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Spheroidal formation preserves human stem cells for prolonged time under ambient conditions for facile storage and transportation

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ABSTRACT

Human stem cells are vulnerable to unfavorable conditions, and their transportation relies on costly and inconvenient cryopreservation. We report here that human mesenchymal stem cells (MSC) in spheroids survived ambient conditions (AC) many days longer than in monolayer. Under AC, the viability of MSC in spheroids remained >90% even after seven days, whereas MSC in monolayer mostly died fast. AC-exposed MSC spheroids, after recovery under normal monolayer culture conditions with controlled carbon dioxide and humidity contents, resumed typical morphology and proliferation, and retained differentiating and immunosuppressive capabilities. RNA-sequencing and other assays demonstrate that reduced cell metabolism and proliferation correlates to the enhanced survival of AC-exposed MSC in spheroids versus monolayer. Moreover, AC-exposed MSC, when injected as either single cells or spheroids, retained therapeutic effects *in vivo* in mouse colitis models. Spheroidal formation also prolonged survival and sustained pluripotency of human embryonic stem cells kept under AC. Therefore, this work offers an alternative and relatively simple method termed spheropreservation versus the conventional method cryopreservation. It shall remarkably simplify long-distance transportation of stem cells of these and probably also other types within temperature-mild areas, and facilitate therapeutic application of MSC as spheroids without further processing.

Mammalian cell culture is generally conducted in humidified incubators with strict conditions including 37 °C and with 5% CO_2, 20% O_2, and routine medium refreshments. Deviations from this standard environment can alter cellular functions and even cause abnormalities and/or death of cells. Human embryonic stem cells (hESC), induced pluripotent stem cells (iPSC), and their progenies are even more vulnerable to suboptimal culture conditions. They easily differentiate, develop aneuploidy, detach or die when any of the standard parameters changes.

Many cell types can be stored only for a short time (1–2 days) in a refrigerator or under ambient condition (AC), *i.e.*, in a sealed vessel filled with corresponding culture medium without refreshments, lacking standard levels of CO_2 and O_2 for mammalian cell culture, and placed at room temperature between 20 °C and 25 °C. Cell viability declines dramatically even after a short storage under these unfavorable conditions, especially AC [1,2]. Thus, cryopreservation is needed for long-term storage and long-distance transportation of cells by preserving cell viability [3], imposing a major limitation for stem cell research and therapeutic applications. In spite of its inconvenience and high cost, cryopreservation has long been taken as the default method with little attempts, if any, to change or simplify it. Another scenario that often involves cell handling under AC is three-dimensional (3D) bio-printing, which prints cells (often in dissociated cell suspension) and supporting materials into biocompatible tissue blocks for application in regenerative medicine, however reduced cell viability is also a major challenge during printing and subsequent transplantation [4].

In 2009, Sakai et al., reported that enzymatically fabricated and degradable microcapsules can be used for production of spheroids from the human hepatoma HepG2 cells with well-defined diameters [5]. Recently, Swioklo, et al., further demonstrated that encapsulation of human adipose-derived stem cells in 1.2% calcium alginate can improve their viability in hypothermic (4 °C–23 °C) conditions for 72 h [6]. However, encapsulation demands additional procedures for optimization when used for stem cell therapy, which may reduce the efficiency and increase the cost [7]. It has been known that spheroidal formation by somatic tissue-derived

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MSC improves cell survival, while increasing differentiation potentials and delaying replicative senescence of the cells in normothermia [8]. Studies have also shown that low to moderately low temperature (hypothermia) protects stemness and suppress apoptosis in neural stem cells and MSC via cold-inducible RNA-binding proteins [9,10].

Here we hypothesized that stem cells cultured in spheroids may tolerate hypothermic conditions such as room temperature more than controls cultured in monolayer. We first tested this hypothesis on mesenchymal stem cells (MSC) derived from hESC or bone marrow and found that spheroidal formation could retain MSC in very high viability under AC or in an extended temperature range (10 °C–37 °C) even after 7–9 days, when monolayer MSC were mostly dead. High viability was also preserved with hESC when stored as spheroids under AC for 4 days. The cytoprotective effect of spheroidal formation is associated with reduced cell metabolism, proliferation, and apoptosis, as revealed by RNA sequencing (RNA-seq) and other assays.

MSC have proved efficacious in treatment of many autoimmune, inflammatory, and degenerative diseases in animal models and clinical trials [11]. Remarkably, we found that AC-exposed MSC, when injected as either single cells or spheroids, retained therapeutic effects on mouse colitis models. Our findings thus have important practical implications for maintaining and working with stem cells under AC, which may serve as an alternative method for their large-scale and long-distance transportation for research and clinical applications.

1. Results

1.1. Spheroidal formation prolongs MSC survival under AC

CT3 hESC [12] were induced to differentiate into MSC (named EMSC) using the method we reported recently [13]. EMSC were then dissociated and allowed to form spheroids ($EMSC_{Sp}$) at different sizes from various numbers of cells/spheroid using the hanging drop method [14] (Fig. S1A). 48 h later, multiple $EMSC_{Sp}$, equivalent of 2×10^6 cells were collected and put into a 1.5-ml centrifuge tube containing 1.5-ml MSC medium, and the tube was placed on a laboratory bench under AC ($EMSC_{Sp\text{-}AC}$) for 7 days ($EMSC_{Sp\text{-}AC/D7}$). As a control, EMSC cultured in monolayer ($EMSC_{ML}$) in a 6-well plate sealed with parafilm and covered with aluminum foil were also placed under AC ($EMSC_{ML\text{-}AC}$) for 7 days ($EMSC_{ML\text{-}AC/D7}$) (Fig. 1A). Sibling EMSC ($EMSC_{sibling}$) were used as a control, which were continuously cultured in monolayer under normal conditions, *i.e.*, in an incubator at 37 °C with 5% CO_2 and 20% O_2 without spheroidal formation and AC exposure. The same definition for sibling controls is used hereafter. $EMSC_{ML\text{-}AC}$ cells quickly shrank and many detached by day 7, which are indications of cell death. In contrast, $EMSC_{Sp\text{-}AC}$ spheroids remained intact and healthy without obvious morphological changes throughout the entire 7 days (Fig. 1A). Histological analysis of sectioned spheroids shows no obvious difference between $EMSC_{Sp\text{-}AC/D7}$ and the control spheroids cultured under the normal conditions, and both spheroids were intact with higher cell density in the periphery than the inner core of the spheroids (Fig. 1B). $EMSC_{Sp\text{-}AC/D7}$ spheroids were dissociated and re-plated for post-AC analyses. The cells reattached well, formed a monolayer (named $EMSC_{Sp\text{-}AC/D7\text{-}ML}$) on gelatin-coated plates under the normal conditions, and demonstrated typical MSC morphology (Fig. 1A). Similar results were obtained with EMSC in spheroids versus monolayer on a horizontal rocker at 20 rounds per min. (to mimic movements during transportation) under AC (data not shown).

Through acridine orange (AO, which labels live cells green) and propidium iodide (PI, which labels dead cells red) assay, we found that almost all of the cells dissociated from the 7-day AC-exposed spheroids ($EMSC_{Sp\text{-}AC/D7}$) were stained green, whereas most of the 7-day AC-exposed monolayer cells ($EMSC_{ML\text{-}AC/D7}$) stained red (Fig. 1A). Quantification of live (AO^+) cells using Cellometer demonstrated that 91.5% of the $EMSC_{Sp\text{-}AC/D7}$ cells were still alive, as compared to 4.4% of viability for the monolayer EMSC under the same conditions ($EMSC_{ML\text{-}AC/D7}$) (Fig. 1C). As another control, dissociated EMSC also died quickly under AC with only 2.7% AO^+ cells left by day 7 ($EMSC_{DISSOC\text{-}AC/D7}$) (Fig. 1C). Even after 9 days under AC, we found that EMSC in spheroids ($EMSC_{Sp\text{-}AC/D9}$) still retained more than 60% of viability (Fig. 1C).

To address the potential effect of cell density per tube, we added different numbers of $EMSC_{Sp}$ into a tube to achieve three densities, *i.e.*, 1 million, 6 million, and 12 millions cells per tube, and stored the spheroids under AC for 3 or 7 days. The cell survival rate was similar among the three groups by day 3, but slightly decreased at the two higher densities by day 7 (Fig. S1B). This indicates that the density of 2 million cells/tube we used throughout the study is within the optimal cell density range. To test whether or not $EMSC_{Sp}$ could tolerate temperatures beyond AC, we stored $EMSC_{Sp}$ at 10, 17, 25, and 37 °C and observed only slight decrease in the rate of survived $EMSC_{Sp}$ cells at 10 °C (Fig. S1B).

Next we tested whether the spheroid-based preservation, which we termed "spheropreservation", can also apply to human bone marrow-derived MSC (BMSC). Indeed, 87.8% of cells dissociated from the spheroids $BMSC_{Sp\text{-}AC/D7}$ were alive (AO^+), whereas almost all cells from the monolayer $BMSC_{ML\text{-}AC/D7}$ were dead (AO^-) (Fig. 1C). Further, we found that 6.1% of cells dissociated from the spheroids $EMSC_{Sp\text{-}AC/D7}$ and 82.4% of cells from the monolayer $EMSC_{ML\text{-}AC/D4}$ were double positive for PI and Annexin V (an apoptotic marker), which are features of late apoptotic and/or necrotic cells (Fig. 1D). Like $EMSC_{sibling}$ in normal monolayer culture, cells dissociated from the spheroids $BMSC_{Sp\text{-}AC/D7}$ also attached well and formed a monolayer with typical MSC morphology under the normal conditions and could be expanded for further analyses (data not shown). Thus, spheropreservation also prolongs the survival of BMSC stored under AC. Similar results were observed with EMSC derived from other hESC lines H9 and Envy [15] (data not shown). Taken together, these data suggest that spheropreservation can reduce cell death on MSC exposed to AC.

We compared the survival rate of $EMSC_{Sp\text{-}AC/D7}$ and $EMSC_{ML\text{-}AC/D7}$ with EMSC thawed after conventional cryopreservation (*e.g.* frozen as cell suspension in liquid nitrogen for 7 days). As expected, EMSC stored in monolayer couldn't tolerate AC for so long. On the other hand, spheropreservation and cryopreservation achieved similar survival rates (90% or higher) from the same number of EMSC (Fig. 1E). This suggests that spheropreservation is as good as (if not better than) cryopreservation for MSC storage within 7 days, and much more convenient and economic than cryopreservation for long-distance transplantation of MSC.

1.2. AC-recovered MSC retains MSC features at the morphological and cellular levels

In order for the AC-exposed MSC to be useful for research and clinical applications, it is critical to assure that exposure to AC does not compromise MSC features. To this end, we determined whether MSC following spheropreservation still retain the MSC features. Indeed, immunostaining on frozen sections of $EMSC_{Sp\text{-}AC/D7}$ and $BMSC_{Sp\text{-}AC/D7}$ spheroids demonstrates that both samples were still highly positive for the MSC markers CD44 and CD90 (Fig. 2A). Further, after re-plating from the spheroids and culture under normal conditions, both $EMSC_{Sp\text{-}AC/D7\text{-}ML}$ and $BMSC_{Sp\text{-}AC/D7\text{-}ML}$ cells also expressed MSC markers CD44, CD73, CD90, and CD105, but not the negative control cocktail of hematopoietic markers (Fig. S2A).