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Prevention of Lethal Osmotic Injury to Cells During Addition and Removal of Cryoprotective Agents: Theory and Technology

Dayong Gao¹ and Xiaoming Zhou²

¹*University of Washington, Seattle, WA*

²*University of Electronic Science and Technology of China, Chengdu,*

¹*USA*

²*China*

1. Introduction

Significant survival of cryopreserved cells became a reality only after the discovery and the use of cell-membrane-permeating cryoprotective agents (CPAs) (e.g. glycerol, Polge et al, 1949). Before freezing, one or various CPAs should be added to cell suspensions to prevent the cells from the cryoinjury during the freezing and thawing processes. Unfortunately, the CPAs, themselves, may have chemical toxicity to cells after thawing at room temperatures (Katkov et al, 1998). Therefore, a post-thaw washing of CPAs is required to remove CPAs from cells prior to scientific or medical applications. However, the addition of CPAs to cells before freezing and the removal of CPAs from cells after thawing may cause serious cell loss and damage if the processes are not properly handled.

“One-step” methods were formerly used for addition/removal CPAs. During the “one-step” CPA addition process, cells are directly (one-step) placed in a solution that is hyperosmotic with respect to the permeating CPA but isosmotic with respect to the impermeable salts/electrolytes. Cells first shrink because of the osmotic efflux of intracellular water and then increase in volume as the CPA permeates and as water concomitantly reenters the cells (as shown in Figure 1a). During the “one-step” CPA removal process, cells with a high intracellular concentration of CPA are directly exposed to an isotonic salt solution without CPA. Cells will swell because of an osmotic influx of extracellular water and then decrease in volume as the CPA diffuses out of the cells and as water concomitantly moves out (as shown in Figure 1b). As a result of these two aspects (i.e. addition and removal of CPAs) of the cryopreservation procedures, the cells may experience severe osmotic volume excursion causing significant cell “osmotic” injury (Sherman, 1973; Mazur and Schneider, 1984, 1986; Penninckx et al, 1984; Leibo, 1986; Crister et al, 1988a, Meryman, 2007).

Several possible reasons for the osmotic injury have been proposed, including (i) 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’ caused cell membrane damage (Muldrew and McGann, 1994); (iii) the minimum volume hypothesis:

cell shrinkage in hyper-osmotic 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 isotonic conditions, they swell beyond their normal isotonic volume and lyse (Mazur et al., 1972).

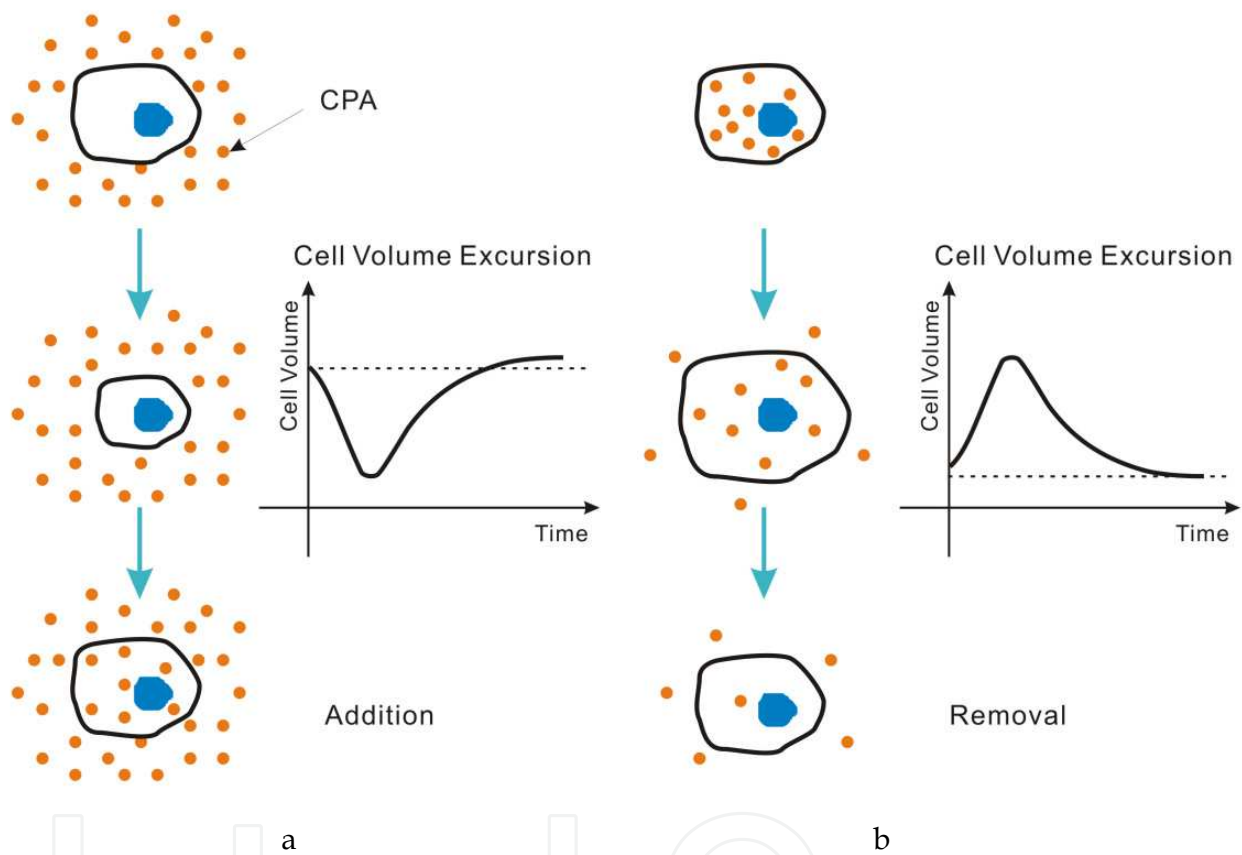


Fig. 1. Cell volume excursion during addition and removal of CPAs

In order to minimize osmotic injury, many efforts have been made and several techniques have been proposed. Basically, people utilize so-called “multi-step methods” instead of “one-step method” for addition and removal of CPAs, and the resulting cell recovery rate can be significantly improved. During the multi-step CPA addition process, solution with high CPA concentration is added into a cell suspension step by step and the CPA concentration in the cell suspension increases slowly and gradually. During the multi-step CPA removal process, an isotonic salt solution is added into the cell suspension step by step, and then by means of centrifugation CPAs in the cell suspension are removed (Figure 2). Although to some extent multi-step method reduces osmotic damage of cells, it is complex to operate, requires more laboratory staffs, and costs more time, which makes the addition and removal procedures more expensive and difficult practically.

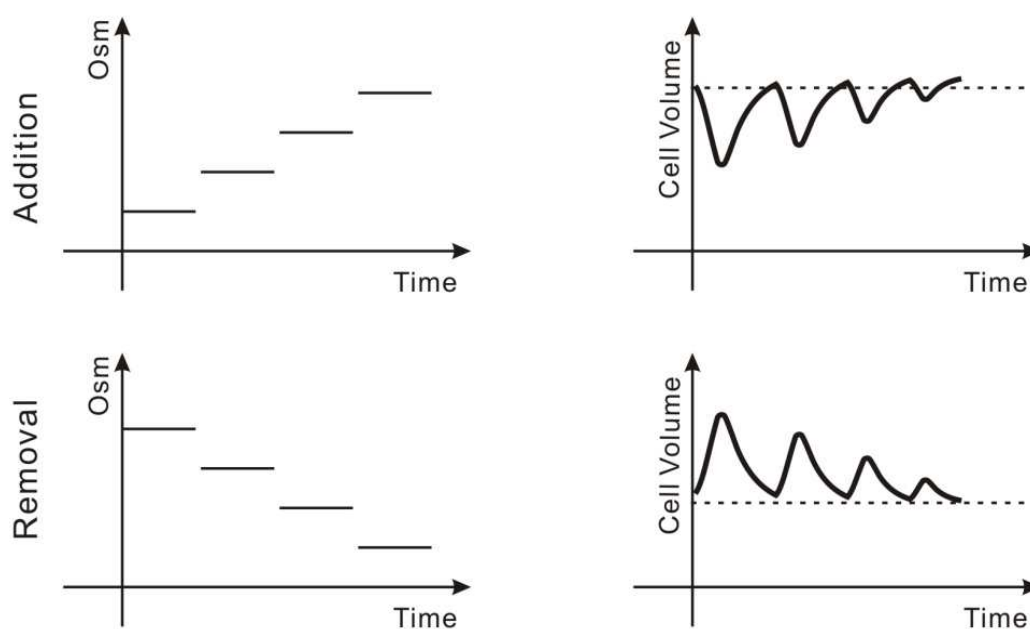


Fig. 2. Multi-step method for addition and removal of a CPA

In the past, attempts to develop procedures for the addition and removal of CPAs have been made based primarily on empirical approaches, i.e. for a given cell type, various temperatures, CPA types and concentrations, and number of procedures or steps for CPA addition and removal were empirically tested to find an acceptable procedure. Typical techniques includes (i) a multi-step addition and multi-step removal of permeating CPAs (Watson, 1979) and (ii) a multi-step addition and two-step removal (using a non-permeating solute as osmotic buffer) of CPAs (Rowe et al., 1968; Mazur and Leibo, 1977; Leibo 1981). New CPA addition-removal methods and automated devices have recently been developed based on fundamental cell membrane transport theory and engineering approaches (Gao, et al, 1995; Gilmore et al, 1997; Katkov, 1998; Myrthe, et al ,2004, Zhou, et al, 2011), which are introduced and discussed in this chapter.

2. Cell membrane transport models and mathematical formulatins

To date, a number of formalisms exist for describing the cell membrane transport process. These include a one-parameter model, a two-parameter model, and a three-parameter model, considering solute-solvent interactions.

- i. one-parameter model (Mazur et al, 1974, 1976),

The one-parameter model utilizes the hydraulic permeability (L_p) of cell membrane as the only parameter to describe the water transport across cell membrane. The model can be formulized as follows.

$$\frac{dV_w^i}{dt} = -L_p A_c (\Pi_e - \Pi_i) \quad (1)$$

where, V_w^i is the volume of intracellular water, A_c is the area of cell membrane surface, Π_e and Π_i are the extracellular and intracellular osmotic pressures.

ii. Two-parameter model

The two-parameter model was firstly presented by Jacob (1932-1933), and further developed by Kleinhans (1998), Katkov (2000) recently. The model utilizes the parameters L_p and P_s (CPA solute permeability) to characterize membrane permeability when water, a permeable solute and a nonpermeable solute are present:

$$\frac{dV_w^i}{dt} = -L_p A_c RT (M^e - M^i) \quad (2)$$

$$\frac{dN_s^i}{dt} = P_s A_c (M_s^e - M_s^i) \quad (3)$$

where N_s is the number of osmoles of solute inside cell, R is the universal gas constant, T is the absolute temperature, M_i and M_e are the intracellular and extracellular osmolality, respectively. The subscript 's' refers to permeable solute, and remaining symbols are as previously defined.

iii. Three-parameter model

The classical formulation of coupled, passive membrane transport was developed by Kedem and Katchalshy (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.

In the model (so called Kedem-Katchalssky transport formalism or KK formalism), a reflection coefficient (σ) was introduced with L_p and P_s to describe water and solute (CPA) transport across the plasma membrane:

$$\frac{dV_c}{dt} = -L_p A_c RT \left[(M_n^e - M_n^i) + \sigma (M_s^e - M_s^i) \right] \quad (4)$$

$$\frac{dN_s^i}{dt} = (1 - \sigma) \bar{M}_s \frac{dV_c}{dt} + P_s A_c (M_s^e - M_s^i) \quad (5)$$

Where V_c is cell volume, \bar{M}_s is the average osmolality of intracellular and extracellular solution, and the subscript 'n' refers to nonpermeable solute, respectively.

The KK formalism used to be the most general of the three mentioned. However, more recent literature suggests that aquaporins in cell membrane are highly selective, with nonionic solute transport occurring mainly through the lipid bilayer or through other channels that are distinct from the aquaporins (Gilmore et al, 1995; Preston et al, 1992). In this case, the estimation of σ as independent parameter may be inappropriate and may not be relevant from a biological stand point (Kleinhans, 1998). By assuming that there is no interaction between water and solute during their transport through the membrane, the value of σ can be determined as $1 - (P_s \bar{V}_s) / (RT L_p)$, where \bar{V}_s = partial molar volume of permeating solute. In this manner, the KK formalism can still get correct result as two parameter model.

In the following context, two examples are demonstrated to show how to use cell membrane transport models and mathematical formulations to develop optimal conditions and technology/instrument for the addition and/or removal of the permeating CPAs in cells. **An important hypothesis** is that the degree of cell volume excursion can be used as an independent indicator to evaluate and predict the possible osmotic injury of the cells during addition and removal of CPAs.

Example 1: Development of optimal “multi-step methods” for addition and dilution of glycerol in human sperm

Glycerol is the most commonly used CPA in the cryopreservation of spermatozoa (Polge et al, 1949; Watson, 1979; Critser et al., 1988a). Glycerol permeability characteristics for human spermatozoa have been very well studied and reported (Du et al, 1994; Gao et al., 1992). The hypothesis above was tested first using the following procedures: (i) to determine sperm osmotic injury as a function of its volume excursion limits (swelling/shrinking) in anisotonic 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.

2.1 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% CO₂, 95% air, 37°C, and high humidity) for ~1h. A total of 5 ul 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; Crister et al., 1988b). A swim-up procedure was performed to separate motile from immotile cells [layering 500ul of modified Tyrode's medium (TALP: Bavister et al., 1983) over 250 ul of semen, incubating for ~1 h in the incubator and carefully aspirating 400 ul of the supernatant in which >95% of spermatozoa were motile]. The motile cell suspensions were centrifuged at 400g for 7min and resuspended in the TALP medium (286~290 Osmol) supplemented with pyruvate (0.01 mg/ml) and bovine serum albumin (4 mg/ml), at a cell concentration of 1×10⁹ cell/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; Noilles 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 in to spermatozoa with a damaged membrane. 6-Carboxyfluorescein diacetate (CFDA; Sigma, Catalog #C5041) is a membrane-permeable compound. After penetrating into cells, it is hydrolysed by intracellular esterase to 6-carboxyfluorescein 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 μ l CFDA (0.25 mg/ml DMSO) and 5 μ l propidium iodide (1 mg/ml water) stock solutions were added to each 0.5ml of the treated sperm suspensions. A total of 1×10^5 spermatozoa per treatment were analyzed 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 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 anisotonic solutions of nonpermeating solutes

The anisotonic solutions, ranging from 40 to 1500 mOsmol, were prepared as follows: hypo-osmotic solutions were made osmotic 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 Instrument, Inc., Needham Heights, MA, USA). The osmotic tolerance of human spermatozoa was evaluated by exposing the cells to the anisotonic solutions. A 10 μ l volume of isotonic cell suspension (286 mOsmol, 1×10^9 cells/ml) was mixed with 150 μ l of each anisotonic solution. After 1 s to 30 min, spermatozoa in each anisotonic solution were returned to near isotonic conditions (272-343 mOsmol) by adding 1500 μ l isotonic TALP medium to 100 μ l of each anisotonic 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 anisotonic exposure. The centrifugal force used in sample preparation was 400 g for 7 min. All experiments were conducted at 22°C.

Thermodynamic modeling and mathematical formulation for glycerol and water permeating 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 KK formalism (shown as equations (4) and (5)) is used here and for the case of a solution consisting of a single permeable solute (e.g. glycerol) the average of extracellular and intracellular cryoprotective agent concentrations (osmolality) can be given as

$$\bar{M}_s = (M_s^e - M_s^i) / \left[\ln(M_s^e / M_s^i) \right]$$

Since human spermatozoa behave as ideal osmometer (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).

$$M_n^i(t) = M_n^i(0) \left(\frac{V(0) - V_b - \bar{V}_s N_s^i(0)}{V(t) - V_b - \bar{V}_s N_s^i(t)} \right) \quad (6)$$

$$M_s^i(t) = \left(\frac{N_s^i(t)}{V(t) - V_b - \bar{V}_s N_s^i(t)} \right) \quad (7)$$

Where V_b = osmotically inactive cell volume (μm^3), and 0 = initial condition ($t=0$). Initial conditions for $V(0)$, $M_n^i(0)$, $M_s^i(0)$, $N_s^i(0)$ 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 1. 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 $\sigma = 1 - (P_s \bar{V}_s) / (RTL_p)$ (Kedem and Katchalsky, 1958), can be calculated. From this equation and the data in Table 1, σ was calculated to be 0.99. This value was used in the present example.

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 $\mu\text{m}/\text{min}/\text{atm}$	Noiles et al. (1993)
Glycerol permeability coefficient	(P_s)	1.68 $\times 10^{-3}\text{cm}/\text{min}$	Gao et al. (1993)

Table 1. Characteristic of human spermatozoa at 22°C

Using equations [4-7] 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 anisotonic solution without glycerol were calculated using equation [4] and [5] with $M_s=0$ and $N_s=0$.

Addition of glycerol

A final 1.00 M glycerol in sperm suspension was achieved by 1:1 (v/v) mixing of the original, isotonic sperm suspension with 2.0M 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 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 1ml 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:

$$V_i = \frac{M_f \times V_o}{M_o - M_f} \times \frac{1}{n} \quad (8)$$

Where M_f = the final CPA 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 CPA 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 0.5-1min.

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

$$V_i = \frac{M_f \times V_o \times n \times M_o}{(nM_o - iM_f)[nM_o - (i-1)M_f]} \quad \text{where } i=1, \dots, n \quad (9)$$

$$V_i = \frac{1}{\lambda n \left(\frac{V_o}{V_{i-1}^*} \right) - 1} \times V_{i-1}^* \quad (10)$$

$$V_{i-1}^* = V_o + \sum V_k \quad \text{where } k=1, \dots, i-1 \quad (11)$$

$$\lambda = \frac{M_o}{M_f} \quad (12)$$

$$\Delta M = \frac{M_f}{n} \quad (13)$$

Where M_f = the final cryoprotective agent concentration in the cell suspension (molarity), M_o = cryoprotective agent concentration in the 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 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:

$$V = \left[\frac{M_o}{M_s} - 1 \right] \quad (14)$$

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

$$V_i = \frac{V}{n-1} = \frac{V_o}{n-1} \left[\frac{M_o}{M_s} - 1 \right] \quad (15)$$

where M_s = cryoprotective agent concentration in the cell suspension (molarity) after $n-1$ step dilutions, M_o = cryoprotective agent concentration 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 $n-1$ step (n steps in total):

$$\Delta M = \frac{M_o}{n} \quad (16)$$

$$V_i = \frac{1}{n(V_o/V_{i-1}^*) - 1} \times V_{i-1}^* \quad \text{where } i=1, \dots, n-1 \quad (17)$$

$$V_{i-1}^* = V_o + \sum V_k \quad \text{where } k=1, \dots, i-1 \quad (18)$$

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 $n-1$ step 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 2 for examples.

Approach 3: Two-step dilution with an osmotic buffer

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 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 [8] or [9]

One-step dilution
Add 2000 μl of isotonic solution directly to 100 μl of cell suspension with 1.0 M glycerol

Table 2. Procedures used in one-step and eight-step removal of 1.0 M glycerol from human spermatozoa

1.	Add 2000 μl of sucrose buffer medium (TALP + sucrose, 600 mOsm) 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.)
	Centrifuge the suspension (400 g for 7 min) and aspirate the supernatant.
	Resuspend the cell pellet with 500 μl of isotonic TALP medium

Table 3. Procedures used in the two-step removal of 1.0 M glycerol from spermatozoa using sucrose as 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 3) (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 2.0M 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 Table 2 and 3. 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 analyzed 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).

2.2 Result

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 5min 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 3 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 anisotonic solutions back to near isotonic solutions. Figures 4 and 5 show the motilities as a function of time after transfer 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 6 compares sperm motilities after a 5 min exposure to the various anisotonic 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

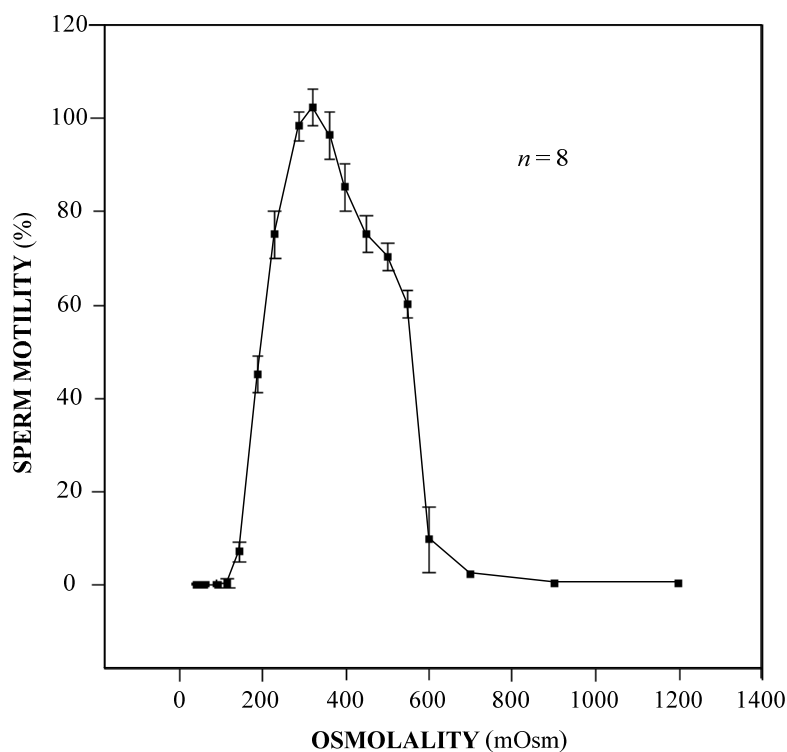


Fig. 3. Percent motility (mean \pm SEM, $n=8$) of human spermatozoa which were abruptly (one-step) exposed to different osmotic conditions for 5 min at 22°C.

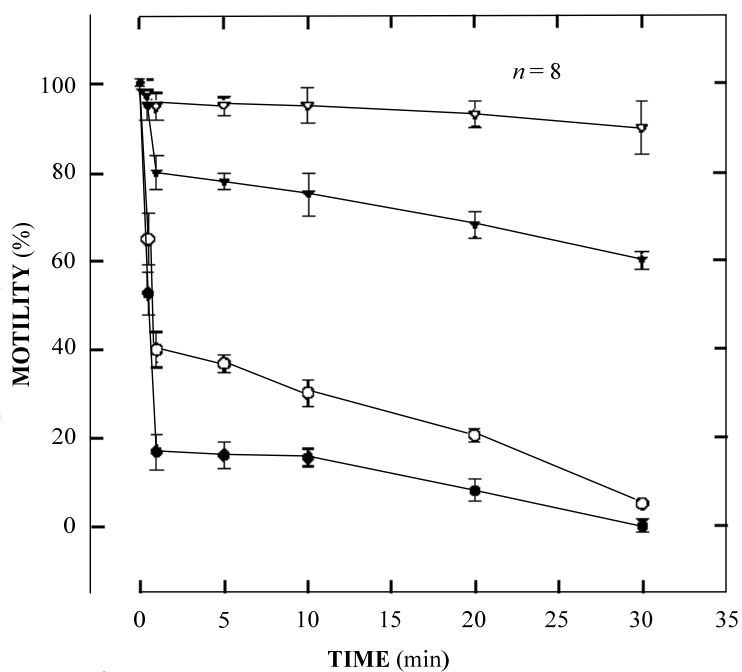


Fig. 4. Percent motility (mean \pm SEM, $n=8$) of human spermatozoa which were abruptly (one-step) returned to near isotonic conditions (305-343 mOsmol) after they had been exposed to different hyperosmotic conditions (TALP +sucrose) for different periods of time. ∇ , 600 mOsmol; \blacktriangledown , 700 mOsmol; \circ , 900 mOsmol; \bullet , 1200 mOsmol.

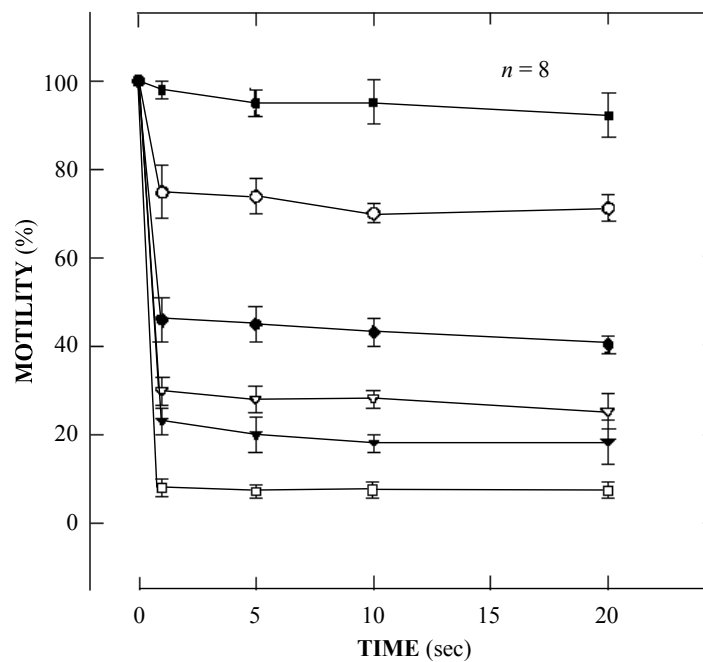


Fig. 5. Percent motility (mean±SEM, $n=8$) of human spermatozoa which were abruptly (one-step) returned to near isotonic conditions (273-284 mOsmol) after they had been exposed to different hyperosmotic conditions (TALP +water) for different periods of time. ■, 240 mOsmol; ○, 215 mOsmol; ●, 190 mOsmol; ▽, 143 mOsmol; ▼, 114 mOsmol; □, 90 mOsmol.

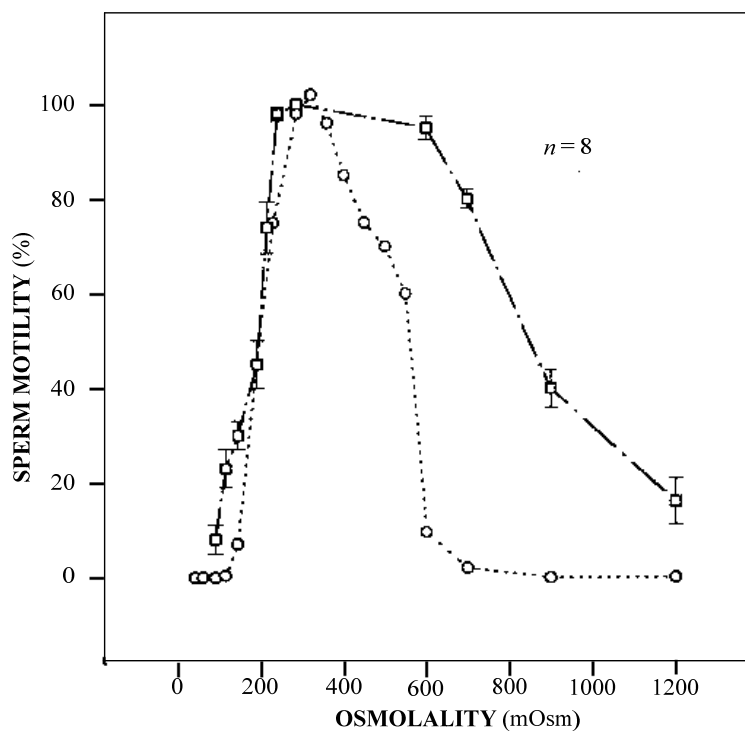


Fig. 6. A comparison of human sperm motility (% mean±SEM, $n=8$) after a 5 min exposure to the various hypo- and hyperosmotic solutions of non-permeating solutions before (○) and after (□) the return to near isotonic conditions (273-343 mOsmol).

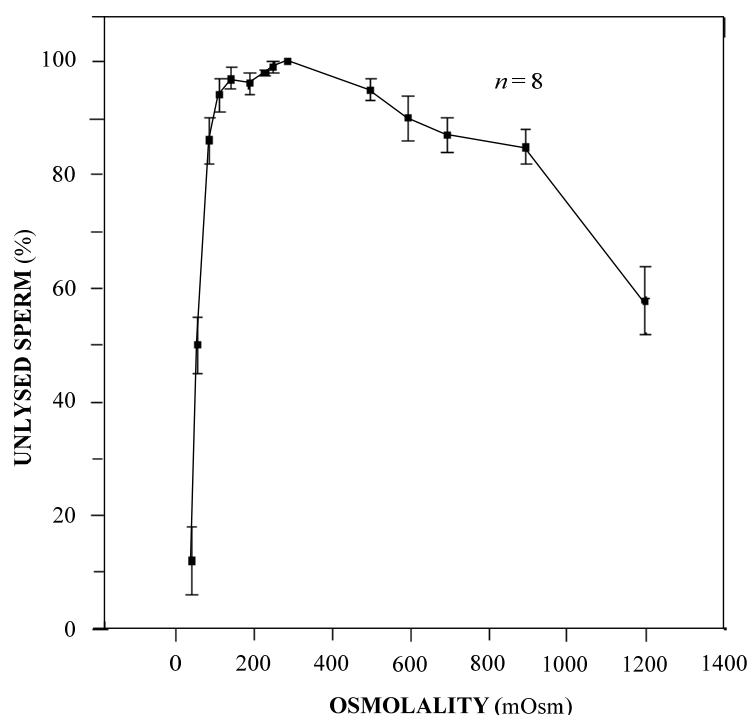


Fig. 7. Membrane integrity (CFDA and propidium iodide stain) (% mean \pm SEM, $n=8$) of human spermatozoa which were abruptly (one-step) returned to near isotonic conditions (273-343 mOsmol) after they had been exposed to different anisotonic conditions for 5 min.

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 7 shows that integrity of the plasma membrane 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 membrane 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 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 anisotonic solutions. In a portion of the spermatozoa, the tail region became configured as a 'zigzag' pattern when exposed to a hyper-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 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 hyper osmolality.) The tail curling was irreversible. The mechanism(s) behind the morphological change is not clearly understood.

Calculated volume excursions associated with exposures to anisotropic solutions

Since it has been shown that human spermatozoa behave as ideal osmometer over most of the range of osmolalities studied here (Du et al., 1993), a direct physical consequence of the exposures to anisotropic 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 8A and B

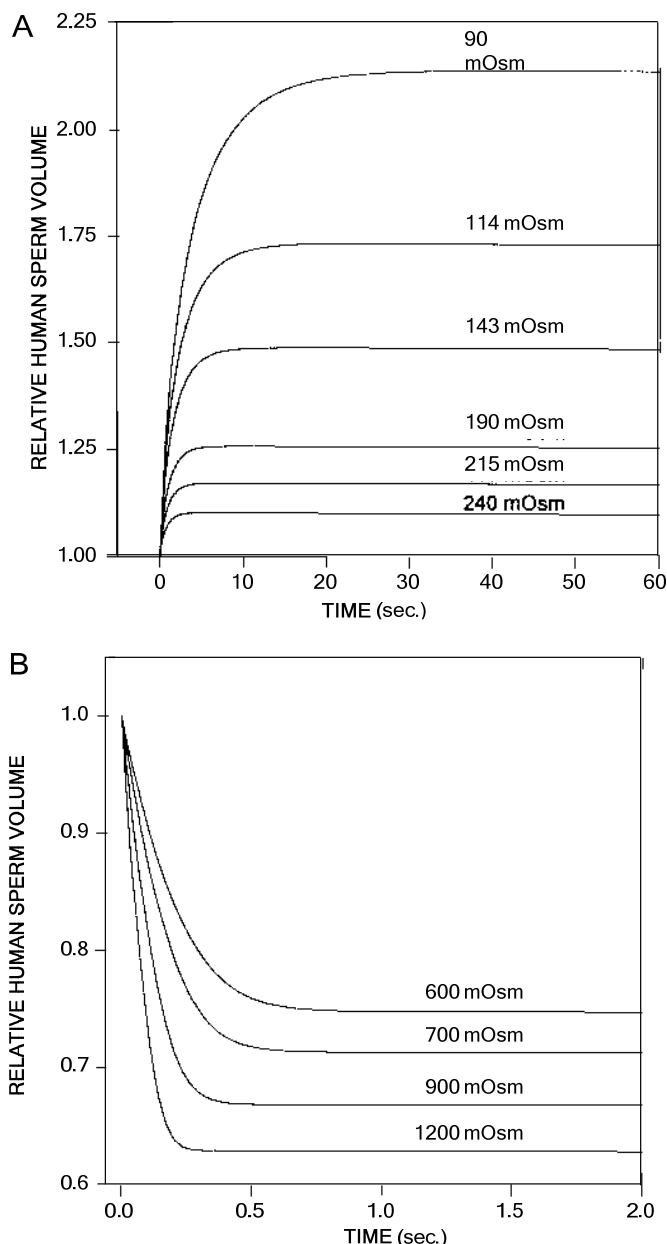


Fig. 8. (A) Calculated relative sperm volume (normalized to an isotonic sperm volume of 1) as a function of time after spermatozoa were one-step exposed to different hypo-osmotic solution containing non-permeating solutes. (B) Calculated relative sperm volume (normalized to the isotonic sperm volume of 1) as a function of time after the isotonic spermatozoa were one-step exposed to different hyperosmotic solutions containing non-permeating solutes.

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 8A and B also show the maximum or minimum volume of spermatozoa when they were osmotically equilibrated with each anisotonic solution. Sperm equilibration volume as a function of extracellular osmolality is shown in Figure 9, which can be calculated using equation (6) (no cryoprotective agent) or obtained directly from Figure 8A and B. To obtain a high ($>95\%$) motility recovery, the lowest and highest osmolalities which human spermatozoa can tolerate (Figures 4 and 5) were found to be close to 240 and 600 mOsmol respectively. At these two osmolalities, the corresponding cell volume at osmotic equilibrium were directly estimated (Figure 9) 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-anisotonic motility recovery. Based on Figure 4, 5 and 9, Figure 10 was plotted, which clearly shows the post-anisotonic injury (motility loss) as a function of osmotic equilibrium volume of spermatozoa in anisotonic 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 10 as follows: $LVL = 0.75 \times$ isotonic sperm volume, $UVL = 1.10 \times$ 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 (6-9) and are shown in Figure 11A 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 example. Based equations (6-9), the kinetics of water and glycerol transport through the

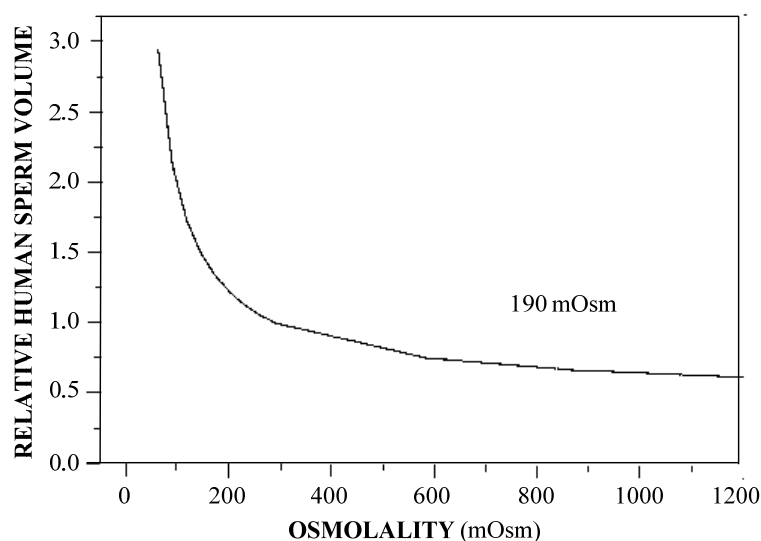


Fig. 9. Calculated relative sperm volume (normalized to the isotonic sperm volume of 1) after spermatozoa were osmotically equilibrated to different anisotonic conditions.

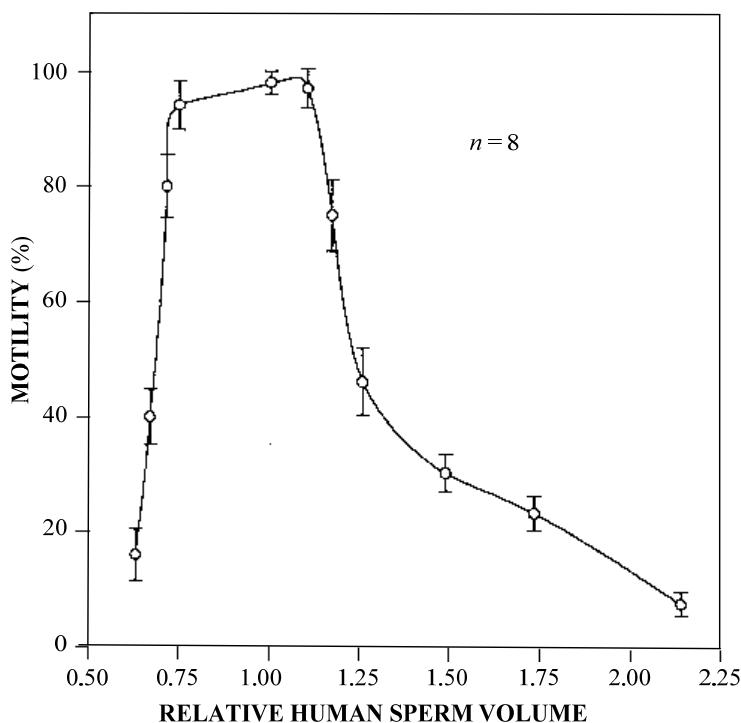


Fig. 10. Post-anisotonic sperm motility recovery as a function of relative sperm volume (normalized to the isotonic sperm volume of 1) in different anisotonic equilibrium states. Human spermatozoa were abruptly (one-step) returned to near isotonic conditions after exposure to anisotonic conditions for 1 min.

sperm membrane were simulated by computer. Figure 12 shows the calculated sperm volume excursion during a one-step or four-step addition of glycerol achieve a final 1.0 M glycerol concentration at 22 C using the FMS and FVS approaches respectively. From Figure 12, 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 loss (<5% loss). Figure 12 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 13, 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 14 shows that a four- or six-step FMS removal procedure was predicted to reduce 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 13 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 11-14, it was also found, from calculations, that human spermatozoa will

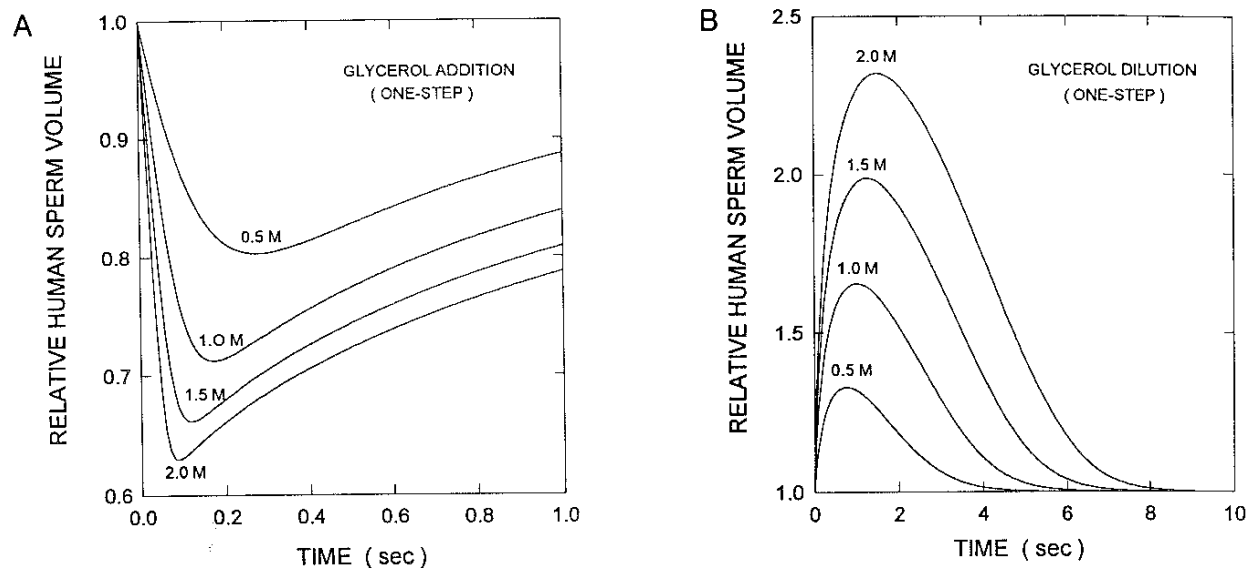


Fig. 11. (A) Calculated relative sperm volume (normalized to the isotonic sperm volume of 1) as a function of time after the isotonic sperm were exposed to different hyperosmotic glycerol solution isotonic with respect to non-permeating solutes (salt). (B) Calculated relative sperm volume (normalized 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 (salt), were one-step exposed to isotonic (286 mOsmol) saline solution without glycerol.

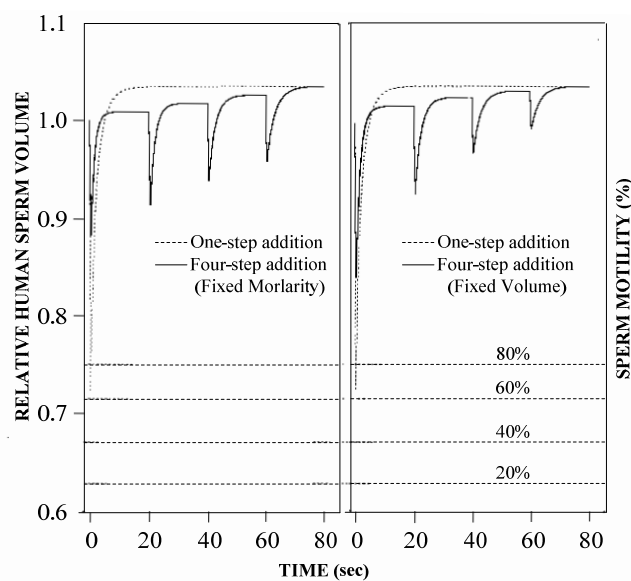


Fig. 12. (left) Calculated relative sperm volume (normalized to the isotonic sperm volume of 1) as a function of time after 1M glycerol was added to spermatozoa by either one-step or four fixed molarity steps. (right) Calculated relative sperm volume (normalized to the isotonic sperm volume of 1) as a function of time 1M 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.

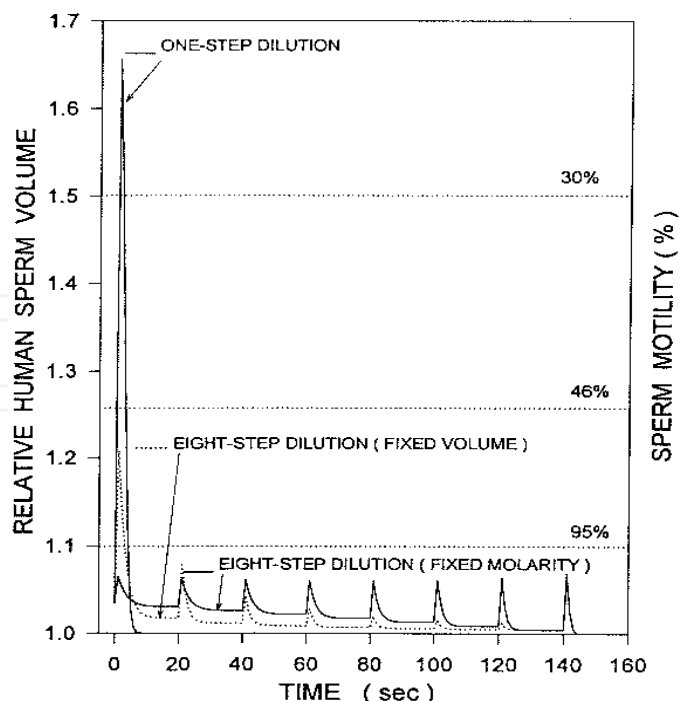


Fig. 13. Calculated relative sperm volume (normalized to the isotonic sperm volume of 1) as a function of time after 1 M glycerol was removed from spermatozoa by one-step, eight fixed-molarity steps or eight fixed-volume steps. The estimates of percent motility recovery as a function of sperm relative volume were obtained from Figure 10 and are indicated in the diagrams.

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 of four-step addition (Figure 12). 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%).

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 solutes; and (ii) the cells in the osmotic buffer are rehydrated by directly transferring them to 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 15. It was predicted that the maximum volume spermatozoa would achieve is 1.25 times (15%) the isotonic cell volume, which is higher than the UVL of human spermatozoa, and could be expected to cause >40% sperm motility loss, as predicted from Figure 10.

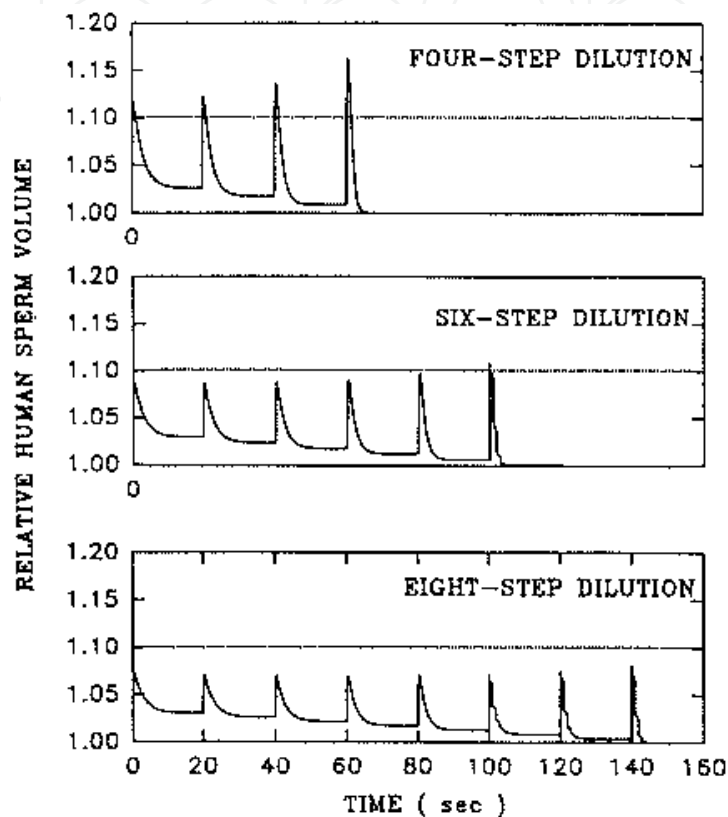


Fig. 14. Calculated relative sperm volume (normalized to the isotonic sperm volume of 1) as a function of time after 1 M glycerol was removed from spermatozoa by four, six and eight fixed-molarity steps. The dotted lines in this figure indicate the upper volume limit, 1.1, below which >95% of spermatozoa can maintain the motility. The four- or six-step dilution results in a cell volume excursion causing >5% motility loss.

Results from experimental examination

Glycerol was added to or removed from human spermatozoa using stepwise procedure to test the theoretical predictions. A one-step addition resulted in ~19.2% sperm motility loss or $81.8 \pm 8.7\%$ ($\bar{X} \pm \text{SEM}$, $n=15$) motility recovery, while the four-step FMS or FVS addition significantly ($P < 0.001$) increased in the motility recovery to $93.5 \pm 5.6\%$ ($\bar{X} \pm \text{SEM}$, $n=15$) or $91 \pm 4.8\%$ ($\bar{X} \pm \text{SEM}$, $n=15$) respectively. During different glycerol removal procedures (c.f. Table 2), <30% ($28.5 \pm 3.8\%$, $n=15$) of motile spermatozoa kept their motility after a one-step removal of 1.0 M glycerol, while the majority of spermatozoa ($92 \pm 8.2\%$, $n=15$) maintained motility after the eight-step FMS removal. In comparison, only $62 \pm 5.8\%$ of spermatozoa maintained motility after eight-step FVS removal. The motility recovery after a two-step

removal of glycerol (Table 3) using sucrose as an osmotic buffer was $43 \pm 5.3\%$ ($\bar{X} \pm \text{SEM}$, $n=15$). The experimental result 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). Over 90% of spermatozoa maintained membrane integrity under all experimental conditions.

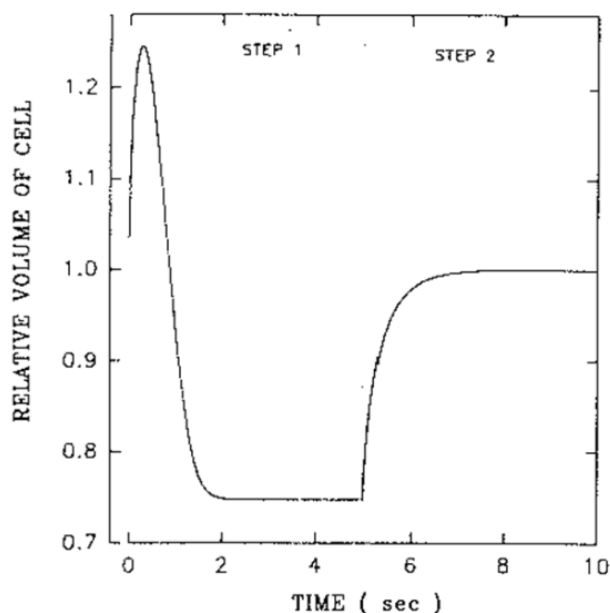


Fig. 15. Calculated relative sperm volume (normalized to the isotonic sperm volume of 1) as a function of time after 1 M glycerol was removed from spermatozoa by two steps using a 'hyperosmotic buffer' solution. Step 1: 1.0 M glycerol was removed from spermatozoa by one -step exposure of spermatozoa 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.

Example 2: Development of a novel dilution-filtration method and instrument to remove glycerol from human red blood cells (RBCs)

Cryopreservation has been widely used today around the world for long term preservation of RBCs. In the USA, the FDA has approved the storage of frozen RBCs at -80°C for as long as 10 years (Meryman, 2007). However, the glycerol in RBCs must be reduced to final concentration below 1% before infusion to prevent hemolysis (Valeri et al, 2001). The step of removing CPAs may cause serious cell loss due to the cell volume excursion induced by osmotic disequilibria (Meryman, 2007). In the past decades, many efforts have been made to improve the process (Rowe et al, 1968; Meryman et al, 1972, 1977; Valeri et al, 1975, 2001; Castino et al, 1996; Arnaud et al 2003).

Currently, multi-step centrifuging methods are most commonly used, and some of them can achieve favorable results (Rowe et al, 1968; Meryman et al, 1972, 1977; Valeri et al, 1975, 2001). However, the procedures are very difficult and time consuming for manual operation due to the large cell suspension volume or high CPA concentration. In addition, most of the systems are not closed and are thus open to contamination (Castino et al, 1996; Valeri et al, 2006). Automatic centrifuging systems may significantly reduce human labor and

contamination (Valeri et al, 2001), but the expensive cost limits their application in many areas. Recently, Dialysis was considered as an alternative method by some researchers (Castino et al,1996; Arnaud et al 2003; Ding et al 2007,2010). It can remove CPAs efficiently; however, due to the non-uniformity of distribution of hollow fibers, the mass transport in dialyzer is too complicated to be controlled, especially in the unsteady state. In addition, dialysis method is not efficient to remove large molecular substances (Daugirdas, et al, 2006), such as cell fragment and the released protein from broken cells. These factors limit the use of dialysis method in some applications.

In clinic, hemofiltration, which involves dilution and filtration to remove toxins from blood, has been proved to have better controllability as well as ability of removing large molecular substances than hemodialysis (Daugirdas, et al, 2006). By referencing to hemofiltration, a dilution-filtration system is developed recently for removing CPAs (Zhou et al, 2011). The closed system helps to avoid contamination to cells, and the continuous and automatic process could provide particular advantage in efficiency especially for large-scale samples. The related research work is introduced in the following.

2.3 Materials and methods

Technical Design

A dilution-filtration system is developed as shown in Fig.16 (Hemofilter: Plasmflo TM AP-05H/L, ASAHI; Pumps: 400F/M1, Watson-Marlow; silicone tubing: 985-75, Pall). For removing CPAs, thawed cell suspension is first transferred into the special blood bag (made by an infusion bag). Then, the suspension is driven by the blood pump to flow circularly among the bag, the mixer and the hemofilter. While going through the mixer, the suspension is quickly diluted by diluent, and the dilution ratio can be controlled to prevent lysis. In the hemofilter, extracellular solution containing CPA is partly ultrafiltrated while cells keep inside. Along with the circulation goes on, CPAs in cell suspension can be removed continuously. The whole process is conducted automatically in a closed system, and thus it is hopeful for this method to reduce human labor as well as the risk of contamination significantly.

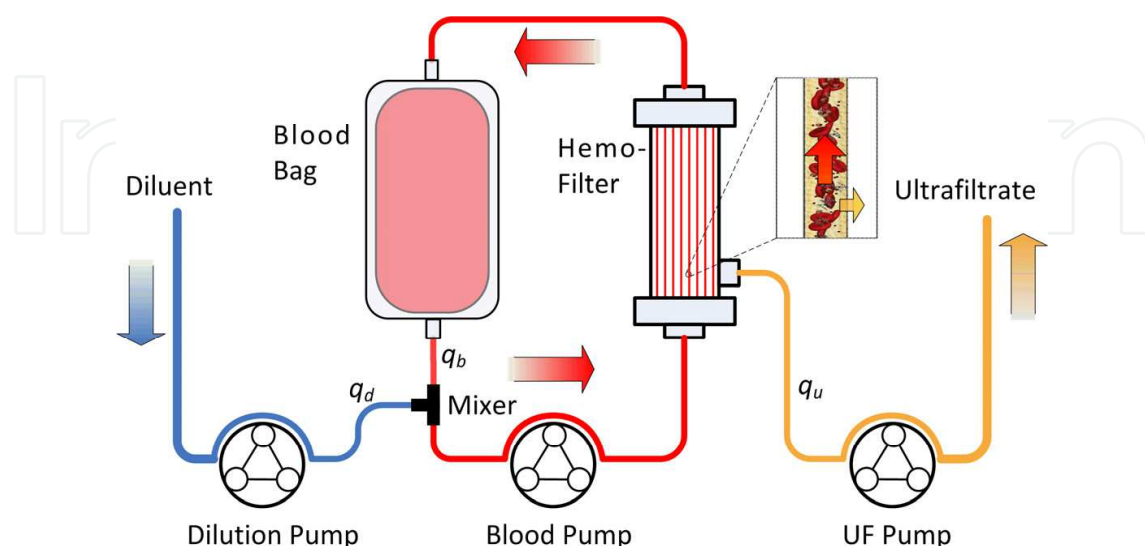


Fig. 16. Principle of the dilution-filtration system. Cell suspension is diluted and ultrafiltrated during circulating in the system, and then the CPAs inside can be continuously removed.

Theory of optimal operation protocol

Optimal operation protocol is defined here as the processes that minimize the operation time (to a final CPA concentration below 10g/L) as well as the osmotic cell volume excursion. A theoretical model was developed to predict the optimal operation protocols under the given experimental conditions (initial CPA concentration, cell density and total volume of cell suspension) and practical constraints. The detailed considerations for this procedure are described below.

Basic Assumptions and Formulation

The theoretical model of the dilution-filtration system is developed (as shown in Fig.17) under the following assumptions: (1) Both intra- and extra-cellular solutions in cell suspension consist of water, a permeable CPA (e.g. glycerol) and an impermeable salt (e.g. NaCl); (2) Blood bag, hollow fibers and their connecting tubing are filled with cell suspension, and cells are uniformly distributed in the suspension; (3) Extracellular solution is diluted/filtrated immediately and evenly at the diluting/filtrating point when cell suspension circulates in the system; (4) Suspension flow is one dimensional, and the convection factors can be neglected.

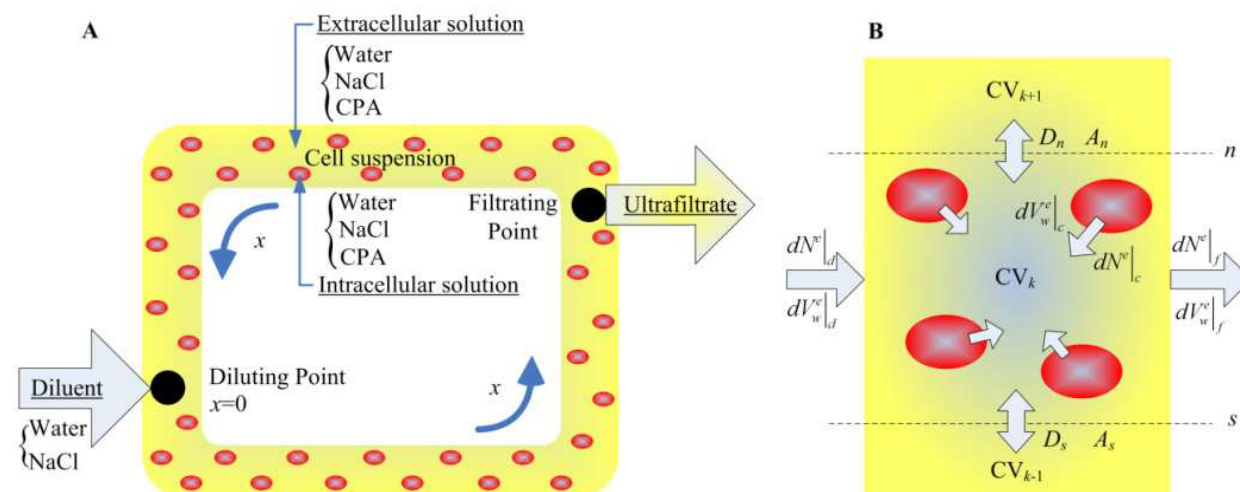


Fig. 17. Theoretical modeling of the system. A: the overall system, and B: a control volume.

Based on the assumptions, a governing equation about the mass transfer process can be derived by focusing on the extracellular solution:

$$\frac{\partial \phi^e}{\partial t} = \frac{1}{A} \frac{\partial}{\partial x} (DA \cdot \frac{\partial \phi^e}{\partial x}) + S \quad (19)$$

where, A refers to effective mass transfer area, D refers to diffusion coefficient, ϕ^e refers to extracellular solute concentration (in osmolality), and S is the mass source/sink term, respectively.

Source/Sink terms

The source/sink term can be derived by temporarily ignored the diffusion term:

$$S = \frac{\overline{d\phi^e}}{dt} = \frac{\overline{d(N^e/V_w^e)}}{dt} = \frac{1}{V_w^e} \frac{\overline{dN^e}}{dt} - \frac{\phi^e}{V_w^e} \frac{\overline{dV_w^e}}{dt} \quad (20)$$

where N^e and V_w^e are the number of osmoles of solutes and water volume in extracellular solution, respectively. The overlines in the equation indicate the given deriving condition. The terms of dN^e and dV_w^e can be further specified as

$$\overline{dN^e} = dN^e|_d - dN^e|_f + dN^e|_c \quad (21)$$

$$\overline{dV_w^e} = dV_w^e|_d - dV_w^e|_f + dV_w^e|_c \quad (22)$$

where the subscripts "d", "f" and "c" refer to the effects of dilution, filtration and cell membrane transport, respectively. According to assumption (2), cell suspension inside the system can be equally divided into a finite number of control volumes (CVs), as shown in Fig.17B. For each CV, the values of the terms in the right hands of equation [21] and [22] can be determined as flows.

i. Dilution/filtration

According to assumption (3), when a CV is going through the diluting point, the extracellular solution will be diluted immediately. Considering the pure filtration method used in the system, it is also assumed that ultrafiltration happens only at a certain location (the filtrating point, shown in Fig.17A), and the ultrafiltrate has the same composition as the extracellular solution. Thus the values of $dV_w^e|_d$, $dN^e|_d$, $dV_w^e|_f$ and $dN^e|_f$ of each CV can be determined as

$$\overline{dV_w^e|_d} = \begin{cases} \frac{Q_d V_{CV}}{Q_b} & \text{CV at the diluting point} \\ 0 & \text{CVs at the other locations} \end{cases} \quad (23)$$

$$\overline{dN^e|_d} = \overline{dV_w^e|_d} \cdot \phi^d \quad (24)$$

$$\overline{dV_w^e|_f} = \begin{cases} \frac{Q_f V_{CV}}{(Q_b + Q_d)(1 + \bar{V}_s \phi_s^e)} & \text{CV at the filtrating point} \\ 0 & \text{CVs at the other locations} \end{cases} \quad (25)$$

$$\overline{dN^e|_f} = \overline{dV_w^e|_f} \cdot \phi^e \quad (26)$$

where Q_f , Q_b and Q_d are the flow rates of ultrafiltrate, cell suspension and diluent, ϕ^d is the solute concentration in diluent, ϕ_s^e is the extracellular CPA concentration, \bar{V}_s is the partial molar volume of the CPA, and V_{CV} is the volume of a CV, respectively.

ii. Transportation across cell membrane

For the ternary system as considered in the present example, the mass transport across cell membrane can be described by the two-parameter formalism [2,3]. The total cell volume is the sum of the water, CPA and cell solid volumes:

$$V_c = V_w^i + V_s^i + V_{cb} \quad (27)$$

where the intracellular CPA volume can be determined as $V_s^i = \bar{V}_s N_s^i$. As soon as cell volume and intracellular solute concentrations are calculated the values of $dN_c^e|_c$ and $dV_w^e|_c$ can be further determined based on mass conservation:

$$dN_s^e|_c = -n_c dN_s^i, \quad dN_n^e|_c = 0 \quad (28)$$

$$dV_w^e|_c = -n_c dV_w^i = -n_c (dV_c - dV_s^i) \quad (29)$$

where n_c is the number of cells in a CV.

Numerical Simulation

With finite volume method, a fully implicit control volume integration of the governing equation will result in a finite difference scheme:

$$\left[a_{k-1} + a_{k+1} + a_k^{old} - (S_p V_{CV})_k \right] \phi_k^e = a_{k-1} \phi_{k-1}^e + a_{k+1} \phi_{k+1}^e + a_k^{old} \phi_k^{eold} + (S_c V_{CV})_k, \quad k = 2, \dots, K-1 \quad (30)$$

where a is the coefficient and K is the total number of CV in the system. The subscript 'k' refers to the kth CV in the system and the superscript 'old' refers to the previous time level. S_c and S_p are the constant portion and gradient of the linearized source term:

$$S_c = \frac{1}{V_w^e} \frac{dN^e}{dt}, \quad S_p = -\frac{1}{V_w^e} \frac{dV_w^e}{dt} \quad (31)$$

The subscript 'k-1' and 'k+1' in equation (30) refer to the previous and next CVs along the x direction, respectively. Noting that the cell suspension flows circularly in the closed system, the 1st CV is followed by the Kth one. Thus

$$\left[a_K + a_2 + a_1^{old} - (S_p V_{CV})_1 \right] \phi_1^e = a_K \phi_K^e + a_2 \phi_2^e + a_1^{old} \phi_1^{eold} + (S_c V_{CV})_1 \quad (32)$$

$$\left[a_{K-1} + a_1 + a_K^{old} - (S_p V_{CV})_K \right] \phi_K^e = a_{K-1} \phi_{K-1}^e + a_1 \phi_1^e + a_K^{old} \phi_K^{eold} + (S_c V_{CV})_K \quad (33)$$

Here, the removal of glycerol from cryopreserved human red blood cells (RBCs) is discussed for an instance. For the ease of discussion, it is further restricted that blood volume keeps constant, i.e. ultrafiltrate flow rate keeps equal to diluent flow rate, although the presented system and model can adapt to more complicated situations. Besides, the concentration of NaCl in diluent and thawed blood are considered to be isotonic (0.29 Osmol/kg water). In

this manner, the basic variables for a simulation consist of the experimental conditions (including the initial blood volume (V_b^0), hematocrit (h^0), and the concentrations of CPA (M_s^0) in extra/intracellular solution) as well as the operation parameters (including the flow rates of blood (Q_b) and diluent (Q_d)). The initial values of the other parameters in the model can be determined as

$$V_{CV}^0 = V_b^0 / K \quad (35)$$

$$V_c^0 = V_{iso} + V_s M_s^0 (V_{iso} - V_{cb}) \quad (36)$$

$$n_c^0 = V_{CV}^0 h^0 / V_c^0 \quad (37)$$

where V_{iso} is the isotonic volume of RBC. When terming the CV at the diluting point ($x=0$) when $t=0$ as the 1st CV (CV_1), the initial location of each CV can be allocated. Then the values of dN^e and dV^e for each CV can be calculated according to equations [21]-[31]. By alternatively calculating the source terms and solving the linearized governing equation, the concentration variation of extra-/intracellular solution as well as the responding cell volume excursion can be simulated. A typical process is shown in Fig.18, in which $V_b^0 = 200\text{ml}$, $h^0 = 30\%$, $M_s^0 = 6.28 \text{ Osmol/kg water}$ (approximately 40% w/v), $Q_b=200\text{ml/min}$, and $Q_d=25\text{ml/min}$.

To quantitatively evaluate the effect of an operation protocol, the maximum cell volume and the total time cost (to a final glycerol concentration below 10g/L (Brecher, 2002)) of the removing process can be taken as criteria for cell recovery rate and removing efficiency, respectively. Then the optimal protocol can be found out by applying different operation parameters to the given experimental conditions and comparing the simulated results. Hereinafter, the diffusion coefficients of glycerol and NaCl in water were set to be $5.43 \times 10^{-10} \text{ m}^2/\text{s}$ and $14.41 \times 10^{-10} \text{ m}^2/\text{s}$, respectively (Ternstorm et al, 1996). The parameters about the dilution -filtration system and RBC membrane are also specified as listed in Table 4 and Table 5. These parameters may be different in various applications and systems.

Sections	Inner volume	Effective area
From the outlet of blood bag to the diluting point	5ml	$1.25 \times 10^{-5} \text{ m}^2$
From the diluting point to the filtrating point	5ml	$1.25 \times 10^{-5} \text{ m}^2$
From the filtration point to the outlet of hemofilter	85ml	$5 \times 10^{-4} \text{ m}^2$
From the outlet of hemofilter to the inlet of blood bag	5ml	$1.25 \times 10^{-5} \text{ m}^2$
Blood bag	Variable	$5 \times 10^{-3} \text{ m}^2$

Table 4. Structural parameters of the dilution-filtration system used in the calculation

Surface area of RBC (A_c)	$135 \times 10^{-12} \text{ m}^2$ ^a
Hydraulic permeability of cell membrane (L_p)	$1.74 \times 10^{-12} \text{ m/Pa/s}$ ^a
Isotonic volume of RBC (V_{iso})	$98.3 \times 10^{-18} \text{ m}^3$ ^a
Solid volume of RBC (V_{cb})	$0.283 \times V_{iso}$ ^a
Glycerol permeability to cell membrane (P_s)	$6.61 \times 10^{-8} \text{ m/s}$ ^a

^a From literature (Papanek, 1978);

Table 5. Membrane parameters of human RBC used in the calculation

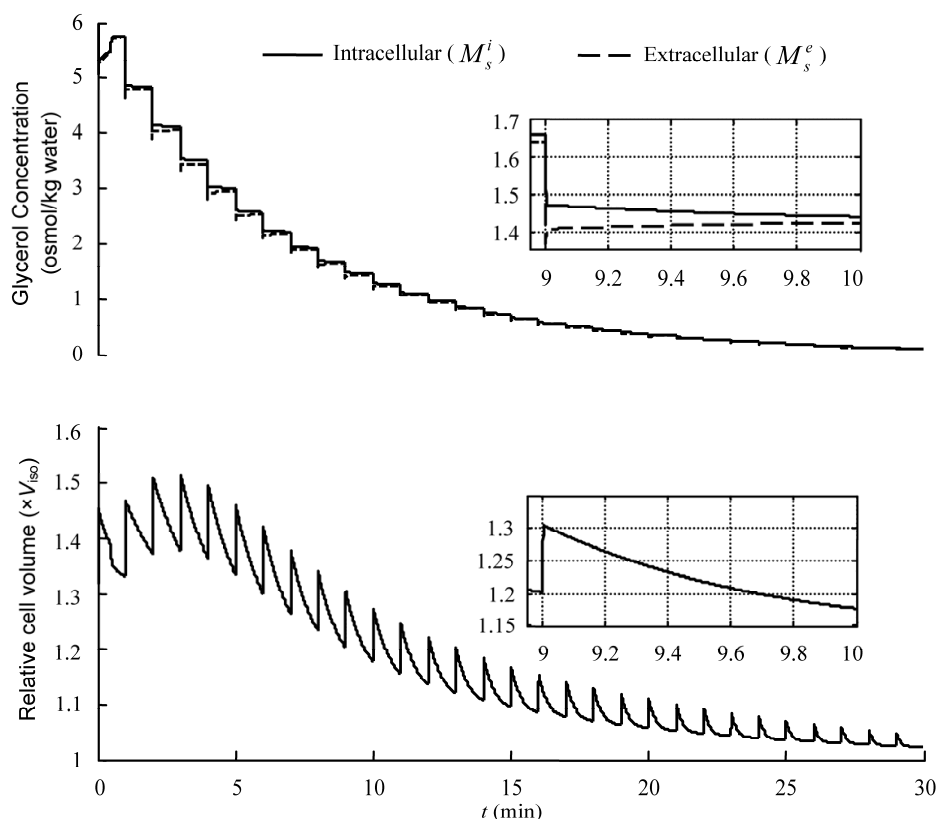


Fig. 18. Simulated glycerol concentration variation and cell volume excursion in CV_1 (initially at the diluting point) during a dilution-filtration process.

Experiments

Venous human blood was collected from healthy, adult blood donors in the Red Cross Transfusion Center of Heifei. For each donor, up to 200ml whole blood was collected into CPDA-1 anticoagulant solution in PVC plastic bag, and stored for up to 24 hours at 4°C . Then it was centrifuged at $1615\times g$ for 4 minutes, and the platelets, leukocytes and plasma were removed to produce a hematocrit of 75 ± 5 percent.

Each of the RBC suspensions was transferred into a 400-ml plastic bag, and then it was glycerolized by 57.1% w/v glycerol solution with a volume ratio of 2:1 (glycerol to blood) to achieve a final glycerol concentration about 40% (w/v) and a hematocrit of 25%-30%. Subsequently the blood bag was covered by PE foam sheet (thickness: 5mm) and then placed into a metal box (size: $200\text{mm}\times 150\text{mm}\times 20\text{mm}$). After 30 minutes of equilibrium, the metal box was transferred to a -80°C freezer (MDF-U52V, SANYON, Japan) and the RBC suspension was frozen gradually. After cryopreservation in the freezer for 2~7 days, the RBC suspension was taken out and thawed in a 37°C water bath for about 10 minutes with gentle agitating.

Each unit of the thawed blood was deglycerolized with the dilution-filtration system as shown in Fig. 16, and the operation protocol was theoretically optimized. A typical experimental conditions ($V_b^0 = 200\text{ml}$, $h^0 = 30\%$, $M_s^0 = 6.28$ Osmol/kg water) was studied first to reveal the general law. To evaluate the effect of each operation parameter, different protocols were applied respectively. Fig.19 shows that time cost is significantly reduced but maximum cell volume grows directly along with diluent flow rate increases, i.e. the washing efficiency can be

improved by applying higher diluent flow rate but more hemolysis may be induced. Thus the diluent flow rate has to be carefully selected to achieve the optimal result. Comparatively, the effect of blood flow rate is not so complicated. Increasing of blood flow rate has little effect on glycerol clearance, but helps to reduce the maximum cell volume excursion.

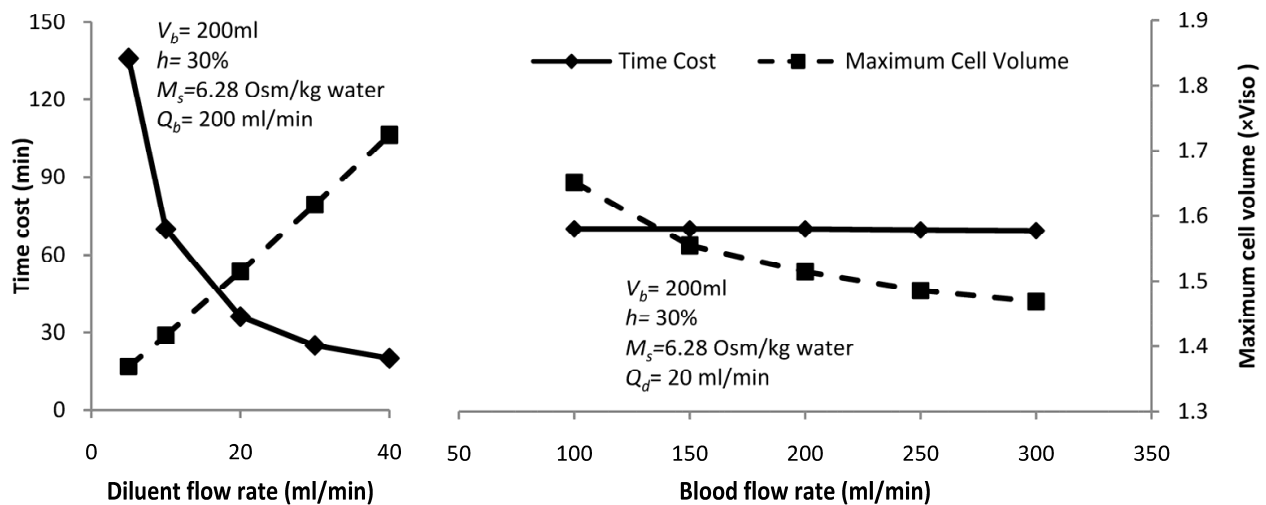


Fig. 19. Variations of time cost (real line and left Y-axis) and maximum cell volume (dash line and right Y-axis) with blood or diluent flow rates as parameters.

On the other hand, the effect of the operation parameters is also highly related to the blood conditions, especially the glycerol concentration. As shown in Fig.20, the same operation protocol ($Q_b=200$ ml/min and $Q_d=20$ ml/min) is applied to several different conditions, in which $V_b^0=200$ ml, $h^0=30\%$, and M_s^0 varies from 0.56 Osmol/kg water (5% w/v) to 6.28 Osmol/kg water (40% w/v). When the glycerol concentration decreases, both the glycerol clearance and the maximum cell volume are reduced (glycerol clearance is defined here as the difference of initial and final numbers of osmoles of glycerol in blood over time cost). This phenomenon indicates us that along with the glycerol concentration drops during washing, diluent flow rate can be continuously increased to speed up the process without inducing extra cell volume excursion.

Based on the analysis above, it can be concluded that to achieve the optimal deglycerolization it is important to: a) use a low diluent flow rate at first, and stepwise increase it as CPA concentration drops; b) always use a high blood flow rate. The detailed operation parameters of the optimal protocol can be found out by the theoretical model with some practical constraints. During the in-vitro experiments, operation protocol for each unit was optimized theoretically according to the specific experimental conditions as well as the following constraints: maximum cell volume: 1.35 times of the isotonic volume (V_{iso}) of RBCs; maximum flow rate of pumps: 200 ml/min and maximum ultrafiltrate flow rate of hemofilter: 40 ml/min. The value of upper cell volume level was conservatively selected in order to achieve the best cell recovery rate, although the washing efficiency may be limited.

Samples were taken before and after deglycerolization. Cell count and hematocrit were measured by a hematology Analyzer (Ac·T diff II TM, Beckman COULTER®) The Freeze-Thaw-Wash (FTW) cell count recovery rates were calculated by comparing the total cell counts

after thawing to that after washing (Valeri et al, 2001). Residual glycerol concentration in the washed blood was measured by a glycerol assay kit (K-GCROL, Megazyme®) and a spectrophotometer (756MC UV-VIS, Scientific Instrument®, Shanghai, China).

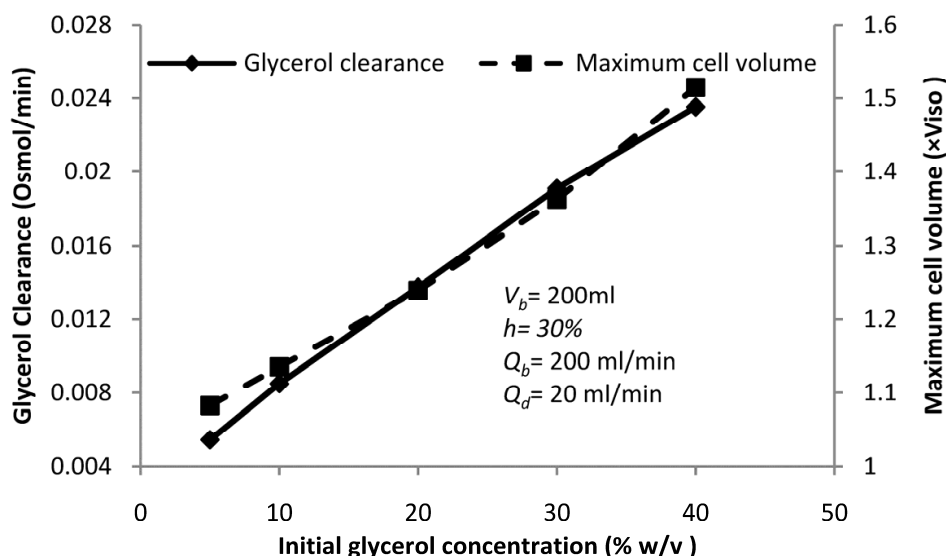


Fig. 20. Variations of glycerol clearance (real line and left Y-axis) and maximum cell volume (dash line and right Y-axis) with glycerol concentration as a parameter.

2.4 Results

A total number of ten units of blood were cryopreserved and deglycerolized by the dilution-filtration method, and the results are shown in Table 6. The residual glycerol concentration (5.57 ± 2.81 g/L, $n=10$) is obviously lower than the standard value (10g/L) indicated by American association of blood banking (AABB). During the optimization of the operation procedures, the maximum cell volume constraints was critically applied ($1.35 \times \text{Viso}$) for the best of cell recovery, and thus the deglycerolizing efficiency is limited. However, each of the unit was processed within an hour, which is similar to the automatic centrifuging method (Valeri et al, 2001). The cell count recovery rate is $91.19 \pm 3.57\%$ ($n=10$). Comparing to the reported methods (Diafiltration method: 70% (Castino et al, 1996), dialysis method, no in vitro data was presented (Ding et al, 2007, 2010), manual centrifuging method: $>80\%$ (Brecher, 2002), and automatic centrifuging method $89.4 \pm 3.0\%$ (Valeri et al, 2001)), the recovery rate indicates an obviously advantage of our method in cell safety.

UNITS	Thawed Blood Volume (ml)	Thawed Blood Hct (%)	Cell Count Recovery (%)	Residual Glycerol (g/L)
1	221.8	30	93.64	2.60
2	204.1	27	85.92	3.39
3	229.8	29	91.64	12.26
4	219.0	23	90.91	4.90
5	217.6	25	91.38	3.39
6	210.0	28	90.76	6.56
7	216.7	24	81.60	3.80
8	200.5	29	94.18	7.34
9	204.5	21	92.24	5.35
10	205.0	30	93.80	6.08
Mean	212.9	27	91.19	5.57
S.D.	9.49	3	3.57	2.81

Table 6. *In-vitro* experiments of deglycerolization with dilution-filtration method

2.5 Discussion

An optimized method for addition and removal glycerol from cryopreserved human spermatozoa has been illustrated as an example. Although the mechanism(s) of the osmotic injury during cryopreservation is not clearly understood, the hypothesis 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. Hence, the procedures used for testing the hypothesis provide a methodology to predict optimal protocols for cryoprotective agent addition/removal..

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 four 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 example, 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 (≥ 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 12 and 13). 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 12 and 13).

In the example, 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. In the example, 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 7, 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 example, based on the information provided in Figure 5. For example, it was found (Figure 7) that >85% of spermatozoa maintained membrane integrity when they were returned to isotonic condition after having been exposed to anisotonic conditions ranging from 90 and 700 mOsmol. The corresponding sperm volume excursion range was 0.7-2.1 times the isotonic sperm volume (Figure 9). From Figures 12 and 13, 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 90.7-2.1 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.

A dilution-filtration system for removing CPAs from cryopreserved cell suspension was also introduced here. The system realized continuous processing of cell suspension and the dilution & filtration were conducted simultaneously, thus it can achieve much better efficiency than traditional multi-step centrifuging methods. Moreover, dilution in the system is conducted to cell suspension flow in tubing but not whole suspension in container, thus the mixing process should be much rapider and then the osmotic disequilibrium during dilution can be significantly reduced.

A theoretical model was established to simulate the specific process. Based on the model, cell volume excursion and the variation of CPA concentration during the dilution-filtration process can be simulated. Theoretical analysis indicates the operation parameters, especially the flow rate of diluent, are critical for the dilution-filtration method. In the previous studies concerning removing CPAs with hollow fibers (Castino et al, 1996; Arnaud et al 2003; Ding et al, 2007, 2010), only the protocols with constant flow rates were discussed. However, it was found to be difficult to balance the requirements in removing efficiency and cell safety. This problem also exists in the presented dilution-

filtration method. Removing efficiency can be improved by using higher diluent flow rate, but the cell recovery rate may be seriously reduced in the way. Besides, when using a constant diluent flow rate, the profile of glycerol concentration is nearly exponential, i.e., the removing efficiency starts at the highest value but gradually decreases as the process going on. However, when using a stepwise increased diluent flow rate, the removing efficiency can be maintained at a high level for a quite long period. Moreover, theoretical analysis also indicates stepwise increasing of the diluent flow rate may not cause any extra cell damage. Therefore, a stepwise increased diluent flow rate is necessary to achieve both high cell recovery rates and efficient glycerol clearance when using the dilution-filtration system. In addition, it was also deduced by the theoretical analysis that the removing effect of an operation protocol is highly related to the initial volumes and cell densities of cell suspensions. Therefore, the optimal operation protocols should be specialized and various from case to case. The theoretical model provides an effective tool to find out the optimal protocols for given applications.

The system was also investigated experimentally with deglycerolization from cryopreserved blood, and the operation procedures were optimized based on the theoretical model. It is clearly indicated by the results that the dilution-filtration method is safe and efficient for deglycerolization from cryopreserved RBCs. Comparing to the automatic centrifuging method, the cell recovery rate and removing efficiency are similar, but the equipment cost of the dilution-filtration system is much lower and thus it can be applied in more areas. We can also believe that with properly selected operation parameters, this system can also be applied to various CPA removal applications. In addition, all the media are processed in a closed system, and thus the system should have further advantages in avoiding contamination. It is hopeful for the cells to have a long shelf life after washing. These suppositions will be verified by further experiments.

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Current Frontiers in Cryobiology

Edited by Prof. Igor Katkov

ISBN 978-953-51-0191-8

Hard cover, 574 pages

Publisher InTech

Published online 09, March, 2012

Published in print edition March, 2012

Almost a decade has passed since the last textbook on the science of cryobiology, *Life in the Frozen State*, was published. Recently, there have been some serious tectonic shifts in cryobiology which were perhaps not seen on the surface but will have a profound effect on both the future of cryobiology and the development of new cryopreservation methods. We feel that it is time to revise the previous paradigms and dogmas, discuss the conceptually new cryobiological ideas, and introduce the recently emerged practical protocols for cryopreservation. The present books, "Current Frontiers in Cryobiology" and "Current Frontiers in Cryopreservation" will serve the purpose. This is a global effort by scientists from 27 countries from all continents and we hope it will be interesting to a wide audience.

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Dayong Gao and Xiaoming Zhou (2012). Prevention of Lethal Osmotic Injury to Cells During Addition and Removal of Cryoprotective Agents: Theory and Technology, *Current Frontiers in Cryobiology*, Prof. Igor Katkov (Ed.), ISBN: 978-953-51-0191-8, InTech, Available from: <http://www.intechopen.com/books/current-frontiers-in-cryobiology/prevention-of-lethal-osmotic-injury-to-cells-during-addition-and-removal-of-cryoprotectants-theory-a>

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China

Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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