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UNIVERSITY OF ALBERTA

Osmotic and Toxic Effects During Freezing and Vitrification of Human Granulocytes

by

Hongyou Yang

A thesis

submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE

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(Signed) Hongyou Yang

Permanent Address: 133 Primrose Gardens

Edmonton, Alberta

Canada, T5T 0R1

Date: December 16, 1991

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The undersigned certify that they have read, and recommend to the Faculty of Graduate studies and Research for acceptance, a thesis entitled OSMOTIC AND TOXIC EFFECTS DURING FREEZING AND VITRIFICATION OF HUMAN GRANULOCYTES submitted by Hongyou Yang in partial fulfillment of the requirements for the degree of Master of Science in Experimental Pathology.

Locksley E. McCann

Robert A. Stinson

Kenneth Wong

Nancy Telford

Date: December 11, 1991

DEDICATION

To the memory of my **mother** and my **father** who always encouraged me to study, and selflessly supported me

To all people who love me

ABSTRACT

Some mammalian cells have resisted attempts at cryopreservation despite many efforts over the past forty years. The hypothesis put forward in this thesis is that the nature of damage to these cells during cryopreservation is unique to these cell types. Specifically, the primary site of injury is not the plasma membrane, as is currently believed to be the case for most other mammalian cell types. Human granulocytes were used as a model because of their sensitivity to osmotic stress and chemical toxicity during cryopreservation.

The nature of injury to granulocytes during freezing and thawing was studied by comparing the damage seen in granulocytes to that found in lymphocytes and fibroblasts after a graded freezing protocol. The assays for membrane integrity and mitochondrial function were used to localize the primary site of injury. Similar results with cytoplasts (created by removing the nuclei and other intracellular organelles from granulocytes) showed that the plasma membrane was not the primary site of cryoinjury in granulocytes.

Since vitrification (solidification without crystallization) has been proposed as an approach for cryopreservation of osmotically sensitive cells and tissues, the recovery of granulocytes after exposure to vitrification solutions was investigated. By varying the temperature of exposure, the rates of addition and dilution, times of exposure and the composition of the solutions, it was possible to differentiate between osmotic and chemical effects. Using five types of vitrification solutions with the same permeability to cells with unique addition and dilution protocols, it was shown that the cell damage encountered with

vitrification solutions is primarily due to chemical toxicity rather than osmotic injury effects. This toxicity could be reduced by a combination of cryoprotectants, but no specific toxicity neutralizer was found.

In summary, this study has demonstrated that the plasma membrane is not the primary site of freezing injury in human granulocytes, and toxicity of vitrification solutions to cells is determined by their chemical nature *per se*. No specific toxicity neutralizer was found in this study, but toxicity of vitrification solutions was be effectively minimized by optimal combinations of cryoprotectants.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACET	acetamide
ANOVA	analysis of variance
ATP	adenosine triphosphate
2,3-B	2,3-butanediol
BME	basal medium eagle
BSA	bovine serum albumin
DNA	deoxyribose nucleic acid
DMSO	dimethyl sulfoxide
EB	ethidium bromide
EG	ethylene glycol
FDA	fluorescein diacetate
FCS	fetal calf serum
HSD	Tukey's studentized range
Lp	hydraulic conductivity
MEM	minimum essential medium
MTT	3-(4,5-dimethylthialzol-2yl)-2,5-diphenyl tetrazolium bromide
PBS	phosphate buffered saline

PG	propylene glycol
R0	rapid addition at 0°C
RRT	rapid addition at room temperature (22°C)
S0	slow addition at 0°C
SEM	standard error of mean
SRT	slow addition at room temperature (22°C)
TSAI	tolerable surface area increment
VS	vitrification solution

CHAPTER I

INTRODUCTION

1.1 Importance of cryopreservation of biological systems

Mammalian cells are assumed to be preserved in liquid nitrogen for 2,000-4,000 years without damage (33) because liquid water does not exist below -130°C and the only reactions that can occur in frozen aqueous systems at -196°C are photophysical events such as the formation of free radicals and the production of breaks in macromolecules as a direct result of "hits" by background ionizing radiation (52). Since storage temperatures below -130°C effectively stop biological time, cryopreservation of biological systems has become invaluable to many fields. Embryo freezing is currently the most valuable use of cryopreservation, especially the preservation of rare and endangered species. The frozen embryos of extinct species can be transferred to the uterus of a closely related species to reincarnate. In experimental biology, cells can be removed from an animal when it is young, frozen, and used as a reference to study changes as an animal ages. The cryopreservation of cells and tissues, such as progenitor bone marrow cells and skin, plays an important role in clinical applications. It is generally believed that organ cryopreservation will be most important in clinical replacement therapy because freezing offers a logical solution to the establishment of organ banks to facilitate coordinating donor availability and recipient need and to permit closer immunological matching (33). Unfortunately, however, organs do not yet survive freezing and thawing.

1.2 Limitations of cryopreservation

Discovery of the cryoprotective properties of glycerol by Polge, Smith, and Parkes in 1949 is a landmark of modern cryobiology (48). Since then, cryoprotective agents have been used to secure the survival of innumerable cells after storage at low, stabilizing temperatures. However, despite this impressive degree of success, few cryopreservation techniques today permit 100% survival after freezing and thawing, and the preservation of many important systems has proven unsatisfactory or unsuccessful. This is because cells or organized tissues cannot tolerate the osmotic stresses and chemical toxicity encountered during the process of cryopreservation, including addition and dilution of cryoprotectants as well as freezing and thawing. Mechanisms of cryoinjury are unclear. There are several hypotheses of slow freezing injury but almost all these are established on damage to the plasma membrane during freezing. Vitrification, a process by which a liquid solidifies without the formation of any crystalline phase, has been suggested as an approach to cryopreservation of cells and organized tissues (31). Because osmotic stresses encountered during slow freezing and extracellular ice formation are avoided by vitrification, it may be possible to cryopreserve cells (which are sensitive to the osmotic stress) and tissues (which are damaged by extracellular ice) by this process. It has been shown that several types of tissues and cells can be successfully cryopreserved by vitrification (50, 64), but this is currently limited to cells and tissues which can survive conventional cryopreservation. Cryoprotectant toxicity is a fundamental obstacle to the full potential of artificial cryoprotection, yet it remains in general a poorly understood phenomenon (21). It is generally assumed that toxicity of a vitrification solution depends on solute concentration and

composition of cryoprotectants; therefore, a mixture of solutes is usually used to reduce this toxicity. On the other hand, toxicity of a vitrification solution is thought to be determined by its osmotic potential (28) and hence toxicity is minimized by formulating solutions with an optimal osmolality.

1.3 **Objectives**

- *Investigation of primary site of cell injury during freezing and thawing.*
- *Identification of some critical factors affecting viability of cells during addition and dilution of cryoprotectants.*
- *Determination of the nature of chemical toxicity of vitrification solutions, and development of vitrification solutions with low chemical toxicity.*

1.4 **Experimental approach**

Human granulocytes were chosen as a model to study the mechanisms of injury because granulocytes are sensitive to osmotic stresses during freezing and thawing, and to chemical toxicity during addition and dilution of cryoprotectants (3, 25). Firstly, human granulocytes, human lymphocytes, and hamster fibroblasts without cryoprotectant were cooled to various subzero temperatures using a graded freezing-protocol, and the recovery of thawed cells were assayed using plasma membrane and mitochondrial activity. Secondly, responses of human granulocytes to this graded freezing technique were compared with those of granulocyte cytoplasts. Cytoplasts are derived from granulocytes by removal of lysosomes and other organelles, but retain the functions (such as

phagocytosis) as intact granulocytes. Thirdly, some critical factors affecting osmotic stresses during cryopreservation procedures were investigated in granulocytes, including rates of addition and dilution, and addition temperatures of various concentrations of propylene glycol. Finally, the osmotic stress and toxicity of different concentrations of individual cryoprotectants in vitrification solutions were studied, and based on this information several types of new vitrification solutions were developed.

CHAPTER II

LITERATURE REVIEW

2.1 **Human granulocytes**

Granulocytes, one type of leukocyte, are present in blood. Granulocytes contain numerous lysosomes and are subdivided into three classes on the basis of their morphology and staining properties. The differences in staining reflect major difference of chemistry and function: Neutrophils are the most common type of granulocytes ($5x10^9$cells/liter blood); they phagocytose and destroy small organisms - especially bacteria. Basophils ($4x10^7$cells/liter blood) secrete histamine to help mediate inflammatory reactions. Eosinophils ($2x10^8$cells/liter blood) help destroy parasites and modulate allergic inflammatory responses (2).

2.2 **Granulocyte transfusion**

In the mid to late 1970s, widespread granulocyte transfusion was used to treat infected patients who responded poorly to the marginally effective antibiotics. Since the beginning of 1980s, the transfusions have been abandoned because the poor efficacy of this therapy was identified by a series of controlled trials, and the majority of patients can now be treated effectively with antibacterial and antifungal agents. However, there still remains a fraction of patients who can clearly benefit from granulocyte transfusions, especially those afflicted with neonatal sepsis (59).

2.3 **Preservation of granulocytes**

2.3.1 *Storage at suprazero temperatures*

It was shown that granulocytes maintained their function for only a few hours after blood collection (35, 57). Normal chemotactic response of granulocytes is maintained longer when granulocytes are stored at 20 to 24°C than those stored at 1-6°C (35). Cold exposure is detrimental to the granulocyte cytoskeleton and impairs its motility and chemotactic response (57).

2.3.2 *Cryopreservation*

By the early 1980s many cryobiologists found that few granulocytes survive freezing to below -75°C (10, 11, 26, 56). Others reported fair functional survival (29) and some have reported high survivals (1, 68). However, since the procedures reported to be successful seem little different from those that fail, and because some reports of success have not proven repeatable, the problems of preserving human granulocytes by freezing seem far from solved. The belief now is that human granulocytes cannot be successfully cryopreserved because granulocytes poorly tolerate anisotonicity associated with cryopreservation (25).

2.4 **Hypotheses of freezing injury**

2.4.1 *Two-factor hypothesis*

Mazur and his co-workers (32) proposed that cells can be damaged by two factors during freezing and thawing. One is that rapidly cooled cells are damaged by the formation of intracellular ice and its subsequent growth by recrystallization during warming. The other is solution effects produced during slow cooling where cells are damaged by the relatively long exposure to the major alterations produced in extracellular solutions. The alternations include concentration of solutes, dehydration of cells, and the precipitation of solutes

caused by conversion of water to ice. However, Mazur *et al* did not mention the nature and the site of freezing injury to cells caused by either of these effects.

2.4.2 *Solute effect*

In 1953 Lovelock (30) suggested that freezing damage in human erythrocytes is a consequence of the increase in electrolyte concentration that occurs during freezing due to the removal of water in the form of ice but that the formation of ice is not in itself damaging. He proposed that high salt concentrations caused direct damage to the plasma membrane which resulted in a net influx of Na^+. Consequently, when the cells are returned to an isotonic medium, they swell beyond their normal isotonic volume and lyse.

2.4.3 *Minimum cell volume*

Meryman (42) demonstrated that red cells suspended in isotonic sucrose begin to hemolyze at the same subzero temperature at which hemolysis is seen in cells initially suspended in isotonic sodium chloride, and concluded that extracellular salt concentration is not the primary factor of injury. He proposed that cellular responses to the high osmolality of the suspending solution is responsible for injury. This hypothesis states that cells behave as osmometers, reversibly shrinking and swelling, over a limited range of osmolality and that they reach a minimum volume at osmolalities near the upper end of that range. Further shrinkage in response to higher osmolalities is resisted, and the resultant stresses damage the plasma membrane. This behavior would be characterized by a flattening of the Boye-van't Hoff plot (a linear relationship

between equilibrium cell volumes and reciprocal osmotic pressure) with increasing solute concentration.

2.4.4 *The Tolerable Surface Area Increment (TSAI)*

Steponkus and Wiest (62) proposed that cell surface changes rather than high solute concentration are the cause of damage in cells exposed to hyperosmotic solutions. Cell shrinkage in response to hyperosmotic solutions reduces the expansion potential of the cell and thus limits the subsequent increase in volume tolerated by the cell during rehydration. The proposed mechanism is the loss of surface membrane by an amount that is dependent on the degree of shrinkage of the cell. It is proposed that, during shrinkage, areas of the plasma membrane first invaginate and then encapsulate. Consequently, vesicles of plasma membrane are internalized and the amount of surface that the cell has available for subsequent expansion is reduced. Thus the cell will lyse upon rehydration if the loss of surface membrane is extensive.

2.5 **Vitrification**

Experience with the cryopreservation of organs has led to the general conclusion that organized tissues present a particularly difficult challenge. With cell suspensions, extracellular ice apparently has no physical effect on the cells other than to concentrate the extracellular solution. On the other hand, in organized tissue, extracellular ice is interstitial and can damage tissue architecture, break intercellular connections and is particularly damaging within the vasculature where extensive endothelial damage can result (22, 46). It is now generally agreed that good preservation of organs probably cannot be

achieved if ice is present within the tissue. Vitrification solutions are aqueous cryoprotectant solutions which solidify without crystallization when cooled at moderate rates to very low temperatures. Moreover, Rall (50) reported that embryos cryopreserved by vitrification are exposed to less concentrated solutions of cryoprotectants for shorter periods of time. Therefore, vitrification has been suggested as an approach for cryopreseration of organized tissues or cells which cannot be cryopreserved using conventional techniques.

In 1937, Luyet successfully vitrified organic colloids and protoplasm with gelatin gels, fresh albumin and with protoplasmic material and proposed that vitrification could be used as a method of cryopreservation (31). However, actual vitrification of biological samples was almost never achieved over the ensuing four decades, because the methods used by Luyet relied on ultrarapid cooling and warming rates rather than on adequate treatment with vitrification solutions. A vitrification solution (8.6 *M* glycerol) was first used to vitrify human erythrocytes successfully in 1968 (51). Nevertheless, the significance of the experiment was overlooked even by the authors of the report due to their reliance on rapid cooling and warming. More than a decade of inactivity followed before the current concepts of cryopreservation by means of vitrification solutions were introduced (14, 15, 18, 23) and successfully applied (4). However, cryopreservation of cells and tissues by vitrification is still limited to those specimens which can be cryopreserved by conventional freezing methods. Organ vitrification has never been achieved. The central problem restricting the use of vitrification for cryopreservation is the high chemical toxicity of vitrification solutions.

2.6 Cryoprotectant toxicity

Cryoprotectant toxicity is a fundamental obstacle to the full potential of artificial cryoprotection, yet it remains in general a poorly understood phenomenon. A model biochemical study of cryoprotectant toxicity was that of Baxter and Lathe (7), which demonstrated that alteration of a specific enzyme (fructose diphosphatase) was the cause of impaired glycolysis after treatment with and removal of dimethyl sulfoxide (DMSO). Fahy and co-workers (16, 17, 20, 24) suggest that the basis for the detrimental effects of cryoprotectants is due to direct "biochemical" injury. Injuries such as inactivation or denaturation of specific enzymes, disruption of transmembrane ionic pumps, or other related perturbations of cellular structure and function, by implication, are most likely due to the direct interaction of the cryoprotectant with proteins and biological membranes. Therefore, Fahy pointed out that chemical toxicity depends on the total concentration and composition of cryoprotectants, and suggested that the toxicity could be reduced by replacing of a fraction of the cryoprotectant with another cryoprotectant (21). However, this hypothesis has been challenged by Langis and Steponkus, who suggested that dehydration-induced alterations in membranes are a primary cause of injury and therefore toxicity of a vitrification solution largely depends on its osmotic potential (28).

2.7 Summary

Cryopreservation of granulocytes is limited because the cells are particularly sensitive to osmotic stresses and chemical toxicity encountered during addition and removal of cryoprotectants and freezing-thawing. The

mechanisms of cryoinjury are unknown although there is general agreement that the plasma membrane is the primary site of injury. Vitrification has been suggested as an approach for cryopreservation of osmotically sensitive cells and organized tissues. However, high osmotic stress and chemical toxicity produced by vitrification solutions limit the application of this technique. Chemical toxicity is the main problem preventing employment of vitrification but the mechanism of this toxicity remains unknown.

CHAPTER III

MATERIALS AND METHODS

3.1 Cell preparation

3.1.1 *Human granulocytes and lymphocytes*

3.1.1.1 Percoll protocol

Human granulocytes and lymphocytes were isolated from the peripheral blood of normal human volunteers. Peripheral blood was collected in heparinized tubes and diluted with a solution of phosphate-buffered saline (PBS) (GIBCO, Grand Island, NY) (10 ml PBS was added to 50 ml blood), and 6% (w/v) dextran was added (seven parts blood to five parts dextran solution). The majority of erythrocytes were removed from the suspension by sedimentation at unit gravity and room temperature for 30 min. The resulting supernatant was washed twice in PBS with 0.15% (v/v) bovine serum albumin (BSA) (Miles Laboratory, Naperville, IL) (PBS/BSA). A stock Percoll (Pharmacia, Sweden) solution was prepared by mixing 90 ml of Percoll with 10 ml of PBS at 10 times isotonic concentration (GIBCO). A working Percoll solution, consisting of 22.5 ml of stock Percoll solution and 13.5 ml of PBS/BSA was centrifuged at 11,800*g* for 2 h to create a continuous density gradient (density, 1.02-1.13 g/ml). The washed cell suspension was layered on continuous density gradients of Percoll and centrifuged at 500*g* for 30 min to separate the cell suspension on the basis of density into bands of erythocytes, granulocytes, lymphocytes, monocytes, and debris at the top in the platelet band. The granulocyte and lymphocyte bands were harvested from the gradient,

washed twice in PBS/BSA, and resuspended in PBS/BSA or minimum essential medium (MEM, GIBCO). The resulting cell suspensions usually contained about 95% granulocytes with erythrocytes as the main contaminant. This protocol was used for preparation of granulocytes and lymphocytes for Chapter IV. The granulocytes for the rest of the experiments were prepared using the Ficoll protocol because it is more convenient in handling large volumes of blood.

3.1.1.2 Ficoll protocol

Human granulocytes were isolated from the buffy-coat of pooled units of peripheral blood from healthy volunteers. Thirty ml of buffy-coat was layered on the top of 15 ml Ficoll-Hypaque (5.7% w/v), and centrifuged at 500*g* for 30 min at 22°C. The supernatant was discarded and any remaining erythrocytes lysed using a solution of 8.27 g/liter NH_4Cl, 0.84 g/liter $NaCO_3$ and 0.0372 g/liter EDTA. Granulocytes were then sedimented by centrifugation at 500*g* for 10 min at 22°C, and the following steps were the same as for Percoll.

3.1.2 *Granulocyte cytoplasts*

Granulocyte cytoplasts were prepared according to the method described by Roos *et al.* (53) (Fig.3.1.), a modification of that described by Wigler and Weinstein (66). Briefly, granulocytes were suspended in 12.5% (w/v, 1.0477 g/ml) Ficoll-70 with 20 μM cytochalasin-B, incubated at 37°C for 5 min, and then this suspension was layered on the top of a discontinuous (16% w/v, 1.0578 g/ml, and 25% w/v, 1.0885 g/ml) Ficoll gradient prewarmed to 37°C for 2 h. Care was taken to adjust osmolality to 300 mosmol/kg in each gradient layer.

After centrifugation at 81,000*g* for 20 min at 33°C, in a rotor prewarmed for 4 h at 37°C, the band of cytoplasts, visible at the interface of the 12.5% and 16% Ficoll layers, was collected, washed four times in MEM containing 0.15%BSA, and resuspended in the same medium.

This method produces vesicles with cytoplasm similar to that of the granulocytes from which they originated (53). Lysosomes and nuclei are engulfed in the lower part of the constricted granulocytes and partitioning occurs with the membrane resealing by adhesion. Consequently, the cytoplasm contained in the upper vesicle - the cytoplast - is that of the original cell. Ross *et al* (53) found that the volumes of cytoplasts were about 100 μm^3, about 20% of that of granulocytes, and that the biochemical composition of the plasma membrane is similar.

3.1.3 *Hamster fibroblasts (V79)*

Hamster fibroblasts were grown in tissue culture as monolayers attached to the bottom of tissue culture flasks (Corning, N.Y), in Eagle's minimal essential medium (MEM; GIBCO) supplemented with 10% (v/v) fetal calf serum (FCS; GIBCO). Cells in the exponential growth phase were detached from the flasks by a 10 min incubation at 37°C with 0.25% (w/v) trypsin, and resuspended in MEM with FCS.

3.2 **Permeability of cells to cryoprotectants and kinetics of cell volume changes exposed to impermeant solutes**

3.2.1 *Permeability*

Mean cell volumes of human granulocytes were measured using a Coulter ZBI electronic particle counter (Coulter Electronics, Hialeah, FL) and the data were stored on an IBM PC microcomputer using a custom interface (35). One hundred μl of granulocytes with $2x10^6$ cells/ml were injected into a vial with 10 ml experimental solutions and the mean cell volume was measured as a function of time of exposure to the solutions.

3.2.2 *Kinetics of cell volume changes*

Cells with a semipermeable plasma membrane respond osmotically to changes in the osmolality of the suspending solution. The Boyle-van't Hoff plot, which describes a linear relationship between equilibrium cell volumes and reciprocal osmotic pressure, was used to compare osmometric behavior of granulocytes and cytoplasts. In addition, the kinetics of cell volume changes on exposure to anisosmotic solutions of impermeant solutes were used to estimate and compare the hydraulic conductivities of the plasma membrane of granulocytes and cytoplasts. A 50 μl aliquot of cells or cytoplasts suspended in an isotonic solution was added to 10 ml of isotonic or hypertonic solutions, and cell volumes were monitored as a function of time. The osmolality of the final solution ranged from 0.3 to 2 osmol/kg. The electronic particle counter was used to record average cell volumes as a function of time. Beads of known volume were used for calibration, giving absolute measurements of cell volume.

A Simplex algorithm and Runge - Kutta numerical integration was used to fit the experimental data to a theoretical description of osmotic water movement across membranes (39). The change of volume with time was expressed as

$$dV/dt = LpART(\pi_i - \pi_e), \qquad (1)$$

where V is cell volume (μm^3), t is time (min), L_p is hydraulic conductivity of the membrane ($\mu m^3/\mu m^2/min/atm$), A is the cell surface area (μm^2), R is gas constant (liter atm/°K-mol), T is absolute temperature (°K), and π_i and π_e are the intra- and extracellular osmotic pressure (osmol/kg), respectively.

The intracellular osmotic pressure π_i was determined from the Boyle-van't Hoff relationship:

$$\pi_i(V-V_d) = \pi_o(V_o-V_d), \qquad (2)$$

$$or \quad \pi_i = \pi_o(V_o-V_d)/(V-V_d), \qquad (3)$$

where V and V_o are cell volumes at osmotic pressures π_i and π_o (isotonic), respectively, and V_d is osmotically inactive volume of the cell.

Substituting equation (3) for π_i in equation (1) gives

$$dV/dt = LpART(\pi_o(V_o-V_d)/(V-V_d) - \pi_e) \qquad (4),$$

and the fitting of this equation to experimental measurements yields best-fit values for L_p and V_d.

3.3 Freezing-protocol: Graded freezing

This technique allows investigation of the effects of slow cooling and rapid cooling rates on cells. Aliquots of cell suspension (0.2 ml) were dispensed into glass tubes (5x10 mm diameter) and immersed in an alcohol bath at -5°C. After 1 min, the samples were seeded to induce ice formation, and another minute was allowed for the removal of the latent heat of fusion. These times have been experimentally optimized. The bath was then cooled at 1°C/min, and at various subzero temperatures samples were either thawed directly in a

water bath at 37°C or cooled rapidly by immersion in liquid nitrogen, stored for 30 min and thawed in water at 37°C.

3.4 **Cell recovery after experimental treatment**

3.4.1 *Assessment of membrane integrity*

Cell recovery was assessed by a membrane integrity test using a combination of fluoresein diacetate (FDA, Sigma), and ethidium bromide (EB, Sigma), with or without a two-h incubation period (55). FDA and EB were used with the granulocyte suspensions at concentrations of 10 μM for each dye, but FDA alone was used with the cytoplasts since these vesicles contained no nucleus, precluding the use of EB, which binds to DNA. The assay was similar to that originally described by Rotman and Patermaster (55), as modified by McGann et al (38). FDA was added to each cell suspension immediately after thawing and the cells were incubated at 22°C for 10 min before assessment by fluorescence microscopy. Samples in a hemacytometer were then examined under a fluorescence microscope (Zeiss) using a mixture of white and UV illumination to determine cell recovery. Intact cells and cytoplasts showed a green fluorescence due to fluorescein retained intracellularly; damaged granulocytes appeared red due to EB staining; damaged cytoplasts showed no fluorescence. Initial cell concentrations were adjusted to give approximately 300 cells in the control samples. This procedure accounts for cells which may lyse during the freeze-thaw process. Cell recovery was expressed as the percentage of cells with fluorescence compared with the control sample.

3.4.2 *MTT.*

3,(4,5-dimethylthialzol-2yl)-2.5-diphenyl tetrazolium bromide (MTT, Sigma, St. Louis, MI) can be used to assess the activity of various dehydrogenase enzymes. Tetrazolium is cleaved in active mitochondria so the reaction occurs only in metabolically active cells. The experimental procedure used for the MTT assay was similar to that described by Mossman (43). After experimental treatment, 100 μl cell suspension ($2x10^6$ cells/ml) and 10 μl MTT solution (5 mg/ml) were added to each well of a 96-well flat-bottom plate (Flow Labs, VA). The plate was incubated at 37°C for 2 h and then centrifuged at 800*g* for 5 min. The media was removed and 100 μl of isopropanol was added to each well. The plate was then shaken for 60 s, and the absorbance in each well was recorded at a wavelength of 570 nm relative to the reference reading at 630 nm (Dynatech, VI). The mean absorbance of eight blank wells was used as the background reading and subtracted from the measured absorbence. Cell recovery was expressed as the mean absorbance of the experimental samples as a percentage of the mean absorbance of the control.

3.5 **Electron microscopy**

Scanning electron microscopy was used to observe differences between granulocytes and cytoplasts. Samples of granulocytes and cytoplasts were fixed immediately after preparation using 2.5% (v/v) glutaraldehyde in Milloning's buffer (16.8 g NaH_2PO_4 $4H_2O$; 3.85 g NaOH; 0.05 g $CaCl_2$; 5.4 g glucose per liter), stored overnight at 4°C, then processed for electron microscopy, which involved washing three times in Milloning's buffer, fixing in 1% osmium tetroxide for 45 to 60 min, and washing in distilled water. The samples were

then dehydrated in a series of ethanol solutions, air-dried, and mounted with silver glue on aluminum stubs later coated with gold. A scanning electron microscope (Phillips-505) was used to examine the samples.

3.6 Statistical analysis

Each value in the figures presents mean and standard error of the mean (SEM) of at least three experiments for each condition. Analysis of variance (ANOVA) was utilized and significant multiple differences were determined by the Tukey's Studentized Range (HSD) test (60). All statistical analyses were carried out by using the Statistical Analysis System Institute and the SAS User's Guide:Statistics, 1988 ed., SAS Institute Inc., Cary, NC.

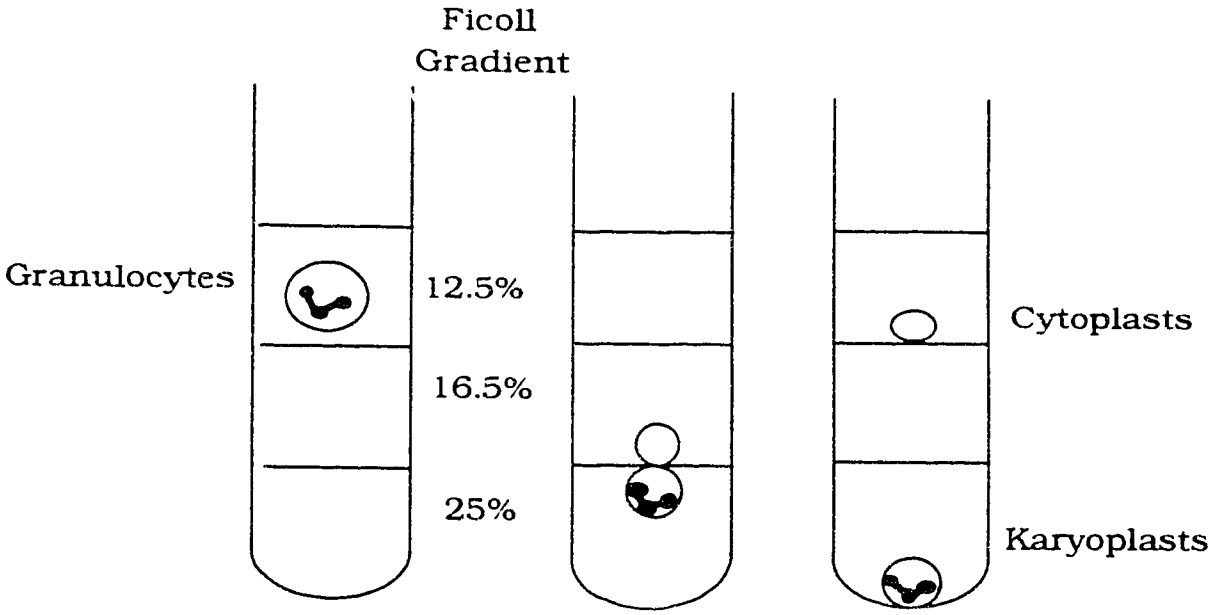


Figure 3.1. Preparation of granulocyte cytoplasts. Granulocyte cytoplasts were prepared from human granulocytes by first layering granulocytes suspended in 12.5% (w/v, 1.0477 g/ml) Ficoll on discontinuous layers of 16% (w/v, 1.0578 g/ml) and 25% (w/v, 1.0885 g/ml) Ficoll. On centrifugation, granulocytes are enucleated, with the denser organelles sedimenting to the bottom of the tube and the cytoplasts remaining at the layering interface.

CHAPTER IV

MANIFESTATIONS OF CELL DAMAGE AFTER FREEZING AND THAWING

4.1 Introduction

It has long been known that freezing and thawing can damage living cells, and there have been many investigations into the nature of this injury. A general conclusion to date is that the plasma membrane is the primary site of freezing injury, although the actual mechanism is unclear (44). Many of the studies on the mechanisms of freezing injury have used assays that test the integrity or function of membranes to assess recovery in cells or membrane model systems. Conditions such as the stresses encountered during freezing and thawing will result in changes which are lethal. As well, there will be a progressive development of damage to other regions within the cell, eventually leading to a loss of all functions associated with a living cell. Measurement of the integrity of the plasma membrane after a freeze-thaw stress will therefore inevitably lead to the conclusion that the plasma membrane is a site of freeze-thaw injury, whether or not damage to the membrane is a primary lesion. It is of interest to investigate the primary sites of freezing injury in order to develop strategies for avoiding damage during cell and organized tissue preservation. The purpose of this work was to investigate the sites of injury by subjecting living cells to a graded freeze-thaw stress and to assess the interrelationships between, and the sequence of changes in, membrane integrity, mitochondrial

function, and reproductive integrity. A comparison of responses of cells immediately after thawing with their responses after an incubation period allows determination of the types of damage evident immediately after thawing, and of the secondary damage that develops.

4.2 **Experimental protocol**

Detailed methods of graded freeze-thaw and assays of MTT and FDA are described in Chapter III. Briefly, aliquots of suspensions of granulocytes, lymphocytes and hamster fibroblasts (0.2ml) in PBS/BSA, without cryoprotectant, were dispensed into glass tubes (5x10 mm) and a graded freeze-thaw protocol was carried out to investigate effects of slow cooling and fast cooling rates on cells. Cell damage was assessed using colony growth for hamster fibroblasts and MTT and FDA assays for ganulocytes, lymphocytes, and hamster fibroblasts.

4.3 **Results**

Cells cooled at 1°C/min to subzero temperatures without cryoprotectant suffer increasing damage as the temperature is lowered as shown in Fig. 4.1. Recovery of hamster fibroblasts measured by reproductive integrity shows that cell damage begins below -10°C and increases at lower temperatures (Fig. 4.1a). Recovery was low for samples cooled to -196°C from the higher subzero temperatures, increased at intermediate temperatures, and subsequently decreased at the lower temperatures. When assayed using MTT, the recovery of hamster fibroblasts was lower than that obtained using the colony growth

assay, and the response of human lymphocytes to the same freeze-thaw stress (Fig. 4.1b) was similar to that hamster fibroblasts. Human granulocytes, however, were significantly more sensitive to injury, with virtually no recovery after the suspension was seeded (initiation of ice nucleation) at -5°C and thawed directly. This granulocyte response is similar to results using phagocytosis as the assay for recovery (13, 27, 40). Fig. 4.2 shows cell recovery measured using FDA immediately after thawing. Hamster fibroblasts show a pattern of recovery with FDA similar to that shown by colony formation, but with a higher level of recovery at all sub-zero temperatures. Human lymphocytes and granulcoytes also showed higher recoveries with FDA than with MTT, but even with FDA, granulocytes showed no recovery when thawed from -196°C. After a 2 h incubation period, cell recoveries for lymphocytes and granulocytes measured using FDA (Fig. 4.2b) were similar to the results using MTT.

4.4 **Discussion**

In cells cooled at 1°C/min in the absence of cryoprotective compounds and thawed directly from a subzero temperature, cell recovery assessed using FDA decreased progressively as the temperature was lowered. The result indicates that the cooling rate of 1°C/min is suboptimal for those cells used, so the cells will respond by osmotic shrinkage during cooling, and the decline in cell recovery is related to changes caused by the removal of water in the form of ice. For cells cooled rapidly to -196°C from the subzero temperature, the recovery after thawing was low at higher temperatures due to intracellular

freezing (40). Recovery increased as the intracellular water content (hence the potential amount of intracellular ice) was reduced by osmotic water efflux, and finally decreased to values limited by cell damage sustained during slow cooling. This supports the concept that the type of damage suffered by cells is dependent on the cooling conditions (34). A post-thaw incubation period allows time for the development of secondary damage, as demonstrated by the experiments with FDA. Impairment of mitochondrial function is evident immediately after thawing under some conditions where the FDA assay shows no damage in intracellular fluorescence. However, on incubation, significant changes are evident using FDA, indicating that the damage develops during the incubation period, which is then manifested as changes detectable using the FDA assay. This type of damage is therefore secondary, resulting from a previous lesion.

A post-thaw incubation period also allows time for the repair of sublethal damage (4) as demonstrated by the difference between the results from the colony growth and MTT assays. In fibroblasts, the colony growth assay for reproductive integrity is a functional test for virtually all active systems within the cell. Cell recovery after freezing to subzero temperatures is higher when measured using colony growth than when measured using MTT. Therefore some of the damage measured by MTT must be repaired during the period of incubation for colony growth. Repair of sublethal freeze-thaw damage has been previously reported for these cells (4).

A comparison of the MTT assay with the other tests for cell recovery reported here (colony growth and FDA) shows that the MTT assay is a very sensitive test to detect freeze-thaw damage, a conclusion supported by the

experiments with granulocytes in which our results with the MTT assay compared well with those using phagocytosis (13, 27, 40) and chemotaxis (13).

Results for the different cell types illustrate the similarity of responses of human lymphocytes and hamster fibroblasts and the different responses of human granulocytes. These observations are consistent with the interpretation (47) that lysosomes are sensitive organelles in granulocytes that are easily activated by the perturbations such as the osmotic stresses encountered during freezing and thawing. The similar responses of two very different cell types - human lymphocytes and hamster fibroblasts-would indicate that these both have similar sites of freezing injury.

Based on these observations a general model of freezing injury to nucleated mammalian cells is consistent with our experimental observations. Disruption of the lysosomes constitutes the primary lesion in cells which are dehydrated during the cooling process. This leads to an early impairment of mitochondrial function. There is evidence that the time course of damage to mitochondria by lysosomal enzymes (45) is much shorter than that of damage to the plasma membrane (12). The plasma membrane is a primary site of freezing injury during rapid freezing when intracellular freezing is either a cause or a result of this damage.

A practical implication of this hypothesis is that two modes of cryoprotection for slow cooling are desirable - one is the reduction of osmotic stresses by colligative action, and the other is the stabilization of the lysosomal membrane.

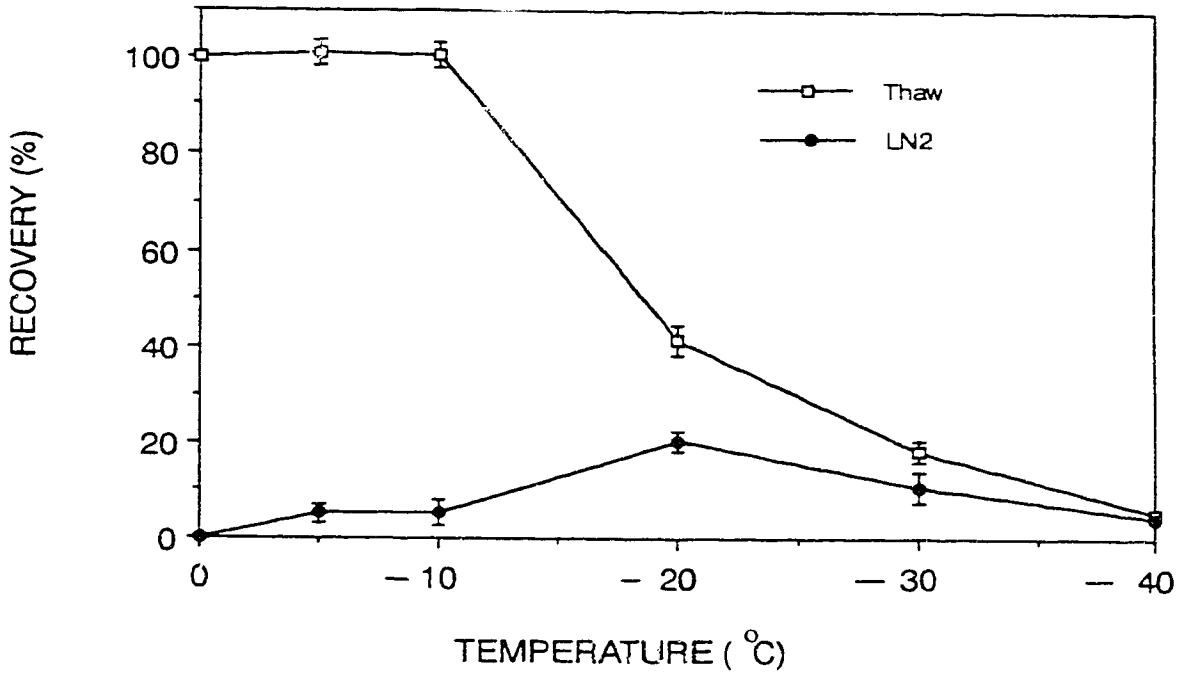


Figure 4.1a. Recovery of hamster fibroblasts after freezing and thawing. Hamster fibroblasts were cooled at 1°C/min to various subzero temperatures and then either thawed by warming at 300°C/min (thaw) or cooled at 600°C/min to -196°C before warming (LN_2). Cell recovery was assessed after thawing using colony growth cells. Values shown are means with vertical bars equal to $\pm$SEM.

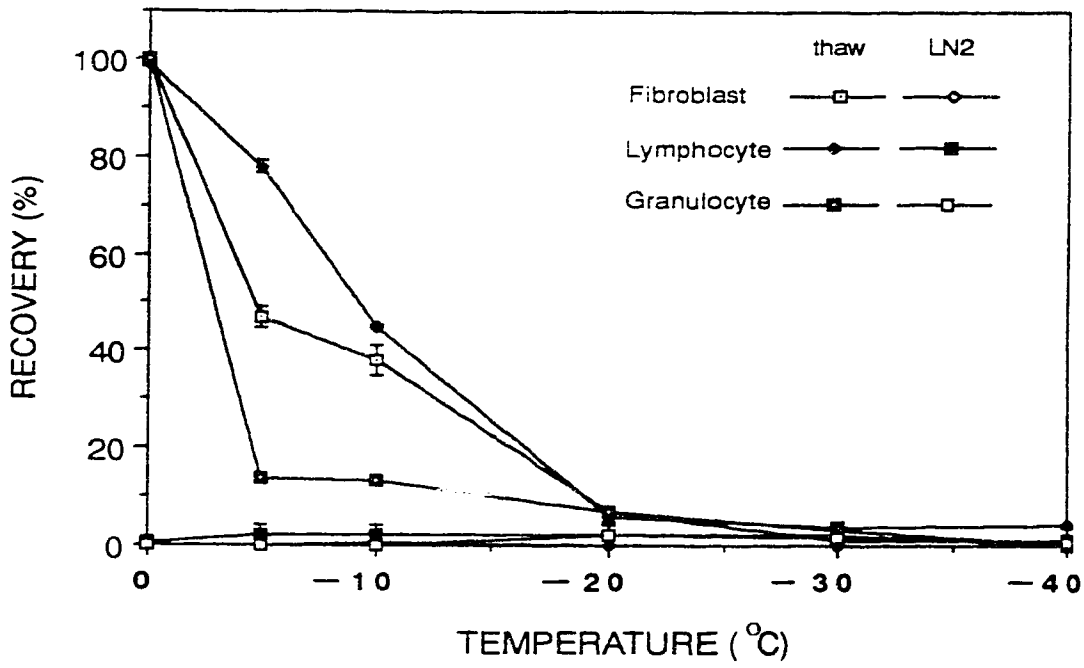


Figure 4.1b. Recoveries of fibroblasts, lymphocytes, and granulocytes after freezing and thawing. The cells were cooled at 1°C/min to various subzero temperatures and then either thawed by warming at 300°C/min (thaw) or cooled at 600°C/min to -196°C before warming (LN_2). Cell recovery was assessed after thawing using MTT. Values shown are means with vertical bars equal to $\pm$SEM.

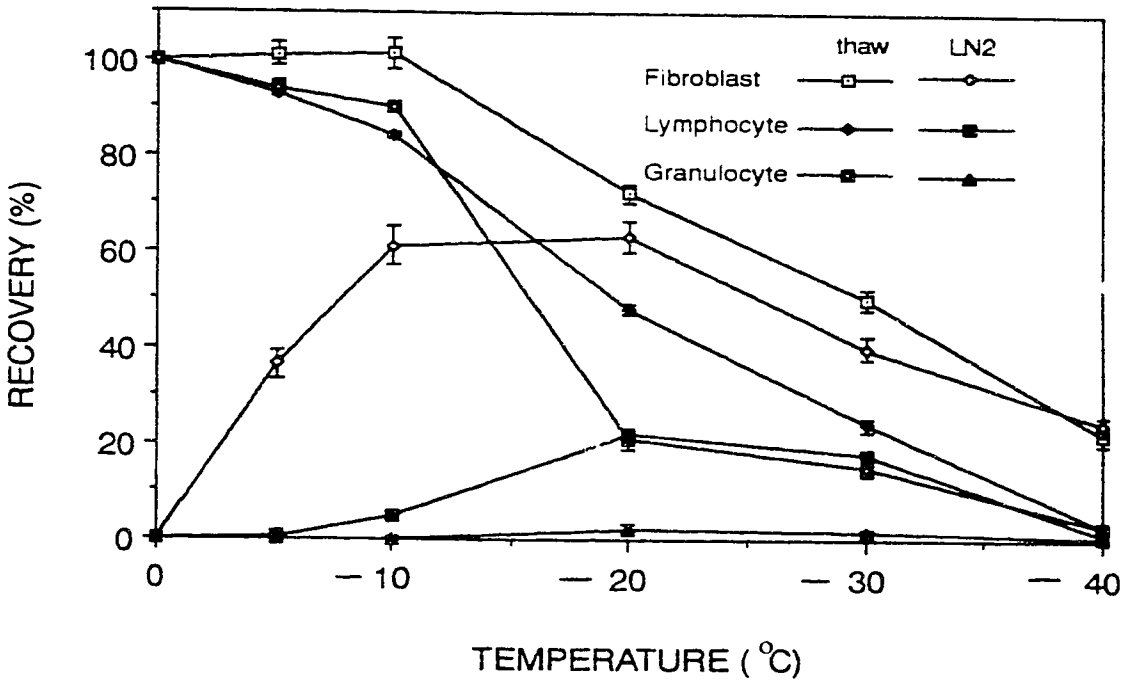
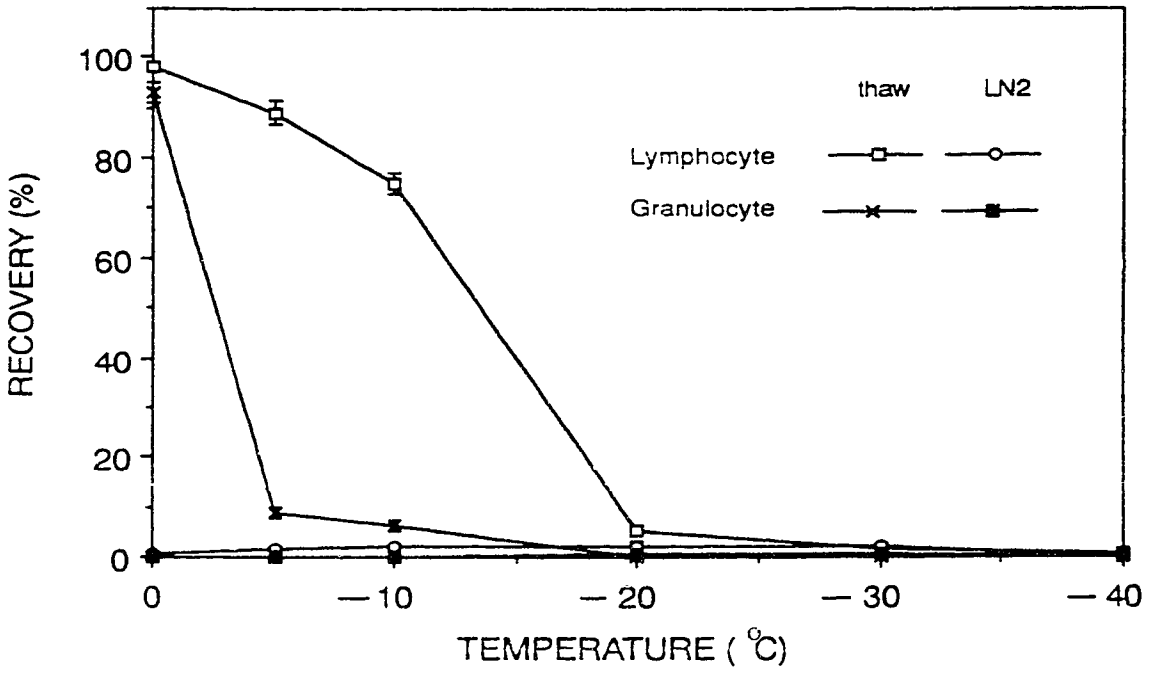


Figure 4.2a. Recoveries of fibroblasts, lymphocytes, and granulocytes after freezing and thawing. The cells were cooled at 1°C/min to various subzero temperatures and then either thawed by warming at 300°C/min (thaw) or cooled at 600°C/min before warming (LN_2). Cell recovery was assessed using FDA immediately after thawing. Values shown are means with vertical bars equal to $\pm$SEM.



Fig[illegible].2b. Recoveries of lymphocytes, and granulocytes after freezing a[illegible]awing. The cells were cooled at 1°C/min to various subzero temperatures and then either thawed by warming at 300°C/min (thaw) or cooled at 600°C/min before warming (LN_2). Cell recovery was assessed using FDA after incubation for 2 h at 37°C. Values shown are means with vertical bars equal to ±SEM.

CHAPTER V

CRYOINJURY IN HUMAN GRANULOCYTES AND GRANULOCYTE CYTOPLASTS

5.1 Introduction

The plasma membrane of human granulocytes is not the primary site of freezing injury but the secondary site because, after thawing, the intact cells were increasingly damaged during 2 h incubation at 37°C. However, it is unknown what causes the damage during the incubation. Rowe (56) and Persidsky (47) proposed that lysosomal activity plays an important part in freezing injury to human granulocytes. It was subsequently suggested that hydrolytic enzymes released from damaged lysosomes subsequently damage the plasma membrane during incubation (38) but this hypothesis lacks direct experimental support.

This study was designed to examine the role of the plasma membrane and organelles in cryoinjury by comparing the low temperature responses of human granulocytes with that of granulocyte cytoplasts. The cytoplasts are vesicles created from granulocytes by depletion of organelles, including lysosomes and nuclei (47, 53, 65), and experiments have shown that the rates of oxygen consumption, hydrogen peroxide generation, cytochrome C reduction, and phagocytosis and killing of bacteria in cytoplasts was comparable to that of granulocytes (53). The retention of these properties indicates the presence of a functional cytoskeleton. Since the cytoplasts share the plasma membrane from

the parent granulocytes, yet contain no lysosomes, cytoplasts provide an excellent model to evaluate the relative importance of the plasma membrane and the lysosomes in cryoinjury to human granulocytes.

5.2 **Experimental protocol**

No cryoprotective compounds were used in these studies. Granulocytes and cytoplasts were suspended in MEM with 0.15% (v/v) BSA. Aliquots (0.2 ml) placed in glass tubes (5x50mm) were cooled and warmed according to the graded freeze-thaw protocol (details in Chapter III). Samples were assessed using FDA immediately after thawing or after 2 h incubation at 37°C.

5.3 **Results**

The scanning electron microscope micrographs of granulocytes and cytoplasts (Fig. 5.1.) show differences in size, but similarities in the appearance of the plasma membrane. Diameters of 9 to 10 μm for granulocytes and 6 μm for cytoplasts were calculated from the scanning electron microscope micrographs, and these correspond well with the values reported by Roos *et al* (53), and with our measurements of cell volume using the electronic particle counter (see Chapter III). Volume distributions for cytoplasts and granulocytes are shown in Fig. 5.2 and indicate pure populations of both particles.

Volume changes due to osmotic shrinkage of granulocytes and cytoplasts in hypertonic solutions are shown in Fig. 5.3. Cell volume was determined by the osmolality of the suspending solution and the osmotically inactive volume of the cell. When the osmotically inactive volume is plotted as a function of the inverse osmotic pressure (a Boyle - van't Hoff plot) as in Fig. 5.4, the cytoplasts

are seen to respond as osmometers in a manner similar to granulocytes. This indicates that the resealing of the plasma membrane during preparation of cytoplasts is sufficient to support normal osmometric behavior. Extrapolation of the Boyle-van't Hoff plots of granulocyte or cytoplast volume to infinite osmotic pressure gives an estimate of the osmotically-inactive volume as shown in Fig. 5.4. A student's t-test indicates no significant difference between the relative osmotically-inactive volumes (V_d) of granulocytes and cytoplasts ($P > 0.05$).

Analysis of the kinetics of osmotic shrinkage in hypertonic solutions (Fig. 5.3.) allow the determination of the hydraulic conductivity (Lp) of granulocytes and cytoplasts as listed in Table 5.1, and provide an additional estimate of the osmotically-inactive volumes. No significant difference in Lp for granulocytes and cytoplasts was observed ($P > 0.05$). The similarities in osmometric behavior and hydraulic conductivity of the plasma membrane in cytoplasts and granulocytes indicate that the plasma membrane of the cytoplast was not significantly altered by the preparation procedures, and conserves the properties of the parent granulocytes after resealing.

When assessed immediately after thawing, granulocytes cooled at 1°C/min became increasingly damaged at lower temperatures (Fig. 5.5a.), similar to observations in Chapter IV. Cytoplasts under similar experimental conditions showed higher recoveries at lower temperatures than granulocytes. The temperature for 50% recovery was -25°C for cytoplasts and -15°C for granulocytes (Fig. 5.5b). For cells cooled rapidly from the intermediate temperature to - 196°C before thawing, recovery was low for granulocytes, but

peaked at an intermediate temperature of -15°C. Maximum recovery for cytoplasts thawed from -196°C was observed at an intermediate temperature of -20°C. This is the same range of temperature for maximum recovery reported for human lymphocytes and hamster fibroblasts (38) in the absence of cryoprotectant.

After a 2 h post-thaw period of incubation at 37°C, granulocyte recovery was virtually eliminated (Fig. 5.5), whereas the period of incubation had little effect on the recovery of cytoplasts (Fig. 5.5b). Under these conditions, the temperature for 50% recovery after immediate thawing remained at -25°C for cytoplasts, but was -4°C for granulocytes. The responses of cytoplasts are very similar to that of human lymphocytes and hamster fibroblasts (38).

5.4 **Discussion**

Cytoplasts are smaller in size than granulocytes, about 20% of the volume and 40% of the surface area, owing to the removal of nuclei, lysosomes, and other organelles. Comparable osmometric responses of granulocytes and cytoplasts, as indicated by the equilibrium Boyle-van't Hoff plots and kinetic measurements of the hydraulic conductivity, illustrate the integrity of the plasma membrane in cytoplasts, evidence of a remarkable ability of the plasma membrane to reseal during preparation of the cytoplasts and to recover the semipermeable properties of the parent granulocytes. The cytoplasts, devoid of nuclei, lysosomes, and other organelles, respond with an osmotically-inactive volume similar to that of intact granulocytes, indicating that the nuclei and other organelles respond osmotically in a similar manner to the whole granulocytes.

The observation that the hydraulic conductivity of cytoplasts is similar to that of granulocytes indicates that the hydraulic conductivity for the membrane-bound organelles are equal or higher than that of the plasma membrane, otherwise these would become rate-limiting during osmotic shrinkage, leading to an effective value of Lp lower for granulocytes than for cytoplasts.

Cytoplasts also retain the ability of granulocytes to hydrolyze FDA, allowing the commonly-used FDA assay for integrity of the plasma membrane to be used with cytoplasts. The esterase enzymes necessary to hydrolyze FDA are therefore present in the cytoplasts, demonstrating that the cytosol retains functional properties of the parent granulocyte. These results, along with the observations of phagocytic activity in cytoplasts, validate the use of cytoplasts as an experimental model system for assessment of the effects of freezing and thawing on the membrane and cytoplasm of human granulocytes.

During slow cooling of a cell suspension water is removed from the extracellular solution in the form of ice, thereby concentrating the solutes in the remaining liquid phase. At the low rate of cooling in the experiments reported here (1°C/min) cells respond to the increasing extracellular osmolality by an osmotically-driven efflux of water. Because of the similarities in the osmotic properties, similar responses in granulocytes and cytoplasts after the graded freezing procedures would be expected if the plasma membrane was the primary site of injury. The responses, however, are distinctly different. After slow cooling in the absence of cryoprotectant and thawing directly from high subzero temperatures, the recovery of granulocytes (Fig. 5.5a) and cytoplasts (Fig. 5.5b) fell to 50% at -15°C and -25°C, respectively, when assessed

immediately after thawing. Under these conditions, both granulocytes and cytoplasts will have lost most osmotically-active water, indicating that the plasma membrane can tolerate significant increases in solute concentration and severe cell shrinkage. It has been shown that human granulocytes are damaged by osmotic stresses when osmolality is greater then 600 mosmol/kg (3, 13), but these results suggest that damage to the plasma membrane is not the primary cause of sensitivity to osmotic stress, and the organelles in human granulocytes are the primary targets of both osmotic stress and freeze-thaw injury.

The recovery of cytoplasts after cooling to -196°C was similar to that reported for human lymphocytes and hamster fibroblasts in Chapter IV, and much higher than that of granulocytes. These observations indicate that the sensitivity to freeze-thaw injury in human granulocytes is related to the presence of granules and other organelles, not to the sensitivity of the plasma membrane to osmotic stresses. The temperature at which maximum recovery occurs in cytoplasts correlates well with the extracellular conditions required for sufficient dehydration of cells to avoid intracellular freezing and to minimize the effects of concentrated solutes as described by McGann (38).

A two h post-thaw incubation at 37°C had no effect on the recovery of cytoplasts, whereas the recovery of granulocytes was significantly and adversely affected. For granulocytes thawed directly from high sub-zero temperatures, the temperature for 50% recovery changed from -15°C to higher than -5°C, indicating that injury sustained during freezing at high subzero temperatures manifested as damage to the plasma membrane during post thaw incubation, similar to the observation of Armitage and Mazur (3). Cytoplasts showed no such effect of post-thaw incubation. Hydrolytic enzymes are released from the

lysosomes to the cytosol after exposure of granulocytes to a stress, and the action of these enzymes becomes evident only after incubation at 37°C (Fig. 5. 5a) as a deleterious effect exerted on the plasma membrane.

In conclusion, cytoplasts are an excellent model system for the study of freeze-thaw injury to the plasma membrane, particularly when used in comparison with intact granulocytes. This approach has been used here to show that the plasma membrane is not the source of sensitivity to freeze-thaw injury consistently observed in human granulocytes. It therefore appears that lysosomes in granulocytes can be activated to release hydrolytic enzymes after a mild osmotic exposure which does not directly damage the plasma membrane integrity. These observations support the hypothesis that organelles, particularly the lysosomes, are the source of sensitivity to cryopreservation in these cells (47). If lysosomes are indeed a primary site of injury, this knowledge should help determine the approach taken in the cryopreservation of cells, such as platelets, where lysosomes are abundant (3, 25). This study therefore has significance in approaches to the cryopreservation of cells with increased sensitivity to the condition s encountered during freezing and thawing.

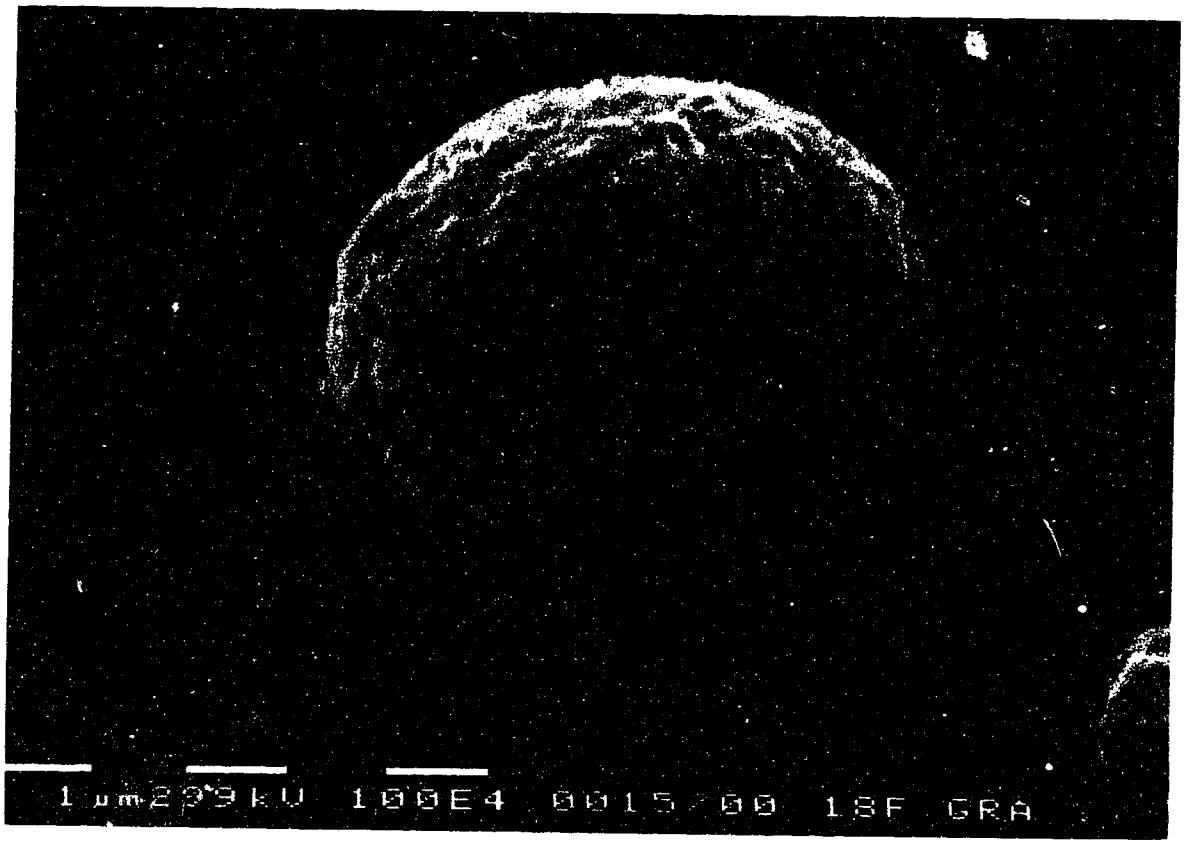


Figure 5.1a. Scanning electron photomicrographs of a granulocyte at a magnification of 10^4. Each scale bar represents 1μ.

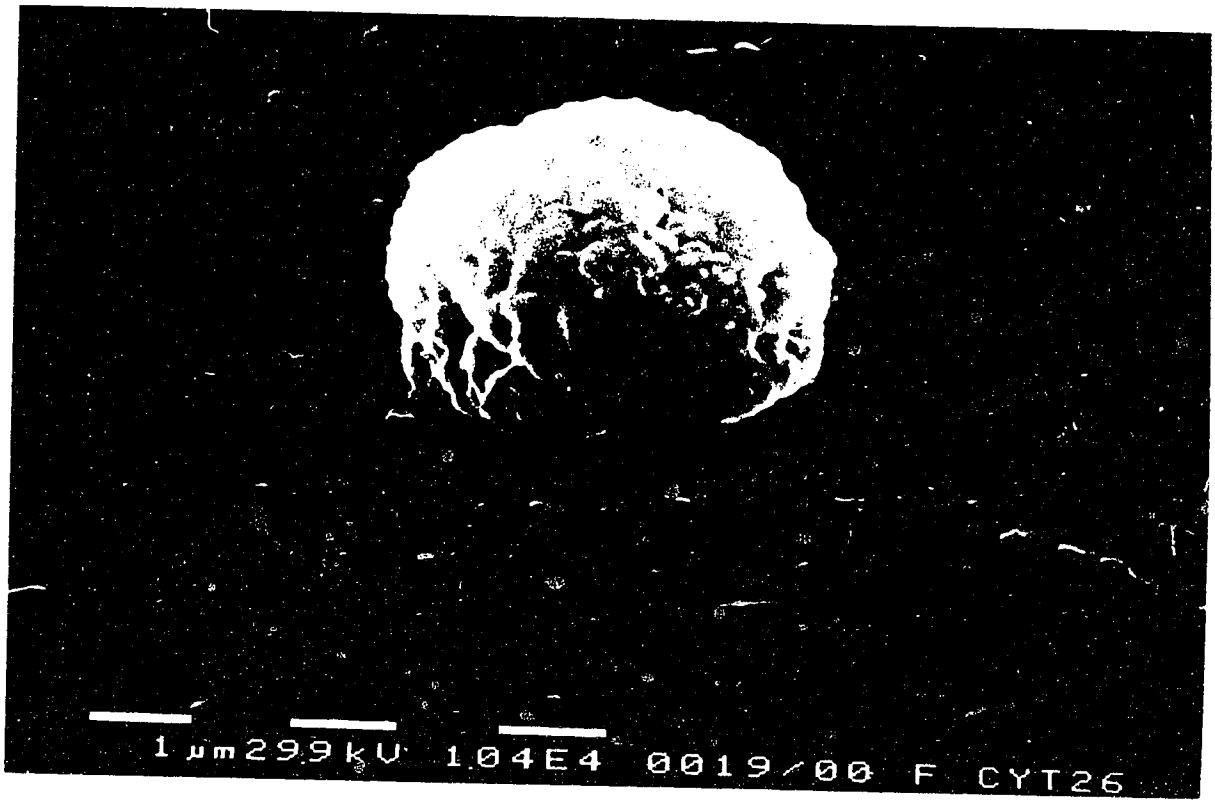


Figure 5.1b. Scanning electron photomicrographs of a cytoplast at a magnification of 10^4. Each scale bar represents 1 μ.

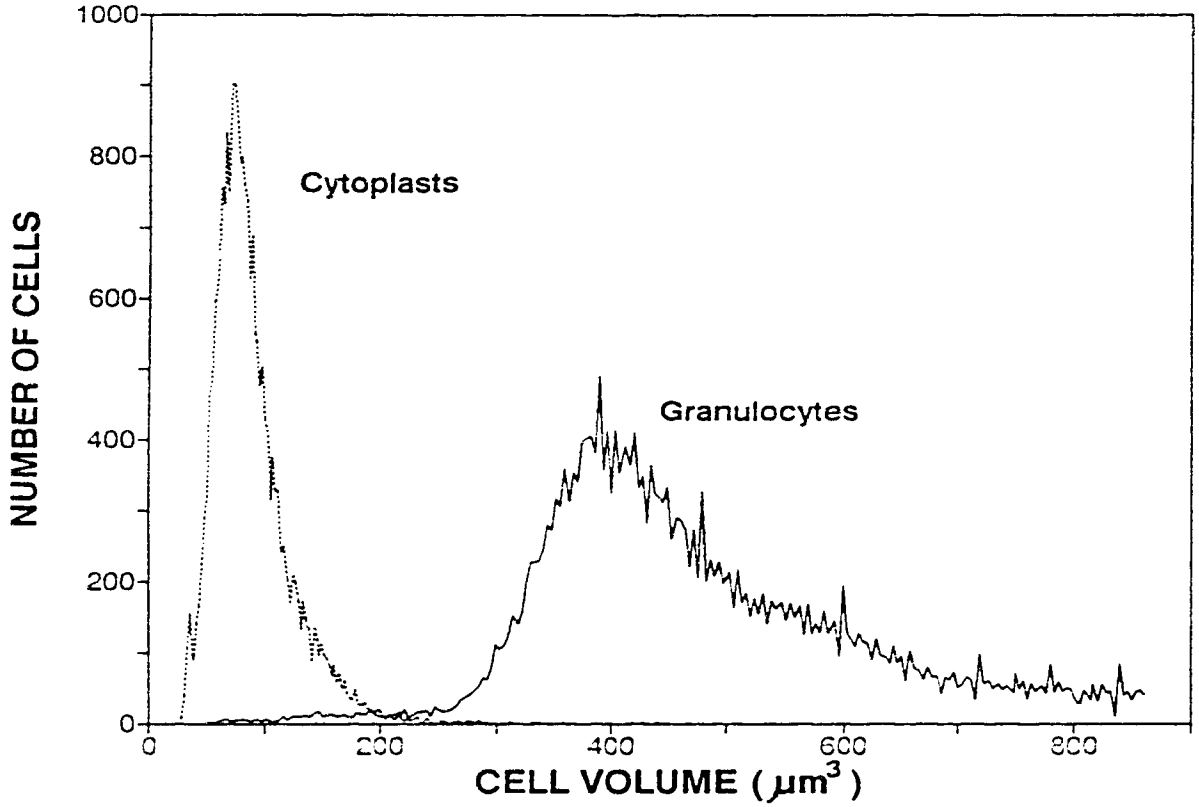


Figure 5.2. Volume distributions of granulocytes and granulocyte cytoplasts measured using an electronic particle counter. The distributions show that pure populations of cytoplasts are produced by the isolation procedure.

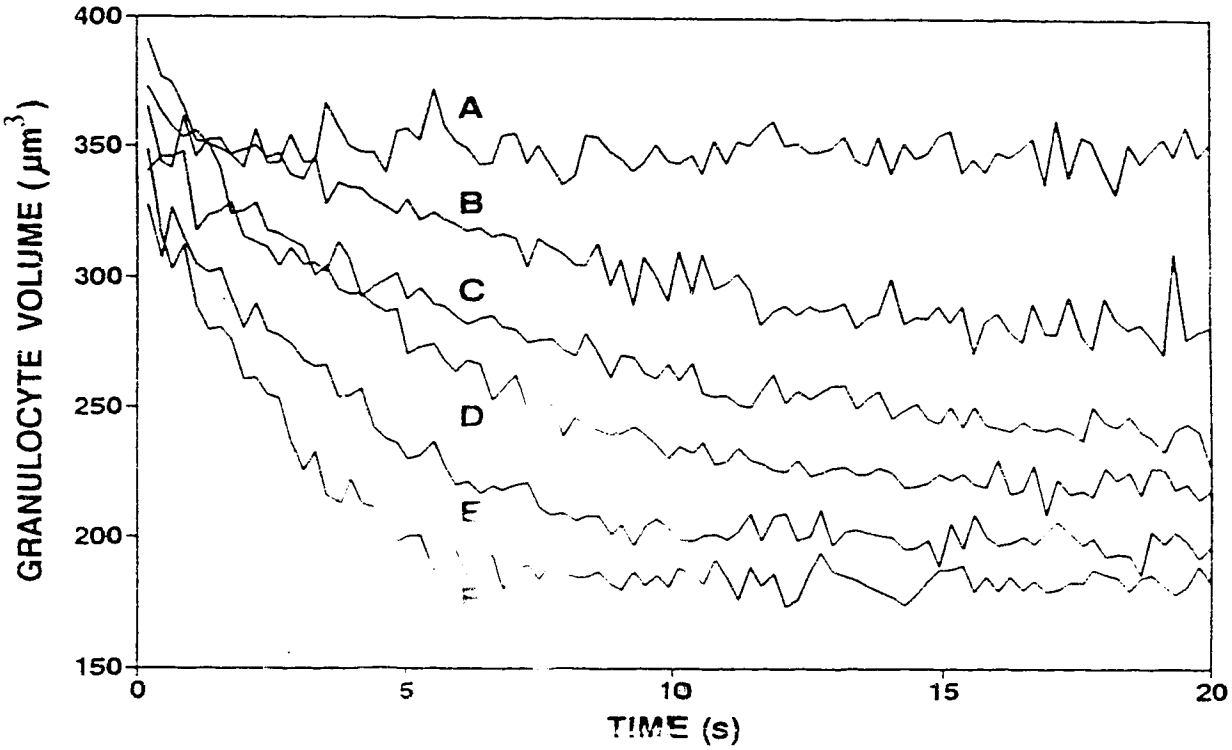


Figure 5.3a. Kinetics of volume changes in granulocytes exposed to hypertonic solutions of impermeant solutes (A, 0.3 osmol/kg; B, 0.58 osmol/kg; C, 0.89 osmol/kg; D, 1.16 osmol/kg; E, 1.4 osmol/kg; F, 2.0 osmol/kg). The cell volumes were monitored as a function of time on an electronic particle counter.

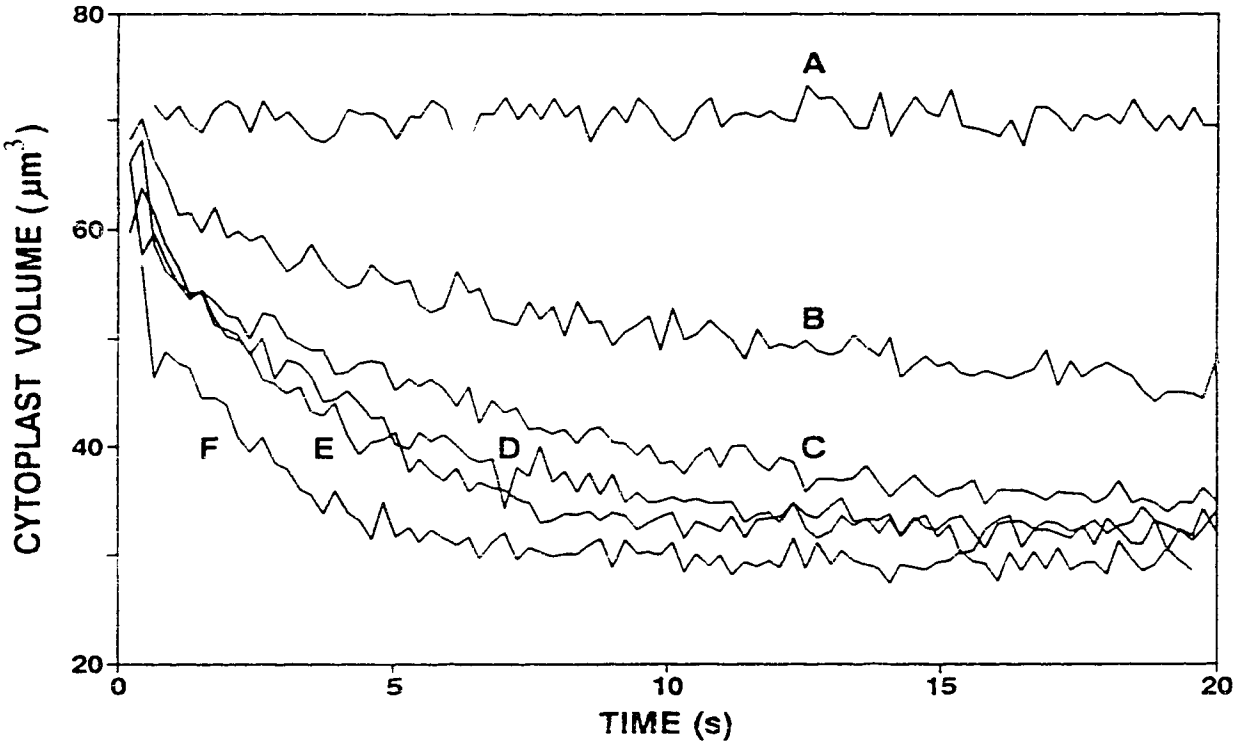


Figure 5.3b. Kinetics of volume changes in cytoplasts exposed to hypertonic solutions of impermeant solutes (A, 0.3 osmol/kg; B, 0.58 osmol/kg; C, 0.89 osmol/kg; D, 1.16 osmol/kg; E, 1.4 osmol/kg; F, 2.0 osmol/kg). The cell volumes were monitored as a function of time on an electronic particle counter.

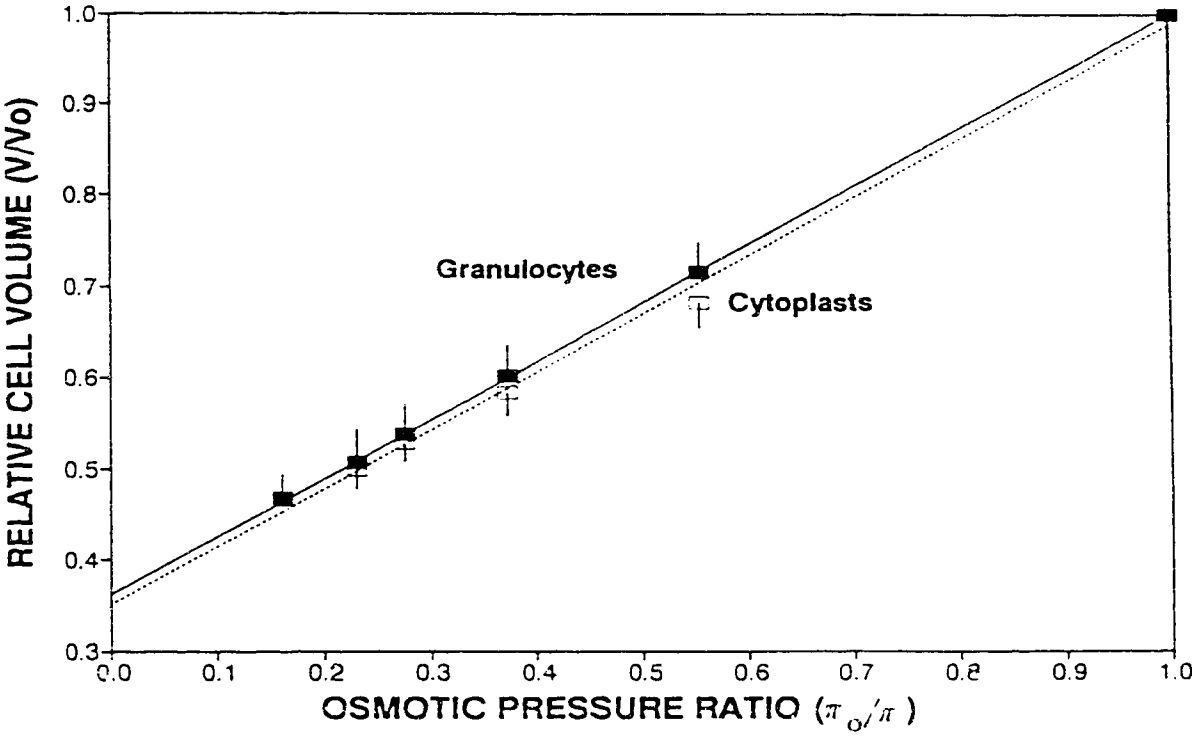


Figure 5.4. Boyle - van't Hoff plots of equilibrium cell volume as a function of inverse external osmotic pressure for granulocytes and cytoplasts. Cell volumes were measured using an electronic particle counter interfaced to a microcomputer. The equilibrium cell volumes (V) and external osmotic pressures (π) are expressed as ratios of the isotonic cell volume (V_o) and isotonic osmotic pressure (π_o). Values shown are means with vertical bars equal to $\pm$SEM.

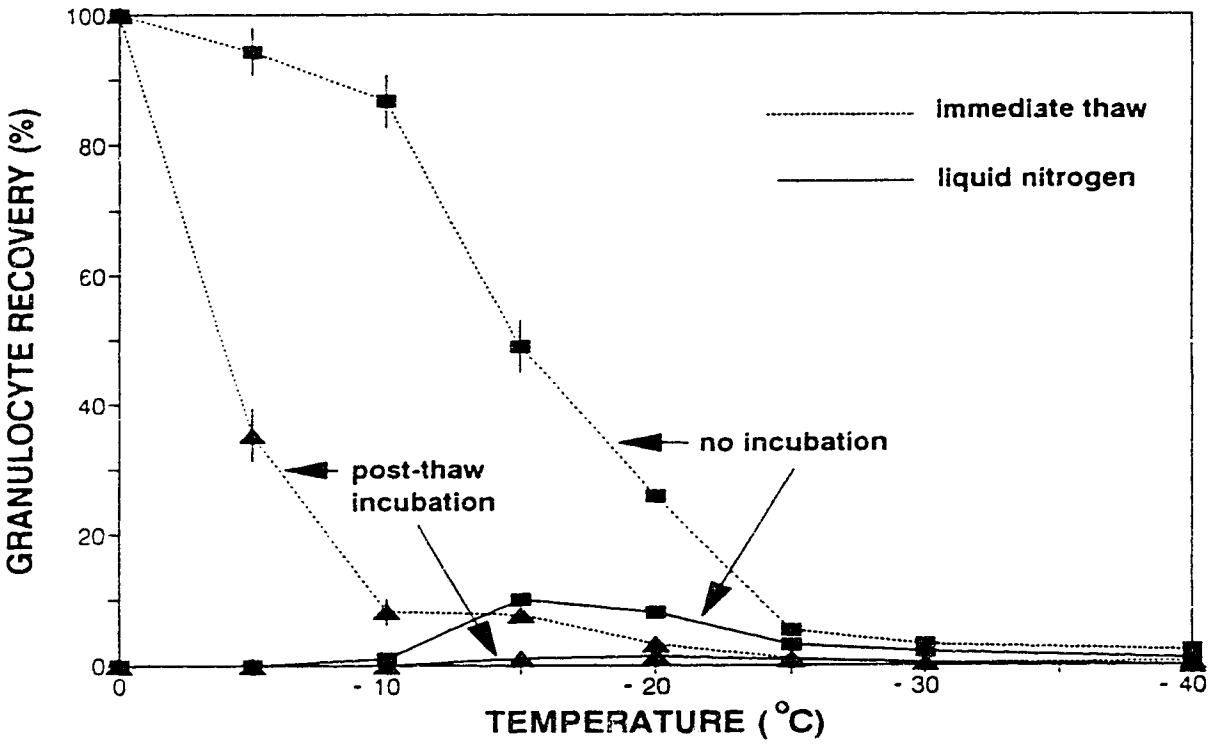


Figure 5.5a. Responses of granulocytes to freezing and thawing. The cells were cooled at 1°C/min to various subzero temperatures and then either thawed by warming at 300°C/min (dotted line) or cooled to -196°C before warming (solid line). Recoveries of granulocytes were assessed using fluorescein diacetate (FDA) and ethidium bromide (EB) either immediately after thawing or after 2 h incubation at 37°C. Values shown are means with vertical bars equal to ±SEM.

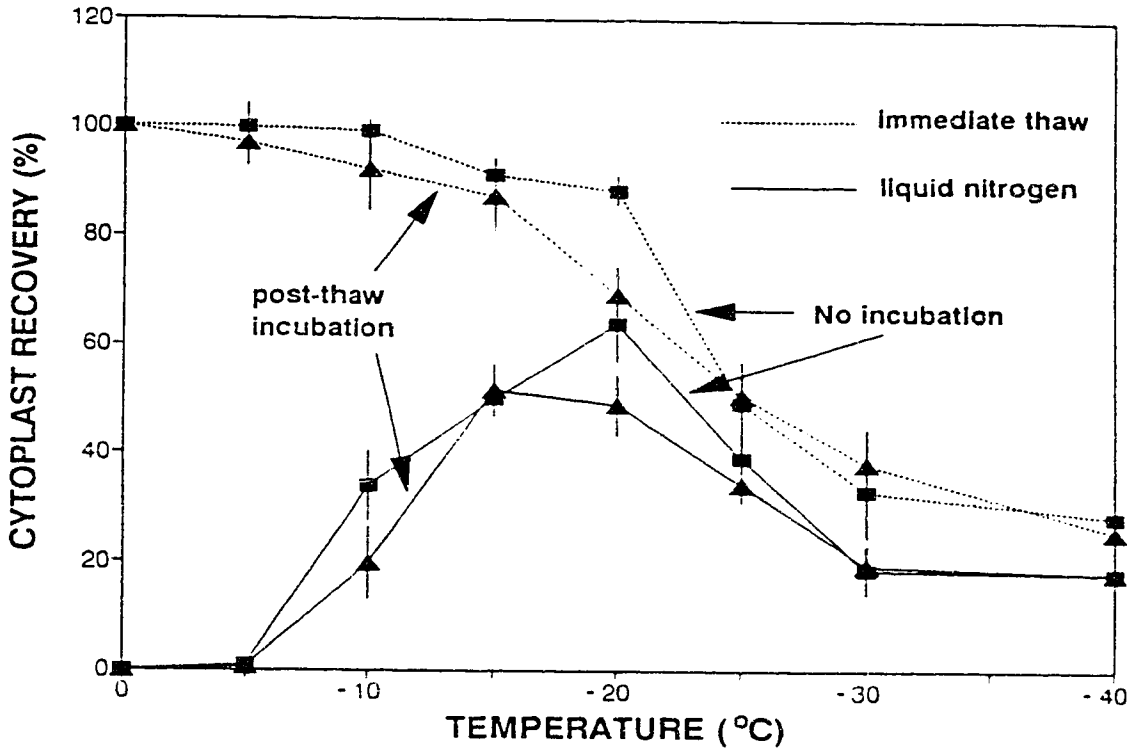


Figure 5.5b. Responses of cytoplasts to freezing and thawing. The cells were cooled at 1°C/min to various subzero temperatures, and then either thawed by warming at 300°C/min (dotted line) or cooled to -196°C before warming (solid line). Recoveries of cytoplasts were assessed using fluorescein diacetate (FDA) either immediately after thawing (squares) or after 2 h incubation at 37°C (triangles). Values shown are means with vertical bars equal to $\pm$SEM.

Table 5.1. Osmotic properties of human granulocytes and cytoplasts

	Granulocytes	Cytoplasts
Isotonic cell volume (μm^3)		
Scanning electron microscopy	382 - 524	113
Electronic counter	448 $\pm$ 31.8	71 $\pm$ 6.32
Osmotically-inactive volume (% of isotonic volume)	36.2 $\pm$ 0.13	35.1 $\pm$ 1.5
Hydraulic conductivity ($\mu m^3/\mu m^2/min/atm$)	0.195 $\pm$ 0.024	0.149 $\pm$ 0.018

CHAPTER VI

RESPONSES OF HUMAN GRANULOCYTES TO HIGH CONCENTRATIONS OF PROPYLENE GLYCOL

6.1 Introduction

It is generally considered that only a few types of nucleated mammalian cells can survive freezing without cryoprotectants because of solution effects, which are lethal to cells during slow freezing (34). The concentration of a cryoprotective additive must be sufficiently high to reduce the solution effects. However, high concentrations of additives normally induce osmotic stress and chemical toxicity. Some types of cells and tissues, for example human granulocytes, are sensitive to osmotic stress and chemical toxicity encountered during addition and dilution of cryoprotectants, and therefore cannot be cryopreserved. To successfully cryopreserve cells, osmotic stress and chemical toxicity must be minimized to an acceptable level. It is generally believed by cryobiologists that osmotic stress is reduced by slow addition and dilution of cryoprotectants and that chemical toxicity is minimized by at lower temperatures. Many types of cells and tissues have been successfully cryopreserved using this technique but cryopreservation of some kinds of cells, for instance, human granulocytes, has not been achieved. This implies that each type of cell has its optimal conditions of cryopreservation.

Human granulocytes maintained better function at 20-22°C than 1-6°C (35) and tolerated higher osmotic stress produced by addition and dilution of

impermeable solutes at 22°C than at 0°C (3). Therefore, some questions were raised whether human granulocytes could tolerate higher osmotic stress induced by addition and dilution of a permeable solute and chemical toxicity of cryoprotectants at 22°C, and what were the other critical factors affecting granulocyte survival during the procedure of cryopreservation.

This study was designed to investigate the factors affecting cell survival during the procedure of cryopreservation. The factors included rates of addition and dilution of different concentrations of propylene glycol (PG) at different temperatures. Human granulocytes were used as a model in the study because of their sensitivity to osmotic stress and chemical toxicity, which will help in the cryopreservation of other type sensitive cells or tissues. PG, a polyalcohol with low toxicity and high permeability across the plasma membrane, was selected for this study because it is used in conventional cryopreservation of living cells, as well as an important component of vitrification solutions. Solutions containing PG have high glass forming tendency and high stability in the amorphous state (8).

6.2 **Experimental protocol**

Permeability of granulocytes to PG at different temperatures was determined from measurements of cell volume (see Chapter III). Different concentrations of propylene glycol solutions were added and diluted at different rates and at different temperatures.

The protocol of addition and dilution of propylene glycol was shown in Fig. 6.1. Equal volumes of 4, 8 and 12 *M* PG solutions were added to 1 ml cell suspensions to get final PG concentrations of 2, 4 and 6 *M* at 22°C and 0°C.

Additions were either rapid (one step: mix cell suspension and PG solution rapidly) or slow (seven steps at 0.03, 0.05, 0.07, 0.11, 0.18, 0.29 and 0.27 ml at intervals of 5 min). Samples were either evaluated with FDA/EB (Chapter III) at 10 min intervals for 60 min or diluted with PBS/BSA in a 10 min incubation. The samples were diluted to 300-400 mosmol/kg with PBS/BSA rapidly (one step) or slowly (dropwise) at 22°C over a period of 40 min. After equilibration for 5 min, samples were centrifuged at 500*g* for 10 min. The supernatant was removed by aspiration and the cells were resuspended in 1 ml of basal medium eagle (BME, GIBCO) and tested for viability.

6.3 **Results**

The volume changes of cells exposed to 1 and 2 *M* PG at 0°C and 22°C were significantly different as shown in Fig. 6.2. The differences indicate that plasma membranes are more permeable to PG at 22°C than at 0°C, and that cells are more shrunken at high concentration than low concentration. This indicates that the osmotic stress exerted on cells during addition at high temperatures is lower than that at low temperature. When was assayed using FDA without dilution, the recovery of the cells exposed to 2 and 4 *M* PG was not decreased for either temperature up to 60 min (Fig. 6. 3). The recoveries for the cells exposed to 6 *M* PG were different from those exposed to 2 and 4 *M* PG and were dependent on the temperatures and addition rates. The cells exposed to 6 *M* PG at 22°C were extremely damaged especially by slow addition (Fig. 6.3a, 3b). The recovery of cells exposed to PG at 0°C at both addition rates showed little decrease after 30 min (Fig. 6.3c, 3d). Fig. 6.4 shows that cell

recovery measured as FDA fluorescence, immediately after removal of PG decreased with increasing concentration of PG. Granulocytes exposed to 6 *M* PG showed higher recovery at 0°C than at 22°C for both rapid dilution and slow dilution.

The recovery of cells after removal of PG was also assayed using MTT. Fig. 6.5 shows that mitochondria of granulocytes were severely damaged during addition and dilution, and the cells exposed to 6 *M* PG almost had no recovery at any treatment condition. Fig.6.6 presents the same data as a function of addition conditions in order to compare dilution rates. The recovery of cells exposed to 2 *M* PG was not dependent on dilution rates ($P > 0.05$) but significantly dependent on addition rates ($P < 0.0001$) and the addition temperature ($P < 0.0001$). For the cells exposed to 4 *M* PG, high recovery was obtained only with slow addition and slow dilution at 22°C, other treatments gave no difference in recovery ($P > 0.05$).

6.4 Discussion

The membrane of human granulocytes remained intact during exposure to 2 and 4 *M* PG in all addition conditions using the FDA assay. The integrity of the cell membrane was slightly decreased at 40 min when 6 *M* PG was added at 0°C and at 15 min when it was added rapidly at 22°C. The integrity of the plasma membrane of cells was completely lost when 6 *M* PG was added slowly at 22°C. Without dilution the FDA assay as a function of time exposure is a truly chemical toxic test because no dilution osmotic stress is involved (25). Therefore, these results indicate that the toxicity of PG is lower at 0°C than at 22°C. However, it is surprising that the recovery after rapid addition of 6 *M*

PG at 22°C was over 50% after up to a 25 min exposure to PG but there was no recovery from exposure to 6 *M* PG after slow addition at the same temperature within 10 min. Obviously rapid addition of 6 *M* PG causes much higher osmotic stress than slow addition. This result suggests that the damage was caused by the final two addition steps, from 4 *M* to 6 *M* PG, even though the osmotic stress caused by 1 *M* increments is not high enough to damage cells if the addition is started in an isotonic solution without PG. Therefore, one must consider a decrease in tolerance of cells in high concentrations of cryoprotectants to additional increments of cryoprotectants. When cells were returned to isotonic solution, and the recovery was assessed using FDA immediately after PG was removed, a high recovery of granulocytes was obtained from exposure to 2 *M* and 4 *M* PG at both temperatures. There was some recovery from exposure to 6 *M* PG at 0°C but only a few cells survived the exposure to 6 *M* PG at 22°C. However, the recoveries evaluated using MTT, were much lower than those assessed using FDA. For recovery from exposure to 4 *M* PG, only cells added and diluted slowly at 22°C gave high recovery. Granulocytes were completely damaged when exposed to 6 *M* PG at both temperatures, 22°C and 0°C.

These observations are consistent with the proposal that the cell plasma membrane was not the primary site of injury as discussed in Chapter IV and V. Fig. 6.6 shows that the survival of granulocytes exposed to 2 *M* PG was significantly dependent on the addition rate ($P < 0.0001$) and temperature ($P < 0.0001$) but not dilution rate ($P > 0.05$) so that attention must be paid to addition procedures. Cells are more permeable to PG at 22°C than 0°C, indicating that more osmotic stress was exerted on cells at 0°C than 22°C when

rapid addition rates were employed. Hence, the recovery from addition at 22°C is significantly higher than addition at 0°C ($P < 0.0001$) at 2 *M* PG. At 4 *M* PG, the osmotic stresses on rapid addition is sufficient to damage the cells at 22°C. However, osmotic stress is not the only factor affecting the recovery of granulocytes because cell damage caused by rapid addition at 22°C was the same as that by slow addition at 0°C ($P > 0.05$). Because granulocytes remain permeable to PG at 0°C, it is obvious that osmotic stress from slow addition (seven steps) at 0°C is certainly lower than that of rapid addition (one step) at 22°C. This suggests that human granulocytes are more susceptible to osmotic stress at 0°C than at 22°C.

Armitage and Mazur (3) also reported the that cells tolerated a higher osmolality of NaCl at 22°C than at 0°C. However, the mechanism of the different tolerance to osmotic stress at different temperatures is not clear. Palm *et al* (45) reported that exposure of human granulocytes to 1-6°C for longer than 24 h can cause a disappearance of microtubules and a disorganization of microfilaments and is associated with the inability of granulocytes to adhere to and spread on foreign surfaces. This is probably due to the decrease of ATP in cells stored at 1 to 6°C which means the cells can remain functional much longer at 22°C. Therefore the lower tolerance of cells to osmotic stress could be due to the cell physiological changes. This study indicates that temperature is a critical factor in the tolerance of human granulocytes to osmotic stress. Further evidence is provided in Fig. 6.6b, which shows that cells exposed to 4 *M* PG were not only sensitive to addition rate and temperature but also to dilution rate. The cells can survive in 4 *M* PG, indicated by MTT assay when slow addition and dilution were carried out at 22°C. This implies that

granulocytes can survive high concentrations of cryoprotectant if optimum conditions are met. Vitrification is a practical alternative for preserving granulocytes since they cannot tolerate slow freezing. A 6 *M* PG solution, which can be practically vitrified, was used in this experiment. Rall (50) proposed that full permeation of cyroprotectants into the cells was not necessary for survival, and furthermore may lead to chemical toxicity and osmotic injury. Partial permeation and osmotic shrinkage concentrates the endogenous cytoplasmic macromolecules and greatly increases the likelihood of intracellular vitrification. Since granulocytes cannot tolerate 6 *M* PG for 10 min, cells were exposed to 6 *M* PG for 5 min instead of 10 min. It was not possible to preserve these cells using PG as a vitrification solution but the experiment showed good prospects for developing a low toxicity vitrification solution, and for cryopreservation of granulocytes by vitrification, since the cells can survive exposure to 4 *M* PG if osmotic stress is avoided.

6.5 **Conclusions**

Human granulocytes can survive exposure and dilution from concentrations up to 4 *M* PG when osmotic stress was reduced during addition and removal of cryoprotectants. In general, addition and dilution of cryoprotectant should be carried out at 22°C rather than at 0°C because the granulocytes are more tolerant to osmotic stress at the higher temperature. Cells in an isotonic solution without PG can tolerate much higher increments in osmolality than those already in PG solution. Therefore, steps during addition must be carefully controlled. The addition rate is more important than the

dilution rate because the recovery of cells exposed to 2 *M* PG is determined by addition rather than the dilution conditions. That granulocytes survived 4 *M* PG provides a hope that a low toxicity vitrification solution may be developed by combinations of PG with other cryoprotectants and, therefore human granulocytes and other osmotically sensitive cells and tissues could be cryopreserved by vitrification.

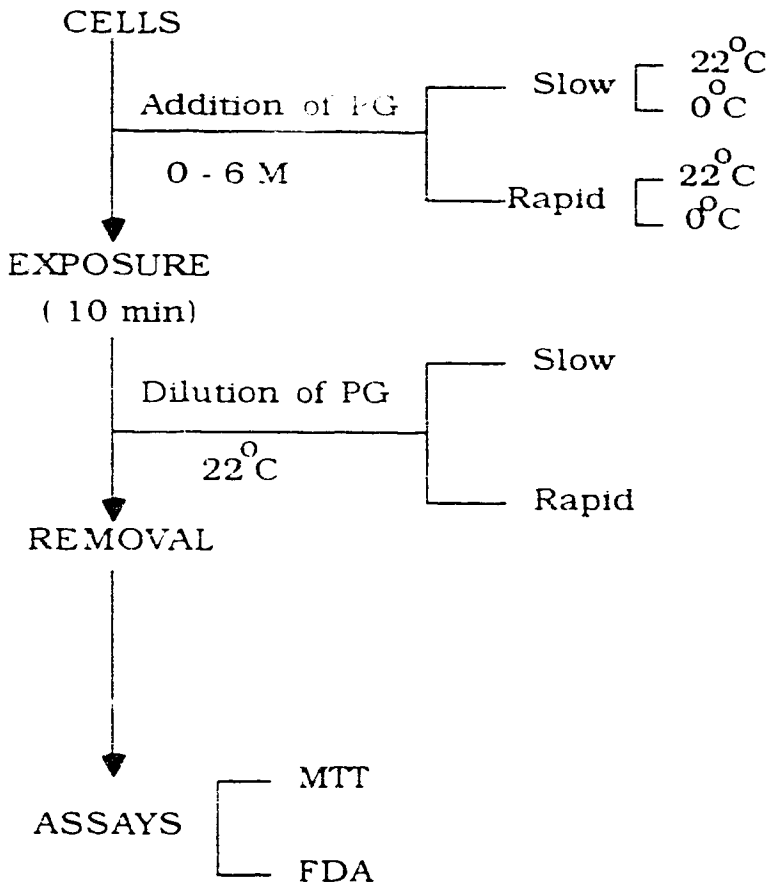


Figure 6.1 Protocol of addition and dilution of propylene glycol (PG)

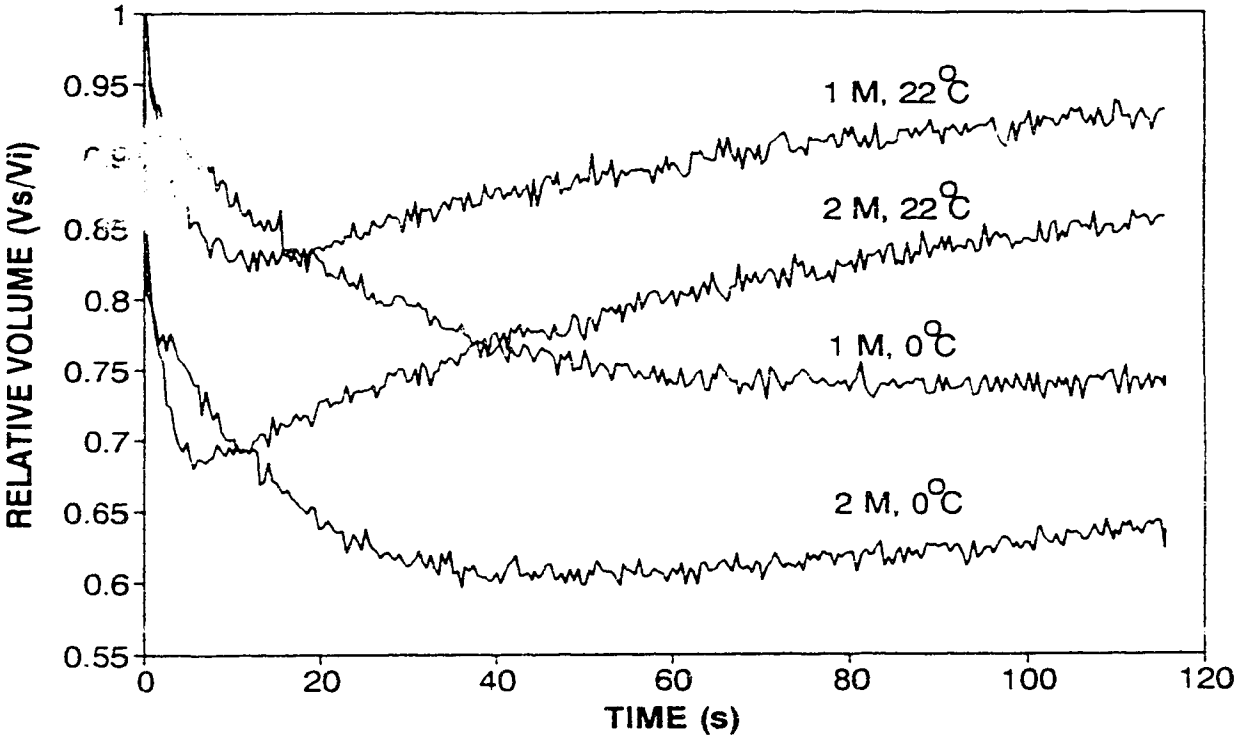


Figure 6.2. Kinetics of volume changes in granulocytes exposed to different concentrations of propylene glycol (PG) at different temperatures. The volume changes were measured as a function of time on an electronic particle counter. V_i is isotonic volume of cells, and V_s is volume of cells exposed into PG.

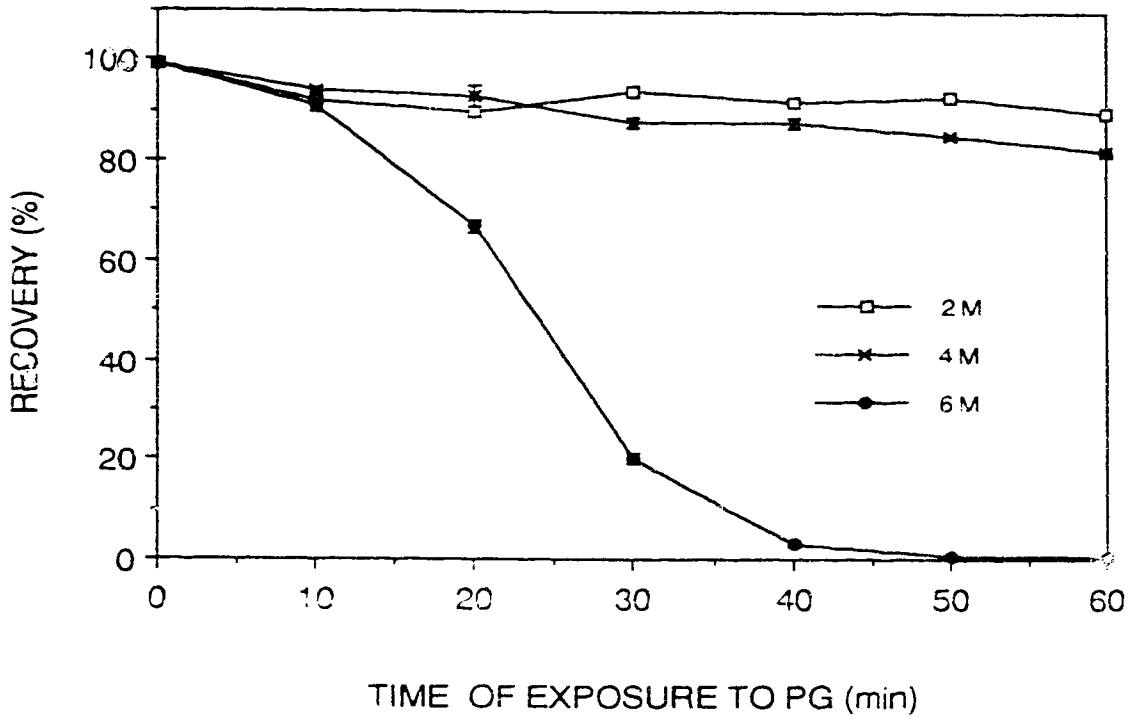


Figure 6.3a. Responses of granulocytes to toxicity of propylene glycol (PG). The cells were exposed to different concentrations of PG by rapid addition (one step) and incubated for various times at 22°C. The cell recoveries were assessed using fluorescein diacetate (FDA) without dilution of PG. Values shown are means with vertical bars equal to ±SEM.

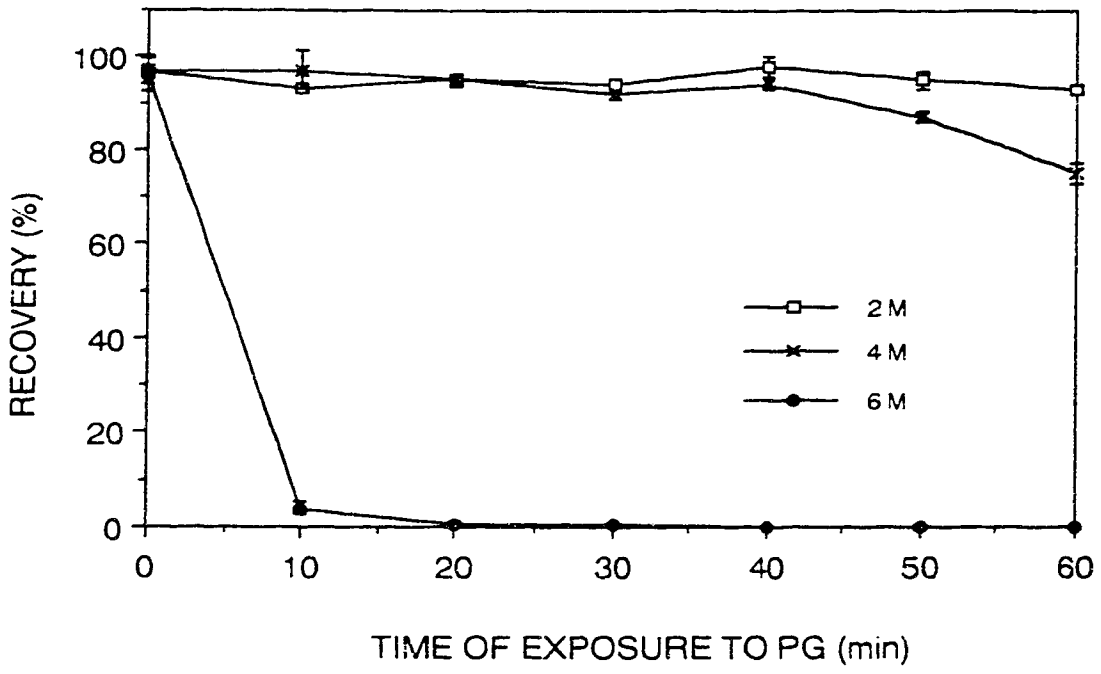


Figure 6.3b. Responses of granulocytes to toxicity of propylene glycol (PG). The cells were exposed to different concentrations of PG by slow addition (seven steps) and incubated for various times at 22°C. The cell recoveries were assessed using FDA without dilution of PG. Values shown are means with vertical bars equal to $\pm$SEM.

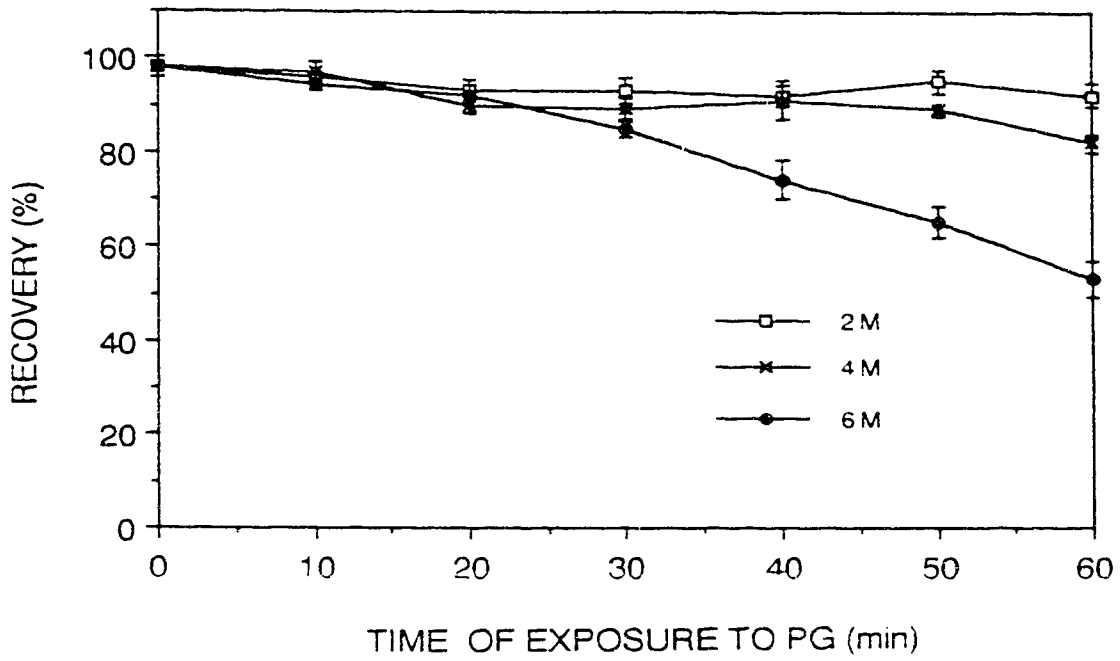


Figure 6.3c. Responses of granulocytes to toxicity of propylene glycol (PG). The cells were exposed to different concentrations of PG by rapid addition (one step) and incubated for various times at 0°C. The cell recoveries were assessed using FDA without dilution of PG. Values shown are means with vertical bars equal to ±SEM.

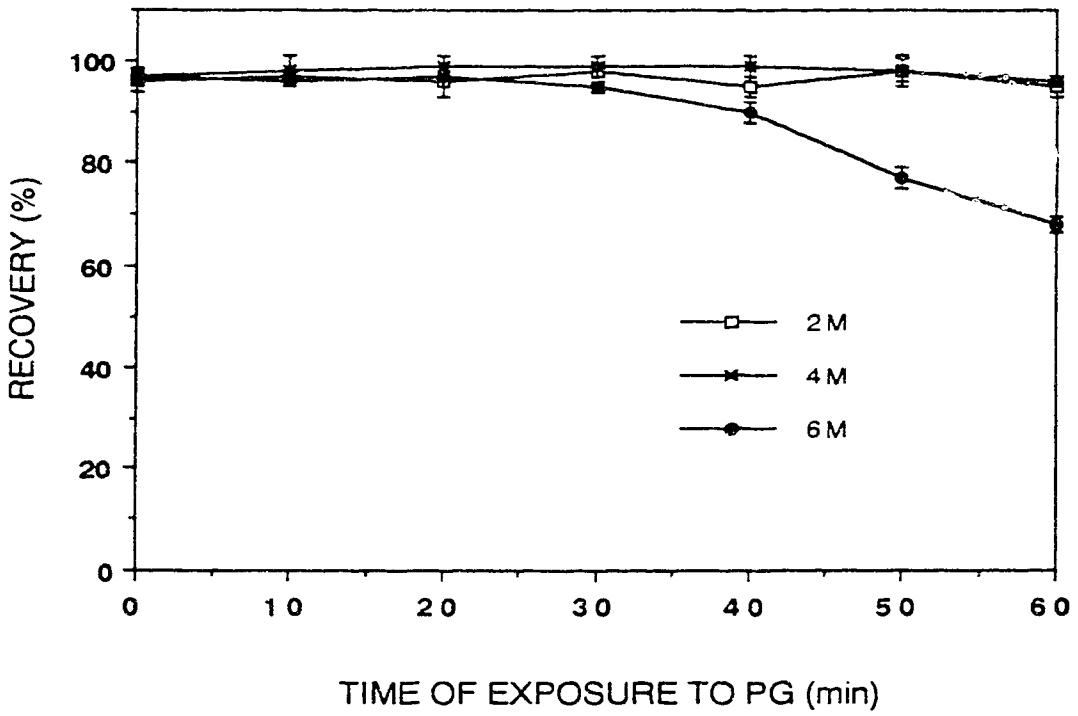


Figure 6.3d. Responses of granulocytes to toxicity of propylene glycol (PG). The cells were exposed to different concentrations of PG by slow addition (seven steps) and incubated for various times at 0°C. The cell recoveries were assessed using FDA without dilution of PG. Values shown are means with vertical bars equal to $\pm$SEM.

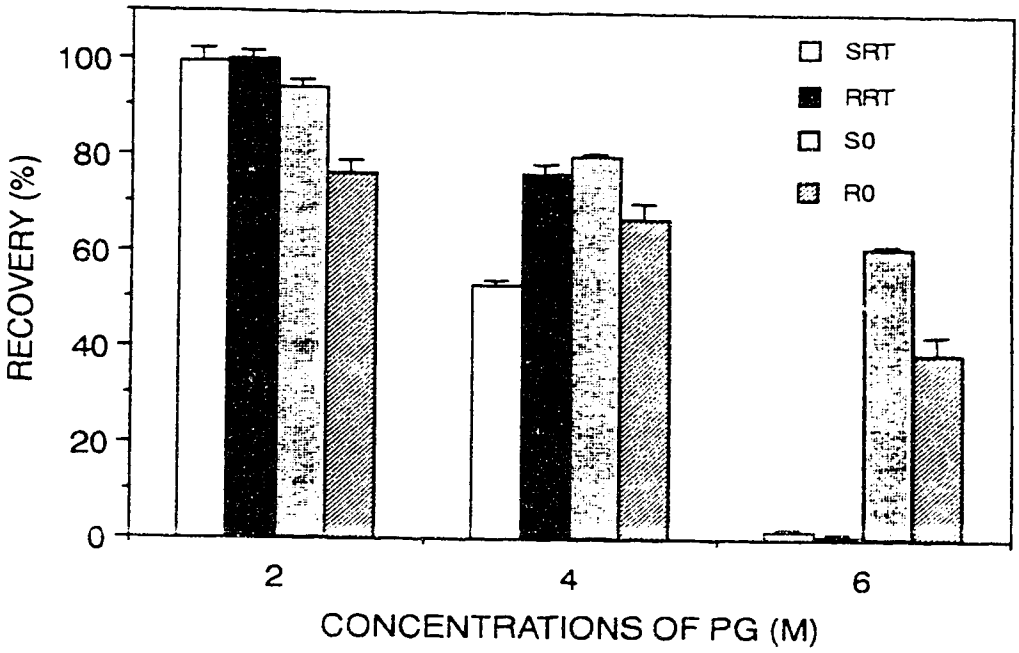


Figure 6.4a. Recoveries of plasma membrane integrity of granulocytes after rapid dilution of propylene glycol (PG). PG was added to granulocytes slowly at 22°C (SRT), rapidly at 22°C (RRT), slowly at 0°C (S0) or rapidly at 0°C (R0). The recoveries of cells were assessed using fluoresein diacetate (FDA) immediately after PG was removed. Values shown are means with vertical bars equal to $\pm$SEM.

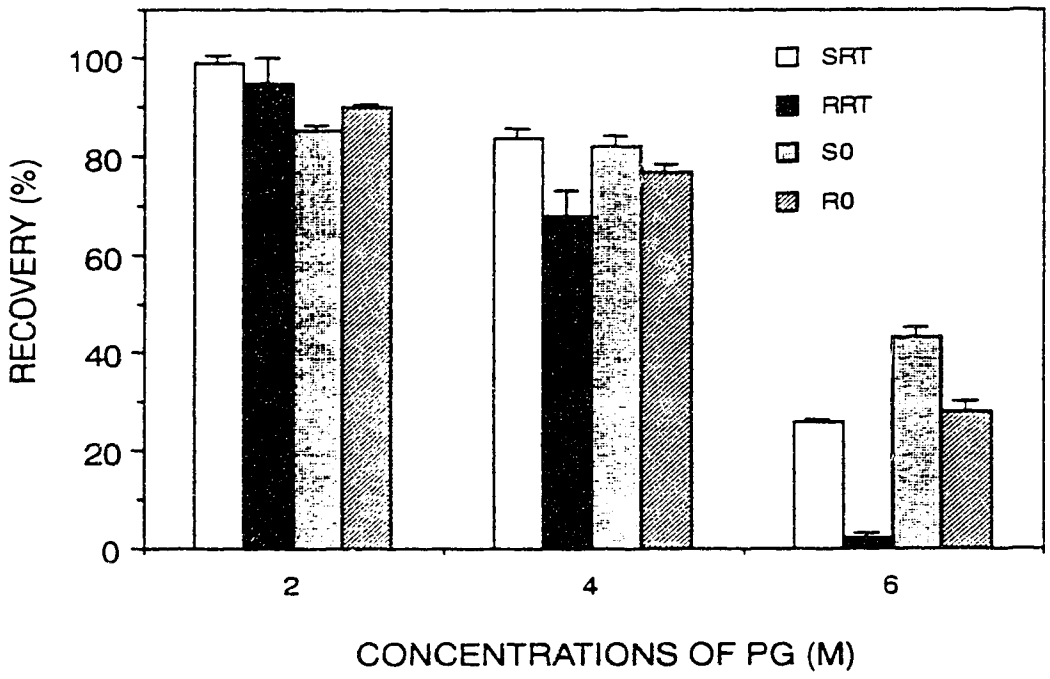


Figure 6.4b. Recoveries of plasma membrane integrity of granulocytes after slow dilution of propylene glycol (PG). Different concentrations of PG were added to granulocytes slowly at 22°C (SRT), rapidly at 22°C (RRT), slowly at 0°C (S0) or rapidly at 0°C (R0). The recoveries of cells were assessed using FDA immediately after PG was removed. Values shown are means with vertical bars equal to ±SEM.

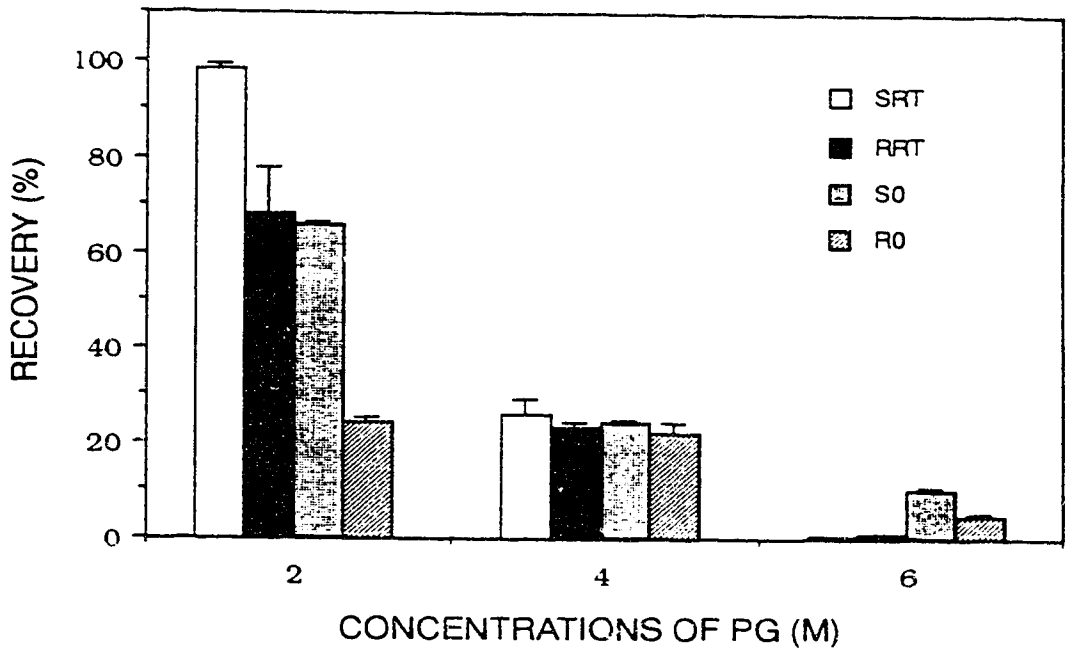


Figure 6.5a. Mitochondrial recoveries of granulocytes after rapid dilution of propylene glycol (PG). Different concentrations of PG were added to granulocytes slowly at 22°C (SRT), rapidly at 22°C (RRT), slowly at 0°C (S0), and rapidly at 0°C (R0). The recoveries of cells were assessed using MTT. Values shown are means with vertical bars equal to $\pm$SEM.

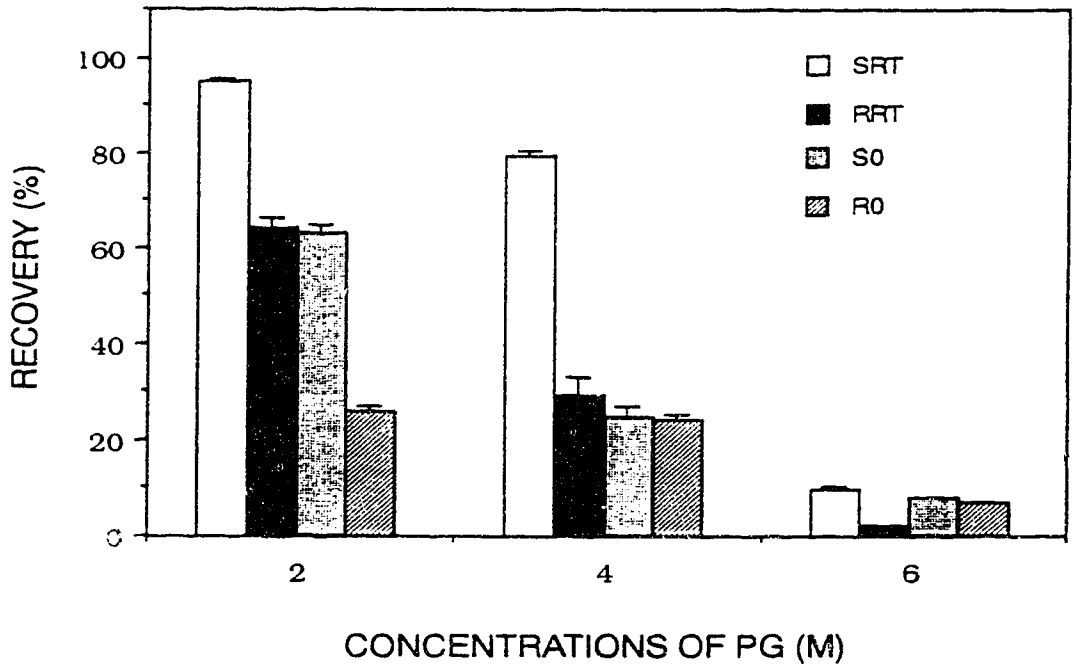


Figure 6.5b. Mitochondrial recoveries of granulocytes after slow dilution of propylene glycol (PG). Different concentrations of PG was added to granulocytes slowly at 22°C (SRT), rapidly at 22°C (RRT), slowly at 0°C (S0), and rapidly at 0°C (R0). The recoveries of cells were assessed using MTT. Values shown are means with vertical bars equal to $\pm$SEM.

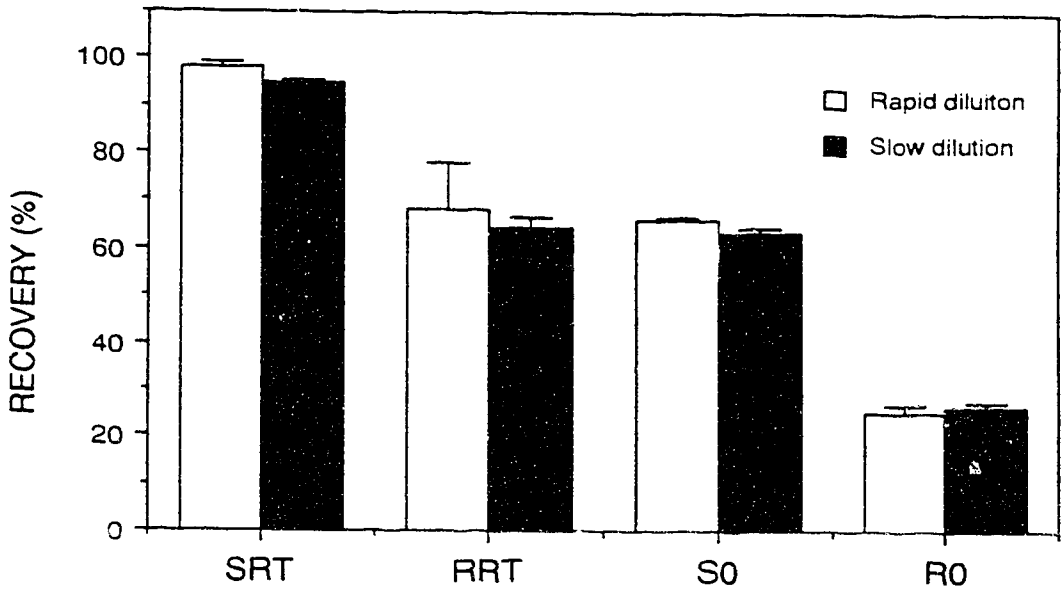


Figure 6.6a. Mitochondrial recoveries of granulocytes exposed to 2 *M* propylene glycol (PG). 2 *M* PG was added to granulocytes slowly at 22°C (SRT), rapidly at 22°C (RRT), slowly at 0°C (S0) and rapidly at 0°C (R0). PG was diluted rapidly or slowly at 22°C. The recoveries were evaluated using MTT. Values shown are means with vertical bars equal to ±SEM.

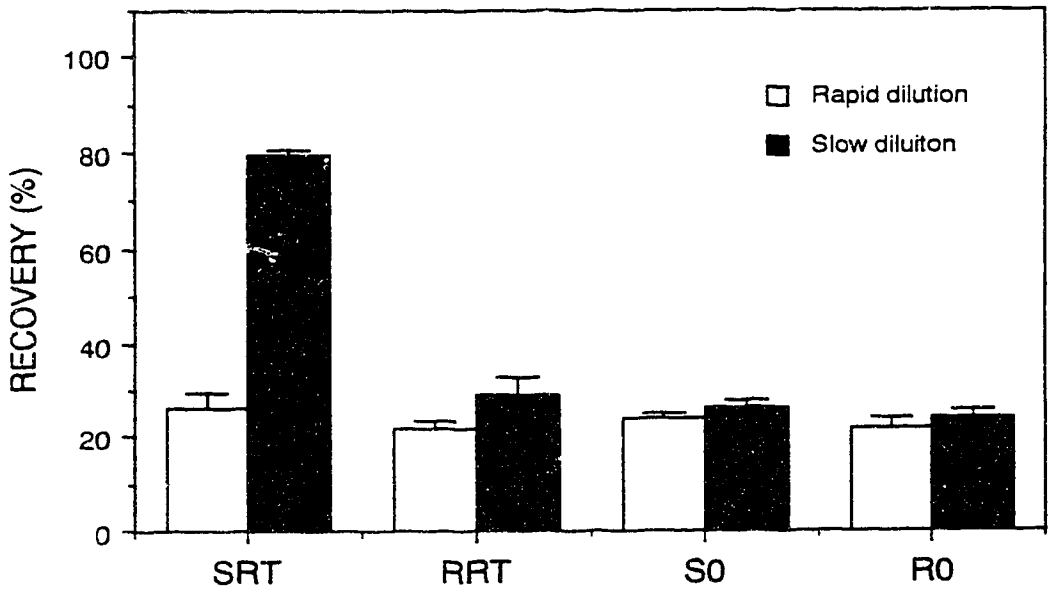


Figure 6.6b. Mitochondrial recoveries of granulocytes exposed to 4 *M* propylene glycol (PG). 4 *M* PG was added to granulocytes slowly at 22°C (SRT), rapidly at 22°C (RRT), slowly at 0°C (S0) and rapidly at 0°C (R0). PG was diluted rapidly or slowly at 22°C. The recoveries were evaluated using MTT. Values shown are means with vertical bars equal to ±SEM.

CHAPTER VII

OSMOTIC STRESS AND CHEMICAL TOXICITY IN HUMAN GRANULOCYTES EXPOSED TO VITRIFICATION SOLUTIONS

7.1 Introduction

Wide application of vitrification for cryopreservation of cells and tissues is limited because of high osmotic stress and chemical toxicity of vitrification solutions. The chemical toxicity has been thought to be the central problem blocking successful cryopreservation by vitrification (20). Although the mechanism of chemical toxicity of cryoprotectants is unknown, toxicity is generally considered to relate to the components in a solution and the total concentration of the solution (7, 21). Several types of cells and tissues have been successfully cryopreserved using a vitrification solution (50, 64) and mouse embryos were vitrified using single cryoprotectants, glycerol or propylene glycol (50). In order to apply the technique to cryopreservation of other sensitive cells and tissues, the osmotic stress and chemical toxicity must be eliminated or reduced to an acceptable level for the cells and tissues. For example, rabbit corneas tolerated 3.4 *M* PG for 20 min at 0°C (53) and human granulocytes tolerated 4 *M* PG when slow addition and dilution were employed at 22°C (67). The 4 *M* PG is not high enough to vitrify at a practical cooling rate and, therefore the combination of 4 *M* PG with other cryoprotectants has been suggested (53, 67) (see Chapter VI). Fahy reported that chemical toxicity of cryoprotectants cannot be reduced by addition of one to another (for example, toxicity of 4 *M* DMSO cannot be reduced by addition of 1 *M* acetamide to 4 *M*

DMSO solution) but could be decreased by replacement of a fraction of one cryoprotectant (for example, 1 *M* of 4 *M* DMSO is replaced by 1 *M* acetamide) (21). This implies that vitrification solutions with low toxicity could be developed by selective combination of solutes. However, Langis and Steponkus proposed that alterations in the membranes are the primary cause of injury to cells during dehydration and, therefore, toxicity of vitrification solutions largely depends on their osmotic potential (16). This argument challenges the approach of reducing chemical toxicity of vitrification solutions since the osmotic potential is determined by the total concentration. In order to apply vitrification to more types of cells and tissues, the cause of chemical toxicity must be clarified because different strategies to minimize chemical toxicity may be required. Langis and Steponkus suggested that the toxicity can be minimized by formulating solutions with an optimum osmolality (28) as opposed to the optimal mixtures of cryoprotectants (16, 17). To this end, dimethyl sulfoxide (DMSO), propylene glycol (PG), 2,3-butanediol (2,3-B), acetamide (ACET) and ethylene glycol (EG) were chosen as components of vitrification solutions because of their valuable cryobiological properties. To distinguish between osmotic and toxic effects on cells the different concentrations of the five cryoprotectants were rapidly added into cell suspensions and incubated for various periods and then rapidly diluted. Based on the information from individual cryoprotectants four types of vitrification solutions have been developed and compared with a standard vitrification solution, termed as VS1 by the author (49). Human granulocytes were used as a model in this study because of their sensitivity to osmotic stress and chemical toxicity.

7.2 Experimental protocol

7.2.1 *Individual cryoprotectants*

All experiments were carried out at 22°C. The protocol of addition and dilution of cryoprotectants is shown in Fig. 7.1. One ml of solutions of 2, 4, and 6 *M* PG, EG, DMSO, ACET, and 2,3-B were added rapidly (one step) into 1 ml cell suspension ($2x10^6$cells/ml), and incubated for 10, 20, 30, 60, and 120 min, and then diluted rapidly. Cells were centrifuged at 500*g* for 10 min and the supernatant was removed, and then the cells were resuspended in 1 ml basal medium eagle (BME, GIBC.). The recoveries were assessed using MTT (see Chapter III).

7.2.2 *Vitrification solutions*

Four types of vitrification solutions (VS) were developed, and they were designated VSY1, VSY2, VSY3, and VSY4 to avoid confusing them with published VSs. Composition of VSs is given in Table 7. 1, and the protocol of addition and dilution of vitrification solutions is shown in Fig. 7.2. Fifteen ml of vitrification solutions was slowly added into 1 ml cell suspension ($2x10^6$cells/ml) using a 2120 Varioperpex II pump (Bromma, Sweden) at 35 μl/drop and the cell suspension was continuously shaken with a wrist action shaker (Model 75, Burrell, PA). The rates of addition and dilution were the same as those for vitrification of human monocytes (64). For the first minute the rate of addition was 0.3 ml/min, 0.6 ml/min for next 5 min and 0.7 ml/min for the last 15 min. After addition the cells were equilibrated for the next 5 min and then slowly diluted. PBS/BSA was added to 5 ml cell suspension at 0.2 ml/min for first 10

min, 0.5ml/min for the next 10 min, 0.75 ml/min for 15 min and 1 ml/min for the last 30 min. Cells were then centrifuged at 500*g* for 10 min, the supernatant was removed, and the cells resuspended in 5 ml BME. Cell recovery was evaluated using MTT.

7.2.3 *4 M mixtures of propylene glycol and 2,3-butanediol*

Addition and dilution protocols were the same as that of VS except that last step was 15 min instead of 30 min for VS because the total concentration was lower than that of VS.

7.2.4 *Statistical analysis*

Analysis of variance and Tukey's Studentized Range (HSD) test (61) were used to determine significant values for the data of VS and the mixtures of PG and 2,3-B.

7.3 **Results**

Fig. 7.3 shows that the recoveries of human granulocytes decreased with increasing concentration of five types of cryoprotectants when the solutes were added rapidly, equilibrated for 10 min, and rapidly diluted at 22°C. The damage to cells was likely to be due to osmotic stress because of such a short incubation time. More damage was seen from EG, and ACET than PG and 2,3-B.

Fig. 7.4 gives the results obtained from incubation for 10, 20, 30, 60, and 120 min after rapid addition of different concentrations of the cryoprotectants. No significant osmotic or toxic effects were seen in Fig. 7.4a. Fig. 7.4b shows

that the initial damage caused by osmotic stresses from five types of 2 *M* solutes was different, but no significant further damage as a function of time (defined as toxic effect from the solutes) during 60 min incubation. Fig. 7.4c illustrates that the initial recoveries due to osmotic stress from 3 *M* solutes were lower than those from 2 *M* solutes, and significant chemical toxicity was caused by PG and 2,3-B even though they induced lower osmotic stresses. No significant chemical toxicity on cells was detected from exposure to EG, ACET, and DMSO after exposure up to 60 min.

Fig. 7.5 shows the chemical toxicity of mixtures of PG and 2,3-B with total concentration of 4 *M* under slow addition, equilibrium for 5 min and rapid dilution at 22°C. Eighty percent of granulocytes recovered from exposure to 4 *M* PG but almost none of the cells survived 4 *M* 2,3-B. The mixture of 1:3 of 2,3-B and PG was not significantly more toxic than 4 *M* PG ($P > 0.05$) but the combination of 2:2 shows significant damage ($P < 0.05$). The results suggest that only the mixture of 1:3 of 2,3-B and PG could be used in vitrification solutions.

The permeability of the five types of vitrification solutions does not show significant differences using an electronic particle counter (Fig. 7.6) (see Chapter III). The results imply that osmotic stresses from the vitrification solutions are not different. Fig. 7.7 gives the results obtained from cells after slow addition, equilibrium for 5 min and slow dilution of vitrification solutions at 22°C. The results show that the recoveries from five kinds of vitrification solutions are different. The VSY2 developed from VS1 using EG instead of ACET gives significant higher recovery than VS1 ($P < 0.05$). Few cells survived VSY1, which consists mainly of PG, 2,3-B and ACET. The recoveries from

VS1, VSY3 and VSY4 are not significantly different ($P>0.05$).

7.4 **Discussion**

The recovery of human granulocytes was decreased with increasing concentration of cryoprotectants used here when rapid addition, equilibrium for 10 min and rapid dilution were carried out at 22°C. The damage was mainly assumed to be due to osmotic stress because of a short incubation time. However, when the incubation time was extended for 20, 30, 60, and 120 min cell damage was dependent not only on solute concentration but duration of incubation as well. For 1 *M* and 2 *M* cryoprotectants no damage was observed within 60 min, which indicates that the cryoprotectants at these concentrations have a low chemical toxicity. The 3 *M* solutes, however, gave different recoveries. With high osmotic stress, EG and ACET showed low toxicity while the PG and 2,3-B with low osmotic stress resulted in higher toxicity to cells. DMSO had low osmotic stress and low chemical toxicity. These results indicate that chemical toxicity is not related to osmotic stress but to the properties of cryoprotectants *per se*.

Human granulocytes survived 4 *M* PG when slow addition and dilution were employed at 22°C but were completely destroyed by exposure to 6 *M* PG for just five min. Therefore, combination of PG with other cryoprotectants, as suggested in Chapter VI, is appropriate. 2,3-B is an excellent glass former and a complete vitrification could be obtained with 3.88 *M* at a cooling rate of 20°C/min (8), so it was combined with PG. Unfortunately, the 4 *M* mixtures containing more than 1 *M* 2,3-B are very toxic. These results demonstrate that chemical toxicity is extremely dependent on the composition of cryoprotectant

solutions because the total concentration of all compositions were the same. Based on the information above, four types of vitrification solutions were developed and were compared with VS1 (49). The recoveries after the five types of vitrification solutions are different. This result indicates that osmotic potential is not an important cause of chemical toxicity because the osmotic stresses of five types of vitrification solutions are not significantly different.

70% of the cells were recovered from VSY2 compared with 30% from VS1. The difference between the two vitrification solutions is that the ACET in VS1 was replaced by EG in VSY2. This implies that EG can reduce the chemical toxicity of the solution significantly better than ACET. However, when PG was replaced by 2,3-B in VSY4 the recovery decreased to the same level as VS1. Those results suggest that chemical toxicity was minimized by optimal combinations of solutes but not by a neutralizer. Although Fahy (16) suggested that cryoprotectant toxicity can be neutralized by a compound (neutralizer) which reduces toxicity of another compound, not enough models have been studied to draw such a sweeping conclusion.

Toxicity is generally thought to be dependent on the concentration of a solution and, therefore, mixtures of cryoprotectants have been proposed (49). Recently Langis and Steponkus argued that toxicity of a vitrification solution depends on its osmotic potential. They believe that alterations in membranes are a primary cause of injury during dehydration, and therefore toxicity can be minimized by formulating solutions with an optimum osmolality (28). This study compared five types of vitrification solutions using human granulocytes as a model and concludes that cell damage on exposure to vitrification solutions is

due to intrinsic chemical toxicity not osmotic stress, and that toxicity can be minimized by optimal cryoprotectant mixtures. The optimal combination of cryoprotectants should be based on the osmotic and toxic properties of individual solutes and may be specific to biological systems. For example, PG and EG are not as effective as the amides in reducing toxicity to kidney slices (17, 20), but mixture of PG and EG with DMSO has a much lower toxicity to human granulocytes than that of DMSO with amides.

7.5 **Conclusions**

Damage to granulocytes from exposure to vitrification solutions is primarily due to the intrinsic chemical toxicity of solutes and not to osmotic stress because the osmotic stresses of vitrification solutions used here on cells were similar. Recovery of granulocytes from VSY2 is significantly different from VS1 when acetamide in VS1 was replaced by ethylene glycol in VSY2. PG-based vitrification solution (VSY3) is not significantly different from VS1, and 2,3-butanediol is too toxic to granulocytes in combination with other cryoprotectants, especially with propylene glycol. Combinations of different cryoprotectants effectively minimize chemical toxicity of vitrification solutions but no specific solute used here reduced the chemical toxicity of any single cryoprotectant. Combinations of cryoprotectants are a potential method for the reduction of chemical toxicity in vitrification solutions.

Table 7. 1. Compositions of vitrification solutions

Component	Final concentration (% w/v and (M))				
	VS1	VSY1	VSY2	VSY3	VSY4
Propylene glycol (PG)	10 (1.3)	20 (2.6)	10 (1.3)	29 (3.8)	—
Ethylene glycol (EG)	—	—	15.5 (2.5)	15.5 (2.5)	15.5 (2.5)
Acetamide (ACET)	15.5 (2.6)	15.5 (2.6)	—	—	—
Dimethyl sulfoxide (DMSO)	20.5 (2.6)	—	20.5 (2.6)	—	20.5 (2.6)
2,3-butanediol (2,3-B)	—	9 (1.0)	—	—	10 (1.1)
Polyethylene glycol (PEG, 8000 WM)	6	6	6 (0.0075)	6	6

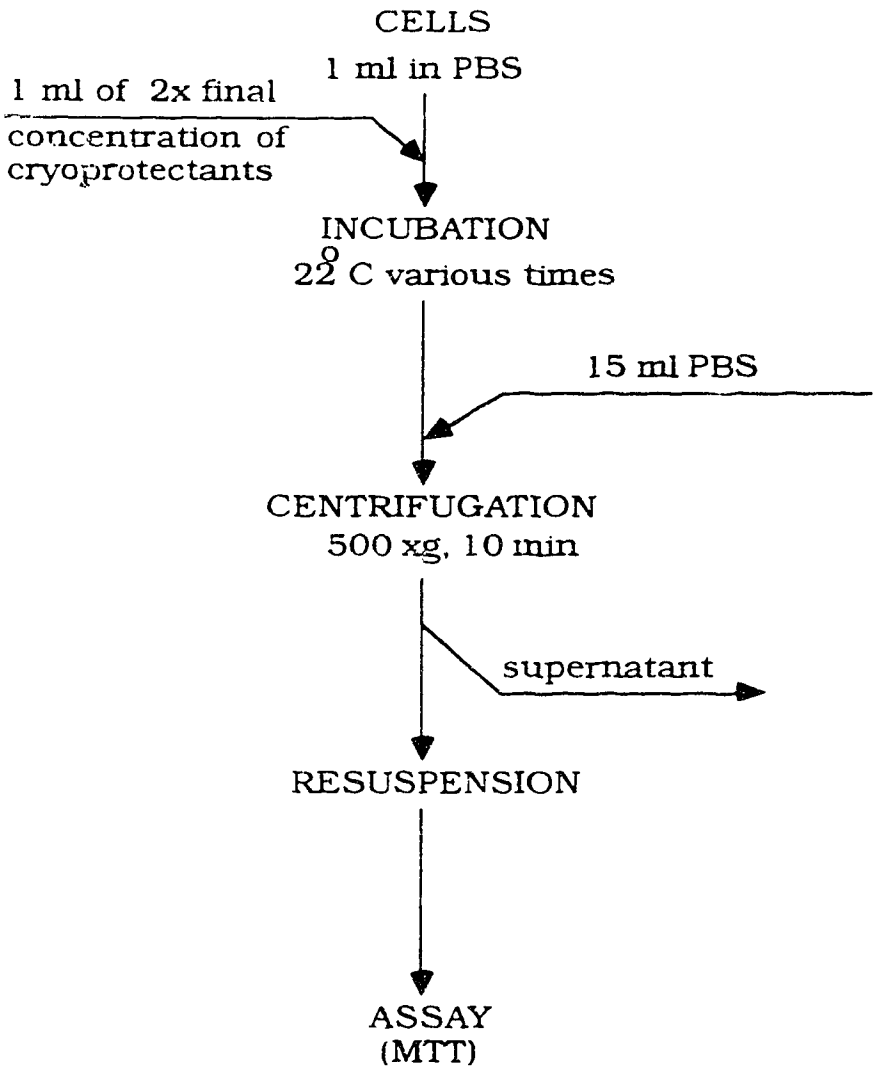


Figure 7.1. Protocol of addition and dilution of different concentrations of propylene glycol, ethylene glycol, acetamide, dimethyl sulfoxide, and 2,3-butanediol.

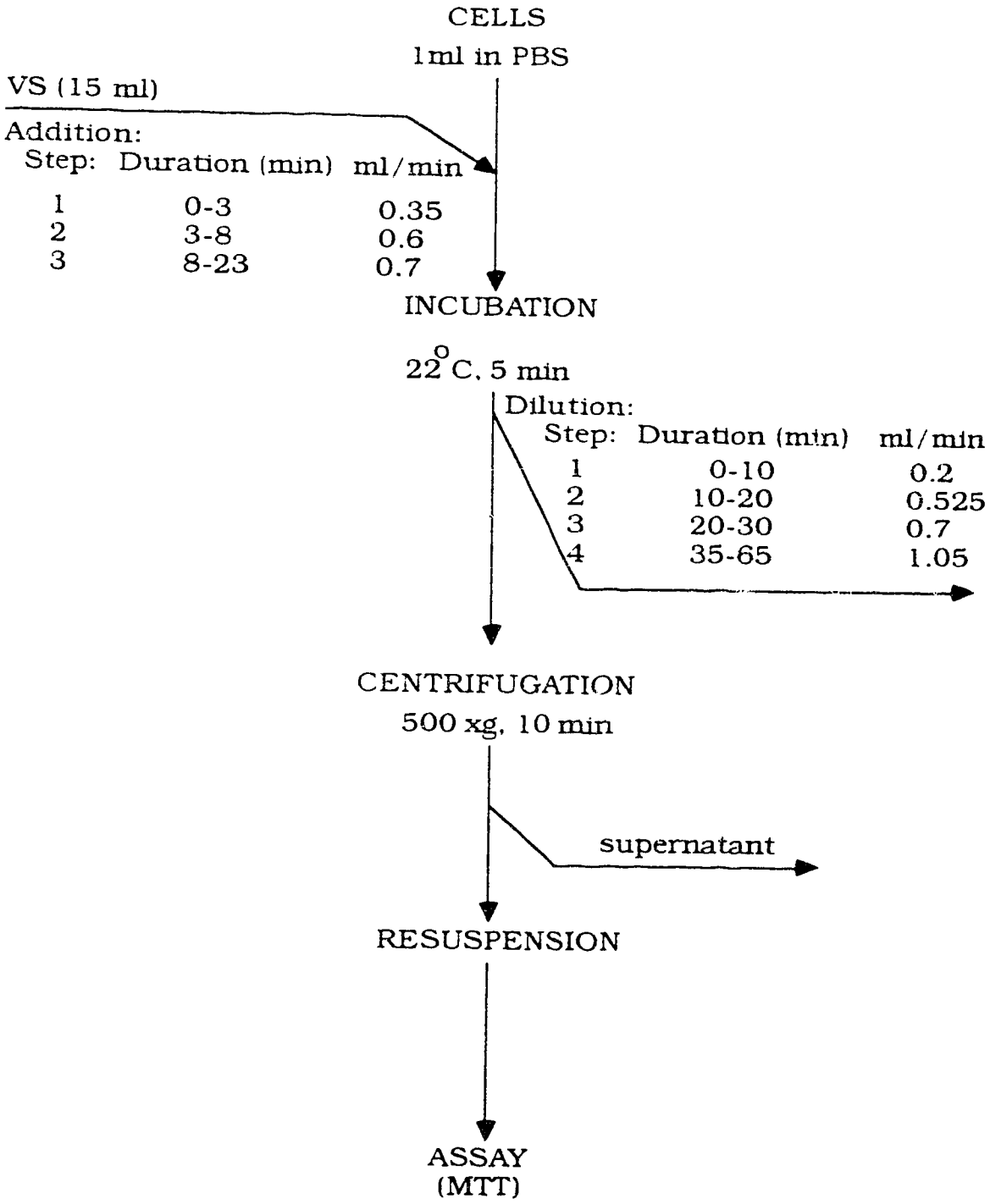


Figure 7.2 Protocol of addition and dilution of vitrification solutions

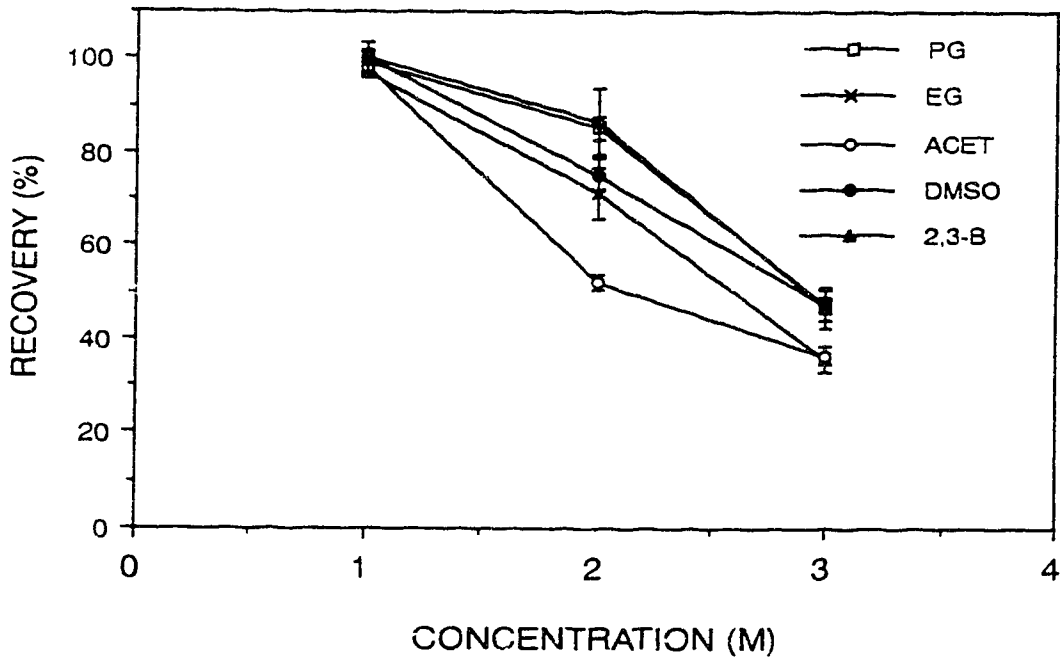


Figure 7.3. Responses of granulocytes to osmotic stresses. Different concentrations of propylene glycol (PG), ethylene glycol (EG), acetamide (ACET), dimethyl sulfoxide (DMSO), and 2,3-butanediol (2,3-B) were rapidly added to granulocyte suspensions, equilibrated for 10 min, and then rapidly diluted. The recoveries of cells were assessed using MTT. Values shown are means with vertical bars equal to $\pm$SEM.

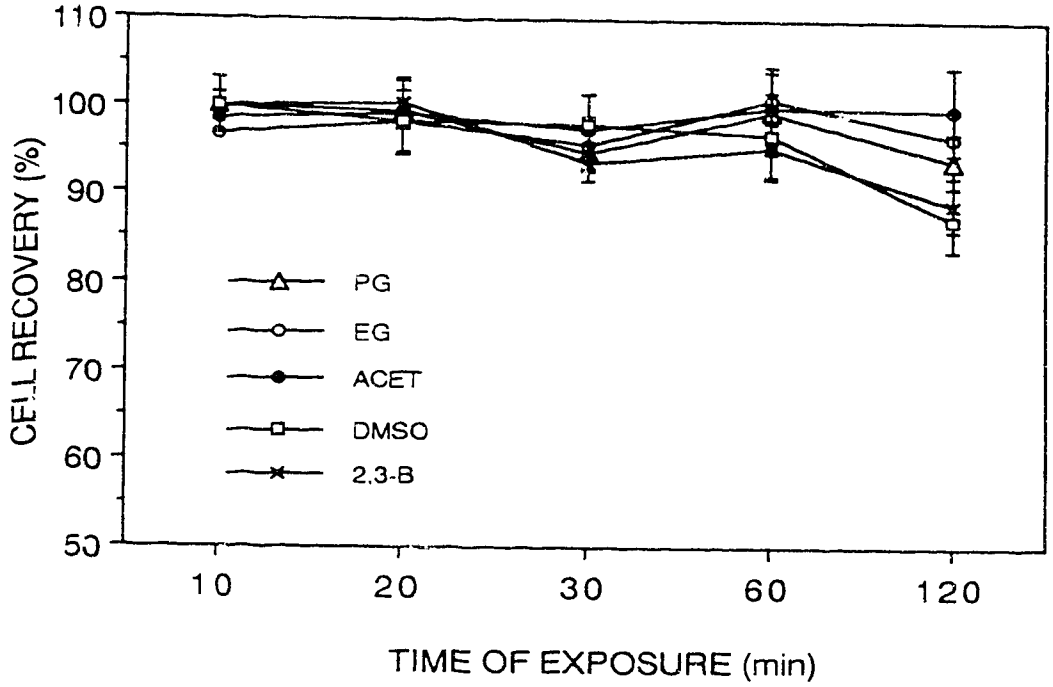


Figure 7.4a. Toxicity of 1 *M* propylene glycol (PG), ethylene glycol (EG), acetamide (ACET), dimethyl sulfoxide (DMSO), and 2,3-butanediol (2,3-B). The solutions were rapidly added to granulocyte suspensions, exposed for various times, and then rapidly diluted. The recoveries of cells were assayed using MTT. Values shown are means with vertical bars equal to ±SEM.

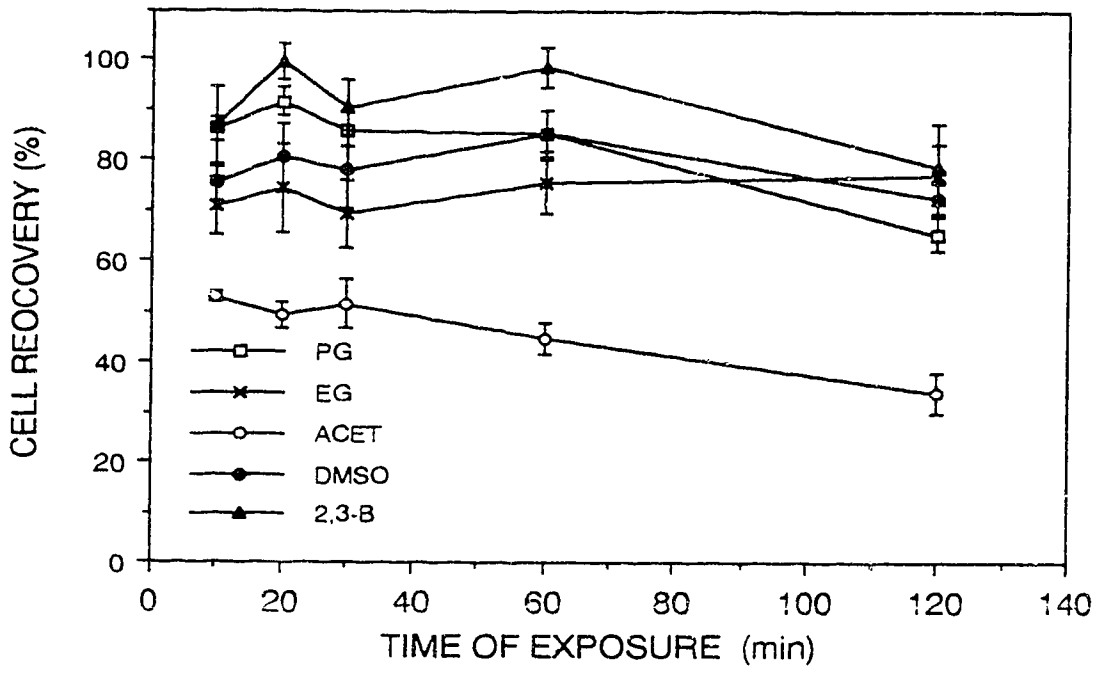


Figure 7.4b. Toxicity of 2 *M* propylene glycol (PG), ethylene glycol (EG), acetamide (ACET), dimethyl sulfoxide (DMSO), and 2,3-butanediol (2,3-B). The solutions were rapidly added to granulocyte suspensions, exposed for various times, and then rapidly diluted. The recoveries of cells were assayed using MTT. Values shown are means with vertical bars equal to ±SEM.

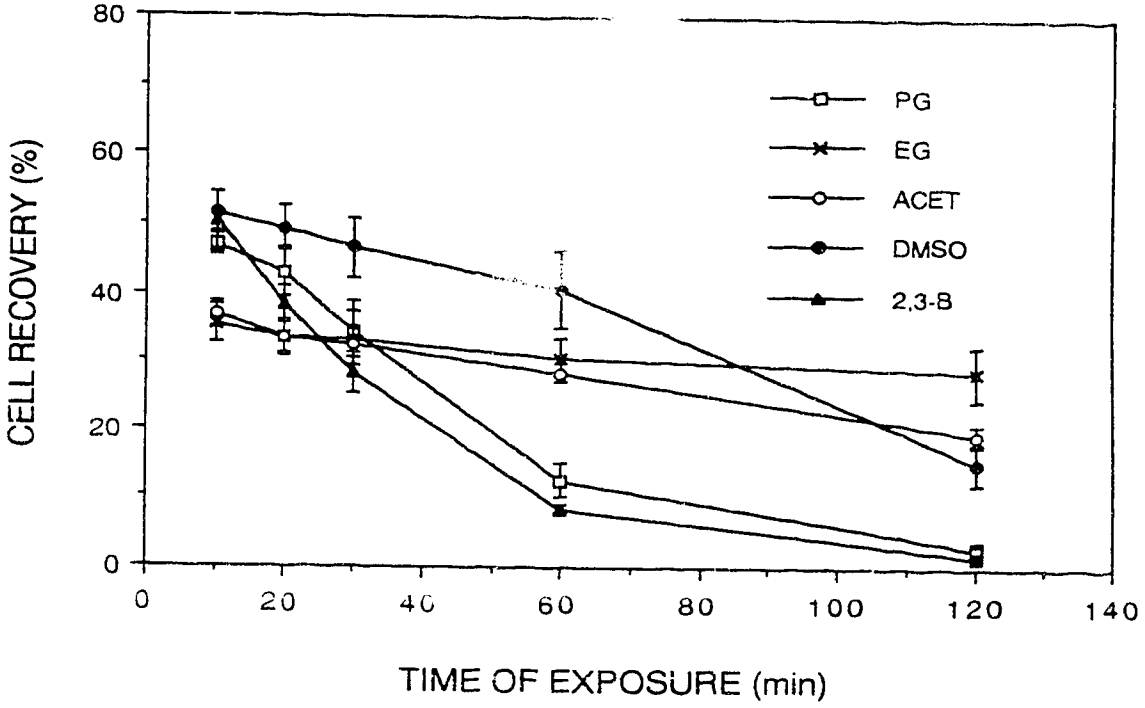


Figure 7.4c. Toxicity of 3 *M* propylene glycol (PG), ethylene glycol (EG), acetamide (ACET), dimethyl sulfoxide (DMSO), and 2,3-butanediol (2,3-B). The solutions were rapidly added to granulocyte suspensions, exposed for various times, and then rapidly diluted. The recoveries of cells were assayed using MTT. Values shown are means with vertical bars equal to ±SEM.

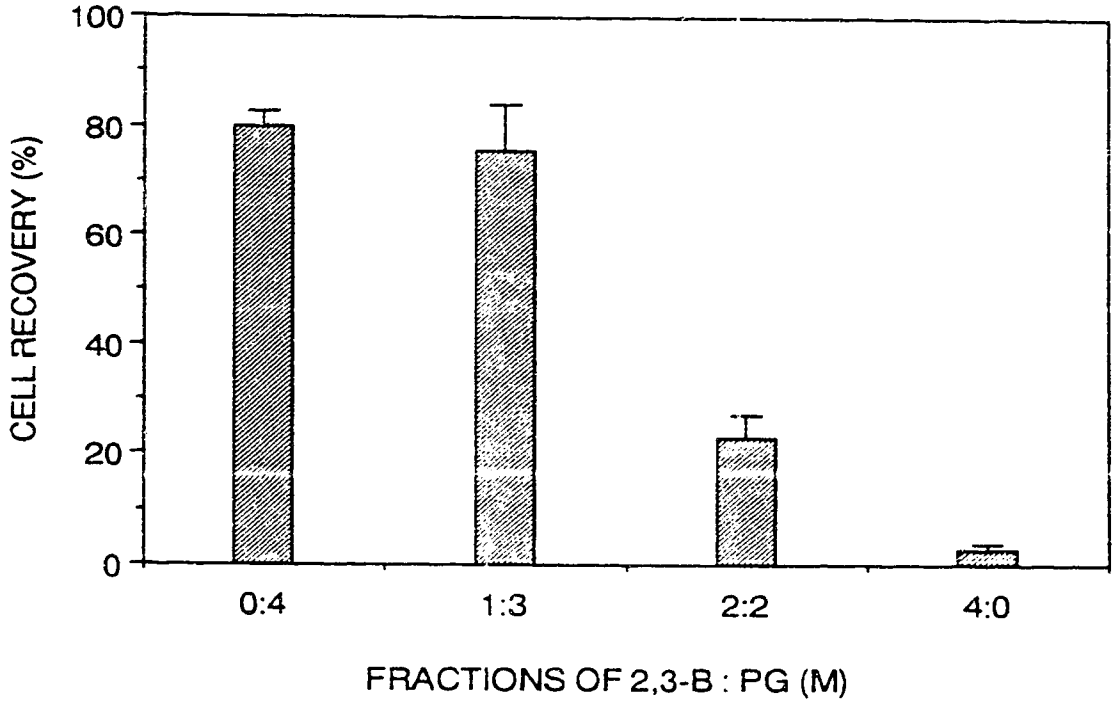


Figure 7.5. Toxicity of different mixtures of propylene glycol (PG) and 2,3 -butanediol (2,3-B). The 4 *M* solutions were slowly added into granulocyte suspensions, incubated for 5 min, and then slowly diluted. The recoveries of cells were evaluated using MTT. Values shown are means with vertical bars equal to $\pm$SEM.

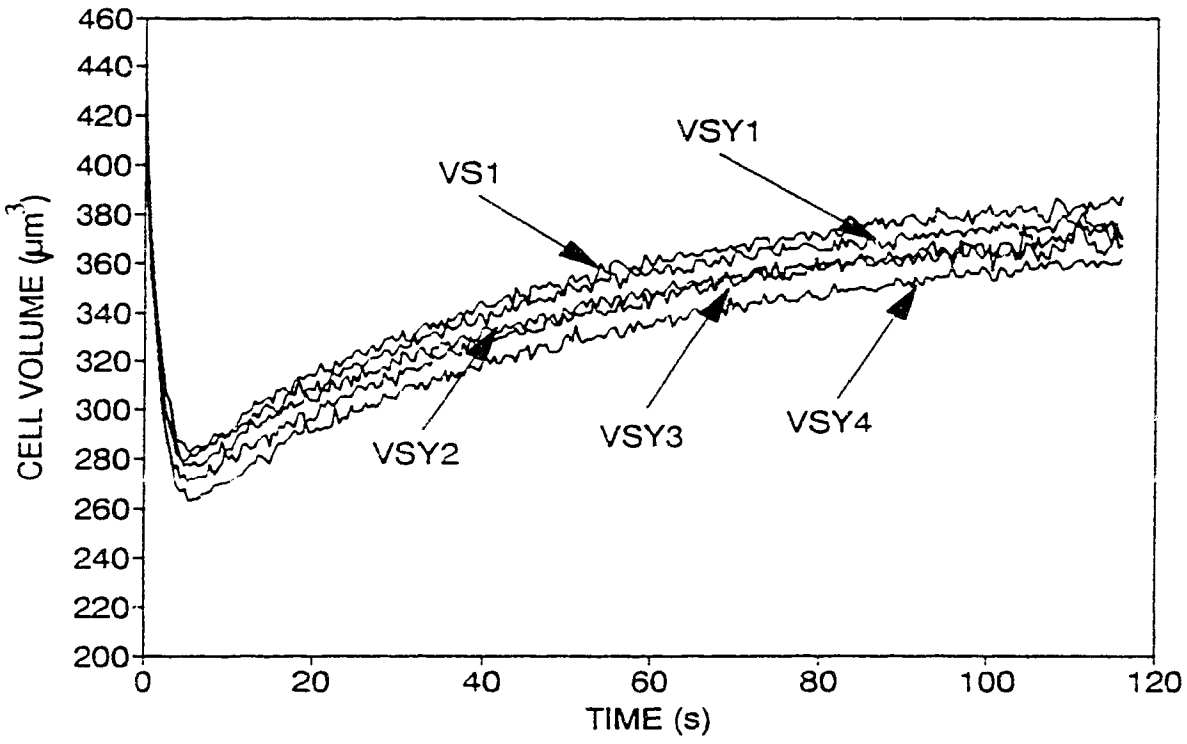


Figure 7.6. Permeability of granulocytes to vitrification solutions (VS). The cell volume changes exposed five types of 2 *M* VSs (VS1, VSY1, VSY2, VSY3, VSY4) were measured as a function of time using an electronic particle counter.

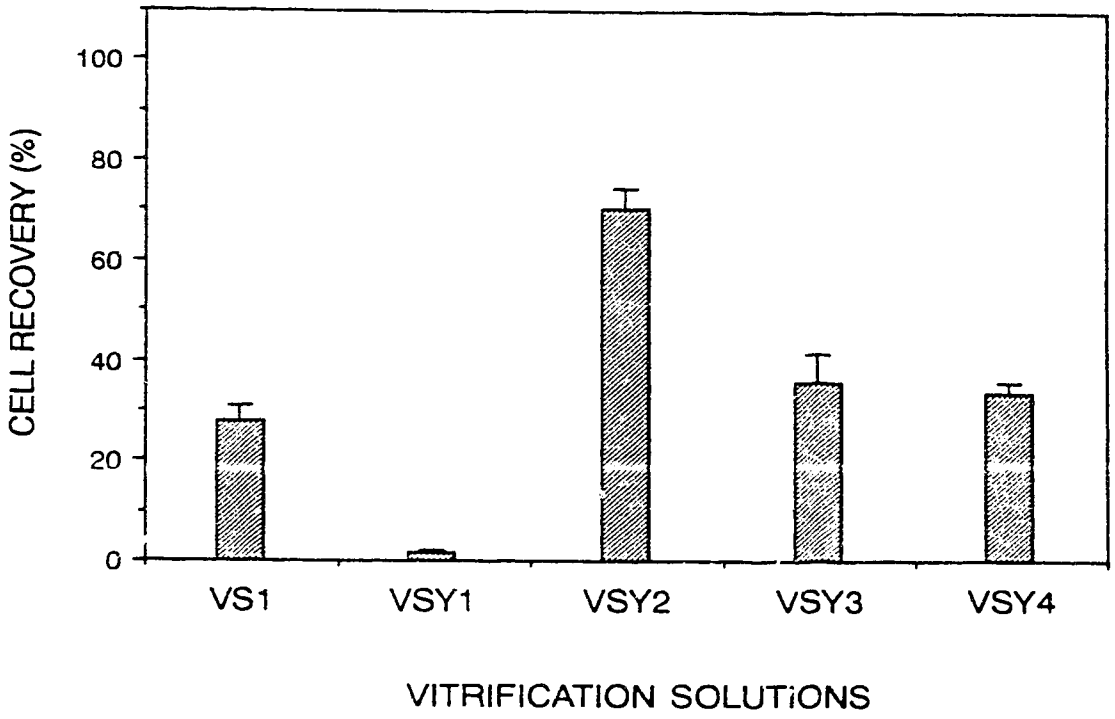


Figure 7.7. Recoveries of granulocytes exposed to vitrification solutions (VS). Five types of VSs (VS1, VSY1, VSY2, VSY3, and VSY4) were slowly added into granulocyte suspensions, equilibrated for 5 min, and then slowly diluted. The recoveries of cells were assessed using MTT. Values shown are means with vertical bars equal to $\pm$SEM.

CHAPTER VIII

DISCUSSION

8.1 Primary site of injury to cells during freeze and thaw

8.1.1 *The plasma membrane?*

During slow freezing human granulocytes were damaged by two factors: solution effects and intracellular ice. The recoveries, based on a FDA assay, were higher immediately after thawing than after 2 h incubation at 37°C. These results suggest that the plasma membrane was damaged during freezing and thawing, but the damage needs time to become evident. However, recovery of human granulocytes after thawing was significantly different from that of lymphocytes and fibroblasts. This implies that human granulocytes are very sensitive to freezing and thawing and that the damage to granulocytes is different from that of the other two types of cells.

Lovelock (30) proposed that freezing damage in human erythrocytes is a consequence of the increase in electrolyte concentration that occurs during freezing. The high salt concentration increases the permeability of the plasma membrane and results in the influx of Na^+. Consequently, when the cells are returned to an isotonic medium, they swell beyond their normal isotonic volume and lyse. However, there is no such evidence of solute loading in granulocytes. A similar result was reported by Armitage and Mazur (3). Neither could Meryman's minimum cell volume nor Steponkus and Wiest's tolerable surface area increment hypotheses interpret the results in this study. According to these

theories the cells will lyse after returning to an isotonic solution if they are damaged during freezing because the plasma membrane is the primary site of freezing damage of cells. It is not surprising that the damage of granulocytes does not fit the these hypotheses because most of the hypotheses were based on a study of the mechanism of cryoinjury in erythrocytes. It is well known that erythrocytes are very simple cells which have no organelles. However, granulocytes are totally different. They have organelles and, therefore the MTT assay, which is a very sensitive test of mitochondrial function was used. The results, compared with those of the FDA assay indicate that mitochondria were damaged earlier than the plasma membrane (see Chapter IV). Therefore, it is concluded that the plasma membrane is not the primary site of freezing injury in granulocytes, and it is further proposed that organelles, specifically lysosomes, are damaged during freezing, resulting in the release of hydrolytic enzymes which may damage the plasma membrane during incubation at 37°C.

8.1.2 *Is primary site of cryoinjury intracellular in human granulocytes ?*

Lysosomes have been studied as indicators of cryoinjury in granulocytes (47, 56). It has been indirectly demonstrated that a striking improvement in cell preservation might result directly from the inhibition of lysosomal enzymes by trypan blue (47) and it was proven that hydrolytic enzymes released from damaged lysosomes showed little destructive activity at low temperatures but quickly destroyed the cell from within at 37°C. However, experimental evidence is not easy to obtain because the activity of the enzymes is complicated, and the enzymes released from damaged granulocytes do not damage intact cells (5). However, it is well known that granulocyte cytoplasts

can be cryopreserved with complete recovery of functional properties although granulocytes cannot be cryopreserved. The cytoplasts can be stimulated to carry out a number of functions (such as phagocytosis) that until now have only been observed in intact cells. Sharing the same plasma membrane and cytoplasm devoid of lysosomes and other organells, cytoplasts is that cytoplasts are an ideal tool to investigate the site of the plasma membrane cryoinjury of human granulocytes.

The results in Chapter V indicate that granulocytes and cytoplasts have similar physiological properties, but their responses to freezing and thawing are completely different. Granulocytes cooled to -10°C had high recoveries based on FDA immediately after thawing but were completely damaged after incubation at 37°C for 2 h. In contrast, recoveries of cytoplasts using the same freezing protocol showed no significant difference whether they received a 2 h incubation at 37°C or not. This result directly demonstrates that the plasma membrane is not the primary site of cryoinjury in granulocytes and all hypotheses proposed based on plasma membrane damage should be ruled out for human granulocytes.

8.2 **Optimum temperatures and rates of addition and dilution of cryoprotectants**

8.2.1 *Temperature dependence of osmotic stress and toxicity*

The majority of cells are destroyed when subjected to the many stresses associated with cooling to, and thawing from, low temperatures (6). Only a very small fraction, perhaps less than 1% of nucleated animal cells survive

unprotected freezing and thawing. Changes in the rate of cooling and thawing modify the survival of all cells but the most important factor influencing survival is the presence or absence of molecules that possess cryoprotective properties. Many types of cells survive freezing and thawing with cryoprotectants, DMSO and glycerol being the most commonly used additives, but still some kinds of cell types do not survive because of sensitivity osmotic stresses encountered during addition and dilution of the additives. Therefore, reduction of osmotic stress has been attempted by reducing both the rate of addition of the cryoprotectant to the bathing medium, and the rate of subsequent dilution of the medium to remove the cryoprotectant.

Because membrane permeability increases with increasing temperature, the osmotic effects of addition and dilution are less at, for example, 22°C than at 0°C. However, any benefit gained by increasing temperature to lower the osmotic stress may be lost because of the increased toxicity of the additive at the higher temperature. Therefore, addition and dilution at 0-4°C have been carried out for almost all types of cells. The results in Chapter VI show that recoveries of human granulocytes based on MTT assay are significantly higher when the addition and dilution were performed at 22°C than those at 0°C. This definitely does not imply that the toxicity of PG is higher at 0°C than at 22°C and, in contrast, the higher toxicity at 22°C is demonstrated by exposure of granulocytes to PG without dilution, which is thought to be a true test of toxicity (25). Therefore, it is concluded that human granulocytes can tolerate higher toxicity, and osmotic stresses associated with addition and dilution at 22°C.

Usually the dilution of a cryoprotectant is thought to be a more important step than the addition, and slow dilution is better than rapid

dilution. The experiments here do not support this assumption. Cells exposed to 2 *M* PG were significantly dependent on addition rates and temperatures but not dilution rates. This implies that cells were irreversibly damaged during the addition of cryoprotectant.

8.2.2 *Tolerance of cells to chemical toxicity of cryoprotectants during slow addition*

Stepwise addition and dilution of cryoprotectants is widely used in procedures of cryopreservation and the concentration of each step is determined according to the tolerance ranges of cells to osmotic stress, which are measured when cells are in isotonic solution. Thus, when cells cannot survive the addition and dilution within those limits, it is usually concluded that the cryoprotectant is toxic to the cells. The results in Chapter VI show that this conclusion is invalid. Granulocytes can tolerate toxicity of up to 6 *M* PG for up to 20 min at 22°C when PG is added rapidly (one step) into cells (in 1x PBS) but when PG was slowly added (seven steps) into cells granulocytes can tolerate only up to 4 *M* PG for 2 h at 22°C, and the cells exposed to 6 *M* PG were completely damaged within 10 min. It is obvious that the cells slowly exposed to 6 *M* PG were damaged during the last two addition steps (from 4 *M* to 6 *M*). These results indicate that the tolerance of granulocytes to chemical toxicity of PG was decreasing during the slow addition.

8.3 **Osmotic stress and chemical toxicity of vitrification solutions**

8.3.1 *Osmotic stress vs. chemical toxicity*

It is generally believed that a central problem using a vitrification as an approach to cryopreservation of biological systems is the toxicity of vitrification solution. Toxicity may depend on solute concentration, and so attention was given to the formulation of vitrification solutions with minimum solute concentration. Accordingly, mixtures of cryoprotectants were used to minimize the concentration of any individual component in the vitrification solution. It has been also proposed that dehydration-induced alterations in membranes are a primary cause of injury and therefore toxicity of a vitrification solution largely depends on its osmotic potential. According to this hypothesis, toxicity can be minimized by formulating solutions with optimum osmolality. The latter interpretation states that cells are damaged by high osmolality but not by any intrinsic toxicity of the vitrification solution. The results in Chapter VII do not support the Langis and Steponkus optimal osmolality hypothesis. Damage to cells initially by osmotic stress was based on the permeability of cells to various cryoprotectants. However, the cell damage with further incubation is not dependent on the initial osmotic stress but toxicity. A further investigation regarding the relationship between osmotic stress and chemical toxicity was carried out using vitrification solutions (see Chapter VII). Based on the permeability of cells to the vitrification solutions, and using identical protocols for addition and dilution, the osmotic stresses of all five types of vitrification solutions are similar; therefore the recoveries of cells were determined by the combination of cryoprotectants. Those results indicate that there is no correlation between osmotic stress and chemical toxicity, and therefore one may conclude that the Langis and Stepunkus optimal osmolality hypothesis is simply not correct.

8.3.2 *Reduction of chemical toxicity of vitrification solutions*

With the reduction of cryoprotectant toxicity being sought for more than a decade, some methods have been developed to forward this aim. Additions and dilutions are carried out at as a low temperature as possible, exposure time at higher concentrations is kept to a minimum, and specific cryoprotectant toxicity neutralizers, such as acetamide, are employed. Using this strategy, successful cryopreservation of several types of cells and tissues has been reached using VS1 (50, 64). The reduction of toxicity of VS1 is claimed to be the result of the employment of a neutralizer acetamide, which is thought to reduce the toxicity of DMSO. This raises the question of whether or not acetamide indeed reduces the toxicity of DMSO in vitrification solutions, and whether there is actually any specific toxicity neutralizer. The results in Chapter VII indicate that acetamide does not effectively reduce toxicity of vitrification solutions, and suggest that there is no specific toxicity neutralizer. The results also show that combination of different cryoprotectants is an effect way to reduce toxicity of vitrification solutions.

CHAPTER IX

CONCLUSION

This study has investigated mechanisms of freezing injury and the reduction of chemical toxicity of vitrification solutions. This was the first study to confirm that, based on a direct evidence, the plasma membrane of cells is not the primary site of freezing injury in human granulocytes. This observation creates opportunities for new strategies in the cryopreservation of these cells.

Granulocytes tolerate higher osmotic stress and toxicity of cryoprotectants at 22°C than at 0°C. Cells in an isotonic solution without PG can tolerate much higher increments in osmolality than those already in PG solution (Chapter VI). Those observations suggest that some types of cells, so-called osmotic and toxic-sensitive cells which cannot be cryopreserved, should be re-examined for the cause of damage during addition and dilution of additives.

Vitrification as an approach to the cryopreservation of cells, tissues, and organs has been many cryobiologists' objective for many years but high chemical toxicity of vitrification solutions is a major obstacle. In this thesis, it was shown that cells exposed to vitrification solutions are damaged by the intrinsic toxicity of vitrification solutions, not by its osmotic potential. Toxicity of vitrification solutions can be minimized by combining cryoprotectants.

In further studies, the protection of organelles, specifically lysosomes, during cryopreservation procedures should be stressed, and reduction of toxicity of vitrification solutions should be carefully investigated using a variety of cell models.

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VITA

Publications resulting from this research:

Papers:

1. Yang, H., Arnaud, F., and McGann, L. E. Cryoinjury in human granulocytes and cytoplasts. *Cryobiology* 28, 1991 (in press).
2. McGann, L. E., Yang, H., and Waterson, M. Manifestations of cell damage after freezing and thawing. *Cryobiology* 25, 178-185 (1988).

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1. Yang, H., and McGann, L. E. Osmotic stress and chemical toxicity in human granulocytes exposed to vitrification solutions. *Cryobiology* 28, 1991 (in press).
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