



Research article

A simple method to improve the quality and yield of human pluripotent stem cell-derived cerebral organoids

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ARTICLE INFO

Keywords:

Human pluripotent stem cell
Cerebral organoids
Necrosis
Mechanical cutting

ABSTRACT

The development of cerebral organoid technology has allowed the human neural tissue to be collected for studying human brain development and neurological diseases. Human pluripotent stem cell-derived cerebral organoids (hCOs) are a theoretically infinite source of fresh human brain tissue for various research purposes. However, hCOs have limitations, including core necrotic cell death. To solve this problem, we tested a simple method, which has been previously overlooked. In this study, we mechanically cut 70-day-old hCOs with a scalpel blade into 2 to 4 pieces, each depending on their original size. After culturing cut hCOs for additional 7 days, their size was less variable and smaller than uncut hCOs and there were no histological differences between uncut and cut hCOs. Note that hypoxia-inducible factor (HIF)–1 α was expressed in the central area of uncut hCOs but not in cut hCOs. Uncut hCOs, therefore, showed broad core areas stained with terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL), whereas cut hCOs did not. In conclusion, this simple mechanical cutting method allowed us to acquire a larger number of hCOs without a necrotic core.

1. Introduction

Human brain development and neurological diseases have traditionally been studied using two-dimensional (2D) cell culture or animal models. Although these models have contributed significantly to these research fields, they also have some limitations. For example, 2D cell culture models do not fully reflect the essential physiology or histology of real tissues and the genetic, physiologic, and histological differences between animals and humans [1, 2]. These limitations can make it difficult to directly apply research results to humans. So, there is a need for models that reflect human characteristics more closely. The recent development of human pluripotent stem cell-derived cerebral organoid (hCO) technology provides a valuable *in vitro* model for human brain development or neurological disease [3]. hCOs form three-dimensional (3D) self-organizing brain tissue samples and are histologically and functionally similar to the human brain, allowing them to overcome the limitations of 2D cell culture or animal models.

However, hCOs also have several limitations, including the formation of necrosis at their inner core when they increase beyond a certain size.

This problem is mainly due to the restriction of the supply of oxygen and nutrients within hCOs [4]. hCOs can also grow to a maximum of about 4mm [5]. In the absence of vascularization, as they increase in size, the permeability of oxygen or nutrients into hCOs decreases. Attempts have been made to solve this problem by modifying culture conditions or engineering approaches to deliver oxygen, nutrients, or signaling molecules deeper into the organoids. Some studies have used bioreactors or microfluidics to help drive the flow of media and distribute nutrients to organoids and these techniques help generate larger and more mature organoids [6, 7]. Other studies have tried growing organoids directly on a microfluidic channel with endothelial cells or signaling molecule carriers to facilitate angiogenesis [8, 9]. Similarly, another study combined tissue-specific cells with mesenchymal cells to generate vascularized organoids [10]. Therefore, to further overcome the limitations of *in vitro* systems, a recent study developed a method to transplant hCOs into adult mice brains to induce vascularization [11]. All these techniques reduce necrotic damage within hCOs by providing oxygen or nutrients through direct diffusion or via blood vessels. Moreover, vascularization can make hCOs to more precisely mimic the *in vivo* brain anatomy and physiology.

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Received 8 February 2021; Received in revised form 7 May 2021; Accepted 15 June 2021

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hCO vascularization is also appropriate to study cerebrovascular structure or function, but in settings requiring only healthy hCOs without necrosis, the problem can be solved more simply. Here, we show a simple method to generate a larger number of hCOs without a necrotic core.

2. Materials and methods

2.1. Human embryonic stem cell culture

A human embryonic stem cell (hESC) line (SNUhES31) was obtained from the Institute of Reproductive Medicine and Population, Medical Research Center, Seoul National University Hospital, South Korea. hESCs were cultured on mouse embryonic fibroblasts treated with 10 $\mu\text{g}/\text{mL}$ mitomycin-C (Roche, Mannheim, Germany). After, these cells were maintained in an hESC medium (20% knockout serum replacement [Life Technologies, Carlsbad, CA, USA], 1% minimum essential media-essential amino acids [MEM-NEAA], Life Technologies, Carlsbad, CA, USA], 1% Glutamax [Life Technologies, Carlsbad, CA, USA], and 7 $\mu\text{L}/\text{L}$ β -mercaptoethanol [Sigma-Aldrich, St. Louis, MO, USA] in DMEM/F-12) with 20 ng/mL basic fibroblast growth factor (bFGF, R&D Systems, Minneapolis, MN, USA). For the feeder-free hESC culture, cells were detached from the feeder cells using 1 mg/mL dispase (Life Technologies, Carlsbad, CA, USA). After, cells were cultured in an Essential-8 medium (Life Technologies, Carlsbad, CA, USA) on Geltrex (Life Technologies, Carlsbad, CA, USA) coated culture plates. Cells were then subcultured as small clusters every 4 days in 0.5 mM EDTA.

2.2. Generation of human pluripotent stem cell-derived cerebral organoids

hCOs were generated from hESCs using a modified protocol [12]. Incubation was then used to briefly dissociate the feeder-free cultured hESCs into single cells by incubation with 0.5 mM EDTA for 4 min. The cells were then incubated with Accutase (Life Technologies, Carlsbad, CA, USA) for 4 min. Next, the cells were plated in 96-well U-bottom ultralow-attachment (ULA) plates (Corning, New York, NY, USA). A density of 9×10^4 cells/ cm^2 in hESC medium containing 5 ng/ml bFGF, 50 μM Y27632, and a Rho-associated protein kinase inhibitor (Tocris Bioscience, Bristol, UK) was used. The day of plating was defined as day 0. On day 2, the medium was exchanged with hESC medium containing 5

ng/mL bFGF and 50 μM Y27632. On day 4, the medium was exchanged to an hESC medium without bFGF or Y27632. On day 6, each embryoid body (EB) was then transferred to a 24-well ULA plate containing 500 μL of a neural induction medium (1% N2 supplement [Life Technologies, Carlsbad, CA, USA], 1% Glutamax [Life Technologies, Carlsbad, CA, USA], MEM-NEAA, and 1 $\mu\text{g}/\text{mL}$ heparin [Sigma-Aldrich, St. Louis, MO, USA] in DMEM/F-12). On day 8, the EBs were fed 500 μL fresh neural induction medium. On day 10, neuroepithelial tissues were transferred to 20 μL matrigel (BD Bioscience, Franklin Lakes, NJ, USA) droplets on a sheet of Parafilm with 3 mm dimples and incubated at 37°C for 1 h. The matrigel droplets were then moved into a 60-mm tissue culture dish (Corning, New York, NY, USA) containing 5 mL of the CO medium without vitamin A (0.5% N2 supplement, B27 supplement minus vitamin A [Life Technologies, Carlsbad, CA, USA], 2.5 $\mu\text{g}/\text{mL}$ human insulin [Roche, Mannheim, Germany], 1% Glutamax, 0.5% MEM-NEAA, and 3.5 $\mu\text{L}/\text{L}$ β -mercaptoethanol in 1:1 mixture of DMEM/F-12 and neurobasal medium [Life Technologies, Carlsbad, CA, USA]). Afterward, the medium was changed every other day. On day 14, the matrigel droplets containing CO were transferred to a 125 mL-sized spinner flask with a CO medium (0.5% N2 supplement, B27 supplement [Life Technologies, Carlsbad, CA, USA], 2.5 $\mu\text{g}/\text{mL}$ human insulin 1%, Glutamax, 0.5% MEM-NEAA, and 3.5 $\mu\text{L}/\text{L}$ β -mercaptoethanol in a 1:1 mixture of DMEM/F-12 and a neurobasal medium). The medium was changed every 7 days. The size was measured using scale bar under the microscope. The circularity was analyzed using Image J software.

2.3. Immunofluorescence staining

COs were fixed in 4% paraformaldehyde at 4°C for 2 h and sequentially immersed in 15% and 30% sucrose solutions, respectively, until the samples sank. Samples were then embedded in an OCT compound (Leica, Wetzlar, Germany) and cryosectioned at 20 μm using a Leica CM1850 cryostat (Leica, Wetzlar, Germany). For immunofluorescence staining, sections were permeabilized and blocked with PBST (0.1% vol/vol Triton X-100 [Sigma-Aldrich, St. Louis, MO, USA] in phosphate-buffered saline [PBS]) containing 10% normal goat serum (Vector Laboratories, Burlingame, CA, USA) at room temperature for 1 h. Next, sections were incubated overnight with primary antibodies in PBST containing 2% normal goat serum at 4°C. Primary antibodies used were as follows: anti-

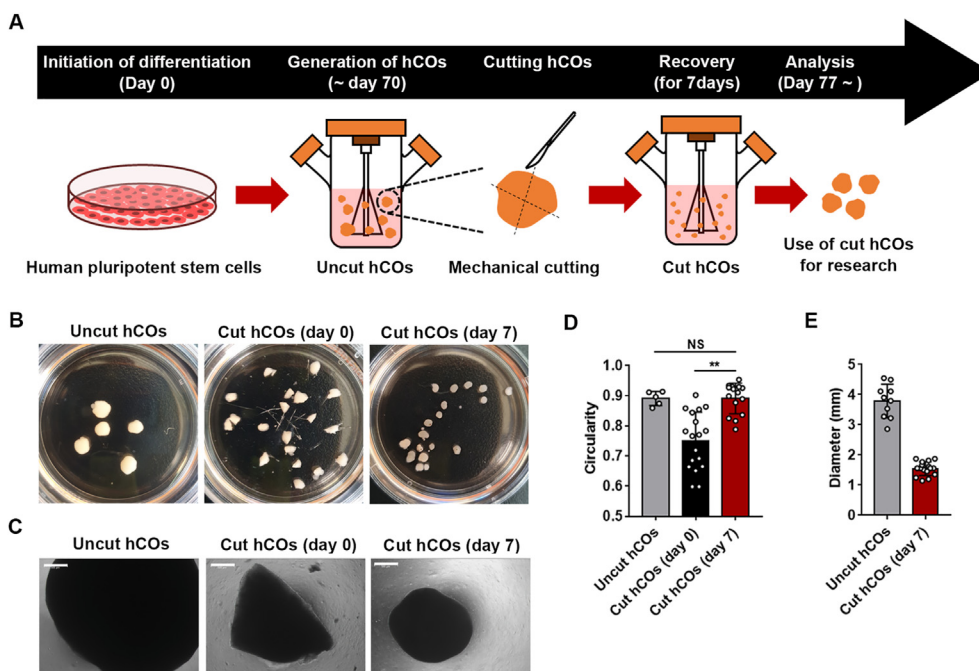


Figure 1. Mechanical cutting of human pluripotent stem cell-derived cerebral organoids (hCOs) (A) Schematic diagram demonstrating mechanical cutting of hCOs. (B) Macroscopic and (C) microscopic images of uncut hCOs and cut hCOs immediately after cutting (day 0) and 7 days after the cutting process. Scale bar: 500 μm . (D) The circularity of uncut and cut hCOs 0 and 7 days after cutting. Values are expressed as the mean \pm S.E.M. (E) Diameters of uncut and cut hCOs 7 days after cutting. Values are expressed as the mean \pm S.E.M.

neuron-specific class III beta-tubulin 1 (TUJ1, mouse, 1:200, R&D systems MAB1195, Minneapolis, MN, USA), anti-sex-determining region Y-box 2 (SOX2, rabbit, 1:100, Cell Signaling 3579, Danvers, MA, USA), anti-T-box brain protein 2 (TBR2, mouse, 1:100, R&D systems MAB6166, Minneapolis, MN, USA), and anti-doublecortin (DCX, rabbit, 1:100, Cell Signaling,

Diego, CA, USA), anti-N-cadherin (rabbit, 1:100, Santa Cruz sc-7939, Dallas, TX, USA), and anti-hypoxia-inducible factor 1- α (HIF1- α ; rabbit, 1:200, Santa Cruz sc-10790, Dallas, TX, USA) was also used. After washing three times with PBST, sections were incubated with secondary antibodies (donkey Alexa Fluor 488 and 568 conjugates [1:500, Life Technologies, Carlsbad, CA, USA]) at room temperature for 3h. After washing three times with PBST, coverslips were mounted on slides using the Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and imaged using a confocal microscope (TCS SP5 II, Leica, Wetzlar, Germany).

2.4. Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining

hCO specimens were fixed in 4% paraformaldehyde at 4°C for 2 h and embedded in a Paraplast wax, followed by the preparation of 4 μ m thick tissue sections. After, we conducted TUNEL staining using an ApopTag *in situ* apoptosis detection kit (Chemicon International, Temecula, CA, USA) according to the manufacturer's protocol. The tissue was then briefly removed from paraffin, washed with PBS (pH 7.6), and treated in 3% H₂O₂ for 30 min at room temperature to inactivate the tissue's endogenous peroxidase. After treatment with the kit's equilibration buffer for 15 min, TdT was then added and incubated for another 90 min at 37°C. Tissue sections were also incubated using the kit's reaction stop buffer at room temperature for 30 min, after which sections were incubated in anti-digoxigenin-peroxidase for 30 min at room temperature, then the color was developed using a diaminobenzidine solution, and the sections were washed with distilled water. Images were obtained using a Panoramic Desk II slide scanner (3DHISTECH Ltd., Budapest, Hungary) and analyzed using Panoramic Viewer software.

2.5. Statistical analyses

The results were reported as means \pm standard error of the mean (S.E.M.). Differences between mean values were analyzed using Student's *t*-test. A *p* < 0.05 was considered significant.

3. Results

3.1. Mechanical cutting of hCOs

We took out 70-day-old hCOs from a spinner flask and mechanically cut them with a sterilized scalpel blade in a Petri dish (Figure 1A). Next, we put back and cultured the cut hCOs in a spinner flask for an additional 7 days for recovery before analysis. To size the hCOs as evenly as possible, they were divided into two to four parts according to their sizes before cutting. When normal round-shaped hCOs were cut into a sharp polygonal shape, the normal round shape was restored after the 7-day recovery period without any manipulation (Figures 1B and 1C). The difference of the circularity was also not significant between uncut and cut hCO 7 days after cutting (Figure 1D). The size of uncut and cut hCOs was 3.79 ± 0.18 mm and 1.55 ± 0.057 mm, respectively (Figure 1E), showing that the size of the hCOs was reduced by cutting. However, the standard error value was also decreased. Taken together, mechanical cutting increased uniformity in size and the number of available hCOs.

3.2. Histological characterization of uncut and cut hCOs

To compare the histological characteristics of uncut and cut hCOs, we examined the expression of several neural markers expressed in hCOs

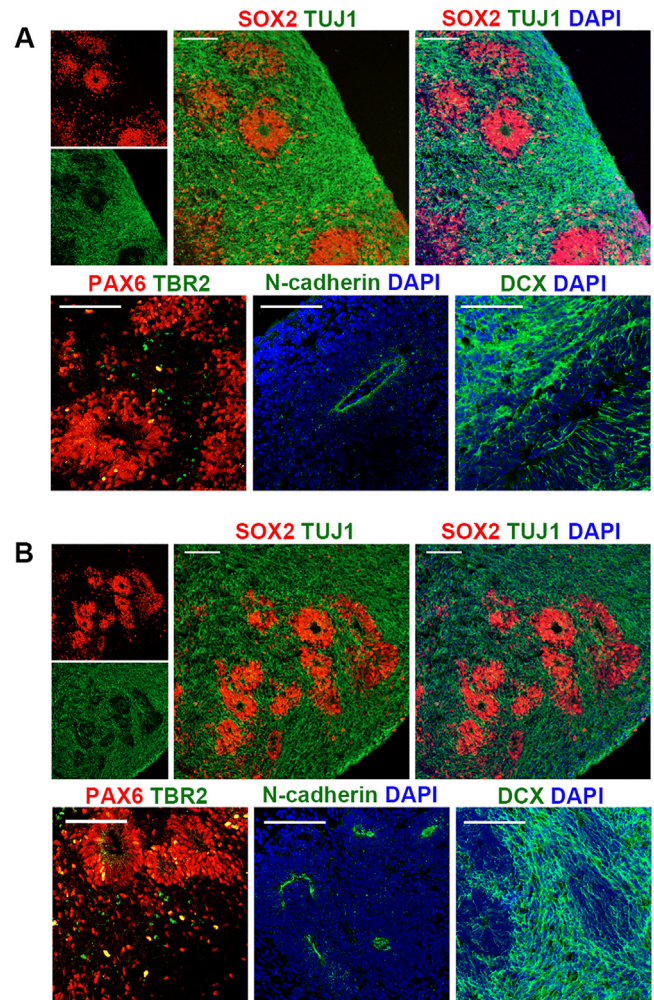


Figure 2. Histological characterization of uncut and cut human pluripotent stem cell-derived cerebral organoids (hCOs) Histologic characterization of (A) uncut and (B) cut hCOs conducted using immunohistochemical staining. SOX2, sex-determining region Y-box 2 (neural progenitor marker, red), TUJ1, neuron-specific class III beta-tubulin 1 (neuronal marker, green), PAX6, paired box 6 (radial glial cell marker, red), TBR2, T-box brain protein 2 (intermediate progenitor cell marker, green), neuronal-specific N-cadherin (green), DCX, doublecortin (neuronal marker, green). Nuclei were stained with DAPI (4',6'-diamidino-2'-phenylindole). Scale bar:100 μ m.

using immunohistochemical staining. In both uncut and cut hCOs, SOX2 (a neural progenitor marker), TUJ1 (a neuronal marker), PAX6 (a radial glial stem cell marker), TBR2 (an intermediate progenitor marker), neural-specific N-cadherin, and DCX (a neuronal marker) were consistently expressed (Figures 2A and 2B) as previously reported [3]. Also, there were no cellular or microscopic structural differences between uncut and cut hCOs. Overall, cutting hCOs induced no histological changes except for a decrease in size.

3.3. Effect of mechanical cutting in the formation of the necrotic core of hCOs

Due to the increase in the size of hCOs by long-term culture, the diffusion of oxygen into hCOs becomes difficult, and this difficulty causes necrosis in their core [13, 14]. Therefore, to confirm the hypoxic state inside the hCOs, we examined the expression of HIF1- α , a marker of hypoxia. Figure 3A shows that HIF1- α was expressed in the core part of uncut hCOs, but not in cut hCOs, suggesting that oxygen penetration was achieved in cut hCOs. Also, to confirm whether neuronal cell death in the core of hCOs was prevented by mechanical cutting, we compared the

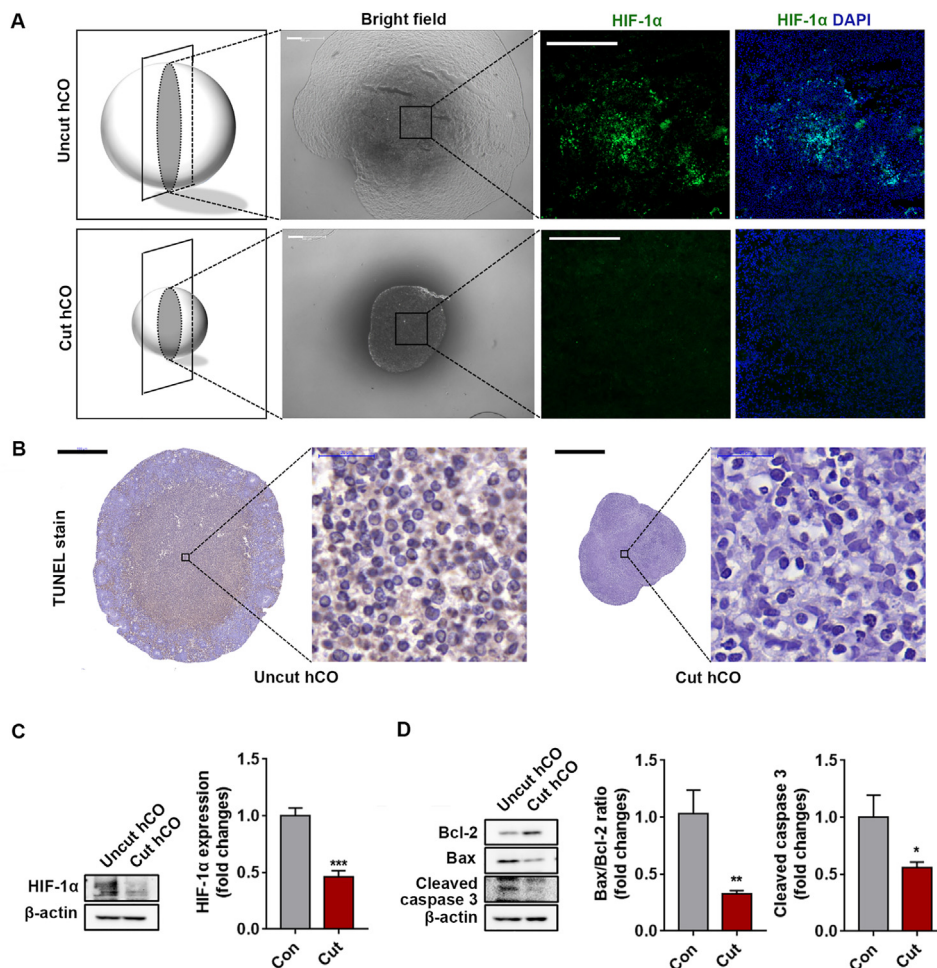


Figure 3. Effect of mechanical cutting on the necrotic core of human pluripotent stem cell-derived cerebral organoids (hCOs) (A) Hypoxia-inducible factor 1- α (HIF1- α), Green) detected with Immunohistochemical staining in uncut and cut hCOs. Nuclei were stained with DAPI (4',6-diamidino-2'-phenylindole). Scale bar, 200 μ m. (B) Representative images of the transferase-mediated dUTP nick end-labeling (TUNEL) staining of tissue sections for uncut and cut hCOs. Scale bar: 500 μ m. (C, D) Tissue lysates of uncut and cut hCOs were subjected to Western blotting using HIF-1 α , Bcl-2, Bax, and cleaved caspase-3 antibodies, respectively. Intensified HIF-1 α , Bcl-2, Bax, and cleaved caspase-3 were determined using densitometry and expressed relative to β -actin, and the Bax:Bcl-2 ratio was calculated. Uncropped blot images were provided as supplementary figure 1 and 2. Values are expressed as the mean \pm S.E.M. of three independent experiments. * p < 0.05; ** p < 0.01; *** p < 0.005 vs. uncut hCOs.

normal and cut hCOs using TUNEL staining. As shown in [Figure 3B](#), uncut hCOs had a wide area of TUNEL positivity, whereas cut hCOs had none. To quantify these results, we examined protein levels coding for hypoxic stress and cell death. As shown in [Figure 3C](#), HIF-1 α expression was significantly decreased in cut hCOs than in uncut hCOs ([Supplementary figure 1](#)). Also, because cell death under conditions of oxygen deprivation was regulated by Bcl-2 family proteins, and caspase-3 activation induces this process, we examined the expression levels of cell death-related proteins. Bax, a pro-apoptotic Bcl-2 family protein, Bcl-2, an anti-apoptotic Bcl-2 family protein, and cleaved caspase-3 were examined [[15](#), [16](#)]. Our data showed that Bcl-2 expression was significantly increased in cut hCOs, while the expression levels of Bax, as well as Bax:Bcl-2 ratio and cleaved caspase-3 were significantly decreased ([Figure 3D](#), [Supplementary figure 2](#)). Overall, mechanical cutting prevented the necrotic core formation by hypoxia in hCOs.

4. Discussion

Organoids, including hCOs, have great potential in enabling the study of human organogenesis and development. Such studies were not possible until recently, primarily due to difficulty in accessing human embryonic material [[17](#), [18](#)]. Organoids can also be used to investigate human-specific disease mechanisms. Also, the use of disease-specific organoids will facilitate the analysis of molecular mechanisms behind the disease, the identification of potentially novel biomarkers, and the development of platforms for drug testing or toxicology studies [[17](#), [19](#), [20](#)]. Furthermore, organoids open up enormous possibilities as a source

for cell therapies and as a potential alternative for whole-organ transplantation [[21](#), [22](#)].

Therefore, to successfully use organoids, keeping them in optimal conditions is fundamental. The lower the quality of organoids, the lower the experimental reliability and scope of use for the organoids. It is also established that the absence of vascularization restricts the supply of oxygen and nutrients deep into organoids by causing core necrosis when the volume of organoids expands beyond the limit of perfusion, which lowers the quality of organoids as an experimental model by limiting their potential for development, maturation, and so on [[13](#)]. Thus, various methods to grow hCOs that use particular devices, cells, or by transplanting them into the brain of experimental animals for vascularization or reduction of necrosis have previously been reported [[6](#), [7](#), [8](#), [9](#), [10](#), [11](#)]. These methods have great experimental value but are complicated or laborious.

Also, because one of the simplest methods to supply oxygen and nutrients to the core of hCOs is to make physical contact between the inside of hCO and the culture medium, in this study, we simply cut hCOs and continued their growth. Our results showed a necrotic core in uncut hCOs that was unobserved in cut hCOs. We expect that during the recovery period after cutting, necrotic cells in the hCO core are dispersed through the truncated section by spinning forces during the culture of cut hCOs in the spinner flask. During this recovery period as well, the cut hCOs were spontaneously recovered to a normal round shape. Cutting hCOs therefore reduced their size but caused no other histological changes, thus increasing their number. Similarly, because the generation of hCOs requires long culture periods and high-cost supplements, a simple method

to increase the number of hCOs indirectly reduces the cost and time required to produce hCOs. This simple cutting process improves the quality and number of this valuable material.

The mechanical cutting of hCOs is underestimated due to its methodological simplicity, but in this study, we have shown its practical usefulness and value. To our knowledge, this is the first report of such a method and this study will contribute to improving the utilization of hCOs and the quality of research results.

Declarations

Author contribution statement

Mu Seog Choe: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Seung Taek Oh, So Jin Kim, Chang Min Bae, Won-Young Choi: Performed the experiments; Analyzed and interpreted the data.

Kyung Min Baek: Contributed reagents, materials, analysis tools or data.

Joong Sun Kim: Performed the experiments; Contributed reagents, materials, analysis tools or data.

Min Young Lee: Conceived and designed the experiments; Wrote the paper.

Funding statement

This work was supported by NRF grants (2018R1D1A1A02044017; 2020R1A5A2017323) and the 4th BK21 project (ERGID) (5199990614732) funded by the Korea Government.

Data availability statement

Data included in article/supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at <https://doi.org/10.1016/j.heliyon.2021.e07350>.

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