ENHANCEMENT OF DIRECT CELL INJURY DURING FREEZING AT-1 TUMOR TISSUES BY USE OF EUTECTIC CRYSTALLIZATION

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INTRODUCTION

Understanding the mechanism of freezing induced cell injury is one of the most important issues in the field of cryobiology as it pertains to the applications of cryopreservation and cryosurgery. A two-factor hypothesis [1] was suggested to explain direct cell injury on the basis of two distinct freezing injury mechanisms – intracellular ice formation (IIF) and dehydration dominate the injury processes during freezing depending on the cooling rate of systems. When the cooling rate is rapid, cellular water nucleates and forms lethal intracellular ice. Otherwise, ice forms in the extracellular solution first and it leads to increased concentration of the unfrozen fraction. The increased concentration induces consequent cellular dehydration due to osmotic pressure difference. If this dehydration is too severe, then a form of toxicity or injury due to the high concentration of electrolytes can injure cells by mechanisms collectively called "solute" effects.

Han and Bischof [2] recently suggested another direct cell injury mechanism associated with eutectic crystallization which extends the solute effects injury mechanism. The eutectic crystallization is defined as simultaneous solidification of unfrozen fraction (i.e. water and solutes) into solids (i.e. hydrates) [3]. They investigated the post-thaw viability of AT-1 cell suspensions in various media and observed a significant viability drops across the eutectic crystallization temperatures of the suspended media. To explain these viability drops, they extended solute effects injury mechanism by including the effect of eutectic crystallization in the suspensions. They also demonstrated the possibility to enhance direct cell injuries during cryosurgery using artificially induced eutectic crystallization by introducing other solutes whose eutectic temperatures are higher than NaCl. However, their work was only performed with cell suspensions, and needed to be verified with tissue systems.

In the present study, the freezing injury mechanism associated eutectic crystallization was verified with Dunning AT-1 rat prostate tumor tissue system. The possibility of inducing eutectic crystallization on a tumor tissue during freezing was examined, and the corresponding freezing injury enhancement was assessed after a freeze/thaw. The results from the present study provide biophysical evidences of the eutectic induced freezing injury in tissue and may lead to improvements in cryosurgical efficacy.

MATERIALS AND METHODS

AT-1 Tumor AT-1 tumors were seeded by subcutaneous injection of $2x10^6$ AT-1 cells in 100µl of Hanks' balanced salt solution in the flank region of ~250g male Copenhagen rats (Harlan-Spraque-Dawley, Inc., Indianapolis, IN). Tumors were grown to a size of 2-3cm in the largest dimension, and harvested from the rats. Using a razor blade or a precision cutter, tumors were sliced in 5mm long, 2mm wide and 0.5mm thick for freezing experiments.

Infusion of Eutectic Inducing Solutions To induce eutectic crystallization during freezing, three different solutes - KNO_3, KCl and NaCl - were prepared in aqueous solution at a half eutectic concentration of each solute (5.5% wt/wt for KNO_3, 9.9% wt/wt for KCl and 11.8% wt/wt for NaCl). The infusion was performed by either 1) the submersion of the tissue samples in the prepared solutions for 15 minutes; or 2) the injection of the solutions to the tissue samples using a hypodermic needle. After the infusion, excessive solutions were removed with absorbent paper towels.

Differential Scanning Calorimetry (DSC) To investigate bio-physical phenomena during freeze/thaw, a DSC (Pyris 1, Perkin-Elmer Corporation, Norwalk, CT) was used. A DSC can provide thermal information of biophysical events within a sample during freeze/thaw as described elsewhere [4].

Directional Solidification Stage A directional solidification stage [5] consists of two constant temperature reservoirs, which are at different temperature and separated by an adjustable gap. The first reservoir was held at suprazero temperature and the second at subzero temperature. The sample rested in 3mm wide and 1mm deep well on a microscope microslide. The glass microslide was moved from the high temperature reservoir to the low temperature one over the gap at a precisely controlled velocity. By appropriately setting the microslide velocity, gap size, and reservoir temperatures, constant cooling rates and precise end temperatures can be imposed on the cell suspension.

Freeze/Thaw Procotol Controlled cooling and thawing rate

could be easily achieved by the DSC and directional solidification stage. Unless otherwise mentioned, cooling and thawing rates were 5ºC/min. For the directional solidification stage, fast thawing rate (~200ºC/min) were employed. To obtain the rapid thawing rate, the glass microslide was removed from the directional solidification stage and quickly placed on an aluminum block at 37ºC.

RESULTS AND DISCUSSION

Induction of Eutectic Crystallization Figure 1 shows DSC thermograms of AT-1 tumor tissues while the samples were frozen to –50°C and thawed back to room temperature. The tissue sample without infusion only generates heat release/absorption peaks associated with water/ice phase change. However, when other solutes (either KCl or NaCl) are infused, the samples generated secondary heat release/ absorption peaks associated eutectic phase change. This confirms that eutectic crystallization can be induced by infusion of solutes into a tissue system.

Enhancement of Freezing Injury Figure 2 shows histology of AT-1 tumor tissues 2 days after freezing experiments on the directional solidification stage. By comparing samples before freezing to -20ºC, the samples with infusion have very similar to the control sample (i.e. no infusion). This suggests that the infusion of these solutes does not injure cells and tissues. However, when the samples were frozen to -20°C which is below the eutectic crystallization temperatures of KNO_3 and KCl, the samples with the infusion are more severely injured than the frozen sample without infusion.

In summary, this agrees with the results of Han and Bischof on cells [2], and suggests that the freezing injury mechanism associated with eutectic crystallization also occurs within a tissue system.

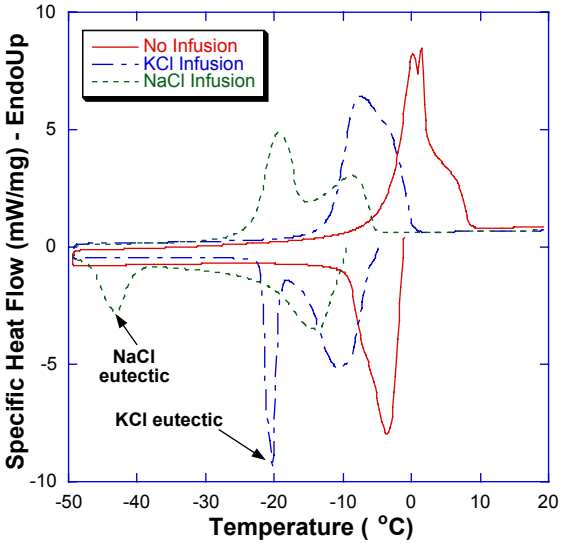

Figure 1: DSC thermograms of AT-1 tumor tissues

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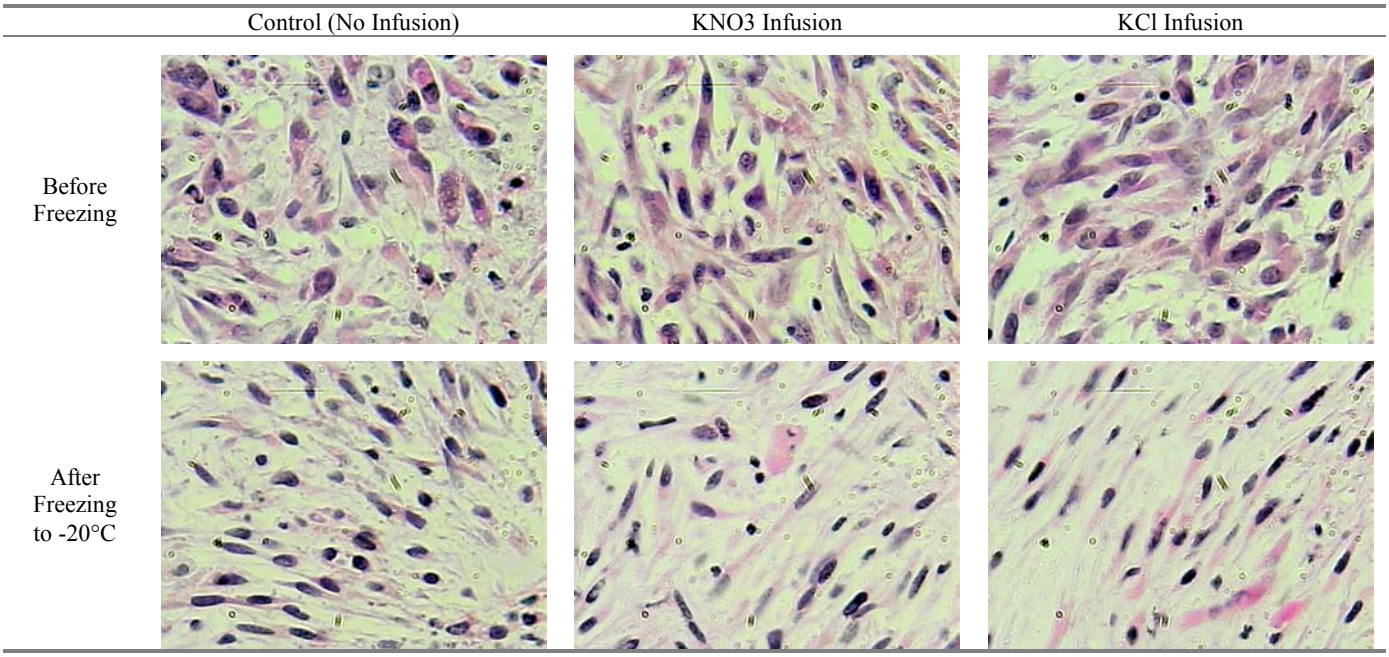

Figure 2: In vitro histology of AT-1 tumor tissues 2 days after freeze/thaw experiments (400X magnification)