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Cryopreservable three-dimensional spheroid culture for ready-to-use systems

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Abstract−Three-dimensional (3D) spheroid culture has applications in many fields as spheroids closely recapitulate physiological conditions. However, spheroid culture and maintenance are time-consuming and unsuitable for urgent situations; therefore, appropriate cryopreservation methods for spheroids are required for their use in an on-demand and ready-to-use manner. We hypothesized that the feasibility of a ready-to-use system relies on diffusion of the preservation solution within spheroids; we thus evaluated the effects of spheroid-forming parameters, such as cell number and culture period, on spheroid viability and functionality. Long-term spheroid culture for seven days interfered with penetration of the cryopreservation solution as it caused cell condensation and extracellular matrix (ECM) secretion, as well as low viability and migratory activity upon replating after storage. However, ready-to-use spheroids, which were cultured for one day and then cryopreserved, showed viability and migration similar to those of non-cryopreserved spheroids, confirming that a short incubation period was suitable for this system. The chondrocyte-based ready-to-use spheroid system designed in this study can be easily applied to regenerative medicine applications that require a large number of cells in the future and can provide information for applying the ready-to-use spheroid system to various cell types.

Keywords: 3D Spheroid Culture, Chondrocytes, Cryopreservation, Ready-to-use System

INTRODUCTION

Three-dimensional (3D) spheroid cultures not only present enhanced functionality compared to 2D cultured cells but are also favorable for delivery via injections. They have thus received much attention in the development of cell-based therapeutics and *ex vivo* tissue modeling [1-4]. The development of various culture dishes for spheroid formation has facilitated the widespread application of spheroids in bioengineering and biotechnology [5,6]. However, spheroid culture is inevitably time-consuming and difficult to perform in large numbers in a short period; therefore, it is not suitable for patients who urgently need a large number of spheroids for treatment [7]. From this viewpoint, a ready-to-use approach using cryopreservable spheroids could be a useful strategy for cell-based therapy, drug development, and biological research. Cryopreservable spheroids can be used immediately after thawing when needed after advance production and cryogenic storage in sufficient quantities, and can be delivered over long distances and periods in a cryopreserved state [8,9]. For their effective use, cryopreserved spheroids should maintain the same characteristics and quality as non-cryopreserved spheroids.

Although there are many methods of cell cryopreservation, including slow cooling, rapid cooling using liquid nitrogen, vitrification, freeze drying, and encapsulation, their application to spheroids is limited [8,-12]. Ice crystals generated during freezing and thawing cause cell damage and death [13]. Crystallization during freezing and recrystallization during thawing can be controlled by the volume and cooling rate of the cryopreservation solution; however, it is difficult to control 3D spheroids, which have relatively larger volumes than those of single cells [8]. While most previous studies have focused on optimizing the cryopreservation solutions and protocols for the cryopreservation of spheroids [11], studies on spheroid formation conditions have not been investigated so far. In particular, the effects of cell concentration, spheroid size, and spheroid culture period on the biological function and viability of spheroids after the freeze-thaw cycle have not been evaluated.

Therefore, in this study, we developed a ready-to-use spheroid system with chondrocytes targeting cartilage tissue treatment and its associated applications. A mesh-integrated ultra-low-attachment (ULA) culture dish was used to simply and precisely control the spheroid size and culture period. The ULA dish can induce spheroid formation through effective cellular assembly by minimizing cell attachment on the surface and promoting cell-cell interactions. A mesh pattern integrated within the dish acts as a microwell structure to ensure that single cells are evenly distributed to generate uniform spheroids. Here, we hypothesized that two parameters, cell number per spheroid and culture period, are important for the interaction between the spheroid and cryopreservation solution and may determine cryopreservable spheroid formation. Therefore, we investi-

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gated the effect of these two parameters on the viability and functionality of ready-to-use spheroids.

EXPERIMENTAL

1. Cell Cultures

Chondrocytes were isolated from the knee joints of New Zealand White rabbits (4-week-old, male; Orient, Korea). Briefly, articular cartilage fragments were chopped into small pieces and digested in 0.05% type II collagenase containing DMEM/high glucose (Hyclone, Utah, USA). After 18-24 h of digestion, the digested tissue was filtered through a cell strainer with 70 μm pore size (Fisher Scientific, Pittsburgh, PA, USA). Phosphate-buffered saline (PBS; pH 7.4) with 1% penicillin/streptomycin (PS, Gibco, Carlsbad, CA, USA) was used for washing and cell isolation, followed by centrifugation at 1,200 rpm for 10 min at room temperature to obtain chondrocytes. The isolated chondrocytes were cultured in DMEM supplemented with 10% FBS (Gibco, Grand Island, USA) and 1% PS in a humidified atmosphere with 5% CO_2 at 37 °C. The culture medium was changed every two days. Chondrocytes were used until passage 3.

All animal experiments for chondrocyte isolation were performed in compliance with the relevant laws and institutional guidelines of the Institutional Animal Care and Use Committee (IACUC) of the Korea Institute of Toxicology (KIT). The experiment was approved by the IACUC (IAC-21-01-0143).

2. Preparation of Chondrocyte Spheroids

Spheroid formation was conducted on mesh-integrated ULA culture dishes specially designed for uniform spheroid formation (Cat No. 2035100, LabToLab Co., Ltd., Daejeon, Korea). Chondrocytes harvested using a standard trypsinization protocol were plated on ULA dishes at different densities (1×10^6, 2×10^6, and 4×10^6 cells/dish). Cultures for spheroid formation were performed for seven days with medium changed every two days. On days one, three and seven, the spheroids were observed under a microscope (Leica DMi8, Leica, Wetzlar, Germany). Spheroids cultured for one and seven days were cryopreserved and analyzed. A schematic representation of the chondrocyte spheroid culture is shown in Fig. 1.

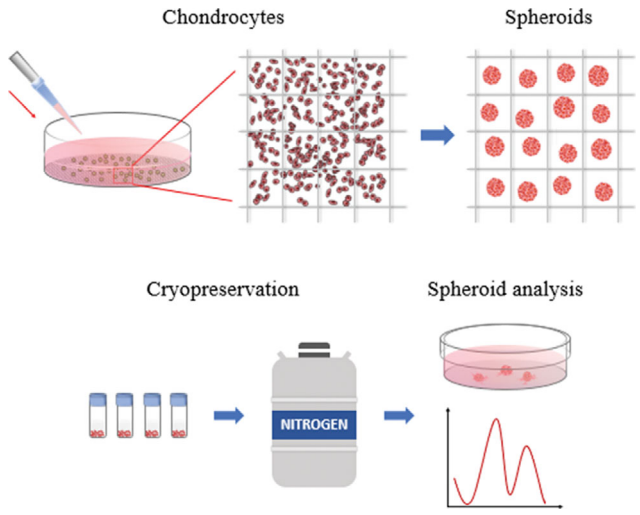

Fig. 1. Schematic illustration showing the development of a cryopreservable and ready-to-use spheroid system.

3. Freezing and Thawing Spheroids

For cryopreservation, the prepared spheroids were harvested from culture dishes using simple pipetting and briefly centrifuged (500 rpm for 2 min). The collected spheroids (4×10^3 spheroid/tube) were gently suspended in 1 ml cryopreservation solution (CS10, Stemcell Technologies, Vancouver, BC, Canada) and transferred to Cryotubes (Thermo Scientific, DK -4000 Roskilde, Denmark). The spheroids were stored at −80 °C for one day and then stored in liquid nitrogen for seven days.

The cryopreserved spheroids were slowly warmed in a water bath (37 °C) for 2 min, the freezing solution with spheroids was mixed, and gently suspended in 9 ml of pre-warmed medium (37 °C). After centrifugation (500 rpm for 2 min), the supernatant was removed and the spheroids were recovered by adding 3 mL of fresh medium. The spheroids were then used in the experiments.

4. Viability Assessment of Viability of the Ready-to-use Spheroids

The viability of cryopreserved spheroids after thawing was assessed in comparison with that of non-cryopreserved spheroids. For live/dead staining, a LIVE/DEAD viability/cytotoxicity kit (Life Technologies, Carlsbad, CA, USA) was used, following the manufacturer's instructions. Spheroids were washed with PBS, incubated in 1 : 1,000 calcein acetoxymethyl (CAM) and 1 : 500 ethidium homodimer-1 (EtHd-1) for 30 min, and directly analyzed using a fluorescence microscope (Leica DMi8). Live cells were identified based on the green fluorescence signals of CAM, whereas dead cells were determined by the red fluorescence signals of EtHd-1.

Ten chondrocyte spheroids were collected and placed in each well of a ULA 96-well flat bottom (Corning, NY 14831 USA) for the cell counting kit-8 assay (CCK-8; Dojindo, Tokyo, Japan). Spheroids were incubated with 100 μL of CCK-8 reagent mixed with DMEM (1 : 10 dilution) for 2 h at 37 °C. The absorbance of the supernatant was measured at 450 nm using a microplate reader (Varioskan Flash; Thermo Fisher Scientific).

5. Spheroid Migration Assay

Ten spheroids were randomly collected and plated on a 100 mm diameter cell culture plate (Thermo Fisher Scientific). The spheroids were observed under a Leica DMi8 microscope. The area covered by the proliferated and migrated cells from the spheroids on days one and three was measured using Leica LAS X and ImageJ software.

6. Statistical Analysis

Statistical analysis was conducted using one-way analysis of variance (ANOVA) and Tukey's multiple comparison post-test using Origin 8 (OriginLab) and Prism 8 (GraphPad, CA, USA). Data are presented as the mean±standard deviation (SD). Statistical differences were determined by p-values and are indicated as * $p<0.05$, ** $p<0.01$, and *** $p<0.001$.

RESULTS

1. Formation of Cryopreservable Chondrocyte Spheroids for the Ready-to-use System

Chondrocyte spheroids were generated by inducing the self-assembly of cells on mesh-integrated ULA culture dishes (Fig. 1).

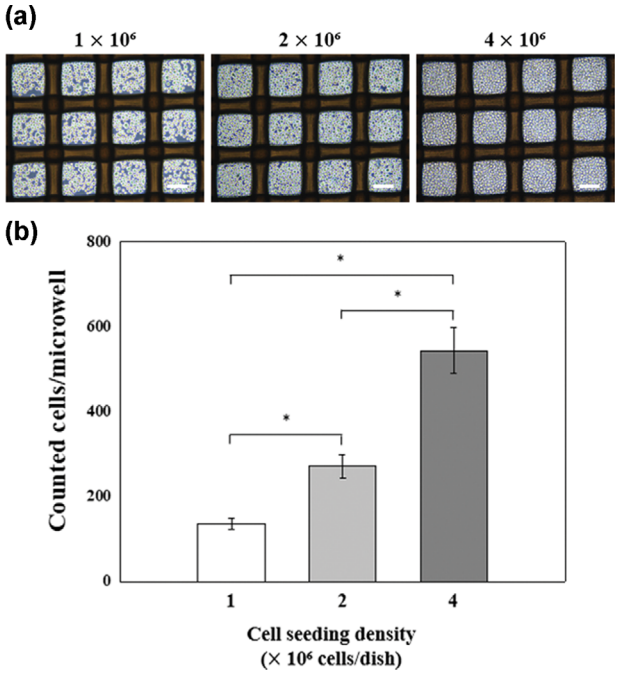

Fig. 2. Seeding of chondrocytes on ultra-low attachment microwell plates to achieve spheroid size control. (a) Phase contrast images of chondrocytes plated at different seeding densities (1×10^6, 2×10^6, and 4×10^6 cells/dish) (Scale bars=250 μm) (b) number of cells placed in one microwell according to varying conditions. '*' indicates statistical significances ($p<0.05$).

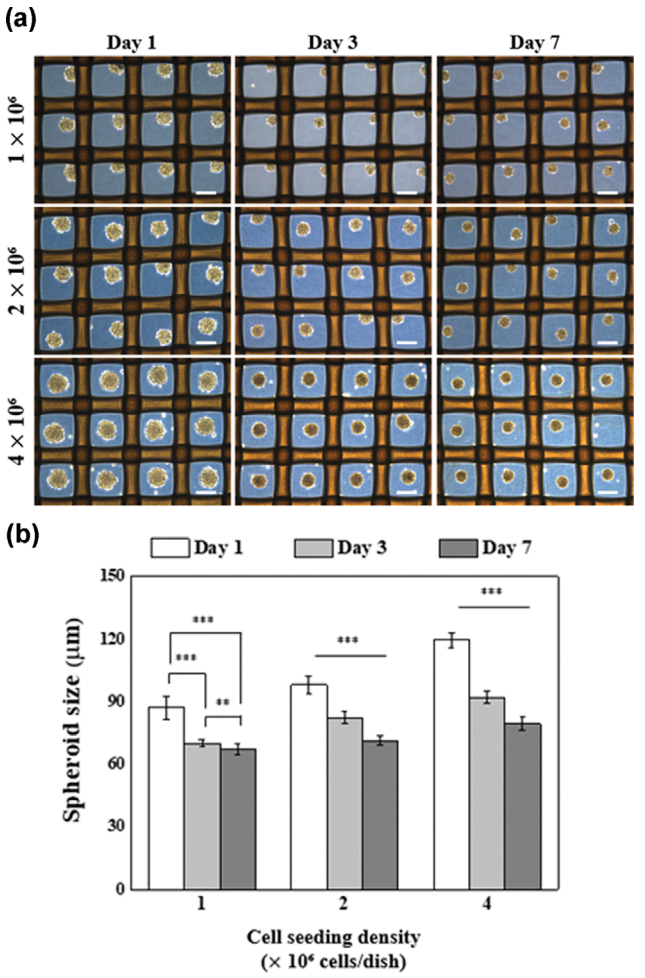

Fig. 3. Formation and culture of spheroids. (a) Phase contrast images of chondrocyte spheroids cultured for seven days with varied seeding densities (Scale bars=250 μm). (b) Diameters of chondrocyte spheroids depending on the number of days cultured. ** ($p<0.01$), * ($p<0.001$) indicated statistical significances.**

Each independent square divided by the integrated mesh pattern acted as a microwell, in which a specific number of cells were assembled to form spheroids. It was hypothesized that the spheroid size could be controlled by adjusting the initial cell concentration, which, along with the spheroid incubation period, could act as an important factor in establishing and optimizing a ready-to-use spheroid system. To evaluate the ready-to-use spheroid system, cryopreservation was performed according to the conventional protocol, and the thawed spheroids were analyzed.

To form spheroids with the designated cell numbers, various initial cell seeding densities (1.0, 2.0 and 4.0×10^6 cells/dish) were used (Fig. 2). As the initial cell seeding density increased, the average number of cells in each microwell appeared to increase (Fig. 2(a)), and 136, 272, and 544 cells were counted in each microwell, respectively (Fig. 2(b)). We then investigated the effect of the initial seeding density and culture time on spheroid size (Fig. 3). Within one day, the chondrocytes self-assembled into spheroids in each microwell. As the number of applied cells increased, the size of the spheroids on day one increased proportionally (87.2±5.4, 98.2±4.1 and 119.5±3.8 μm in the groups of 1.0, 2.0 and 4.0×10^6 cells/dish, respectively). However, with the extended incubation period up to day seven, the cells in the spheroids showed an overall tendency to condense, and the spheroid size decreased to approximately 70 μm in all groups.

2. Viability Assessment of Viability of the Ready-to-use Spheroids

To establish the ready-to-use spheroid system, we first checked the viability of the spheroids after the freezing and thawing processes. The one-day-old fresh spheroids formed by one day of culture and not cryopreserved, presented good viability with rarely observed dead cell signals regardless of cell seeding densities (Fig. 4(a)). In contrast, one-day-old cryo-spheroids, meaning the ready-to-use spheroids formed by one day of culture, which underwent the cryopreservation and thawing processes, showed more dead cell signals as the seeding density increased (Fig. 4(b)). A quantitative viability test using the CCK-8 assay was conducted to further investigate the effect of seeding density on the viability of one-day-old ready-to-use spheroids (Fig. 4(c)). The one-day-old fresh spheroids showed increased cell viability in proportion to seeding density. This appears to be attributed to the increased number of cells constituting the spheroids. However, one-day-old cryopreserved spheroids showed values of approximately 0.3 regardless of the increase in cell number. This suggests that spheroid size has a negative effect on cell viability in the ready-to-use system. We conducted the same assays with spheroids cultured for seven days (Fig. 5). Although most cells in the seven-day-old fresh spheroids showed

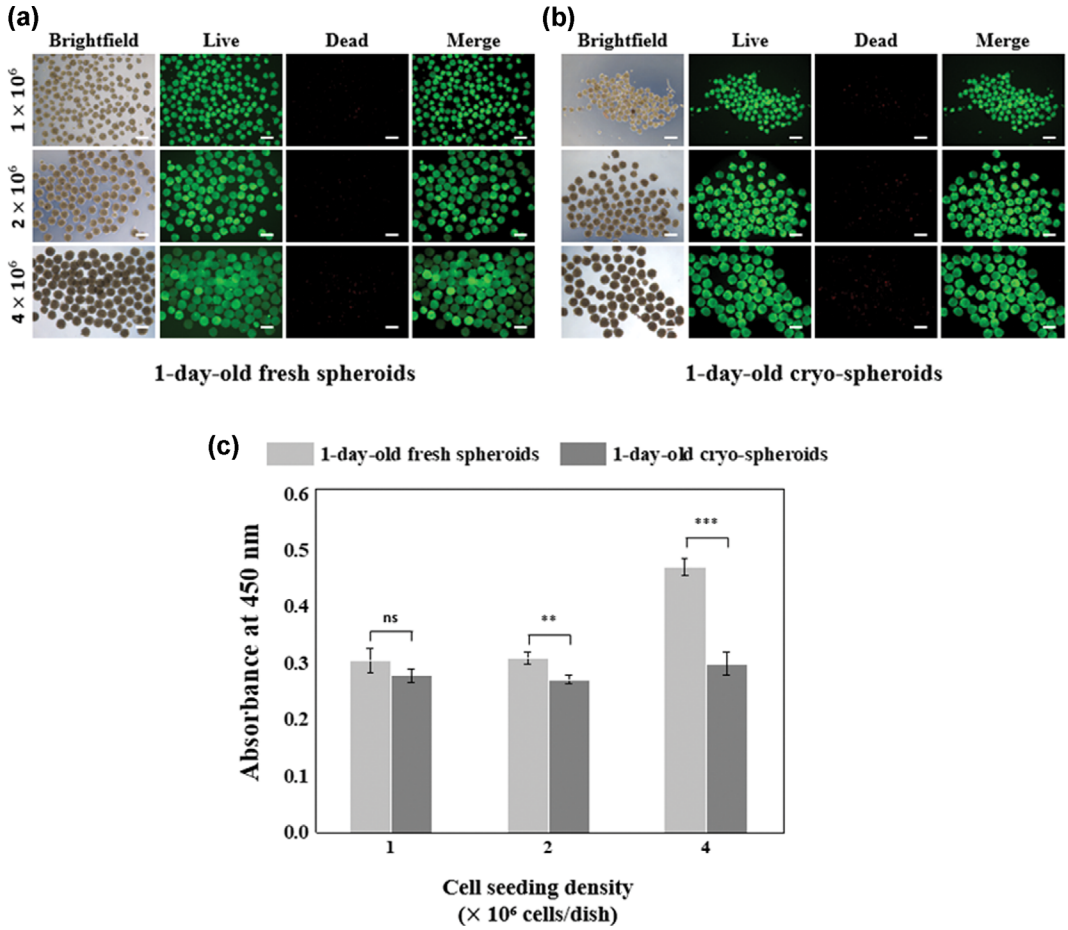

Fig. 4. Viability of the prepared ready-to-use spheroids after one day of culture. Phase contrast and live/dead staining images of (a) 1-day-old fresh spheroids and (b) 1-day-old cryo-spheroids (Scale bars=250 μm). (c) Quantitative assessment of viability in the spheroids analyzed using the cell counting kit-8 (CCK-8) (=p<0.01, ***=p<0.001, ns=no significance).**

live cell signals, increased dead cell signals were detected under conditions of 2 and 4×10^6 cells/dish (Fig. 5(a)). The seven-day-old cryo-spheroids representing the ready-to-use system showed a high level of dead signals at 2 and 4×10^6 cells/dish and markedly decreased live cell signals (Fig. 5(b)). In the quantitative viability test, reduced cell viability was observed after applying the ready-to-system. After comparing Figs. 4 and 5, we found that a shorter culture period is more suitable for the ready-to-use chondrocyte system.

3. Assessment of Cell Migratory Activity in the Ready-to-use Spheroids

Migration of chondrocytes from the ready-to-use spheroids was assessed according to the variables of seeding density (1.0, 2.0, and 4.0×10^6 cells/dish) and spheroid culture period (1 and 7 days). The one-day-old spheroids exhibited chondrocyte migration after they were attached and cultured for seven days of culture period. At all seeding densities, cells gradually migrated around the spheroids (Fig. 6(a)). Similar to the non-cryopreserved spheroids, the one-day-old cryo-spheroids presented gradual cell migration over seven days of culture, indicating that migratory activity was well maintained in ready-to-use spheroids. The measured coverage area by the one-day-old fresh spheroids increased as the cell seeding density increased by 22.6±6.2, 36.3±5.7, and $72.0\pm12.6\times10^3$ μm^2, respectively. Although a proportional increase in covered area was observed with the ready-to-use spheroids as 17.4±5.4, 15±2.7 and $47.6\pm8.9\times10^3$ μm^2, the ready-to-use spheroids showed a decrease in area compared to the freshly cultured spheroids. However, by day three, it was difficult to find differences in the coverage area between ready-to-use and non-preserved spheroids, proving that migration of the one-day-old ready-to-use spheroids may be slow immediately after thawing but is recovered over time (Fig. 6(c), (d), (e)). We noticed significantly reduced migratory activity in ready-to-use spheroids prepared after seven days of culture, as shown in Fig. 7. Although the seven-day-old fresh spheroids exhibited migratory activity over time in all varied seeding density groups, the measured area from the seven-day-old cryo-spheroids remained below $15.5\pm5.8\times10^3$ μm^2 regardless of the seeding density and culture time. These results suggest that the condition of seven-day spheroid culture is not suitable for establishing a ready-to-use system using chondrocytes.

DISCUSSION

Chondrocytes have been widely explored for articular cartilage regeneration [2,10,14-16]. With advances in cell culture methods, chondrocyte culture has also undergone changes. Chondrocyte cul-

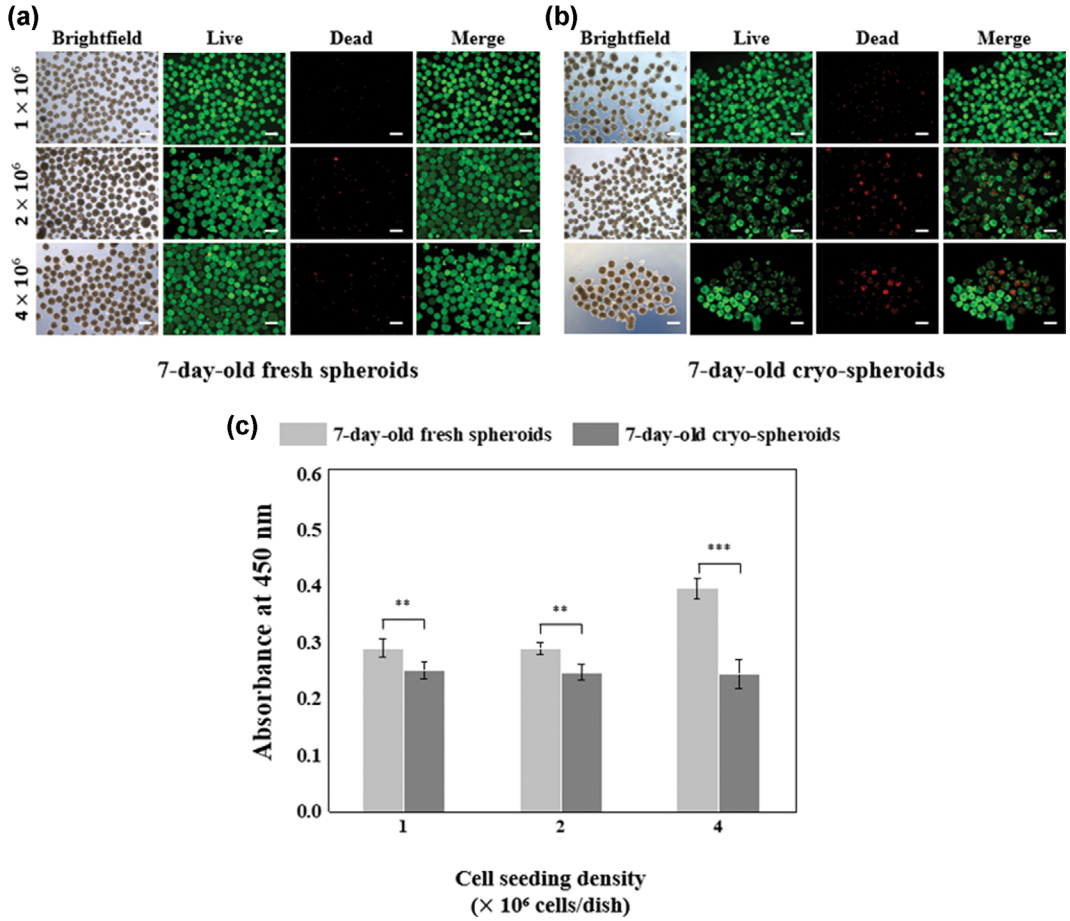

Fig. 5. Viability of the prepared ready-to-use spheroids after seven days of culture. Phase contrast and live/dead staining images of (a) 7-day-old fresh spheroids and (b) 7-day-old cryo-spheroids (Scale bars=250 μm). (c) Quantitative assessment of viability in the spheroids analyzed using the cell counting kit-8 (CCK-8) (=p<0.01, ***=p<0.001).**

ture was first initiated in flat plastic dishes for 2D cell culture; however, several limitations are associated with 2D cell culture. In particular, chondrocytes lose their differentiated phenotype during 2D cell culture, which is indicated by the loss of type II collagen synthesis [17-20]. This shortcoming has led medical and scientific experts to recognize the reduced accuracy of their experimental outcomes, which has increased the need for the invention and utilization of a more advanced form of cell culture known as 3D cell culture. Indeed, many studies have reported that chondrocytes cultured in a 3D microenvironment demonstrated a higher growth rate, realistic morphology, and the expression of several genes that were not expressed in 2D culture [21]. The 3D cultured cells form natural cell-to-cell interactions and synthesize extracellular matrix (ECM), as they do *in vivo* [22]. In addition, numerous reports have confirmed that 3D cultured cells showed higher regeneration efficiency than that of 2D cultured cells in osteochondral defect models [16,18,23].

As the development and utilization of 3D cell culture technology is rapidly expanding, research on cryopreservation methods suitable for 3D cultured cells or tissues has recently attracted increasing attention. One of the most widely used cryopreservation methods is the slow-freezing method [24]. This method is divided into four main steps: (1) mixing of the cryopreservation solution with spheroids, (2) storage at low temperature, (3) mild thawing, and (4) removal of cryopreservation solution from the spheroids [11]. In these steps, the fatal factors that threaten cell recovery include the toxicity of cryoprotectant chemicals and ice crystals during the freeze-thaw cycle. Thus, most previous studies focused on cryopreservation solutions or four-step protocols to develop new cryopreservation methods. However, these approaches alone hinder optimization of spheroid cryopreservation because they are heterogeneous and inherently have limited internal mass-transfer properties [9,25]. Therefore, in this study, we focused on spheroid formation conditions that are more suitable for cryopreservation, to avoid or overcome the limitations of conventional cryopreservation methods.

Cellular functions within spheroids are strongly correlated with spheroid size [26]. During spheroid formation, spheroid size can be easily controlled by varying the cell concentration [27]. There is an appropriate size limitation for spheroid formation because oxygen and nutrients must be able to penetrate effectively, even inside the spheroids [9]. Similarly, during cryopreservation, spheroid size is considered important because the cryopreservation solution must penetrate evenly into the spheroid. Thus, in this study, to confirm the effect of spheroid size and culture period on spheroid survival

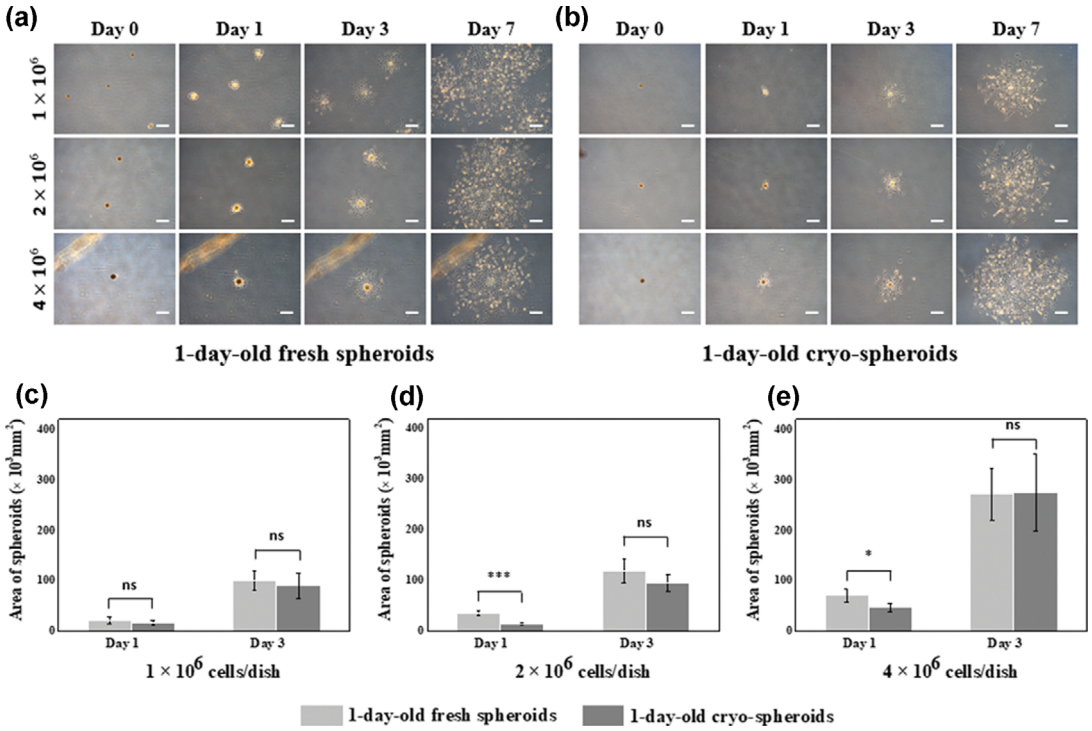

Fig. 6. Migratory activity of the prepared ready-to-use spheroids after one day of culture. (a) Phase contrast images of 1-day-old fresh spheroids and migrated cells (Scale bars=250 μm). (b) Phase contrast images of 1-day-old cryo-spheroids and migrated cells (Scale bars=250 μm). (c)-(e) Area covered by the spheroids according to the different preparation conditions (*=p<0.05, **=p<0.01, ***=p<0.001, ns=no significance).

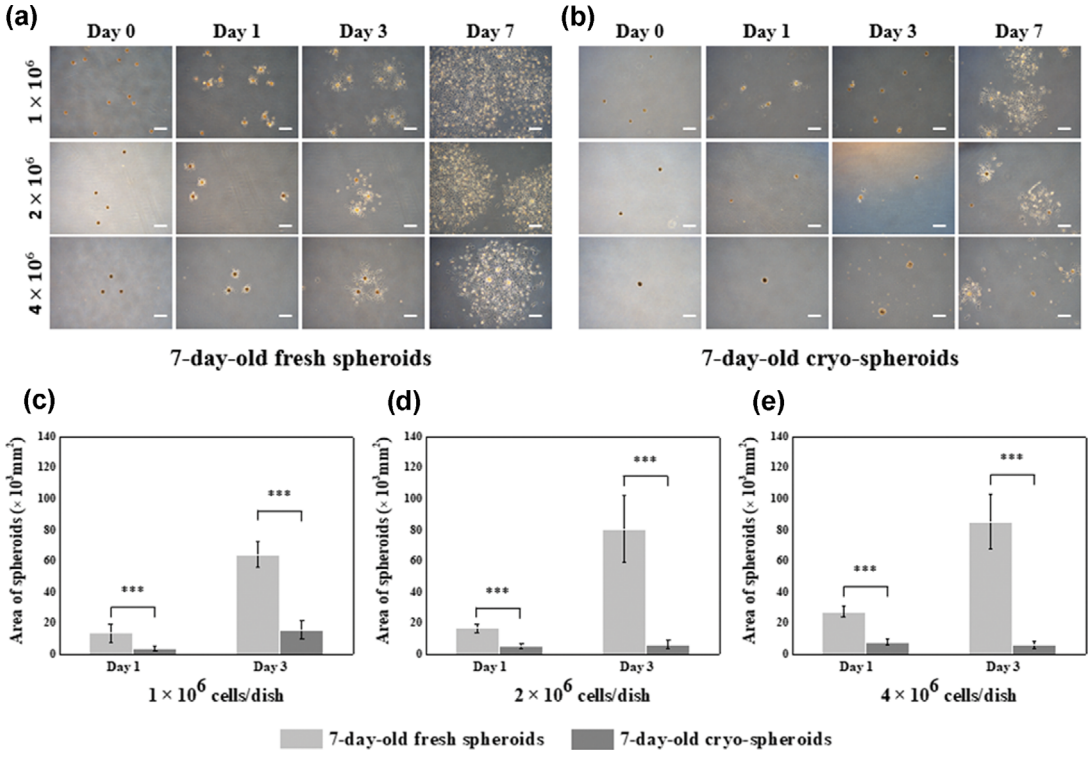

Fig. 7. Migratory activity of the prepared ready-to-use spheroids after seven days of culture. (a) Phase contrast images of the 7-day-old fresh spheroids and migrated cells (Scale bars=250 μm). (b) Phase contrast images of the 7-day-old cryo-spheroids and migrated cells (Scale bars=250 μm). (c)-(e) Area covered by the spheroids according to the different preparation conditions (*=p<0.05, **=p<0.01, ***=p<0.001, ns=no significance).

rate after cryopreservation, spheroid formation and culture was performed at various cell concentrations over one to seven days. As shown in Fig. 3, as the seeding concentration increased, the number of cells in the wells increased, and the size of the formed spheroids also increased (day 1). However, as the culture period increased, spheroid size decreased (day 7). This time-dependent decrease in spheroid size was most significant for the largest spheroids formed from the highest number of cells. For the cryopreserved spheroids at one day (one-day-old cryo-spheroids), there was no significant difference in the survival rate before and after freezing in the small-sized spheroid groups (Fig. 4). However, the survival rate after thawing was significantly reduced in the largest spheroid group. This result is probably because the cryopreservation solution did not efficiently penetrate the large spheroids. In contrast, after cryopreservation following seven days of culture, the survival rate decreased in all groups regardless of size, despite the decrease in spheroid size. In long-term cultured spheroids, the cells become denser and more tightly aggregated owing to the secreted ECM [28]. It is considered that such a change in the internal environment of the spheroids hinders penetration of the cryopreservation solution into the spheroids despite their decreased size. These results imply that the spheroid size and culture period are important factors in establishing a ready-to-use spheroid system.

In the development of cell therapy products using spheroids, engraftment is an important issue for achieving a therapeutic effect. We evaluated the possibility of ready-to-use spheroids sprouting from the transplanted site and integrating into the host tissue by confirming the migration of chondrocytes from spheroids (Figs. 6 and 7). Similar to the viability results, migration was also affected by cell seeding density and culture period. As described above, a ready-to-use system using cryopreservation was not properly achieved as the culture period of chondrocyte spheroids increased. For one-day-old ready-to-use spheroids, it would be relatively easy to induce penetration of the cryopreservation solution; accordingly, even after thawing, they showed normal migration activity similar to that of the control group. Collectively, our results demonstrate the importance of inducing an appropriate interaction between spheroids and the cryopreservation solution when developing a ready-to-use spheroid system, which is largely dependent on the spheroid culture period. Further, this study proves that seeding density is a variable that determines the number of delivered cells.

CONCLUSIONS

We successfully developed a ready-to-use spheroid system for large-scale production and immediate application to various biological purposes by modulating the cell seeding density and culture period. This study demonstrates that a longer culture period is not favorable for the development of a ready-to-use spheroid system, as it may limit the effective infiltration of the cryopreservation solution into spheroids owing to enhanced cell condensation and ECM secretion. Through a migration test representing engraftment at the delivery site, we partially demonstrated that the developed ready-to-use system could efficiently produce spheroids in large amounts in advance for therapeutic purposes. Therefore, the ready-to-use spheroid system is promising for pharmacotoxicological study and autologous chondrocyte implantation treatment.

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AUTHOR CONTRIBUTIONS

T.T.T and S.W.K, conceived and planned the experiments. T.T.T and K.H.P contributed to sample preparation. T.T.T carried out the experiments, process the experiment data, draft the manuscript and designed the figure. T.T.T, S.H.E, K.H.P and Y.B.L were involved in analyzing data. J.J.S contributed to the histological evaluation. T.T.T and K.H.P performed the measurement, Y.B.L wrote the manuscript with input from all authors. S.W.K and K.M.H supervised the project.

CONFLICT OF INTEREST

The authors declare no competing interests.

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