

SHORT COMMUNICATION

Vitrification of Biological Cells Using a Cryogenic Fine Solid Particulate Spray

Jun ISHIMOTO*

Graduate School of Information Sciences, Tohoku University, Sendai 980-8577, Japan

We have developed a cryopreservation method that uses a single-component super-adiabatic two-fluid de Laval nozzle for the continuous production of solid nitrogen particles with a micro-nano-order particle size. The cell viability results showed that use of the solid nitrogen spray improved cell viability at the time of thawing by more than 20% compared with the conventional liquid nitrogen immersion freezing method. By using the present dynamic spray cooling method that avails of the synergistic effect of impingement heat transfer, convective heat transfer, and evaporation latent heat transfer of solid nitrogen particles, the new vitrification method reduces the cell membrane disruption caused by ice nucleation, crystal growth, and volume expansion.

KEYWORDS: cryogenics, solid nitrogen spray, vitrification, cell viability

1. Introduction

Although a great deal of effort has been expended in improving cell cryopreservation [1, 2], a considerable amount of specially designed cryopreservation solution [3–7] is still required and low cell viability at the time of thawing remains a serious problem. Accordingly, availing of the newly developed high-speed vitrification cryopreservation system, we propose an ultra-high heat flux vitrification (quenching) method that uses a micro-nano fine solid nitrogen (SN₂) particulate spray [8, 9].

Our results show that use of this vitrification method to freeze biological cells minimizes cell membrane disruption and DNA damage due to ice nucleation, crystal growth, and volume expansion. This technology also reduces the amount of cryoprotective solution required. SN₂ has considerable cooling enthalpy (cooling capacity) and is a suitable refrigerant for vitrification involving high-speed quenching because it is generally harmless to living tissue and cell membranes.

We have developed a cryopreservation method that uses a single-component super-adiabatic two-fluid de Laval nozzle for the continuous production of SN₂ particles with a micron-order particle size (micro-nano SN₂ particles). In this technique, micro-nano SN₂ particles are generated by adiabatic expansion of a high-speed subcooled two-phase liquid nitrogen (LN₂) flow (Fig. 1). When the flow reaches the internal section of the de Laval nozzle, the subcooled LN₂-gaseous nitrogen (GN₂) two-phase flow is atomized and an LN₂ droplet is generated at the nozzle throat. High-speed ultra-fine SN₂ particles are continuously generated due to the freezing of the LN₂ droplet induced by rapid adiabatic expansion of the transonic subcooled two-phase GN₂-LN₂ flow passing through the de Laval nozzle.

To enhance the solid phase nucleation and solid particle atomization, an ultrasonic oscillator is attached to the tip side of the nozzle. Additionally, to further enhance the SN₂ particle atomization process, a spiral device is used in the nozzle aperture. Application of this cryogenic micro-nano SN₂ particulate spray flow to vitrification cryopreservation technology is a novel approach. When the cryogenic solid particles collide with the object being cooled and undergo a high-speed phase change to the vapor phase, nonwetted cooling of the heat transfer surface (the cryotube surface) has been almost completed (Fig. 2). The phase change rate and vitrification rate become as fast as those of a smaller particle size.

In contrast, in the conventional cooling method in which the object to be frozen is directly immersed into the cryogenic liquid, when the cryogenic liquid makes contact with the vial surface, the vicinity of the surface locally transitions to the film boiling state. Because the thermal conductivity of the vapor film (gaseous phase) is much lower than that of the liquid phase, the vapor film between the cryogenic liquid and the wall surface decreases the cooling heat transfer rate between the cryogenic liquid and the vial surface. Heat transfer characteristics are degraded in the film boiling state by the transition from the nucleate boiling state. This is particularly evident for cryogenic liquids because the large temperature difference between the refrigerant and the surface of the object being cooled readily generates the film boiling state. Thus, the conventional liquid immersion method is at a disadvantage regarding high-speed cooling.

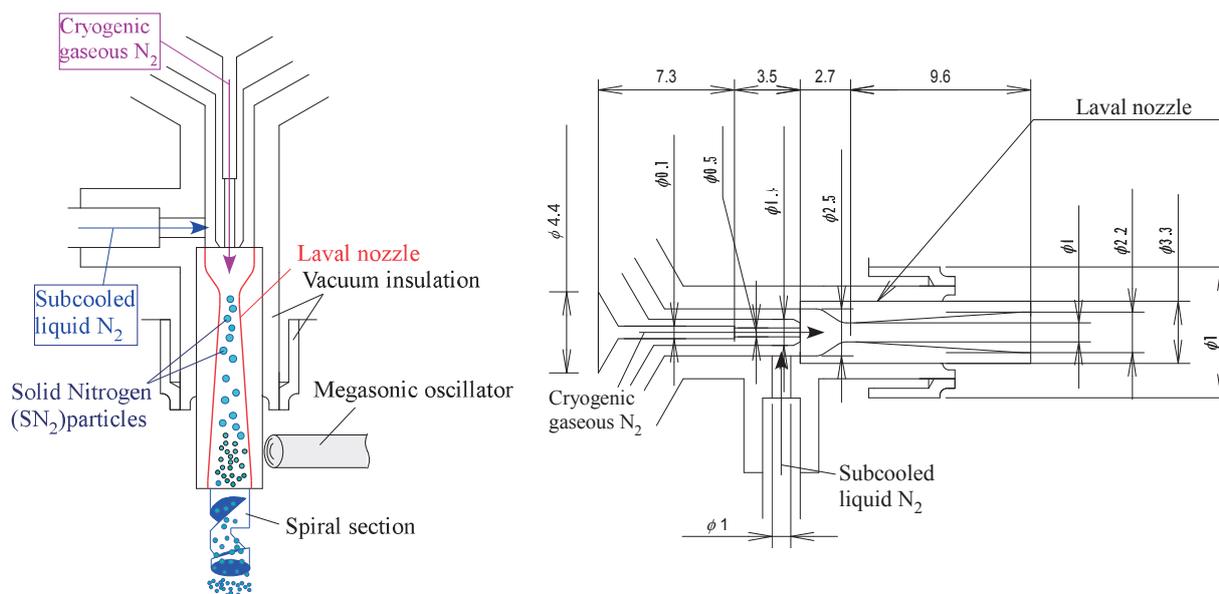


Fig. 1. Geometry of the two-phase de Laval nozzle for cryogenic fine solid nitrogen particle production.

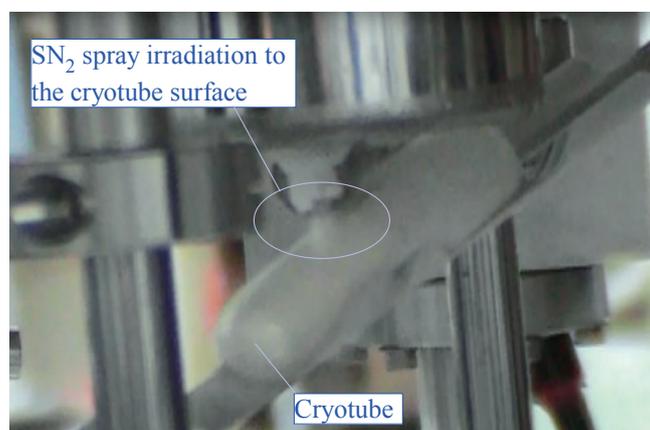


Fig. 2. Vitrification of biological cells using the fine solid nitrogen particulate spray flow.

Use of solid particulate spray cooling improves contact heat transfer, specifically that of the latent heat of melting during the phase change from solid to liquid, and the latent heat of vaporization during the phase change from liquid to vapor. There are two main differences in the latent heat transfer characteristics between solid and liquid. (1) The liquid (droplet) has only the evaporation latent heat when it changes phase from the liquid phase into the gas phase (the latent heat of vaporization of LN₂ is 214.0 kJ/kg). (2) Solid particle cooling takes advantage of the following thermophysical principle: “The latent heat of melting (SN₂ melting latent heat of 25.56 kJ/kg) + The vaporization latent heat during the vapor phase change from the liquid phase.” Therefore, use of SN₂ improves the latent heat transfer cooling characteristics by about 12% compared with LN₂. In fact, when SN₂ undergoes the phase change from solid to gas, the phenomenon is similar to sublimation. Additional cooling by sublimation latent heat transfer occurs with solid particles.

2. Experimental Method

We experimentally measured the ultra-high heat flux cooling characteristics of the SN₂ particulate spray. Immediately after spray particle impingement of about 5.0 s, the ultra-high heat flux cooling characteristics reached a critical heat flux of 1.0×10^5 W/m². The maximum freezing rate obtained was 25.8 K/s. The present method can maintain a fast freezing rate because, as new solid particles collide with the cryotube surface, additional latent cooling occurs, completing the vitrification.

To confirm the actual cryobiological performance of the present cryogenic fine SN₂ particulate spray cooling method, we investigated the vitrification of A549 cancer cells. First, 5.08×10^6 of harvested A549 cells were diluted in 4 ml of RPMI 1640 and further diluted 4.5 times to reach a concentration of 1.0×10^6 cells/ml. Then, 1500 μ l was dispensed into each of 10 cryotubes with cryopreservation solution. Each cryotube was then cryopreserved using either

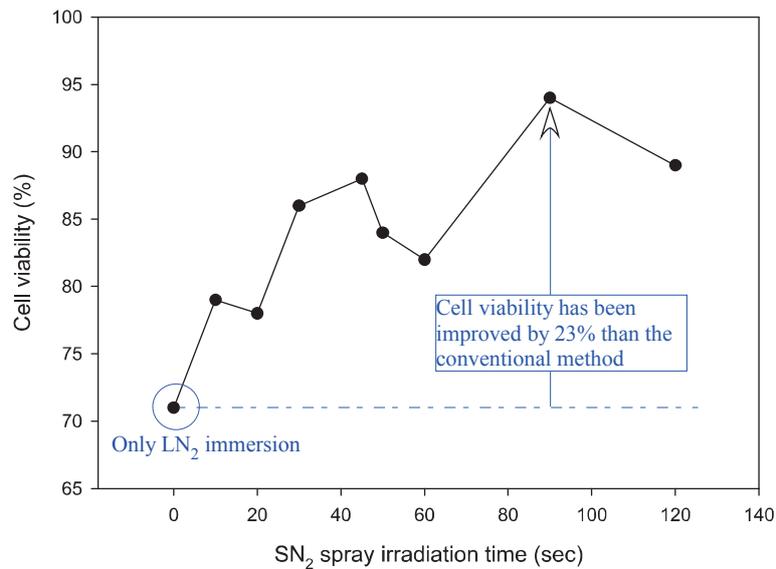


Fig. 3. Relationship between the solid nitrogen particulate spray irradiation time and A549 cancer cell viability.

the SN₂ spray or the conventional LN₂ immersion technique. After the vitrification, the cells in each cryotube were thawed in a water bath at 310 K. The cells were pipetted up and down five times and diluted in 9 ml of RPMI 1640. Then, the cells were centrifuged for 2 min and the supernatant was removed by suction. The cells were suspended in 2 ml of RPMI 1640 and 10 μ l of cells in suspension and 10 μ l of trypan blue (Bio-Rad) were mixed by pipetting. The cell viability was measured by the cell counter.

3. Results and Discussion

Figure 3 shows the qualitative measurement result of the relationship between the solid nitrogen particulate spray irradiation time and A549 cancer cell viability. The cell viability results showed that use of the SN₂ spray improved cell viability at the time of thawing by more than 20% compared with the conventional LN₂ immersion freezing method (Fig. 3). Thus, by using the present dynamic spray cooling method that avails of the synergistic effect of impingement heat transfer, convective heat transfer, and evaporation latent heat transfer of SN₂ particles, the new vitrification method reduces the cell membrane disruption caused by ice nucleation, crystal growth, and volume expansion.

In contrast to the conventional technique, our method does not show a decreased vitrification rate during the freezing process. Additionally, our high-speed vitrification method can reduce the amount of cryopreservation solution required. One of the important considerations before the present high-speed vitrification cryopreservation technology is applied to extremely delicate human stem cells such as induced pluripotent stem cells is its effect on cell viability. Crucially, our results found improved cell survival at the time of thawing. The method is also expected to greatly reduce the cryopreservation fluid volume compared with the conventional LN₂ immersion method because it enables faster vitrification. It may be possible to apply this low-cell damage cryopreservation technology to the vitrification of ova, sperm cells, and hematopoietic cells [10]. It could even be a useful frozen storage technology by helping to minimize the loss of taste texture of fresh food.

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