Regular Article

Calcium Peroxide-Containing Polydimethylsiloxane-Based Microwells for Inhibiting Cell Death in Spheroids through Improved Oxygen Supply

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Multicellular spheroids are expected to be used for *in vivo*-like tissue models and cell transplantation. Microwell devices are useful for the fabrication of multicellular spheroids to improve productivity and regulate their size. However, the high cell density in microwell devices leads to accelerated cell death. In this study, we developed O₂-generating microwells by incorporating calcium peroxide (CaO₂) into polydimethylsiloxane (PDMS)-based microwells. The CaO₂-containing PDMS was shown to generate O₂ for 3 d. Then, CaO₂-containing PDMS was used to fabricate O₂-generating microwells using a micro-molding technique. When human hepatocellular carcinoma (HepG2) spheroids were prepared using the conventional microwells, the O₂ concentration in the culture medium reduced to approx. 67% of the cell-free level. In contrast, the O₂-generating microwells maintained O₂ at constant levels. The HepG2 spheroids prepared using the O₂-generating microwells had a larger number of live cells than those prepared using the conventional microwells. In addition, the O₂-generating microwells rescued hypoxia in the HepG2 spheroids and increased cell viability. Lastly, the O₂-generating microwells were also useful for the preparation of multicellular spheroids of other cell types (*i.e.*, MIN6, B16–BL6, and adipose-derived stem cells) with high cell viability. These results showed that the O₂-generating microwells are useful for preparing multicellular spheroids with high cell viability.

Key words oxygen generator; multicellular spheroid; tissue engineering; hypoxia

INTRODUCTION

Culturing cells under three-dimensional conditions induces more *in vivo* tissue-like cellular characteristics than culturing cells under monolayer conditions and is expected to be used for tissue models and cell transplantation.¹⁾ Multicellular spheroids, which are cell aggregates prepared by culturing cells on non-adhesive surfaces, are typical examples of cells cultured under three-dimensional conditions.^{2,3)} Multicellular spheroids have been used as tumor tissue models^{4–6)} and as formulations for transplantation of islet β cells,⁷⁾ hepatocytes,⁸⁾ and mesenchymal stem cells,⁹⁾ among others.¹⁰⁾

High-quality spheroids for cell transplantation need to be prepared in large quantities. For example, it has been reported that 300000 islets, which are spheroid-like cell aggregates, were required for a single patient to obtain therapeutic effects in patients with type 1 diabetes.¹¹⁾ In addition, spheroid size is one of the factors determining the cellular functions of spheroids^{12,13)}; thus, uniformly sized spheroids should be prepared. Microwell devices, in which a number of microwells are placed on a low-adhesive material, such as polydimethylsiloxane (PDMS), are useful for preparing a large number of size-controlled spheroids. We reported previously that uniformly sized spheroids were prepared in large quantities using PDMS-microwell devices.^{14,15)}

Spheroids contain densely packed cells, which results in poor oxygen permeability into their core region.^{16,17)} In addition, during the culture of a large number of spheroids using

microwell devices, it is predicted that oxygen concentration in the culture medium will decrease because of excess oxygen consumption by cells. Factors, such as poor oxygen permeability and excess oxygen consumption, inevitably induce hypoxia and cell necrosis in the spheroids. Therefore, hypoxic conditions in spheroids should be prevented in order to prepare highly functional spheroids for cell transplantation.

Calcium peroxide (CaO₂) is an oxygen generator that reacts with water to produce hydrogen peroxide. Then, the hydrogen peroxide decomposes into oxygen and water.¹⁸⁾ Coronel *et al.* reported that PDMS mixed with CaO₂ gently and continuously generated oxygen, and the cell viability and insulin secretion of rat islets was improved by culturing them using the CaO₂-containing PDMS.^{19,20)} Therefore, we assumed that the CaO₂-containing PDMS-microwell can be used to prepare spheroids with high cell viability.

MATERIALS AND METHODS

Chemicals The PDMS prepolymer (Silpot 184) and curing agent were purchased from Toray-Dow Corning Co., Ltd. (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM, low glucose type) and Hank's balanced salt solution (HBSS) were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Fetal bovine serum (FBS), 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE), penicillin and streptomycin (PS), and penicillin, streptomycin, and glutamine (PSG) were purchased from Thermo Fisher Scien-

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tific (Waltham, MA, U.S.A.). Trypan blue stain solution was purchased from Nacalai Tesque Inc. (Kyoto, Japan). Trypsin was purchased from Becton Dickinson and Co. (Mansfield, MA, U.S.A.). Mono azo rhodamine (MAR) was obtained from Goryo Chemical (Hokkaido, Japan). DMEM (high glucose type), calcium peroxide, and collagenase (derived from *Clostridium histolyticum*) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Monothioglycerol was purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). All other chemicals were of the highest grade commercially available.

Cell Culture Human hepatocellular carcinoma HepG2 cells were purchased from ATCC (Rockville, MD, U.S.A.) and cultured in DMEM (low glucose) supplemented with 10% heat-inactivated FBS, 0.2% NaHCO_3 , and PSG. Mouse insulinoma MIN6 cells were kindly provided by Professor Junichi Miyazaki (Graduate School of Medicine, Osaka University, Osaka, Japan) and cultured in DMEM (high glucose) supplemented with 10% heat-inactivated FBS, 0.3% NaHCO_3 , PS, and 0.5 mM monothioglycerol. Mouse melanoma B16-BL6 cells were obtained from RIKEN BRC (Tsukuba, Japan) and cultured in DMEM (low glucose) supplemented with 10% heat-inactivated FBS, 0.2% NaHCO_3 , PSG, and 2 g/L glucose. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 .

Animals Six-week-old male ICR mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). All protocols for the animal experiments were approved by the Animal Experimentation Committee of the Kyoto University Graduate School of Pharmaceutical Sciences.

Isolation of Adipose-Derived Stem Cells (ASCs) Fat pads were isolated from the abdominal cavity of ICR mice euthanized by cervical dislocation. The fat pads were minced to <1-mm pieces in HBSS using forceps and digested in 1 mg/mL collagenase in HBSS for 90 min at 37°C. Then, the digested samples were filtered through a 100- μm filter and centrifuged ($300 \times g$, 5 min, 20°C). The supernatant containing fat cells was discarded and the red blood cells (RBCs) were lysed using RBC lysis buffer (155 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM ethylenediaminetetraacetic acid (EDTA)). After centrifugation ($300 \times g$, 5 min, 20°C), the precipitates were seeded in culture plates, and non-adherent cells were removed one day after seeding. The adherent cells were used as ASCs.

Measurement of O_2 Generation from CaO_2 -Containing PDMS CaO_2 was mixed with PDMS prepolymer containing 10% curing agent at 0, 5, 15, and 25% of CaO_2 , and 1 g of this mixture was poured into a 50-mL tube and cured at 60°C for 2 h. Then, 5 mL of water was added to the solidified CaO_2 -containing PDMS and the concentration of O_2 in the liquid was measured using a dissolved oxygen meter (Multimeter HQ30d, TOADKK Co., Ltd., Tokyo, Japan) at 1, 2, 4, 6, 8, 12, 24, 48, and 72 h after the addition of water. The liquid was replaced with fresh water every 24 h.

Fabrication of O_2 -Generating Microwells O_2 -Generating microwells were fabricated as described in our previous manuscript¹⁴⁾ with some modifications. In brief, the PDMS prepolymer containing CaO_2 (0, 5, 15, and 25%) and the curing agent were poured into the microwell mold and cured at 60°C for 2 h. Then, the sheet containing the O_2 -generating microwells was peeled off and 34-mm-diameter circles (800

microwells/circle) were punched out. The O_2 -generating microwell circles were placed into the wells of 6-well plates with 1 mL PDMS prepolymer containing 10% curing agent. Following incubation at 60°C for 2 h for anchorage to prevent from floating of microwell circle, the system was sterilized by irradiation with UV light (253.7 nm) for 1 h.

Preparation of Spheroids Cells harvested using 0.25% (w/v) trypsin-EDTA solution were resuspended in culture medium at a cell density of 2.5×10^6 cells/3 mL. Then, 2.5×10^6 cells were added on each O_2 -generating microwell circle that had been placed in the 6-well plates and incubated at 37°C in a humidified atmosphere containing 5% CO_2 . The culture medium was changed 1 h after the addition of cells and every 24 h thereafter. After 72 h of incubation, the spheroids obtained were used for experiments.

Characteristics of Spheroids The diameter of the spheroids was measured using a microscope with an image analysis system (BZ-X710, KEYENCE, Osaka, Japan). Twenty spheroids were collected using a micropipette and were dispersed using 0.25% (w/v) trypsin-EDTA. After staining with trypan blue solution, the number of live cells was measured by counting the unstained cells using microscopy, and the number of live cells in each spheroid was calculated. The cell viability in spheroids could not be calculated because dead cells were hardly liberated from cell debris after dispersion of the spheroids.

Live/Dead Imaging The spheroids were stained with 1 μM CFSE and 5 $\mu\text{g/mL}$ propidium iodide in Opti-MEM (30 min, 37°C). After washing three times with phosphate-buffered saline (PBS), the spheroids were fixed with 4% paraformaldehyde in phosphate buffer (Nacalai Tesque, Inc.) overnight at 4°C. After fixing, the spheroids were optically cleared using the ScaleS method.²¹⁾ In brief, the spheroids were incubated with ScaleSQ(0) (22.5% (w/v) D[−]-sorbitol and 9.1 M urea [pH 8.4]) for 24 h at 4°C and then with ScaleS4(0) (40% (w/v) D[−]-sorbitol, 10% (w/v) glycerol, 4 M urea, and 20% (v/v) dimethyl sulfoxide) for 24 h at 4°C. The optically cleared spheroids were observed using confocal fluorescence microscopy (A1R MP microscope, Nikon, Tokyo, Japan).

Estimation of the Hypoxic Environment around Each Spheroid The culture medium of the spheroids was collected every 24 h, and the O_2 concentration in the medium was measured using a dissolved oxygen meter. At 1 or 48 h after addition of cells to the microwells, the culture medium was replaced with fresh culture medium containing 1 μM MAR. After incubation for 24 h, the spheroids were dispersed using trypsin-EDTA (0.25% (w/v)) in PBS. The fluorescence intensity of the cells was measured using flow cytometry using a Gallios Flow Cytometer (Beckman Coulter, Miami, FL, U.S.A.), and the mean fluorescence intensity (MFI) was calculated. The data were analyzed using Kaluza software (version 1.0; Beckman Coulter).

Statistical Analysis Differences among multiple groups were statistically evaluated using one-way ANOVA followed by the Tukey–Kramer test. p -Values <0.05 were considered statistically significant.

RESULTS

CaO_2 -Containing PDMS Continuously Generates Oxygen in Water After water was added onto PDMS containing

Fig. 1. CaO₂-Containing PDMS Slowly Releases Oxygen

(A) O₂ release from PDMS containing 0% (black circle), 5% (white circle), 15% (triangle), or 25% (square) CaO₂ for 24 h. Results are expressed as the mean $\pm$ standard deviation of three independent experiments. (B) Repetitive oxygen release from PDMS containing 0, 5, 15, or 25% CaO₂ evaluated at 24, 48, and 72 h. Results are expressed as the mean $\pm$ standard deviation of three independent experiments. * p < 0.05 vs. 0%, † p < 0.05 vs. 5%, ‡ p < 0.05 vs. 15%.

Fig. 2. Microwells Formed Using PDMS Containing Different Amounts of CaO₂

(A) Images of O₂-generating microwells. (B) Sectional images of O₂-generating microwells. Scale bar = 100 μ m. (Color figure can be accessed in the online version.)

0, 5, 15, and 25% CaO₂, which had been cured at the bottom of the tubes, the O₂ concentration in the water was measured for 24 h (Fig. 1A). The O₂ concentration increased as the amount of CaO₂ increased. Next, to estimate ongoing O₂ release, the water was replaced every 24 h and the O₂ concentration was measured 24 h after each replacement (Fig. 1B). At all time points, the O₂ concentration increased as the amount of CaO₂ increased. Therefore, PDMS containing CaO₂ generated O₂ for at least 72 h after soaking in water.

Fabrication of O₂-Generating Microwells O₂-generating microwells were fabricated using a micro-molding technique.¹⁴⁾ Figure 2A shows images of the microwell circles of PDMS containing 0, 5, 15, and 25% CaO₂. The translucent PDMS became opaque upon the addition of CaO₂. Observation of sectional images confirmed that the O₂-generating microwells had microwell structures (Fig. 2B).

O₂-Generating Microwells Attenuate the Reduction in Oxygen Concentration during Fabrication of HepG2 Spheroids HepG2 cells were cultured in the O₂-generating microwells for 72 h. The O₂ concentration in the culture medium was measured every 24 h before the medium was changed (Fig. 3). Twenty-four hours after the start of incubation using conventional microwells (without CaO₂), the O₂ concentration

Fig. 3. O₂-Generating Microwells Prevent O₂ Decrease in the Culture Medium of HepG2 Spheroids

O₂ concentration in the culture medium of HepG2 spheroids at 24, 48, and 72 h. Results are expressed as the mean $\pm$ standard deviation of three independent experiments. * p < 0.05 vs. 0% (control).

in the culture medium decreased by 67% in the presence of spheroids compared to that in the cell-free group. In contrast, the O₂-generating microwells containing CaO₂ at 15 and 25% attenuated the reduction in O₂ concentration. At 48 and 72 h,

Fig. 4. Improvement in Hypoxic Core Region and Viability of HepG2 Spheroids Using O₂-Generating Microwells

(A) Images of HepG2 spheroids fabricated using O₂-generating microwells. Scale bars = 100 μm. (B) Diameter of HepG2 spheroids fabricated using O₂-generating microwells. Results are expressed as the mean ± standard deviation of one hundred measurements. (C) Number of live cells in HepG2 spheroids fabricated using O₂-generating microwells. Results are expressed as the mean ± standard deviation of five measurements. **p* < 0.05. (D) Mean fluorescence intensity of MAR (hypoxia-sensitive fluorescence probe)-treated HepG2 cells in spheroids fabricated using O₂-generating microwells. The fluorescence intensity was measured using flow cytometry. Results are expressed as the mean ± standard deviation of four independent experiments. **p* < 0.05 vs. 0% (control). (E) Fluorescence images of HepG2 spheroids fabricated using O₂-generating microwells. Live and dead cells were stained with CFSE (green) and propidium iodide (PI, red), respectively. Scale bar = 100 μm. (Color figure can be accessed in the online version.)

there was a tendency for an increase in oxygen concentration using the O₂-generating microwells, but the differences were not significant.

O₂-Generating Microwells Rescue Hypoxia in HepG2 Spheroids and Improve Cell Viability HepG2 spheroids were successfully prepared by culturing HepG2 cells in O₂-generating microwells for 72 h (Fig. 4A). The size of the spheroids increased as the amount of CaO₂ mixed with the microwells increased (Fig. 4B). Dispersion of the spheroids with 0.25% (w/v) trypsin–EDTA followed by trypan blue staining showed that the number of live cells in the HepG2 spheroids increased as the amount of CaO₂ mixed with the microwells increased (Fig. 4C). Next, the O₂ status in the HepG2 spheroids was determined using a hypoxia-sensitive probe MAR, which irreversibly becomes fluorescent under hypoxic conditions and is locked into cells.²²⁾ The MAR-treated HepG2 spheroids were dispersed, and the fluorescence intensity derived from MAR was measured using flow cytometry (Fig. 4D). The HepG2 cells in spheroids cultured for 1 d in O₂-generating microwells showed lower fluorescence intensity than those cultured in conventional microwells. The HepG2 cells in spheroids cultured for 3 d in O₂-generating microwells showed that the fluorescence intensity decreased as the

amount of CaO₂ mixed with microwell increased. However, graphical evidence showing the hypoxic status in the core region of the spheroids was not obtained because the converted MAR was leaked out from the cells during the optical clearing process. In addition, the live/dead staining of the optically cleared spheroids showed that some live cells were observed at the core region of the spheroids cultured using O₂-generating microwells, whereas few live cells were observed when cultured using control microwells (Fig. 4E). These results suggest that the O₂-generating microwells improve the hypoxic state and viability of the cells in the core region of the HepG2 spheroids.

O₂-Generating Microwells Improve Cell Viability of Spheroids Prepared Using Various Types of Cells To confirm whether the O₂-generating microwells are useful for preparing spheroids of cells other than HepG2, MIN6, B16–BL6, and ASC cells were used to prepare spheroids in the O₂-generating microwells. The MIN6 spheroids were successfully formed unless O₂-generating microwells containing 25% CaO₂ were used (Fig. 5A). MIN6 cells produced spheroids of the largest diameter (Fig. 5B) and containing the highest number of live cells when using O₂-generating microwells with 5% CaO₂ (Fig. 5C). The B16–BL6 spheroids were hardly formed

Fig. 5. Spheroid Size and Live Cell Number of Spheroids Increase Using Appropriate O₂-Generating Microwells

(A) Images of MIN6, B16-BL6, and ASC spheroids fabricated using O₂-generating microwells. Scale bars = 100 μm. (B) Diameter of MIN6, B16-BL6, and ASC spheroids fabricated using O₂-generating microwells. Results are expressed as the mean ± standard deviation of 100 measurements. **p* < 0.05 vs. 0% (control). (C) Number of live cells in MIN6, B16-BL6, and ASC spheroids fabricated using O₂-generating-microwells. Results are expressed as the mean ± standard deviation of five measurements. **p* < 0.05 vs. 0% (control).

when the O₂-generating microwells with 0, 5, 15% CaO₂ were used. However, the B16-BL6 spheroids with high cell viability were formed when the O₂-generating microwells containing 25% CaO₂ were used. For ASC cells, the diameter of the spheroids and content of live cells increased as the amount of CaO₂ in the microwells increased. These results suggest that O₂-generating microwells containing the proper amount of CaO₂ are useful for the fabrication of a variety of cell spheroids with high cell viability.

DISCUSSION

A fabrication technique is required to prepare highly functional multicellular spheroids for tissue models and cell transplantation. Spheroid functionality is closely related to the viability of the cells in spheroids. It is reasonable to speculate that the cell viability and engraftment rate after cell transplantation is correlated with the cell viability under *in vitro* conditions. In this study, we developed novel O₂-generating microwells, which rescued hypoxia-induced cell death in spheroids by continuously supplying O₂. In general, hypoxia in the core region of spheroids causes necrosis and decreases cellular function.^{16,17)} Moreover, when a large number of spheroids are prepared using microwell devices, the reduction in O₂ concentration in the culture medium caused by excess oxygen consumption worsens hypoxia in the spheroids (Fig. 3). The novel approach of this study showed that the O₂ supply to the spheroids using the O₂-generating microwells prevented the

reduction in O₂ concentration under culture conditions (Fig. 3) and rescued cell death in the spheroids (Fig. 4E). These results ensure that a greater number of viable cells can be transplanted when spheroids are prepared using the O₂-generating microwells. Coronel *et al.* reported that the cell viability and insulin secretion of rat islets after transplantation to diabetic rats were improved by culturing the islets on the CaO₂-containing PDMS discs.²⁰⁾ Therefore, we think it reasonable to speculate that the cell viability and transplantation rate of the spheroids prepared using the O₂-generating microwells are higher than those prepared using the conventional microwells. Thus, the O₂-generating microwells developed in this study can accelerate studies using multicellular spheroids, including those using *in vivo*-like tissue models and cell transplantation.

Various approaches to rescue cell death in spheroids have been reported. We and other groups have reported that the incorporation of gelatin microspheres into spheroids increases oxygen permeability and inhibits cell death in the spheroids.^{23–25)} However, these approaches could not solve the problem of decreasing O₂ concentration under culture conditions. Other groups have used a bioreactor with a large volume of medium for the preparation of spheroids.²⁶⁾ However, this system could not control the spheroid size. Although the O₂-generating microwells would not be expected to increase O₂ permeability into the spheroids, hypoxia in the spheroids was improved by using these microwells (Fig. 4D). Perfusion systems were also used to supply O₂ for large-scale cell culture. In comparison to the perfusion systems, the O₂-generating

microwells are low in preparation cost, easy to use and require no specialized equipment. Therefore, O₂-generating microwells are expected to become a superior approach to minimize cell death in spheroids.

Cells in *in vivo* tissues are supplied with O₂ through blood vessels. On the other hand, cells in spheroids cultured in the O₂-generating microwells are supplied with O₂ from the surface of the spheroids. The difference in the direction of the O₂ supply may have some effects on cell phenotypes. The O₂ concentration in the core region of the spheroids could be limitedly increased by the O₂ supply from the surface of the spheroids. In this study, the O₂-generating microwells increased the O₂ concentration in the culture medium during HepG2 spheroids preparation and the viability of HepG2 cells in the core region of the spheroids was improved (Fig. 4). However, the O₂ concentration in the core region of spheroids would be lower than that of the peripheral region because O₂ was consumed by the cells in the peripheral region before O₂ reached to the core region. Therefore, in the hypoxic core region of the spheroids, the phenotypes of the cells could be different from those of the cells in *in vivo* tissues.

PDMS containing CaO₂ slowly and continuously generated O₂ for at least 3 d (Fig. 1). Pedraza *et al.* reported that a PDMS disc containing CaO₂ generated O₂ for 2 weeks.¹⁹⁾ PDMS is hydrophobic and CaO₂ in PDMS would have difficulty reacting with H₂O; this would be one reason for the slow and continuous O₂ generation. This system also inhibits the toxicity of CaO₂. H₂O₂, the intermediate compound during O₂ generation from CaO₂, is toxic to cells.²⁷⁾ However, the slow reaction between CaO₂ and H₂O prevented H₂O₂ accumulation in the culture medium because H₂O₂ is quickly decomposed before accumulation. The H₂O₂ concentration in the culture medium was less than 2.5 μM (data not shown), which would be barely toxic to HepG2 cells. Excess O₂ generation might also damage the cells in spheroids.²⁸⁾ However, the O₂ concentration showed no obvious increase when the O₂-generating microwells were used (Fig. 3). Therefore, O₂-generating microwells can be useful devices for the preparation of highly functional spheroids.

We prepared spheroids of four types of cells, namely, HepG2, MIN6, B16–BL6, and ASC (Figs. 4, 5). The optimal CaO₂ concentrations in the O₂-generating microwells were found to be dependent on the cell type. When using the O₂-generating microwells containing 25% CaO₂, the number of live MIN6 cells in the spheroids decreased, whereas the number of live HepG2, B16–BL6, and ASC cells increased. In particular, B16–BL6 spheroids were formed only when the O₂-generating microwells containing 25% CaO₂ were used. When the conventional microwells or the O₂-generating microwells containing 5 or 15% CaO₂ were used for the formation of B16–BL6 spheroids, the color of the culture medium changed from red to yellow after 24 h culture, indicating the pH of the medium decreased because of the accumulation of lactate. From these results, it was suggested that B16–BL6 cells performed anaerobic respiration because of the lack of O₂ and that the cells were under severe hypoxic conditions. The energy produced by the anaerobic respiration is not sufficient for the cells and the resulting low pH would be toxic to cells. Thus, B16–BL6 spheroids were not formed due to cell death under such conditions. On the other hand, the color of the culture medium remained red when the cells were cultured using

the O₂-generating microwells containing 25% CaO₂. These results suggest that a sufficient O₂ supply by the O₂-generating microwells containing 25% CaO₂ prevented severe hypoxia-induced B16–BL6 cell death. On the other hand, hyperoxia produces reactive oxygen species in mitochondria, and they modulate multiple redox-sensitive cellular functions, including apoptosis, survival responses and cellular growth.^{29,30)} In this study, the local O₂ concentration at the interface of the microwells and the spheroids was not measured, but spheroids could possibly be exposed to hyperoxia when the O₂-generating microwells containing 25% CaO₂ were used. MIN6 spheroid could not be formed in the O₂-generating microwells containing 25% CaO₂ because of the toxicity of reactive oxygen species (ROS) produced under hyperoxic conditions. It was reported that hyperoxic exposure increased ROS production and apoptotic cells in rat islets.³¹⁾ Therefore, when spheroids are prepared using O₂-generating microwells, the optimal CaO₂ concentration is needed to be checked. The CaO₂ concentration in O₂-generating microwells can arbitrarily be adjusted, and the concentration could be optimized depending on cell types in future studies.

In general, hypoxia-induced cell death occurs in the core region of spheroids. However, dead or dying cells stained with propidium iodide (PI) or PI and CFSE, respectively, were found not only in the core region, but also in the peripheral region of HepG2 spheroids (Fig. 4E). The limited nutrient supply as well as excessive O₂ production near the surface of the O₂-generating microwells could cause cells death even in the peripheral region of the spheroids. Most HepG2 spheroids were observed in hemispherical shapes, but not in symmetrical spherical shapes. It can be speculated from these shapes that HepG2 cells settled down to the bottom of the microwells and the basal surface of the spheroids attached directly to the microwell surface. PDMS is a hydrophobic polymer, and the nutrients in the medium are hardly supplied from the basal side of the spheroids. In addition, the local O₂ concentration would be very high on the surface of the O₂-generating microwells, and this high O₂ concentration would result in the production of reactive oxygen species, which could induce cell death even in the peripheral region of the spheroids. Therefore, the results suggest that some cells in the peripheral region of the spheroids underwent cell death.

In this study, we fabricated O₂-generating microwells with PDMS containing CaO₂. The O₂-generating microwells prevented the decrease in O₂ concentration in the culture medium and rescued cell death in the spheroids. O₂-generating microwells can be useful for fabricating highly functional spheroids.

Conflict of Interest The authors declare no conflict of interest.

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