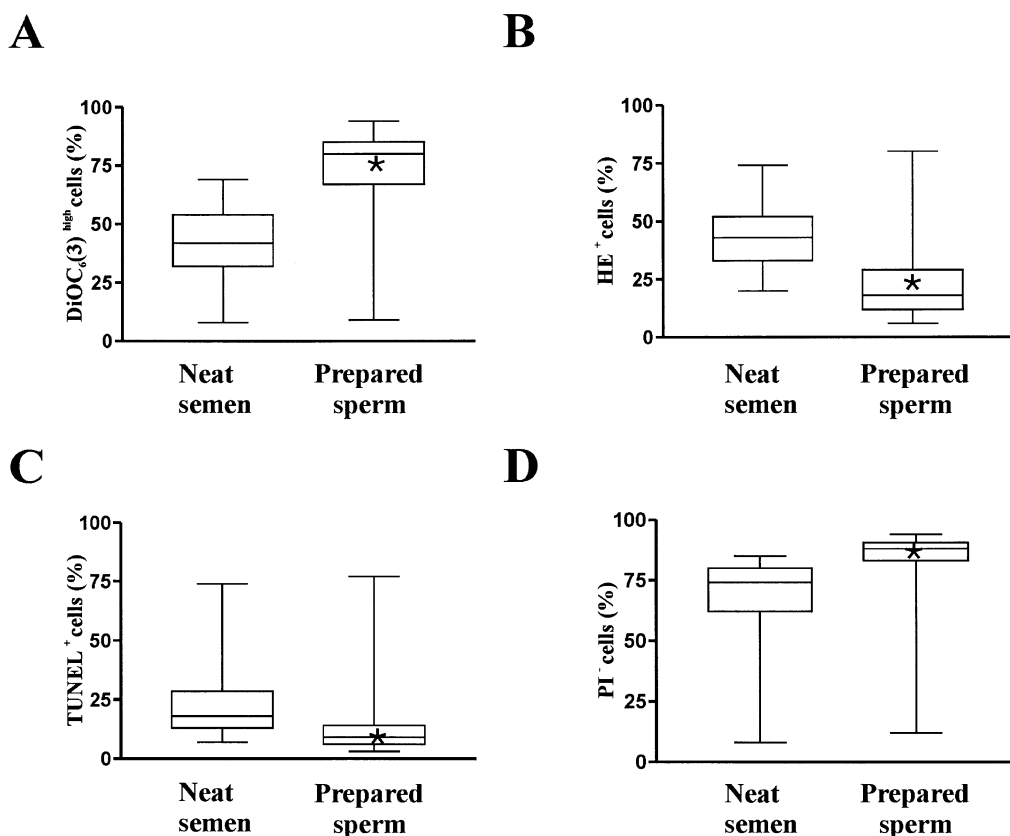
A

B

C

D

Figure 5. Comparison of percentage of $DiOC_6(3)$ high cells (**A**), HE^+ cells (**B**), $TUNEL^+$ cells (**C**), PI^- cells (**D**) in neat semen and prepared sperm from the same ejaculates. Both neat semen and prepared sperm after PureSperm® gradient from the same ejaculates were stained with $DiOC_6(3)$ ($n = 45$) (**A**) or with HE ($n = 45$) (**B**), or with TUNEL ($n = 31$) (**C**), or with PI ($n = 20$). Values are medians (horizontal bars) with 25–75% interquartile ranges (boxes) and minimum–maximum values (I). *$P < 0.0005$ between neat semen and prepared sperm for each analysis (Wilcoxon matched pair test).

As expected from sperm isolated by classical Percoll gradient (Donnelly *et al*., 2000), the sperm fraction obtained using PureSperm® gradient contained more sperm characterized by the high $\Delta\psi_m$, less ROS generation, less DNA fragmentation and they were more viable than sperm initially present in the neat semen. PureSperm® gradient is a Percoll-based density gradient in isotonic salt solution used to enhance semen fertilizing ability applied in medically assisted reproductive procedures. This gradient, containing silanized silica particles, efficiently separates sperm subpopulations and allows for a good recovery of sperm with a high motility and quality characteristics (Yamamoto *et al*., 1997; Ding *et al*., 2000). Another method, a swim-up sperm preparation requiring more repeated manipulations, may be responsible for the high content of ROS (Agarwal *et al*., 1994; Aitken, 1994) seen in deficient sperm not detected in our study. Also, we showed that sperm separated by PureSperm® gradient had only few signs of DNA fragmentation. In contrast, using the swim-up method one could not avoid getting sperm with DNA strand breaks (Host *et al*., 2000a). These findings are particularly important for the assisted reproductive technologies such as IVF and ICSI, requiring sperm with enhanced DNA integrity in order to avoid the inadvertent use of DNA-damaged sperm. Since preparation with PureSperm® gradient removed most of the damaged cells, the forward motility of prepared sperm lacked correlation with ROS production, DNA fragmentation or loss

of viability (Figure 6). Importantly, prepared sperm with high $\Delta\psi_m$ always correlated with forward motility, thus confirming the strong link between the functional status of mitochondria and sperm cell quality.

Additional evidence supporting the evaluation of cell death markers to test male infertility is provided by the negative correlation between DNA fragmentation in neat semen and IVF fertilization rate (Table III; Host *et al*., 2000b). In contrast to others (Sun *et al*., 1997) we did not detect any negative correlation between DNA fragmentation and the fertilization rate in prepared sperm, perhaps because they used the swim-up preparation. Our study determined that fertilization rate significantly correlated with the proportion of sperm with a high $\Delta\psi_m$ both in neat semen and after separation. Thus, the evaluation of $\Delta\psi_m$ appears to be the most efficient test of human sperm assuring successful IVF. This is not surprising, because values of $\Delta\psi_m$ link mitochondrial function to motility of sperm. Indeed, concentration of motile sperm was one of the most significant parameters in predicting the chance of natural conception (Larsen *et al*., 2000) and was significantly and positively correlated with the fertilizing ability of sperm *in vitro* (Zollner *et al*., 1999).

To conclude, we observed that detection of $\Delta\psi_m$ changes is the most efficient test to evaluate sperm quality during the preparation steps for IVF. The $\Delta\psi_m$ determine functional mitochondria that have been related mainly to sperm motility.
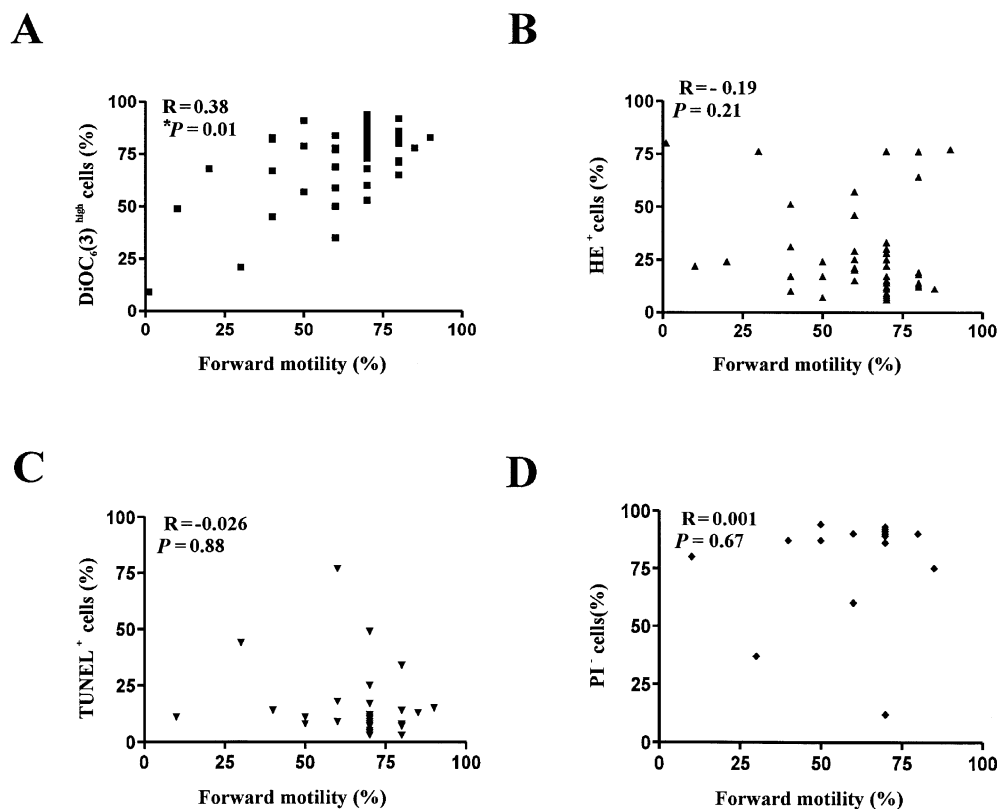
A

B

C

D

Figure 6. Relationship between forward motility and percentage of $DiOC_6(3)$ high cells (**A**), HE^+ cells (**B**), $TUNEL^+$ cells (**C**), PI^- cells (**D**) in prepared sperm. R indicates the Spearman correlation factor.

Table III. Correlation of cytofluorometric markers in sperm before (a) and after (b) PureSperm® gradient with fertilization rate

% of sperm		n	Spearman coefficient *r*	P value
$DiOC_6(3)$ high	a	46	0.45	0.001
	b	45	0.30	0.04
HE^+	a	46	−0.069	NS
	b	45	−0.11	NS
$TUNEL^+$	a	47	−0.45	0.001
	b	32	−0.01	NS
PI^-	a	33	0.28	NS
	b	20	0.16	NS

NS = not significant.

Our data indicate the $\Delta\psi_m$ value could represent an important determinant of high quality sperm in neat semen and prepared sperm of infertile men, predicting successful IVF.

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