

Protective effects of pressurized xenon gas against cold damage in a cell monolayer

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Objective: Although cold storage (0–4°C) is used for all organs, many tissues, and specific cell types, it is currently limited for a short period of time that they can be preserved. To extend their preservation periods, a new protective agent needs to be devised. The protective effects of pressurized Xe gas against the damaging effects of cold storage on a cell monolayer were investigated.

Methods: The test samples comprised human dermal fibroblast monolayers incubated in a culture dish at 4.5×10^4 cells/dish for 24 hours. The samples were pressurized with between 0–1.0 MPa Xe gas and maintained at 4°C for 24 hours. Pressure-dependent cell activity was evaluated. Subsequently, the effects of 0.5 MPa pressurized Xe gas on cellular activity following the cold storage for 0–72 hours of the monolayers in culture medium were investigated.

Results: The optimum Xe gas pressure for preserving human fibroblasts was determined to be 0.5 MPa. Long-term high cell activity was maintained under pressurized Xe gas conditions, regardless of the storage solution, compared to under non-Xe gas conditions, although the highest protective effect of pressurized Xe gas was observed with ET-Kyoto solution. None of the experimental conditions tested resulted in the production of clathrates.

Conclusion: An Xe gas pressure appropriate for the cold storage of human dermal fibroblast monolayers does not cause the production of clathrates, reduces cell damage due to low temperature, and may extend cell storage time.

Key words: protective effect, cold storage, fibroblast monolayers, preservation solution, pressurized Xe gas

Introduction

Successful medical transplantation requires an established preservation technique for a wide array of cells, tissues, and organs. Freezing, or cryopreservation, is an effective means of preserving samples for an extended period of time, but the types of biological samples that can be cryopreserved is currently limited. Therefore, all organs, many tissues, and specific cell types are preserved by cold storage at 0–4°C.^{1,2}

The low temperatures used in cold storage stop active transport but also cause metabolic imbalances and cell damage.³ Inactivation of the Na⁺-K⁺ pump has been reported to be a leading cause of such damage, i.e., cold damage⁴ because it stops active transport, resulting in an accumulation of Na⁺ in the cell and leading to cell damage

due to intracellular dysfunction. Consequently, preservation solutions such as University of Wisconsin (UW) solution and ET-Kyoto (ETK) solution⁵ are necessary to reduce cold damage and improve the transplant success rate. Using current techniques, tissue can only be preserved for up to 0.5–3 weeks and organs for up to 1–2 days. We therefore investigated whether or not xenon (Xe) gas can augment the protective effects of preservative solutions.

Xe gas has been used in many clinical fields because it is deemed inactive and biologically safe. Since 1951, Xe has been used as an anesthetic, and the isotopes ¹²⁷Xe and ¹³³Xe are currently used for computed tomography examination of the cardiovascular and respiratory systems.^{6,7} Xe gas has a relatively high solubility in water,⁸ and the pressurized dissolution of Xe gas in water

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results in water molecules re-orienting around the Xe atoms and thus becoming structured. This structurization restricts the migration of water molecules in the liquid state and inhibits the transfer of water in and out of cells. Inclusion compounds (Xe clathrates) are produced when Xe is confined in a cage of structuralized water molecules resulting from an increase in Xe pressure.⁹ The clathrate is formed when a guest molecule 380–700 pm in diameter (e.g., Xe) is surrounded by a lattice of the host molecule (e.g., water). Xe is one of more than 120 known guest molecules, ranging from rare gases to organic compounds that cause clathrate formation in water.¹⁰

There have been a number of studies in plants and food on the structuralization of water by Xe. In one study, plant cells were preserved for an extended period of time, and it was concluded that an increase in water viscosity is one reason why low temperature inhibits metabolism. Xe gas dissolves in intracellular water, causing the water to structuralize. Investigation of how this structuralization inhibits metabolism showed that structuralization decreases the speed of protoplasmic streaming.¹¹ A study aimed at maintaining the freshness of vegetables during storage showed that the inhibition of metabolism by water structuralization reduced the degradation of agricultural products.¹² Another study showed that water structuralization in bacteria attached to bean sprouts inhibited bacterial division.¹³ A report on water structuralization by Xe, and its effect on the replication of food surface and subsurface bacteria, concluded that Xe inhibits the replication of bacteria located up to several millimeters beneath the surface of food.¹⁴ The effects of clathrate hydrate formation on the inhibition of microbial replication have also been investigated.^{15,16} Although few studies on the effects of Xe on animal cells have been published, it has been reported that pressurized Xe resulted in the adsorption of Xe in the cell membrane, suggesting that Xe had a protective effect and helped maintain cell membrane integrity at various temperatures.¹⁷ Studies to date have shown that plant cells can be preserved for a long period of time at 15°C by inhibiting their metabolic activity with Xe, but few studies have demonstrated the protective effect of Xe towards animal cells.

Our goal was to clarify the protective effect of Xe gas at an optimized pressure on human dermal fibroblast monolayer samples in cold storage at 4°C. Typical solutions for preservation, such as UW solution and ETK solution, were combined with pressurized Xe gas; for comparison, samples were also preserved in culture medium. The relationship between cell activity after cold storage in the presence and absence of Xe gas and

storage time was evaluated, as was the effect of these preservation solutions vs. culture medium. Finally, the use of Xe gas to extend cell storage time was evaluated.

Materials and Methods

Experimental samples

Samples were prepared using human dermal fibroblast (Fb Cells, CSC-2F0; Cell Systems, Kirkland, WA, USA) monolayers. Dulbecco's Modified Eagle Medium (DMEM) (Gibco 12320-032; Life Technologies, Waltham, MA, USA) with 10% fetal bovine serum (Gibco 26140 Lot No. 1215196) and 1% antibiotics (Gibco antibiotic/antimycotic 15240-062) was used as the cell culture medium. A 2-ml cell suspension containing 4.5×10^4 cells was added to a culture dish (3294, 35-mm diameter; Corning, New York, NY, USA) and cultured in an incubator (MCO-19AIC-PJ; Panasonic Healthcare, Tokyo) at 37°C in 5% CO₂ for 24 hours. Duplicate samples were prepared and left untreated to provide control samples.

Experimental device

The experimental device is shown in Figure 1. A pressure-resistant test chamber (TVS-1; Taiatsu Techno, Tokyo, Japan), 45-mm internal diameter, 60-mm high, internal volume approximately 100 ml, pressure-resistant up to 10 MPa, made of stainless steel (SUS316), was used for the experiments involving pressurized Xe gas at 99.995% purity (Tokyo Rare Gas, Tokyo). Four experimental samples (in 35-mm culture dishes) could be placed in the chamber simultaneously. The chamber was connected to a stainless steel extension pipe in turn connected to a pressure regulator attached to an Xe gas cylinder. The chamber was immersed in a water-filled reservoir made from polystyrene foam and maintained at 4°C using a CTR-220 temperature control water circulator (Komatsu-Yamato, Tokyo). The samples in the chamber were pressurized with between 0–1.0 MPa Xe gas using the cylinder regulator.

Cell-activity evaluation

Cell activity was determined using water-soluble tetrazolium salt (WST-1) (Takara Bio, Shiga) by measuring the quantity of formazan produced by mitochondrial enzymatic activity using a Gene Quant 1300 spectrophotometer (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).¹⁸ WST-1 was added to the sample, the sample was incubated for 2 hours (37°C, 5% CO₂), then the absorbance of the sample supernatant was measured. Cell activity was calculated from the sample

absorbance divided by the absorbance of the control sample, as shown in the equation.

$$\text{Cell activity} = \frac{\text{absorbance of samples after cold storage}}{\text{absorbance of control samples}} \times 100 (\%)$$

Cell activity dependence following cold storage on the Xe gas pressure

The chamber was cooled in a refrigerator at 4°C before adding the samples. Four samples were removed from the incubator (37°C, 5% CO₂), the DMEM was removed, the cells were washed with 1 ml of Hank's Balanced Salt Solution (Gibco Cat. No. 24020-117) containing 1% antibiotics, and then 1 ml of DMEM without phenol red

was added to each sample. The 4 culture dishes were stacked in the chamber (Figure 2), then the chamber was closed, placed in the water reservoir, and maintained at 4°C. The chamber was filled with ambient air (0 MPa gauge pressure), pressurized with Xe gas to a gauge pressure of 0.2–1.0 MPa, and then the samples in the chamber were stored in an MIR-254-PJ refrigerator (Panasonic Healthcare, Tokyo) at 4°C for 24 hours. No Xe gas was added to the chamber during storage to maintain gas pressure.

At the end of the storage period, the chamber was removed from the refrigerator. The Xe gas was gradually released using a decompression program to prevent cell damage caused by the expansion of bubbles (Figure 3).

The program cycled through decompression and fixed pressure and was based on decompression methods used in hyperbaric oxygen therapy. Pressure readings were recorded during the gradual decompression steps and used to ensure constant gaseous expansion for each decompression pressure. The samples were removed from the chamber and maintained in an incubator at 37°C for 2 hours to allow recovery of cellular functions. Next, the sample medium was replaced with 1 ml of DMEM and 100 μl of WST-1, and the samples were incubated for 2 hours. The absorbance of the supernatant of each sample was measured spectrophotometrically.

The cell activity inhibitory effects

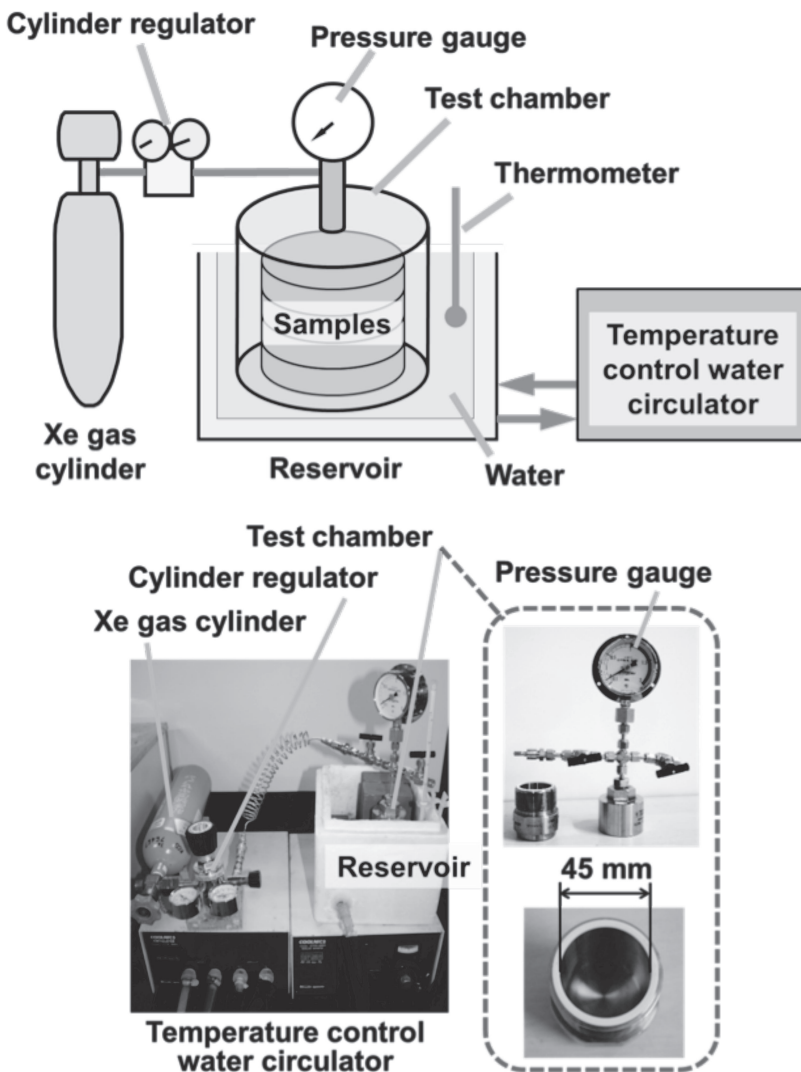


Figure 1. Experimental apparatus for the cold storage of samples

Samples were pressurized with Xe gas in a pressure-resistant stainless steel test chamber that could accommodate 4 samples (35-mm culture dishes) simultaneously. The chamber was immersed in a water-filled polystyrene foam reservoir maintained at 4°C using a temperature control water circulator. The samples in the chamber were pressurized with between 0–1.0 MPa Xe gas using the cylinder regulator.

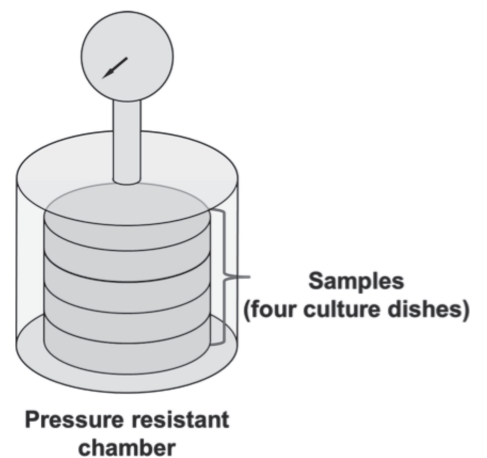


Figure 2. Placement of the experimental samples in the test chamber

Four culture dishes were stacked and placed in the chamber.

of N₂, which does not cause the structuralization of water in the pressure range used in the present study (0–1.0 MPa), and of Xe gas were compared. The experiments were performed using 0.2, 0.5, and 1.0 MPa N₂. Slightly different pressures were used for Xe gas (0.2, 0.3, 0.4, 0.5, 0.6, and 1.0 MPa). Cell activity after cold storage was evaluated using the WST-1 assay.

Pressurized Xe gas effect and the cell activity time dependence in cold storage

Four samples were removed from the incubator (37°C, 5% CO₂), the DMEM was removed, then the cells were washed with 1 ml of Hanks' solution. One milliliter of DMEM without phenol red, ETK solution (Otsuka Pharmaceutical, Tokyo) or UW solution (ViaSpan; Astellas Pharma, Tokyo) was added to each sample. Four samples were stacked and placed in the pressure-resistant chamber (Figure 2). An initial pressure of 0.5 MPa was chosen based on the results of the experiments described in the preceding section. Samples in ambient air (0 MPa) were also prepared to provide cell activity reference samples and were placed in another chamber. All the samples in chambers were stored in a refrigerator at 4°C for 0–72 hours.

The test and control sample chambers were removed from the refrigerator at the end of the storage period. The Xe gas was gradually decompressed from the test samples and the samples were removed from the pressure chamber. For samples incubated with UW solution, the solution was removed, the samples were washed with Hanks' solution, 1 ml of DMEM was added, and then the samples were incubated for 2 hours (37°C, 5% CO₂).

Next, the sample medium was removed, 1 ml of DMEM and 100 μl of WST-1 were added, and the samples were incubated for an additional 2 hours. The absorbance of the supernatant of each sample was measured spectrophotometrically. Control samples were prepared by adding DMEM, ETK solution, or UW solution to samples that were not subsequently pressurized with Xe gas at 4°C for 15 minutes, then were evaluated for cell activity in the same manner as the experimental samples.

The presence or absence of clathrates in a sample was confirmed by reducing the pressure during cold storage and observing the samples visually. Our preliminary experiments showed that the presence of clathrates in a solution can be confirmed visually immediately after removal of the sample from cold storage. The pressure of Xe gas decreases by more than 10% during cold storage if clathrates are produced in the samples, but decreases by no more than 10% if clathrates are not produced and the Xe gas simply dissolves. Clathrates produced during cold storage are retained for more than 5 minutes after decompression.

Results

The relationship between cell activity after cold storage for 24 hours and the pressure of Xe (or N₂) gas is shown in Figure 4. The vertical axis shows cell activity and the horizontal axis shows the pressure of Xe (or N₂) gas. The error bars show the Standard Deviation (SD) for $n = 12$. Zero MPa Xe means that the chamber was filled with air.

Cell activity increased as the pressure of Xe gas increased from 0 to 0.5 MPa then decreased as the pressure

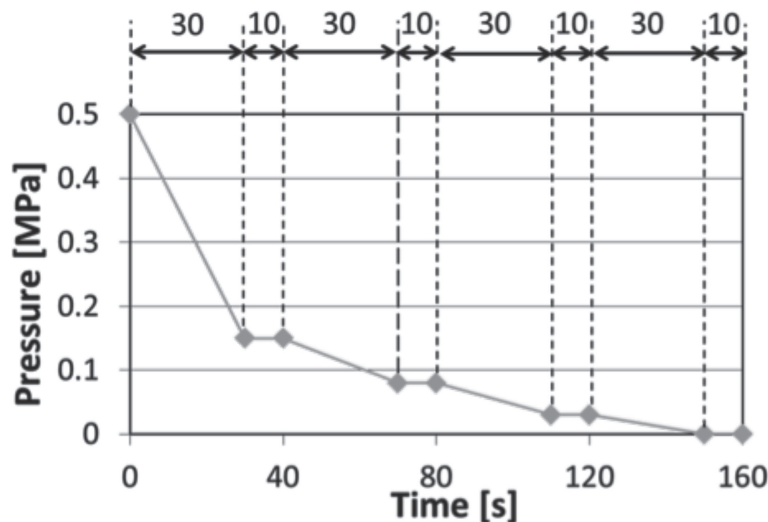


Figure 3. Decompression diagram

Pressure and time are shown on the vertical and horizontal axes, respectively.

increased from 0.5 to 1.0 MPa. Maximum cell activity was observed at 0.5 MPa ($69.3 \pm 9.9\%$). The differences in cell activity between 0 MPa ($4.3 \pm 2.5\%$) and 0.3 ($53.3 \pm 2.4\%$), 0.4 ($63.8 \pm 8.0\%$), 0.5, or 0.6 ($64.2 \pm 6.5\%$) MPa were statistically significant (Kruskal-Wallis H test, $P < 0.05$), whereas the difference in cell activity between 0 and 0.2 ($8.9 \pm 4.4\%$), or 1.0 ($29.1 \pm 10.8\%$) MPa were not. The differences in cell activity if the samples were treated with N_2 gas between 0 MPa (= 0 MPa Xe) and 0.2, 0.5, and 1.0 MPa were not significant (Kruskal-Wallis H test, $P > 0.05$). In all cases, cell activity was low. In addition, some clathrates were produced using 0.6 and 1.0 MPa Xe; and under these conditions, there was no cell activity. These data were, therefore, eliminated from the calculation of cell activity because cell activity was significantly decreased under conditions that resulted in clathrate production.

The effect of pressurized Xe gas on cell activity after cold storage at 4°C in each solution as a function of storage time is shown in Figures 5A–C. The vertical axis shows cell activity and the horizontal axis shows the storage time. The error bars show SD for $n = 12$.

Figure 5A shows that there was no difference in the activity of cells stored in DMEM for up to 12 hours under pressurized Xe gas. In all the cases, the cell activity was very high ($>90\%$). However, cell activity decreased in all cases with increasing storage time. The cell activity following storage under pressurized Xe gas ($69.3 \pm 9.9\%$ [mean \pm SD]) was higher than that following storage in

the absence of Xe gas ($1.7 \pm 2.9\%$) after 24 hours. Figure 5B shows no difference in the activity of cells stored in ETK solution for up to 24 hours, regardless of the gas used. In all cases, cell activity was relatively high ($>55\%$). Longer storage times resulted in decreased cell activity, but the activity of cells stored under pressurized Xe gas was higher than that of cells stored in the absence of Xe gas ($49.7 \pm 4.1\%$ vs. $0.3 \pm 0.8\%$ after 60-h storage). Figure 5C shows that the activity of cells stored in UW solution for up to 3 hours under Xe gas or in the absence of Xe gas remained very high ($>95\%$), but in both cases cell activity decreased with increasing storage time ($44.9 \pm 10.0\%$ under Xe gas vs. $5.0 \pm 5.1\%$ for in the absence of Xe gas after 9-h storage).

Regardless of the storage medium (48 h $>$ DMEM $>$ 12 h, 72 h $>$ ETK solution $>$ 12 h, 12 h $>$ UW solution $>$ 3 h), the cell activities of samples stored under pressurized Xe gas were higher than those of cells stored in the absence of Xe gas (not pressurized). Xe gas pressurization in conjunction with ETK solution helped maintain high cell activity for an extended period of time. No clathrates were produced in samples pressurized with 0.5 MPa Xe gas.

Discussion

The results indicate that the cell activity following cold storage under pressurized Xe gas (0.3–1.0 MPa) was higher than that of cells stored in the absence of Xe gas (0 MPa), and that the degree of cell activity also depended

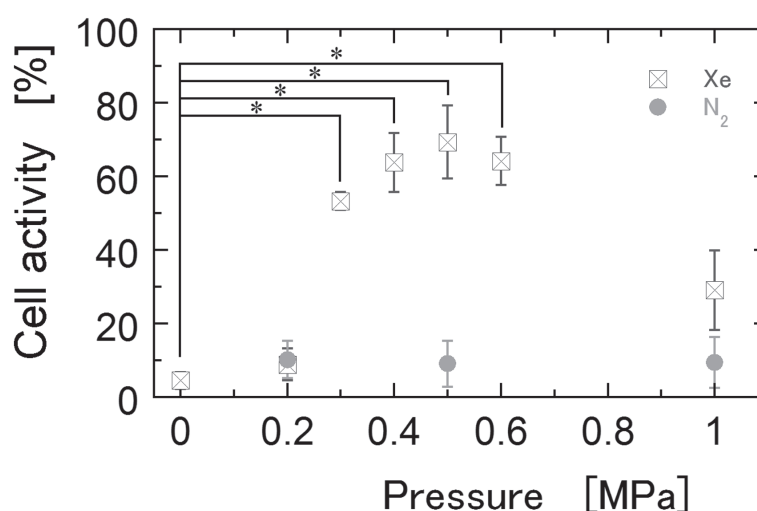
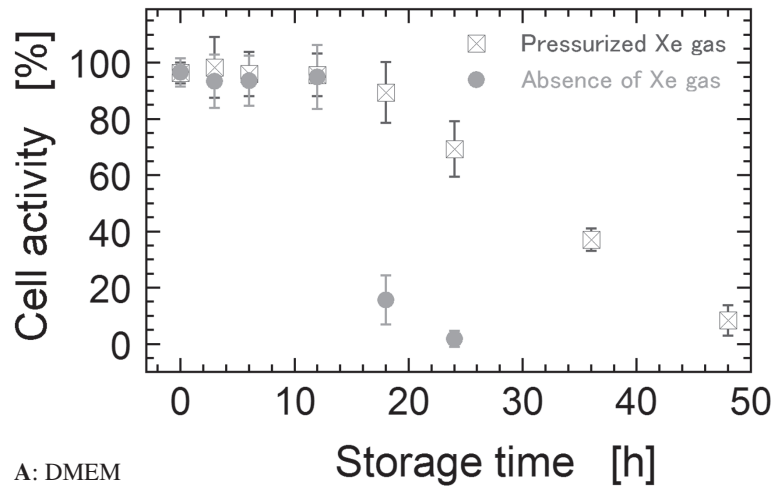
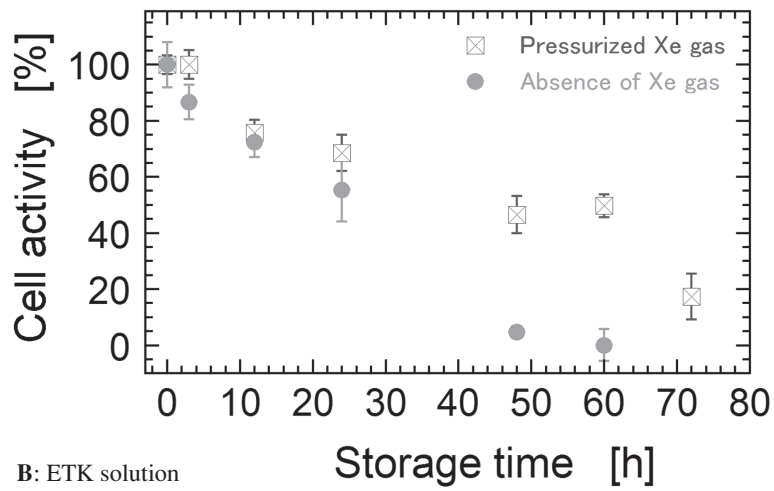


Figure 4. Relationship between cell activity after cold storage for 24 hours and pressure of Xe and N_2 gas

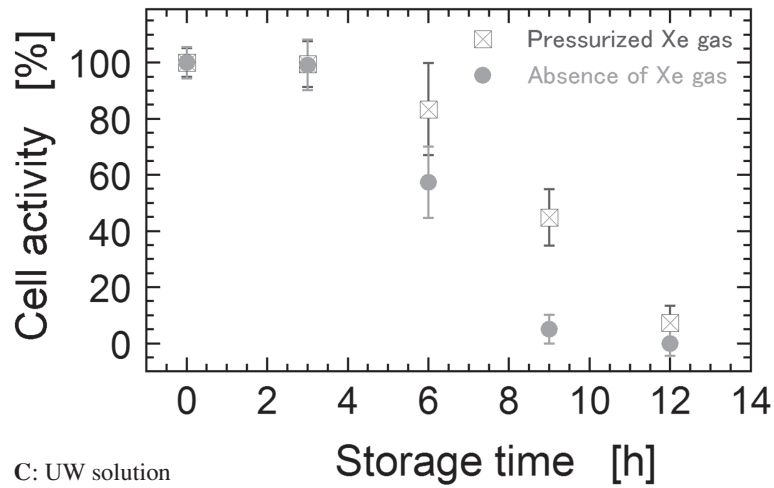
Cell activity is provided on the vertical axis, and Xe (or N_2) gas pressure is provided on the horizontal axis. Error bars show the standard deviation (SD). Asterisks show a statistically significant difference in cell activity (Kruskal-Wallis test, $*P < 0.05$ for $N = 12$).



A: DMEM



B: ETK solution



C: UW solution

Figure 5. Effect of pressurized Xe gas with 0.5 MPa on cell activity after cold storage of human dermal fibroblast monolayers at 4°C in different solutions as a function of storage time

Cell activity is shown on the vertical axis and storage time on the horizontal axis. Error bars show SD for N = 12.

on the pressure (Figure 4). In contrast, cell activity after cold storage under pressurized N₂ gas (0.2, 0.5, 1.0 MPa) or 0 MPa were comparable and, therefore, not dependent on pressure. Cell activity after cold storage reflects the cellular protective effect of the gas against cold damage to cells. No effect was observed at 0 MPa, allowing the data obtained under this condition to be regarded as control data. The protective effect of Xe gas increases with increasing pressure, suggesting that the protective effect arises from the structuralization of water when Xe gas is dissolved under pressure. Pressurized N₂ gas does not exert a protective effect, probably because water is not structuralized by pressurized N₂ gas in the experimental range tested (0–1.0 MPa).¹⁹ The results in the present study show that pressurized N₂ gas did not damage cells (Figure 4). It is known that the viscosity of water generally increases when water is structuralized. When intra- and extra-cellular water is structuralized by pressurized Xe gas, it is believed that compound diffusion, or the transfer of water through the cell membrane, is physically inhibited by the increased viscosity, resulting in decreased cell damage due to edematization.

It is also believed that the viscosity of water is proportional to the concentration of dissolved Xe, therefore, viscosity should increase with increasing Xe pressure. This was experimentally confirmed using CO₂, a hydrophobic gas like Xe, by observing that an increase in the concentration of dissolved CO₂ increases the viscosity of water.²⁰ Based on this finding, one would expect the cellular protective effect to increase with increasing Xe pressure. However, our results show a maximum cellular protective effect at a pressure of 0.5 MPa Xe, and that the effect decreases at pressures above 0.5 MPa (Figure 4), suggesting that an effect other than the structuralization of water is involved. A previous study showed that Xe gas has a protective effect on mitochondrial function and an inhibitive effect on the Ca²⁺-induced opening of mitochondrial permeability transition pores.²¹ This suggests that mitochondria can be protected by chemical actions of Xe gas within the cell, thus possibly improving cellular activity after cold storage. Such chemical factors may also be involved in the present results because it is thought that the activity of these chemical factors requires an optimal concentration of Xe gas and thus is dependent on the pressure of Xe gas.

Our results also suggest that clathrate production in samples stored under 0.6 and 1.0 MPa pressurized Xe gas led to cell damage in cold storage. Clathrate production increases extracellular solute concentrations because water structuralization removes free water

molecules from the solvent.²² The concentration of intracellular solutes is increased by an increase in extracellular solute concentration, resulting in cell damage. This is comparable to salt injury caused by intracellular dehydration due to extracellular freezing at sub-zero temperatures.²³ Therefore, initial pressures of 0.6 and 1.0 MPa, which may lead to clathrate production, are not suitable for cell preservation, whereas an initial Xe gas pressure of 0.5 MPa is appropriate for the cold storage of cells.

Cell activity was higher under pressured Xe gas than in the absence of Xe gas as the storage time increased (Figure 5). The ETK solution maintained the highest cell activity with increased storage time, followed by the DMEM and UW solutions. The enhanced cell activity with ETK solution was likely due to the protective action on the cell membrane of trehalose, a non-reducing disaccharide in the solution. Part of the protective effect was also due to the compositions of these preservative solutions. The UW solution mimics the composition of intracellular fluid (Na⁺ < K⁺), and the DMEM and ETK solutions mimic the composition of extracellular fluid (Na⁺ > K⁺). According to a previous study, cold storage of dermal cells in RPMI-1640 medium, that mimics the composition of extracellular fluid, was more effective than the UW solution.²⁴ Therefore, preservative solutions that mimic the composition of extracellular fluid may be suitable for the storage of dermal fibroblasts; and the composition of an effective preservation solution may need to be altered, depending on the cell or organ type.

The decreased cell activity after 3 hours of storage in ETK solution (Figure 5B) may reflect the time required for active transport to stop. This time is solution-dependent and is shorter when cells are preserved in ETK solution than in other solutions (Figures 5A, C). Because slightly higher cell activity is maintained under pressurized Xe gas vs. in the absence of Xe gas, dissolved Xe gas may help preserve cell activity.

The limitation of this study is that although the optimal pressure was determined using a human dermal fibroblast and DMEM in this research, there were no findings of whether or not the optimal pressure for other types of cells or storage solutions is 0.5 MPa. This warrants further investigations.

In conclusion, Xe gas has a pressure-dependent protective effect on the 4°C cold storage of human dermal fibroblast monolayers. Maximum protection is obtained at a pressure of 0.5 MPa Xe. Cellular storage time can be extended by pressurizing the sample with Xe gas using conditions that do not produce clathrates, regardless of the preservation solution used.

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