Improvement of cooling rate during cryopreservation of living cells

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Abstract. Cryopreservation is used in long term storage and transportation. Human ES / iPS cells could be useful in regenerative medicine. The problem of using cryopreservation for storing and transporting human ES / iPS cells is that the survival rate of these cells is low during the freezing and thawing process. Better cooling rates and cryoprotectants are needed for improvement of the cell survival rate. The growth of the ice crystals causes dehydration, deformation, contraction and increase of the electrolytic concentration. When cooled fast enough the cells freeze in the vitrification state and the ice crystals don’t have time to form. Immersion in liquid nitrogen is necessary to achieve a high cooling rate. To achieve a higher cooling rate than the current state, it was reported in our previous study that the effect of the surface condition on the cooling rate during cryopreservation was investigated. It was confirmed that the cooling rate is improved by covering the cooling subject with a stainless steel mesh. However, the difference in behavior of the vapor bubble during film boiling with surface condition has not been clarified yet. In order to investigate the boiling state with high cooling rate, visualization of film boiling on the surface of cooling object was performed using a high-speed camera. It was confirmed from the image analysis results that the size and frequency of the vapor bubble were changed with the surface condition.

1. Introduction

Cryopreservation is utilized in the storage and transportation of living tissues and cells. ES (embryonic stem) / iPS (induced pluripotent stem) cells are expected to utilized in the reproduction medicine. For the application of the cryopreservation to human ES / iPS cells, there is a problem that the cell viability after freezing and thawing process is low compared with that of mouse ES / iPS cells [1]. Therefore, the improvement of cell viability of the cells during the cryopreservation is required. The cell viability can be influenced on the cooling rate and the concentration of cryoprotectant. On the other hand, there are few reports for the effective cooling method during cryopreservation and the possibility of the contribution to the cryopreservation by thermal and fluid engineering is large. Generally, the cell viability after freezing and thawing process depend on the cooling rate shown in Figure 1 [2]. This figure shows the qualitatively relationship between the survival rate of cells and the cooling rate. This curve is related to the freezing pattern of the ice crystal. It is known that there are three freezing patterns: extracellular freezing, intracellular freezing and vitrification. In the case of the low cooling rate indicated region A, the extracellular freezing occurs. As the cooling rate increases, the freezing pattern changes to the intracellular freezing. In region C most of ice crystals are intracellular freezing. Many cells are physically damaged by ice crystals sharpened inside the cells. At higher cooling rate indicated region D, the freezing pattern changes to vitrification. The actual cooling rate value corresponding to each region depends on the cell type and size. For example, it was reported that the cooling rate value corresponding
Survival rate of cells
Cooling rate
A
B
C
D

Figure 1. The qualitatively relationship between the survival rate of cells and the cooling rate [2].

to point B is about 1 °C/min for ova cell and about 100 °C/min for red blood cell[3].
To increase the cell viability, the cooling rate should be collected at the appropriate value indicated region B or D shown in Figure 1. Especially, in the region D, it is expected that the cell viability becomes high as the increase of the cooling rate. Therefore, the choice of the higher cooling rate is one of the effective methods to improve the viability. However, there is some problems for the realization of the higher cooling rate. In fact, the practical method of the cooling by the high cooling rate is the direct immersion in the cryogenic fluid such as liquid nitrogen. In the direct immersion in the liquid nitrogen, the severe film boiling occurs around the cooling medium and then the cooling medium is covered with much vapor. So the heat transfer from the cooling medium to the liquid nitrogen becomes low. To resolve this problem, it was reported in the previous study [4] that the improvement of the heat transfer during the direct immersion cooling was tried to change the surface condition of cooling medium. The stainless steel mesh covered the cooling medium and the variation of the heat transfer with the size of mesh was investigated by the temperature measurements. It was confirmed that the temperature in the surface of cooling medium during film boiling was decreased with the stainless steel mesh and the case with 60 mesh/inch was most effective compared with the case without the mesh. The temperatures at the surface and the center (4 mm from the surface) of the cooling medium were measured and the cooling rate was calculated. The instantaneous maximum cooling rates were about 7000 °C/min in the surface and 600 ~ 800 °C/min in the center. For the cooling rate at the surface, the improvement by the mesh was not confirmed. On the other hand, for the center the cooling rate with the mesh was improved about 15 % compared with the case without the mesh. However, the relationship between the variation of the surface condition by the mesh and the heat transfer and fluid dynamics of film boiling around cooling medium have not been clarified. The investigation of the vapor behavior near the surface of cooling medium is necessary to evaluate the effect of the variation of surface condition.

In the present study, the vapor behavior around the surface of the cooling medium immediately after immersion in liquid nitrogen is visually observed using high speed video camera. A copper rod with a square cross section is used as the cooling medium and the transient film boiling state around the copper rod after direct immersion in liquid nitrogen is observed. For the images taken by the high speed video camera, the vapor behavior is tried to investigate quantitatively using an image analysis for the variation of brightness value due to passage of vapor. The relationship between the vapor behavior and the heat transfer during film boiling is examined.

2. Experimental apparatus and procedure
The schematic illustration of the experimental system is shown in Figure 2. This system consists of a cryostat, a test section (a jig and a copper block), a high speed video camera and a light source. The vapor behavior around the copper block in cooling by the high cooling rate. To achieve the high cooling rate, the cooling method by direct immersion in liquid nitrogen is used.

2.1. Cryostat
The picture of the liquid nitrogen cryostat is shown in Figure 3. This cryostat has a liquid nitrogen bath, a vacuum insulating bath and three optical windows. In the present study, two optical windows are used
for the visual observation. The light source is located on one side outside of windows and the high speed camera is located on the opposite side as shown in Figure 2. The effective diameters of these windows are 45 mm.

2.2. Test section
In our previous study [3], The cryo-vial with agar was used for the cooling medium for the temperature measurement experiment. On the other hand, in the present study, the copper rod with a square cross section is used as the cooling medium to make it easier to observe the vapor behavior near copper surface. The size of copper block is 10 x 10 x 48 mm. Two types of copper blocks are prepared. One is the copper block only (without mesh), and another one is covered with stainless steel mesh (with mesh). The specification of the mesh is 60 mesh/inch and the mesh covers the entire side surface. When the copper block is immersed in liquid nitrogen, the vapor layer and vapor bubble generate and cover with copper block. As a result, the visual observation may not be clear. And so the jig is prepared to be able to observe the boiling state only from one side of the copper block through the optical window.

2.3. Experimental procedure
For the experiment, the jig is located in the liquid nitrogen bath and is filled with liquid nitrogen. The liquid nitrogen is saturated state in atmospheric pressure (77 K, 101 kPa). The jig has a gap with 13 mm x 20 mm and the copper block is dropped along the gap to the bottom. After the block arrives at the bottom, the visual observation by the high speed video camera is started. The frame rate is 2000 FPS. To drop the copper block accurately in the jig, the experiment is conducted with the upper side of the cryostat open to the atmosphere.

3. Results and Discussions
3.1. Qualitative film boiling behavior near the copper block surface
pictures of the film boiling behavior near the one side surface of the copper block are shown in Figures 4. Figure 4(a) is the case of without mesh, and Figure 4(b) is the case of with mesh (60 mesh/inch). The position of the copper block in each picture is indicated by a white broken line. The vapor layer and large vapor bubble can be seen on the right side of the copper block. In these pictures, for the purpose of the explanation, the area from the lower end to the upper end of the copper block is divided into four regions as shown in region (1) – (4).
It is confirmed that there are some differences in the vapor behavior with and without mesh. In the case of without mesh shown in Figure 4(a), the large amount of vapor generate in region (1). In region (2) and (3), the vapor rise along the copper block and the vapor bubble coalesce and grow as the vapor rise. These vapor behaviors repeat periodically and eventually leave the copper block as the large vapor bubble. On the other hand, in the case of with mesh shown in Figure 4(b), the amount of vapor in region (1) is less than those in the case of without mesh. It is found in region (2) and (3) that the vapor rise in contact with the mesh. As the vapor grow as they rise, but the vapor layer is thinner than that in the case of without mesh. As a result, the vertically long vapor layer is formed along the stainless steel mesh.
3.2. Image analysis using variation of brightness value

The vapor behavior taken by the visual observation is quantitatively evaluated using image analysis for the variation of brightness. The analytical area is shown in Figure 5. In the visualization pictures taken in the present study, the vapor is displayed in dark colors. So, the brightness value is low where there is vapor bubble or vapor layer, and the brightness value is high where there is liquid nitrogen. Consequently, the amount of vapor and the vapor behavior can be quantitatively analyzed by calculating the number of pixels for each brightness value in the designated analysis area. Image analysis function is included with the high speed camera control software ‘Photron FASTCAM Viewer (PFV)’. The analytical area is indicated by the red square in Figure 5. This area is 20 mm from the top of copper block and the range is up to 10 mm from the copper block surface. There are about 6000 pixels in the red square area.

The time series variations of the brightness value are shown in Figures 6. The vertical axis is number of pixels, horizontal axis is time and brightness value is represented by color bar. Originally, the brightness value is analyzed the ranges from 0 to 255, but these results indicate ranges from 0 to 125 where the significant variation has confirmed. In these graphs, low brightness value indicated cold colors and relatively high brightness value indicated warm colors. So, when there are many cool colors areas, it means that there is a lot of vapor. It is confirmed from comparison of both graphs shown in Figures 6 that there are many cool colors areas in the case of without mesh. And then focusing on the peak in the

Figure 4. Pictures of the film boiling behavior near the copper block. Frame rate is 2000 FPS.

Figure 5. Analytical area for the image analysis.
cool colors areas, there is variation of peak height in the case of without mesh. On the other hand, in the case of with mesh, the peak height is low and its value is relatively stable. Additionally, the frequency of the vapor behavior is slightly higher. Therefore, it is considered that the vapor generation is suppressed and the vapor behavior is promoted by the stainless steel mesh.

Next, the difference in vapor layer thickness is analysed. The other analytical area is shown in Figure 7. To evaluate the vapor layer thickness, the analyses are performed in all twelve regions. Each analytical region is indicated by red square and there are about 600 pixels in each region. In the vertical direction, the area from the lower end to the upper end of the copper block is divided into four regions. And in the horizontal direction, the analytical regions are set at positions from 3mm, 6 mm and 9 mm from the copper block surface.

The analytical results are shown in Figure 8 and Figure 9. The numbers (1) – (12) attached to each graph correspond to the numbers shown in Figure 7. The vertical axis is number of pixels, horizontal axis is time and brightness value is represented by color bar. In the case of without mesh shown in Figure 8, it can be seen that at all positions 3 mm from the copper surface indicated as region (1) – (4) there are much vapor. The amount of vapor increase as the vapor rise and the growth of the large vapor bubble at least 3 mm in diameter is confirmed. On the other hand, in the case of with mesh shown in Figure 9, it is confirmed that the amount of vapor is less even at the positions 3 mm from the copper surface. There is no significant growth of vapor at the region (2), (3) and (4) though in the bottom at region (1), the amount of vapor is relatively much due to coalescence with the vapor generated at the bottom of the copper block. Additionally, at the region (8), (11) and (12), the amount of vapor is much compared with those in Figure 8. It is considered that in these regions the departure of the vapor bubble from the copper block occurs. Therefore, it is confirmed that in the film boiling immediately after direct immersion in liquid nitrogen, the generation of the vapor is suppressed by covering the cooling medium with stainless steel mesh. And the effect of the stainless steel mesh promotes the vapor behaviour and improves the heat transfer.

![Figure 6](image6.png)  
(a) Without mesh (copper block only)  
(b) With mesh (60 mesh/inch )  

**Figures 6.** The time series variations of the brightness value in the analytical area shown in Figure 5.

![Figure 7](image7.png)  

**Figure 7.** Analytical area for the image analysis.
Figure 8. The time series variations of the brightness value in the analytical area shown in Figure 7. In the case of without mesh.

Figure 9. The time series variations of the brightness value in the analytical area shown in Figure 7. In the case of with mesh (60 mesh/inch).
4. Summary
The vapor behavior around the surface of the cooling medium immediately after immersion in liquid nitrogen is visually observed using high speed video camera. The relationship between the vapor behavior and the surface condition of cooling medium with the stainless steel mesh is investigate using the image analysis by the variation of brightness value. It is quantitatively confirmed that the generation of the vapor is suppressed by covering the cooling medium with stainless steel mesh.

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