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Scientific Officer
Patrick Curran, CAPT, MC, USN
Naval Medical R&D Command
Director of Research and Development
Bethesda, Maryland 20814-50440

Reference: N00014-91-C-0044

Dear Captain Curran:

Enclosed is the Eighth Triannual Report for Contract N00014-91-C-0044, entitled "Cellular and Tissue Injury During Nonfreezing Cold Injury and Frostbite". This Report covers the period from May through August 1993.

If you have any questions about the Report or the research, please contact me at 404 952-1660.

Respectfully submitted,

Kelvin G.M. Brockbank, Ph.D.
Director, Research and Development

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*CELLULAR AND TISSUE INJURY
DURING NONFREEZING COLD INJURY AND FROSTBITE*

EIGHTH TRIANNUAL REPORT: MAY-AUGUST, 1993

We have continued our investigations in the following five major areas:

- (a) the effects of cold-induced membrane phase changes on the function of transmembrane ion pumps;
- (b) the mechanism of metabolic inhibition under cold, acidotic conditions;
- (c) the influence of these conditions on the function of skeletal and smooth muscle;
- (d) the role of free radicals in reperfusion injury in human cells; and
- (e) developing methods for amelioration of cell and tissue damage during non-freezing injury and frostbite, based on the results of the aforementioned studies.

Abstracts and publications presented since April 1993 are listed in Appendix 1. The text of the abstracts and publications described in this report may be found in Appendix 2.

1. IMPACT OF ALTERATIONS IN MEMBRANE FLUIDITY IN CELL CHILLING SENSITIVITY AND FUNCTION

It is well established that lowering the temperature leads to a phase change in membrane phospholipids from liquid-crystalline to gel state. The phase transition has been purported to induce decreases in the function of transmembrane ion pumps, activate phospholipases, and reduce the barrier function of the cellular membranes. We examined the hypothesis that the higher the membrane phase transition temperature, the greater the chilling sensitivity of the cells.

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We measured the membrane phase transition of human cells by Fourier transform infrared spectroscopy (FTIR). The cellular membrane was monitored by measuring the shift in the peak of the CH_2 symmetric stretch band (located at 2853 cm^{-1}). The CH_2 bands are associated primarily with the phospholipids. In parallel studies, we examined the chilling sensitivity of the cell lines by measuring leakage of cytosolic enzymes into the media. The membrane phase transition temperature was related to the chilling sensitivity of the cells: the higher the membrane phase transition temperature, the greater the chilling sensitivity of the cells. A manuscript describing the results has been submitted for publication.

Further studies on the effects of hypothermia were undertaken using platelets. Initial studies determined that chilling platelets to 20°C resulted in spontaneous aggregation. Platelet aggregation correlated with the change in platelet membrane fluidity. A manuscript describing the results is currently in press. We are currently studying the effects of agonist-induced aggregation on chilled platelets to critically examine whether chilled platelets are also hypersensitive to agonists. High concentrations of the agonist ADP resulted in the same amount of aggregation in platelets at 37 and 20°C . In contrast, low concentrations of ADP caused greater aggregation of platelets at 20°C than at 37°C . We are investigating whether the different metabolic pathways activated during ADP-induced aggregation are similar in chilled and normothermic platelets. These studies will help to determine more precisely the role of platelets in chilling-induced injury of tissues, and whether specific metabolic pathways of the platelets need to be blocked to avoid spontaneous and agonist-induced platelet activation during chilling.

2. THE MECHANISM OF METABOLIC INHIBITION UNDER COLD, ACIDOTIC CONDITIONS

Preliminary results in bovine aortic endothelial cells indicated that low pH inhibited lactate production and heat generation. ATP levels were also decreased after incubation at low pH. These results have been confirmed through further experiments. In addition, it was noted that carbon dioxide release at low pH was decreased by about 40% compared to pH 7.4. Heat production per mole of carbon dioxide released was not affected by pH. These results suggest that low pH inhibits both glycolysis and mitochondrial oxidation. Therefore, under acidotic conditions, it is likely that endothelial cells will have a decreased capacity for ATP

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production, which could affect metabolic and regulatory functions of these cells in tissues.

We have begun development of protocols for measurement of release of vasoactive substances produced by endothelial cells under conditions of hypothermia and low pH. The substances include the vasoconstrictor endothelin and the vasodilators prostacyclin and endothelium-derived relaxation factor (nitric oxide). We have begun collecting samples for measurement of prostacyclin release. These studies will help establish which cellular mechanisms of homeostasis are altered by hypothermia in the vasculature.

3. THE INFLUENCE OF COLD AND ACIDOTIC CONDITIONS ON THE FUNCTION OF SMOOTH MUSCLE

Measurements of smooth muscle function using rabbit jugular vein rings has continued. Additional measurements have confirmed the preliminary results that at 20°C the vein rings are more sensitive to norepinephrine while responsiveness to norepinephrine, as indicated by the maximum tension achieved during contraction, is decreased relative to 37°C. For other agonists the results varied. Temperature had no effect on either sensitivity or maximum tension for contraction induced by bradykinin, while both sensitivity and maximum tension for contraction induced by histamine were decreased at 20°C relative to 37°C. Finally, smooth muscle relaxation to acetyl choline was diminished at 20°C.

In order to study the effects of rewarming after hypothermia in the vasculature, the response of veins exposed to 20°C and then rewarmed to 37°C has also been measured. Maximum tension for contraction induced by norepinephrine increased back to 37°C levels from the lower tension measured at 20°C. Sensitivity also returns to control levels. There was no change for contraction induced by bradykinin, while the maximum tension for histidine-induced contraction returned to original 37°C levels. However, the decreased sensitivity of veins for histidine at 20°C remained unchanged. Surprisingly, there was a large increase in relaxation induced by acetyl choline: the extent of relaxation was greater after rewarming than in controls which had remained at 37°C.

The results obtained so far indicate that exposure to low temperature causes significant changes in the response of vein rings to agonists for both contraction and relaxation. Some of the changes are reversed quickly on rewarming, while others are not reversible within the time frame of the experiments. The results suggests that upon chilling there may be an increased vasoconstriction of the vasculature, which would encourage vascular stasis, anoxia, and acidosis in the surrounding tissues. The vascular effects appear to be quickly reversed upon rewarming.

4. ROLE OF FREE RADICALS IN HYPOTHERMIC INJURY IN HUMAN TISSUES

Oxygen free radical formation upon reperfusion of ischemic tissues and organs has been implicated in tissue damage in several animal models. However, the role of free radicals in human tissues is a matter of debate in the literature. As part of our studies characterizing the effects of cold induced injury in human hemopoietic cell lines, we noted that the addition of hydrophobic free radical scavengers increased the survival of cells incubated at 4°C. In contrast, hydrophilic free radical scavengers did not increase the survival of cells incubated at 4°C. Since hydrophobic free radical scavengers would be associated primarily with cell membranes, we hypothesized that the chilling damage associated with free radical formation is associated with the cellular membranes. We are currently investigating the hypothesis by developing assays that will allow for a direct measurement of lipid peroxidation products from cell membranes. The products are the result of free radical formation and damage to cell membranes. The levels of lipid peroxidation in cells incubated at 4°C with and without free radical scavengers will be measured to confirm the formation of free radicals during the incubations, and to determine whether the radicals formed are responsible for the observed decrease in cellular viability over time.

5. AMELIORATION OF CELL AND TISSUE DAMAGE DURING NON-FREEZING COLD INJURY AND FROSTBITE

We initially investigated the feasibility of using antifreeze peptides to prevent frostbite damage due to ice crystal formation. Antifreeze peptides are found in the blood of Antarctic fish and are vital in the prevention of freezing injury in the fish

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Brockbank, K.G.M.

by inhibiting the growth of ice crystals. Studies using recombinant antifreeze peptides were performed under conditions of slow freezing to determine whether the peptide can alleviate the effects of frostbite in tissues. The results obtained suggest that this particular antifreeze peptide can not alleviate the effects of frostbite. There are a number of other classes of antifreeze peptides which have better ice crystal control and hence may prevent tissue damage associated with ice formation. The results of the study have been submitted for publication.

APPENDIX 1

Appendix 1
Publications Listed by Research Area

1. The effects of cold-induced membrane phase changes on the function of transmembrane ion pumps. (Articles and Abstracts)

Hypothermic-induced injury in endothelial cells: mechanisms and clinical applications. *Cryobiology* (1993) (In Press).

Correlation between membrane phase transition and chilling-induced injury in cells. *Transplantation Proceedings* (1993) (In Press).

Correlation between chilling-induced injury in human cells and phospholipid membrane phase transition temperature. Presented at the 2nd International Congress of the Society for Organ Sharing in Vancouver, Canada (1993) (Abstract).

The role of platelets in chilling-induced injury. New York Academy of Sciences (1993) (In Press).

Chilling-induced injury in hemopoietic cell lines correlates with phospholipid membrane phase transition temperature. *Cryobiology* (1993) (Abstract In Press).

Membrane phase transition temperature predicts chilling sensitivity in hemopoietic cell lines. Congress on Cell and Tissue Culture (1993) (Abstract).

2. The mechanism of metabolic inhibition under cold acidotic conditions. (Abstracts)

Hemoglobin and Fructose-6-phosphate Prevent pH-induced Dissociation of Phosphofructokinase in Intact Red Blood Cells. Presented at 30th Annual Meeting of the Society for Cryobiology, Atlanta, Georgia. *Cryobiology* (1993) (Abstract In Press).

Metabolic Studies of Bovine Aortic Endothelial Cells. Presented at 30th Annual Meeting of the Society for Cryobiology, Atlanta, Georgia. *Cryobiology* (1993) (Abstract In Press).

3. Influence of environmental conditions on skeletal and smooth muscle function. (Abstracts and Articles)

The Effect of 2,3-Butanedione 2-monoxime (BDM) on Ventricular Trabeculae from the Avian Heart. *J. Muscle Resh. Cell. Motil.* (Submitted for Publication).

BDM (2,3, Butanedione Monoxime) Induces Calcium Release from Sarcoplasmic Reticulum of Crustacean Striated Muscle by a Mechanism that does not Involve Chemical Phosphatase Activity. *J. Muscle Resh. Cell. Motil.* (Submitted for Publication).

Effects of Inorganic Phosphate (pi), Orthovanadate (Vi), and 2,3 Butanedione Monoxime (BDM) on Contraction of Triton Skinned Fibers from Rabbit and Lobster Muscle. Biophysical Society (1993) (Abstract).

Butanedione Monoxime (BDM) Induces Calcium Release from the Sarcoplasmic Reticulum of Crustacean Striated Muscle Fibers. IUPS (1993) (Abstract).

BDM (2,3, Butanedione Monoxime) Induces Calcium Release from Sarcoplasmic Reticulum of Crustacean Striated Muscle. Presented at the XXXII Congress of the International Union of Physiological Sciences, Glasgow, Scotland (1993) (Abstract).

The Effects of Ionic Strength and Trimethylamine N-oxide on Cross-Bridge Kinetics of Skinned Muscle Fibers of the Rabbit. To be Presented at the Annual Meeting of the Biophysical Society, New Orleans, Louisiana (1994) (Abstract).

4. The role of free radicals in reperfusion injury. (Abstracts)

The role of oxygen radicals in chilling-induced injury of hemopoietic cell lines. Presented at the 30th Meeting of the Society for Cryobiology in Atlanta, Georgia. Cryobiology (1993) (Abstract In Press).

The role of oxygen radicals in chilling-induced injury of hemopoietic cell lines: A model for reperfusion injury. Presented at the 2nd International Congress of the Society for Organ Sharing in Vancouver, Canada (1993) (Abstract).

5. Amelioration of cell and tissue damage during non-freezing cold injury and frostbite. (Abstracts and Articles)

Type I antifreeze protein attenuates dimethylsulfoxide-induced cryoprotection in hemopoietic cells. *Transplantation Proceedings* (1993) (In Press).

Type I antifreeze protein effects cell recoveries following cryopreservation by modulation of ice formation. Presented at the 2nd International Congress of the Society for Organ Sharing in Vancouver, Canada (1993) (Abstract).

Synergistic interaction of low molecular weight polyvinylpyrrolidones and dimethylsulfoxide during cell cryopreservation. *Transplantation Proceedings* (1993) (In Press).

Synergistic interaction of low molecular weight polyvinylpyrrolidones and dimethylsulfoxide during cell cryopreservation. Presented at the 2nd International Congress of the Society for Organ Sharing in Vancouver, Canada (1993) (Abstract).

Type I antifreeze protein attenuates dimethylsulfoxide-induced cryoprotection in hemopoietic cells. 30th Meeting of the Society for Cryobiology in Atlanta, Georgia. *Cryobiology* (1993) (Abstract In Press).

APPENDIX 2

Effects of Hypothermia upon Endothelial Cells: Mechanisms and Clinical Importance.

T.N. Hansen, P.E. Dawson and K.G.M. Brockbank
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Abstract

The endothelial cell is vital in the regulation of blood vessel wall structure, vasomotor tone, and thrombogenicity. Hypothermic temperatures alter both the physiological and biochemical dynamics of endothelial cells. However, there has been no systematic investigation of the influence of cold temperatures upon endothelial cell biology. This review summarizes the current clinical areas of interests, identifies the problems, and addresses the fundamental requirement for further research in endothelial cell cryobiology.

Introduction

The endothelium is the principal regulator of the vasculature. Endothelial cells are continuously interacting with components of the blood and with smooth muscle cells of the vessel wall (39, 61). Understanding the pathophysiology of hypothermic tissue injury demands an assessment of the complex interactions between the endothelial, smooth muscle, and blood cells. Such studies using

cell culture, isolated blood vessels, and vascularized organs should allow the rational design of preventative and therapeutic protocols to counter the adverse effects of accidental (uncontrolled) hypothermia and iatrogenic (controlled) hypothermia.

The observations that hypothermia in the absence of ice formation reduces tissue metabolic demands and can delay or offset cell death have increased its use clinically and within the laboratory (4, 52). However, hypothermic temperatures with and without ice formation can result in cell death (40). Local cooling of the vasculature results in reduced blood flow due to an associated increase in blood viscosity and local vasoconstriction (28, 37, 38). Intravascular stasis may develop, which will result in localized tissue anoxia (38) and metabolic acidosis (40). The situation may be further compounded by the formation of extracellular ice which, upon thawing, damages the intima of blood vessels and in particular leads to the loss of endothelial integrity (8, 40, 51). In addition, extracellular ice can induce tissue edema on thawing. The loss of endothelial control and tissue edema compromises the body's ability to re-open the microvasculature (the "no reflow" phenomenon), leading to enhanced tissue ischemia and cell death. These cold and freezing injuries are manifested in situations of uncontrolled and controlled hypothermia associated with trench foot and cardioplegia, respectively.

Exposure to relatively cold temperatures alters the

physiological and biochemical characteristics of cells with loss of transmembrane ion gradients and membrane barrier functions as well as activation of phospholipases (23, 27, 50). Some reports have suggested that organ injury after cooling can be attributed to hypothermia-induced damage to endothelium and the vessel wall (1, 2, 6, 11, 24, 34, 48, 59). Numerous reports have emphasized the importance of endothelial cell preservation in the prevention of post-transplantation diseases (1, 11, 13, 31, 36, 42, 46, 56, 59). Although hypothermic conditions are commonly encountered in clinical situations (Table 1), little is known about the effects of chilling and hypothermia on endothelial cells. This review outlines the present knowledge of the physical and biochemical responses of endothelial cells to hypothermia.

Morphologic studies of cold stored blood vessels suggest that endothelial cells can tolerate short-term storage at 4°C. However, with increasing cold storage times, the endothelial cells swell and demonstrate cytoplasmic vacuolation. With even longer durations of storage cytoplasmic organelles become disrupted, the endothelial cell nuclei become hyperplastic, and the endothelium detaches (5, 48, 58). In perfused human hearts (5) and rat livers (41), the endothelium detached after 3 to 8 hours at 4°C, while in human kidneys (20), dog saphenous veins (9), rabbit aortas (58), and rat lungs (48), it detached after 1 to 4 days.

There are a number of cellular changes associated with the

pathophysiology of cold-induced injury of endothelial cells (Table 2). Hochachka (23) has suggested that at low temperatures there is a loss of cell energy and that transmembrane ion pumps become inactivated. The result is a substantial change in intracellular ion concentrations. An increase in cytosolic free calcium concentration ($[Ca^{2+}]_i$) could result in disruption of the cellular cytoskeleton and activation of phospholipases, proteases, and endonucleases (43, 45), which would further compromise membrane and organelle functions. Endothelial cells chilled to 22°C and stimulated with bradykinin or thapsigargin have a significantly higher $[Ca^{2+}]_i$ compared to endothelial cells at 37°C (60). In contrast, unstimulated basal $[Ca^{2+}]_i$ remained unchanged. A change in $[Ca^{2+}]_i$ could activate phospholipases which, in turn, may cause the rapid release of internal calcium stores (3, 53, 54).

Chilling not only changes the function of the membrane associated enzymes, but is also associated with physical changes in the membrane. In general, cell membranes at normothermia are in a liquid-crystalline phase and upon cooling undergo a transition to a gel phase (14, 50). It has been shown that the higher the phase transition temperature of the cell membrane, the greater the chilling sensitivity of the cell (14, 22, 47). Studies employing various cell types have shown that chilling-induced injury is biphasic, related to the membrane phase transition temperature (32, 33). Various mechanisms explaining the chilling sensitivity of cells involving the cell membrane have been proposed, including

increased leakage of ions (7), membrane ion pump shutdown (26, 30), and phospholipase A₂ activation (27, 44). The membrane phase transition temperature for whole cells has been measured by Fourier transform infrared spectroscopy (FTIR) (14, 22). Human umbilical vein endothelial cells (HUVEC) have a membrane phase transition centered at 15.9°C. This membrane phase transition temperature has been linked with high short-term survival in other cell types when incubated at 4°C (22). In addition, the ability of HUVEC to survive at 23 and 4°C has been measured (Figure 1). Incubation of cells at 23°C resulted in a linear and gradual decrease in surviving cells with time, a phenomenon not noted at 4°C. Seventy nine percent of the endothelial cells survived at 4°C for 48 hours, in agreement with the prediction based on the membrane phase transition temperature. However, beyond 48 hours at 4°C the cell survival rate dropped. This delayed endothelial cell injury is confirmed by morphological studies, which have documented cell damage after 40 hours or more at 4°C (20, 48, 58).

In conclusion, there is little information on the functional consequences of cold storage and hypothermia in endothelial cell cultures *in vitro* and even less on their performance in whole vessel preparations or *in vivo*. In view of the importance of endothelial cells in vascular biology and thus in applied cryobiology, major research efforts are required.

Acknowledgement

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Figure 1. Human umbilical endothelial cell survival curve after 37, 23 and 4°C incubation. The cells were incubated for various lengths of time at 37°C (open bars), room temperature (about 23°C) (hatched bars), and 4°C (cross-hatched bars) in Dulbecco's modified Eagle's medium (D-MEM) containing 10% fetal bovine serum and 20 mM HEPES, pH 7.4 at 37°C. After incubation, the medium was removed and replaced with serum free endothelial basal medium (Clonetics). After 1 hour at 37°C, the medium was replaced with endothelial growth medium (Clonetics) containing ³H-glycine. The cells were incubated for 24 hours at 37°C, harvested, and the incorporated radioisotope determined by scintillation counting. DPM: disintegrations per minute. *: p < 0.05 compared to 37°C control; **: p < 0.05 compared to room temperature (23°C) sample; n = 8.

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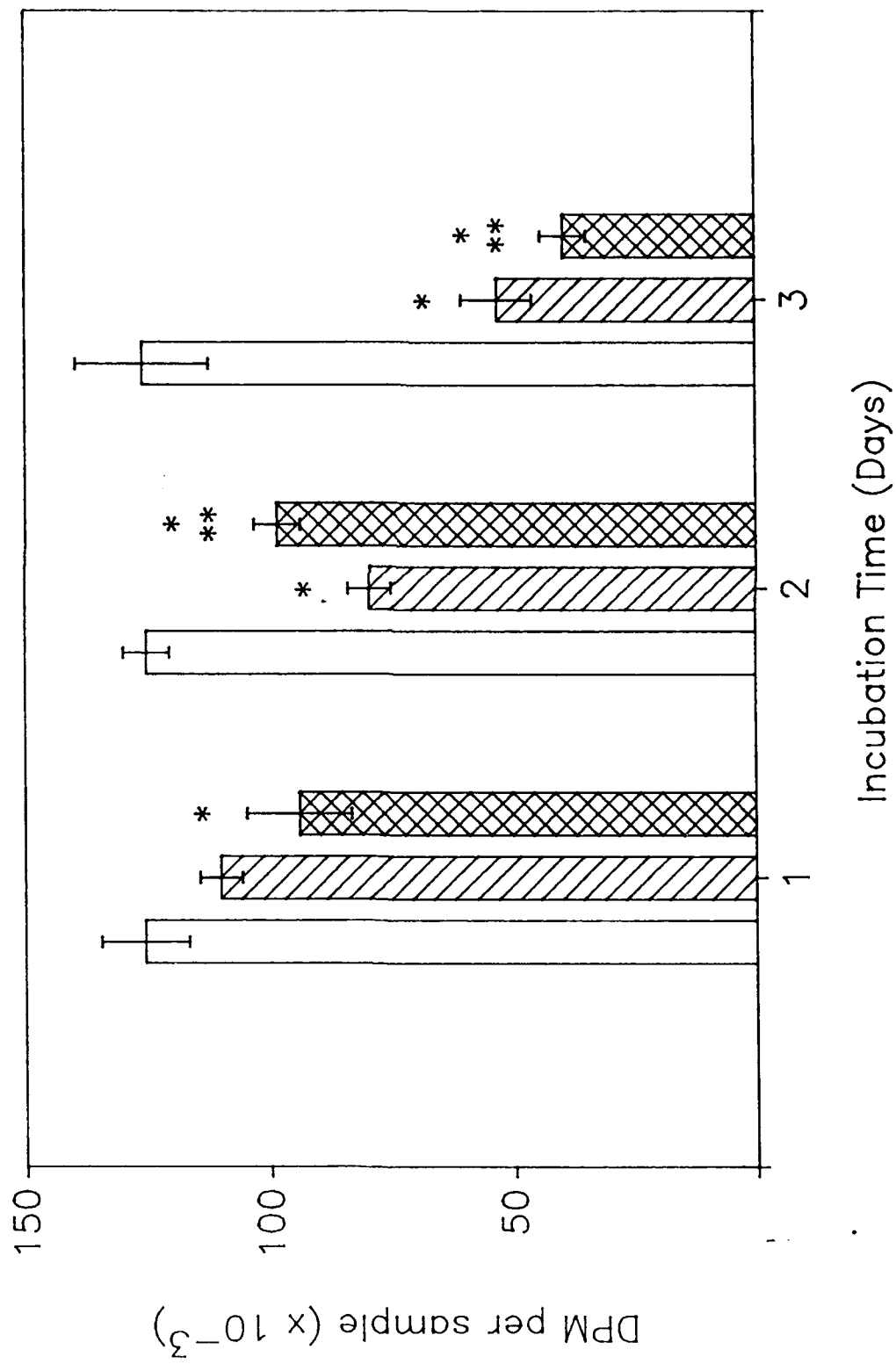
(1943) .

Table 1: Clinically important situations in which endothelial cells are exposed to low temperatures.

Area	References
Perfusion and storage solution formulation	2, 12, 15, 29, 57
Tissue and organ storage	41, 48, 58
Cryopreservation	10, 16, 17, 19, 35, 49
Accidental hypothermia	24, 28, 38, 62
Deep hypothermia	
Trench foot	
Immersion foot	
Frostbite	
Clinically induced hypothermia (surgical procedures)	6, 21, 25, 55

Table 2: Cellular Changes Associated with Low Temperature Exposure of Endothelial Cells.

Cellular Change	References
Loss of energy charge	23
Membrane pump shutdown	23, 26, 30
Cytosolic ion concentrations	7, 23, 40
Enzyme activation	3, 27, 43, 44, 45, 53, 54
Eicosanoid metabolism	17, 19, 60
Cytoskeletal disassembly	18
Free radical formation	48
Membrane phase transition	14, 22, 47, 50
Membrane barrier function loss	7, 50
Cell and organelle swelling; Cell detachment and death	5, 9, 20, 41, 48, 58



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The role of platelets in chilling-induced injury.

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Supported by Grant # N00014-91-C-0044 from the Office of Naval Research.

Hypothermia in the absence of freezing can lead to extensive tissue injury. In vitro studies have suggested that platelets may play a role in tissue damage during peripheral cold injury to humans. For example, washed platelets aggregated spontaneously with stirring when cooled either to 20°C and tested at 20°C or to 4°C and tested at 37°C^{1,2} (Table 1).

We initially hypothesized that chilling of platelets would result in an increase in free intracellular calcium. The increase could be due to either increased membrane permeability³ or to inhibition of transmembrane ion pumps⁴. Analysis of cytosolic free calcium revealed similar levels of calcium in platelets at 37 and 20°C, and calcium concentrations were only slightly elevated in platelets after a 3 hour pre-incubation at 4°C (Table 1). The values did not change during 30 minute incubations. Thus, spontaneous aggregation of chilled platelets was not associated with an initial increase of intracellular calcium concentrations.

Alternately, chilling-induced platelet aggregation may be caused by phospholipase A_2 activation in chilled preparations^{5,6}. The lipase cleaves phosphatidylcholine to form arachidonic acid, which in turn is metabolized to thromboxane A_2 . Accumulation of thromboxane B_2 , the stable hydrolysis product of thromboxane A_2 , was observed in platelets incubated at 37°C after 60 minutes, but not in chilled platelets (Table 1). Yet, the platelets at 20°C and pre-incubated at 4°C had greater spontaneous aggregation than the platelets at 37°C, suggesting that the aggregation associated with chilling is not due to an initial production of thromboxane A_2 .

Platelet sensitivity upon chilling is associated with a physical decrease in the membrane fluidity. Isothermal experiments in which the membrane fluidity of platelets is altered suggest an increase in platelet aggregation with a decrease in fluidity^{7,8}. FTIR analysis of the platelet membrane revealed a monotonic decrease in the CH₂ vibrational peak position with temperature (Figure 1). The data suggest that the platelet membrane underwent a broad phase transition as it was cooled, with a mid-point at $18.7 \pm 1.05^{\circ}\text{C}$ ($n = 12$), close to the temperature at which maximal spontaneous platelet aggregation is observed². This correlation suggests that the increased platelet aggregation induced by chilling is due to a thermotropic decrease in the platelet phospholipid fluidity.

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Figure Legend

Membrane Phase Transition of Human Platelets Measured by Fourier Transform Infrared Spectroscopy.

Top: FTIR spectra of platelets. At each temperature a spectrum was obtained consisting of the average of 32 scans. Four peaks in the C-H stretch region were identified: CH_3 asymmetric stretch at 2960 cm^{-1} , CH_2 asymmetric stretch at 2920 cm^{-1} , CH_3 symmetric stretch at 2870 cm^{-1} , and CH_2 symmetric stretch at 2850 cm^{-1} . The CH_3 stretch peaks are associated primarily with protein, while the CH_2 stretch peaks are associated primarily with lipids¹⁰. Two spectra are presented, one taken at 39°C (dashed line) and the other at 4°C (dotted line), showing the thermotropic shift in the lipid peaks. Bottom: Graph of the CH_2 symmetric stretch peak center versus temperature. The graph was used to calculate the membrane phase transition, and revealed a monotonic shift in the lipid peak with temperature, with a slight plateau around the liquid-crystalline to gel phase transition temperature of 18.7°C .

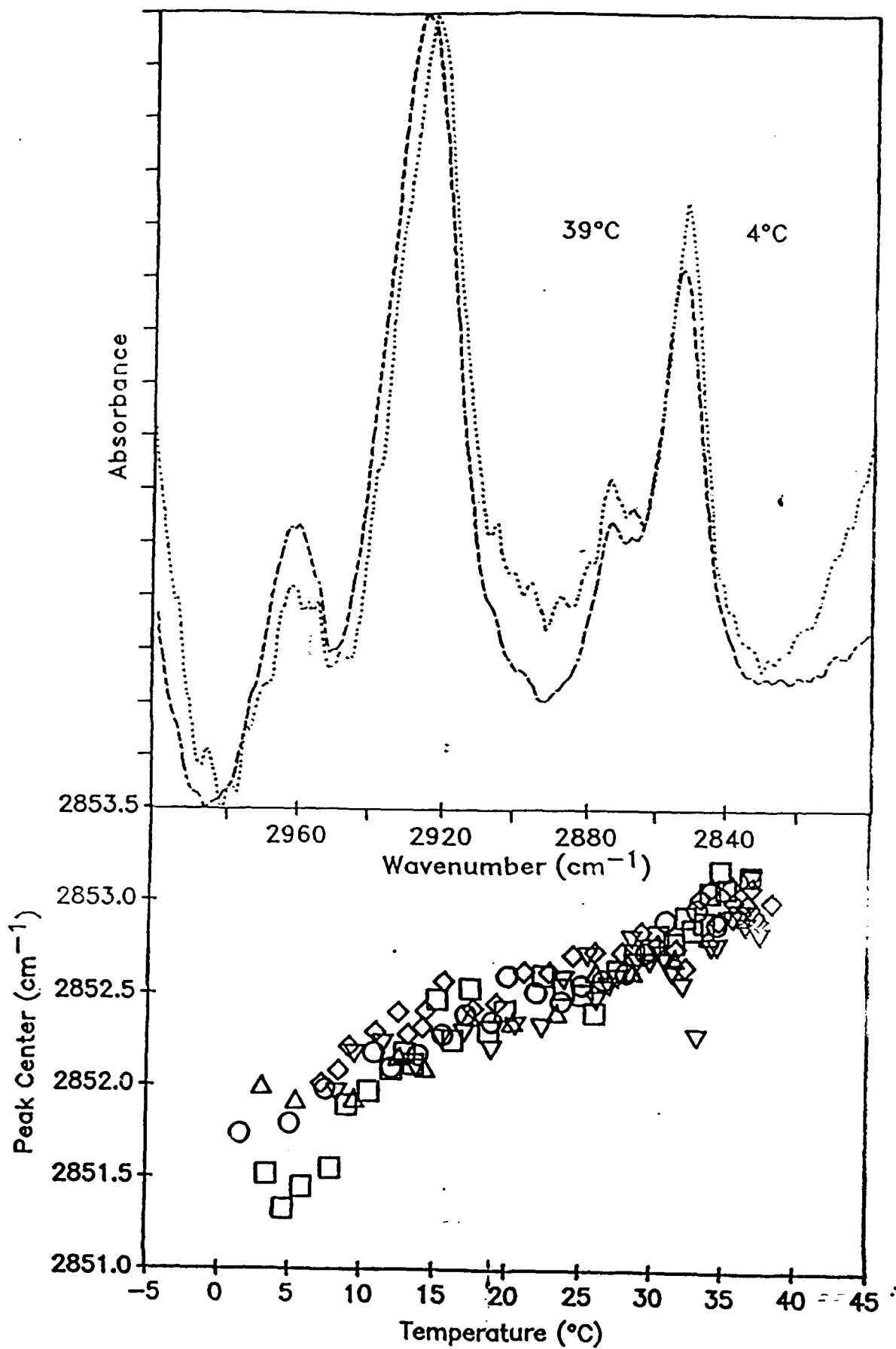
Table Legend

Spontaneous aggregation of washed platelets was measured after 5 minutes in an aggregometer (Chrono-log Corporation, Havertown, PA USA). 3×10^8 platelets/ml containing 1 mM CaCl_2 and 14 $\mu\text{g/ml}$ fibrinogen were tested for aggregation in the absence of agonist either at 37°C with or without a 4°C pre-incubation of 3 hours or at 20°C. Cytosolic free calcium concentrations were determined using the fluorescent probe Fura-2⁹.

Thromboxane concentrations were determined by radioimmunoassay at 0 and 60 minutes after incubation (E.I. Du Pont de Nemours & Co., Boston, MA USA). The values presented are the mean $\pm$ 1 standard error of the mean. Unless indicated, $n=3$. ** $p \leq 0.01$, * $p \leq 0.05$ compared to 37°C controls.

**Spontaneous Aggregation, Cytosolic Free Calcium Concentration, and Thromboxane
Production in Washed Platelets.**

Incubation Conditions	Time (min)	% Aggregation (n)	Cytosolic [Ca ²⁺] (nM)	Thromboxane B ₂ (pmole/ml)
37°C	0	3.1 ± 0.7 (19)	71 ± 2.9	3.18 ± 0.54
	60			19.2 ± 0.96
20°C	0	16.9 ± 4.4 (11)**	66 ± 3.9	2.2 ± 0.65
	60			2.1 ± 0.05
4°C → 37°C	0	13.8 ± 3.9 (10)*	94 ± 2.1	<1.21 ± 0.00
	60			3.05 ± 0.08



Subject Index

Hypothermia, Cell Membrane, Non-freezing cold injury, FTIR, lactate dehydrogenase, mitochondrial dehydrogenase.

Title

Correlation between chilling-induced injury in human cells and phospholipid membrane phase transition.

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Abstract

The physical transition of the cell membrane phospholipids from liquid-crystalline to gel phase has been proposed as a mechanism for chilling-induced injury in cells in the absence of ice formation. We investigated the role of the membrane phase transition temperature in relation to cell survival after chilling to 4°C. The thermotropically induced membrane phase transition temperature was measured using Fourier transform infrared spectroscopy (FTIR). Survival was assessed by a combination of Trypan blue exclusion, lactate dehydrogenase leakage into the supernatant, and mitochondrial activity. The data suggested that the higher the membrane phase transition, the greater the chilling sensitivity of the cell line. For example, MOLT-4, an acute human lymphoblastic leukemia, had a mean membrane phase transition of 20.2°C and K-562, a chronic human myelogenous leukemia, had a mean membrane phase transition of 14.9°C. MOLT-4 cells were very sensitive to chilling, with 29% survival after 24 hours at 4°C based on Trypan blue exclusion, compared to K-562, with 68% survival after the same incubation period. In addition, KG-1a, a human acute myelogenous leukemia, had a membrane phase transition temperature of 9.2°C, and had 82% survival after 24 hours at 4°C. These data support the hypothesis that relatively high membrane phase transitions correlate with increased cell sensitivity to chilling.

Introduction

Chilling in the absence of ice crystal formation can lead to cell injury and death. Many mechanisms have been proposed for chilling-induced injury, including a role for the physical transition of the membrane phospholipids from liquid-crystalline to gel phase¹. Relatively high membrane phase transition temperatures have been correlated with greater chilling sensitivity². Mechanisms by which the membrane phase transition may cause cellular injury include the phase separation of lipids into domains of gel phase lipids surrounded by liquid-crystalline phase lipids¹. It has been proposed that such phase separation-induced membrane defects may lead to a loss of membrane barrier function (i.e., leakiness)³. In vitro studies also suggest that non-freezing cold injury and death in cells may be caused by the activation of phospholipases at the membrane phase transition point^{4,5}.

We investigated the role of the membrane phase transition temperature in relation to cell survival after chilling to 4°C. In this study three hemopoietic cell lines were employed: KG-1a, an acute myelogenous leukemia from bone marrow; K-562, a chronic myelogenous leukemia; and MOLT-4, an acute lymphoblastic leukemia from peripheral blood. The membrane phase transition of the three cell lines was monitored using Fourier transform infrared spectroscopy (FTIR)². In a parallel study, cell survival after chilling was also determined. The results suggest that the lipid

phase transition of these hemopoietic cell lines correlates with chilling sensitivity of these cells.

Materials and Methods

Three human hemopoietic cell lines were investigated: KG-1a (American Type Culture Collection (ATCC) CCL 246.1), K-562 (ATCC CCL 243), and MOLT-4 (ATCC CRL 1582). The cells were maintained at 37°C in a 5% CO₂ atmosphere in either Iscove's modified Dulbecco's medium (KG-1a) or RPMI-1640 (K-562 and MOLT-4), supplemented with 10% fetal bovine serum. The cells were changed to fresh media one day prior to experimentation.

Cellular membrane phase transitions were monitored by FTIR. Spectra were collected on a Perkin-Elmer FTIR 1600 microscope fitted with a Peltier cooling stage. The microscope allowed for small sample size (about 80 cells in the beam) without loss of sensitivity. The cells were pelleted and placed between two BaF₂ windows. One window had a groove etched out with a thermocouple placed inside the groove near the sample. Three spectra, each consisting of the average of 32 scans, were collected at each temperature. The thermotropic shift in the CH₂ symmetric peak stretch, centered around 2850 cm⁻¹, was monitored. The center of the peak was calculated based on a "center of gravity" routine using the top 10% of the peak².

In parallel experiments, cells were incubated at either room temperature (about 23°C) or at 4°C for cell survival analysis. Samples were taken every 24 hours. For the experiments, 5×10^5

cells/ml were incubated in T-25 flasks in media buffered with 20 mM HEPES. Cell survival after incubation at room temperature or 4°C was determined by Trypan blue (TB) exclusion, lactate dehydrogenase (LDH) leakage into the supernatant, and mitochondrial dehydrogenase activity. TB exclusion was determined by diluting cells 1:10 in Trypan blue and counting cells excluding the dye in a hemocytometer. Survival was calculated by comparing the number of TB-negative cells after incubation to pre-incubation values. LDH activity was determined by the rate at which NADH is converted to NAD in the presence of pyruvate. The reaction was monitored at 340 nm using a Beckman DU-64 Spectrophotometer. LDH activity was measured in cell-free supernatant and in lysed (sonicated) samples. Cell survival was calculated as the ratio of supernatant LDH to total LDH activity. MTT (thiazoly blue) was obtained from Promega Corporation (Madison, WI, USA). Mitochondrial dehydrogenase activity was determined by the conversion of MTT tetrazolium salt to a blue formazan derivative following the manufacture's instructions. The reaction was monitored at 590 nm using a Titertek Multiscan MCC/340 96 well plate reader. Cell survival was calculated as the ratio of mitochondrial dehydrogenase activity in the post-chilling samples compared to pre-incubation activity.

Results

FTIR spectra were collected for all three cell lines. A typical spectrum for K-562 (Figure 1) revealed four major absorption peaks². Similar spectra were collected from the other cell lines. The center of the CH₂ symmetric peak stretch, located at 2850 cm⁻¹, was measured. A graph of the thermotropically-induced shift in the peak center was used to calculate the phase transition temperature of the phospholipid membranes (Figure 2). The calculated membrane phase transition temperature for the three cell lines is presented in Table 1. Cell survival after 24 hours at 4°C, measured by Trypan blue exclusion, suggests that the higher the membrane phase transition temperature, the greater the susceptibility to chilling (Table 1).

To examine further the correlation between the membrane phase transition and the chilling sensitivity of the cell lines, cell survival after 4°C incubation was measured by LDH leakage into the supernatant and by mitochondrial dehydrogenase activity (Figures 3 and 4). Both assays revealed that the cell lines incubated at room temperature had a decrease in survival over 3 days. However, MOLT-4 cells showed a much greater decrease, with less than 20% of the cells surviving. All three cell lines showed a greater decrease in survival over time after incubation at 4°C, when compared to the room temperature incubated cells. Again, the MOLT-4 cells, with the highest membrane phase transition temperature, were the most

sensitive to chilling, while the K-562 cell line was slightly more sensitive to chilling than the KG-1a cell line. When the samples were corrected for cell counts, no difference in mitochondrial dehydrogenase activity was noted (data not shown), demonstrating that the assays were an indication of live cells.

Discussion

The data presented suggest that the membrane phase transition temperature of the three hemopoietic cell lines examined is inversely proportional to chilling sensitivity in the cell line. Using Chinese hamster fibroblasts as a model, Kruuv and coworkers^{6,7} suggested a biphasic effect of chilling-induced injury, based on the membrane phase transition temperature of the fibroblasts, with greater injury at temperatures below the phase transition temperature. Consistent with their predictions, incubation of the hemopoietic cell lines at either room temperature or at 4°C resulted in a decrease in cell survival after 3 days, with a much greater decrease in survival for the cell lines incubated below the membrane phase transition temperature (Figures 3 and 4).

Although the results suggest a correlation between chilling sensitivity of the cell lines and their membrane phase transition temperature, the data do not reveal what the mechanism by which chilling-induced injury occurs. It has been suggested that the chilling-induced injury is associated with a loss of membrane-linked functions and ion gradients⁸. A decrease in ion membrane gradients would most likely be due to a combination of loss of membrane barrier function and a shutdown of membrane ion pumps⁹. In vitro and in situ preparations have also suggested that phospholipases may be activated at the membrane phase transition temperature^{4,5,10}. It is possible that activation of phospholipases

occurred in the MOLT-4 cell line incubated at room temperature, which was near their membrane phase transition temperature (Table 1). Even though the cells incubated at 4°C were not near the membrane phase transition temperature, where less phospholipase activity would be expected^{4,5}, the broad phase transition temperature measured in the cell lines suggests that some activation of phospholipases could have occurred.

In summary, the membrane phase transition temperature in three hemopoietic cell lines was measured by FTIR. The transition temperature was inversely related to the chilling sensitivity of the cells. Although no specific mechanism of chilling-induced cellular injury and death was determined in this study, the data suggest an association with loss of cell membrane-linked functions. Further investigations to determine the mechanism(s) of chilling-induced nonfreezing cold injury in hemopoietic cell lines are in progress.

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Figure Legend

Figure 1: FTIR spectra for K-562 cells. Two spectra are presented, one at 39°C (dashed line), and the other at 0.7°C (solid line). Four peaks in the C-H stretch region were identified: CH₃ asymmetric stretch at 2960 cm⁻¹, CH₂ asymmetric stretch at 2920 cm⁻¹, CH₃ symmetric stretch at 2870 cm⁻¹, and CH₂ symmetric stretch at 2850 cm⁻¹. The CH₃ stretch peaks are associated primarily with proteins, while the CH₂ stretch peaks are associated primarily with lipids². The CH₂ stretch peaks shifted with temperature.

Figure 2: Thermotropic induced shift in the CH₂ symmetric stretch peak center of K-562 cells. The peak center was calculated using a "center of gravity" routine². Each point represents the mean ± 1 SD of the peak center of 3 different spectra. The membrane phase transition temperature was calculated from the graph as the midpoint of the thermotropic shift in peak center. The shift in the peak center was gradual, suggesting a broad phase transition.

Figure 3: Chilling-sensitivity of three hemopoietic cell lines based on lactate dehydrogenase leakage from cells. LDH activity was measured in cell free supernatant and in lysed preparations after incubation at either room temperature (top) or 4°C (bottom). The data indicate that MOLT-4 are more sensitive to chilling than K-562 and KG-1a. Each point represents the mean ± 1 SD (n=3).

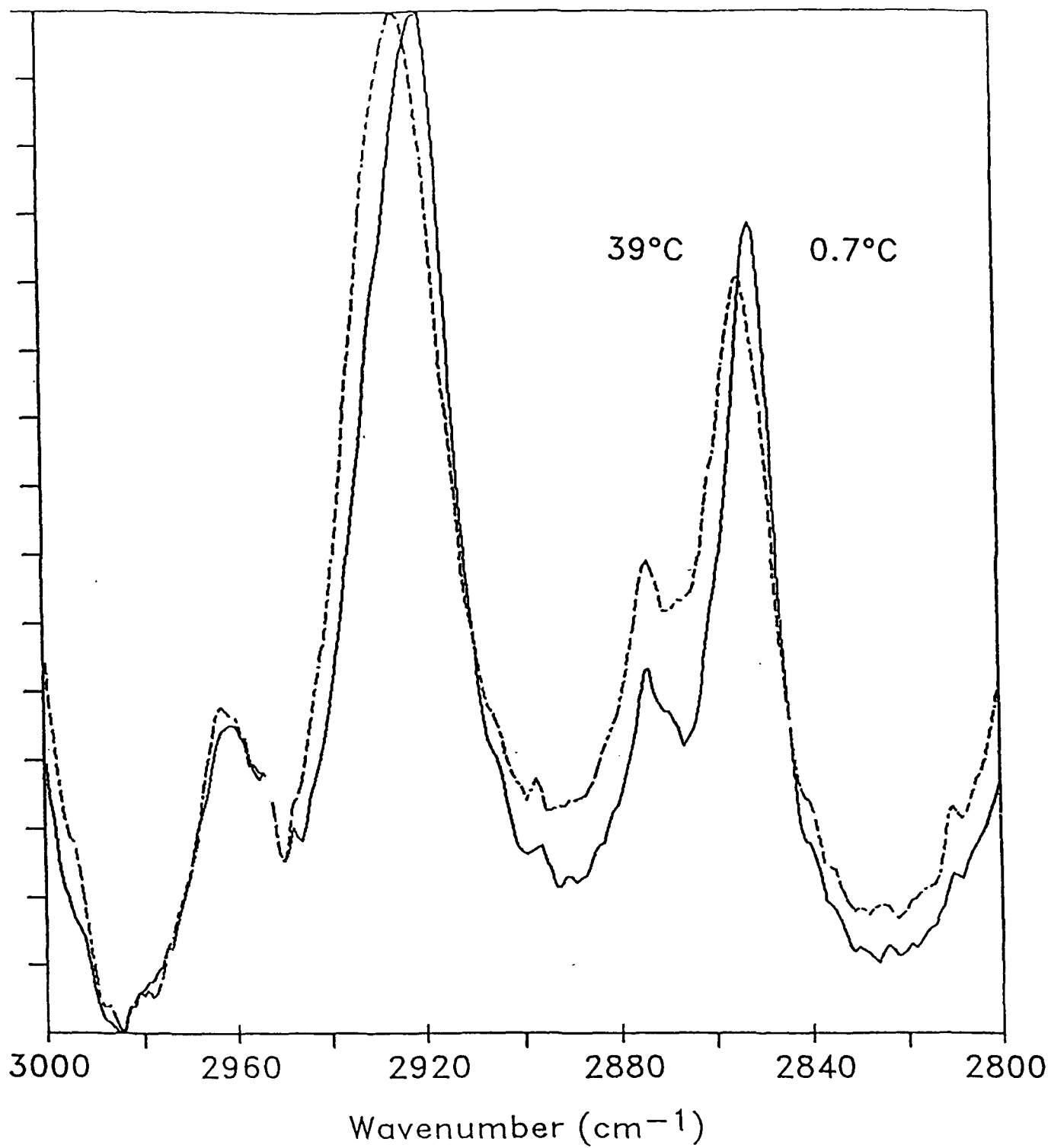
Figure 4: Chilling-sensitivity of three hemopoietic cell lines based on mitochondrial dehydrogenase activity. Mitochondrial dehydrogenase activity was measured in samples after incubation at either room temperature (top) or 4°C (bottom). KG-1a were the least sensitive to chilling, while MOLT-4 were the most sensitive to chilling. Each point represents the mean $\pm$ 1 SD (n=3).

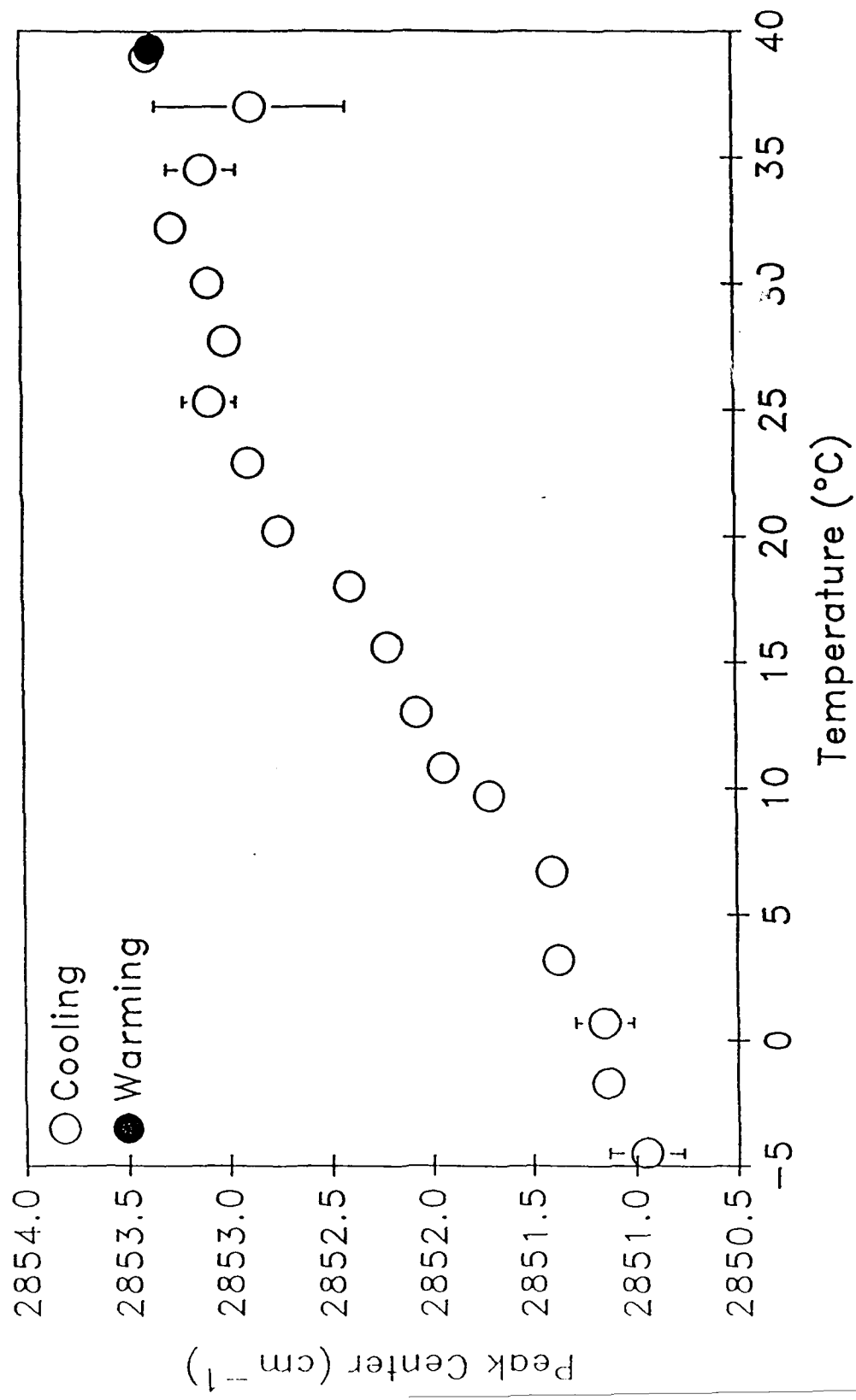
Table 1: Thermotropic membrane phase transition temperatures and cell survival of three hemopoietic cell lines.

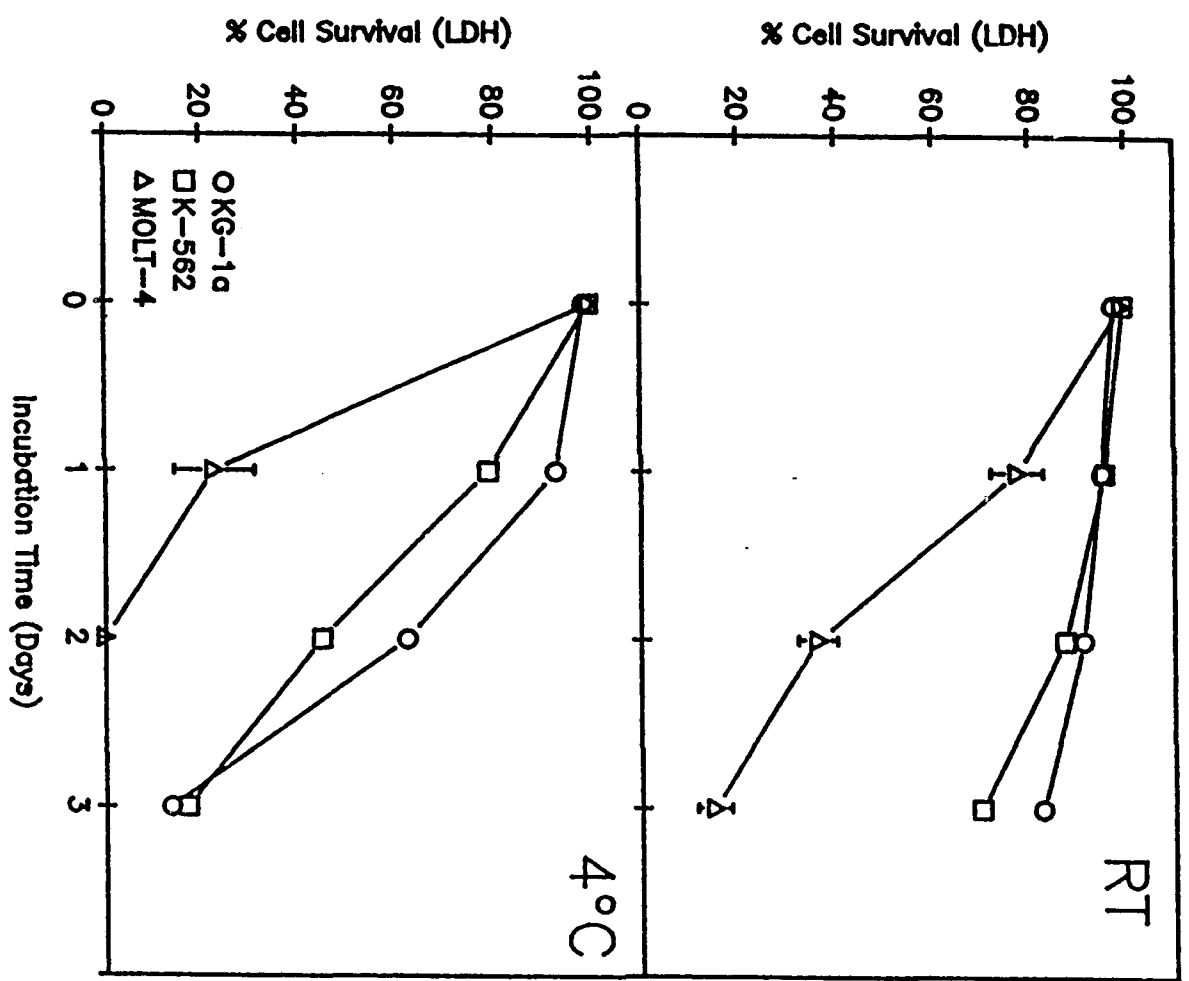
Cell Line	Membrane Phase Transition ($^{\circ}\text{C}$) ¹	% Survival ²	
		RT	4 $^{\circ}\text{C}$
KG-1a	9.1 $\pm$ 0.0	84	82
K-562	14.9 $\pm$ 1.9	97	68
MOLT-4	20.2 $\pm$ 3.1	60	29

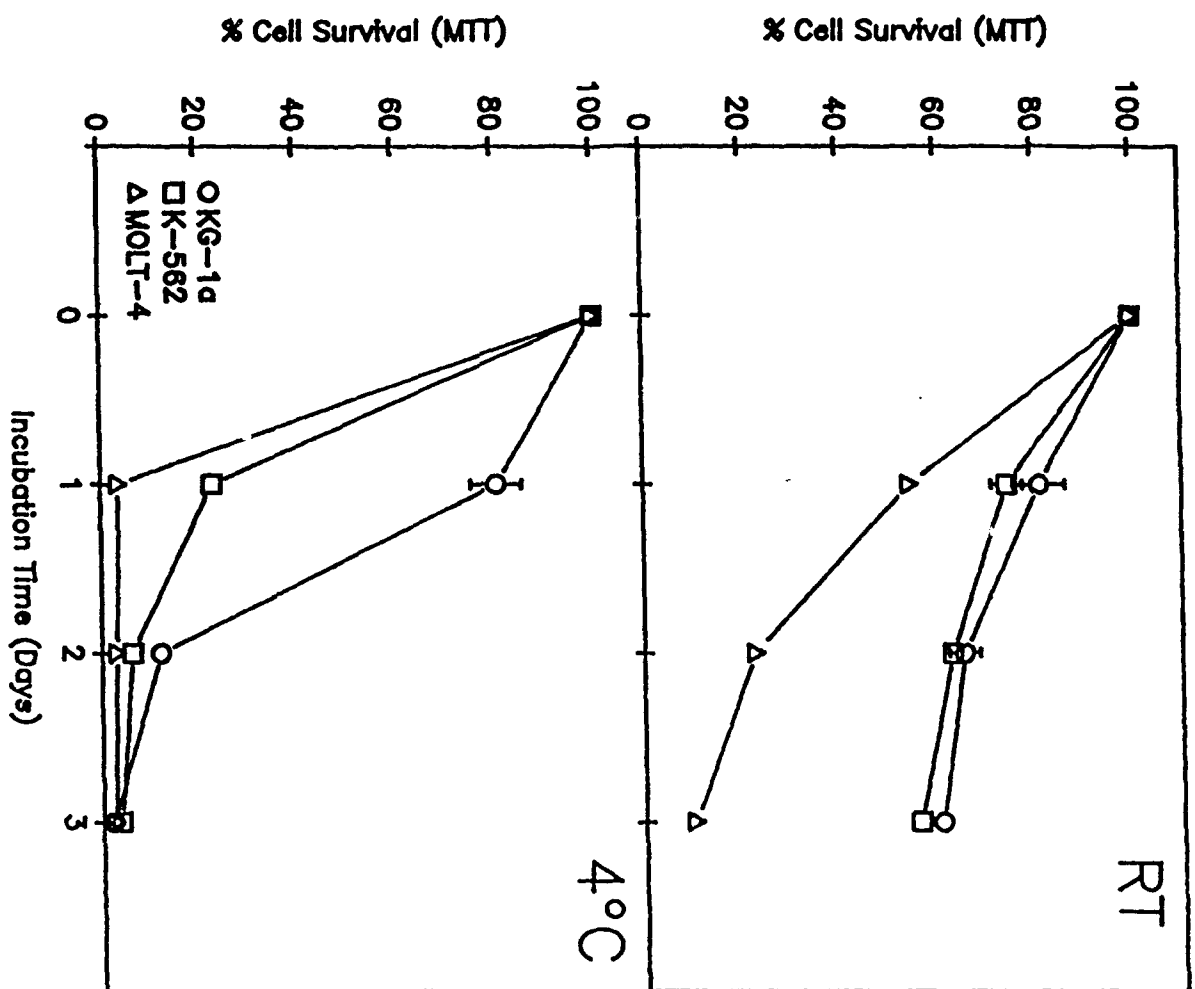
1: Mean $\pm$ 1 SD; n=3.

2: Mean cell survival of 2 independent experiments was measured by Trypan blue exclusion after 24 hours incubation at either room temperature (RT) or at 4 $^{\circ}\text{C}$.









Chilling-induced injury in hemopoietic cell lines correlates with phospholipid membrane phase transition temperature.

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Chilling in the absence of ice formation can lead to cell death. Many mechanisms have been proposed for chilling-induced cell death, including a role for the physical transition of the membrane phospholipids from liquid-crystalline to gel phase. Relatively high membrane phase transition temperatures have been correlated with greater chilling sensitivity. We investigated the role of the membrane phase transition temperature in relation to cell survival after chilling to 4°C. In this study two hemopoietic cell lines were employed: MOLT-4, an acute human lymphoblastic leukemia, and K-562, a chronic human myelogenous leukemia. MOLT-4 cells were very sensitive to chilling, with less than 20% survival after 24 hours at 4°C, compared to K-562, with greater than 80% survival after the same incubation period. We tested the hypothesis that the greater chilling sensitivity of MOLT-4 was associated with a higher membrane phase transition temperature. The thermotropically induced membrane phase transition temperature in both cell lines was measured using Fourier transform infrared spectroscopy (FTIR). Although the liquid-crystalline to gel phase transition was broad in both cell lines, the phase transition was about 5°C higher for MOLT-4 ($20.2 \pm 3.1^\circ\text{C}$, $n=3$) than for K-562 ($14.9 \pm 1.9^\circ\text{C}$, $n=3$). Further investigation of the role of membrane phase transition temperature and cell survival during chilling was performed by incubation of the hemopoietic cell lines with membrane fluidizers. MOLT-4 survival was not affected by the presence of fluidizers. In contrast, K-562 survival was enhanced. These data support the hypothesis that higher membrane phase transitions correlate with greater cell chilling sensitivity. Supported by Contract N00014-91-C-0233 from the Office of Naval Research.

RTM Sts 5/7/93

AH X/S/Lease 4/27/93

KB KOMB 4/26/93

Submitted Cryo '93

Membrane Phase Transition Temperature Predicts Chilling Sensitivity in Hemopoietic Cell Lines.

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The physical transition of the cell membrane phospholipids from liquid-crystalline to gel phase has been proposed as a mechanism for chilling-induced injury in cells in the absence of ice formation. We investigated the role of the membrane phase transition temperature in relation to cell survival after chilling to 4°C. The thermotropically induced membrane phase transition temperature was measured using Fourier transform infrared spectroscopy (FTIR). Survival was assessed by a combination of Trypan blue exclusion, lactate dehydrogenase leakage into the supernatant, and mitochondrial activity. The data suggested that the higher the membrane phase transition, the greater the chilling sensitivity of the cell line. For example, MOLT-4, an acute human lymphoblastic leukemia, had a mean membrane phase transition of 20.2 and K-562, a chronic human myelogenous leukemia, had a mean membrane phase transition of 14.9. MOLT-4 cells were very sensitive to chilling, with less than 20% survival after 24 hours at 4°C, compared to K-562, with greater than 80% survival after the same incubation period. These data support the hypothesis that relatively high membrane phase transitions correlate with increased cell sensitivity to chilling. Supported by Contract N00014-89-C-0233 from the Office of Naval Research.

The role of oxygen radicals in chilling-induced injury of hemopoietic cell lines.

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Non freezing cold injury can lead to cell death. One proposed mechanism of injury during chilling is the formation of oxygen radicals. We examined the role of oxygen radicals in induction of cell damage during low temperature storage. Two hemopoietic cell lines were tested: MOLT-4, an acute human lymphoblastic leukemia, and K-562, a chronic human myelogenous leukemia. MOLT-4 are more chilling sensitive than K-562. The cells were incubated at either room temperature or at 4°C in the presence of either a hydrophilic free radical scavenger (dimethyl sulfoxide or catalase) or a hydrophobic free radical scavenger (α -tocopherol or butylhydroxyanisole (BHA)). Little or no increase in cell survival was observed after incubation with hydrophilic scavengers, while a large increase in cell survival was observed after incubation at 4°C in the presence of hydrophobic free radical scavengers. Since hydrophobic free radical scavengers associate with cell membranes, the data suggest that the site of free radical damage in these cell lines is in the cell membrane. FTIR measurements of the thermotropically induced membrane phase transition in the presence of α -tocopherol or BHA were undertaken to determine whether the increased cell survival at 4°C in the presence of the hydrophobic free radical scavengers could be explained by increased membrane fluidity. α -tocopherol fluidized the cellular membrane of MOLT-4, but not of K-562, while BHA did not fluidize the membrane of either cell line. These data suggest that oxygen radical induced-membrane damage plays a major role in the pathophysiology of non freezing cold injury. Supported by Contract N00014-91-C-0233 from the Office of Naval Research.

RTM Ats 5/1/93

AH JS 4/27/93

KB KMB 4/26/93

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The Role of Oxygen Radicals in Chilling-Induced Injury of Hemopoietic Cell Lines: A Model for Reperfusion Injury

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Non-freezing cold injury can lead to cell death. One proposed mechanism of injury during chilling is the formation of oxygen radicals. We examined the role of oxygen radicals in induction of cell damage during low temperature storage. Two hemopoietic cell lines were tested: MOLT-4, an acute human lymphoblastic leukemia, and K-562, a chronic human myelogenous leukemia. MOLT-4 are more chilling sensitive than K-562. The cells were incubated at either room temperature or at 4°C in the presence of either a hydrophilic free radical scavenger (dimethyl sulfoxide or catalase) or a hydrophobic free radical scavenger (α -tocopherol or butylhydroxyanisole (BHA)). Little or no increase in cell survival was observed after incubation with hydrophilic scavengers, while a large increase in cell survival was observed after incubation at 4°C in the presence of hydrophobic free radical scavengers. Since hydrophobic free radical scavengers associate with cell membranes, the data suggest that the site of free radical damage in these cell lines is in the cell membrane. These data suggest that oxygen radical induced-membrane damage plays a major role in the pathophysiology of chilling induced injury. Furthermore, K-562 and MOLT-4 can be used as models to screen potential adjuncts of oxygen radical scavengers for reperfusion and cold storage solutions. Supported by Contract N00014-91-C-0044 from the Office of Naval Research.

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Type I Antifreeze Protein Effects Cell Recoveries
Following Cryopreservation by Modulation of Ice
Formation

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Type I antifreeze peptide (AFP) has an α -helical structure that allows it to bind to ice. The peptide prevents ice growth and concomitant damage in fishes inhabiting ice-laden waters. We used a recombinant form of the peptide to examine whether it may help increase cell survival after cryopreservation under various conditions. We used two human cell types as models: KG-1a, a promyeloblastic cell line, and red blood cells (RBC). KG-1a were cryopreserved with the permeating cryoprotectant dimethyl sulfoxide and cooled slowly. The addition of 25 to 1000 $\mu\text{g/ml}$ AFP to the cryoprotectant solution resulted in a dose-dependent decrease in KG-1a recovery after thawing. RBC were cryopreserved with the extracellular cryoprotectant hydroxyethyl starch by plunging in liquid nitrogen. The addition of low concentrations of AFP (0 to 100 $\mu\text{g/ml}$) resulted in a gradual increase in RBC recovery. Higher concentrations of AFP (up to 1500 $\mu\text{g/ml}$) resulted in decreased RBC recovery, compared to controls with no AFP added. Cryomicroscopy of KG-1a cells indicated that cells both with and without AFP darken by about -20°C during cooling. Upon warming, samples frozen in the presence of AFP had smaller ice crystals in the extracellular space than cells frozen in the absence of AFP. Cryomicroscopy of RBC demonstrated that 60 $\mu\text{g/ml}$ AFP inhibited recrystallization during warming. Thus, higher AFP concentrations were expected to result in increased red cell survival by reducing ice recrystallization even further. Addition of 1500 $\mu\text{g/ml}$ AFP to the RBC greatly reduced ice recrystallization in the extracellular milieu. However, massive ice growth was induced around the red cells. These observations suggest that inhibition of ice recrystallization by AFP in the extracellular milieu may cause increased cell-associated ice crystal growth, resulting in greater cell loss. Supported by Contract N00014-91-C-0044 from the Office of Naval Research.

Type I antifreeze protein attenuates dimethylsulfoxide-induced cryoprotection in hemopoietic cells.

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Recombinant type I antifreeze protein (AFP) can reduce the hemolysis of human red blood cells cryopreserved with hydroxyethyl starch by partial inhibition of ice crystal growth in the extracellular space during warming (Carpenter and Hansen (1992) Proc. Natl. Acad. Sci. 89: 8953). In this report, AFP was tested during slow cooling of nucleated cells in combination with the permeating cryoprotectant dimethylsulfoxide (Me_2SO). KG-1a, a human promyeloblast cell line, was used as a model for nucleated hemopoietic cells. Recovery of cell function was determined by measuring DNA synthesis using a ^3H -thymidine incorporation assay. Me_2SO dose-response studies (0.5-5% v/v) demonstrated a correlation between cell recovery and increasing Me_2SO concentrations ($1.2 \pm 0.41\%$ to $74.1 \pm 3.70\%$, $n=4$). KG-1a cells were optimally cryopreserved in the presence of 5% Me_2SO , cooled at $3^\circ\text{C}/\text{min}$ and thawed by immersion in a 37°C water bath. The addition of 25 to 1000 $\mu\text{g}/\text{ml}$ AFP to the cryoprotectant solution resulted in a dose-dependent decrease in cell recovery after thawing. Cryomicroscopy indicated that cells both with and without AFP darken by about -20°C . Upon warming, samples frozen in the presence of AFP had smaller ice crystals in the extracellular space than cells frozen in the absence of AFP. These observations suggest that the decreased recoveries of cells frozen in the presence of AFP is associated with modulation of ice crystal size or structure by AFP. Supported by Contract N00014-91-0044 from the Office of Naval Research.

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Subject Index

Nucleated Cell Cryopreservation, Antifreeze protein, dimethylsulfoxide, Cryoprotectant, ice crystal modulation, KG-1a, Cryomicroscopy.

Title

Type I Antifreeze Protein Attenuates Cell Recoveries Following Cryopreservation

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Abstract

Type I antifreeze peptide (AFP) has an α -helical structure that allows it to bind to ice. The peptide prevents ice growth and concomitant damage in fishes inhabiting ice-laden waters. A recombinant form of the peptide was used to examine whether it may help increase cell survival after cryopreservation under various conditions. The peptide was examined using KG-1a cells, a human promyeloblastic cell line, as a model. KG-1a cells were cryopreserved with the permeating cryoprotectant dimethylsulfoxide and cooled slowly. The addition of 25 to 1000 $\mu\text{g/ml}$ AFP to the cryoprotectant solution resulted in a dose-dependent decrease in cell recovery after thawing. Cryomicroscopy of KG-1a cells indicated that cells both with and without AFP darken by about -20°C during cooling. Upon warming, samples frozen in the presence of AFP had smaller ice crystals in the extracellular space than cells frozen in the absence of AFP. Previous work with red blood cells revealed that high concentrations of AFP fostered ice crystal growth around the cells, resulting in decreased post-thaw recoveries (J.F. Carpenter and T.N. Hansen (1992) *Proc. Natl. Acad. Sci. USA* 89: 8953). These observations suggest that inhibition of ice recrystallization by AFP in the extracellular milieu may cause increased ice crystal growth associated with the cells, resulting in greater cell loss.

Introduction

Cryopreserved biological material can be damaged by ice crystal formation¹. Recent efforts in low temperature storage have focused on vitrification, where the cells or tissues are preserved in a manner to avoid any ice formation². However, the cryoprotectant concentrations necessary to achieve vitrification may be toxic. In addition, some ice formation may occur during the cooling or warming process. Knight and Duman³ suggested that many of the problems associated with ice formation during cryopreservation might be limited by the addition of antifreeze protein (AFP) to the cryoprotectant mixture.

AFPs are a class of molecules that bind to ice crystals and prevent further ice growth⁴. A recombinant form of the winter flounder type I AFP was successfully used to improve red blood cell cryopreservation during rapid cooling in the presence of the extracellular cryoprotectant hydroxyethyl starch⁵. Low concentrations of AFP (5-160 µg/ml) enhanced cryopreserved red cell survival during suboptimal thawing, while higher concentrations of AFP caused a decrease in cell survival. Cryomicroscopy suggested that AFP at low concentrations caused a partial inhibition of ice recrystallization. However, higher concentrations of AFP induced preferential growth of ice crystals around the red blood cells.

Nucleated cells are best cryopreserved using slow cooling rates and the cell permeating cryoprotectant dimethylsulfoxide (Me₂SO). We decided to examine whether AFP could have

beneficial effects during cryopreservation of cells using slow cooling rates and permeating cryoprotectants. As AFP is a relatively large molecule (3300 Da) and would not be expected to enter the cells, the protein would be confined to the extracellular milieu. KG-1a cells, a human mylogenous leukemia cell line from bone marrow, were used as a model. The cells were cryopreserved in the presence of differing concentrations of Me₂SO and AFP. The results showed that AFP did not improve cell recoveries after cryopreservation, and that high concentrations of AFP (>100 µg/ml) caused a great reduction in cell viability.

Materials and Methods

Purified (> 99% homogeneity) antifreeze peptide was a gift from Dr. J.E. Villafranca (Agouron Pharmaceuticals, Inc., La Jolla, CA). The recombinant protein was produced by expression of a synthetic gene in Escherichia coli. The protein has a molecular weight of 3300 Da, is comprised of 37 amino acids, and has an amino acid sequence identical to that of the natural Type I antifreeze protein from winter flounder (Pseudopleuronectes americanus), which is designated as HPLC-6⁶.

KG-1a cells (American Type Culture Collection CCL 246.1) were maintained at 37°C in a 5% CO₂ atmosphere in Iscove's modified Dulbecco's medium supplemented with 10% fetal bovine serum. One day before cryopreservation, the cells were split into fresh media at a density of 2×10^6 cells/ml.

Prior to freezing, the cells were transferred to Dulbecco's modified Eagle's medium (D-MEM) supplemented with 2% fetal bovine serum at a final concentration of 1×10^6 cells/ml, pelleted (300 g; 10 minutes) and resuspended in a cryoprotectant solution containing Me₂SO (0 to 5% v/v), antifreeze peptide (0-1000 µg/ml) and D-MEM supplemented with 2% fetal bovine serum. The cells were cooled to 4°C, frozen at -3°C/min to -80°C, and then stored in the vapor phase of liquid nitrogen. An unfrozen control was also prepared as a reference for viability of the cells after freezing. The cells were thaw by immersion in a 37°C water bath, washed twice to remove the cryoprotectant, and then tested for viability.

Cell viability was determined by ^3H -thymidine incorporation. For each vial frozen, 4 samples of 100 μl each were placed in wells of a flat bottom microtiter plate, and 100 μl of 2.5 $\mu\text{Ci/ml}$ ^3H -thymidine in D-MEM supplemented with 5% fetal bovine serum was added. The cells were incubated (6 hours, 37°C, 5% CO_2 atmosphere), harvested onto filter paper, and the radioactivity counted.

The cells were also monitored visually using a liquid nitrogen-cooled microscope stage (Interface Techniques Co., Cambridge, MA), with a computer temperature control. The samples (about 2 μl) were cooled at -3°C/min to -45°C, -100°C/min to -130°C and then warmed at 100°C/min to +20°C. The ice crystals were monitored using a video camera (Sony CCD-IRIS) connected to a video printer (Sony UP-5000). Micrographs were taken at the time of freezing, about 5°C below the freeze, and during the melt.

The data are expressed as the mean $\pm$ 1 standard deviation of at least 4 samples. To determine any differences within a group, the data were tested statistically by one way analysis of variance (ANOVA). Student-Newman-Keuls (SNK) multiple comparison test for differences within a group was also used.

Results

The post-thaw viability of KG-1a cells, measured by ^3H -thymidine incorporation, was improved by increasing the concentration of Me_2SO from 0 to 5% during cryopreservation in a dose dependent manner (Figure 1). The best cryopreservation results were obtained with 5% Me_2SO . The addition of AFP resulted in a decrease in KG-1a cell recoveries. Cells cryopreserved with 5% Me_2SO had a significant decrease in post-thaw viability with the addition of AFP (ANOVA, $F(6,15) = 65.042$; $P < 0.001$). SNK multiple comparison test for differences between AFP concentrations revealed that, except for samples cryopreserved with 25, 50, or 100 $\mu\text{g/ml}$ AFP, the samples were all significantly different ($p < 0.05$) from each other. Similar results were obtained for KG-1a cells cryopreserved in 3% Me_2SO (ANOVA, $F(6,15) = 25.025$; $P < 0.001$; SNK test $p < 0.05$). Cells cryopreserved with 2% Me_2SO also showed a significant decrease in viability with addition of AFP (Kruskal-Wallis ANOVA, $F(6)$, $P = 0.033$), while there was no difference in viability at lower concentrations of Me_2SO .

Cryomicrographs of KG-1a cells in 5% Me_2SO with 0 or 20 $\mu\text{g/ml}$ AFP revealed that the media froze around -17 to -20°C . The freezing point was lower than for the larger volume samples frozen in the vials, which froze above -10°C . The cells quickly darkened after the media froze, suggesting intracellular ice formation. About 5°C below the freezing point, the fine ice crystals became more defined in samples without AFP (Figure 1). The ice structure did not change upon further cooling to -130°C . During warming, ice crystal growth occurred in the samples frozen without AFP, while minimal crystal growth was observed in samples containing

25 $\mu\text{g/ml}$ AFP. By the melting point, large ice crystals were present in the samples frozen without AFP, and smaller ice crystals were present in the samples with AFP. Similar results were obtained when KG-1a cells were observed during cryopreservation with higher concentrations of AFP.

Discussion

The cryoprotective effects of permeable cryoprotectant is dose dependent⁷. The data revealed that increasing Me₂SO concentrations from 0 to 5% (v/v) increased the post-thaw viability of KG-1a cells. Surprisingly, the addition of AFP to the cryoprotectant media did not improve the post-thaw viability of the cells, and, in fact, caused a dose dependent decrease in viability. These results were statistically significant. The decrease in viability was especially great at AFP concentrations greater than 100 µg/ml (Figure 1). These data are in agreement with those obtained for red blood cells cryopreserved in the presence of the non-permeating cryoprotectant hydroxyethyl starch, where concentration above 160 µg/ml AFP caused a decrease in post-thaw red cell survival⁵. In contrast, however, low AFP concentrations enhanced red blood cell survival.

Carpenter and Hansen⁵ proposed that cryopreservation of red blood cells in the presence of high concentrations of AFP cause increased injury by preferential growth of ice crystals around the cells. Low concentrations of AFP were suggested to be beneficial by a partial inhibition of ice crystal growth in the extracellular space. A similar mechanism of injury may occur in the KG-1a cells cryopreserved in the presence of Me₂SO. Because cryopreservation by slow cooling of cells in the presence of Me₂SO allows ice formation and growth to occur during both the cooling and the warming phases, the decrease in cell viability with the addition of AFP suggests that additional deleterious effects may have occurred. The mechanism of injury by addition of AFP may be similar to that observed in red cells cryopreserved at high concentrations

of AFP, i.e., increased ice formation associated with the cells. However, we were not able to observe such a mechanism using cryomicroscopy, since ice formation was encouraged by the cryopreservation regime.

In summary, the addition of AFP to a cryoprotectant solution used in slow cooling of cells results in decreased post-thaw viability. The decrease in viability may be due to an increase in ice formation associated with the cells, consistent with a proposed mechanism of damage in cryopreservation by rapid cooling⁵.

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Figure Legend

Figure 1: KG-1a post-thaw viability after cryopreservation with varying concentrations of dimethylsulfoxide and antifreeze protein. The best viability results were obtained after cryopreservation with 5% Me₂SO. The addition of AFP resulted in statistically significant decreases in cell recovery. AFP concentrations of 25-100 µg/ml were the least deleterious.

Figure 2: Photomicrographs of KG-1a in 5% dimethylsulfoxide frozen and thawed with and without antifreeze peptide. Photomicrographs were taken before cooling (1), upon freezing (2), about 5°C below the freeze (3), about -10°C during thawing (4), and at the melt (5). Much ice growth occurred in the samples without AFP, while little ice growth occurred in samples containing AFP during cooling or during the subsequent warming. In samples with 800 µg/ml AFP, the cells did not appear to freeze until the thaw, suggesting that ice formation associated with the cells during thawing and caused decreased post-thaw viability. Scale bar = 10 µm.

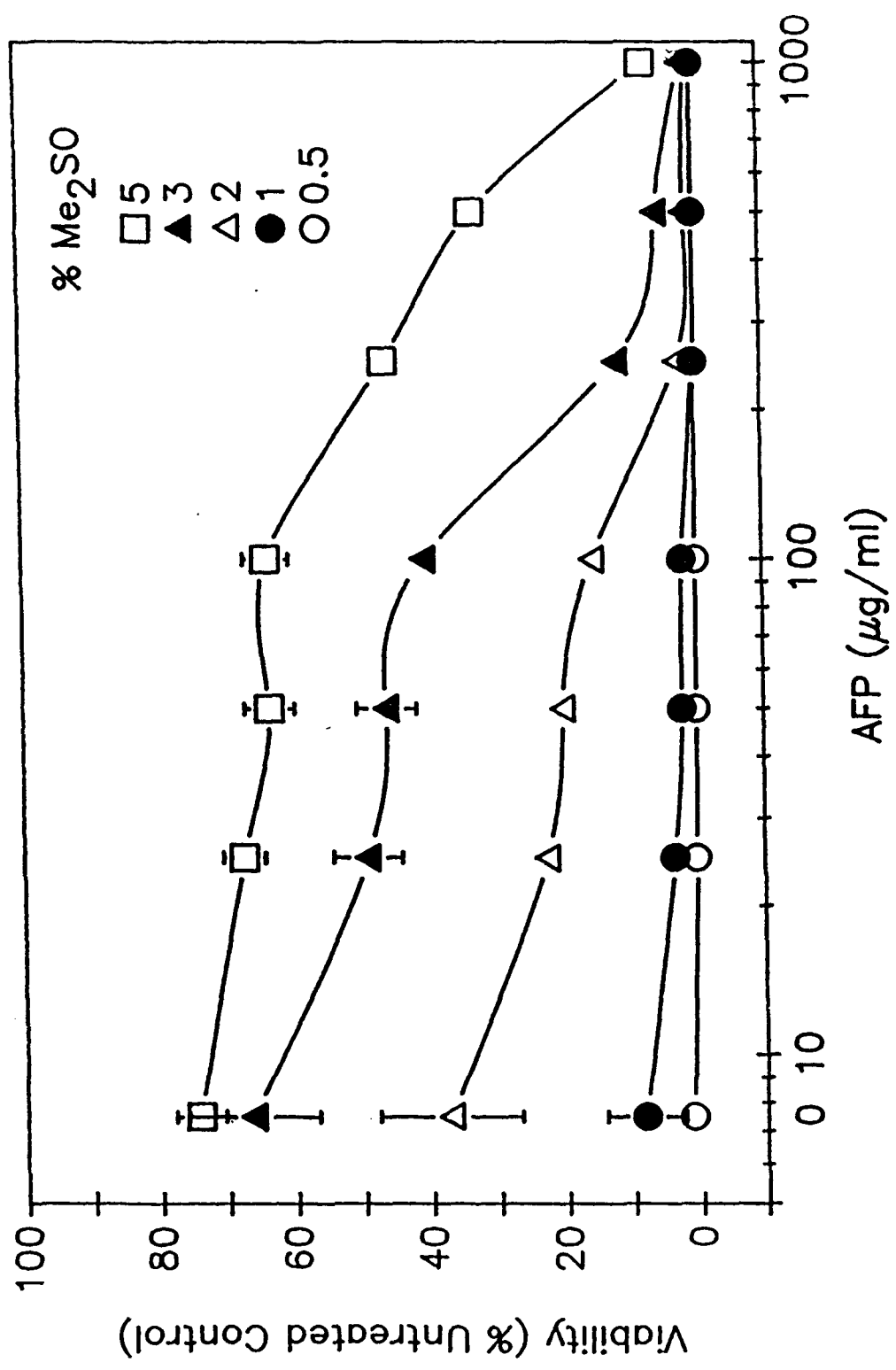


FIG. 1

Synergistic Interaction of Low Molecular Weight Polyvinylpyrrolidones and Dimethylsulfoxide During Cell Cryopreservation

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Development of cryopreservation methods for organs requires low toxicity combinations of cryoprotectants. Dimethylsulfoxide (DMSO) the most effective and commonly employed cell penetrating cryoprotectant has been associated with cytotoxic effects during organ preservation. The objective of our research program is to identify combinations of cryoprotectants which permit reduction of DMSO concentrations. Polyvinylpyrrolidone ($[C_6H_9NO]_n$) has been tested as a non-cell penetrating extracellular cryoprotectant using a variety of cell and tissue models. In this report polyvinylpyrrolidones (PVP) ranging from 10 to 1,450 kilodaltons were tested in combination with DMSO. The human promyeloblast cell line, KG-1a, was employed as a cell model. Dose response studies with a 40kd PVP demonstrated that there was a dose dependent enhancement of cell survival during slow rate cryopreservation. Doses greater than 10% (w/v) demonstrated decreased survival. Cryopreservation of KG-1a in the absence of DMSO using 10% PVP varying in molecular weight demonstrated optimal cell survival with 40kd PVP. In the presence of DMSO there was optimal cell survival with both 10 and 40kd PVP. Combinations of 1% DMSO with PVP demonstrated a synergistic effect on cell survival. Further experiments demonstrated that cell losses during cryopreservation at PVP molecular weights of 630 and 1,450kd could, at least in part, be attributed to high solution viscosity. In conclusion, relatively low molecular weight and low viscosity PVPs synergistically interact with relatively low concentrations of DMSO to enhance cell survival during cryopreservation. Supported by Contract N00014-91-0044 from the Office of Naval Research.

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Correlation Between Chilling-Induced Injury in Human Cells and Phospholipid Membrane Phase Transition Temperature

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Chilling in the absence of ice formation can lead to cell death. Many mechanisms have been proposed for chilling-induced cell death, including a role for the physical transition of the membrane phospholipids from liquid-crystalline to gel phase. Relatively high membrane phase transition temperatures have been correlated with greater chilling sensitivity. We investigated the role of the membrane phase transition temperature in relation to cell survival after chilling to 4°C. In this study two hemopoietic cell lines were employed: MOLT-4, an acute human lymphoblastic leukemia, and K-562, a chronic human myelogenous leukemia. MOLT-4 cells were very sensitive to chilling, with less than 20% survival after 24 hours at 4°C, compared to K-562, with greater than 80% survival after the same incubation period. We tested the hypothesis that the greater chilling sensitivity of MOLT-4 was associated with a higher membrane phase transition temperature. The thermotropically induced membrane phase transition temperature in both cell lines was measured using Fourier transform infrared spectroscopy (FTIR). Although the liquid-crystalline to gel phase transition was broad in both cell lines, the phase transition was about 5°C higher for MOLT-4 ($20.2 \pm 3.1^\circ\text{C}$, $n=3$) than for K-562 ($14.9 \pm 1.9^\circ\text{C}$, $n=3$). Further investigation of the role of membrane phase transition temperature and cell survival during chilling was performed by incubation of the hemopoietic cell lines with membrane fluidizers. MOLT-4 survival was not affected by the presence of fluidizers. In contrast, K-562 survival was enhanced. These data support the hypothesis that higher membrane phase transitions correlate with greater cell chilling sensitivity. Supported by Contract N00014-91-C-0044 from the Office of Naval Research.

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Subject Index

POLYVINYLPYRROLIDONE; Cryopreservation with, Synergistic Interactions, Molecular Weight Comparison.

DIMETHYLSULFOXIDE; Cryopreservation with, Synergistic Interactions, Concentration Reduction.

CRYOPRESERVATION.

Title

SYNERGISTIC INTERACTION OF LOW MOLECULAR WEIGHT
POLYVINYLPYRROLIDONES WITH DIMETHYLSULFOXIDE DURING CELL
CRYOPRESERVATION

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Summary

The objective of our research program is to identify combinations of cryoprotectants which minimize cytotoxic effects. Polyvinylpyrrolidones (PVP) ranging in molecular weight from 10,000 to 1,450,000 daltons were tested in combination with 0%-5% dimethylsulfoxide (DMSO) during cryopreservation of a human promyeloblast cell line, KG-1a. A dose dependent enhancement of cell survival occurred during slow rate cryopreservation with PVP. PVP concentrations greater than 10% (w/v) demonstrated decreased cell survival. At each concentration of DMSO tested the highest cell survival was observed with either 10 or 40 kDa PVP. Combinations of 1% DMSO with PVP demonstrated a synergistic effect on cell survival. Further experiments demonstrated that cell losses during cryopreservation at PVP molecular weights of 630,000 and 1,450,000 daltons could, at least in part, be attributed to high solution viscosity. Cryopreservation of KG-1a cells in the absence of DMSO, using 10% PVP of various molecular weights, demonstrated optimal cell survival with 40 kDa PVP. In conclusion, relatively low molecular weight and low viscosity PVPs synergistically interact with low concentrations of DMSO to enhance cell survival during cryopreservation.

Introduction

Development of cryopreservation methods for organs requires the combination of cryoprotectants at concentrations which are not toxic. Dimethylsulfoxide (DMSO) is both the most effective and the most common cryoprotectant employed for long-term frozen storage of cells, tissues, and organs. DMSO had the lowest cytotoxic effects when compared with two

alternative cryoprotectants (ethylene glycol and glycerol) in animal kidney models (1-3).

Identification of alternative cryoprotectants which permit the reduction of DMSO concentrations would further minimize the risks of cytotoxicity to both the organ being preserved and the graft recipient.

In this report polyvinylpyrrolidones (PVP, $[C_6H_7NO]_n$), varying in molecular weight between 10,000 and 1,450,000 daltons, either alone or in combination with DMSO, were investigated as cell cryoprotectants. An acute human myelogenous leukemia-derived cell line with a promyeloblast phenotype, KG-1a (4), was used to enable rapid screening of cryoprotectant combinations in anticipation of employing selected combinations for preservation of tissues and organs in the future.

Materials and Methods

KG-1a promyeloblast cells (American Type Culture Collection CCL 246.1) were cultured in Iscove's modified Dulbecco's medium plus 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere consisting of 5% CO₂ in air. Sixteen hours prior to cryopreservation, the cells were split into T-25 culture flasks containing 9 ml of culture medium at 2×10^6 cells/ml. The KG-1a cells were harvested by centrifugation and were resuspended in Dulbecco's modified Eagle's medium (DMEM) plus 2% FBS. Cell counts were performed using a hemacytometer after making a 1:10 dilution of the cell suspension in trypan blue. One ml aliquots containing 10^6 cells were then placed in 5 ml polypropylene snap cap tubes. These tubes were centrifuged (300 g, 10 min) and 800 µl of supernatant was removed. At this point in each experiment, unfrozen cells were analyzed as 100% reference controls for viability after freezing. Duplicate samples of experimental cells were then resuspended in 800 µl of each cryoprotectant solution to be tested. Four types of polyvinylpyrrolidone (GAF Chemicals Corp, Texas City, TX), varying both in molecular weight and viscosity in solution, were employed. PVP C-15 had an average molecular weight of 10,000 daltons -- there was no information available on viscosity. Plasdone C-30 had an average molecular weight of 40,000 daltons and a K-value (viscosity) of 31. PVP K-90 had an average molecular weight of 630,000 daltons and a K-value of 94.6. Plasdone K-120 had an average molecular weight of 1,450,000 daltons and a K-value of 117.8. DMSO was obtained from Fisher Scientific, Atlanta, GA.

Before freezing, the tubes were cooled on ice to +4°C and placed in a precooled Cryomed freezing chamber, and cooled at -3°C/min to -80°C. The frozen samples were stored in the vapor phase of liquid nitrogen for at least 16 hours. All samples were thawed by immersion in a 37°C water bath. Once completely thawed, the cryoprotectant solutions were eluted by addition of 2 ml of DMEM plus 2% FBS and mixed by inversion. The cells were then pelleted by centrifugation and resuspended in 800 µl of DMEM plus 2% FBS.

For each tube of control or experimental thawed cells, at least 4 wells of a 96 well flat-bottom microtiter plate were loaded with 100 µl of the cell suspension and 100 µl of DMEM plus 2% FBS containing 0.25 µCi of ³H-thymidine (86.10 Ci/mmol specific activity). The plate was incubated at 37°C for six hours. The cells were then harvested onto filter paper using a Titertek Cell Harvester. After air drying, the filters were placed in scintillation vials with 100 µl deionized water and 10 ml Cytoscint. The vials were allowed to sit in the dark overnight and then read on a Beckman Scintillation Counter.

The data are expressed as the mean radioactive disintegrations per minute (DPM) per well $\pm$ 1 standard error. Statistical analyses were performed using one-way analysis of variance (ANOVA). The Student-Newman-Keuls test was used for multiple comparisons, $p < 0.05$ indicates statistical significance.

Results

Dose response studies with 10,000 and 40,000 dalton PVP demonstrated that there was a dose-dependent enhancement of cell survival during preservation in the presence of 0%-2%

DMSO (Fig. 1). Addition of PVP to higher concentrations of DMSO generally had a less dramatic enhancing effect on cell survival (Figs. 1 and 2). Doses greater than 10% PVP (w/v) demonstrated a general decrease in cell recovery (Fig. 1). Experiments to determine whether this was due to increasing solution viscosity were not performed. Cryopreservation of KG-1a cells in the absence of DMSO using 10% PVP varying in molecular weight demonstrated optimal cell survival with 40,000 dalton PVP (Fig. 2). In the presence of DMSO there was maximum cell survival with both the 10,000 and 40,000 dalton PVP at each DMSO concentration tested (Fig. 2). Combinations of 1% DMSO with relatively low molecular weight PVPs demonstrated that there was a synergistic effect on cell survival, while combinations of 2% DMSO with relatively low molecular weight PVPs demonstrated an additive effect on cell survival (Fig. 2). Small enhancements of cell survival were observed at 5% DMSO with the low molecular weight PVPs (Fig. 2). Comparison of cell recoveries in samples cryopreserved with DMSO and low molecular weight PVPs with samples cryopreserved with DMSO and high molecular weight PVPs demonstrated statistically significant decreases using high molecular weight PVPs (Fig. 2). Further experiments demonstrated that cell losses similar to those obtained during cryopreservation at PVP molecular weights of 630,000 and 1,450,000 daltons occurred if the cells were simply placed in the cryoprotectant solution and processed without the freezing step (Fig. 3). These results suggest that the KG-1a cells were not being pelleted adequately by centrifugation in the presence of high molecular weight PVPs due to high solution viscosity.

Discussion

Polyvinylpyrrolidone has been tested as a non-penetrating extracellular cryoprotectant using a variety of cell models. PVP was shown to be an extremely effective cryoprotectant during the rapid freezing of erythrocytes (5-8). However, PVP has a long retention time in the recipient's tissues following systemic administration (9) and for this reason is not currently employed clinically for erythrocyte cryopreservation.

Low molecular weight PVPs have also been successfully employed during slow rate cryopreservation of nucleated mammalian cells, including lymphocytes (10,11), bone marrow stem cells (12,13), murine hemopoietic cell lines (14), and Chinese hamster ovary cells (15). In this study the human promyeloblast cell line, KG-1a, was employed as a model to determine whether combinations of DMSO and PVPs, ranging from 10,000 to 1,450,000 daltons, would have beneficial effects on cell survival during cryopreservation. The results demonstrated a synergistic interaction of PVPs and 1% DMSO and an additive interaction of 2% DMSO and low molecular weight PVPs (10,000 - 40,000 daltons). High molecular weight PVP's resulted in cell loss during processing without cryopreservation. The cell loss was probably due to the cells not being pelleted adequately by centrifugation in the high viscosity PVP solutions.

The addition of either 10,000 or 40,000 dalton PVPs to 3% DMSO solutions raised cell survival to values observed with 5% DMSO cryopreservation (Fig. 1), indicating that DMSO concentrations can be reduced to 3% in the presence of low molecular weight PVPs. These results suggest that combination of DMSO and low molecular weight PVP may be of

benefit for cryopreservation of more complex biological structures, such as organs. The possibility that further reduction of DMSO concentrations can be achieved by addition of other macromolecules, such as chondroitin sulphate, alginates, or agaroses, which are cryoprotectants only in the presence of cell permeating cryoprotectants (18,19), to cryoprotectant solutions containing PVP and DMSO needs to be tested.

An explanation of why the addition of PVP should permit reduction of DMSO concentration is not available because the molecular bases of freezing damage are not understood (reviewed, 16,17). Both intracellular and extracellular ice formation can cause cell damage. The situation is further complicated by solution effects, which can be divided into at least four discrete events. During freezing, water is removed from cells to form extracellular ice, solutes concentrate as water is removed to form ice, cell volumes decrease, and solutes precipitate. DMSO is highly permeable to cell membranes and is believed to interact primarily with the bulk solvent properties of water on a colligative basis. PVPs, due to their high molecular weights, are excluded from cells. Colligative mechanisms of action are usually not considered to be of importance for PVP-induced cryoprotection, rather colloidal osmotic pressure exerted by PVP and alterations in the activity of unfrozen water by hydrogen bonding to PVP have been considered.

In conclusion, relatively low molecular weight and low viscosity PVPs either additively or synergistically interact with low concentrations of DMSO to enhance cell survival during cryopreservation. Combination of low molecular weight PVPs with DMSO permits reduction of the DMSO concentrations required to obtain optimal cell survival.

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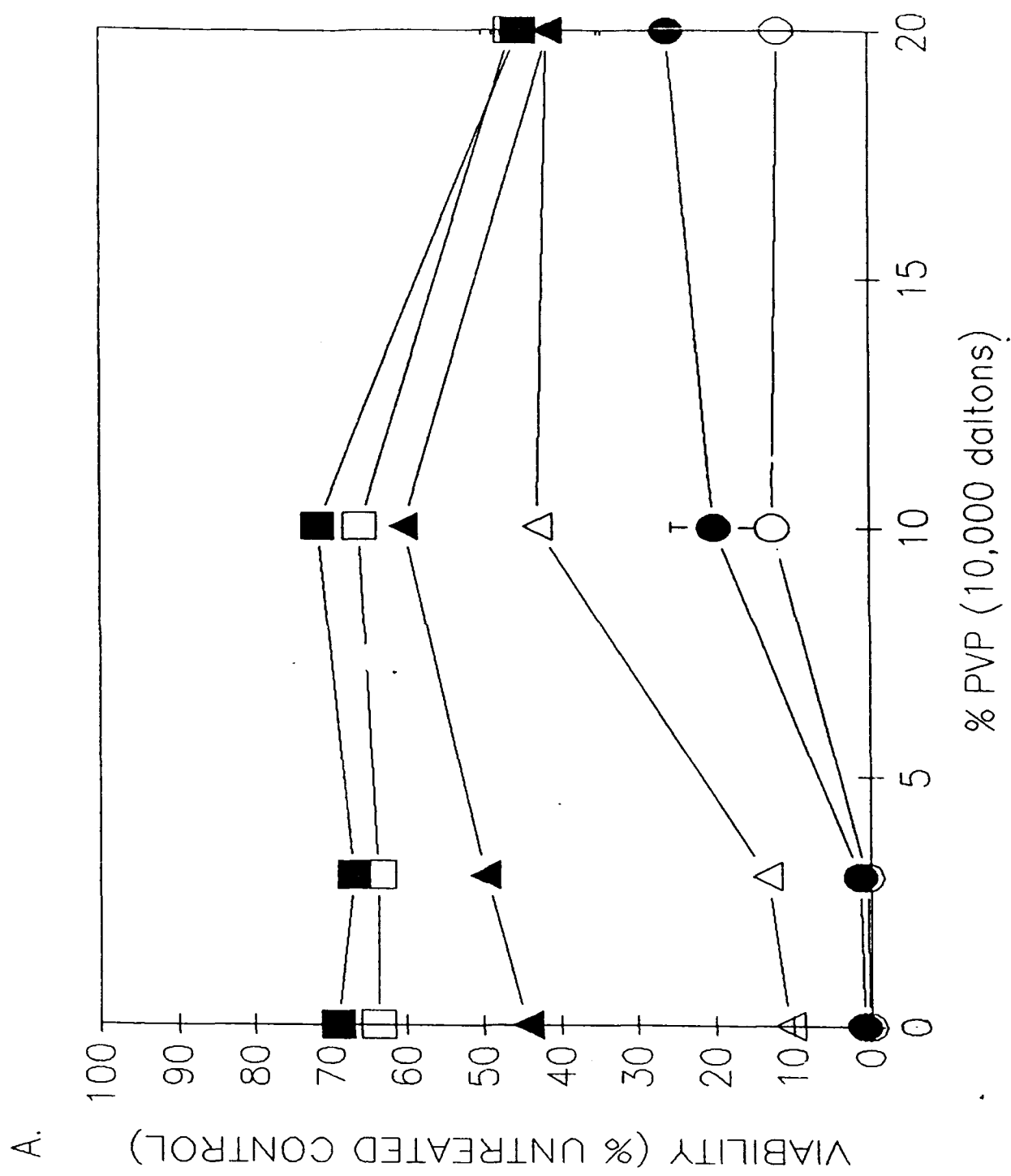
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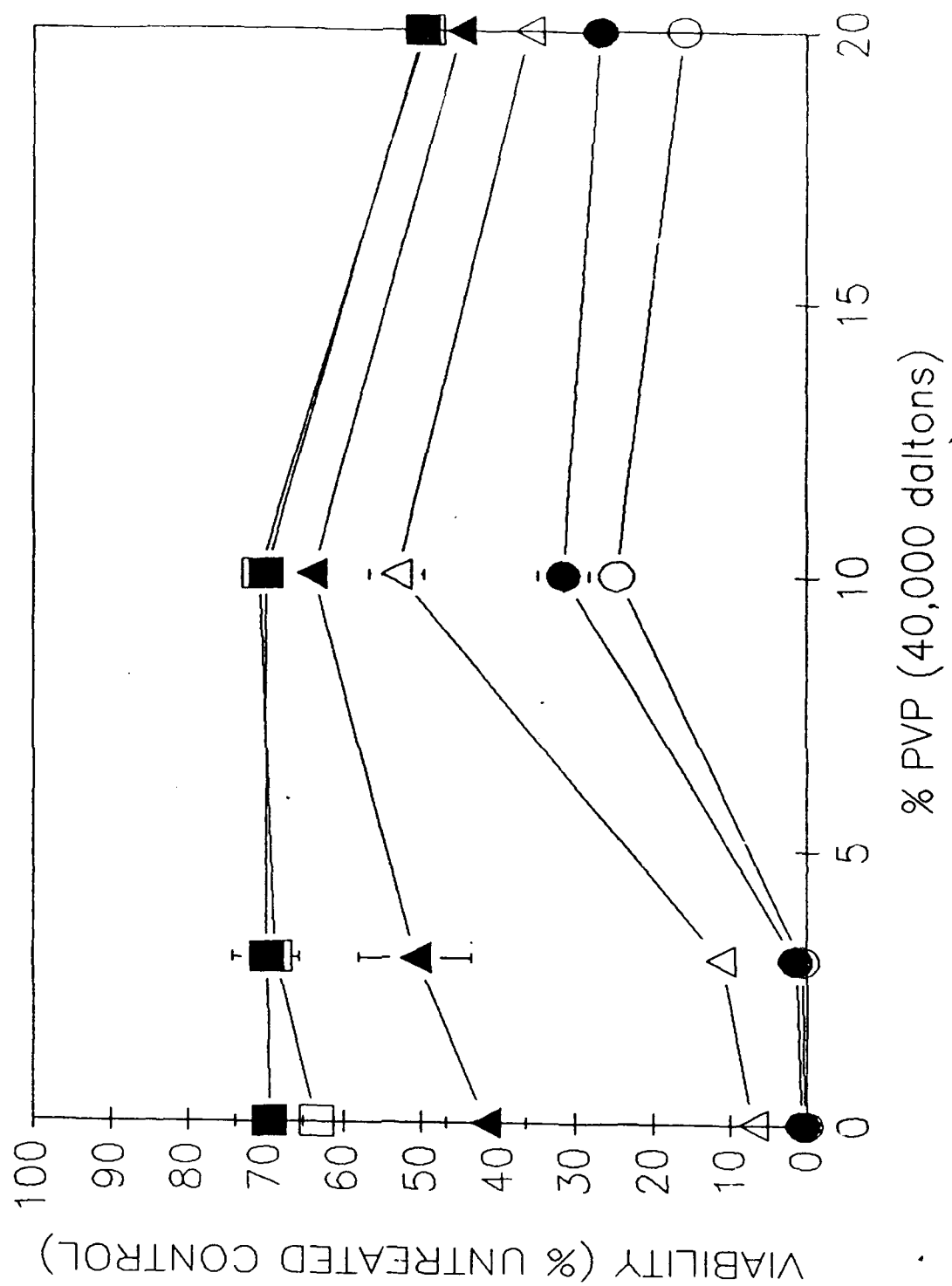
Fig. 1. PVP dose responses in combination with 0% DMSO (○), 0.5% DMSO (●), 1% DMSO (△), 2% DMSO (▲), 3% DMSO (□), and 5% DMSO (■). The experiments were performed with (A) 10,000 dalton PVP and (B) 40,000 dalton PVP. The data are expressed as the mean $\pm$ 1 se, n = 3.

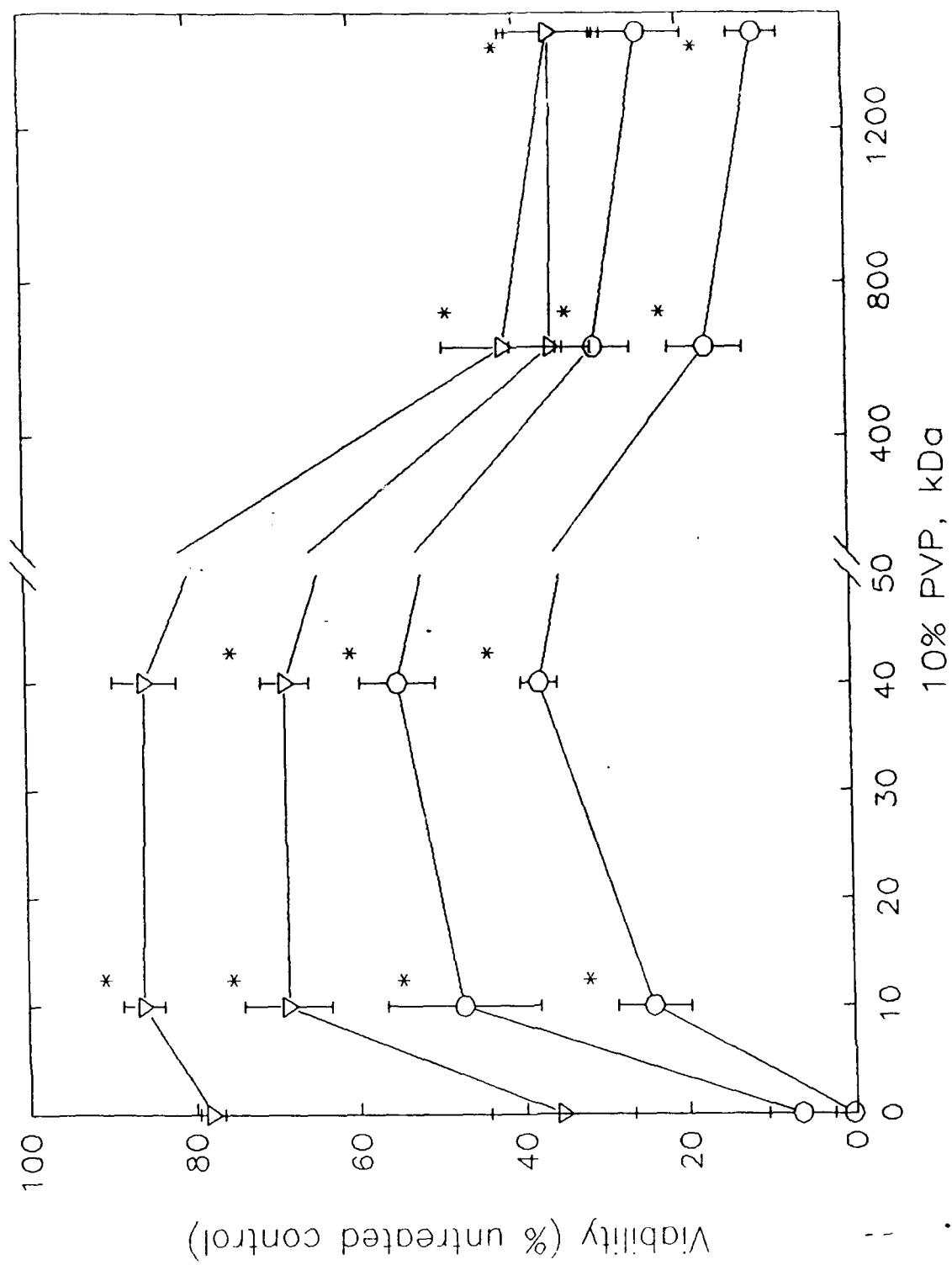
Fig. 2. Effect of PVP molecular weight during cryopreservation upon cell recovery. 10% PVP (W/V) was combined with 0% (○), 1% (●), 2% (△), and 5% DMSO (▲). The data are expressed as the mean $\pm$ 1 se, n = 3. * p <0.05 compared to controls without PVP addition.

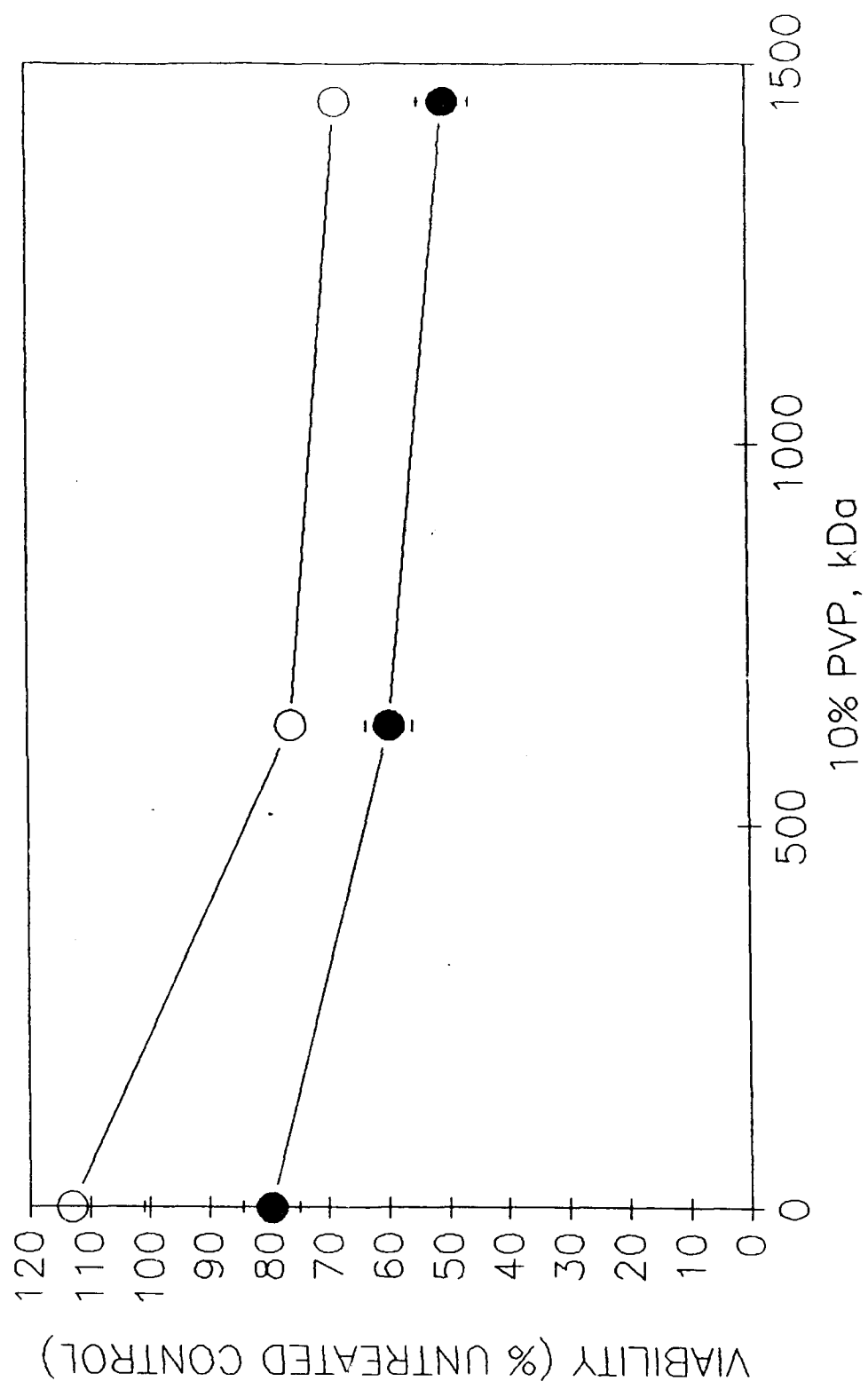
Fig. 3. Effect of high molecular weight PVPs upon cell recovery without cryopreservation. All samples were handled in the same way as in cryopreservation experiments except that cryopreservation was not performed. Viability was measured either by trypan blue exclusion (○), n = 3, or by ³H-thymidine incorporation (●), n = 4. * p <0.05.



B.







Metabolic Studies of Bovine Aortic Endothelial Cells

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We are currently using bovine aortic endothelial cells as an *in vitro* model for studies of non-freezing cold injury. The studies presented in this report were performed to determine the baseline values for metabolic heat production, carbon dioxide release, and respiratory quotient (moles CO₂ released/mole oxygen consumed) in these cells. Cells were detached from flasks with trypsin, centrifuged, then resuspended in Hank's balanced salt solution with 50% Percoll to increase medium density. 2 to 5 X 10⁶ cells per 500 μ l suspension were placed in the ampule of a differential scanning calorimeter (Hart Scientific) and metabolic heat was measured after thermal equilibration (about 30 minutes). CO₂ release was calculated from the increase in heat production after addition of a CO₂ trap to the ampule. The cells produced 15.7 ± 1.2 pW/cell at 37°C and pH 7.4. They also released 20 ± 6 amol CO₂/cell/s. This is equivalent to 742 ± 180 kJ/mole CO₂, a value higher than the heat of combustion of glucose (468 kJ/mole CO₂) or palmitate (623 kJ/mole CO₂), indicating that a substantial portion of the heat production in these cells is not related to CO₂ production. Pressure changes within the measurement ampule showed that the respiratory quotient was about 0.8, indicating that mitochondrial oxidation is consuming a more reduced substrate than glucose, possibly a fatty acid. These results show that energy metabolism of endothelial cells as measured calorimetrically is in concert with traditional measures such as O₂ consumption. This work was supported by Office of Naval Research Grant #N00014-91-C-0044.

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