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Continuous Assessment of Human Spermatozoa Viability During Cryopreservation

S. N. MOHAMMAD, C. L. BARRATT, I. D. COOKE, AND H. D. MOORE

From the University of Sheffield, Department of Obstetrics and Gynaecology, Jessop Hospital for Women, Sheffield, United Kingdom.

ABSTRACT: Cryomicroscopy has enabled direct observation of freezing and thawing of human spermatozoa. When used with a fluorescent viability kit, sperm membrane damage was not apparent down to temperatures of −5°C, but significant damage occurred after thawing (55% of spermatozoa had damaged membranes). Semen samples were cooled or frozen to temperatures (at decrements of 10°C) from 0°C to −110°C. At all these temperatures the proportion of live to membrane-damaged cells remained constant. Samples held at temperatures above −30°C were not adversely affected. Below −30°C there was a gradual increase in the proportion of membrane-damaged cells on thaw and a decrease in the number of live cells recovering motility. At temperatures between −50°C and −60°C there was an equal proportion of live motile, immotile, and membrane-damaged cells. It is concluded that some irreversible damage to spermatozoa was a result of freezing processes in cells frozen to −30°C or less, but most of the cryodamage was incurred during thawing, possibly due to recrystallization.

Key words: Sperm, cryomicroscopy, fluorescent viability probes, cryosurvival.

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There is a large variability in the extent of damage semen samples experience during cryopreservation. Those donor samples displaying good cryosurvival can be selected for donor insemination (DI), but problems still remain when no selection is possible, e.g., cancer patients (Mortimer, 1994). Moreover, use of frozen semen for insemination has yielded significantly lower fertility rates than fresh semen (Bordson et al, 1986; Marshburn et al, 1992).

Our understanding of cryodamage has been reviewed by Hammerstedt et al (1990) and Mazur (1984). Hypotheses exist for events such as "cold shock," intracellular ice damage and solution effects, but there is little direct evidence for their mechanisms (Hammerstedt et al, 1990). Investigations of cells at low temperatures in the semisolid or frozen state are difficult as the thaw process (which involves a reversal of the previously mentioned events) has to be completed before damage can be assessed. Different combinations of freezing and thawing rates, cryoprotectants, sample containers, and sample preparation methods have been compared for human sperm by assessing changes in membrane integrity, viability, morphology, and motility (e.g., Taylor et al, 1982; Mahadevan and Trouson, 1983; Keel et al, 1987; Oettle et al, 1992; Henry et al, 1993; Verheyen et al, 1993). Optimum protocols yield only 50% human spermatozoa recovery after the freeze/thaw process. This could be due to the various compartments of the spermatozoa responding differently to the cryopreservation protocol (Hammerstedt et al, 1990).

In this study, we report preliminary results to determine at what stage of the freeze/thaw cycle damage occurs to human spermatozoa. The use of fluorescent probes, such as the Molecular Probes[1] Live/dead® *Ferti*light™ Sperm Viability Kit (L-7011), are useful in distinguishing between live and dead cells (Garner et al, 1994). Garner and Johnson (1995) have successfully used this kit on semen samples from bulls, boars, rams, mice, and men. They determined that the kit has the advantage over other viability stains of a rapid and stable response that is not based on enzyme substrate conversion. Also, as the SYBR14 (live stain) targets DNA, the stain is easily quantifiable and compared to other nucleic acid stains easy to use as it excites within the visible spectrum. The *Ferti*light Kit does not have the associated problems of other dyes that are affected by glycerol (Garner et al, 1986). This makes it more desirable for use when studying cryopreserved semen whose main cryoprotectant is glycerol. Propidium iodide has been extensively used to estimate dead-sperm populations (Centola et al, 1990; Graham et al, 1990; Kramer et al, 1993). It is membrane impermeable and only stains the nucleus of cells whose mem-

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Correspondence to: H. D. Moore, Department of Obstetrics and Gynaecology, Jessop Hospital for Women, Leavygreave Rd., Sheffield S3 7RE, United Kingdom.

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[1] Molecular Probes Inc., 4849 Pitchford Ave., Eugene, Oregon 97402-0144. Supplied by Cambridge Science Ltd.

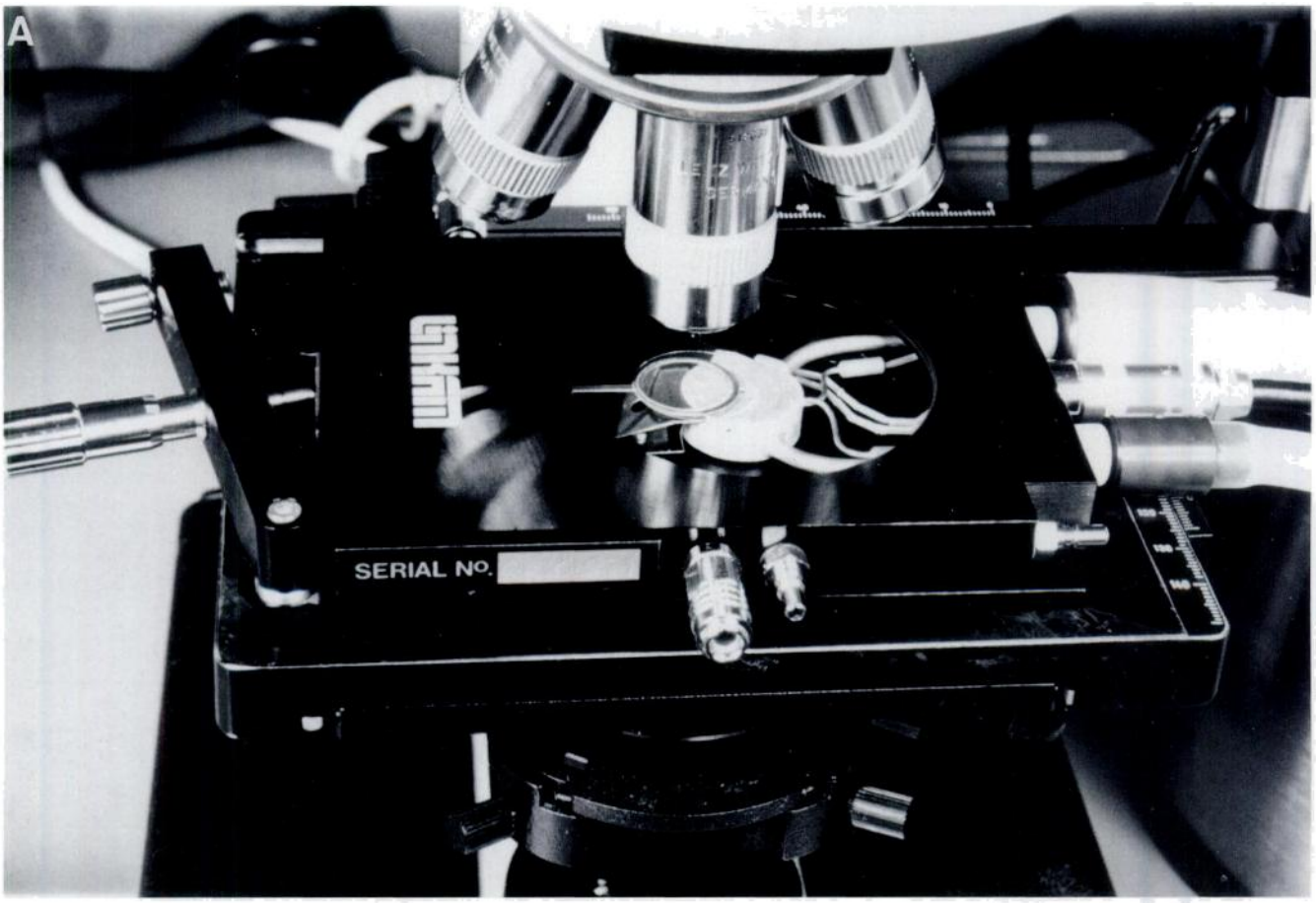

FIG. 1. Photograph of cryostage equipment. **(A)**, The cryostage is mounted on the microscope stage. It is linked to the liquid nitrogen dewar and three stage control units.

branes have been compromised. The kit therefore allows live and dead cell counts to be made on fresh and cryopreserved samples, enabling direct comparison to be made of different cryopreservation protocols.

The experiments reported here involved simple live/dead counts of human spermatozoa, performed on a commercially available cryostage designed by Linkam (Linkam Scientific Instruments Ltd., Surrey, United Kingdom). Unlike Garner and Johnson (1995), who used a flow cytometer, here we were able to perform single-cell analysis. By using the cryostage it was possible to observe on-going freezing and thawing effects on sperm viability, as previously reported by Holt et al (1988, 1992).

Methods and Materials

Preparation of Semen

Semen samples, from recruited donors of proven fertility, were obtained by masturbation and left to liquefy for 20 minutes at 37°C in an incubator. Those with a minimum motile concentration of 30 × 10^6/ml were selected for the study. The samples were then washed in Earle's balanced salt solution (EBSS, Life Technologies Ltd., Middlesex, United Kingdom) and 0.3% bovine serum albumin (BSA, Sigma Aldrich Co. Ltd., Dorset, United Kingdom) and a swim-up performed (Mortimer, 1994). The swim-up preparations were again washed (500 × *g* for 6 minutes) and the subsequent pellets were resuspended in 1 ml of fresh media and adjusted to a motile concentration of 50 × 10^6/ml. The proportion of motile spermatozoa in these samples was greater than 98% (average path velocity > 25 μm/second) as determined by computer-assisted semen analysis (CASA, Hamilton Thorne Motility analyzer (HTA) 2030 vers. 7, Beverly, Massachusetts). All semen analyses and CASA were performed as described by Clements et al (1995). Standard settings were used for the HTA, with a frame rate of 20 at 25/seconds, stage temperature 37°C, sample in a Microcell® chamber (20-μm depth), and motility parameters measured that included: VAP (μm/second), VCL (μm/second), VSL (μm/second), ALH (μm), LIN (%), STR (%), and sort fraction.

Fluorescent Staining and Dilution With Cryoprotectant

The general method for staining with the *Fertil*ight Kit was as follows. The SYBR14 (1-mmol dye reagent in 100% DMSO)

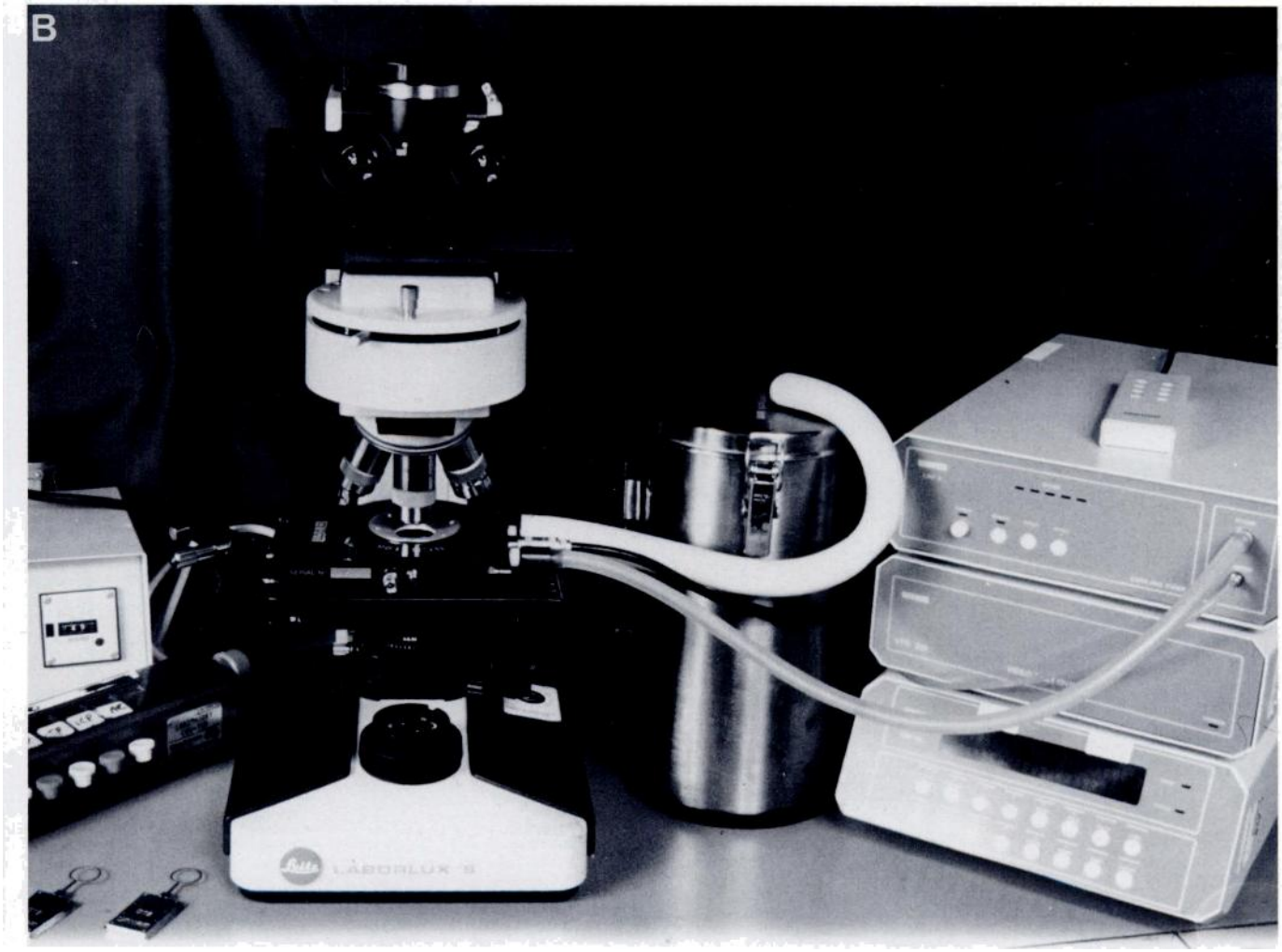

FIG. 1. (B), Photograph of the stage in detail. A sample is held on a sample holder that has been inserted through the stage door on the left. To the right of the stage the connections to the dewar and control units can be seen. The silver block is located in the center of the stage below the objective lens. A lid is placed over the stage leaving an orifice just sufficient for the objective to fit in.

green vital permeable nucleic acid probe was diluted to a concentration of 1:10 in DMSO. A 1-μl volume of this stock solution was added to 1 ml of washed semen and incubated at 37°C (5% carbon dioxide) for 10 minutes. A 5-μl volume of propidium iodide stock solution (2.4 mmol in water) was then added and the sample incubated for a further 10 minutes. An equal volume of glycerol (7.5%) egg-yolk citrate buffer (GEYB) was then added stepwise to the entire sample and mixed gently (final cell concentration 25 × 10^6/ml). This procedure was followed for the first and second experiments as described below. During the third experiment, the kit was added to the samples at the time of use.

Preparation and Programming of the Cryostage and Assessment of Samples

The Linkam BCS196 cryostage was used, mounted on the Leitz Laborlux S fluorescent microscope (Fig. 1A). The stage contains a silver block whose temperature is controlled via the TMS92 unit at rates between 0.1°C/minute (minimum) and 100°C/minute (maximum). The cryostage was prepared by running a short profile entered into the TMS92 programmer. The stage was cooled at 10°C/minute to −40°C and warmed at the same rate back to 30°C. This removed most of the condensation in the stage chamber. A 10-μl drop of the sample was then placed on a 20-mm round coverslip and, using vacuum tweezers, a 15-mm round coverslip was overlaid. The coverslip with sandwiched sample was then inserted through the stage door on its side using the sample holder (Fig. 1B). The freeze/thaw rate profile for the sample was then programmed into the controller. All experimental profiles were based on the "standard" profile used during programmed freezing of routine semen samples (Fig. 2A). Using a 488-nm filter, it was possible to observe live green motile and immotile spermatozoa and red immotile dead cells at 200× magnification. No cells were exposed to UV light for more than 30 seconds.

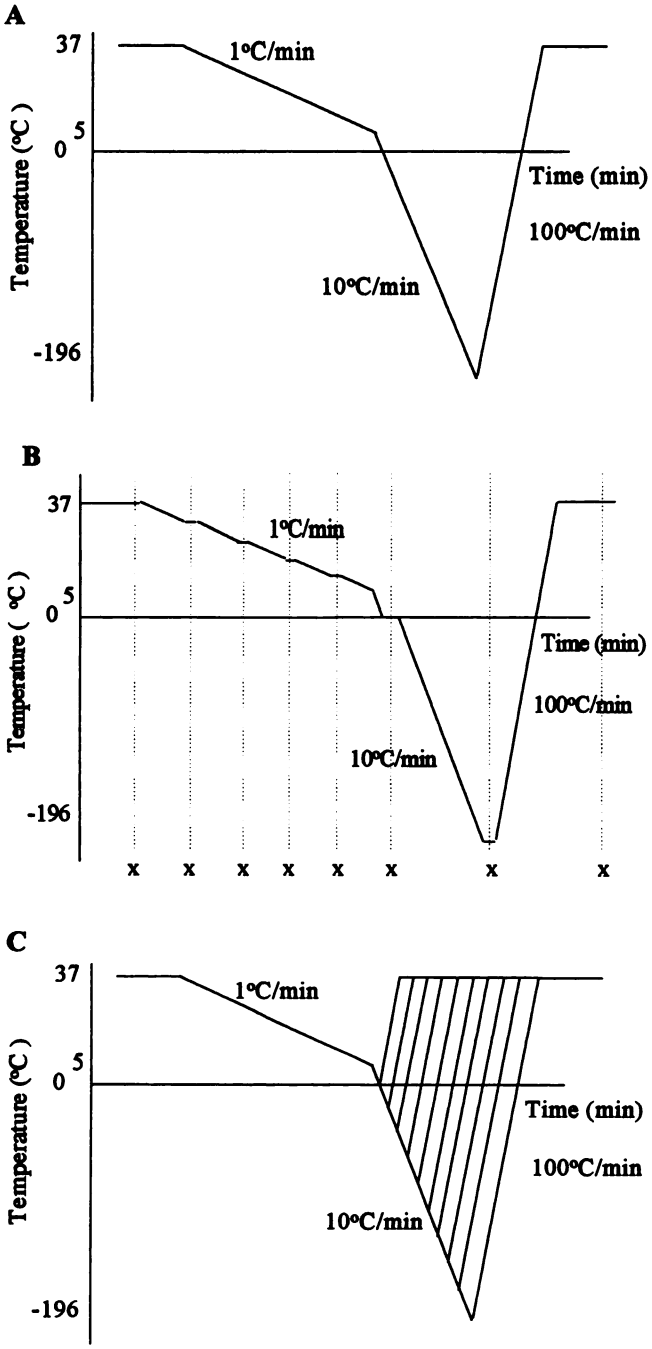

FIG. 2. Cryopreservation temperature profiles for the different experiments. **(A)**, Normal profile. **(B)**, Sketch of profile for experiment 2. **(C)**, Sketch of profile for experiment 3.

Experiment 1—The Effect of Fluorescent Dye on the Spermatozoa Cells

A comparison was made on two samples from different donors over 20 hours of their motility with and without addition of the kit and also with or without the cryoprotectants when kept at room temperature. Samples were also frozen with or without the kit and their post-thaw motility checked. These samples were frozen in a Planer Programmable Freezer (KRYO 10 Series III, controlled rate, Fig. 2A) and thawed at room temperature. All of the motility characteristics obtained on the Hamilton Thorne were assessed. As only two samples were used, no statistical analysis was performed on this data.

Experiment 2—Cell Viability During a Freeze and Thaw Cycle

At 5°C intervals, during a standard cooling and freezing profile, 200 spermatozoa were counted (Fig. 2B). This was repeated on four semen samples from different donors. The specimens were held for 2-minute intervals at each temperature, the cells being counted in the second minute. Counts were only taken until −5°C, when the sample had frozen at −70°C, and after the thaw to 37°C. The samples were held for 5 minutes at post-thaw 37°C before a cell count was attempted.

Experiment 3—Determination of Temperature at Which Cell Damage Occurs

Samples were cooled or frozen to defined temperatures (10°C steps) and returned at a thaw rate of 100°C/minute to 37°C (Fig. 2C). Counts were taken for 200 cells pre-freeze at 37°C, at low temperature, and post-thaw at 37°C for the final count after 5 minutes. The samples were held for 5 minutes at each temperature and the cells counted in the last minute. Experiment temperatures were randomly chosen to avoid any artifacts in the methodology. This experiment was performed on three different donor samples.

Results

Experiment 1

Addition of the kit did not produce greater differences in cell motility as compared to the differences observed due to the different treatments (Fig. 3). For example, when the percentage of overall motile sperm (Fig. 3B) are compared for samples 1086 and 1096, with and without the kit, all the samples have high motilities at the start of the experiment (between 60 and 80%, light bars) and much reduced motilities after 20 hours (between 10% and 20%, darker bars). None of the motility characteristics appeared unusual in the presence of the kit compared to its absence.

Experiment 2

Figure 4 shows the mean different populations of cells, represented as a percentage of the 200 cells counted, at each temperature and indicates that until −5°C few sperm had been damaged due to cooling. At −110°C, the vast majority of the cells were still live (all of these were immotile as the sample had frozen). On thawing to 37°C, 45% of the cells remained viable, of which 6% were immotile.

Experiment 3

The Wilcoxon test was applied to these samples and no significant difference was noted at $P < 0.05$ (perhaps due

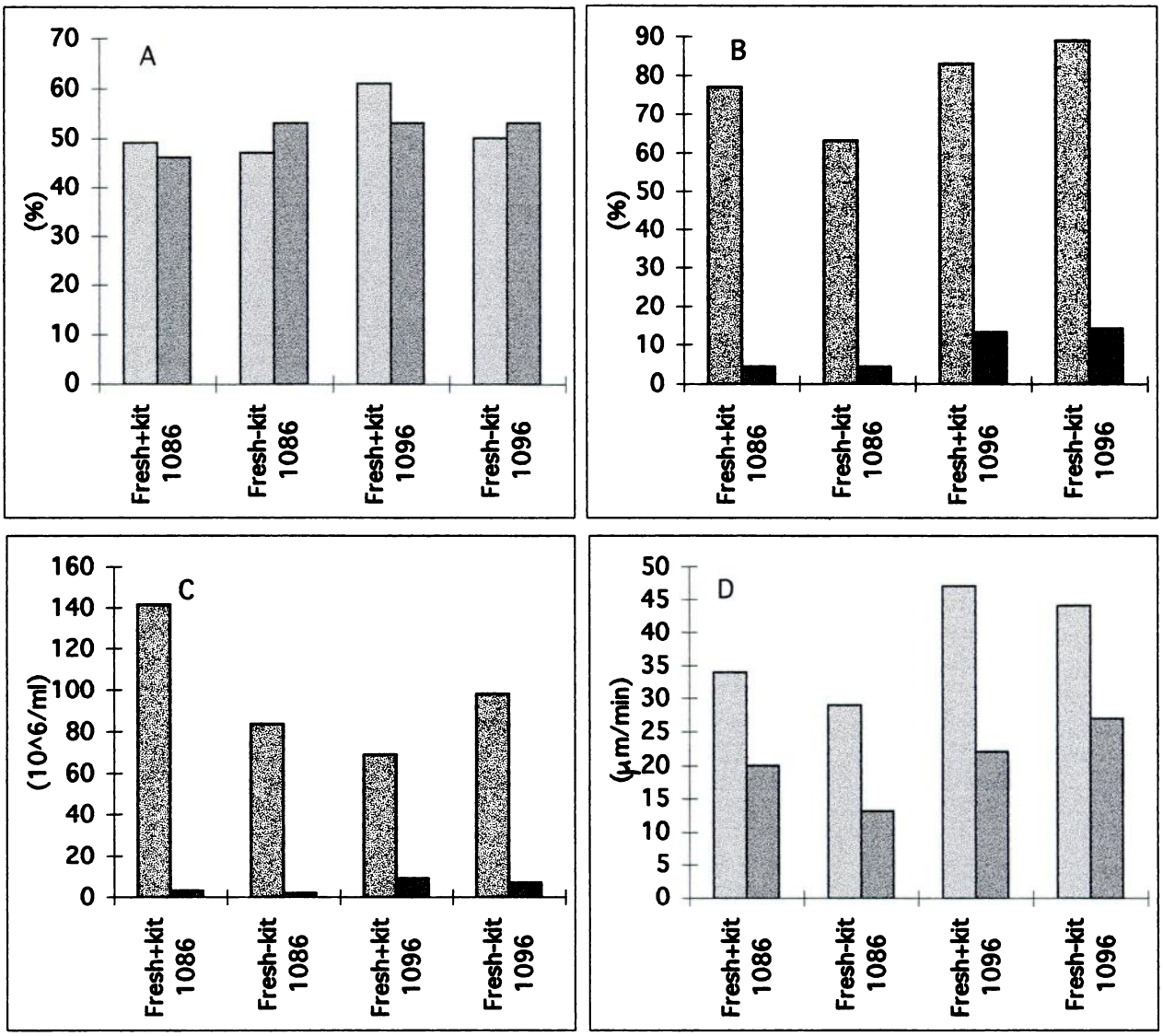

FIG. 3. Changes in motility characteristics over 20 hours for two semen samples (1086 and 1096), with (+) and without (−) kit. **(A)**, Linearity (LIN, %). **(B)**, Motile sperm (%). **(C)**, Concentration of motile sperm (10^6/ml). **(D)**, Average path velocity (µm/minute). Light bars represent start (0 hours) and dark the finish (20 hours).

to the sample number), but the data did show strong trends. In all of the samples pre-freeze, the maximum percentage of dead cells was 15% (Fig. 5A). Samples cooled down to −30°C showed little effect in the ratios of live to dead cells at those temperatures, but from there down to the freeze, the vast majority of cells were immotile but live (Fig. 5B). When the samples were thawed, an apparent difference was noted on samples taken down to −30°C or those taken to temperatures from −70°C or lower, the former of which had high ratios of live to dead cells, while the latter showed a 3:2 ratio in favor of dead cells (Fig. 5C). Between these temperatures few cells were dead, but a gradually increasing number of immotile cells were observed.

Discussion

It was necessary to use washed, swim-up, and further washed spermatozoa with the fluorescent probes to get a consistent debris-free preparation for analysis. Debris hindered accurate cell counts and motility determination by increasing the number of collisions occurring in the field of view and making visual discrimination difficult. Although washing can affect the motility of sperm, addition of albumin can protect such changes (Makler and Jakobi, 1981), and pregnancy rates, the ultimate objective of these treatments, are unaffected (Cumming, 1988). Care was taken to ensure that a suitable concentration of cells was

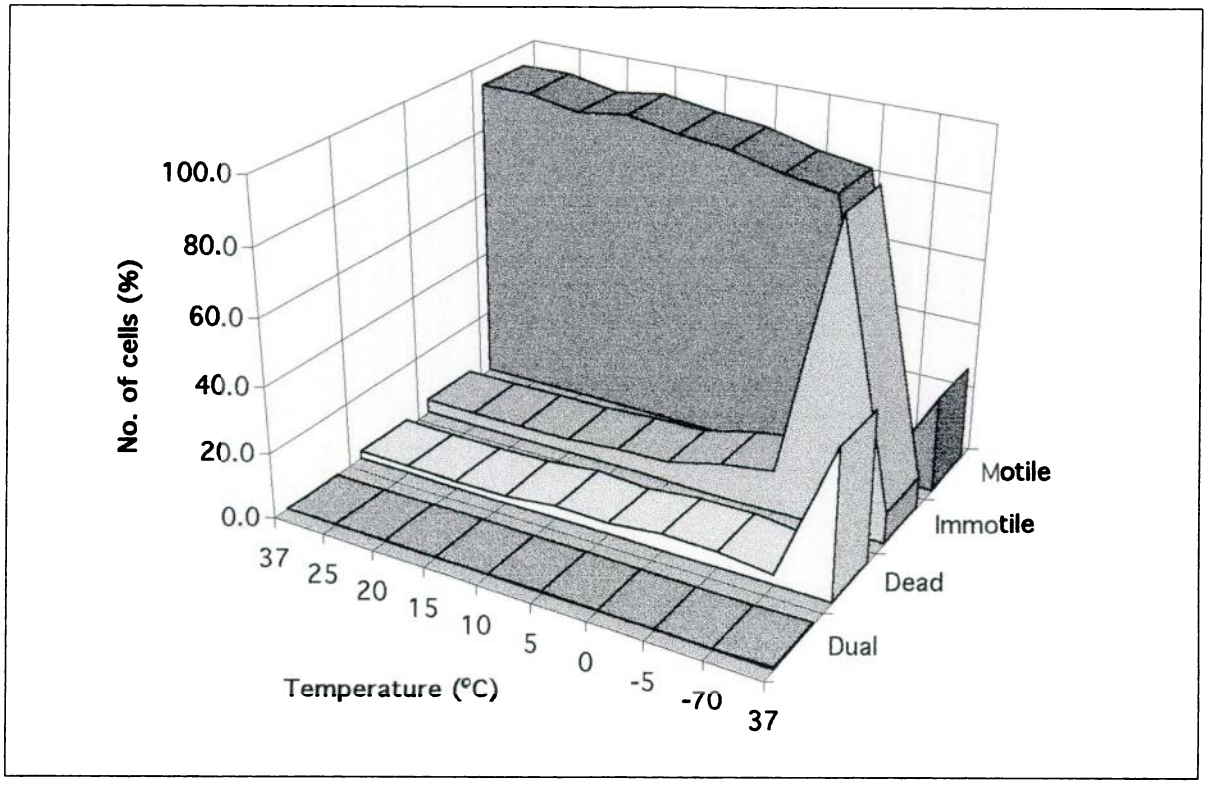

FIG. 4. Numbers of live (motile/immotile), dead, and dual-stained cells during a freeze/thaw profile (experiment 2).

used so that accurate cell counts could be reported for motile cells, a particular problem with fresh samples at 37°C. Moreover, by having a low concentration, at least three fields of view were counted to obtain the 200 cells per temperature reading. Washing has previously been shown to result in altered motility of cells (Perez-Sanchez et al, 1994). Our preliminary study indicated that sperm motility was not consistently affected by the viability probes. Few fluorescent probes have been assessed for their effect on cell motility, although many have been used for measuring viable spermatozoa (e.g., Centola et al, 1990). Previous studies comparing fresh and washed human spermatozoa samples have shown no significant difference between post-thaw survival (unpublished observation).

The proportion of sperm surviving freeze/thawing on the cryostage, as assessed by the viability probe, was about 45%. This compares well with sperm survival when frozen in straws (Mohammad and Moore, unpublished data; Taylor et al, 1982; Verheyen et al, 1993). From our observations of the changes in fluorescence, it appeared that the damage to the cells occurred during the thaw rather than the cooling or freezing processes. As the viability probes only determined whether the cells were intact or not, more subtle forms of damage, e.g., influx or leakage of ions or enzymes, were not apparent.

The proportion of cells permeable to propidium iodide increased after the samples had been frozen. At temperatures between −30°C and −40°C, the cells appeared frozen under the microscope. It has been shown that at these temperatures unfrozen high-solute concentration channels exist between the ice crystals and that these channels freeze at much lower temperatures (Mazur, 1984; Hammerstedt et al, 1990). Samples frozen to temperatures of −30°C to −40°C did not display as much damage as those frozen to −70°C or below, as indicated by the consistent post-thaw damage in the latter. It has been suggested that the membranes of cells lying across unfrozen diluent are damaged by high-solute concentrations (Holt and North, 1994). However, our observations indicated that the crystals formed on freezing were much smaller than the spermatozoa, so that most, if not all, of the cells should have been exposed to these channels. Furthermore, cells frozen between −30°C and −70°C had a higher tendency to be immotile and non-viable. This could be a reflection of the increasing concentration of the surrounding solutes. As the samples were held for a few minutes at these temperatures for equilibration, this could also reflect a degree of recrystallization damage (Hammerstedt et al, 1990). Indeed, much of the thaw damage may be attributed to this phenomenon, as even with rapid thaw rates, recrystallization was still obvious under the cryomicroscope.

Other investigators have used cryostages in combination with fluorescent probes (Holt et al, 1988), but in this

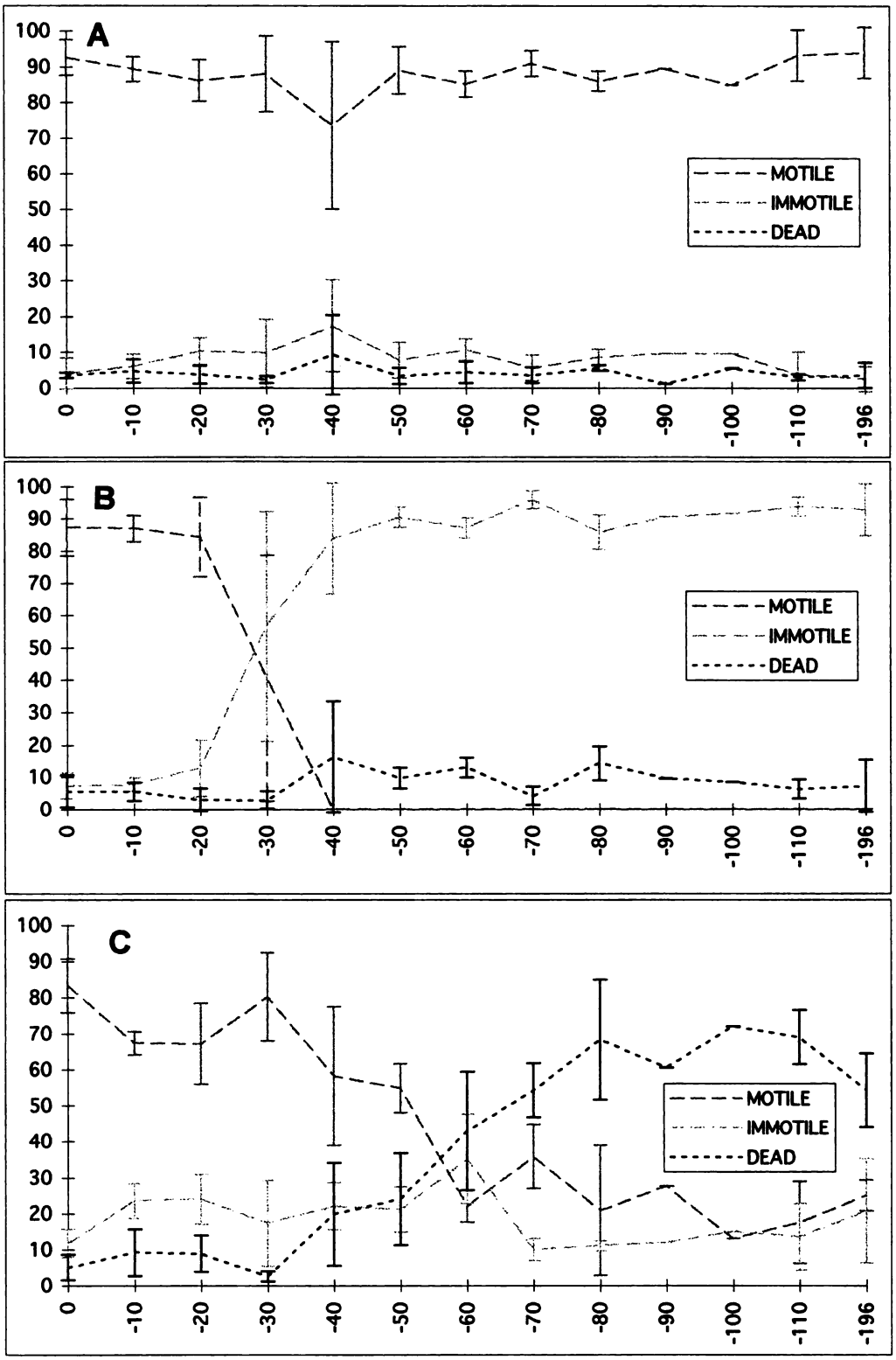

FIG. 5. Proportion (%) of different populations of cells (200 spermatozoa counted for each value) before **(A)**, at **(B)** and after **(C)**, a cool/freeze and thaw (± standard deviation) cycle. In graphs **(A)** and **(C)** all the cells were held at 37°C (experiment 3).

study the preparation of the samples with the use of the *Ferti*Light™ Sperm Viability Kit was very straightforward (Garner et al, 1994). Moreover, the cryostage had few of the problems associated with open cryostage systems (e.g., condensation effects) (Schwartz and Diller, 1982). However, there are a number of points that require further investigation. It is not known how permeable the fluorescent probes are at low temperatures. If the permeability of propidium iodide (PI) was low, then a longer time would be required to allow the probe to diffuse into the cells before making the observations. On thaw, a small proportion of cells appeared to be dual-stained (both red and green) or orange, and these were believed to be moribund as, when left, they fluoresced red only. This may indicate that insufficient time had been left for the PI to enter the cells. Samples frozen on the cryostage had freezing points 10°C lower than those frozen without the kit. This could have been due to the freezing properties of the fluorescent dye itself or its contribution to the overall sample freeze. It is possible that the DMSO in which the vital dye was dissolved lowered the temperature but it was in relatively small quantities (1 μm) compared to the sample (1 ml).

It should be possible to observe single cells during freeze/thaw at higher magnification than in this study. As yet, it is not possible to remove or add media to the cells while they are in the Linkam cryostage. This would be advantageous for some cryobiological observations and when designing cryopreservation protocols.

In conclusion, damage to human spermatozoa cells has been shown to be associated with thawing effects. Fluorescent viability probes have been successfully used with a cryostage to study freeze/thaw damage of human spermatozoa.

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