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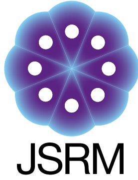

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Original Article

Hydrogen gas improves proliferation and mitochondrial activity of human adipose-derived stem cells after cryopreservation

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A B S T R A C T

The objective of this study is to evaluate the effect of hydrogen gas on the biological functions of human adipose-derived stem cells (hADSC) in cryopreservation. hADSC were cryopreserved by a commercial cell preservation solution in the presence of hydrogen gas. After cryopreservation at −80 °C, the viability, initial attachment morphology, and biological parameters of cells cryopreserved were evaluated to compare with those of cells cryopreserved in the absence of hydrogen gas. The hydrogen concentration in the cell preservation solution was 2.0 ppm immediately after preparation and after that decreased with time. The presence of hydrogen gas permitted cells to significantly increase the proliferation of cells in addition to the percent initial adhesion. The number of cells in the spread state was significantly high compared with that of hydrogen gas-free cryopreserved cells. The cell cycle measurement with the flow cytometry and measurement of intracellular reactive oxygen species (ROS) were performed to demonstrate an enhanced cell cycle and a decreased ROS production. In the cell cycle assay, the percentage of cells in the mitotic phase increased. The presence of hydrogen gas decreased hydroxyl radicals immediately to a significantly great extent after thawing. It is concluded that the presence of hydrogen gas during cryopreservation is promising to improve the biological behavior of cells after cell thawing in terms of cells viability, proliferation or metabolic activity.

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1. Introduction

The hydrogen molecule of the lightest weight is known to have a high kinetic energy [1]. One of the major industrial applications of hydrogen has extensively been used in "light current fields, such as semiconductors, silicon wafers, and electronic components". In addition, the demand for hydrogen energy has been increasingly noted not only for space exploration applications, but also for hydrogen stations and other hydrogen energy sources. On the other hand, recently this molecular hydrogen has been paid attention for the function in living organisms. As one of the typical studies, Osawa *et al.* report that molecular hydrogen selectively scavenges reactive oxygen species (=ROS) [2]. The ROS generation gives cells a damage or functional loss, which often causes a big problem related in cell transplantation therapy. The hydrogen molecules is a reagent to have a strong reduction activity [2,3]. It is expected that the presence of hydrogen molecules chemically reduces the ROS production, resulting in an improved cell viability and functions.

This study is undertaken to investigate the effect of molecular hydrogen on the viability, proliferation activity, and metabolic activity of cells. Following cryopreservation of hADSC in the presence or absence of hydrogen gas, the cell behavior was evaluated to compare with that of cells cryopreserved without hydrogen gas. We examine the ROS generation of cells cryopreserved in the presence of hydrogen gas. The cell behavior was also assessed in terms of cell morphology, cell cycle and stress fiber formation.

2. Materials & methods

2.1. Cell culture

hADSC cells (ATCC, SCRC-4000™) were purchased from ATCC Cell Bank (American Type Culture Collection). The cells were

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cultured in α-Minimum Essential Medium (αMEM, Invitrogen Corp., California, USA) with 10 vol% fetal bovine serum (FBS, HyClone™, SH30910.03, GE Healthcare Life Sciences, England) and 1 vol% penicillin streptomycin (09367e34, Nacalai Tesque, Inc., Kyoto, Japan) at 37 °C in a humidified atmosphere with 5% CO2 -95% air at an initial density of 1.3×10^4 cells per cm^2. After incubation, cell suspension was prepared. The number of live cells in the cell suspension was determined by the hemocytometer. Cell viability was calculated from the number of cells immediately after thawing. The number of adherent cells was determined from the number of cells at 3 h after incubation.

2.2. Cell cryopreservation in the presence of hydrogen gas

Fig. 1 shows the preparation procedure of cell suspension in the preservation solution with hydrogen gas for cryopreservation. Cell preservation solution (CELLBANKER 1, Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan) was placed in an aluminum bag (CCK-1, GL Sciences Inc., Tokyo, Japan) and filled with hydrogen gas. The aluminum bag containing the cell preservation solution and hydrogen gas was then shaken for 5 min. The hydrogen-saturated preservation solution was removed and mixed with the cell suspension prepared in 2.1 in a cryotube, which was immediately placed in the aluminum bag and filled with hydrogen gas. The aluminum bag with cryotube was then frozen at −1 °C/min to −80 °C. The cryotube was removed from the aluminum bag and stored under liquid nitrogen at −150 °C or lower.

2.3. Measurement of hydrogen concentration and redox potential

Hydrogen concentration in the cell preservation solution was measured by Gas chromatography (GC7100TW, J-Science LAB Co., Ltd., Kyoto, Japan), using a MS-5A 60/80 mesh Packed Column (3 mm I.D. × 2.0m, J-Science LAB Co., Ltd., Kyoto, Japan) and column temperature of 70 °C. The carrier gas was argon gas at 120 kPa and the thermal conductivity detector was used as the detector. The concentration was determined by creating a calibration curve using a gas of known concentration. 1 μl of cell preservation solution (prepared in 2.2) was injected into the GC/TCD and the concentration of hydrogen was calculated from the calibration curve.

Redox potential in the cell preservation solution was measured at 25 °C using redox electrometer (TPX-999Si, Tokokagaku Co., Ltd., Tokyo, Japan). Experiments were independently performed 4 times for each sample unless otherwise mentioned.

2.4. Determination of ROS

The amount of total ROS in the cells was measured using an assay kit (C10444, CellROX™ Green Reagent, Invitrogen, Camarillo, CA). The measurement method was performed as described in the manual of the kit. The thawed cells were seeded into 96 multi-well plate. After removing the supernatant, 100 μL of medium was added and CellROX™ was added to a final concentration of 5 μM, and the cells were incubated at 37 °C for 30 min. The cells were then washed three times with PBS, and the fluorescence intensity was measured with a plate reader (SpectraMax i3x, Molecular Devices Japan Co., Ltd., Tokyo, Japan). The measurement wavelength was 485nm/520 nm.

The amount of hydroxyl radicals in the cells were measured using a measurement kit (16055, Cell Meter™ Mitochondrial Hydroxyl Radical Detection Kit, COSMO Bio, Tokyo, Japan). The measurement method was performed as described in the manual of the kit. The thawed cells were seeded into 96 multi-well plate. After removing the supernatant, 100 μL of the kit's reagent (Working Solution) was added and incubated at 37 °C for 1 h. Then, the cells were washed 3 times with PBS. After washing, 100 μL of the kit's reagent (Component B) was added and fluorescence intensity was measured in a plate reader (SpectraMax i3x, Molecular Devices Japan Co., Ltd., Tokyo, Japan). The measurement wavelength was 540nm/590 nm.

2.5. Analysis of cell morphology

The thawed cells were cultured for 0, 3 and 18 h. Images were then acquired using an optical microscope (BZ-X700, Keyence Corp., Osaka, Japan). The area and perimeter of the cells were measured using BZ-X700 Analyzer software (Keyence Corp., Osaka, Japan).

2.6. Cell cycle measurement

Cell cycle was measured using a measurement kit (559619, BrdU Flow Kit, Becton Dickinson). The measurement method was performed as described in the manual of the kit. The thawed cells were seeded into 6 wells, and after 0, 3, and 18 h of incubation, 20 μL of 1 mM BrdU was added to each well and incubated for 3 h. The supernatant was then discarded and the cell suspension was prepared according to the manual. The obtained samples were analyzed with a flow cytometer (BD FACSCanto™ II Flow Cytometer, BD Biosciences). Experiments were independently performed 3 times for each sample.

2.7. Actin staining

For actin staining, the thawed cells were seeded in 6 wells and incubated for 3 and 18 h. After culture, cells were washed twice with PBS. The cells were then fixed with 3.7% formaldehyde, washed with PBS and Triton X-100, and incubated with Phalloidin

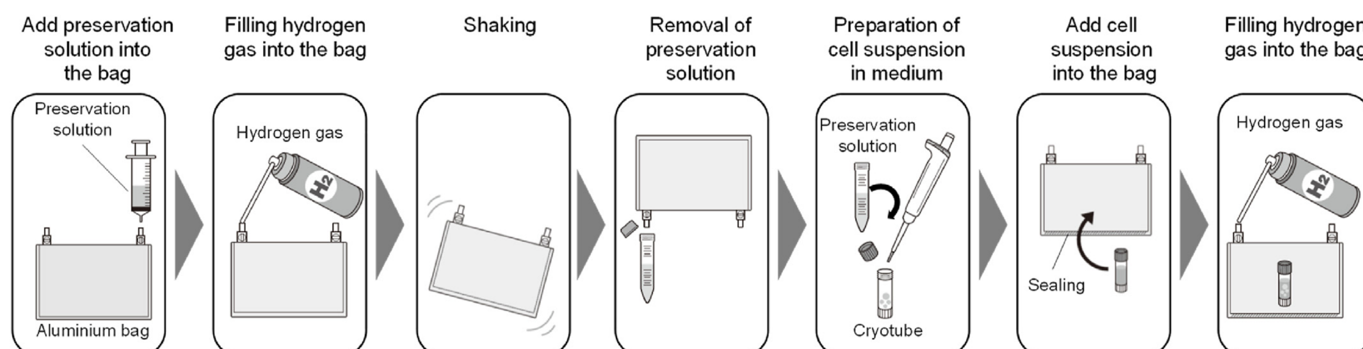

Fig. 1. A schematic illustration to prepare cell suspension in the presence of hydrogen gas.

for 20 min at room temperature. The cells were then washed with PBS and 300 nM DAPI was added and allowed to stand at room temperature for 5 min. The cells were then washed with PBS and observed using a fluorescence microscope (BZ-X700, Keyence Corp., Osaka, Japan).

2.8. Statistical analysis

All the data were statistically analyzed and expressed as the mean ± the standard error of the mean. The data were analyzed by student t-test or Tukey's test to determine the statistically significant difference while the significance was accepted at $p < 0.05$.

3. Results

3.1. Cell culture

Table 1 shows the hydrogen concentration and redox potential in the cell preservation solution. The filling of hydrogen gas significantly increased the hydrogen concentration in the solution. On the other hand, the redox potential significantly decreased from 92 mV to −393 mV.

3.2. Cell cryopreservation in the presence of hydrogen gas

Fig. 2 shows the time profile of hADSC proliferation after cryopreservation with hydrogen gas. Cells cryopreserved with hydrogen gas showed an enhanced proliferation compared with those done without hydrogen gas (Fig. 2A). The cells 4 days after incubation were almost in the confluent condition.

For the case of hydrogen-containing culture, the number of cells attached 3 h after incubation was higher than that of hydrogen-free cryopreserved. Cells cryopreserved with hydrogen gas showed the doubling time around 18 h whereas those without hydrogen did a longer doubling time after 24 h. There was no lag time of cell proliferation during the initial 1 day after culturing. On the contrary, the number of cells once decreased and then increased after some lag time for cells cryopreserved without hydrogen gas.

3.3. The viability and initial attachment of cells

Fig. 3 shows the viability of cells frozen and thawed in hydrogen-containing cell preservation solution. There was no significant difference in the survival percentage between cells cryopreserved in the presence and absence of hydrogen.

Fig. 4 shows the initial adhesion rate of cells frozen and thawed in hydrogen-containing cell preservation solution. The presence of hydrogen gas increased the percent initial adhesion of cells cryopreserved.

3.4. Generation of ROS

Fig. 5A shows the amount of total ROS of cells cryopreserved with hydrogen gas. After 18 h incubation in the absence of hydrogen gas, the amount of total ROS increased. On the other

Table 1
Hydrogen concentration and redox potential of cell preservation solution.

	Hydrogen content (ppm)	Redox potential (mV)
Presence	1.2 ± 0.1[a]	−393 ± 59[a]
Absence	0.0 ± 0.0[a]	92 ± 9[a]

[a] Average ± standard deviation.

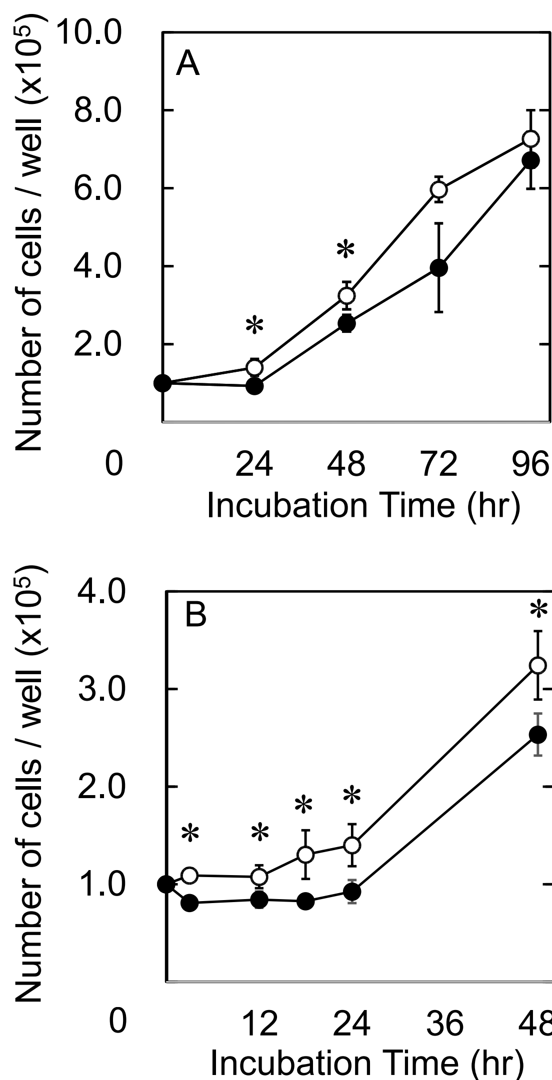

Fig. 2. Proliferation profile of hADSC cryopreserved in the presence (○) or absence of hydrogen gas (●) 0, 24, 48, 72 and 96 h (A) and 0, 3, 12, 18, 24 and 48 h after incubation (B). *, $p < 0.05$; significant difference between the two groups at the corresponding time.

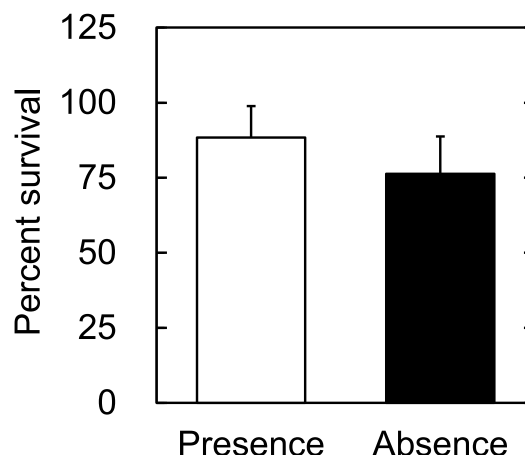

Fig. 3. Percent survival of hADSC cryopreserved in the presence (□) or absence of hydrogen gas (■) immediately after thawing.

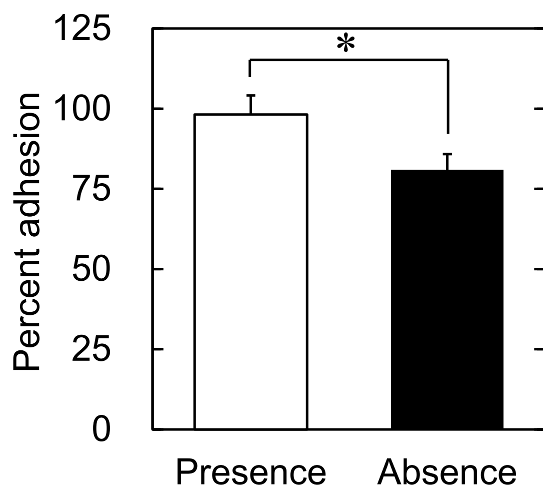

Fig. 4. Percent adhesion of hADSC cryopreserved in the presence (□) or absence of hydrogen gas (■) 3 h after incubation. *, $p < 0.05$; significant difference between the two groups.

hand, the total ROS of cells incubated in the presence of hydrogen did not increase.

Fig. 5B shows the amount of hydroxyl radicals of cells cryopreserved with hydrogen gas. The amount of hydroxyl radicals tended to degrease for cells cryopreserved with hydrogen gas. The amount of hydroxyl radicals reduced for cells cryopreserved with hydrogen gas reduced to a significantly great extent compared with that of cells without hydrogen.

3.5. Morphology of cells shape

Fig. 6 shows the percent morphology of hADSC cryopreserved in the presence of hydrogen gas. The number of spherical cells was significantly lower for in cells cryopreserved in the presence of hydrogen, irrespective of incubation time.

3.6. Flow cytometry of cell cycle

Fig. 7 shows the percent BrdU uptake rate of cells frozen and thawed in hydrogen-containing cell preservation solution. After 18 h incubation, the hydrogen gas presence allowed cells to increase the percent BrdU uptake.

3.7. Morphology of actin staining

Fig. 8 shows the actin immunofluorescence images of cells. The more clear formation of actin fibers was observed for cells cryopreserved in the presence of hydrogen. This result was well corresponded with that of Fig. 6. Comparing between Fig. 8B and D, the area stained with actin was much larger for cells cryopreserved in the presence of hydrogen gas.

4. Discussion

The present study demonstrates that the presence of hydrogen gas allowed cells to maintain the proliferation during their cryopreservation. The hydrogen reduced the level of ROS which is one of the factors to damage the cell activity. The cells cryopreserved in the presence of hydrogen gas showed a reduced lag time before cell proliferation compared with those without hydrogen gas. It is reported that ROS gives cells various functions, such as the apoptosis, induction senescence, and to enhance or stagnate the cell cycle

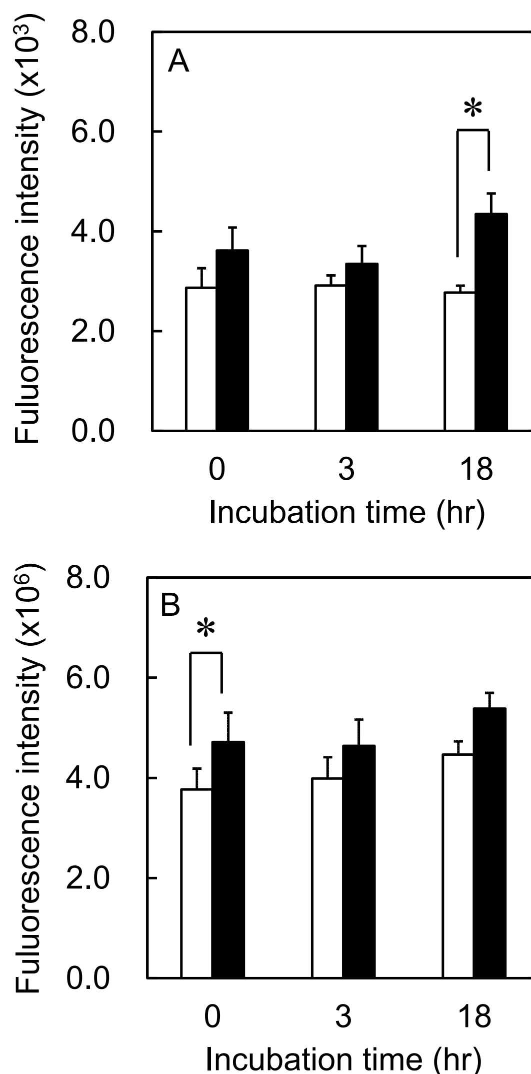

Fig. 5. ROS fluorescence intensity of hADSC cryopreserved in the presence (□) or absence of hydrogen gas (■): (A) Total ROS and (B) hydroxyl radicals. *, $p < 0.05$; significant difference between the two groups at the corresponding time.

[3–5]. The presence of hydrogen gas in cryopreservation had no effect on the survival rate of cells (Fig. 3). However, hydrogen gas was effective in increasing the percentage of initial cell adhesion (Fig. 4) and enhancing that of extended cell shape (Fig. 5) [6–9].

When cell suspensions with hydrogen gas added to the preservation solution was left at atmospheric pressure, the hydrogen concentration in the preservation solution decreased. Therefore, the hydrogen concentration in the storage solution was maintained by using an aluminum bag that prevents the permeation of hydrogen gas.

The hydrogen concentration in cell suspensions frozen to −80 °C in the aluminum bag filled with hydrogen gas was 1.2 ppm (Table 1). Furthermore, the concentration of hydrogen gas in the gas phase in the aluminum bag was proportional to the concentration of hydrogen in the cell suspension (data not shown). The cryotubes were then removed from the aluminum bag and stored below −150 °C. The hydrogen concentration of the cell suspension removed was 0.0 ppm.

Since cell proliferation increased in a hydrogen concentration-dependent manner in cell suspensions (data not shown), the following experiments were performed at the highest hydrogen concentration of 1.2 ppm.

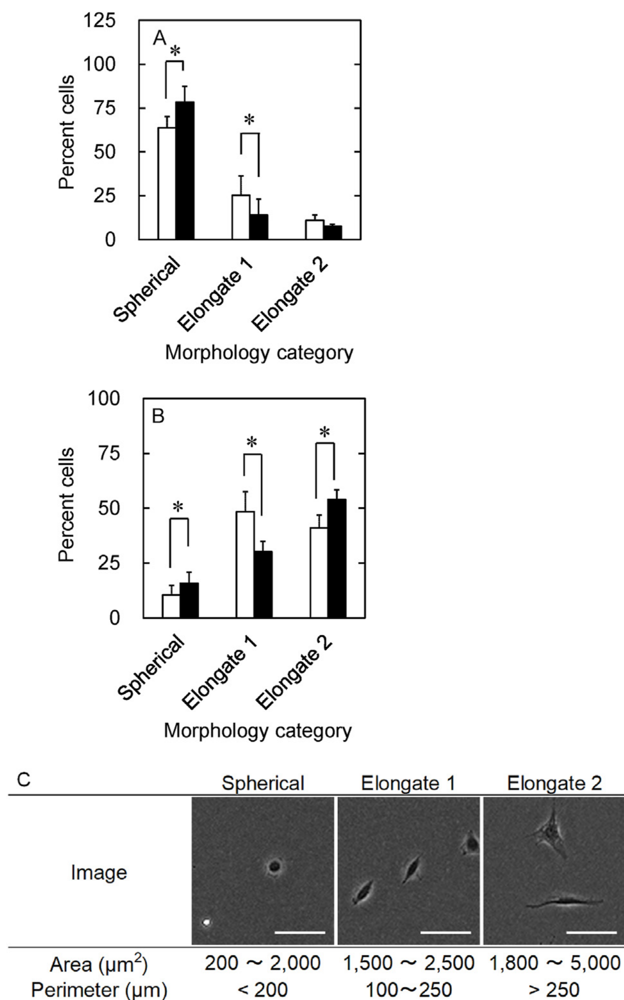

Fig. 6. Percent morphology of hADSC cryopreserved in the presence (□) or absence of hydrogen gas (■) 3 (A) and 18 h after incubation (B). *, $p < 0.05$; significant difference between the two groups. (C) Morphological category of cells in terms of area and perimeter. Scale bar is 200 μm.

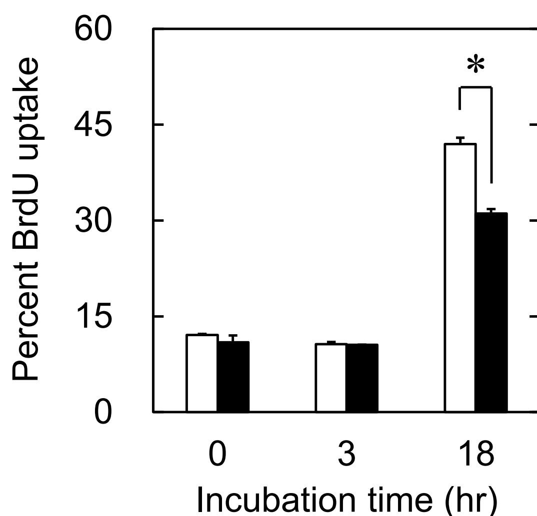

Fig. 7. Percent BrdU uptake of hADSC cryopreserved in the presence (□) or absence of hydrogen gas (■) 0, 3 and 18 h after incubation. *, $p < 0.05$; significant difference between the two groups at the corresponding time.

In cells frozen under normal conditions without hydrogen, the number of cells that initially adhered after seeding maintained for a certain time (lag time) and then shifted to the logarithmic growth phase, which is a general cell proliferation. However, the addition of hydrogen not only improved the initial adhesion of seeded cells after thawing, but also gradually increased the number of cells for 6, 12, and 18 h (Fig. 2B). The fact that cells proliferate without lag time during hydrogenation was considered to be a phenomenon unique to hydrogen.

The absence of lag time could be caused by an increase in cell cycle activity. The absence of lag time may be due to an enhanced cell cycle, which is suggested to be related to the cell cycle, although there is a time lag compared to the cell cycle results (Fig. 7).

It is reported that helium gas increased the viability and initial adhesion of cells [10]. This result is different from that of hydrogen gas, suggesting that the biological effects of hydrogen gas were different from those of noble gases.

There was no significant difference between the cell viability of 88% in the presence of hydrogen and 76% in the absence of hydrogen (Fig. 3). It is possible that the higher survival rate of cells in the absence of hydrogen is one of the possible reasons.

It is well recognized that the frozen process often causes an increased ROS production. It is well known that when mammalian cells are exposed to a low temperature stress, the mitochondrial respiration is impaired to accumulate ROS in the cells [4], which sometimes causes the cell damage. Hydrogen molecules selectively scavenged hydroxyl radicals generated immediately after thawing (Fig. 5B). It is well recognized that hydroxyl radicals are highly toxic to cells. It is highly conceivable that the reduction in hydroxyl radicals affected cell adhesion after 3 h of culture. In addition, the total ROS amount was also reduced after 18 h incubation, when cells proliferation more actively (Fig. 5A). It is considered that the amount of hydroxyl radicals (Fig. 5B) is the result of a direct reduction in hydrogen molecules. On the other hand, it is likely that the difference in the total ROS (Fig. 5A) is due to the indirect effect of hydrogen molecules. It is conceivable that the time lag of the change in the total ROS amount is not due to a direct effect of molecular hydrogen, but an indirect effect while results from an improved mitochondrial activity [11,12].

It is well known that the molecular hydrogen reacts specifically with hydroxyl radicals [2]. It is apparent from Fig. 5B that the amount of hydroxyl radicals is reduced by the hydrogen gas treatment in cryopreservation compared with hydrogen treatment immediately after cell thawing process. The hydroxyl radicals generated gives cells a damage or function loss [11]. It is noted that molecular hydrogen can specifically react to remove hydroxyl radicals. Considering the findings, it is likely that the significant reduction of hydroxyl radicals immediately after thawing contributes to the improvement of cell functions, such as an enhanced initial adhesion of cells, an increased cell number, the enhancement of cell cycle and the increase in the actin content.

It is reported that the majority of ROS in cells was produced in mitochondria, and the ROS themselves caused damage to mitochondria [11]. The amount of ROS was reduced by hydrogen, which causes a reduced damage to mitochondria.

It is demonstrated that hydrogen improves the mitochondrial biosynthesis [12]. Hydrogen would reduce the cold stress during the freeze and thaw process, resulting in a decrease in the mitochondrial negative action. It is demonstrated that low ROS levels activate signaling via p53 antioxidant function to enhance cell proliferation [13]. Therefore, the presence of hydrogen reduced ROS in freeze-thawed cells, which led to an enhanced cell proliferation.

Fig. 6 shows that for cells cryopreserved in the presence of hydrogen gas the percentage of spherical cells decreased with time. A decreased spherical cell means an increased spreated cell.

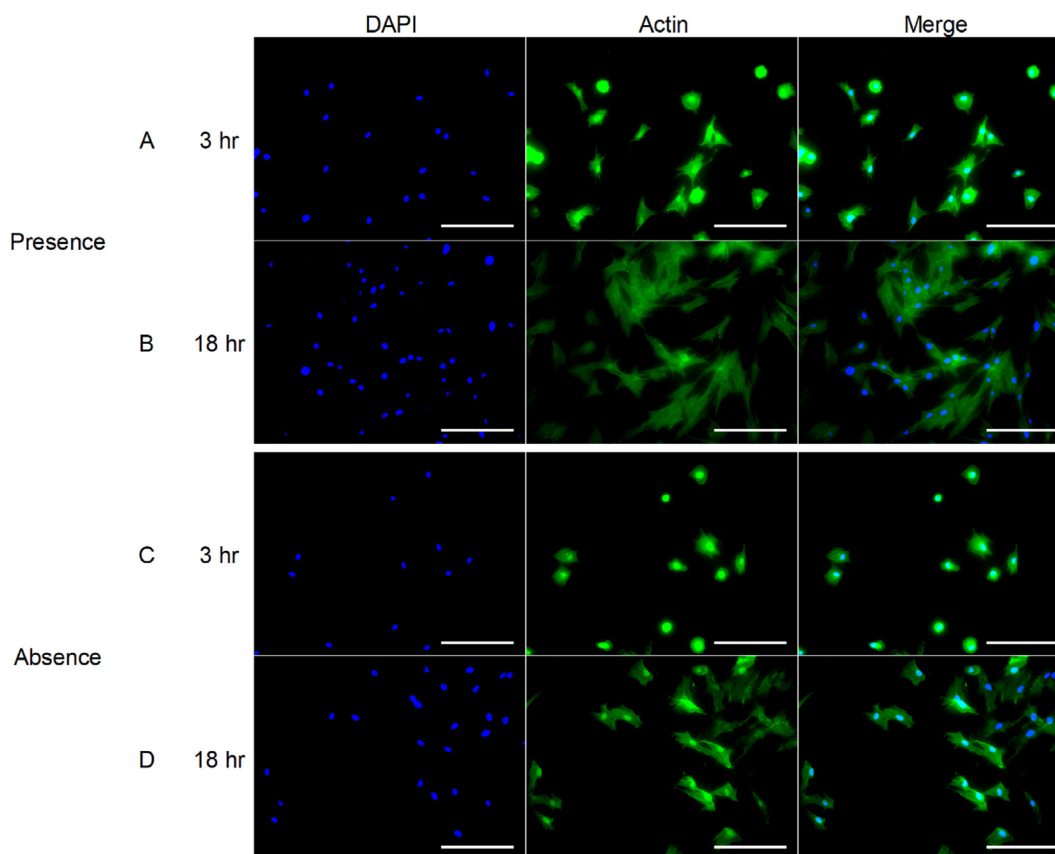

Fig. 8. Actin immunofluorescence images of hADSC cryopreserved in the presence (A, B) or absence of hydrogen gas (C, D) 3 and 18 h after incubation. Scale bar is = 200 μm.

The result in Fig. 6 supports an increase in the number of adherent cells. This result is supported by the actin staining image (Fig. 8). After 18 h of incubation, the total percentage of Elongated 1 and 2 cells was significantly higher for cells cryopreserved in the hydrogenated condition. It is reported that hydrogen affects the morphology of injured cells [8]. Taken together, hydrogen gas has an effect on the cell morphology in the cryopreservation condition. This may be a new effect of hydrogen gas on the cell morphology.

It is apparent in Fig. 7 that the BrdU significantly increased for cells frozen and thawed in the presence of hydrogen 18 h after incubation. This suggests that the hydrogen gas has an influence on the cell cycle and division shown in Figs. 2B and 8. This finding is supported by the finding that ROS promotes the cell cycle [5].

There are some reports to evaluate the effect of hydrogen on the living organisms and the hydrogen ameliorates damage to living organisms [14–16]. However, few researches have been reported on the effect of hydrogen on the biological activity of cells. When hydrogen was added to normal cell cultures, no increase in cell number or changes in morphology was observed (data not shown). Therefore, it is possible that the small effect of hydrogen on cells may be due to a reduced damage. Hydrogen behaves differently from other gases, and the improvement in cell adhesion is unique for hydrogen, and even if the difference is small, we believe this study experimentally confirm that hydrogen has a beneficial effect on the cryopreservation of cells.

Considering a high permeability property of molecular hydrogen, the hydrogen treatment will be available for the cryopreservation of cell aggregates, such as 3-dimensional cell spheroids and organoids. This study demonstrates that the hydrogen gas may improve the biological function of cells by reducing the amount of ROS in the cell, although further experiment need to optimize the treatment condition of hydrogen gas.

5. Conclusions

The present research demonstrates that hydrogen gas had a good effect on the biological functions of hADSC in cryopreservation. When hADSC were cryopreserved by a commercial cell preservation solution, the presence of hydrogen gas increased the percent initial adhesion of cells cryopreserved. The cell cycle and intracellular ROS measurements demonstrate an enhanced cell cycle and a decreased ROS production. In conclusion, the presence of hydrogen gas during cryopreservation is promising to improve the biological behavior of cells after cell thawing in terms of cells viability, proliferation or metabolic activity.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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