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Hyperosmotic Tolerance of Human Spermatozoa: Separate Effects of Glycerol, Sodium Chloride, and Sucrose on Spermolysis[1]

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ABSTRACT

Hyperosmotic stress, which cells experience during the freezing process, and its release during the warming process are both related to cryoinjury. To define optimal cooling or warming rates and prevent osmotic injury to human sperm, information is required regarding the osmotic tolerance of the cells as a function of 1) time, 2) temperature, 3) type of solute, and 4) solute concentration. Human sperm samples were divided into three aliquots. The aliquots were equilibrated at 0, 8, and 22°C, respectively. Different hyperosmotic solutions were prepared by addition of either a permeating cryoprotective agent (glycerol) or nonpermeating solutes (sucrose, non-ionic; or NaCl, ionic) to isotonic Mann's Ringer solution. Aliquots of the prepared solutions were equilibrated at 0, 8, and 22°C, respectively. A small volume (2.5 μl) of each sperm aliquot was quickly mixed with 50 μl of each hyperosmotic solution at the corresponding temperature. After times ranging from 5 s to 5 min, 10 μl of each hyperosmotic cell suspension was abruptly returned to an isosmotic environment by mixing with 500 μl of Mann's Ringer solution at the corresponding temperature. The plasma membrane integrity of cells after exposure to hyperosmotic stress and after return to isosmotic conditions was measured by a dual staining (carboxyfluoroscein diacetate and propidium iodide) technique and flow cytometry. The morphology of the treated cells was observed by scanning electron microscopy of freeze-substituted sperm.

The results indicate that human spermatozoa exhibited a significant posthypertonic lysis/injury, i.e., loss of membrane integrity, when returned to isosmotic conditions after exposure to hyperosmotic solutions of NaCl or sucrose. The higher the hyperosmolality, the more serious the cell injury. The majority of the cells (> 50%) lost membrane integrity when the osmolality was ≥ 2000 mOsm. In contrast, if the sperm were not returned to isosmotic conditions, the majority of the sperm in the hyperosmotic solutions appeared to maintain membrane integrity. For a given higher hyperosmolality (> 1000 mOsm), posthypertonic spermolysis was reduced with a decrease of temperature. Cell survival was also affected by time of cell exposure to hyperosmotic environments before cells were returned to the isotonic condition. The shorter the time, the higher the cell survival. When exposed to hyperosmotic glycerol solutions that were isotonic with respect to electrolytes, few cells lost their membrane integrity if the osmolality of glycerol was < 3000 mOsm. For a fixed high osmolality (> 3000 mOsm), the lower the temperature, the higher the percentage spermolysis. At the highest glycerol concentration in this study, i.e., 4694 mOsm, the percentage spermolysis was 17%, 10%, and 2% at 0°C, 8°C, and 22°C, respectively. Spermolysis caused by the removal of glycerol from the cells depended on the means by which the cells were returned to isotonic conditions. A one-step return to isotonic conditions resulted in serious spermolysis, while a multi-step (nine-step) procedure significantly reduced the spermolysis. The scanning electron micrographs showed the distinct morphology of the spermatozoa experiencing the different osmotic conditions. The abnormality of spermatozoa that underwent posthypertonic treatment was demonstrated especially clearly.

INTRODUCTION

Cryopreservation of human spermatozoa is extensively used in artificial insemination and in vitro fertilization programs [1]. However, there is an apparent contradiction between the concept of preservation and experimental results showing that more than 50% of spermatozoa are usually injured by the cryopreservation process itself [2,3]. The mechanism of the sperm injury is not clearly known, but it is generally believed that cryoinjury is related to the hyperosmotic stress [4–7] experienced by cells during the cooling process and the release of this stress during the warming process. When a cell suspension is cooled, extracellular solutes—both ionic/nonionic solutes and cryoprotective agents (CPAs)—become progressively more concentrated as cooling proceeds and water precipitates as ice. This creates a hyperosmotic environment for the cells, which

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may cause 1) changes in pH as buffer salts reach their solubilities [8], 2) increasing cellular dehydration resulting in a cross-linking of the intracellular structures [9], and 3) weakening protein-lipid complexes in the cell membrane and increasing phospholipid losses [4]. All of these are potential mechanisms that may affect cell viability. These possible effects that may cause cell injury as a result of concentration of the solutes have been characterized collectively by Mazur et al. [6] as "solution effects." Mazur et al. suggested that the solution effects on cells are greatly enhanced in a slow cooling process, during which the time of exposure of cells to a concentrated solution is prolonged. In addition to solution effects on cells during the cooling and thawing processes, it has been noted that a change in the cells' osmotic environment that can cause injury is induced by addition of CPAs to cells before cooling and subsequent removal of CPAs from the cells after thawing [10, 11].

To prevent osmotic injury to cells during cryopreservation, one needs to know the osmotic tolerance of the cells as a function of 1) time, 2) temperature, 3) type of solute, and 4) concentration of the solute. Osmotic tolerance varies among cell types and within cell type, among species [12–14]. Therefore, to optimize cryopreservation techniques for human spermatozoa, a series of experiments was conducted to investigate the hyperosmotic tolerance of the sperm to three different solutes: 1) glycerol, a most commonly used permeating CPA in cryopreservation of human sperm [1, 3, 10, 15–17]; 2) NaCl, a nonpermeating and ionic solute commonly used in physiological media; and 3) sucrose, a nonpermeating and nonionic solute commonly used as an "osmotic buffer" during the removal of CPAs [18].

MATERIALS AND METHODS

Preparing Suspensions of Spermatozoa

Human semen was obtained by masturbation from healthy donors after at least 2 days of sexual abstinence. Samples were allowed to liquefy in an incubator (5% CO_2: 95% air, 37°C, and high humidity) for approximately 1 h. Five microliters of the liquefied semen was used for a computer-assisted semen analysis (CASA) via CellSoft (Version 3.2/C, CRYOResources, Ltd., New York, NY) [19, 20]. A swim-up procedure [21] was performed to separate motile from immotile cells. The motile cell suspensions were contrifuged at 400 × g for 10 min and were resuspended in Mann's Ringer solution [22] at a cell concentration of 1 × 10^9 cells/ml.

Preparing Hyperosmotic Solutions

Different hyperosmotic solutions (Table 1) were prepared by adding glycerol, sucrose, or NaCl into isosmotic Mann's Ringer solution (286 mOsm). Since the molality of NaCl, sucrose, or glycerol in the solutions was known, the corresponding osmolality was calculated by using polynomial interpolation [23] of the osmolality data published by Scatchard et al. [24]. It was assumed that osmolalities generated by the different solutes in a solution are additive without interaction between the solutes, i.e., that the summation of the osmolalities is equal to the total osmolality of the solution. The error resulting from this assumption is small. For instance, in the case of the addition of NaCl to Mann's Ringer (286 mOsm), this leads to an overestimate of the total solution osmolality that does not exceed 1.4% in the case of the most concentrated solution used in the present study. The total osmolality (< 2000 mOsm) of some solutions was also checked through use of a freezing-point-depression osmometer (Advanced DigiMate Osmometer, Model 3D2; Advanced Instruments, Inc., Needham Heights, MA).

Exposing Human Sperm to Hyperosmotic Environments

The hyperosmotic solutions were equilibrated to 0, 8, or 22°C. The sperm suspension (1 × 10^9 cells/ml) was di-

TABLE 1. Osmolality of NaCl, sucrose, and glycerol solutions.

NaCl solutions	Molality of NaCl (mol/kg)*	Additional osmolality† (mOsm)	Total osmolality‡ (mOsm)
1	0	0	286
2	0.149	276.2	562.2
3	0.450	830.8	1116.8
4	0.744	1379.0	1665.0
5	1.042	1949.0	2235.0
6	1.340	2540.0	2826.0
7	2.084	4078.0	4364.0
Sucrose solutions	**Molality of sucrose (mol/kg)***	**Additional osmolality† (mOsm)**	**Total osmolality‡ (mOsm)**
1	0	0	286.0
2	0.300	307.0	593.0
3	0.900	970.4	1256.4
4	1.500	1707.0	1993.0
5	2.100	2515.0	2801.0
Glycerol solutions	**Molality of glycerol (mol/kg)***	**Additional osmolality† (mOsm)**	**Total osmolality‡ (mOsm)**
1	0	0	286.0
2	0.300	301.0	587.0
3	0.600	606.0	892.0
4	1.200	1218.0	1504.0
5	1.800	1839.0	2125.0
6	2.400	2463.8	2749.8
7	3.000	3095.8	3381.8
8	4.500	4694.0	4980.0

*Molality of NaCl, sucrose, or glycerol was calculated assuming Mann's Ringer solution = H_2O, i.e. the unit mol/kg means mol/(kg Mann's Ringer)
†Osmolality of NaCl, sucrose, and glycerol solutions (i.e., "Additional osmolality") is equal to "Total osmolality" minus 286 mOsm (osmolality of Mann's Ringer solution).
‡Total osmolality of each solution was computed by assuming that osmolalities are additive without interaction between the solutes. The error resulting from this assumption is small. For instance, in the case of the addition of NaCl to Mann's Ringer (286 mOsm), this leads to an overestimate of the total solution osmolality that does not exceed 1.4% in the case of the most concentrated NaCl solution (i.e., NaCl solution 7).

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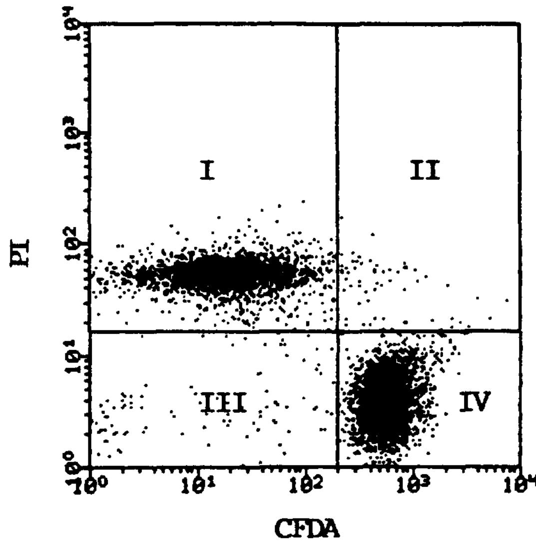

FIG. 1. Scatter diagram showing the different populations of human spermatozoa with CFDA/PI staining (CFDA or PI as labeled in the diagram means the intensity of the CFDA or PI staining). Quadrant I: lysed sperm population (PI-positive); Quadrant II: sperm with dual staining (PI + CFDA); Quadrant III: debris (no staining); Quadrant IV: intact sperm population (CFDA-positive).

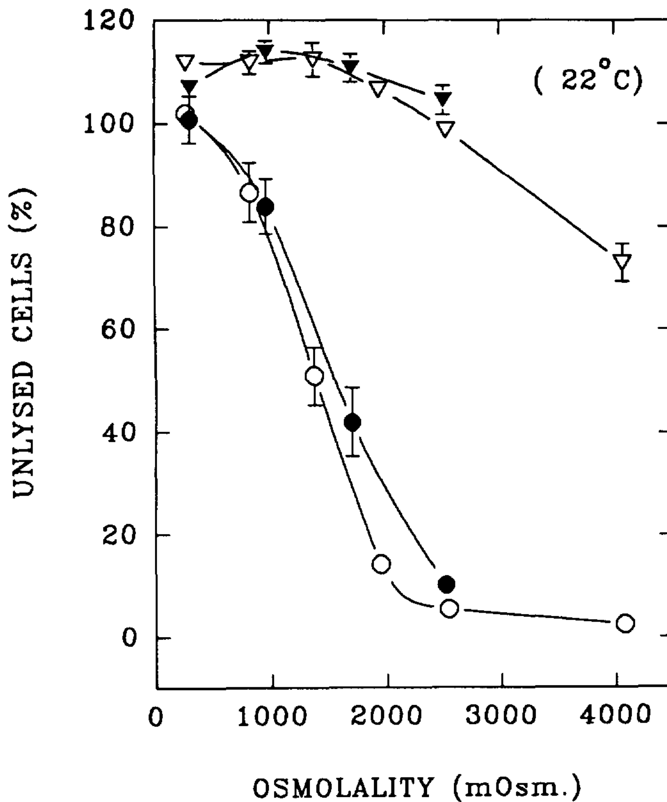

FIG. 2. Normalized percentage (Mean ± SD, n = 6) of unlysed spermatozoa exposed to hyperosmotic solutions at 22°C. Open circles: returned to isotonic condition after 5-min exposure to hyperosmotic NaCl solutions; filled circles: returned to isotonic condition after 5-min exposure to hyperosmotic sucrose solutions; open triangles: exposure to hyperosmotic NaCl solutions only; and filled triangles: exposure to hyperosmotic sucrose solutions only.

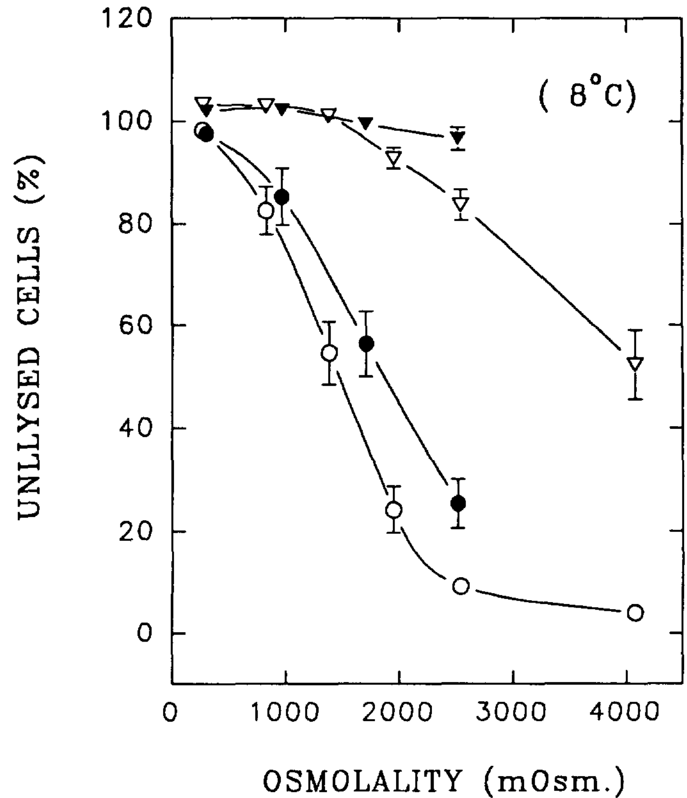

FIG. 3. Normalized percentage (Mean ± SD, n = 6) of unlysed spermatozoa exposed to hyperosmotic solutions at 8°C. Open circles: returned to isotonic condition after 5-min exposure to hyperosmotic NaCl solutions; filled circles: returned to isotonic condition after 5-min exposure to hyperosmotic sucrose solutions; open triangles: exposure to hyperosmotic NaCl solutions only; filled triangles: exposure to hyperosmotic sucrose solutions only.

vided into three aliquots, and the aliquots were equilibrated to three different temperatures (0, 8, or 22°C), respectively, by the following methods. 1) 22°C: This was the initial temperature for all cell samples because the cell suspension was prepared at room temperature (22°C). 2) 8°C: A test tube containing the cell sample (0.2 ml) was first placed in a glass beaker (100-ml capacity) with 50 ml water at 22°C. The beaker with the cell sample was then put in an 8°C cold room. It took approximately 30 min for the sample to reach 8°C (average cooling rate: 0.5°C/min) as measured by a digital thermometer (Model 2176A, Omega Engineering, Inc., Stamford, CT) with copper-constantan thermocouples. 3) 0°C: A test tube containing the cell sample (0.2 ml) was first placed in a glass beaker (100-ml capacity) with 50 ml water at 22°C. The beaker and cell sample were then put in an 8°C cold room. After 35 min in the 8°C cold room, the beaker with the cell sample was transferred into an ice-water mixture bath. It took approximately 10 min for the cell sample to achieve 0°C (average cooling rate: 1°C/min).

Two and one half microliters of each sperm aliquot was added and quickly mixed with 50 μl of each hyperosmotic solution at the corresponding temperatures. After times

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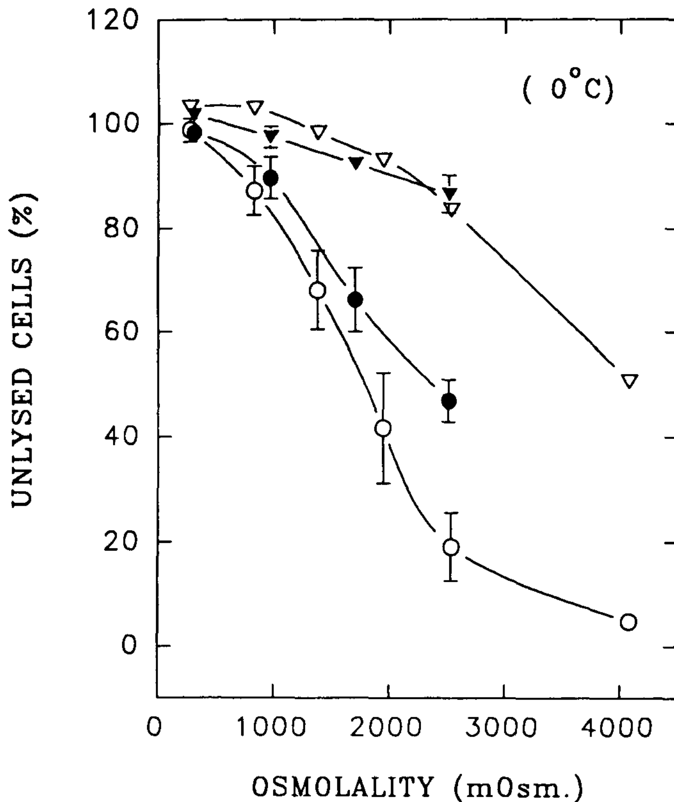

FIG. 4. Normalized percentage (Mean ± SD, n = 6) of unlysed spermatozoa exposed to hyperosmotic solutions at 0°C. Open circles: returned to isotonic condition after 5-min exposure to hyperosmotic NaCl solutions; filled circles: returned to isotonic condition after 5-min exposure to hyperosmotic sucrose solutions; open triangles: exposure to hyperosmotic NaCl solutions only; filled triangles: exposure to hyperosmotic sucrose solutions only.

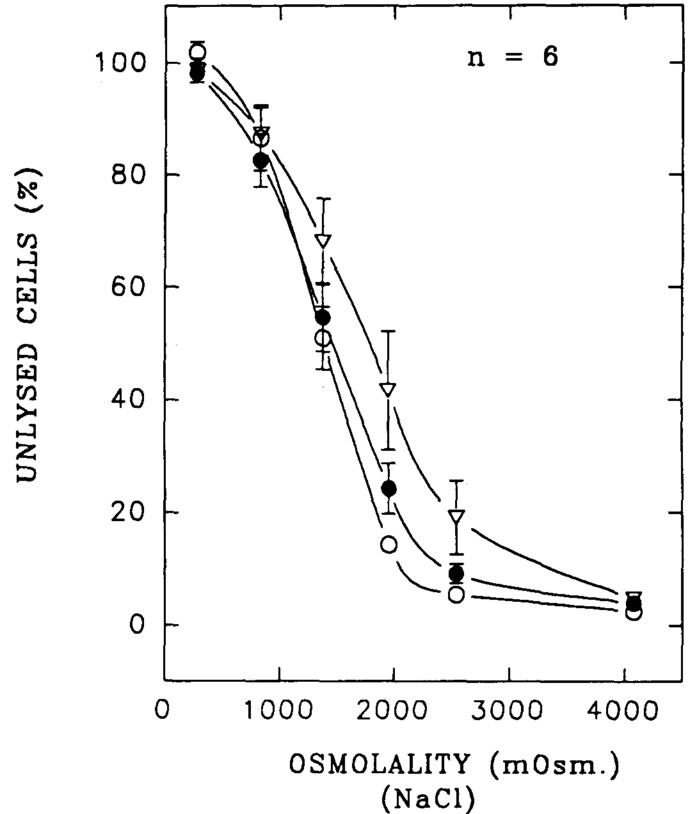

FIG. 5. Normalized percentage (Mean ± SD, n = 6) of unlysed spermatozoa exposed to hyperosmotic NaCl solutions at 0°C (open triangles), 8°C (filled circles), and 22°C (open circles), respectively, and returned to isotonic condition.

ranging from 5 s to 5 min, 10 μl of each hyperosmotic cell suspension was abruptly (one-step) returned to an isotonic environment by diluting and mixing with 0.5 ml of Mann's Ringer solution at the corresponding temperatures. One microliter of each sperm aliquot was used as a normal untreated control. The cells in 1.5 M glycerol solution at 22°C were returned to isotonic condition by either a one-step or a nine-step dilution procedure, i.e., adding and mixing 0.5 ml of Mann's Ringer solution in nine steps (5, 5, 10, 10, 20, 30, 70, 120, and 230 μl) into 10 μl of cell suspension with 1.5 M glycerol. Plasma membrane integrity of the cells in the diluted suspensions or in the remaining hyperosmotic suspensions as well as membrane integrity of the normal control cells was measured by a dual staining technique and flow cytometry.

Determining Percentage of Intact Cells by Flow Cytometry

A methodology for the assessment of sperm membrane integrity, using dual fluorescent staining and flow cytometric analysis, was developed by Garner et al. [25] and previously validated in our laboratory [26, 27]. Propidium iodide (PI) is a bright-red, nucleic acid-specific fluorophore (PI was purchased from Sigma Chemical Co., St. Louis, MO; cat. #P4170) that does not readily permeate an intact plasma membrane of the sperm. Therefore, only sperm cells with damaged membranes are stained by PI. 6-carboxyfluorescein diacetate (CFDA) is a membrane-permeable compound. After penetrating into the cells, it is hydrolyzed by intracellular esterase to 6-carboxyfluoroscein (CF), which is a bright-green, membrane-impermeable fluorophore (CFDA was purchased from Sigma Chemical Co.; cat. #C5041). When CFDA is added into the cell suspension, membrane-intact spermatozoa fluoresce bright green [25]. Five microliters CFDA (0.25 mg/ml dimethylsulfoxide) and 5 μl PI (1 mg/ml H_2O) stock solutions were added to each 0.5 ml of the treated sperm suspensions. A total of 1×10^5 spermatozoa per treatment was analyzed via a FACStar Plus Flowcytometer (Becton, Dickinson, CA). The cells with CFDA staining and without PI staining were considered intact cells. The percentage of intact cells was determined for each treatment. A scatter diagram illustrating the different populations of sperm with CFDA/PI staining is shown in Figure 1. Details for the flow cytometer settings are as follows. 1) Gates were set using forward and 90° light-scatter signals at acquisition to exclude debris and aggregates; 2) instrument alignment was performed daily with fluorescent mi-

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crobead standards to standardize sensitivity and setup; 3) photomultiplier settings were adjusted with unstained spermatozoa, and electronic compensation was adjusted for spectral overlap with individually stained cells; and 4) excitation was at 488 nm from a 4-Watt Argon laser operating at 200 mW. Fluorescein emission intensity was measured via a 530/30 bandpass filter, PI intensity via a 630/22 bandpass filter.

Determining Intracellular Water Loss

It has been estimated [21] that under isotonic condition (isotonic osmolality, M_i = 286 mOsm), 59% of total sperm cell volume is water volume (V_{iw}) and 15% of the water volume is osmotically inactive (V_a). Because human sperm have been shown to be a good representative of an ideal osmometer [28], when the spermatozoa are exposed to a solution with a nonpermeating solute osmolality, M, the corresponding water volume (V_w) at osmotic equilibrium can be determined by solving the following equation:

$$\frac{M}{M_i} = \frac{V_{iw} - V_a}{V_w - V_a} \quad (1)$$

or

$$V_w = \frac{(V_{iw} - V_a) M_i}{M} + V_a \quad (2)$$

Dividing two sides of equality (2) by V_{iw} yields:

$$\frac{V_w}{V_{iw}} = \left[1 - \frac{V_a}{V_{iw}}\right] \frac{M_i}{M} + \frac{V_a}{V_{iw}}$$

Because V_a = 15% V_{iw} [21], one can get:

$$\frac{V_w}{V_{iw}} = 0.85 \times \frac{M_i}{M} + 0.15 \quad (3)$$

Therefore, the fraction (F) of cell water lost can be estimated as:

$$F = 1 - \frac{V_w}{V_{iw}} = 0.85 \times \left[1 - \frac{M_i}{M}\right] \quad (4)$$

Examination of Specimens Using Scanning Electron Microscopy

Human sperm suspended in different osmotic solutions were concentrated by centrifugation (400 × *g*). The concentrated cell suspensions were placed in capillary tubes (0.5 mm in diameter), sealed (Critoseal; Monojet Scientific, St. Louis, MO), and quench-frozen in liquid nitrogen. The capillary tubes were fractured under liquid nitrogen, and approximately 1-cm-long segments were rapidly transferred to vials containing absolute ethanol at −80°C. Specimens were freeze-substituted in absolute ethanol at −80°C for 7 days, warmed to room temperature, and then rinsed with absolute ethanol [29, 30]. Specimens within glass straws were critical point-dried. Glass straws were fractured, and both pieces of glass straws and the contents were mounted on aluminum stubs. Samples were sputter-coated with gold-palladium and examined through use of a JEOL JSM-840 scanning electron microscope (JEOL, Ltd., Tokyo, Japan) at 10 kV accelerating voltage [30].

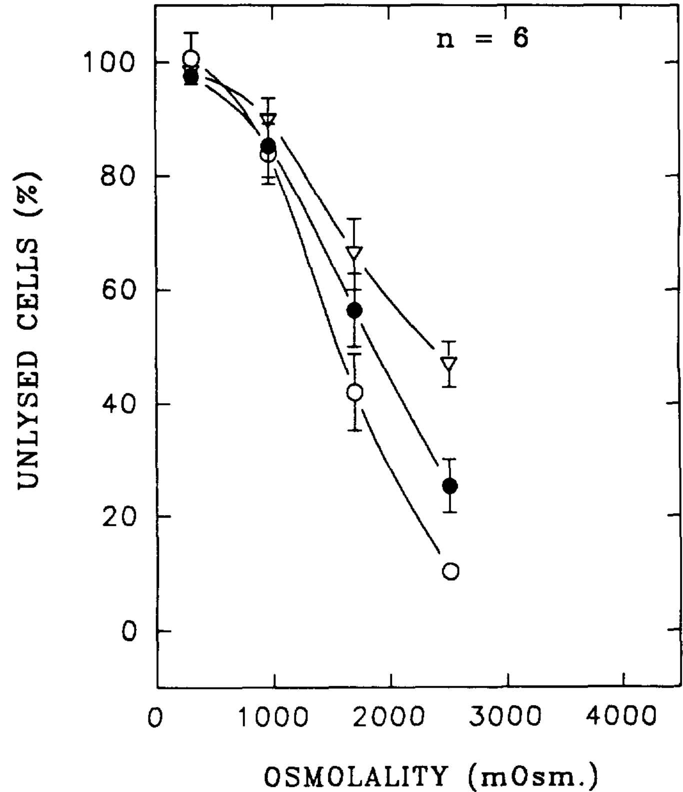

FIG. 6. Normalized percentage (Mean ± SD, n = 6) of unlysed spermatozoa exposed to hyperosmotic sucrose solutions at 0°C (open triangles), 8°C (filled circles), and 22°C (open circles), respectively, and returned to isotonic condition.

Statistical Analysis

Data were analyzed using the General Linear Models procedure of the Statistical Analysis System [31]. Comparisons were conducted through use of a protected LSD (least significant difference) approach [31, 32].

RESULTS

The percentage of unlysed spermatozoa in the hyperosmotic treatment groups was normalized to the percentage of intact cells in corresponding isotonic control cell suspensions. The osmolality of NaCl, sucrose, or glycerol in hyperosmotic solutions was defined as the total osmolality of the solutions minus 286 mOsm (osmolality of Mann's Ringer solution).

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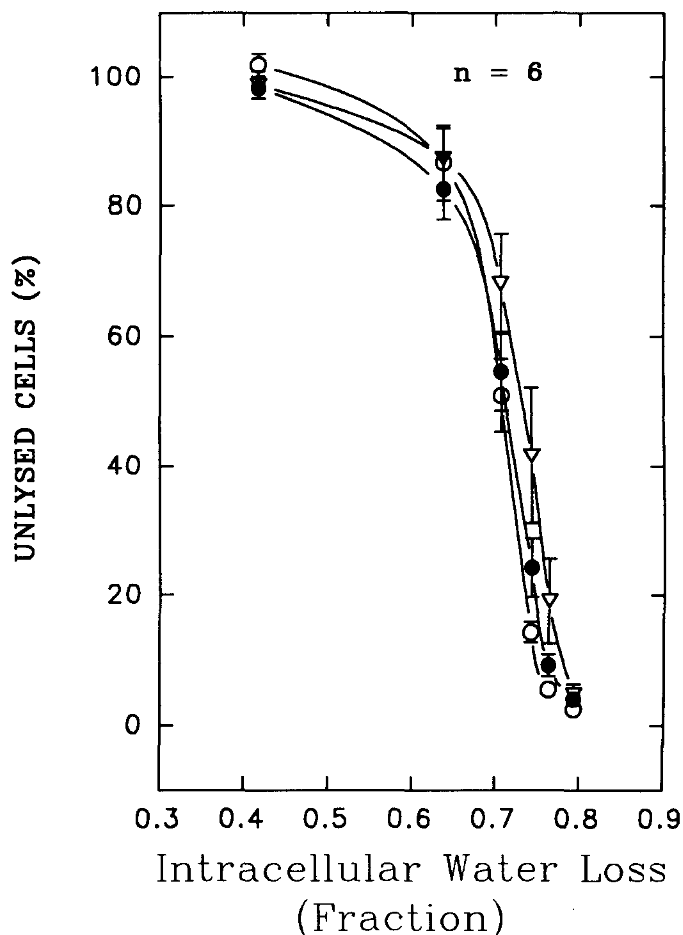

FIG. 7. Normalized percentage (Mean ± SD, n = 6) of unlysed spermatozoa as a function of intracellular water loss. The cells were exposed to hyperosmotic NaCl solutions at 0°C (open triangles), 8°C (filled circles), and 22°C (open circles), respectively, for 5 min and then one-step returned to isotonic condition.

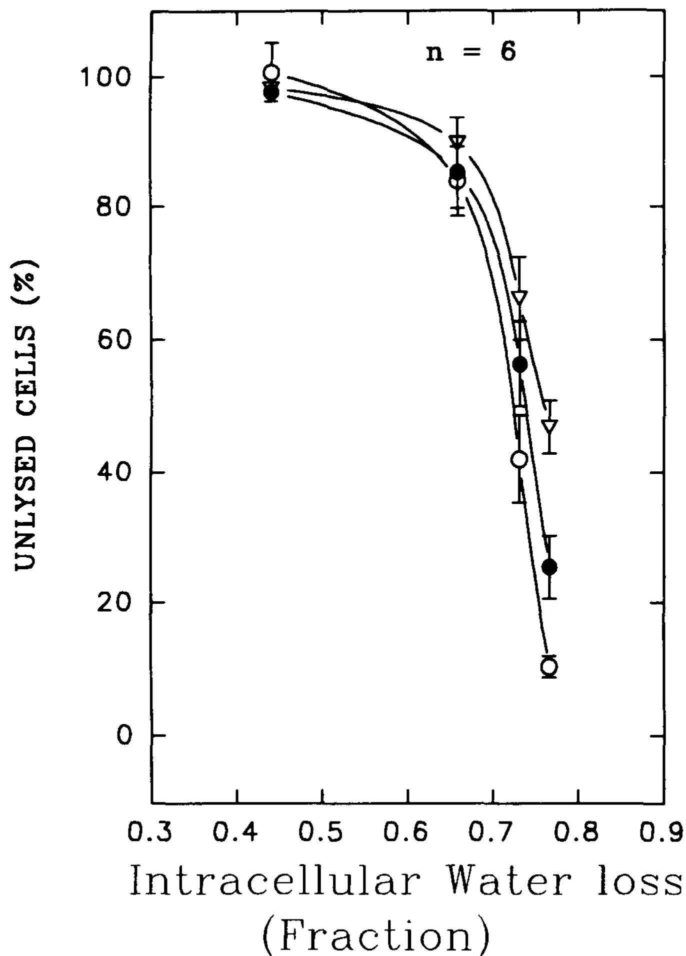

FIG. 8. Normalized percentage (Mean ± SD, n = 6) of unlysed spermatozoa as a function of intracellular water loss. The cells were exposed to hyperosmotic sucrose solutions at 0°C (open triangles), 8°C (filled circles), and 22°C (open circles), respectively, for 5 min and then one-step returned to isotonic condition.

Exposing Human Sperm to Hyperosmotic Environments

It was noted that the slow cooling process alone, which was used to cool sperm samples from 22°C to 8°C or 0°C, did not cause membrane damage of the sperm as measured by the dual-staining technique. The data regarding effects of NaCl and sucrose osmolalities on the survival of human spermatozoa at 22, 8, and 0°C are shown in Figures 2, 3, and 4, respectively. The sperm appeared to maintain their membrane integrity in the hyperosmotic NaCl or sucrose solutions ($p < 0.001$) if they were not returned to isotonic conditions. In contrast, sperm showed a significant loss of membrane integrity when returned to isotonic conditions after 5-min exposure to hyperosmotic solutions of NaCl or sucrose. Similar phenomena have been observed for other cell types [12, 14, 33], and this kind of cell injury has been defined as post-hypertonic injury [33].

Posthypertonic spermolysis. There was a significant effect of NaCl/sucrose hyperosmolality on post-hypertonic spermolysis ($p < 0.001$). The greater the hyperosmolality, the more serious the cell injury (Figs. 2–4). The majority of cells (> 50%) lost their membrane integrity when the hyperosmolality was ≥ 2000 mOsm. In addition, post-hypertonic injury varied between solutes (i.e., ionic vs. nonionic, Figs. 2–4) ($p < 0.001$); sucrose was less injurious than NaCl. Post-hypertonic spermolysis taking place at different temperatures is shown in Figures 5 and 6. There was no main effect of temperature on post-hypertonic spermolysis ($p = 0.087$). However, there were significant interactions ($p < 0.001$) between 1) temperature and hyperosmolality, 2) temperature and type of solute, and 3) temperature and dilution treatment. This indicates that spermolysis is highly influenced by the interaction among temperature and other factors. For a given higher hyperosmolality (> 1000 mOsm), post-hypertonic spermolysis was reduced with a decrease of temperature ($p < 0.001$) (cf. Figs. 5 and 6). The corresponding water loss in human sperm in the different hyperosmotic solutions was calculated using equation 4. Figures 7 and 8 show a correlation between post-hypertonic spermolysis and fraction of cell water loss.

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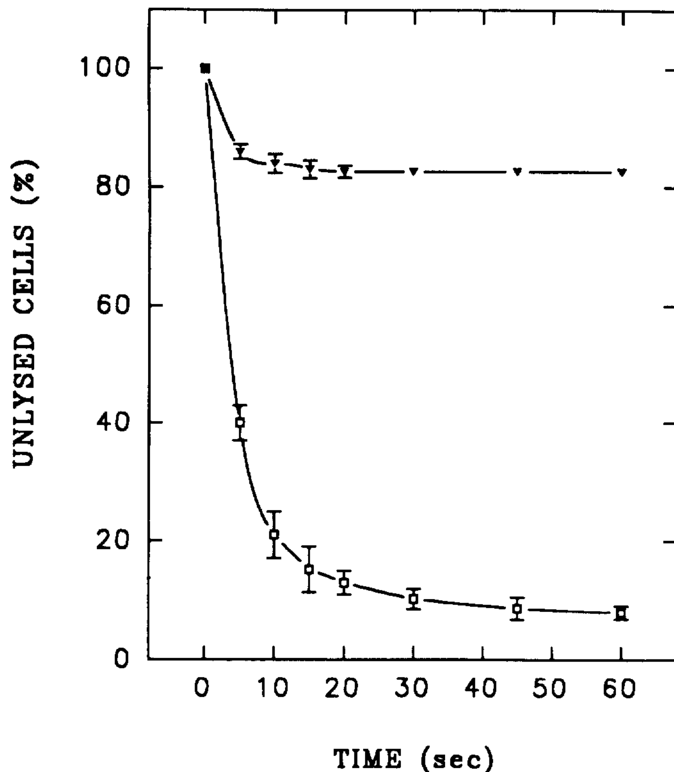

FIG. 9. Normalized percentage (Mean ± SD, n = 6) of unlysed spermatozoa as a function of the time of sperm exposure to 1000 (filled triangles) and 2300 mOsm (open squares) NaCl solutions at 22°C before the cells were returned to isotonic condition.

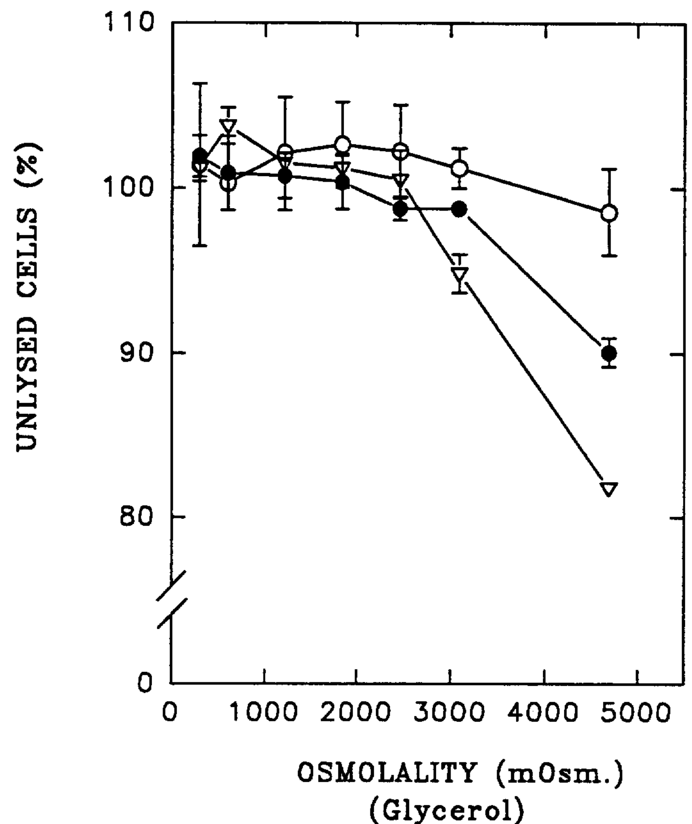

FIG. 10. Normalized percentage (Mean ± SD, n = 6) of unlysed spermatozoa after 20-min exposure to hyperosmotic glycerol solutions at 0°C (open triangles), 8°C (filled circles), and 22°C (open circles), respectively.

Post-hypertonic spermolysis was increased quickly with an increase of intracellular water loss. The time of cell exposure to hyperosmotic environments before cells were returned to the isotonic condition was found to be important for cell survival. As shown in Figure 9, when exposed to hyperosmotic NaCl solutions, cells rapidly lost their membrane integrity within 30 s from the beginning of the exposure. For this time period, the shorter the exposure, the greater the percentage of cell survival. It should be mentioned that in the present study, the minimum cell exposure time (5 s) is longer than the calculated time required for cell dehydration to achieve osmotic equilibrium because of the high water permeability of the sperm [26].

Spermolysis caused by glycerol. When exposed to hyperosmotic glycerol solutions, cells retained membrane integrity if the osmolality of glycerol was < 3000 mOsm (Fig. 10). For glycerol at an osmolality > 3000 mOsm, spermolysis increased with an increase of glycerol concentration ($p < 0.001$) and a decrease of temperature ($p < 0.001$). At the highest concentration of glycerol used in this study (i.e., 4980 mOsm), the percentage spermolysis was 17%, 10%, and 2% at 0°C, 8°C, and 22°C, respectively. Spermolysis caused by removal of glycerol from the cells depended on the means by which the cells were returned to isotonic conditions. As shown in Figure 11, for a cell suspension with 1.5 M glycerol, the one-step return (see *Ma-*

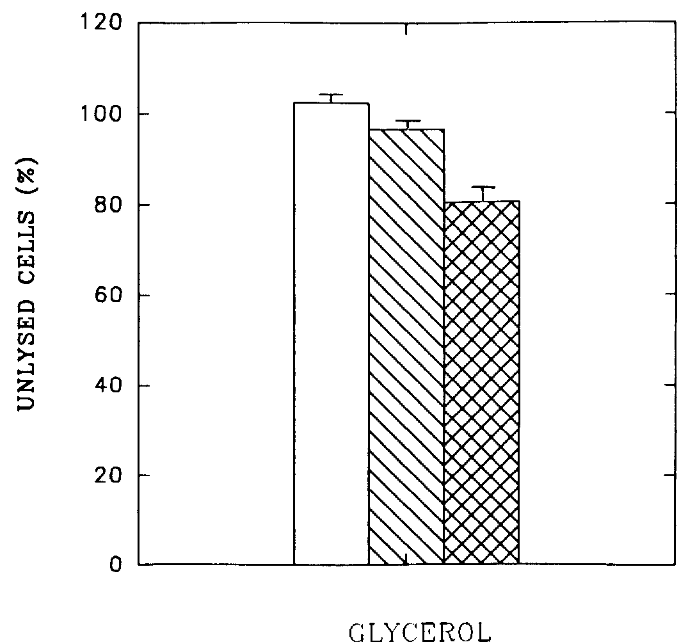

FIG. 11. Normalized percentage (Mean ± SD, n = 6) of unlysed spermatozoa returned or not returned to isotonic condition after 20-min exposure to Mann's Ringer solution with 1.5 M glycerol. Open column: not returned to isotonic condition; striped column: nine-step returned to isotonic condition; cross-hatched column: one-step returned to isotonic condition.

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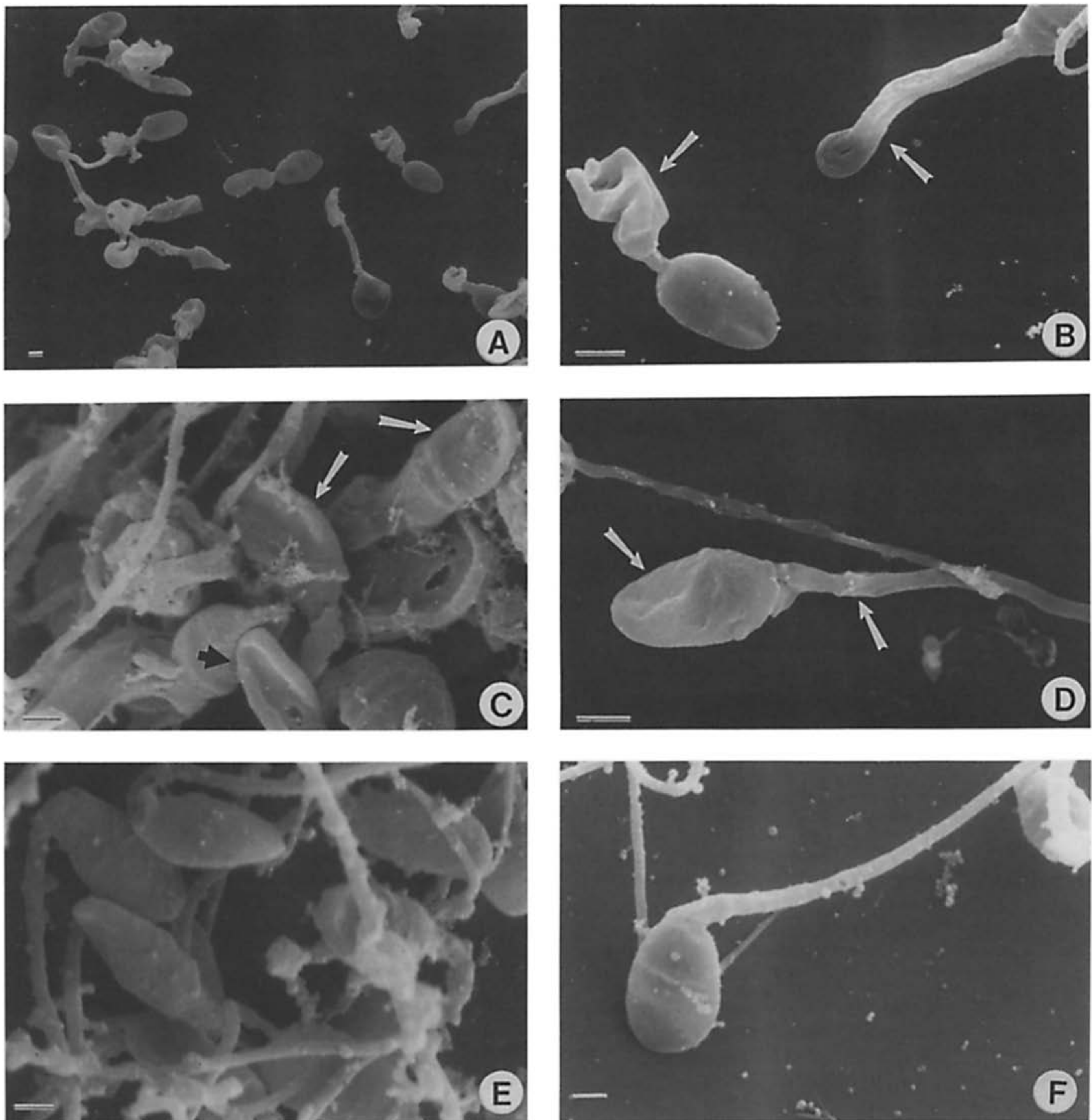

FIG. 12. Scanning electron micrographs showing a comparison of relative volumes and morphology of human spermatozoa in different osmotic conditions. The volume and morphology of the sperm were preserved by a freeze-substitution technique [29, 30]. (A) and (B): Swollen cells in 100 mOsm NaCl solution (white arrows point out the swelling, curling and bending of the sperm tails). (C) and (D): Shrunken cells in 2650 mOsm NaCl solution (white arrows indicate very rough membrane surface of sperm head and tails; a black arrow indicates the folding of the sperm head). (E) and (F): Sperm showing normal volume in isotonic Mann's Ringer solution. Scale bar = 1 μm.

terials and Methods) to isotonic conditions (Mann's Ringer solution) could cause over 20% spermolysis on average, while the nine-step procedure significantly reduced spermolysis (3% spermolysis on average; $p < 0.001$).

Morphology of the Treated Human Sperm

Scanning electron micrographs of human sperm treated by the different methods are shown in Figures 12 and 13. Figure 12 shows a comparison of relative volumes and the

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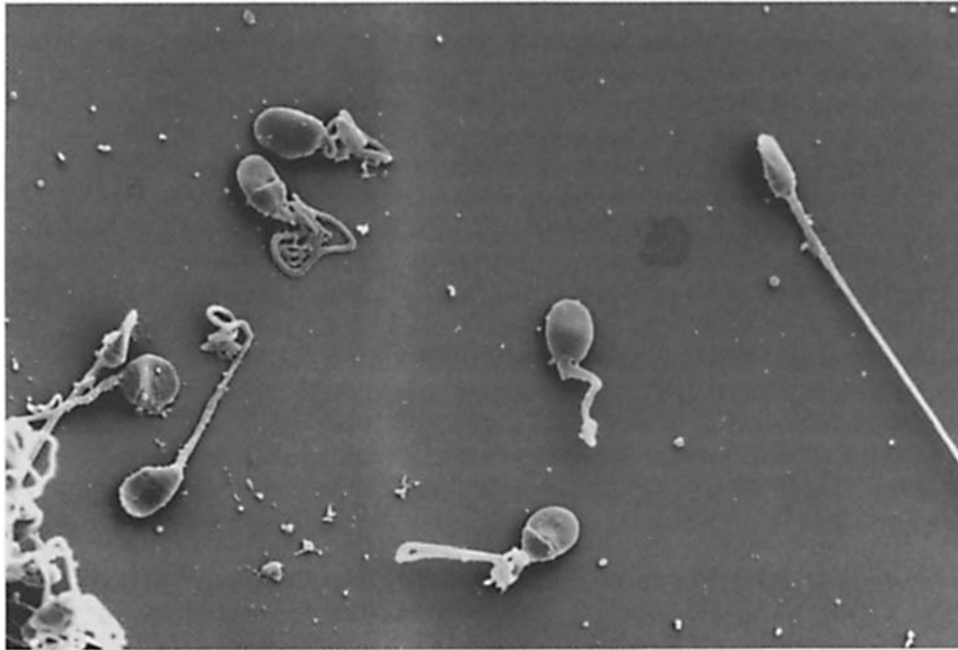

FIG. 13. Scanning electron micrograph (×2000) showing the distinct morphology (curling and bending of the tails) of spermatozoa returned to isotonic Mann's Ringer medium after a 5-min exposure to hypertonic (2650 mOsm) NaCl solution.

morphology of the sperm in hypotonic, hypertonic, or isotonic conditions. Figures 12A and 12B show swollen human spermatozoa with the curling and/or bending of the tails in a hypoosmotic NaCl solution (100 mOsm). Shrunken sperm in a hyperosmotic NaCl solution (2650 mOsm) are shown in Figure 12, C and D. Human sperm with normal volume in the isotonic Mann's Ringer solution are shown in Figure 12, E and F. In contrast, the sperm that were re-

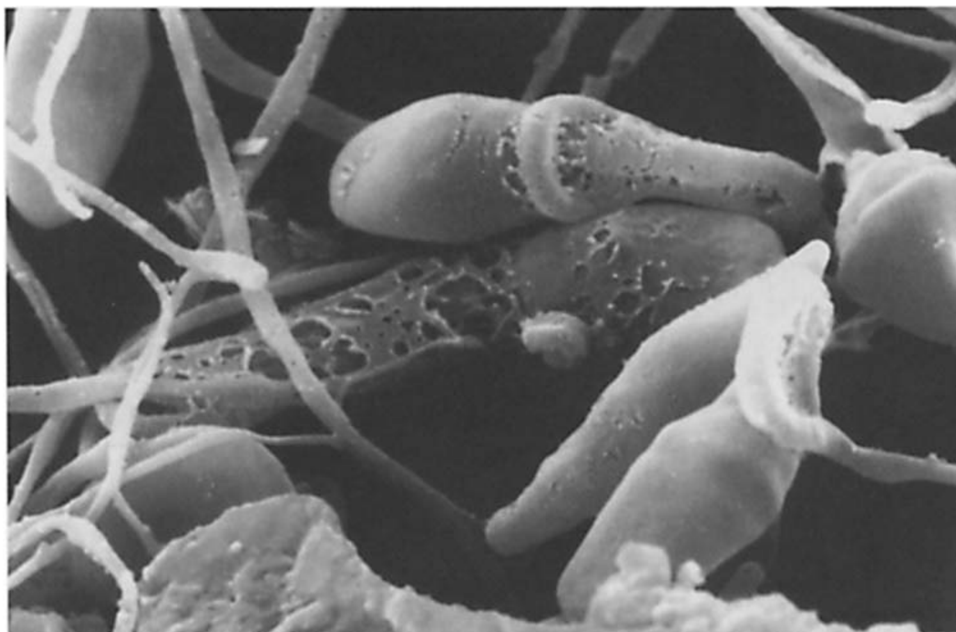

FIG. 14. Scanning electron micrograph (×10 000) showing the swollen spermatozoa in 1.5 M glycerol solution, which is isosmotic with respect to electrolytes.

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turned to isotonic Mann's Ringer medium after a 5-min exposure to 2650 mOsm NaCl solution exhibited an abnormal morphology (curling or bending of the tails) as shown in Figure 13. The sperm in 1.5 M glycerol solution, which was isoosmotic with respect to electrolytes, were swollen as shown in Figure 14. The volumetric expansion of sperm in the glycerol solution resulted from a net glycerol permeation into the cells to achieve osmotic equilibrium.

DISCUSSION

Human spermatozoa were damaged when they were returned to isotonic conditions after they had been exposed at 0, 8, 22°C to hypertonic NaCl/sucrose solutions. Cell damage was expressed as a loss of membrane integrity as measured by CFDA and PI staining. For a given solute (NaCl or sucrose), post-hypertonic spermolysis depended significantly on the hyperosmolality of the solute.

In a hyperosmotic solution with nonpermeating solutes, if there is no damage or no irreversible change in the sperm plasma membrane during the dehydration process, then theoretically the cells should maintain membrane integrity after a return to isotonic conditions. However, this hypothesis was not supported by the present experimental results (Figs. 5 and 6), indicating that the membrane may undergo irreversible change and/or damage during the dehydration process. As shown in Figures 7 and 8, there is a correlation between post-hypertonic spermolysis and the degree of cell water loss. A sharp drop in the percentage of surviving sperm started when the cells lost approximately 65% of their intracellular water; this is consistent with previous results for the other cell types [5]. When exposed to 3000 mOsm NaCl or sucrose (total osmolality is 3000 + 286 = 3286 mOsm), the sperm lost nearly 80% of their cell water; and more than 80% of the cells were lysed after being returned to an isotonic condition. Morphologically, as shown in Figure 12, C and D, the flattening, cupping, and wrinkling of the sperm head and tail regions reflect the severe shrinkage of sperm in a hyperosmotic solution. The rough surfaces of shrunken cell membranes may result from an interaction between the shrinkage of the cell membrane and a resistance of the cytoskeleton or other constitutive intracellular structures.

Although several hypotheses [4–6, 34, 35] have been invoked to explain the cause of post-hypertonic injury, the mechanism whereby an increase in extracellular solute concentration damages cells is still not known clearly; but this is of fundamental importance to theories of slow-freezing injury [6, 9, 36, 37] and to any attempt to distinguish between osmotic and toxic effects of high concentrations of solutes. Finding's from the present study were the following. 1) Osmotic damage resulting in a loss of membrane integrity is related to the severe shrinkage of sperm in hyperosmotic solution. However, the cells appeared to maintain membrane integrity in the shrunken state. Serious cell membrane damage can be detected only after the cells are returned to isotonic conditions. 2) Post-hypertonic lesion of human spermatozoa was found to be reduced with a decrease of temperature as shown in Figures 5 and 6. The sperm can tolerate a greater degree of dehydration at lower temperature. 3) Post-hypertonic injury of human sperm was shown to be a function of the time of cell exposure to a hypertonic environment before return to isotonic conditions (Fig. 9). The shorter the time, the higher the percentage cell survival. This indicates that cells can tolerate severe shrinkage for a short time.

When the sperm were returned to isotonic conditions after exposure to the hyperosmotic solution, curling and bending of the sperm tails were observed as shown in Figure 13. It was noted that curling and bending of the sperm tails also occurred when the sperm were simply exposed to hypotonic solutions, as shown in Figure 12, A and B. In both these cases, curling and bending of the sperm tails took place when sperm in a relatively hyperosmotic solution were exposed to a relatively hypoosmotic environment. The mechanism causing the curling and bending of the tails is still unknown.

When exposed to hyperosmotic glycerol solutions, the sperm first shrink because of dehydration and then increase in volume as the glycerol permeates and water concomitantly reenters the cell [38]. The injury to sperm undergoing shrinking and reswelling processes in the glycerol solutions was much less serious than the post-hypertonic injury caused by NaCl or sucrose, as shown in Figures 5, 6, and 10 (Note: in these experiments, the cells all experienced the shrinking and reswelling processes). This may be attributable to the fact that the sperm in glycerol solutions experienced severe shrinkage/dehydration for only a very short period of time because of the high glycerol permeability of human sperm [27]; as shown in Figure 9, sperm can tolerate short-time severe shrinkage or dehydration. This finding may also be due to the protective effect of glycerol on the cell membrane [39–42]. Spermolysis in solutions with relatively high glycerol concentration (> 2500 mOsm; Fig. 10) might result from severe shrinkage and subsequent reswelling of the sperm and/or the potential toxicity of glycerol [10] to the cells. It was noted that spermolysis in glycerol solutions was exacerbated with a decrease of temperature (Fig. 10). One possible explanation for this result is that, for a given glycerol concentration, shrinkage of the sperm is more serious at a lower temperature than that at a higher temperature because the activation energy of glycerol permeability to the human sperm membrane is five times higher than that of water (11.76 kcal/mol vs. 2.4 kcal/mol [26, 27]).

The temperature dependence of posthypertonic damage has obvious cryobiological significance. During the slow cooling process, cells may experience over 8000 mOsm hyperosmotic stress for a long time at low temperatures because of the precipitation of extracellular water as ice [43]. The hyperosmotic stress is released during the warming

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process. In conventional cryopreservation, human sperm are cooled (−1 to −100°C/min) from room temperature to −80°C, sometimes with a seeding procedure at −5°C [3, 10, 15–17], and then transferred into liquid nitrogen (−196°C). From the current study it has been found that for human spermatozoa, post-hypertonic spermolysis decreases with the reduction of temperature between 22°C and 0°C (Figs. 5 and 6). If post-hypertonic spermolysis is further reduced or at least not increased at subzero temperatures, this result may be used to explain, in part, why a slow cooling process is successful and is currently used for cryopreservation of sperm cells [3] (post-hypertonic spermolysis at subzero temperatures was not investigated in the present study). In contrast, it has been found that the temperature dependence of post-hypertonic damage of human granulocytes is different from that of human spermatozoa. With a decrease of temperature, the granulocyte's post-hypertonic injury increases [14]. This may be relevant to the reasons for the relative lack of success to date in cryopreservation of the human granulocyte [44].

An important question that remains unanswered is whether human sperm are damaged during shrinkage or during subsequent rehydration. The dependence of sperm survival at any temperature (0, 8, or 22°C) on the dilution of the medium after cell exposure to hyperosmotic NaCl or sucrose solutions (Figs. 2–4) suggested that the damage occurred as the shrunken cells were returned to isosmotic conditions (i.e., during rehydration). As mentioned above, one hypothesis is that the cell plasma membrane had been irreversibly changed during shrinkage and that it was this irreversible change that caused spermolysis only after the shrunken cells were returned to isotonic condition. Several questions remain unaddressed. First, what is the mechanism of the irreversible membrane change (membrane fusion, loss of membrane lipids, denaturation of protein, or cross-linking of cytoskeleton, etc.)? Second, why does spermolysis under hyperosmotic condition become significant only when the hyperosmolality exceeds 3000 mOsm? Third, why is post-hypertonic spermolysis a function of temperature? Finally, why is ionic hyperosmotic stress more damaging than nonionic hyperosmotic stress? These problems form the basis for current ongoing and future investigations.

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