ENHANCEMENT OF CRYOINJURY BY USE OF EUTECTIC CRYSTALLIZATION OF AN AMINO ACID ADJUVANT

by

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ABSTRACT

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Enhancement of cryoinjury by use of eutectic formation was investigated to improve the efficacy of cryosurgery. Although the enhancement of cryoinjury associated with eutectic crystallization of sodium chloride (NaCl) has been demonstrated, its advantage can be maximized with adjuvants which are biocompatible and have higher eutectic temperature than NaCl. In this study, the feasibility of glycine, which is less toxic and have higher eutectic temperature than NaCl, was tested. Two groups of MCF-7 human breast cancer cell were suspended in isotonic saline with glycine, then were frozen and thawed on a directional solidification stage in the same protocol except that eutectic crystallization was initiated in only one group. The results show that the

post-thaw viability drops from 86% to 38.6% upon the initiation of eutectic crystallization at a high temperature (-8.5°C). The results also confirm that enhancement of direct cell injury during freezing can be achieved by eutectic crystallization.

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CHAPTER 1

INTRODUCTION

1.1 Background of the cryosurgery

Cryosurgery is a minimally invasive surgical technique to destroy malignant or unwanted tissues by localized freezing. The primary clinical objective of cryosurgery is to eradicate malignant tissue with the intent to minimize adjacent normal tissue damage. However, challenges rise from incomplete tissue destruction near the iceball edge, where tissues are frozen but not completely destroyed. Therefore, there is a gap between the complete destruction zone and the edge of the iceball. This gap makes a surgical margin inevitable (i.e. freezing bigger volume of tissue than targeted tissue). This will result in unwanted cryoinjury of the adjacent tissues [1-2]. However, sparing adjacent tissues, blood vessels and nerves from cryoinjury is imperative rather than optional in certain surgical situations. Besides adjacent tissue damage, the gap abates the capability of intraoperative imaging of the cryolesion. Therefore,

enhancement of cryoinjury within the iceball is highly desirable without enlarging the volume of frozen tissue.

1.2 Enhancement of cryoinjury

To enhance the cryoinjury within cryolesion, the following adjuvants have been investigated: chemo-therapeutic agents, tumor necrosis factor-α (TNF- α), and antifreeze protein (AFP). Clarke et al. [3] showed that cryoinjury of human prostate cancer (PC-3) cells enhancement can be achieved by exposing PC-3 cells on 5-fluorouracil (5-FU), a chemo-therapeutic agent, for 2 and 4 days with the following freezing. Enhancement of direct cell injury by use of thermophysical adjuvants [4-5] and enhancement of vascular injury induced by inflammatory adjuvants has been studied [6]. The thermophysical adjuvants are usually salts which can induce eutectic crystallization. The inflammatory adjuvants like TNF- α, a cytokine, is known to promote inflammation, endothelial injury, and apoptosis [7]. Koushafar et al. [8] reported that during freezing/thawing cycle with AFP, cellular destruction has significantly increased. Moreover, both thermophysical and inflammatory adjuvants are believed to have the potential to enlarge the complete destruction zone and make the cryolesion and the edge of iceball closer.

1.3 Phase change behavior in biological solutions

Understanding the phase change behavior with biomedical solutions during freezing and thawing is important for the development and enhancement of the cryosurgery. Luyet [9] used a microscope to investigate patterns of ice formation of various biological solutions. Rey [10] used differential thermal analysis (DTA) to investigate the exothermic or endothermic phenomena of the frozen solutions. His research found that during thawing, there was a second heat release around -24°C. He also concluded that the heat release was accompanied with the eutectic phase change, where the solute and the remaining unfrozen water crystallized as a single component.

Eutectic crystallization is defined as simultaneous solidification of unfrozen fractions into solids [11]. Greaves [12] reported that during the freezing, eutectic crystallization occurred with supercooling only, but during thawing, melting occurred at the thermodynamic equilibrium eutectic temperature of that solution. Luyet [13] used a cryomicroscope to investigate the primary crystallization of different solutions and eutectic crystallization of various biological solutions. Han et al. [14] observed the eutectic crystallization in water-NaCl binary solutions and reported that when the samples were cooled below -40°C, the eutectic crystallization could be observed and the amount of eutectic was proportional to the concentration of the sample.

1.4 Objective

Kristiansen [15] investigated the effects of eutectic crystallization on liposome in NaCl-water solutions by the leakage of fluorescent dye. He also reported that eutectic crystallization would increase the leakage which was similar to cell membrane injury after freezing. In a recent study, Han et al. [5] also reported that the viability of AT-1 cell suspensions significantly dropped with the occurrence of eutectic crystallization within the suspended media. The destruction of AT-1 cells enhanced when the cells were frozen and thawed with eutectic crystallization. They concluded that direct cell injury during freezing could be enhanced by infusing other solutes, whose eutectic temperatures are higher than that of sodium chloride, thus improving the incomplete killing zone of the iceball.

The effectiveness of cryoinjury enhancement using eutectic crystallization is limited by the eutectic temperature, which is the threshold of cryoinjury. Moreover, the salts become harmful at high concentrations. The advantages of the eutectic freezing with a high eutectic temperature are to enhance the effect of cryosurgery and to prevent cryoinjury to the surrounding tissue. To maximize the efficacy of this procedure, better adjuvants should be identified which have higher eutectic temperatures and are less toxic at high concentrations than other salts.

The primary objective of this research is to test the feasibility of a new type of amino acid adjuvant, and to produce preliminary data. In this study,

glycine ($C_2H_5NO_2$) will be tested, whose eutectic temperature is approximately -3.6°C and less toxic at high concentrations [16-18]. To achieve these objectives, the following two specific aims will be attained. The first will be to quantify the enhancement of cellular destruction by eutectic crystallization of glycine. The second will be to visualize the opacity change due to the eutectic crystallization. The reason why visualizing the opacity change because it is useful to monitor the eutectic phase change interface.

CHAPTER 2

MATERIAL AND METHODS

2.1 Cells and reagents

MCF-7 human breast cancer cells were cultured in vitro. The waste generated during culturing was washed by a phosphate buffered saline (PBS) solution. Immersing cultured MCF-7 cells in trypsin for 10 minutes caused these cells to separate from a culture flask. They were then suspended in a 5% fetal bovine serum (FBS)-supplemented culture medium (D-MEM/F12, Life technologies, Grand Island, NY). The cells were consistently collected during their rapidly growing phase. After separation and after adding a cell culture medium, the cells were pelleted by centrifuge and the excess medium was removed. Glycine (Sigma-Aldrich, St. Louis, MO) was dissolved in isotonic saline by 10% (weight/weight-saline) at room temperature because visualization of the eutectic phase change was easier and because qualitative features of the thermogram were independent of the concentration over a certain range [18]. Details of the characterization of frozen glycine solutions should refer to other

studies [19-22]. The cell pellet was re-suspended in a 10% (w/w) glycine-isotonic saline (approximately 200 µl) before the experiment. The suspensions were stored in a 1.5 ml microcentrifuge tube on ice (approximately 4°C) for no longer than 3 hours. The viability of the cell suspensions without freezing was checked before and after each experiment as a control.

2.2 Directional solidification stage

The directional solidification stage [23] (see Appendix B) consisted of two constant temperature blocks. One of the blocks was held at a temperature, T_H (see Appendix A), above the phase change temperature. The other block was held at a temperature, T_L, below the phase change temperature. The sample was then placed on a glass microslide (25 mm in width, 75 mm in length and 1 mm in depth) with a machined cavity. During freezing, the microslide was driven from the high temperature block to the low temperature block over a gap distance at a precisely controlled velocity. From equation (1), the desired cooling/thawing rate could be obtained by appropriately setting the temperature at each block, the gap distance, and the velocity of the microslide. A CCD B/W video camera (XCST50, Sony, Tokyo, JP) was mounted above the directional solidification stage which was connected to the frame grabber (NI-PCI-1410, National Instruments, Austin, TX) and controlled via LabVIEW to visualize the eutectic phase change during freezing. Figure 1 represents the schematic diagram of directional solidification stage.

$$CR = \frac{(T_H - T_L) \times V}{D} \quad (1)$$

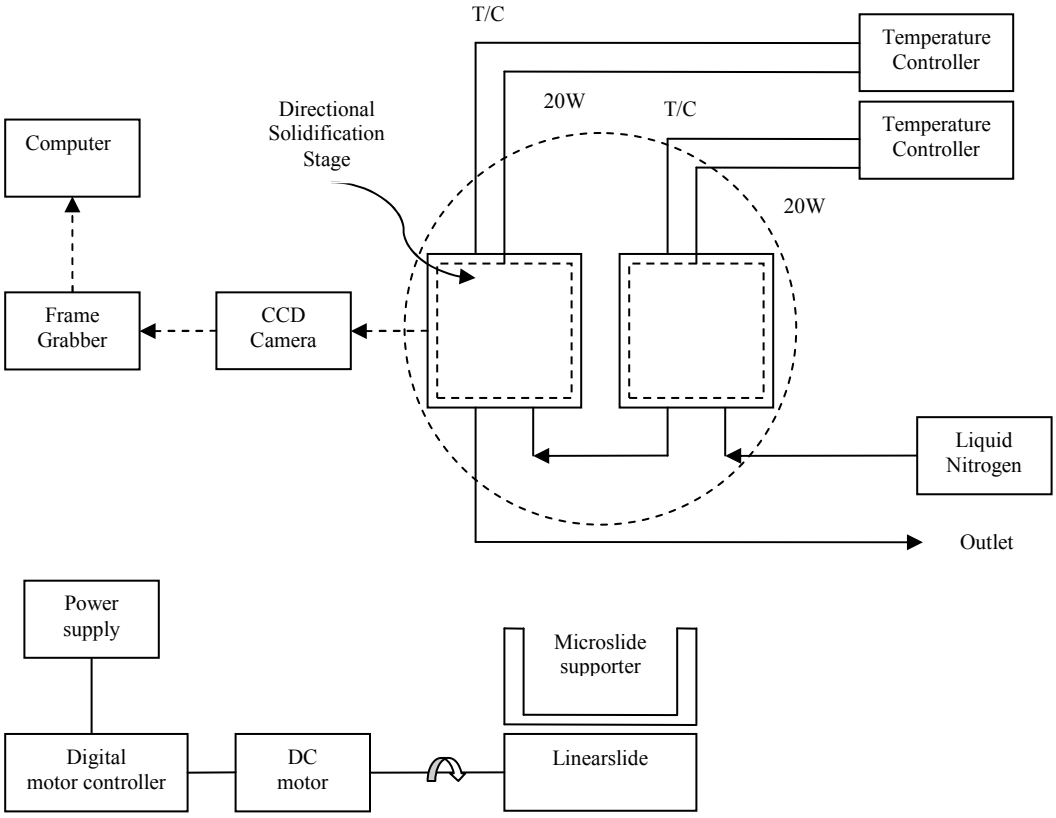

Figure 1.Schematic diagram of directional solidification stage.

2.3 Microscopic Visualization

A microscope (BX51, Olympus, Center Valley, PA) was used to visualize the eutectic phase change during freezing. Initially, a 10% glycine-isotonic saline was placed on the glass microslide and then viewed through the microscope. Later, a thin copper plate was immersed in liquid nitrogen for at least 3 minutes then placed on the top of the microslide to lower the

temperature of the microslide. This was done without touching the sample. Thus, the sample temperature on the microslide could be lowered enough for eutectic crystallization to be initiated spontaneously.

2.4 Design of experiments

The design of experiments is to investigate the MCF-7 cells' post-thaw viability changes to the eutectic crystallization of 10% glycine-isotonic saline. Two groups of cells were suspended in 10% glycine-isotonic saline. The procedure was (i) cooling the samples from 4°C to -8.5°C at 5 °C/min, (ii) holding at -8.5°C for 3 minutes and (iii) thawing back to 30°C at 100 °C/min (n=3 for each group). The main difference between the two suspension groups was that eutectic crystallization was initiated in one group at the beginning of the holding step (ii) by touching the edge of the samples with a pre-cooled needle. The pre-cooled needle was submerged in liquid nitrogen at least 3 minutes. A three minute holding time was long enough for the eutectic crystallization to propagate across the entire sample. The end temperature -8.5°C was lower than the thermodynamic equilibrium eutectic temperature of glycine-water. The occurrence of the eutectic crystallization was visualized by a distinct optical change from transparent to opaque in the medium. After the experiments, the viability of each group will be assessed (see appendix D).

CHAPTER 3

RESULTS

3.1 Microscopic Visualization of eutectic crystallization

The corresponding microscopic images of the eutectic phase change of 10% glycine-isotonic saline during freezing are shown in Figure 2. In Figure 2 (A), the sample is placed on the microscope at room temperature in its initial state; (B) after placed the pre-cooled copper plate on top of the microslide, the temperature goes down, ice crystals grow until the concentration of the unfrozen fraction (denoted with “U”) between the ice crystals reaches the eutectic concentration; (C) with the temperature goes down, eutectic crystallization started to propagate from the left hand side (denoted with →); (D) eutectic crystallization (denoted with “Eu.”) propagated through out the whole sample.

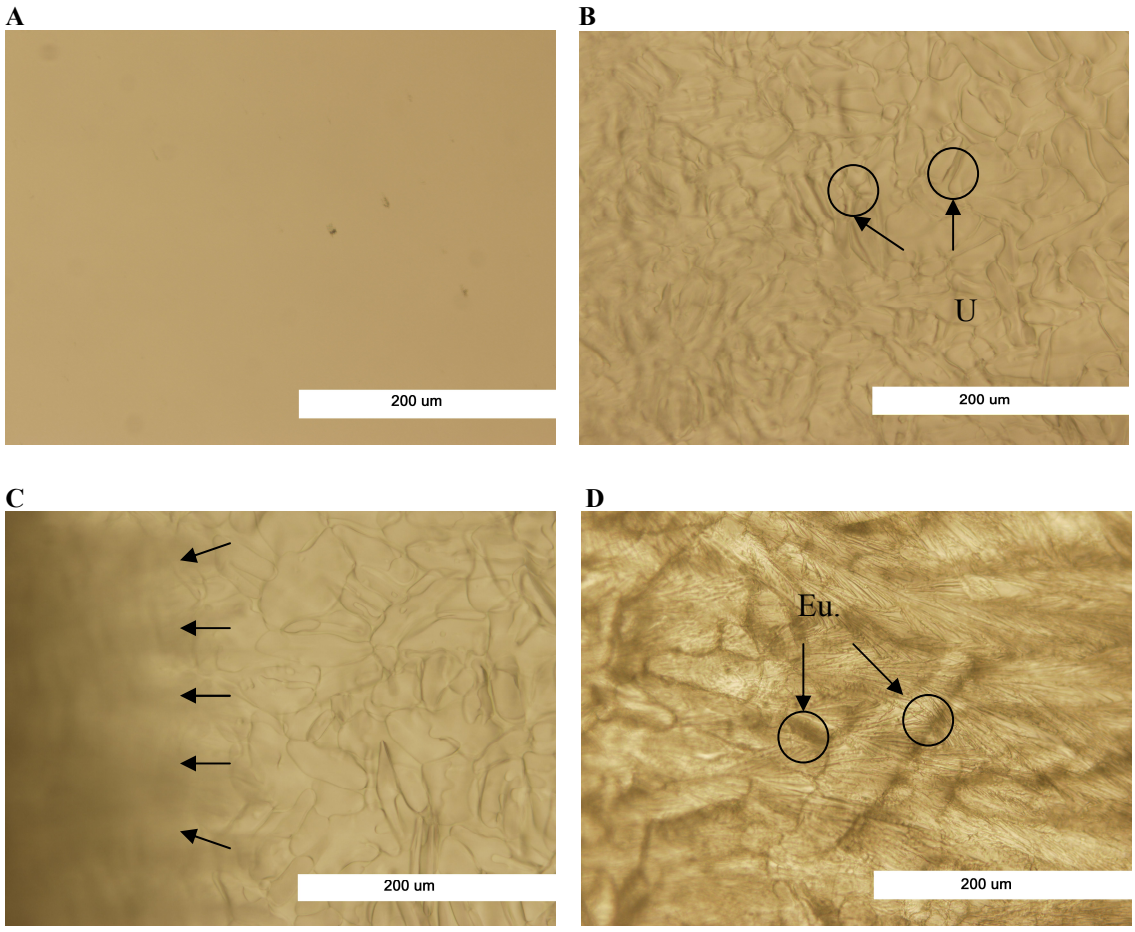

Figure 2. Microscope images of 10% glycine-isotonic saline during freezing. (A) sample before freezing; (B) complete ice formation; (C) propagation of eutectic crystallization; (D) complete eutectic crystallization.

3.2 Macroscopic visualization of eutectic crystallization in directional solidification stage

The phase change images of a 10% glycine-isotonic saline with and without eutectic crystallization during freezing are shown in Figure 3. In the first group of Figure 3 (A) ~ (D), the sample was cooled from 4°C to -8.5°C and held at -8.5°C for 3 minutes without eutectic crystallization (denoted with "Eu.") initiated spontaneously. In the second group of Figure 3 (E) ~ (I), the sample is cooled from 4°C to -8.5°C with the eutectic crystallization initiated by a pre-cooled needle. In Figure 3(A) the sample was held at 4°C; (B) when microslide moved from high temperature block (T_H) to low temperature block (T_L) over a gap, ice started to from during freezing; (C) ice formed completely at low temperature block; (D) there was no initiation of the eutectic crystallization within 3 minutes; (E) the sample was held at 4°C; (F) ice started to form during cooling; (G) complete ice formation at -8.5°C; (H) initiation of the eutectic crystallization by use of a pre-cooled needle; (I) eutectic crystallization propagated through out whole sample within 3 minutes.

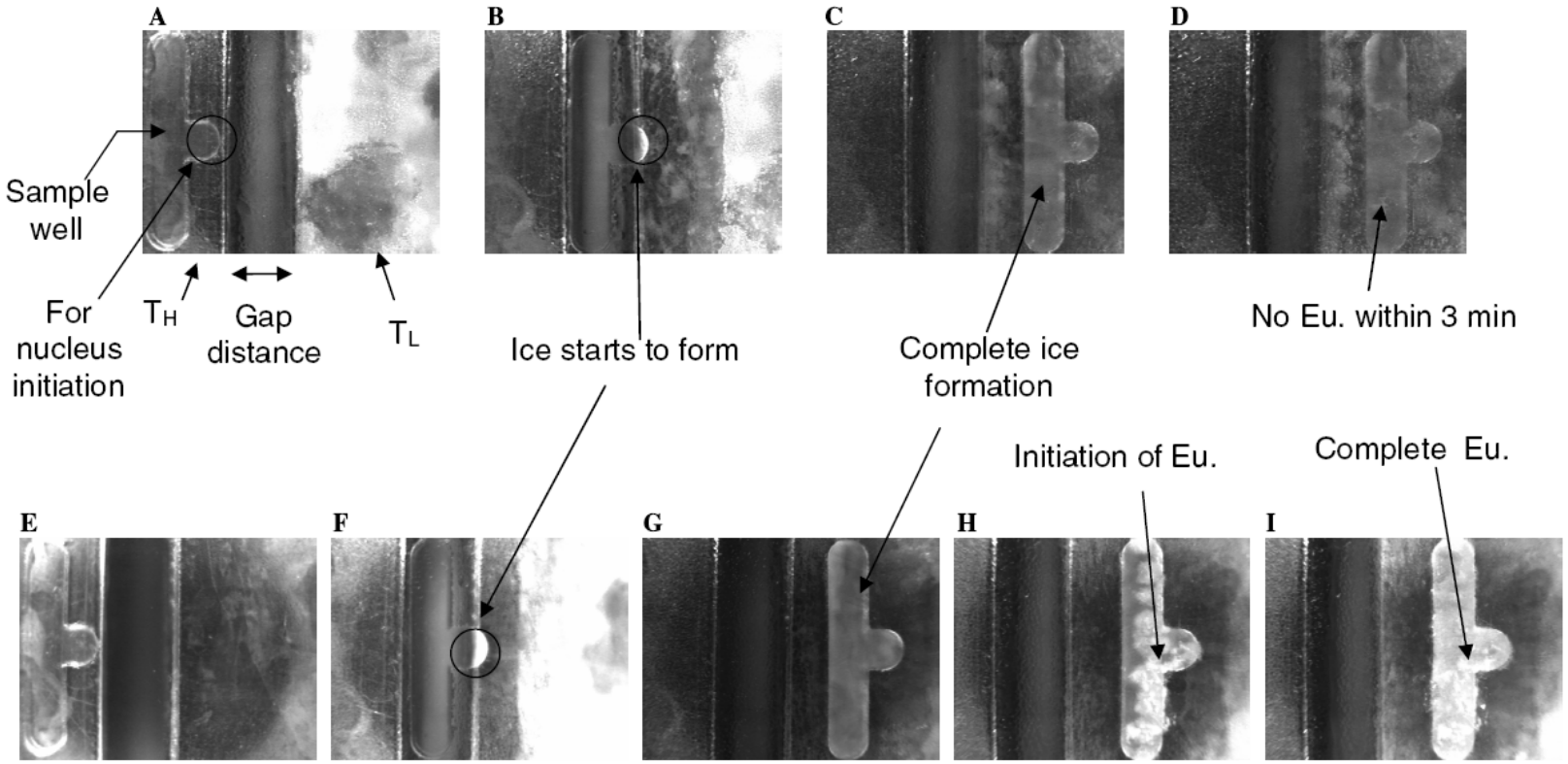

Figure 3. Images of 10% glycine-isotonic saline during freezing with and without eutectic crystallization initiated. Group (A) ~ (D) are without eutectic crystallization initiated during freezing; group (E) ~ (I) are with eutectic crystallization initiated by a cold needle during freezing. (A) initial state of the sample at 4°C; (B)ice starts to from during freezing; (C) complete ice formation at -8.5°C; (D) no initiation of the eutectic crystallization within 3 minutes;(E) initial state of the sample at 4°C; (F) ice starts to form during freezing; (G) complete ice formation at -8.5°C; (H) initiation of the eutectic crystallization by a cold needle; (I) complete eutectic crystallization within 3 minutes.

3.3 Viability assay

The images of viability assay (see Appendix D) are shown in Figure 4. The viability assay of control was checked before and after each experiment. After freezing/thawing (F/T) MCF-7 in 10 % glycine-isotonic saline with and without eutectic crystallization, the viability was also been checked. Figure 4 (A) shows the total cells in control group; (B) dead cell in control group; (C) total cells after F/T without eutectic crystallization; (D) dead cells after F/T without eutectic crystallization; (E) total cells after F/T with eutectic crystallization; (F) dead cells after F/T with eutectic crystallization.

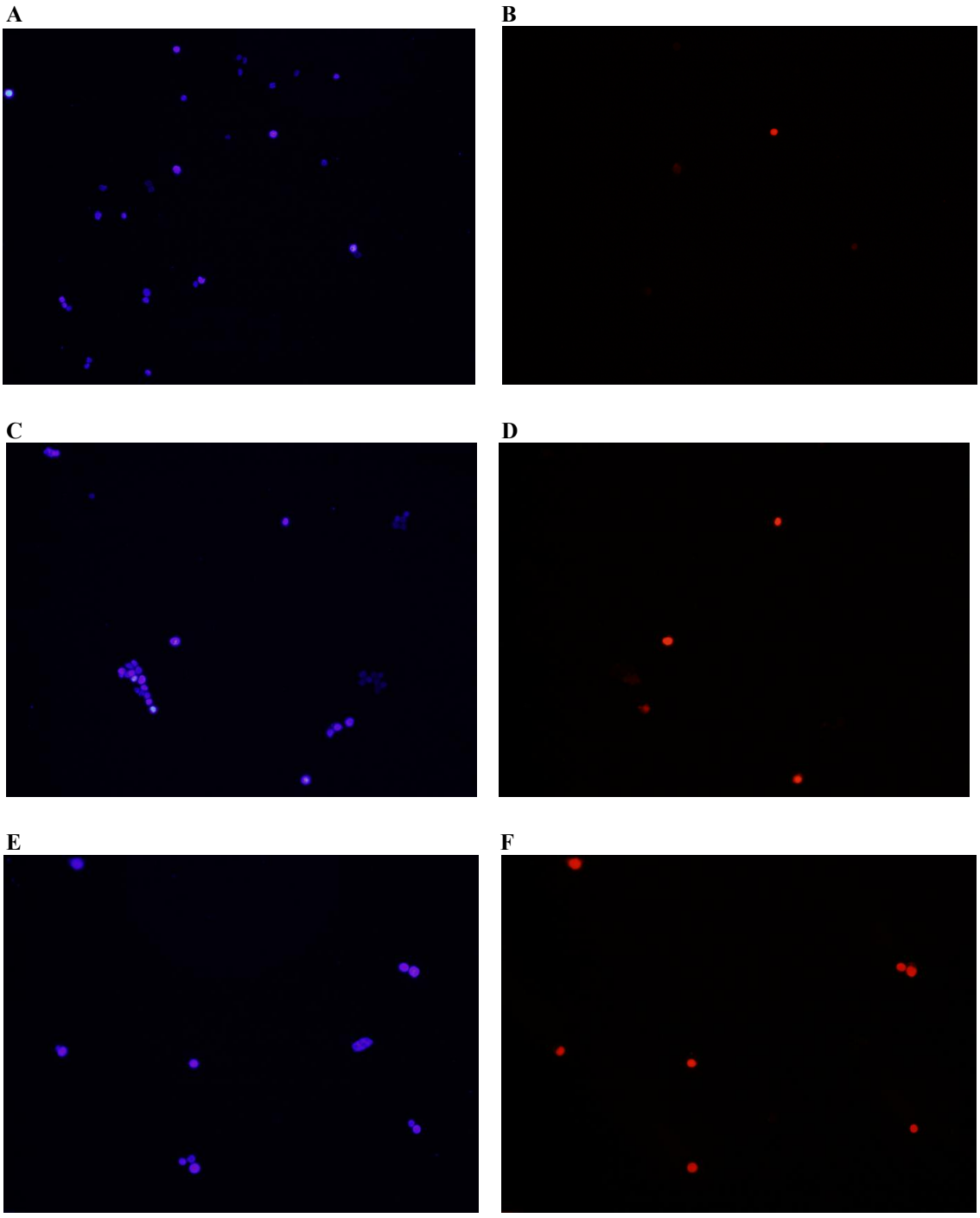

Figure 4. Viability assay of MCF-7 cell suspensions in 10% glycine-isotonic saline. (A) total cells in control group; (B) dead cell in control group; (C) total cells after F/T without eutectic crystallization; (D) dead cells after F/T without eutectic crystallization; (E) total cells after F/T with eutectic crystallization; (F) dead cells after F/T with eutectic crystallization.

Figure 5 shows the post-thaw viability changes of MCF-7 cell suspensions in a 10% glycine-isotonic saline with eutectic crystallization initiated during the freezing/thawing protocol. The viability of the control is 91.9±1.77% (n=3). After freezing/thawing goes to -8.5°C without eutectic formation, the viability of samples decreases to 86±2.07% but the decrease is not statistically significant (* $p=0.06$) (n=3).This change may be due to glycine has cryoprotective effects in certain combinations [16]. When eutectic crystallization is initiated in the sample during the same freezing/thawing protocol to -8.5°C, the viability drops (** $p<0.001$) to 38.6±1.02% (n=3). Both groups have undergone the same freezing/thawing protocol but the group with eutectic crystallization initiated suggests that there may be other cell injury associated with eutectic formation during freezing.

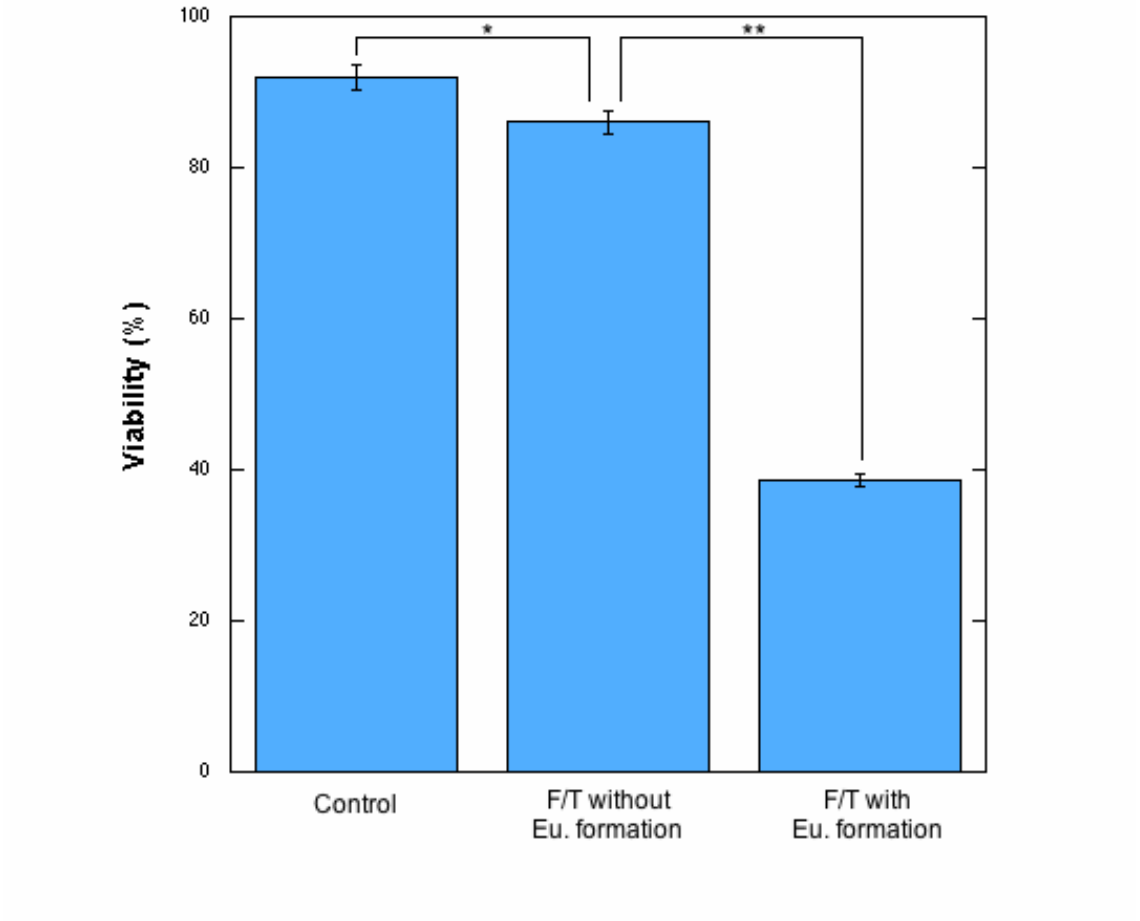

Figure 5. Post-thaw viability changes due to the eutectic crystallization in 10% glycine-isotonic saline. Samples without eutectic formation are frozen to -8.5°C at 5°C/min, then held at -8.5°C for 3 min, and thawed back to 30°C at 100°C/min. Samples with eutectic formation underwent the same freezing/thawing protocol except the eutectic crystallization initiated during 3 min holding time at -8.5°C by use of a pre-cooled needle. (n=3 for each experimental group).

CHAPTER 4

DISCUSSION

4.1 Eutectic phase change of glycine

Representative DSC thermograms of various glycine-water concentrations ranging from 0.5 to 15% w/v are shown in Figure 6 [18]. Here samples were cooled at 0.5°C/min, then warmed at 0.1°C/min. From Figure 6, the thermodynamic equilibrium eutectic temperature is approximately -3.6°C. By adding NaCl into glycine-water solution, the eutectic temperature will change due to increased electrolytes. The initiation of the eutectic formation required for supercooling beyond the thermodynamic equilibrium eutectic temperature has also been confirmed in this study. Although the data are not shown here, setting the end temperature at -10°C, the eutectic crystallization on a glycine-isotonic saline will be initiated spontaneously within 1 minute. The eutectic crystallization can also be initiated by increasing the holding time at certain temperatures lower than the thermodynamic equilibrium eutectic temperature. Other than lowering the end temperature and increasing the holding time the

eutectic crystallization can also be initiated by using a pre-cooled needle which provides supercooling has also been confirmed in this study. This work also supports the idea that eutectic crystallization consists of nucleation and crystal growth. By comparing the microscopic pictures of eutectic crystallization in NaCl-water solution [14] and glycine-isotonic saline in this study, the eutectic structure in glycine-isotonic saline ternary system appears more complicated than in NaCl-water binary system. Also, the dendritic patterns in frozen glycine-isotonic saline are much more obvious than in a frozen NaCl-water solution even though the end temperature and concentration are different.

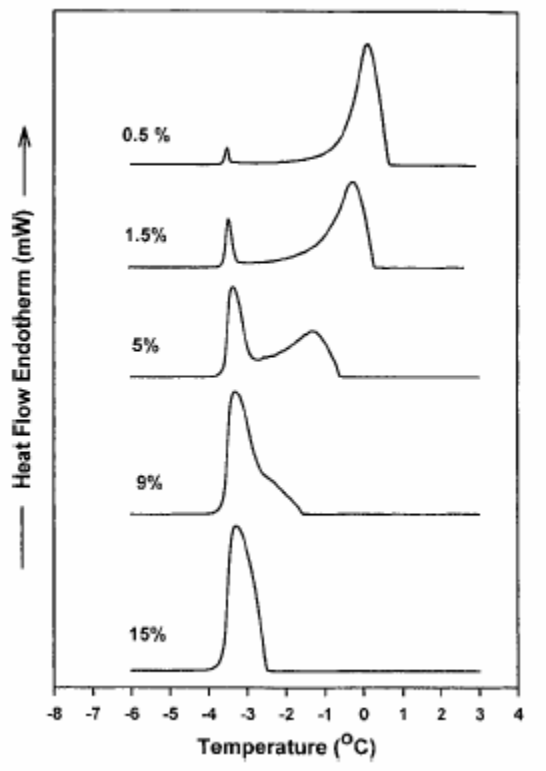

Figure 6. DSC thermograms of various concentrations of frozen glycine solutions cooled at 0.5°C/min. Heating rate 0.1°C/min.

4.2 Enhancement of cryoinjury by eutectic phase change

As shown in the results, the viabilities of MCF-7 cell suspensions decrease with eutectic formation within the suspended media. Meanwhile, the eutectic crystallization initiated at a high eutectic temperature (-8.5°C) was achieved by adding glycine-isotonic saline into cell suspension media. With the eutectic crystallization initiated during freezing, the gap between the complete destruction zone and the edge of the iceball will be decreased. With a higher eutectic temperature, this implies that the damage to the surrounding tissues/cells of the freezing region can be reduced. However, after freezing/thawing samples to -8.5°C with eutectic formation, the viability does not drop significantly. With eutectic crystallization initiated, it does enhance the cryoinjury but around 40% of cells are still alive. Hence, further study is required to enhance the cellular destruction by use of eutectic freezing with high a eutectic temperature. Except for the traditional cryoinjuries, this result supports the fact that there is an additional freezing injury associated with eutectic formation. This may be due to either mechanical damage to the cell membrane by eutectic crystalline structure or by eutectic crystallization in the intracellular space or both [5].

CHAPTER 5

SUMMARY

The cryoinjury associated with eutectic crystallization on MCF-7 breast cancer cells during freezing/thawing was investigated and the eutectic phase change behavior visualized. The conclusions are summarized as following:

1) Enhancement of direct cell injury during freezing can be achieved by adding other solutes whose eutectic temperatures are higher than sodium chloride. This would be helpful to improve the ability of intraoperative imaging of the cryolesion and to abate the gap distance between the complete killing zone and incomplete killing zone.
2) Further study is required to verify additional freezing injury mechanisms due to eutectic formation; and to enhance the effect of eutectic formation of different adjuvants whose eutectic temperatures are higher than the NaCl-water system.

APPENDIX A

NOMENCLATURE

1. Nomenclature

CR	Cooling rate, °C/minute.
D	Gap distance between the edges of the high and low-temperature blocks, mm.
T	Temperature, °C.
V	Velocity of the microslide, mm/minute.

2. Subscripts

H	High-temperature block.
L	Low-temperature block.

APPENDIX B

EXPERIMANTAL APPARATUS

1. Main stage

Two aluminum blocks (2 x 2 x1 inch) mounted on a support structure composed of an aluminum base (10 x 9.5 x 0.6 inch). Oxygen-free copper blocks (2 x 2 x 0.55 inch) are mounted above the aluminum blocks. Each copper block has two 0.28-inch-diameter passages for liquid nitrogen. In addition, a thin 661-Ω (2 x 2 inch) thermofoil heater (HK5169R661L12, Minco, Minneapolis, MN) is sandwiched between each copper block and 0.035 inch thick copper plate. Lord adhesive (3135A/B, Ellsworth Adhesives, Germantown, WI) was chosen to glue the thermofoil heater between copper block and copper plate with temperatures ranging from -195°C to 93°C in usage.

Two thermo-couples (T-type, Omega, Stamford, CT) were attached on the surface of each copper plate with CC high temperature cement (CC high temperature cement, Omega, Stamford, CT) which temperatures ranging is from -200°C to 843°C.

2. Temperature control and measurement system

Two temperature controllers (CT15111, Minco, Minneapolis, MN) were used to adjust the temperature of each surface. The output from the temperature controllers was 3.5A at 250VAC at 25°C; then derates to 1.25A at 55°C. The temperature accuracy is ±0.25% of span ±1°C. The temperature resolution is 1°C or 0.1°C which is selectable. The control box (377-1098-ND Digi-key corporation, Thief River Falls, MN) was chosen to hold two temperature controllers which contain one 8 Amp slow-blow fuse (RadioShack, Fort Worth, TX) for the power entry module, two 3.5 Amp slow-blow fuses (576-022903.5H, Mouser ELECTONICS, Mansfield, TX) for heater #1and heater #2, two 0.25 Amp slow-blow fuses (576-0313.250H, Mouser ELECTONICS, Mansfield, TX) for controller #1and controller #2.

3. Linear slide and motorized system

The Unislide (MB2509, Velmex, Inc, Bloomfield, NY) with leadscrew in 0.05 in/rev was used as the foundation of the motorized positioner combined with a DC motor coupling to its rotating handle. The dc motor (BX230A-30, Oriental Motor, Torrance, CA) has a 60 mm (2.36 inch) square motor size, a 30W in output power, and a 30:1 gear ratio. The speed range is from 3 to 3000r/min and 8-step speed setting in speed control mode. A digital data setter (OPX-1A, Oriental Motor, Oriental Motor, Torrance, CA) was used to control the

speed of the motor. Acrylic supporter were fixed on the linearslide to guide the microslide back and forth between two blocks.

4. Image recording system

The CCD B/W video camera (XCST50, Sony, Tokyo, JP) was used for image capturing via LabVIEW.

5. Liquid nitrogen tank

The MVE liquid nitrogen tank (Lab-20, Sementanks, Washington, PA) with discharge device was used for liquid nitrogen storage.

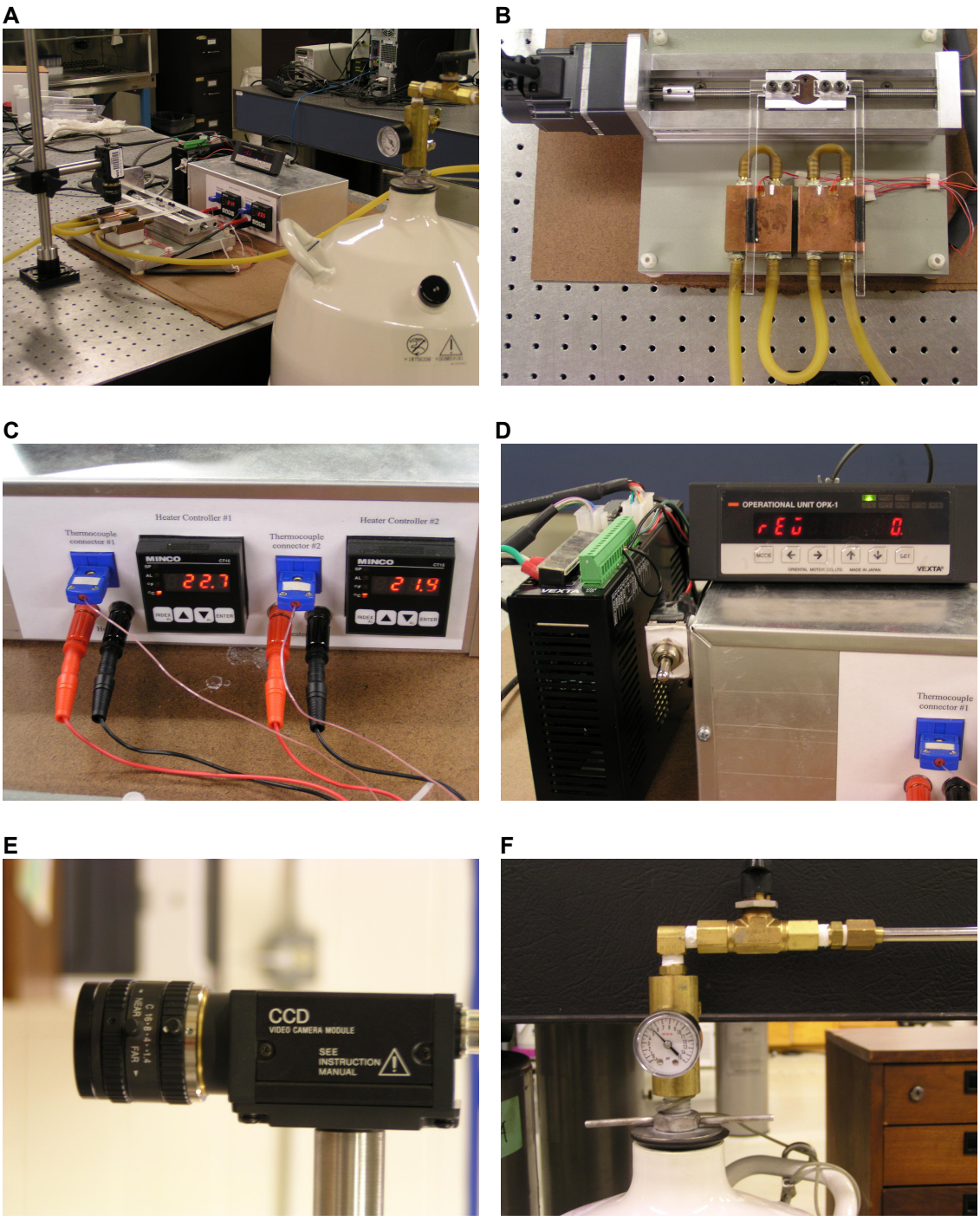

Figure 7. Directional solidification stage. (A) whole system; (B) main stage with motor and linear slide; (C) temperature control and measurement system; (D) motorized control system; (E) CCD camera; (F) liquid nitrogen tank.

APPENDIX C

STANDARD OPERATION PROCEDURE FOR DIRECTIONAL SOLIDIFICATION STAGE

1. Decide the desired cooling rate.

2. Using a speed conversion table based on equation (1), calculate the speed of motor in rpm.

   The speed conversion is as follows:

$$1\text{rpm} \times 0.05 \frac{\text{in}}{\text{rev}} = 1/30\,\text{rpm} \times 1.27 \frac{\text{mm}}{\text{rev}} = 0.04233 \frac{\text{mm}}{\text{min}}$$

Table 1. Speed conversion.

T_H (°C)	T_L (°C)	ΔT (°C)	Gap distance (mm)	Cooling rate (°C/min)	Speed of slide in mm/min (output)	Speed of motor in r/min (input)	Integer speed in r/min (input)
5	-5	10	6	5	3	70.87	71
10	-10	20	6	10	3	70.87	71
15	-15	30	6	20	4	94.49	94
20	-20	40	6	50	7.5	177.17	177

   a) Enter T_H and T_L.
   b) Enter gap distance.
   c) Enter desired cooling rate.
   d) Get motor speed in rpm.

3. Set speed for motor.

   a) Press MODE.
   b) Press ↓ (After pressing ↓, it will indicate the operation data No. 0).
   c) Press SET.
   d) “P┤0000000” – This data is disabled due to speed control mode is set.
   e) Press SET.

f) Press ←/→ to move digit, ↑/↓to change digit number.
g) Press SET (After press set, ⊾⌐90 indicates torque limit, usually 100%).
h) Press SET.

4. Sample preparation.

5. Set temperature on each controller.

a) Press INDEX.
b) Press ▲/▼ to adjust to the desired temperature.
c) Press ENTER to retain the prescribed value.
d) Check Input Correction

Table 2. Input correction for temperature controllers.

Set Temperature	4°C	-10°C	-20°C	-40°C
Input Correction For Controller #1 (Left hand)	-0.4			
Input Correction For Controller #2 (Right hand)		-0.2	-0.6	-0.4

e) Check Pid value.
1) Pb:3
2) rES:0.1
3) rtE:0.01

6. Make valve of the liquid nitrogen tank ¼ turn.

7. Drop pure ethanol on the cold temperature base.

8. Regulate the valve until the temperature reaches steady state.

9. Turn on the switch for motor: + moving from left to right.

- moving from right to left.

10. Run experiment.
11. Clean up the stage after the experiment.

APPENDIX D

STANDARD OPERATION PROCEDURE FOR VIABILITY ASSAY

1. Collect cell with one full flask.

    a) Dump the media.
    b) Add 8ml PBS to wash the waste twice (then dump out PBS).
    c) Add 1/2ml Trypsin to wash the flask then dump out Trypsin.
    d) Add 2ml Trypsin then put it in incubator for 10 minutes.
    e) Add 8ml 5% fetal bovine serum (FBS)-supplemented culture medium.
    f) Centrifuge it in 1000rpm, 3 minutes.
    g) Dump out all the media.

2. Resuspend it with 10% glycine isotonic saline (200µl) in microtube.

3. Put the microtube in a container with ice in it.

4. 25µl of cell suspension media will be place in the machined cavity of the microslide.

5. Set T_H=4°C, T_L=-8.5°C, gap distance=6 mm, CR=5°C/min.

6. Three sets of experiment (n=3 for each experiment).

        (i) Control.
        (ii) Freezing/thawing with eutectic formation.

    a) Cool sample from 4°C to -8.5°C at 5 °C/min.
    b) Initiate eutectic crystallization by touch the edge of the sample with a pre-cooled needle which has immersed in liquid nitrogen for 3 minutes.
    c) Thawing back to 30°C by using water bath.

        (iii) Freezing/thawing without eutectic formation.

    a) Cool sample from 4°C to -8.5°C at 5 °C/min.

b) Holding at -8.5°C for 3 minutes.
c) Thawing back to 30°C by using water bath.

7. Take 10µl of the cell suspensions after the experiment + 1µl of dye then put in microtube and shake it.

8. Culture it in incubator for 15 minutes.

9. Pipette out the sample from the microtube and placed on the microslide with coverslip.

10. Using Fluorescence Microscope for viability assay.

    a) Using DAIP filter to count total cells.
    b) Using TRITC filter to count dead cells.
    c) Each field should have around 200 cells and the viability of the control should be around 90%.
    d) The field should be the same before and after changing filter

APPENDIX E

COOLING RATE VERIFICATION

Verification of the design cooling rate and actual cooling rate are presented in the following table and charts.

$$\text{Design CR} = \frac{\Delta T \times V}{D} = \frac{(T_H - T_L) \times V}{D}$$

Table 3. Cooling rate verification.

T_H (°C)	T_L (°C)	ΔT (°C)	D (mm)	V (mm/min)	Actual Ave. T (°C)	Design CR (°C/min)	Actual CR (°C/min)
4	-40	44	6	0.682	21.55	5	4.83
4	-40	44	6	1.363	21.4	10	10.52
4	-40	44	6	2.727	21.2	20	20.13
4	-40	44	6	6.818	21.3	50	50.3

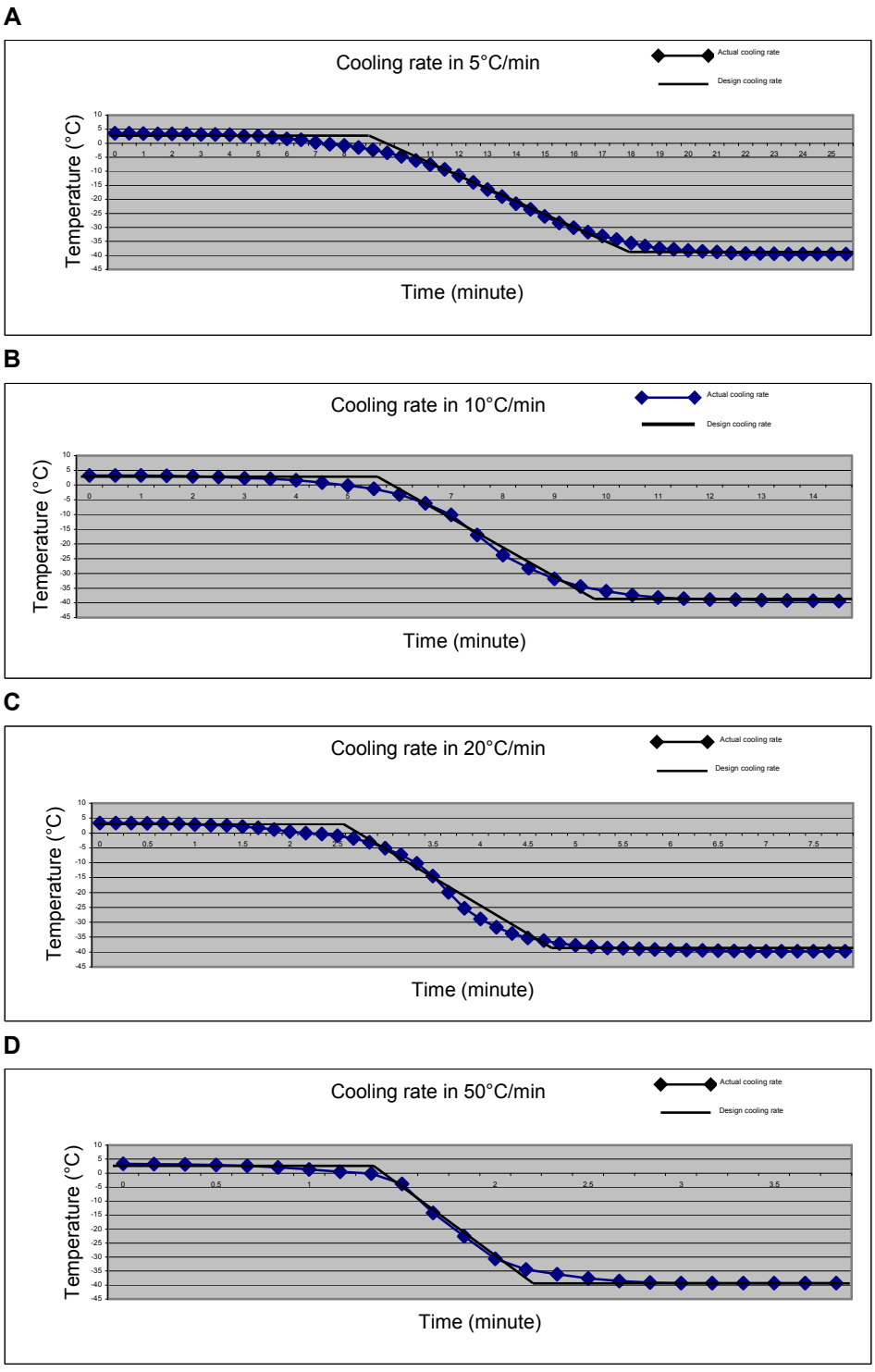

Figure 8. Cooling rate verification. (A) cooling rate at 5°C/min; (B) cooling rate at 10°C/min; (C) cooling rate at 20°C/min; (D) cooling rate at 50°C/min.

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BIOGRAPHICAL INFORMATION

After completing his undergraduate study in Mechanical Engineering, Chuo-Li decided to pursue a higher degree in a related field. He started his graduate study at the University of Texas at Arlington, Dept. of Mechanical Engineering in May 2004. He focused on Biomechanical Engineering because it is desperately needed all over the world. He began his research in enhancement of cryosurgery with Dr. Han his second semester his graduate study. During his research, he not only designed and developed an experimental apparatus, but also quantified the enhancement of cellular destruction during the freezing/thawing procedure. Furthermore, through out his coursework and research, he gained broad-based experience and a hands-on skill-set required for the successful design and research of innovative engineering solutions and advanced technology-driven strategies. He also proven himself capable to deliver engineering expertise, solutions, and research designed to meet the specialized requirements of biomechanical engineering. He will receive his degree in M.S.M.E. at the University of Texas at Arlington, in August 2006. After graduation, he has decided to devote himself to a career in a related engineering field.