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Prevention of osmotic injury to human spermatozoa during addition and removal of glycerol*

D.Y.Gao[1], J.Liu[1,2], C.Liu[1], L.E.McGann[1,3], P.F.Watson[1,4], F.W.Kleinhans[1,2], P.Mazur[1,5], E.S.Critser[1,6] and J.K.Critser[1,6,7]

[1]Cryobiology Research Institute, Methodist Hospital of Indiana, Indianapolis, IN 46202, [2]Department of Physics, Indiana University – Purdue University at Indianapolis, IN 46202, USA, [3]Department of Pathology, University of Alberta, Edmonton, Alberta, Canada T6G 2R8, [4]Department of Veterinary Basic Sciences, The Royal Veterinary College, London, NW1 OTU, UK, [5]Biology Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831 and [6]Departments of Physiology and Biophysics and Obstetrics and Gynecology, Indiana University School of Medicine, Indianapolis, IN 46202, USA

[7]To whom correspondence should be addressed at: Cryobiology Research Institute, Methodist Hospital of Indiana, 1701 North Senate Boulevard, Indianapolis, IN 46206–1367, USA

Use of a cryoprotective agent is indispensable to prevent injury to human spermatozoa during the cryopreservation process. However, addition of cryoprotective agents to spermatozoa before cooling and their removal after warming may create severe osmotic stress for the cells, resulting in injury. The objective of this study was to test the hypothesis that the degree (or magnitude) of human sperm volume excursion can be used as an independent indicator to evaluate and predict possible osmotic injury to spermatozoa during the addition and removal of cryoprotective agents. Glycerol was used as a model cryoprotective agent in the present study. To test this hypothesis, first the tolerance limits of spermatozoa to swelling in hypoosmotic solutions (iso-osmotic medium diluted with water) and to shrinkage in hyperosmotic solutions (iso-osmotic medium with sucrose) were determined. Sperm plasma membrane integrity was measured by fluorescent staining, and sperm motility was assessed by computer-assisted semen analysis before, during and after the anisosmotic exposure. The results indicate firstly that motility was much more sensitive to anisosmotic conditions than membrane integrity, and secondly that motility was substantially more sensitive to hypotonic than to hypertonic conditions. Based on the experimental data, osmotic injury as a function of sperm volume excursion (swelling or shrinking) was determined. The second step, using these sperm volume excursion limits and previously measured glycerol and water permeability coefficients of human spermatozoa, was to predict, by computer simulation, the cell osmotic injury caused by different procedures for the addition and removal of glycerol. The predicted sperm injury was confirmed by experiment. Based on this study, an analytical methodology has been developed for predicting optimal protocols to reduce osmotic injury associated with the addition and removal of hypertonic concentrations of glycerol in human spermatozoa.

Key words: glycerol/human spermatozoa/osmotic injury

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Introduction

Significant survival of cryopreserved cells became a reality only after the discovery and use of cryoprotective agents (Polge *et al.*, 1949). The majority of cryoprotectants currently used are chemicals to which the cell membrane is permeable (so-called 'permeating cryoprotective agents'). Two important procedures related to the use of permeating cryoprotective agents in the cryopreservation of cells are (i) the addition of a cryoprotective agent to the cells before freezing, and (ii) the removal of the cryoprotective agent from the cells after freezing and thawing. In the first case, when a cell is placed in a solution that is hyperosmotic with respect to the permeating cryoprotective agents (e.g. glycerol) but isotonic with respect to the impermeable salts, it first shrinks because of the osmotic efflux of intracellular water and then increases in volume as the solute (glycerol) permeates and as water concomitantly re-enters the cell. Subsequently, in the second case, when cells with a high intracellular concentration of cryoprotectant are exposed to an isotonic solution, they will swell because of an osmotic influx of extracellular water and then decrease in volume as glycerol diffuses out of the cells and as water concomitantly moves out. As a result of these two aspects of the cryopreservation procedure, the cells experience osmotic stress which may cause significant 'osmotic' cell injury (Sherman, 1973; Mazur and Schneider, 1984, 1986; Penninckx *et al.*, 1984; Leibo, 1986; Critser *et al.*, 1988a).

In order to minimize osmotic injury, several techniques have been used, including (i) a multi-step addition and multi-step removal of permeating cryoprotective agents (Watson, 1979) and (ii) a multi-step addition and two-step removal (using a non-permeating solute as osmotic buffer) of the cryoprotective agents (Rowe *et al.*, 1968; Mazur and Leibo, 1977; Leibo, 1981). To date, attempts to develop procedures for the addition and dilution of cryoprotective agents have been based primarily on empirical approaches, i.e. for a given cell type, various temperatures, cryoprotective agent types and concentrations,

and a number of procedures or steps for cryoprotectant addition and removal are tested to find an acceptable procedure.

The high osmotic sensitivity of human spermatozoa and the corresponding marked post-hyperosmotic injury of spermatozoa have been recently reported (Gao *et al.*, 1993; Curry and Watson, 1994). The reasons for this injury are not clear. One consequence of anisosmotic exposure is cell volume excursion. The objective of this study was to test the hypothesis that the degree of human sperm volume excursion can be used as an independent indicator to evaluate and predict the possible osmotic injury of spermatozoa during addition and removal of cryoprotective agents. Glycerol was used as a model in this study because it is the most commonly used cryoprotectant in the cryopreservation of spermatozoa (Polge *et al.*, 1949; Watson, 1979; Critser *et al.*, 1988a), it is an effective cryoprotectant for many other cell types, and also because it is the only cryoprotective agent whose permeability characteristics for human spermatozoa have been reported (Du *et al.*, 1994; Gao *et al.*, 1992). The hypothesis was tested using the following procedures: (i) to determine sperm osmotic injury as a function of its volume excursion limits (swelling/shrinking) in anisosmotic solutions containing only non-permeating solutes without glycerol; (ii) to simulate, by computer, the kinetics of water–glycerol transport through the sperm plasma membrane and to calculate the sperm volume excursion during different glycerol addition and removal processes using membrane transport equations and previously determined sperm membrane permeability coefficients for glycerol and water; (iii) combining information obtained from procedures i and ii, to predict sperm osmotic injury caused by different procedures of glycerol addition and removal; and (iv) to perform experiments to test the predictions. If the hypothesis is confirmed, the above procedures also provide a methodology for predicting optimal protocols to reduce the osmotic injury associated with the addition and removal of high concentrations of glycerol in human spermatozoa.

Materials and methods

Preparation of sperm suspension

Human semen samples were 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 ~1 h. A total of 5 µl of the liquefied semen were used for a computer-assisted semen analysis (CASA) using CellSoft (Version 3.2/C, CRYO Resources, Ltd, Montgomery, NY, USA) (Jequier and Crich, 1986; Critser *et al.*, 1988b). A swim-up procedure was performed to separate motile from immotile cells [layering 500 µl of modified Tyrode's medium (TALP; Bavister *et al.*, 1983) over 250 µl of semen, incubating for ~1 h in the incubator and carefully aspirating 400 µl of the supernatant in which >95% of spermatozoa were motile]. The motile cell suspensions were centrifuged at 400 *g* for 7 min and resuspended in the TALP medium (286–290 mOsmol) supplemented with pyruvate (0.01 mg/ml) and bovine serum albumin (4 mg/ml), at a cell concentration of 1×10^9 cells/ml.

Assessment of human sperm membrane integrity

A methodology for the assessment of sperm membrane integrity, using dual fluorescent staining and flow cytometric analysis, has been developed by Garner *et al.* (1986) and previously validated in our laboratory (Gao *et al.*, 1992, 1993; Noiles *et al.*, 1993). Propidium iodide (catalogue no. P4170; Sigma Chemical Co., St Louis, MO, USA) is a bright red, nucleic acid-specific fluorophore which permeates poorly into spermatozoa with intact plasma membrane, but is able to diffuse readily into spermatozoa with a damaged membrane. 6-Carboxyfluoroscein diacetate (CFDA; Sigma, Catalog #C5041) is a membrane-permeable compound. After penetrating into cells, it is hydrolysed by intracellular esterase to 6-carboxyfluoroscein which is a bright green, membrane-impermeable fluorophore (Garner *et al.*, 1986). When CFDA is added into the cell suspension with membrane-intact spermatozoa, the cells fluoresce bright green (Garner *et al.*, 1986). Thus 5 µl CFDA (0.25 mg/ml DMSO) and 5 µl propidium iodide (1 mg/ml water) stock solutions were added to each 0.5 ml of the treated sperm suspensions. A total of 1×10^5 spermatozoa per treatment were analysed using a FACStar Plus Flow cytometer (Becton Dickinson, Rutherford, NJ, USA). The cells with CFDA staining and without propidium iodide staining were considered as intact cells. The percentage of intact cells was determined for each treatment.

The flow cytometer settings used for the experiments were (i) the gates were set using forward and 90° light scatter signals at acquisition to exclude debris and aggregates; (ii) instrument alignment was performed daily with fluorescent microbead standards to standardize sensitivity and setup; (iii) photomultiplier settings were adjusted with unstained spermatozoa, and electronic compensation adjusted for spectral overlap with individually stained cells; (iv) excitation was at 488 nm from a 4 W argon laser operating at 200 mW. Fluorescein emission intensity was measured using a 530/30 nm bandpass filter, and propidium iodide intensity using a 630/22 nm bandpass filter.

Determination of osmotic injury as a function of sperm volume excursion in anisosmotic solutions of non-permeating solutes

The anisosmotic solutions, ranging from 40 to 1500 mOsmol, were prepared as follows: hypo-osmotic solutions were made by diluting TALP medium with reagent grade water; hyperosmotic solutions were made by adding sucrose to TALP medium (sucrose and the solutes in TALP medium are essentially membrane-impermeable compounds). The final osmolality of each solution was measured and checked using a freezing-point depression osmometer (Advanced DigiMate Osmometer, Model 3D2; Advanced Instruments, Inc., Needham Heights, MA, USA). The osmotic tolerance of human spermatozoa was evaluated by exposing the cells to the anisosmotic solutions. A 10 µl volume of isotonic cell suspension (286 mOsmol, 1×10^9 cells/ml) was mixed with 150 µl of each anisosmotic solution. After 1 s to 30 min, spermatozoa in each anisosmotic solution were returned to near isotonic conditions (272–343 mOsmol) by adding 1500 µl isotonic TALP medium

to 100 μl of each anisosmotic cell suspension. Sperm motility and plasma membrane integrity were measured by CASA and CFDA–propidium iodide dual fluorescent staining techniques respectively before and after the anisosmotic exposure. The centrifugal force used in sample preparation was 400 *g* for 7 min. All experiments were conducted at 22°C.

Thermodynamic modelling and mathematical formulation for glycerol and water permeation across the human sperm membrane

The next step was to compute the osmotic cell volume excursions associated with the addition and removal of hyperosmotic solutions of the permeating cryoprotectant glycerol to suspensions of human spermatozoa in isotonic saline. The classical formulation of coupled, passive membrane transport was developed by Kedem and Katchalsky (1958) using the theory of linear irreversible thermodynamics. The formulation includes two coupled first-order non-linear ordinary equations which describe the total transmembrane volume flux and the transmembrane permeable solute flux respectively. For the case of a solution consisting of a single permeable solute (e.g. glycerol) and other impermeable solutes the equations are

$$J_v = \frac{1}{A_c}\frac{dV(t)}{dt} = -L_p[(C^e_{salt} - C^i_{salt}) + \sigma(C^e_{CPA} - C^i_{CPA})]RT \quad [1]$$

$$J_{CPA} = \frac{1}{A_c}\frac{dN_{CPA}}{dt} = \overline{C}_{CPA}(1-\sigma)J_v + P_{CPA}(C^e_{CPA} - C^i_{CPA}) \quad [2]$$

where J_v = total volume flux, V = sperm volume (μm^3), t = time (s), N = mole number of solute, A_c = sperm surface area (μm^2), L_p = water permeability coefficient of human sperm membrane (μm/min/atm), C = concentration of solute (osmolality), J_{CPA} = cryoprotective agent (glycerol) flux across the cell membrane, superscript e = extracellular, superscript i = intracellular, R = universal gas constant [0.08207 l×atm/(mole×K)], T = absolute temperature (in Kelvin), σ = reflection coefficient of cell membrane to the cryoprotant, P_{CPA} = cryoprotective agent permeability coefficient of cell membrane (μm/min), and $\overline{C}_{CPA}$ = the average of extracellular and intracellular cryoprotective agent concentrations (osmolality):

$$\overline{C}_{CPA} = (C^e_{CPA} - C^i_{CPA})/[\ln(C^e_{CPA}/C^i_{CPA})].$$

Since human spermatozoa behave as ideal osmometers (Du *et al.*, 1993), intracellular concentrations of impermeable solute (salt) and permeable solute (cryoprotective agent) can be calculated as previously described (Mazur and Schneider, 1984):

$$C^i_{salt}(t) = C^i_{salt}(0)\left(\frac{V(0) - V_b - \overline{V}_{CPA}N^i_{CPA}(0)}{V(t) - V_b - V_{CPA}N^i_{CPA}(t)}\right) \quad [3]$$

$$C^i_{CPA}(t) = \left(\frac{N^i_{CPA}(t)}{V(t) - V_b - \overline{V}_{CPA}N^i_{CPA}(t)}\right) \quad [4]$$

where V_b = osmotically inactive cell volume (μm^3), $\overline{V}_{CPA}$ = partial mole volume of cryoprotective agent (μm^3), N = mole number, and 0 = initial condition (t = 0). Initial conditions for $V(0)$, $C^i_{salt}(0)$, $C^i_{CPA}(0)$, $N^i_{CPA}(0)$ are known based on each experimental condition or protocol. In the computer simulations, it was assumed that extracellular concentrations of permeating or non-permeating solutes were constant, and that the mixture of solutions during the glycerol addition and removal was instantaneous, i.e. the mixing time = 0.

Human sperm volume, surface area, V_b, water and glycerol permeability coefficients have been determined and previously published (Gao *et al.*, 1992; Kleinhans *et al.*, 1992; Noiles *et al.*, 1993; Du *et al.*, 1994). The values of these parameters are shown in Table I. Assuming that there is no interaction between water and glycerol during their transport through the sperm membrane (or in other words, water and glycerol penetrate the cell membrane independently), the value of $\sigma = 1-(P_{CPA}\overline{V}_{CPA})/(RTL_p)$ (Kedem and Katchalsky, 1958), where $\overline{V}_{CPA}$ = partial molar volume of glycerol (0.071 l/mol), can be calculated. From this equation and the data in Table I, σ was calculated to be 0.99. This value was used in the present study.

Using equations [1–4], kinetics of glycerol/water transport across the sperm plasma membrane as well as the cell volume excursion during different glycerol addition and removal procedures were calculated using a commercial differential equation solver, SLAB (Civilized Software, Inc., Bethesda, MD, USA). The sperm volume excursion and water transport through the membrane of cells in anisosmotic solutions without glycerol were calculated using equations [1] and [3] with C_{CPA} = 0 and N_{CPA} = 0.

Addition of glycerol

A final 1.0 M glycerol in sperm suspension was achieved by 1:1 (v/v) mixing of the original, isotonic sperm suspension with 2.0 M glycerol solution which contains an isotonic (non-permeating solute) salt concentration. Two approaches for mixing the 2.0 M glycerol solution with the sperm suspension

Table I. Characteristics of human spermatozoa at 22°C

Surface area	(A)	120 μm^2	Kleinhans *et al.* (1992)
Volume	(V)	34 μm^3	Kleinhans *et al.* (1992)
Osmotically inactive volume	(V_b)	16.6 μm^3	Kleinhans *et al.* (1992), Du *et al.* (1993)
Water permeability coefficient	(L_p)	2.4 μm/min/atm	Noiles *et al.* (1993)
Glycerol permeability coefficient	(P_s)	1.68 × 10^{-3} cm/min	Gao *et al.* (1993)

were used, i.e. a fixed-volume-step (FVS) approach and a fixed-molarity-step (FMS) approach:

Approach 1: fixed-volume-step addition

A 2.0 M glycerol solution was added stepwise to the sperm suspension, and the volume of the 2.0 M glycerol solution added in each step was constant. For example, to make a four-step addition of 1 ml of 2.0 M glycerol solution to a 1 ml isotonic sperm sample, 0.25 ml of 2.0 M glycerol solution would be added four times to the isotonic sperm suspension. The time interval between any two steps was 0.5–1 min.

In the general case, the volume of cryoprotective agent stock medium added to cell suspension in each step can be calculated by the following equation:

$$V_i = \frac{M_f \times V_o}{M_o - M_f} \times \frac{1}{n} \quad [5]$$

where M_f = the final cryoprotective agent concentration (molarity) in the cell suspension, M_o = cryoprotective agent concentration (molarity) in the original stock cryoprotective agent medium, n = total number of steps, i = i^{th}-step addition, V_o = the original volume of isotonic cell suspension, and V_i = the volume of cryoprotective agent stock medium added into cell suspension at the i^{th} step.

Approach 2: fixed-molarity-step addition

Glycerol-containing medium was added stepwise into the cell suspension in such a way that the glycerol molar concentration in the cell suspension was increased by a fixed amount after each step of addition. For example, to increase the molarity by 0.25 M in each of four steps, 0.14, 0.19, 0.27 and 0.4 ml of 2.0 M glycerol stock solution should be added (step by step, four steps in total) to 1 ml of the sperm suspension. The time interval between any two steps was 0.5–1 min.

In the general case, the volume of cryoprotective agent stock medium added to cell suspension at the ith step can be calculated by the following equation:

$$V_i = \frac{M_f \times V_o \times n \times M_o}{(nM_o - iM_f)\,[nM_o - (i-1)M_f]} \quad \text{where } i = 1, \ldots, n \quad [6]$$

or

$$V_i = \frac{1}{\lambda n (V_o / V^*_{i-1}) - 1} \times V^*_{i-1} \quad [7]$$

$$V^*_{i-1} = V_o + \sum V_k \quad \text{where } k = 1, \ldots, i-1 \quad [8]$$

$$\lambda = \frac{M_o}{M_f} \quad [9]$$

$$\Delta M = \frac{M_f}{n} \quad [10]$$

where M_f = the final cryoprotective agent concentration in the cell suspension (molarity), M_o = cryoprotective agent concentration in original stock cryoprotective agent medium (molarity), n = total number of steps, i = i^{th}-step addition, V_o = the original volume of isotonic cell suspension (ml), ΔM = increment of glycerol molarity in cell suspension after each step of glycerol addition, V^*_{i-1} = the total volume of cell suspension before the i^{th}-step addition, V_i = volume of cryoprotective agent stock medium added to cell suspension at the i^{th} step.

Removal of glycerol

To dilute the concentrated glycerol in the sperm suspension and remove glycerol from the cells, an isotonic solution without glycerol was added stepwise to the suspension. The FVS approach, FMS approach, and a two-step osmotic buffer approach were used for the dilution.

Approach 1: FVS dilution

Given the volume of the sperm suspension (V_o) with an initial cryoprotective agent concentration (M_o), the total volume of isotonic solution required to dilute the cryoprotective agent concentration from M_o to M_s can be calculated by the following equation:

$$V = V_o \left[\frac{M_o}{M_s} - 1 \right] \quad [11]$$

Table II. Procedures used in one-step and eight-step removal of 1.0 M glycerol from human spermatozoa

Eight-step dilution	
Fixed-volume-step method	Fixed-molarity-step method
Add 100 µl of isotonic TALP seven times to 100 µl of sperm suspension to achieve a final glycerol concentration of 0.125 M. After centrifugation, 710 µl of supernatant is taken off. The remaining cell suspension is 90 µl	(1) Stepwise add 14.3, 19, 26.6 and 40 µl of isotonic TALP medium to 100 µl of sperm suspension with 1.0 M glycerol; (2) centrifuge the cell suspension at 400 *g* for 5–7 min; (3) remove 170 µl of the supernatant; stepwise volume add 10, 20 and 60 µl of isotonic solution to the remaining 30 µl of sperm suspension. After the seven dilution steps, the glycerol concentration in the sperm suspension is 0.125 M. The final suspension volume is 120 µl
The final sperm suspensions (90 or 120 µl) were further diluted by adding 180 µl of TALP solution. The time interval between any two steps was ~0.5–1 min. The volume of diluent added in each step was calculated using equation [5] or [6]	
One-step dilution	
Add 2000 µl of isotonic solution directly to 100 µl of cell suspension with 1.0 M glycerol	

Using the FVS approach, the volume of isotonic solution which needs to be added to the cell suspension at the i^{th}-step during the first $n-1$ steps (n steps in total) can be calculated as follows:

$$V_i = \frac{V}{n-1} = \frac{V_o}{n-1}\left[\frac{M_o}{M_s} - 1\right] \quad [12]$$

where M_s = cryoprotective agent concentration in the cell suspension (molarity) after $n-1$ step dilutions, M_o = cryoprotective agent concentration in initial sperm suspension (molarity), n = total number of steps, i = the i^{th}-step addition, V_o = original volume of cell suspension (ml) and V_i = volume of isotonic solution added into cell suspension at the i^{th} step. After $n-1$ steps of addition of isotonic solution into the cell suspension, the diluted sperm suspension was centrifuged (400 g for 5–7 min), and then the sperm pellet was resuspended in an isotonic solution, which results in the last (n^{th}) step removal of glycerol from the cells.

Approach 2: FMS dilution

Concentrated glycerol in the sperm suspension was diluted stepwise by addition of an isotonic solution. The decrement in the molarity of glycerol after each step dilution was fixed. In the general case, the following equation can be used to calculate the volume of isotonic solution added to cell suspension at the i^{th} step during the first $n-1$ steps (n steps in total):

$$\Delta M = \frac{M_o}{n} \quad [13]$$

$$V_i = \frac{1}{n(V_o/V^*_{i-1})-1} \times V^*_{i-1} \quad \text{where } i = 1, \ldots, n-1 \quad [14]$$

$$V^*_{i-1} = V_o + \Sigma V_k \quad \text{where } k = 1, \ldots, i-1 \quad [15]$$

where ΔM = the decrement in the glycerol molarity in the spermatozoa after each stepwise addition of the isotonic solution, M_o = cryoprotective agent concentration (molarity) in the initial sperm suspension, n = total number of steps, i = i^{th}-step addition, V_o = original volume of cell suspension, V^*_{i-1} = the total volume of cell suspension before the i^{th}-step addition and V_i = volume of isotonic solution added into cell suspension at i^{th} step. After $n-1$ steps of addition, the cryoprotective agent concentration in the cell was diluted to ΔM. Then spermatozoa were transferred to isotonic conditions, which is the last (the n^{th}) step removal of glycerol, see Table II for examples.

Approach 3: Two-step dilution with an osmotic buffer

In the first step, glycerol was directly removed by transferring cells to a hyperosmotic medium (osmotic buffer, TALP with sucrose) containing no glycerol but only non-permeating solutes (salts and sucrose), and in the second step spermatozoa in the osmotic buffer were directly transferred to an isotonic solution (TALP), (Table III) (Rowe *et al.*, 1968; Mazur and Leibo, 1977; Leibo, 1981).

Experimental examination of the predicted osmotic injury during addition/removal of glycerol

Medium (TALP) with 2.0 M glycerol was added either in one step or stepwise (using FVS or FMS approaches) to an equal volume of the isotonic sperm suspension to achieve a final 1.0 M glycerol concentration at 22°C. The glycerol in the spermatozoa was removed/diluted by a one-step or stepwise addition (using FVS or FMS approaches) of TALP medium, with or without an osmotic buffer (sucrose), to the cell suspension. Some detailed procedures for the removal of glycerol are described in Tables II and III. Sperm motility and plasma membrane integrity were measured before and after the different glycerol addition and removal procedures by CASA and the dual staining technique and flow cytometry respectively.

Statistical analysis

Data were analysed using standard analysis of variance approaches with the General Linear Models procedure of the Statistical Analysis System (Spector *et al.*, 1985). Comparisons were conducted using a protected LSD (least significant difference) approach (Zar, 1984).

Results

The percentage of spermatozoa which maintained motility or plasma membrane integrity after each treatment was normalized to that of untreated, isotonic control samples and the data are so presented.

Determination of osmotic injury as a function of sperm volume excursion

Human spermatozoa were exposed for 5 min to hyper- or hypo-osmotic solutions of sucrose and TALP salts ranging in concentration from 60 to 1200 mOsmol, and their motilities were then determined by CASA while still in those solutions. Figure 1 shows that sperm motilities dropped significantly when the osmolality was >50 mOsmol above or below isotonic (286 mOsmol). Motilities approached zero when the osmolalities were <200 or >600 mOsmol.

The next step was to compare these motilities with those observed after spermatozoa were transferred from these anisosmotic solutions back to near isotonic solutions. Figures 2 and 3 show the motilities as a function of time after transfer

Table III. Procedures used in the two-step removal of 1.0 M glycerol from human spermatozoa using sucrose as an osmotic buffer

1.	Add 2000 µl of sucrose buffer medium (TALP + sucrose, 600 mOsmol) to 100 µl of sperm suspension with 1.0 M glycerol. (The total length of time spermatozoa were in contact with sucrose was 0.5 min before centrifugation)
2.	Centrifuge the suspension (400 g for 7 min) and aspirate the supernatant
3.	Resuspend the cell pellet with 500 µl of isotonic TALP medium

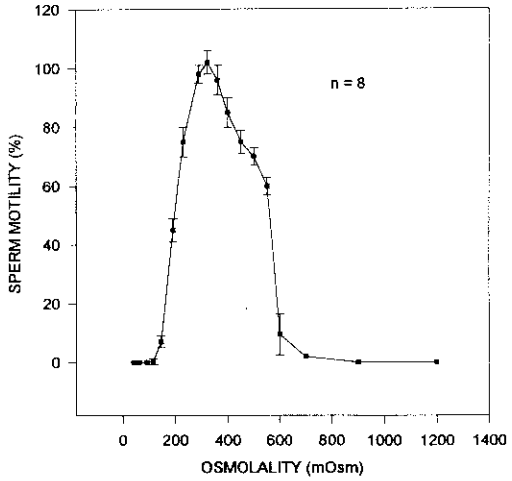

Figure 1. Percent motility (mean ± SEM, n = 8) of human spermatozoa which were abruptly (one-step) exposed to different osmotic conditions for 5 min at 22°C without being returned to isotonic conditions.

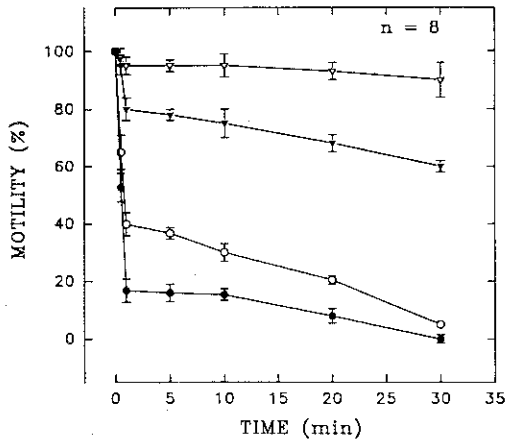

Figure 2. Percent motility (mean ± SEM, n = 8) of human spermatozoa which were abruptly (one-step) returned to near isotonic conditions (305–343 mOsmol) after they had been exposed to different hyperosmotic conditions (TALP + sucrose) for different periods of time. ▽, 600 mOsmol; ▼, 700 mOsmol; ○, 900 mOsmol; ●, 1200 mOsmol.

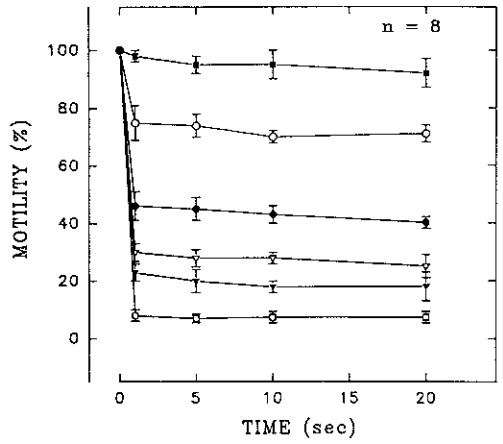

Figure 3. Percent motility (mean ± SEM, n = 8) of human spermatozoa which were abruptly (one-step) returned to near isotonic conditions (273–284 mOsmol) after they had been exposed to different hypo-osmotic conditions (TALP + water) for different periods of time. ■, 240 mOsmol; ○, 215 mOsmol; ●, 190 mOsmol; ▽, 143 mOsmol; ▼, 114 mOsmol; □, 90 mOsmol.

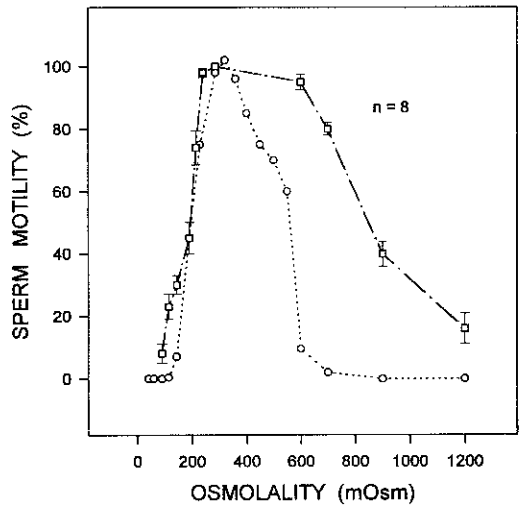

Figure 4. A comparison of human sperm motility (% mean ± SEM. n = 8) after a 5 min exposure to the various hypo- and hyperosmotic solutions of non-permeating solutions before (○) and after (□) the return to near isotonic conditions (273–343 mOsmol).

from hyperosmotic or from hypo-osmotic exposures respectively. In both cases, the more the initial exposure departed from isotonicity, the greater the damage upon return to isotonicity. Most, or all, of the damage was evident in the first 30 s after the return, although in the case of transfer from hypertonic solutions to near isotonic, there was a further slight and gradual decline over the ensuing 30 min.

Figure 4 compares sperm motilities after a 5 min exposure to the various anisosmotic solutions before and after the return to near isotonic conditions. The reduction in the motilities of spermatozoa exposed to hypo-osmotic media was not affected by the return to isotonic media, but most of the apparent loss of motility of spermatozoa in hyperosmotic media of between 286 and 600 mOsmol was reversed when spermatozoa were returned to near isotonic. For example, although only 10% of spermatozoa were motile in 600 mOsmol solutions, 95% of spermatozoa were motile after return to isotonic media. The return to near isotonic became especially damaging, however, when the initial hyperosmotic concentration was >600 mOsmol.

Figure 5 shows that integrity of the plasma membranes of spermatozoa (as assessed by CFDA/propidium iodide) was substantially more resistant to wide excursions from isotonicity than was motility. Thus, >90% of those spermatozoa exposed to a 90 mOsmol salt solution retained intact plasma membranes after return to near isotonic, whereas <10% remained motile both before and after return to isotonic. Loss of plasma membrane integrity in 50% of the spermatozoa occurred only

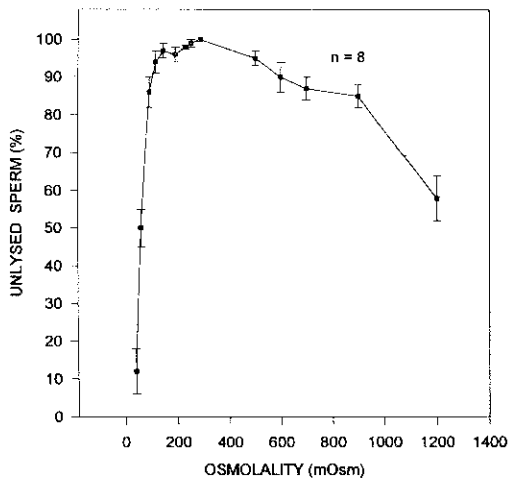

Figure 5. Membrane integrity (CFDA and propidium iodide stain) (% mean ± SEM, $n = 8$) of human spermatozoa which were abruptly (one-step) returned to near isotonic conditions (273–343 mOsmol) after they had been exposed to different anisosmotic conditions for 5 min.

when spermatozoa were exposed to a 60 mOsmol solution, a figure that agrees with a previous report (Noiles *et al.*, 1993); that loss occurs whether or not spermatozoa are returned to isotonic. This has been interpreted to represent lysis from the attainment of a cell volume in excess of that tolerated by the surface area of the plasma membrane.

Using light microscopy, morphological changes in sperm cells were observed during the exposure to anisosmotic solutions. In a portion of the spermatozoa, the tail region became configured as a 'zigzag' pattern when exposed to a hyper-osmotic solution. The tails of some spermatozoa curled when exposed to a hypo-osmotic solution. The pattern of sperm tail curling in hypo-osmotic solutions was osmolality dependent, which is consistent with a previous report (Jeyendran *et al.*, 1984). In addition, the curling of sperm tails occurred not only when the isotonic spermatozoa were exposed to hypo-osmotic solutions, but also when spermatozoa were exposed to a relative hypo-osmotic condition. (For example, the shrunken spermatozoa in hyperosmotic solutions were returned to iso-osmotic conditions. Iso-osmolality was 'hypo' relative to a given hyperosmolality.) The tail curling was irreversible. The mechanism(s) behind the morphological change is not clearly understood.

Calculated volume excursions associated with exposures to anisosmotic solutions

Since it has been shown that human spermatozoa behave as ideal osmometers over most of the range of osmolalities studied here (Du *et al.*, 1993), a direct physical consequence of the exposures to anisosmotic conditions is major excursion in cell volume. The kinetics of volume excursion of spermatozoa in these hypo- and hyperosmotic solutions (containing only non-permeating solutes) were calculated and are plotted in Figure 6A and B respectively, indicating that only a short time was required for human spermatozoa to achieve osmotic equilibration (<1 s for shrinking, and ≤30 s for swelling). Figure 6A and B also show the maximum or minimum volume of spermatozoa when they were osmotically equilibrated with each anisosmotic solution. Sperm equilibration volume as a function of extracellular osmolality is shown in Figure 7, which can be calculated using equation [3] (no cryoprotective agent) or obtained directly from Figure 6A and B. To obtain a high (>95%) motility recovery, the lowest and highest osmolalities which human spermatozoa can tolerate (Figures 2 and 3) were found to be close to 240 and 600 mOsmol respectively. At these two osmolalities, the corresponding cell volumes at osmotic equilibrium were directly estimated (Figure 7) to be ~1.1 (for 240 mOsmol) and 0.75 (for 600 mOsmol) times the isotonic sperm volume, indicating that spermatozoa can only swell or shrink in a relatively narrow range to maintain high post-anisosmotic motility recovery. Based on Figures 2, 3 and 7, Figure 8 was plotted, which clearly shows the post-anisosmotic injury (motility loss) as a function of osmotic equilibrium volume of spermatozoa in anisosmotic solutions. Defining lower volume limit (LVL) and upper volume limit (UVL) as cell volumes at which 5% of motile spermatozoa may irreversibly lose their motility, or, reciprocally, 95% of spermatozoa maintain their motility, one can obtain the LVL and UVL values for human spermatozoa from Figure 8 as follows: LVL = 0.75×isotonic sperm volume, UVL = 1.10×isotonic sperm volume.

Prediction of optimal protocols for glycerol addition/removal

The kinetics of human sperm volume excursion during one-step addition and removal of 0.5–2.0 M glycerol were calculated using equations [1–4] and are shown in Figure 9A and B respectively. The higher the glycerol concentration, the longer the time period taken for sperm volume recovery and the greater the volume excursion.

Two different approaches, i.e. fixed-volume-step (FVS) and fixed-molarity-step (FMS), for the addition/removal of glycerol in spermatozoa were considered and used in the present study. Based on equations [1–4], the kinetics of water and glycerol transport through the sperm membrane were simulated by computer. Figure 10 shows the calculated sperm volume excursion during a one-step or four-step addition of glycerol to achieve a final 1.0 M glycerol concentration at 22°C using the FMS and FVS approaches respectively. From Figure 10, a one-step addition of glycerol to spermatozoa was predicted to cause ~20% sperm motility loss because the minimum volume which the cells would achieve during this glycerol addition was ~72% of the original cell volume, i.e. below the LVL (75% or 0.75×isotonic sperm volume). In contrast, a four-step FMS glycerol addition was predicted to be able to prevent sperm motility loss (<5% loss). Figure 10 also shows a comparison between a four-step FVS and FMS approach. A four-step FVS method was predicted to cause a lower minimum volume than a four-step FMS method. From Figure 11, a one-step removal of 1.0 M glycerol was predicted to cause >70% motility loss, because the maximum cell volume during the glycerol removal was calculated to be in excess of 1.6 times the isotonic cell volume, which is much higher than the UVL (1.1×isotonic sperm volume). Figure 12 shows that a four- or six-step FMS removal procedure was predicted to reduce

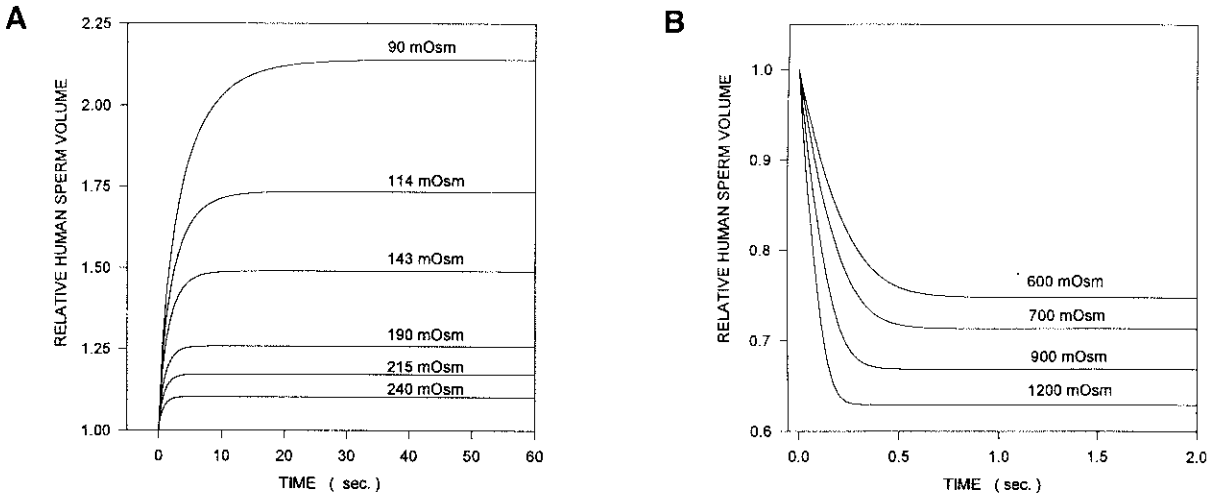

Figure 6. (**A**) Calculated relative sperm volume (normalized to an isotonic sperm volume of 1) as a function of time after spermatozoa were one-step exposed to different hypo-osmotic solutions containing non-permeating solutes. (**B**) Calculated relative sperm volume (normalized to the isotonic sperm volume of 1) as a function of time after the isotonic spermatozoa were one-step exposed to different hyperosmotic solutions containing non-permeating solutes.

sperm motility loss significantly, but these still may cause ≥5% motility loss, while an eight-step FMS removal was predicted to be able to prevent sperm motility loss (<5% loss). Figure 11 also shows a comparison between an eight-step FMS and an eight-step FVS removal procedure. An eight-step FVS removal was predicted to cause a maximum cell swelling >1.2×isotonic cell volume (>UVL), while the maximum cell volume during an eight-step FMS removal was predicted to be much lower than the UVL, indicating the eight-step FVS removal is not as good as an eight-step FMS. Based on the data presented in Figures 9–12, it was also found, from calculations, that human spermatozoa will rapidly achieve an osmotic equilibrium (within 15 s) during any stepwise addition or removal of glycerol. For example, from the calculations, human spermatozoa achieve osmotic equilibrium within 15 s after each step addition of glycerol by either one-step or four-step addition (Figure 10). This indicates that only a short time interval between steps of glycerol addition/removal is required for cells to achieve corresponding osmotic equilibration volume after each step of glycerol addition and removal.

In the analysis above, sperm osmotic injury (motility loss) caused by different glycerol addition/removal procedures has been predicted and a four-step FMS addition and an eight-step FMS removal of 1.0 M glycerol were found to be acceptable protocols to prevent sperm motility loss (<5% loss).

Theoretical evaluation of two-step glycerol removal using an osmotic buffer

A two-step removal of cryoprotective agent from human spermatozoa using a non-permeating solute as an osmotic buffer has been previously used to avoid osmotic injury in other cell types (Rowe *et al.*, 1968; Leibo and Mazur, 1978; Watson, 1979). The steps involved in this approach are (i) the cryoprotective agent is directly removed and cell swelling is reduced by transferring cells with the cryoprotective agent to a hyperosmotic medium (osmotic buffer) of non-permeating

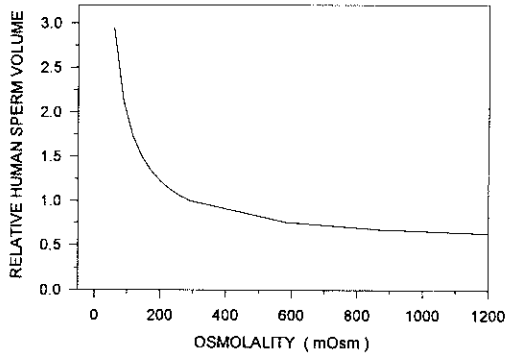

Figure 7. Calculated relative sperm volume (normalized to the isotonic sperm volume of 1) after spermatozoa were osmotically equilibrated to different anisosmotic conditions.

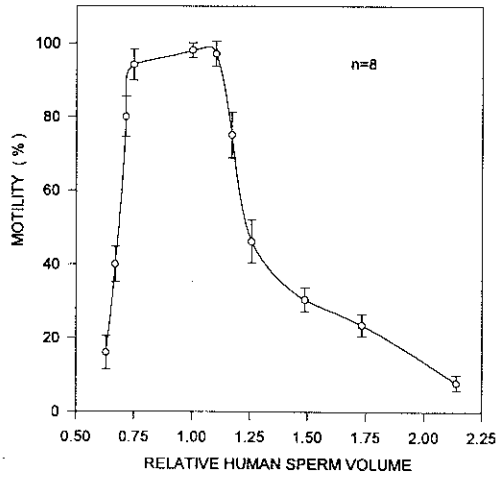

Figure 8. Post-anisosmotic sperm motility recovery as a function of relative sperm volume (normalized to the isotonic sperm volume of 1) in different anisosmotic equilibrium states. Human spermatozoa were abruptly (one-step) returned to near isotonic conditions after exposure to anisosmotic conditions for 1 min.

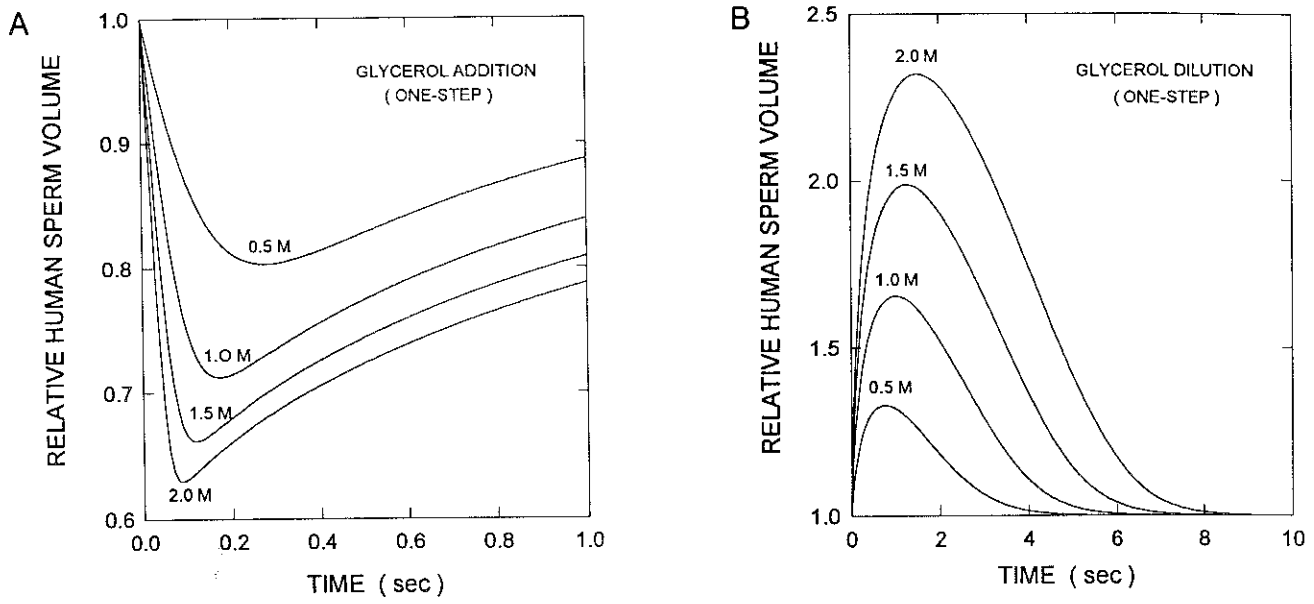

Figure 9. (**A**) Calculated relative sperm volume (normalized to the isotonic sperm volume of 1) as a function of time after the isotonic sperm were exposed to different hyperosmotic glycerol solutions isotonic with respect to non-permeating solutes (salts). (**B**) Calculated relative sperm volume (normalized to the isotonic sperm volume of 1) as a function of time after spermatozoa, which had been pre-equilibrated with different hyperosmotic glycerol solutions isotonic with respect to non-permeating solutes (salts), were one-step exposed to isotonic (286 mOsmol) saline solution without glycerol.

solutes; and (ii) the cells in the osmotic buffer are rehydrated by directly transferring them to an isotonic solution. Since current results showed that 600 mOsmol was the hyperosmotic upper tolerance limit for human spermatozoa to maintain 95% motility, the osmolality of the osmotic buffer medium should not exceed 600 mOsmol. Using this limiting criterion, a hyperosmolality of 600 mOsmol would be expected to provide the maximum 'buffer effect' to reduce sperm volume swelling during the first step of glycerol removal. Sperm volume excursion during this two-step glycerol removal process was calculated and is shown in Figure 13. It was predicted that the maximum volume spermatozoa would achieve is 1.25 times (125%) the isotonic cell volume, which is higher than the UVL of human spermatozoa, and would be expected to cause >40% sperm motility loss, as predicted from Figure 8.

Results from experimental examination

Glycerol was added to or removed from human spermatozoa using stepwise procedures to test the theoretical predictions. The percentage motility of human spermatozoa after either a one-step or four-step FMS or FVS addition of glycerol is shown in Figure 14. A one-step addition resulted in ~19.2% sperm motility loss or 81.8 ± 8.7% ($\bar{X}$ ± SEM, n = 15) motility recovery, while the four-step FMS or FVS addition significantly ($P < 0.001$) increased the motility recovery to 93.5 ± 5.6% ($\bar{X}$ ± SEM, n = 15) or 91 ± 4.8% ($\bar{X}$ ± SEM, n = 15) respectively. Figure 15 shows the effects of different glycerol removal procedures (c.f. Table II) on motility loss. After a one-step removal of 1.0 M glycerol, <30% (28.5 ± 3.8%, n = 15) of motile spermatozoa kept their motility, while the majority of spermatozoa (92 ± 8.2%, n = 15) maintained motility after the eight-step FMS removal. In comparison, only

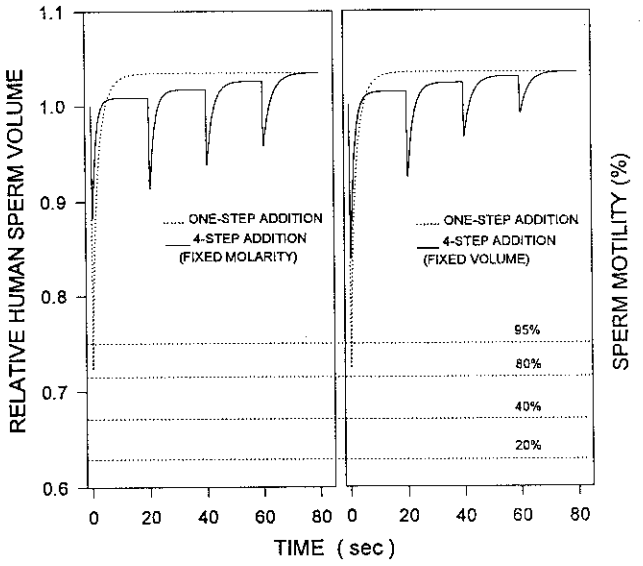

Figure 10. (**left**) Calculated relative sperm volume (normalized to the isotonic sperm volume of 1) as a function of time after 1 M glycerol was added to spermatozoa by either one-step or four fixed-molarity steps. (**right**) Calculated relative sperm volume (normalized to the isotonic sperm volume of 1) as a function of time after 1.0 M glycerol was added to spermatozoa by either one step or four fixed-volume steps. The estimates of percent motility recovery as a function of sperm relative volume were obtained from Figure 8 and are indicated in the diagrams.

62 ± 5.8% of spermatozoa maintained motility after eight-step FVS removal. The motility recovery after a two-step removal of glycerol (Table III) using sucrose as an osmotic buffer was 43 ± 5.3% ($\bar{X}$ ± SEM, n = 15). The experimental

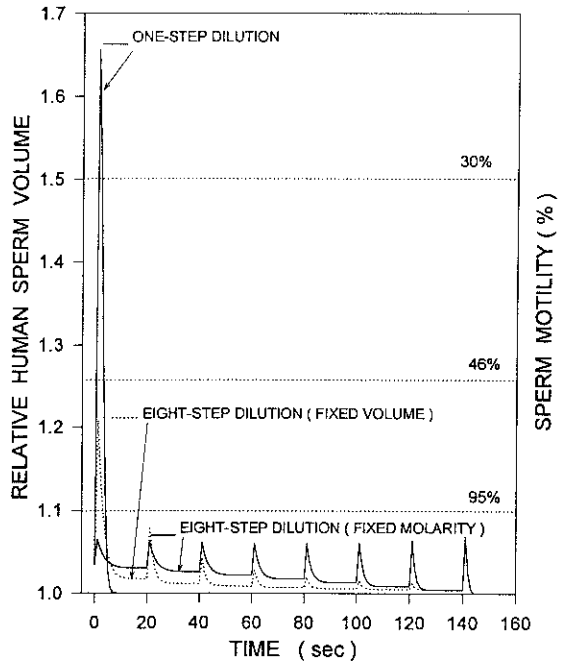

Figure 11. Calculated relative sperm volume (normalized to the isotonic sperm volume of 1) as a function of time after 1.0 M glycerol was removed from spermatozoa by one-step, eight fixed-molarity steps or eight fixed-volume steps. The estimates of percent motility recovery as a function of sperm relative volume were obtained from Figure 8 and are indicated in the diagrams.

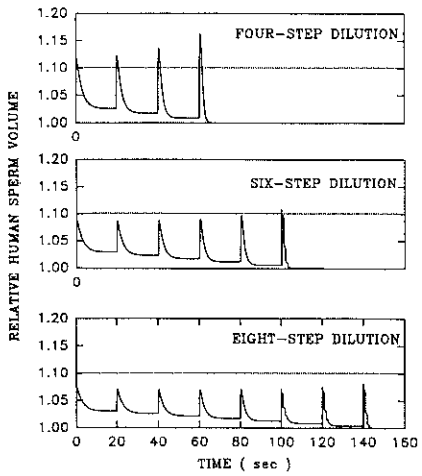

Figure 12. Calculated relative sperm volume (normalized to the isotonic sperm volume of 1) as a function of time after 1.0 M glycerol was removed from spermatozoa by four, six and eight fixed-molarity steps. The dotted lines in this figure indicate the upper volume limit, 1.1, below which >95% of spermatozoa can maintain their motility. The four- or six-step dilution results in a cell volume excursion causing >5% motility loss.

results agreed well with the predictions generated from the computer simulations. Data analyses indicated that the different glycerol removal procedures caused different motility losses ($P < 0.001$ between any two procedures).

Figure 16 shows the membrane integrity of human spermatozoa after addition/removal of 1.0 M glycerol using the different

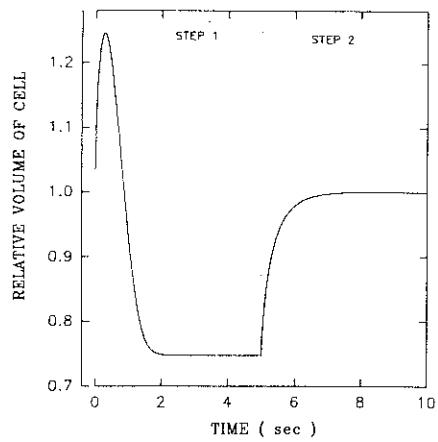

Figure 13. Calculated relative sperm volume (normalized to the isotonic sperm volume of 1) as a function of time after 1.0 M glycerol was removed from spermatozoa by two steps using a 'hyperosmotic buffer' solution. Step 1: 1.0 M glycerol was removed from spermatozoa by one-step exposure of spermatozoa to 600 mOsmol hyperosmotic (salt+sucrose) solution without glycerol. Step 2: Spermatozoa in the 600 mOsmol solution were returned to isotonic condition (286 mOsmol) in one step.

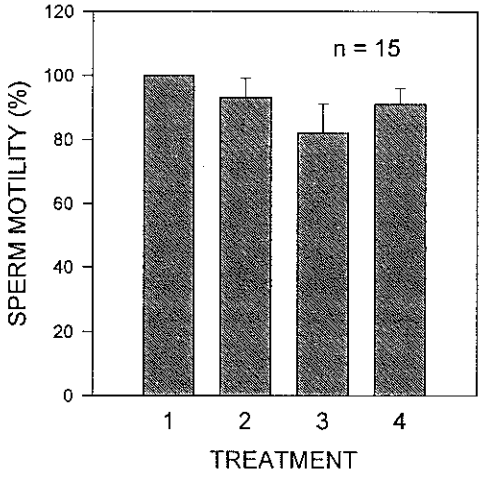

Figure 14. Experimental results: motility recovery (% mean ± SEM, $n = 15$) of human spermatozoa after addition of 1.0 M glycerol. (1) Original spermatozoa in isotonic condition; (2) after four fixed-molarity step addition of 1.0 M glycerol; (3) after one-step addition of 1.0 M glycerol; (4) after four fixed-volume step addition of 1.0 M glycerol.

procedures. Over 90% of spermatozoa maintained membrane integrity under all experimental conditions.

Discussion

Addition of glycerol to human spermatozoa before cooling and its removal from spermatozoa after warming create osmotic stress to the cells resulting in cell injury. Several possible reasons for the osmotic injury have been proposed, including (i) mechanical rupture of the cell membrane in hypo-osmotic conditions (i.e. expansion lysis); (ii) the water flux hypothesis: frictional force between water and potential membrane 'pores' causes cell membrane damage (Muldrew and McGann, 1994);

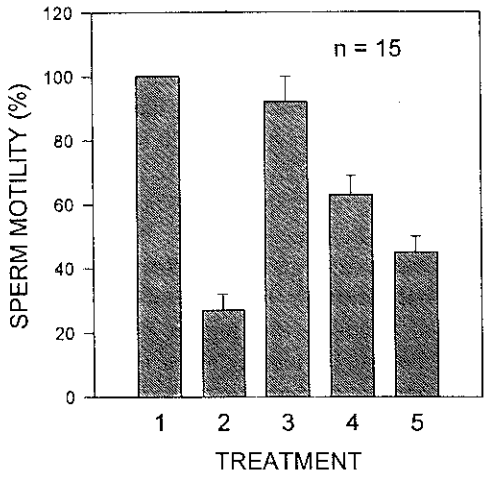

Figure 15. Experimental results: motility recovery (% mean ± SEM, n = 15) of human spermatozoa after removal of 1.0 M glycerol from spermatozoa. (1) original spermatozoa in isotonic solution; (2) after one-step removal of 1.0 M glycerol; (3) after eight fixed-molarity step removal of 1.0 M glycerol; (4) after eight fixed-volume step removal of 1.0 M glycerol; (5) after two-step removal of 1.0 M glycerol using a 'hyperosmotic buffer'.

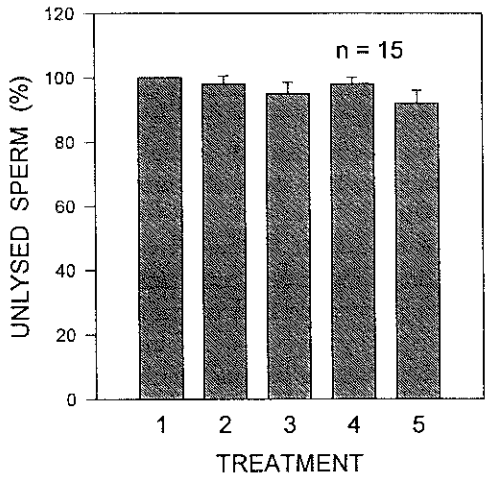

Figure 16. Experimental results: membrane integrity (CFDA and propidium iodide stain) (% mean ± SEM, n = 15) of human spermatozoa after addition and/or removal of 1.0 M glycerol. (1) Original spermatozoa in isotonic solution; (2) after four fixed-molarity step addition of 1.0 M glycerol; (3) after four fixed-molarity step addition and eight fixed-molarity removal of 1.0 M glycerol; (4) after one-step addition of 1.0 M glycerol; (5) after one-step addition and one-step removal of 1.0 M glycerol.

(iii) the minimum volume hypothesis: cell shrinkage in hyperosmotic condition is resisted by cytoskeleton components, and the resultant interaction between shrunken cell membrane and the cytoskeleton damages the cells (Meryman, 1970); (iv) the maximum cell surface hypothesis: the cell shrinkage induces irreversible membrane fusion/change, and hence the effective area of cell membrane is reduced; when returned to isotonic condition, the cells lyse before their normal volume is recovered (Steponkus and Wiest, 1979); and (v) the solute loading hypothesis: hyperosmotic stress causes a net leak/influx of non-permeating solutes; when cells are returned to iso-osmotic conditions, they swell beyond their normal isotonic volume and lyse (Mazur *et al.*, 1972). Although the mechanism(s) of the osmotic injury which occurs to human spermatozoa during cryopreservation is not clearly understood, the hypothesis proposed in the present study has been tested and confirmed, i.e. human sperm volume excursion can be used as an indicator to predict possible osmotic injury to spermatozoa during glycerol addition and removal processes.

The FVS, multi-step procedure for the addition of glycerol to human spermatozoa before cryopreservation is a conventional, commonly used technique, i.e. 'drop by drop' (stepwise) addition of a solution with a relatively high glycerol concentration (the volume of each 'drop' is roughly constant) to the spermatozoa or sperm suspension in order to achieve a 0.6–1.0 M glycerol concentration in the final sperm suspension. In practice, the frozen–thawed sperm samples containing glycerol are either washed for intrauterine insemination or for in-vitro fertilization or directly transferred into the lower female reproductive tract for artificial insemination (e.g. intercervical insemination). In both cases, the glycerol is abruptly removed from spermatozoa by direct exposure to near isotonic conditions. In the present study, it was predicted by computer simulation, and confirmed experimentally, that a one-step removal of glycerol would cause a high frequency of sperm motility loss even without freezing. Based on the results, the FMS removal ($\geq$8 steps) of 1.0 M glycerol is recommended. Within the scope of the present investigation, a four-step FMS addition of glycerol to spermatozoa to achieve a final 1.0 M glycerol concentration and an eight-step FMS removal of 1.0 M glycerol from spermatozoa were predicted and shown to be acceptable procedures which minimize osmotic injury. From calculations, the minimum or maximum cell volumes after each step of FVS addition or removal were shown to be unequal, some of which may exceed the lower or upper volume limits of the cells. In contrast, from calculations, the minimum or maximum cell volumes after each step of FMS addition or removal of glycerol were shown to be relatively even (Figures 10 and 11). For a fixed number of steps, the minimum or maximum of cell volume excursion during glycerol addition or removal using the FMS approach is much smaller than that using the FVS approach (see Figures 10 and 11).

In the current study, it was postulated that the sperm osmotic injury as a function of cell volume excursion must be determined to predict the optimal glycerol addition and removal procedures. However, the definition and determination of 'sperm injury' is dependent upon the assays used. For example, in the present study, sperm motility was used as a standard of sperm viability because of its relatively high sensitivity to osmotic changes and the requirement of sperm motility for functional viability. If sperm membrane integrity was chosen as the endpoint to evaluate the sperm viability, as shown in Figure 5, different osmotic tolerance limits would be obtained. One can readily repeat the same procedures to predict the extent to which spermolysis is caused by the different glycerol addition/removal procedures used in the present study, based on the information provided in Figure 5. For example, it was found (Figure 5) that >85% of spermatozoa maintained

membrane integrity when they were returned to isotonic conditions after having been exposed to anisosmotic conditions ranging from 90 and 700 mOsmol. The corresponding sperm volume excursion range was 0.7–2.1 times the isotonic sperm volume (Figure 7). From Figures 10 and 11, it can be seen that a one-step addition and one-step removal of 1.0 M glycerol would result in a minimum relative sperm volume of 0.72 and maximum volume of 1.68 respectively, which did not exceed the sperm volume excursion range (0.7–2.1 times relative volume) for maintaining >85% sperm membrane integrity. Based on this information, one can predict that the majority (>85%) of spermatozoa would maintain membrane integrity even using one-step addition and one-step removal of glycerol. Again, this prediction was consistent with experimental results (Figure 16).

Since the hypothesis proposed in the present study was confirmed by experimental data, the procedures used for testing the hypothesis provide a methodology to predict optimal protocols for cryoprotective agent addition/removal. This methodology has several advantages when compared with an empirical approach, as delineated below.

An understanding of basic cell biological properties

Cell volume excursion limits are biological properties of a given cell type, which can be readily determined using anisosmotic solution containing only non-permeating solutes (e.g. sucrose). These properties can be generally used for optimizing addition and removal of a cryoprotective agent as well as developing optimal procedures for preventing cell osmotic injury during cooling and warming processes. These limits may be temperature dependent.

Water and cryoprotective agent permeability coefficients of cells are also biological properties of a given cell type. With known water and glycerol permeabilities as well as cell osmotic injury as a function of cell volume excursion, one can predict optimal procedures for addition and removal of any glycerol concentration (or of other cryoprotective agents, given their permeability values) at particular temperatures. The advantages of using a relatively high cryoprotective agent concentration are that cell cryosurvival rate is less dependent on the cooling and warming rate (Mazur, 1984), and that the cells may possibly be vitrified at a feasible cooling rate.

High optimization capability

Having determined human sperm membrane transport properties as a function of temperature, the kinetics of water and glycerol transport through the human sperm cell membrane during many possible glycerol addition and removal processes at different temperatures can be readily simulated by computer. Important information can be obtained, such as (i) the minimum time interval between glycerol addition and removal steps required for glycerol and water transport through the cell membrane to achieve osmotic equilibrium and (ii) the least number of steps required to prevent osmotic injury, neither of which are readily obtained by empirical approaches, especially if one wants to change cryoprotective agent concentration or temperature.

The present study has shown that if the tolerance of human spermatozoa to anisosmotic solutions of non-permeating solutes is known and the water and cryoprotectant (e.g. glycerol) permeability coefficients for human spermatozoa are also known, protocols can be computed for adding and removing that cryoprotectant to minimize loss in motility. Such an approach has also been quite successful for mammalian embryos (Mazur and Schneider, 1984, 1986). Using a similar approach, Arnaud and Pegg (1990) illustrated how to predict possible optimal protocols for glycerol or propylene glycol addition to and removal from human platelets. Whether the approach will be equally successful for spermatozoa and cells from other species depends on the degree to which the underlying assumptions are met. One assumption is that the injury in response to exposure to anisosmotic conditions is due solely to the osmotic volume excursions of the cells. A second is that the volume excursions are reciprocally related to the osmolalities to which the cells are exposed (i.e. the cells behave as ideal osmometers). A third is that the cryoprotectant plays no role in cell survival other than its contribution to the osmotic response. It is possible, for example, that chemical toxicity of the cryoprotectant may also play a role in the survival. The approach used here actually provides a powerful way of testing that possibility. Toxicity is usually reduced at lower temperatures. If one determines the permeability coefficient to the cryoprotectant at lower temperatures, one could use the procedures described here to reoptimize the protocols to minimize osmotic damage from the addition and removal of the cryoprotectant at the lowered temperature, thereby allowing separate study of osmotic damage versus true chemical toxicity.

One of the significant findings from this study was that plasma membrane integrity is substantially more resistant to osmotic volume excursion than is motility. This suggests that motility is affected by the osmotic volume responses of cellular structures (other than the plasma membrane) that are directly or indirectly involved in motility. One possibility is the mitochondria. What our data do not show, and therefore where future work needs to be directed, is the cellular and molecular cause of the loss of motility.

It should be mentioned that there were several factors which might have affected accuracy of the results in the present study. One was the relatively low sperm number (10×10^6 in total) used in each anisosmotic test to determine upper and lower volume excursion limits for the human spermatozoon. The second factor was the finite time required to mix the cell suspension completely with an anisosmotic solution. For example, when a 2 M glycerol solution is added to an equal volume isosmotic cell suspension in one step, it takes at least 0.2 s (McGann *et al.*, 1982) of mixing them to make a 1 M glycerol concentration in the final cell suspension. Some of the spermatozoa may be transiently exposed to higher glycerol concentrations (between 1 and 2 M) during the initial mixing period (0.2 s), which may cause these spermatozoa to shrink to a minimum volume lower than predicted because of the high water permeability of human spermatozoa. (In the computer modelling and calculation, the mixing time was assumed to

be 0; hence, all sperm cells were assumed to be exposed to a 1 M glycerol condition instantaneously.)

It has generally been found that the attainment of high survival rates of cryopreserved mammalian cells requires cryoprotectant concentrations of $\geq$1.0 M. Current procedures for the cryopreservation of human spermatozoa generally limit the concentration of glycerol to $\leq$0.8 M to avoid damage from glycerol, and motilities after cryopreservation are generally only 30–40%. The approach described here permitted the addition and removal of 1.0 M glycerol without damage, and it may permit the innocuous addition and removal of still higher concentrations. The use of these higher concentrations of cryoprotectant may well yield higher survivals after cryopreservation.

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