Pergamon

Int. J. Heat Mass Transfer. Vol. 41, No. 13, pp. 1907–1915, 1998

Printed in Great Britain
0017-9310/98 $19.00+0.00

PII: S0017-9310(97)00368-2

Influence of fish antifreeze proteins on the freezing of cell suspensions with cryoprotectant penetrating cells

HIROSHI ISHIGURO†
Institute of Engineering Mechanics, University of Tsukuba, Tsukuba, 305, JAPAN

and

BORIS RUBINSKY
Department of Mechanical Engineering, University of California at Berkeley, Berkeley, CA 94720, U.S.A.

(*Received* 30 *August* 1996 *and in final form* 21 *October* 1997)

Abstract—This study aims at understanding the influence of antifreeze proteins (AFPs) from fishes on the freezing of biological cells in relation to cryopreservation from the viewpoint of bioheat and mass transfer. Human red blood cells were frozen in the physiological salines including 20% v/v glycerol with and without 20 mg ml^{-1} AFPs. Due to the addition of AFPs, the morphology of the ice crystal changed dramatically from a dendritic appearance into a spicular appearance. The spicular ice crystal caused strong mechanical interaction between the ice crystals and the red blood cells, and the resultant complete destruction of the red blood cells. This suggests that the morphology of the ice crystal and its mechanical interaction with the cells influence the cell survival through the freezing. © 1998 Elsevier Science Ltd. All rights reserved

1. INTRODUCTION

Cryopreservation, the preservation of biological cells by freezing, is one of the promising areas to contribute to medical engineering and bio-industry and is expected to develop. However, the technique and the work on cryopreservation are mostly empirical. Cryopreservation could benefit from fundamental researches of bioheat and mass transfer. Thermal problems in cryopreservation might also be attractive and challenging subjects to the field of heat and mass transfer.

It is a well-known fact that cell survival through freezing and thawing depends upon the thermal history during cooling and warming, the type and concentration of cryoprotectant, and the type of cells frozen. To improve the viability of cells, the compounds called cryoprotectants are added to cell suspensions before freezing. Cryoprotectants are classified in two categories [1]: a type penetrating cells and a type nonpenetrating cells, and the degrees of their effects are different. For example, glycerol is one of the most common cryoprotectants penetrating cells. Although the details of the mechanism of the slow-freezing injury to cells and the mechanism of protection of cells by cryoprotectants are not yet clear [2–4], discovery of more effective additives to improve the viability of cells is also one of the important subjects in the area of cryobiology.

In nature, organisms surviving cold ambient conditions prevent the freezing of their body fluids by unique mechanisms [5]. Some amphibians and reptiles living over the winter increase the percentage of dissolved solutes, such as salts, sugars and glycerol in their blood to colligatively lower its freezing temperature. Interestingly, some cold-water fishes living in the polar oceans and the north-temperate oceans and certain cold-tolerant species of insects synthesize biological antifreezes for the freezing avoidance, which depress the freezing point of the body fluids non-colligatively. The freezing point of seawater is $-1.9°C$, and those of typical marine fishes without antifreezes are $-0.8°C$ due to low molecular weight solutes, mainly sodium chloride in the body fluids. Freezing in fishes is lethal. However, the freezing in the Antarctic fishes with antifreezes does not occur at temperatures above $-2.2°C$ [6].

The antifreeze compounds are glycopeptides and polypeptides, and are known generally as antifreeze proteins (AFPs). These antifreeze proteins depress the melting point (equilibrium freezing point) colligatively on the basis of molar concentration in solution. However, they lower the freezing point non-colligatively and much more effectively. Up to the protein concentration of 20 mg ml^{-1}, the freezing point is lowered almost over one hundred times more than the depression of the melting point and the depression of the freezing point due to the antifreeze proteins tends

* Author to whom correspondence should be addressed.

NOMENCLATURE

AFPs antifreeze proteins
G temperature gradient [K mm^{-1}]
H cooling rate [K s^{-1}]
MW molecular weight
OP antifreeze proteins from ocean pout
PS + Gly red blood cell suspension using physiological saline with 20% v/v glycerol
PS + Gly + AFPs PS + Gly + OP and PS + Gly + WF
PS + Gly + OP red blood cell suspension using physiological saline with 20% v/v glycerol and 20 mg ml^{-1} OP
PS + Gly + WF red blood cell suspension using physiological saline with 20% v/v glycerol and 20 mg ml^{-1} WF
T_c temperature of low-temperature block [K]
T_h temperature of high-temperature block [K]
V moving velocity of sample [mm s^{-1}]
W warming rate [K s^{-1}]
WF antifreeze proteins from winter flounder.

to be nearly saturated at high protein concentrations. This means that the antifreeze proteins can depress the freezing point significantly without substantially increasing their osmotic pressure. The effect of glycerol as a cryoprotectant was discovered by chance historically and glycerol is also found in the bodies of organisms surviving cold ambient conditions. The antifreeze proteins may be also expected to have possibility to improve the way of preservation of biological cells. The addition of antifreeze proteins to cell suspension was found to be effective in preserving cells by means of vitrification [7] and keeping cells in cold storage [8]. Therefore, it is of interest to understand the effect of the antifreeze proteins on cryopreservation of biological cells.

On the other hand, investigation of microscopic behavior of the ice crystal and the cells during the freezing of cell suspension is important in relation to the mechanism of slow-freezing injury of cells [4, 9, 10]. The influence of glycerol added to physiological saline with red blood cells was studied on the microscopic structures during the freezing of cell suspensions. Due to the addition of glycerol, the morphology of the ice crystals growing in the cell suspension changed from a cellular appearance into a dendritic appearance. In physiological saline alone, the red blood cells were pushed aside by the growing solid–liquid interface and were accumulated and packed in the solution channels between the cellular ice crystals. In contrast, the red blood cells were not moved and remained dispersed in physiological saline with glycerol because the ice crystal grew around the red blood cells, deforming flexibly the freezing interface in response to the shapes of the cells. The results suggested that during the freezing of red blood cell suspension the cells in physiological saline with glycerol experiences less mechanical forces than in physiological saline alone. It was proposed that the patterns of interaction between the ice crystals and the red blood cells are classified into two categories: 'flexible' type and 'inflexible' type [9, 10].

To understand the influence of the fish antifreeze proteins on the freezing of cells, experiments were performed with suspension of human red blood cells with a hematocrit of 1% in this study. Physiological saline with 20% v/v glycerol, and physiological saline with 20% v/v glycerol and 20 mg ml^{-1} antifreeze proteins were used as solutions. Antifreeze proteins from two kinds of fishes were used. The directional solidification method was used for freezing, and the technique allows independent control of temperature gradient and advancing velocity of temperature gradient at the solid–liquid interface. Microscopic behavior of the ice crystals and the red blood cells during freezing of cell suspensions was observed using a light microscope, and (1) the morphological characteristics of the ice crystals and (2) the interaction between the ice crystals and the red blood cells were investigated. In addition, the cell viability after freezing and thawing was measured. The results showed antifreeze proteins increase mechanical damage to cells.

2. EXPERIMENTAL MATERIALS

Suspensions of human red blood cells were frozen by the directional solidification method. The hematocrit, a volume fraction of red blood cells in a suspension, was 1% to facilitate to observe microscopic behavior of the ice crystals and the red blood cells. Three different solutions were used: (1) physiological saline (0.154 M NaCl) with 20% v/v glycerol alone, (2) physiological saline with 20% v/v glycerol and 20 mg ml^{-1} antifreeze proteins from the winter flounder (*Pseudopleuronectus americanus*), and (3) physiological saline with 20% v/v glycerol and 20 mg ml^{-1} antifreeze proteins from the ocean pout (*Macrozoarces americanus*). Both the fishes inhabit in the north-temperate oceans and the antifreeze proteins

are isolated from the fishes' serums. Glycerol concentration of 20% v/v is in the range of glycerol concentration practically used in cryopreservation of human red blood cells [1]. 20 mg ml^{-1} antifreeze proteins have obvious effects on the preservation of cells in vitrification [7] and the cold storage of cells [8], and the amount is the one that saturates nearly the depression of freezing temperature due to the addition of antifreeze proteins [6]. Also, the antifreeze proteins cannot penetrate the cells due to their high molecular weights.

Fish antifreeze proteins are classified into two groups: the antifreeze glycopeptides (MW2600–33 700) and the antifreeze polypeptides. The antifreeze polypeptides are classified into three classes: type I AFPs (alanine-rich (60 mol%), MW3300–6000), type II AFPs (cysteine-rich, MW14 000–16 000), and type III AFPs (no cysteines and not rich in alanine, MW5000–6700) [11]. The antifreeze proteins from the winter flounder and those from the ocean pout utilized in this study, respectively, belong to the type I and type III, and these antifreeze proteins were selected because they were the ones that could be obtained commercially.

Fresh human blood collected in Na-heparinized tubes was washed twice by a centrifuge with isotonic buffered saline to isolate the packed red blood cells. The red blood cells were stored at 4°C, suspended in isotonic buffered saline at nearly physiological hematocrit, and they were used within 5 days. Prior to each experiment the red blood cells were washed once with isotonic buffed saline and once with physiological saline in a centrifuge at room temperature, and the packed red blood cells were prepared. Physiological saline with 20% v/v glycerol and the red blood cells were mixed at room temperature by shaking the container, and were left to equilibrate osmotically for 30 min. For materials with antifreeze proteins, 20 mg ml^{-1} antifreeze proteins were also added to the physiological saline with glycerol containing red blood cells, mixed by shaking the container, and left for 30 min.

3. EXPERIMENTAL METHODS

A light microscope with a directional solidification stage and a video system were used to observe and record the process of directional solidification of test materials [4, 9, 10, 12]. The directional solidification technique allows independent control of temperature gradient and its advancing velocity at the solid–liquid interface. The schematic of directional solidification stage is shown in Fig. 1. It consists of two copper blocks kept at predetermined constant temperatures by a distributive flow of liquid nitrogen vapor and heating elements connected to a temperature-control system. The width of gap between the two blocks equals 2.4 mm. Their temperatures are set at a temperature, T_h, above the freezing temperature of the material and a temperature, T_c, below the freezing temperature.

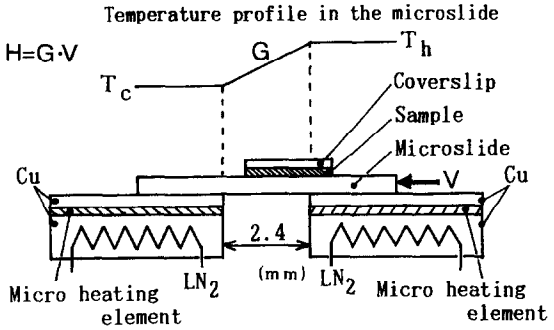

Fig. 1. Schematic of the directional solidification stage.

A sample is set between a glass microslide and a glass coverslip, and they are moved on the surfaces of the stage at a specified velocity using a traverse system driven by a motor. Mechanical pressure assures good mechanical and thermal contacts between the microslide and the two copper blocks. Consequently, a linear temperature distribution is almost realized in the sample on the microslide across the gap between the two blocks due to such high thermal conductivity of the microslide that the convective heat transfer by moving the microslide may be negligible. In this study the temperature gradient in the freezing sample is promised to be expressed by the superficial temperature gradient G, as shown in Fig. 1, which is defined as the temperature difference divided by the width of gap. The actual temperature profile on the microslide has moderate changes near T_h and T_c predominantly because of heat conduction in the glass microslide. This means that the actual temperature gradient, maximum temperature gradient which the sample experiences, is somewhat smaller than the superficial one. The preliminary experiment for measurement of temperature on the microslide showed that in the range of cooling and warming rates in this study the actual gradient of the linear temperature profile realized in the sample equaled about 60% of the superficial temperature gradient and the moving velocity of the sample had negligible influence on the value of temperature gradient. Cooling of the sample is continuously carried out by moving the microslide from the higher temperature copper block to the lower temperature copper block. Cooling rate H is expressed in terms of the product of G and V. Cooling rate H is also superficial, with the actual cooling rate equaling about 60% of the superficial cooling rate. Warming of the sample can be conducted by moving the microslide in the reverse direction under the same temperature gradient as in cooling.

The video system consists of a video camera attached to the microscope, a video timer, a video cassette recorder, and a monitor. Table 1 indicates the experimental conditions for observation of the directional solidification process of cell suspensions. The moving velocity of microslide was changed between 0.15 and 2.50 mm min^{-1} on condition that

Table 1. Experimental conditions for observation of freezing process of cell suspensions

Solution	T_c (°C)	T_h (°C)	G (°C mm^{-1})	V (mm min^{-1})	H (°C min^{-1})
PS+Gly	−42	6	20.0	0.17, 0.55, 1.02, 1.53, 2.08, 2.50	3.4, 11.0, 20.4, 30.6, 41.6, 50.0
	−54	6	25.0	0.22, 0.86, 1.62	5.6, 21.6, 40.5
PS+Gly+WF	−43	5	20.0	0.18, 0.47, 0.50, 0.61, 0.97, 1.00, 1.07, 1.37, 1.47, 1.49, 1.50, 1.58, 1.94, 2.03, 2.11, 2.44, 2.50	3.5, 9.4, 10.1, 12.2, 19.4, 20.1, 21.5, 27.5, 29.4, 29.9, 30.0, 31.6, 38.9, 40.5, 42.2, 48.7, 50.0
PS+Gly+OP	−43	5	20.0	0.16, 0.51, 1.04, 1.50, 2.03	3.2, 10.2, 20.9, 30.0, 40.5

the temperature gradient was kept nearly constant, 20°C mm^{-1}, for the most part. These conditions mean that the cooling rate ranged from 3 to 50°C mm^{-1}.

The viability of cells after freezing and thawing was also measured using the similar sample to that in observing the solidification process. The sample was cooled in the predetermined thermal conditions (G, V), kept on the colder block for 10 min, and then warmed rapidly. Table 2 shows the experimental conditions for measurement of viability. The cell survival of morphological integrity was visually examined to assess the viability of cells. The number of red blood cells before freezing was counted in about ten spots on the microslide, and the number of cells to count per spot ranged roughly from 100 to 250. The number of cell survival retaining a morphologically intact appearance after freezing and thawing was similarly counted in the same spots. The viability was defined as the ratio of the latter number to the former. Table 2 indicates that $T_c = -50$°C and −80°C were chosen for $T_h = 15$°C, and that the temperature gradient, G, and the moving velocity of the sample, V, were varied. The temperature of −80°C is the ternary eutectic point of physiological saline with glycerol.

4. RESULTS AND DISCUSSION

It was found that two different AFPs have a similar effect. The addition of 20 mg ml^{-1} AFPs remarkably changed the morphological structure of the ice crystal and the pattern of the interaction between the ice crystals and the red blood cells in the freezing process. In addition, it decreased the cell survival through the freezing and thawing. In all the following photos, freezing advances from left to right, an isotherm corresponds to a lengthwise vertical line, and red blood cells look like small round particles. Also, it was observed that the freezing temperature of the solution with 20 mg ml^{-1} antifreeze proteins was about 1°C lower than that without them.

4.1. *Morphological characteristics of ice crystals*

The ice crystal in PS+Gly+AFPs had a spicular ice appearance, which was very different from the dendritic appearance of the ice crystal in PS+Gly. Figure 2 shows the morphological changes of the ice crystals for various moving velocities of samples. The basic structure of ice crystal in PS+Gly was dendritic, and the area occupied by the ice became larger in the region of lower temperature. The ice crystal structure (shape, size, directionality, etc.) was not so much well-ordered at low moving velocity of the sample in Fig. 2(a-1). As shown in Figs. 2(a-2) and (a-3) an increase in the moving velocity regularized the arrangement of arms, and made the ice crystal structure better ordered. Simultaneously the dendritic ice crystal became finer and had a higher-order structure with higher-order arms.

In contrast the ice crystal in PS+Gly+WF basically had a unique spicular structure with strong directionality, and the morphology was very different from that in PS+Gly. The bundle of spicular ice crystals in Figs. 2(b-1), (b-2) and (b-3) grew intermittently in the direction of the ice spicules. After the growth of the spicular ice crystal, new spicular ice crystals occurred on the side wall of the spicular ice crystals, and grew in the unfrozen solution between them rapidly. These spicular ice crystals were very slender, and were thin-

Table 2. Experimental conditions for measurement of viability of cells after freezing and thawing

Solution	T_c (°C)	T_h (°C)	G (°C mm^{-1})	H (°C min^{-1})	W (°C min^{-1})	Viability (%)
PS+Gly	−50	15	27	26	290	58
	−80	15	40	26	427	51
	−80	15	40	100	427	82
PS+Gly+WF	−80	15	40	424	427	0
PS+Gly+OP	−50	15	27	26	290	0
	−50	15	27	99	290	0

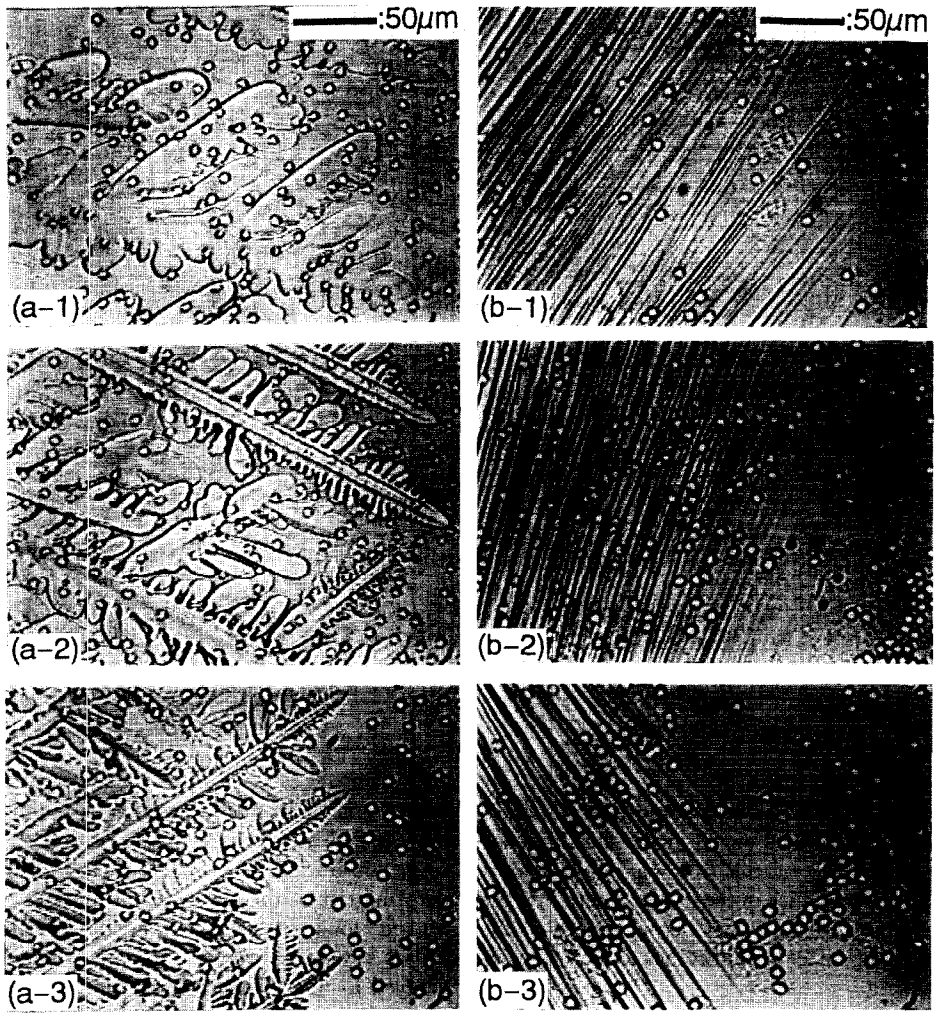

Fig. 2. Ice crystals and red blood cells during freezing. (a-1,2,3): PS+Gly, (b-1,2,3): PS+Gly+WF; (a-1) $H = 11.0°C\ min^{-1}$, $G = 20.0°C\ mm^{-1}$, $V = 0.55\ mm\ min^{-1}$; (a-2) $H = 20.4°C\ min^{-1}$, $G = 20.0°C\ mm^{-1}$, $V = 1.02\ mm\ min^{-1}$; (a-3) $H = 50.0°C\ min^{-1}$, $G = 20.0°C\ mm^{-1}$, $V = 2.50\ mm\ min^{-1}$; (b-1) $H = 9.40°C\ min^{-1}$, $G = 20.0°C\ mm^{-1}$, $V = 0.47\ mm\ min^{-1}$; (b-2) $H = 20.1°C\ min^{-1}$, $G = 20.0°C\ mm^{-1}$, $V = 1.00\ mm\ min^{-1}$; (b-3) $H = 40.5°C\ min^{-1}$, $G = 20.0°C\ mm^{-1}$, $V = 2.03\ mm\ min^{-1}$.

ner than the diameter of a red blood cell. It is important to note that the spicular ice crystal structure did not depend on the advancing velocity of the temperature gradient.

The remarkable difference in morphological characteristics between the ice crystals in PS+Gly and PS+Gly+WF is caused by the difference in formation mechanism of ice crystal. The dendritic ice crystal in PS+Gly is formed by the morphological instability of solid–liquid interface, which is driven by the constitutional supercooling degrees. The constitutional supercooling degrees are given as different degrees between the temperature of solution and the equilibrium freezing temperature corresponding to the concentration of solute in the solution, and are a function of the advancing velocity of temperature gradient. With an increase in the advancing velocity, the ice crystal in PS+Gly becomes finer to acquire a more stable structure.

On the other hand, the morphology of the ice crystal in PS+Gly+AFPs is influenced dominantly by the AFPs. The details of the mechanism is unknown, and at present the mechanism is interpreted by the following model for antifreeze solutions [6]. The ice crystal has a shape of a hexagonal prism with a basal plane $\{0001\}$ and a prism face $\{10\bar{1}0\}$. Antifreeze proteins are adsorbed selectively on the prism face by the hydrogen bond, and it inhibits more water molecules from attaching the prism face. The hydrogen bond between the antifreeze proteins and the ice lattice results from the structural match between the spacing of potential hydrogen-bonding side chains, polar residues, in the antifreeze proteins and the spacing of water molecules on the ice lattice. The adsorption of

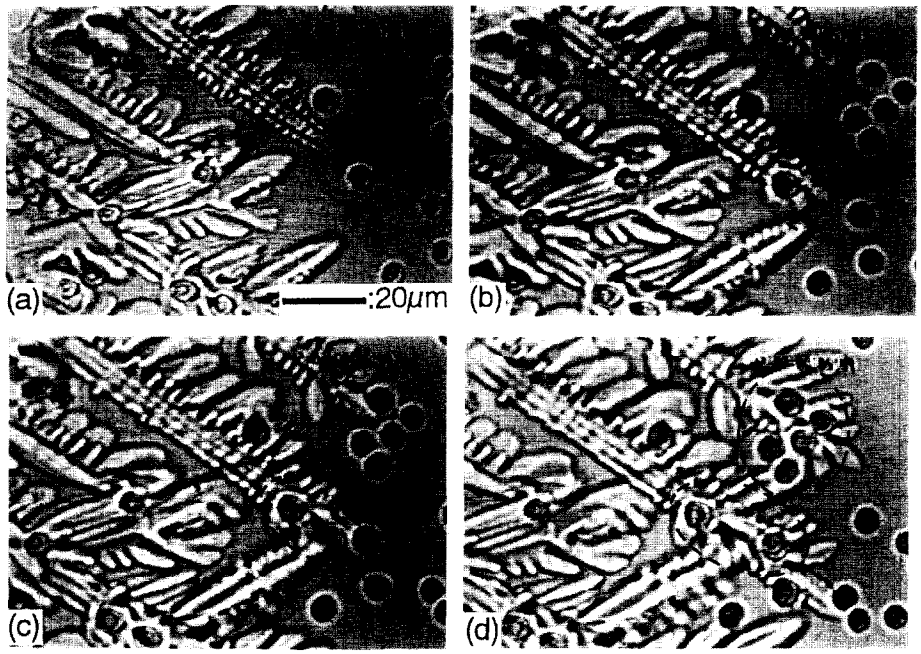

Fig. 3. Interaction between red blood cells and solid–liquid interface near the tip of ice crystal (PS + Gly). $H = 40.5°C\ min^{-1}$, $G = 25.0°C\ mm^{-1}$, $V = 1.62\ mm\ min^{-1}$.

antifreeze proteins causes microscopic deformation of the solid–liquid interface on the prism face, and the resultant depression of the freezing point, and it inhibits the growth of ice in the *a*-axis. Therefore, the ice crystal grows in the *c*-axis perpendicular to the basal plane, and the ice crystal comes to have a spicular structure.

4.2. *Interaction between ice crystals and red blood cells*

Figure 2 also shows the distinctive patterns of the interaction between the ice crystals and the red blood cells. In PS + Gly the freezing interface was deformed flexibly in response to the shapes of the red blood cells, and the ice crystals grew around the cells. Red blood cells were often observed to exist at the branching point of the dendritic ice crystal. This indicates that the presence of the red blood cells promotes the branching of the ice crystal. Figure 3 shows an example of the consecutive branching process of the ice crystal tip due to the presence of the red blood cells. Time passes in order of Figs. 3(a), (b), (c) and (d). For instance, attention should be given to the interaction between the ice crystal and the cells marked in the photos. Furthermore, Fig. 4 shows the states of the ice crystals and the red blood cells in the regions of lower temperature than near the tip of the ice crystal. The red blood cells were kept dispersed with clear appearances and out of contact with one another.

In PS + Gly + AFPs, the red blood cells near the tip of the ice crystal were moved ahead by the spicular ice crystals growing in the direction of higher temperature. This pattern of interaction between the ice crystal and the red blood cells is very different from that in PS + Gly, where the red blood cells remain to be still during the freezing because of the flexible deformation of the ice-solution interface. However, the red blood cells became trapped between the spicular ice crystals in the colder region, and they were arrayed in line. Furthermore, some red blood cells were seen to be deformed with the growth of ice crystals, and their appearances became unclear. The movement and deformation of the red blood cells indicate that the red blood cells experience mechanical forces by the spicular ice crystals. In contrast, the results for PS + Gly suggests that the red blood cells experience less mechanical forces from the ice crystal.

Classification of the patterns of interaction between the ice crystals and the red blood cells was proposed in our previous publications [9, 10]. The patterns of interaction were classified into two categories: 'inflexible' type and 'flexible' type. In the former type, the red blood cells are moved by the growing ice crystal, and in the latter the ice crystal does not move red blood cells and grows deforming the freezing interface in response to the cells. The ice crystal in the former type of interaction exerts mechanical forces (compression, shearing, etc.) on the cells while the ice crystal in the latter is considered to exert less or no mechanical forces on the cells. According to the classification, the pattern of the interaction between the ice crystals and the cells in PS + Gly + AFPs belongs to the category of 'inflexible' type while that in PS + Gly belongs to the category of 'flexible' type.

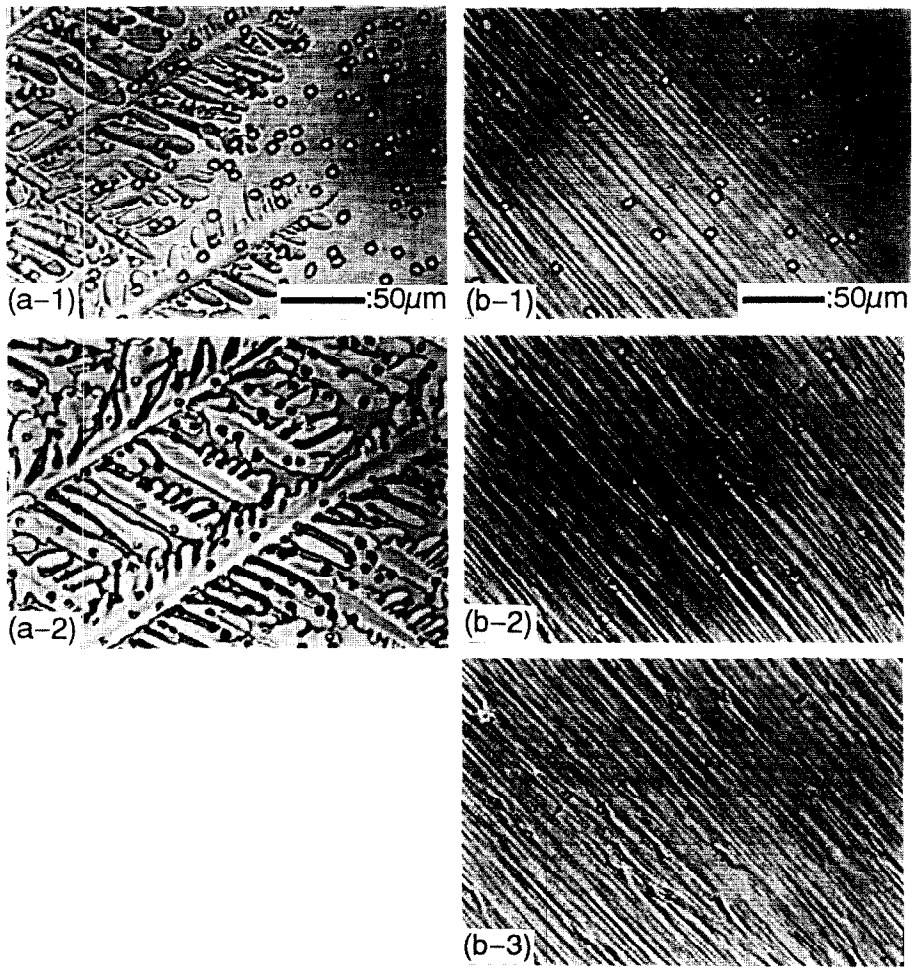

Fig. 4. Ice crystals and red blood cells on the colder region away from the tip of ice crystal. (a-1,2): PS+Gly; $H = 31.2°C\ min^{-1}$, $G = 20.4°C\ mm^{-1}$, $V = 1.53\ mm\ min^{-1}$; (a-2) is in the colder region about 0.25 mm away from (a-1); (b-1,2,3): PS+Gly+OP; $H = 27.1°C\ min^{-1}$, $G = 20.0°C\ mm^{-1}$, $V = 1.36\ mm\ min^{-1}$; (b-2) and (b-3) are located in the colder regions about 0.25 mm and 0.50 mm away from (b-1), respectively.

4.3. *Deformation and destruction of red blood cells due to spicular ice crystals in PS+Gly+AFPs*

The red blood cells trapped between the spicular ice crystals were deformed and destroyed by direct mechanical forces from the growing ice crystal. An example of deformation and destruction of the red blood cells is shown in Fig. 5 as time-variation of the states of red blood cells and ice crystals in the fixed region during the freezing of the sample. After the red blood cells of clear round appearances were deformed into flat appearances, they became unclear and disappeared. This mode of destruction of red blood cells was observed frequently.

4.4. *Red blood cells after thawing*

Figure 6 shows examples of the red blood cells immediately after the thawing. The lowest temperature which the sample experiences equals the temperature, T_c of the colder block. In PS+Gly many red blood cells survived the freezing and thawing, and were kept dispersed with morphologically intact appearances although some red blood cells have ghost-appearances. In contract to this, any survivals of red blood cells did not exist in PS+Gly+AFPs. The addition of 20 mg ml^{-1} AFPs completely destroyed the red blood cells.

4.5. *Cell survival after freezing and thawing*

The viability of cells after freezing and thawing was measured quantitatively to assess the influence of addition of AFPs. Table 2 also shows the results. More than 50% of the red blood cells in PS+Gly survived the freezing and thawing in three different thermal conditions. The viability was 82% in $H = 100°C\ min^{-1}$ while those in $H = 26°C\ min^{-1}$ were about 50–60%. The dependence of the viabilities on cooling rates is consistent with those reported in the past [1]. In PS+Gly+AFPs the viability of cells equaled zero in the range of this study. Therefore, the addition of antifreeze proteins in this study has a

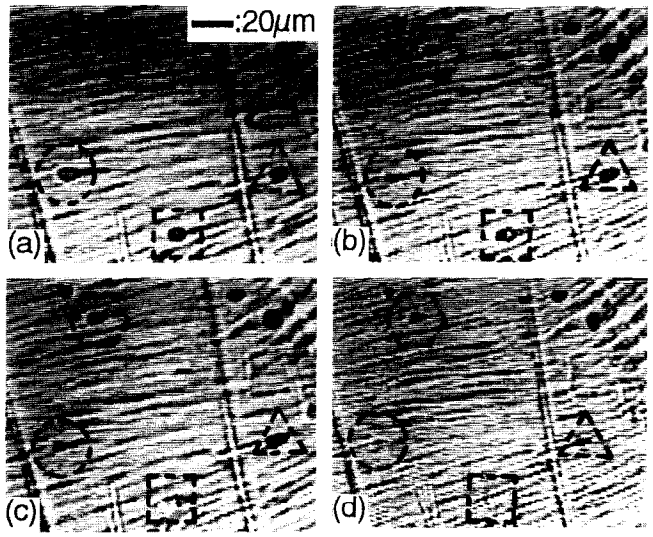

Fig. 5. Example of deformation and destruction of red blood cells due to interaction between red blood cells and spicular ice crystals (PS + Gly + OP). $H = 3.17°C\ min^{-1}$, $G = 20.0°C\ mm^{-1}$, $V = 0.16\ mm\ min^{-1}$; (b), (c), and (d) are about 15 s, 19 s, and 32 s later than (a), respectively.

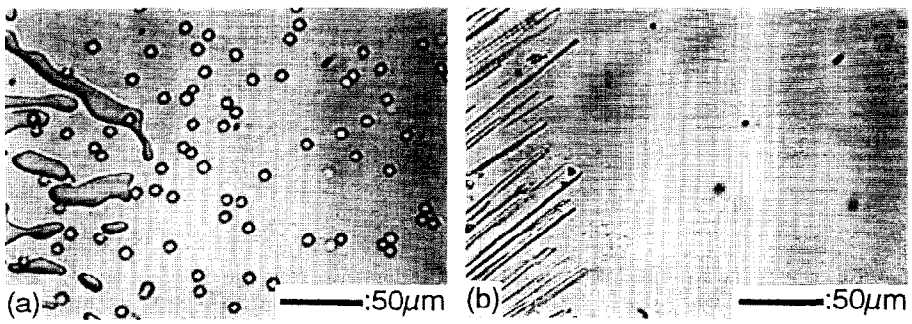

Fig. 6. Red blood cells immediately after thawing (a) PS + Gly, (b) PS + Gly + WF.

negative effect on improving cryopreservation of red blood cells in PS + Gly.

The concentration of 20 mg ml^{-1} WF and OP corresponds to about 5×10^{-3} mol l^{-1}, and the molarity is very much lower compared with those of sodium chloride and glycerol. This means that the amount of 20 mg ml^{-1} antifreeze proteins has little influence on the osmolarity of the solution, which is decided by the molarities of solutes in the solution. Therefore, it should not affect the ability of PS + Gly + AFPs to protect chemically the red blood cells during the freezing, which is induced by the presence of glycerol. However, complete destruction of the red blood cells due to the addition of 20 mg ml^{-1} antifreeze proteins results from the inflexible mechanical-interaction between the ice crystals and the red blood cells. This suggests that the morphology of the ice crystal and its mechanical interaction with the cells are among the factors influencing the cell damage through the freezing.

5. CONCLUSIONS

Experiments were performed to investigate the influence of fish antifreeze proteins on the freezing of suspensions of human red blood cells with glycerol from the viewpoint of bioheat and mass transfer. Microscopic behavior of the ice crystals and the red blood cells during the freezing was observed, and the cell viability after freezing and thawing was measured. It was found that two different antifreeze proteins, WF and OP, have a similar effect. The conclusions are as follows:

(1) The ice crystal in PS + Gly + AFPs had a spicular appearance, which was very different from the dendritic ice crystal appearance in PS + Gly. The spicular ice crystal structure did not depend on the advancing velocity of the temperature gradient. In contrast the dendritic ice crystals became finer and had a higher-order structure with an increase in the advancing velocity. This is attributed to the difference

in the mechanism of ice crystal growth, and shows that the influence of the AFPs on ice crystal morphology is dominant.

(2) The red blood cells in PS+Gly+AFPs were moved ahead by the spicular ice crystals and became trapped between them. With the growth of the ice crystals these red blood cells became deformed and destroyed due to the interaction between the ice crystal and the cells. This interaction is different from that in PS+Gly, where the red blood cells were not moved by the ice and were kept dispersed because of the ice-solution interfacial deformation in response to the cells.

(3) The addition of 20 mg ml^{-1} antifreeze proteins completely destroyed the red blood cells through the freezing and thawing. The increased damage of the red blood cells results from the inflexible mechanical interaction between the ice crystals and the cells. The addition of antifreeze proteins has a negative effect on cryopreservation of the red blood cells.

(4) The pattern of the interaction between the ice crystals and the cells in PS+Gly+AFPs belongs to the category of 'inflexible' type while that in PS+Gly belongs to the category of 'flexible' type.

(5) The experimental results in this study suggests that the morphology of the ice crystals and the mechanical interaction between the ice crystals and the cells are among the factors influencing the cell damage through the freezing of cell suspension.

More research into the interaction between ice crystals and red blood cells, the microscopic mechanism of cell damage and quantitative studies is required.

Acknowledgement—The assistance of Dr T. K. Eto in the arrangement of experiments is acknowledged.

REFERENCES

1. McGrath, J. J., Preservation of biological material by freezing and thawing. In *Heat Transfer in Medicine and Biology*, Vol. 2, Chap. 20, *Analysis and Application*, ed. A. Shitzer and R. C. Eberhart. Plenum Press, New York, 1984.
2. Mazur, P. and Rigopoulos, N., Contributions of unfrozen fraction and of salt concentration to the survival of slowly frozen human erythrocytes: influence of warming rate. *Cryobiology*, 1983, **20,** 274–289.
3. Pegg, D. E. and Diaper, M. P., The unfrozen fraction hypothesis of freezing injury to human erythrocytes: a critical examination of the evidence. *Cryobiology*, 1989, **26,** 30–43.
4. Ishiguro, H. and Rubinsky, B., Mechanical interactions between ice crystals and red blood cells during directional solidification. *Cryobiology*, 1994, **31,** 483–500.
5. Franks, F., *Biophysics and Biochemistry at Low Temperatures*. Cambridge University Press, New York, 1985, pp. 112–126.
6. DeVries, A. L., The role of antifreeze glycopeptides and peptides in the freezing avoidance of antarctic fishes. *Comparative Biochemistry and Physiology*, 1988, **90**(B-3), 611–621.
7. Rubinsky, B., Arav, A. and DeVries, A. L., The cryoprotective effect of antifreeze glycopeptides from antarctic fishes. *Cryobiology*, 1992, **29,** 69–79.
8. Rubinsky, B., Mattioli, M., Arav, A., Barboni, B. and Fletcher, G. L., Inhibition of Ca^{2+} and K^{+} currents by antifreeze proteins. *American Journal of Physiology*, 1992, **262** (Regulatory Integrative Comp. Physiol. 31), R542–R545.
9. Ishiguro, H. and Rubinsky, B., Microscopic behavior of ice crystals and biological cells during directional solidification of solution with cells (in Japanese). *Transactions of the Japan Society of Mechanical Engineers*, 1994, **60** (572B), 1349–1355.
10. Ishiguro, H. and Rubinsky, B., Morphological microstructures during directional solidification of suspensions of human red blood cells. *Proceedings of 10th International Heat Transfer Conference*, Vol. 4, ed. G.F. Brighton, 1994, pp. 43–48.
11. Ananthanarayanan, V. S., Antifreeze proteins: structural diversity and mechanism of action. *Life Chemical Report*, 1989, **7,** 1–32.
12. Rubinsky, B. and Ikeda, M., A cryomicroscope using directional solidification for the controlled freezing of biological material. *Cryobiology*, 1985, **22,** 55–68.