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Reversible growth arrest of 3D tumor spheroids stored in oxygen absorber-induced anoxia

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Abstract. Multicellular tumor spheroids models are of increasing interest in preclinical studies and pharmacological evaluation. However, their storage and transport is often a limitation because it requires adapted and expensive procedures. Here, we propose a very simple method to store 3D spheroids, using a procedure based on oxygen absorber-induced anoxia. We report that oxygen absorbers allow generating an anoxic environment for spheroid storage in culture plates. Oxygen absorber-induced anoxia fully and reversibly arrests spheroid growth for 4 days at 37°C and up to 18 days at 4°C. We then show that the response to etoposide is comparable in spheroids preserved in conditions of absorber-induced anoxia at 4°C and spheroids kept in normoxia at 37°C. These results represent a major improvement that should simplify the storage, transport and use of 3D spheroids.

Introduction

To reproduce the three-dimensional (3D) organization and the cell-cell and cell-matrix features that are found in normal or tumor tissues, cells can be cultured as 3D aggregates, also called spheroids. Over the last decade, spheroids have been recognized as essential 3D culture models for high-throughput screening and pharmacological evaluation (1). They are also of utmost interest in the field of tissue engineering because they represent basic bricks that can be used to generate original cell assemblages and organization, and to produce larger tissues (2).

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Spheroids derived from primary cells or immortalized cancer cell lines are often made using the classical hanging drop or centrifugation methods (3,4). Although these techniques are robust and their variability is acceptably low, there are potential reproducibility issues linked to various classical experimental problems and batch-to-batch variability. Storage in liquid nitrogen, in the presence of a cryoprotectant, has been used for brain cell and hepatocyte spheroids with successful preservation of morphological markers and functionality (5,6). However, most researchers rely on repeated custom production, depending on the need. Alternatively, spheroids can be ordered from companies that deliver standardized and perfectly controlled biological material, usually shipped in conditions that maintain the microtissues at 37˚C during transit. In such case, temperature stabilization and shipping efficiency become critical issues.

Several reports have shown that spheroid growth results in the generation of a hypoxia and nutrient gradients (7-9) where cells localized in the inner region exit the cell cycle and enter a G0 quiescent state. The induced cell quiescence in the spheroid central region mimics the situation observed *in vivo* in microtumor domains and the subsequent resistance to classical chemotherapeutic agents (10,11). Moreover, we recently demonstrated that oxygen partial pressure is a rate-limiting parameter for cell proliferation in 3D spheroids (12). Thus, we hypothesized that culturing spheroids in anoxic conditions could be an efficient way to induce their reversible growth arrest. In order to make the procedure as simple and inexpensive as possible, we investigated whether oxygen absorbers could be used to generate an anoxic environment for microtissues. Oxygen absorbers can efficiently reduce oxygen concentration to less than 0.0001%. Consequently, their composition and packaging has been adapted for a very large range of applications (13). Currently, oxygen absorbers are used to preserve many food products (bread, meat, fish and seafood, fruits, nuts, cheese) from food spoilage due to aerobic microorganism proliferation and to prevent fat oxidation. They are also used in the pharmaceutical industry to improve molecule protection and safety. Oxygen absorbers are also employed to generate oxygen-free environments to control and eradicate museum insect pest and to preserve art works (14,15).

Here, we report that in oxygen absorber-induced anoxia, spheroid growth can be reversibly stopped at 4˚C for up to 18 days. Moreover, after anoxic storage, they can be successfully used in pharmacological assays. This oxygen absorber-based method to reversibly stop cell proliferation could represent a tremendous advance in the field of 3D microtissue engineering with obvious immediate applications for spheroid storage and shipment.

Materials and methods

Cell culture. HCT116 colon adenocarcinoma cells (ATCC) were cultured in DMEM (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% foetal calf serum (FCS), 2 mM/l glutamine and penicillin/streptomycin in a tissue culture incubator (humidified atmosphere of 5% CO_2 at 37˚C). Spheroids were prepared as previously described(9). Briefly, 500 cells/well were distributed in ultra-low attachment 96-round bottom well plates. After centrifugation at 200 g for 6 min, plates were placed in the tissue culture incubator. After 3 days, each well contained a single spheroid. The spheroid maximal area was determined by automated measurement with the High Content Screening ArrayScan Cellomics® platform (Thermo Fischer Scientific Inc.).

Packaging with oxygen absorbers and oxygen concentration measurement. For anoxic storage, each 96-well plate was placed in an ATCO Biocult P plastic bag with or without (control) an ATCO® Biosystem 96P oxygen absorber (Fig. 1A). The bag was immediately heat-sealed and placed in the tissue culture incubator (storage at 37˚C) or in a cold room (storage at 4˚C). The ATCO® Biosystem 96P oxygen absorbers used in this study were specifically designed for 96-well plates. The residual oxygen concentration in the bag was determined using a Servomex 570A oximeter (accuracy in the range of ± 0.1%; Servomex, Norwood, MA, USA). The oximeter probe was inserted in the sealed bag in an airtight manner and was positioned either outside or inside the 96-well plate. The residual oxygen level was calculated as the mean of at least three measurements at each time point. At the end of the storage period in anoxic conditions, bags were opened and plates returned to the tissue culture incubator.

Pharmacological evaluation. Spheroids were prepared in normoxia and then stored at 4˚C in oxygen absorber-induced hypoxia (0% oxygen) for 4, 7 or 14 days and then transferred to normoxia (21% oxygen at 37˚C) for 24 h. This recovery time was chosen in order to use spheroids of approximately 350-400 μm in diameter to allow comparison with controls (spheroids of similar size cultured in normoxia). To test their response to etoposide, 100 μl of culture medium per well was mixed with 100 μl of culture medium containing etoposide (Sigma-Aldrich, St. Louis, MO, USA). In each plate, six different concentrations were obtained by serial dilution (4-6 wells/spheroids for each concentration). Spheroid size was determined with the High Content Screening ArrayScan Cellomics® platform after 72 h-incubation with etoposide. The half maximal inhibitory concentration (IC_{50}) was calculated using the Prism® software.

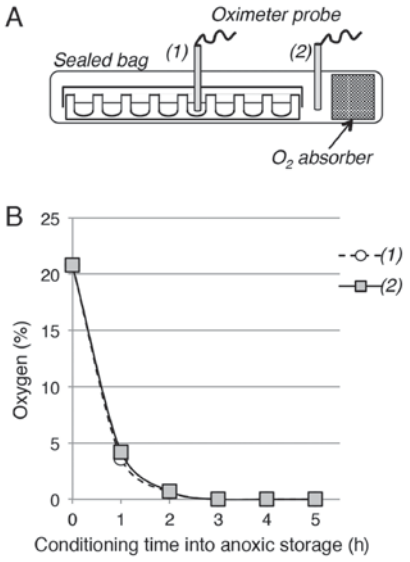

Figure 1. Oxygen absorbers to create an anoxic environment for 96-well plates. (A) Schematic representation of the experimental setting. The oxygen absorber ATCO Biosystem 96P is placed with a 96-well plate inside a plastic bag that is heat-sealed. The oximeter needle probe is positioned inside (1) or outside (2) the plate. (B) Residual oxygen concentration inside (1) and outside the plate (2) at different time points after the bag was sealed.

Results and Discussion

Use of an oxygen absorber to generate an anoxic environment for 96-well plates. Multi-well plates are the most commonly used cell culture disposable material to produce and transfer 3D spheroids. To analyse whether oxygen absorbers could be used to generate a hypoxic environment for spheroids cultured in 96-well plates, the oxygen concentration was measured with an oximeter needle probe positioned either on one side or in the middle of the 96-well plate packaged with an oxygen absorber in a heat-sealed plastic bag (Fig. 1A). The oxygen concentration measured inside the bag and in the plate rapidly decreased from 20.8% to ~4% after 1 h, to 0.7% after 2 h and nearly to 0% after 3 h (Fig. 1B). This indicates that this very simple experimental setting allows the fast and efficient removal of oxygen to generate an anoxic environment compatible with cell culture disposable material, such as 96-well plates.

Oxygen absorber-induced anoxia reversibly arrests spheroid growth at 37˚C. The effect of absorber-induced anoxia on spheroid proliferation was then analysed with the experimental setting depicted in Fig. 2A that was used for all the experiments of this study. After 3 days in normoxia at 37˚C, each 96-well plate that contained one HCT116 colon adenocarcinoma spheroid per well was placed, with or without (control) one ATCO Biosystem 96P oxygen absorber, in a plastic bag that was heat-sealed and put back in the tissue culture incubator for 4 or 11 days. Visual inspection under an inverted microscope of spheroids after return to normoxic conditions (Fig. 2B, upper and lower panels, respectively) showed that control spheroids (no oxygen absorber) kept growing during storage and after removal from the bag. Conversely, growth of spheroids packed with the oxygen absorber was completely inhibited by anoxia. Upon return to normoxia, spheroid growth resumption was observed only after anoxic storage for 4 days,

Table I. Determination of the IC_{50} for etoposide in spheroids stored in anoxic conditions.

	IC_{50} (μM)	
Anoxic storage duration	Control	Anoxia at 4˚C
4 days	1.4	2.3
7 days	2.1	2.4
14 days	1.6	1.7

Spheroids were prepared in normoxia and then stored at 4˚C in oxygen absorber-induced hypoxia (0% oxygen) or in normoxia (21% oxygen at 37˚C; controls) for 4, 7 or 14 days. Spheroid volume was determined at increasing concentratzions of etoposide (4 to 6 spheroids for each concentration). Curve fitting allowed calculating the IC_{50}. IC_{50}, half maximal inhibitory concentration.

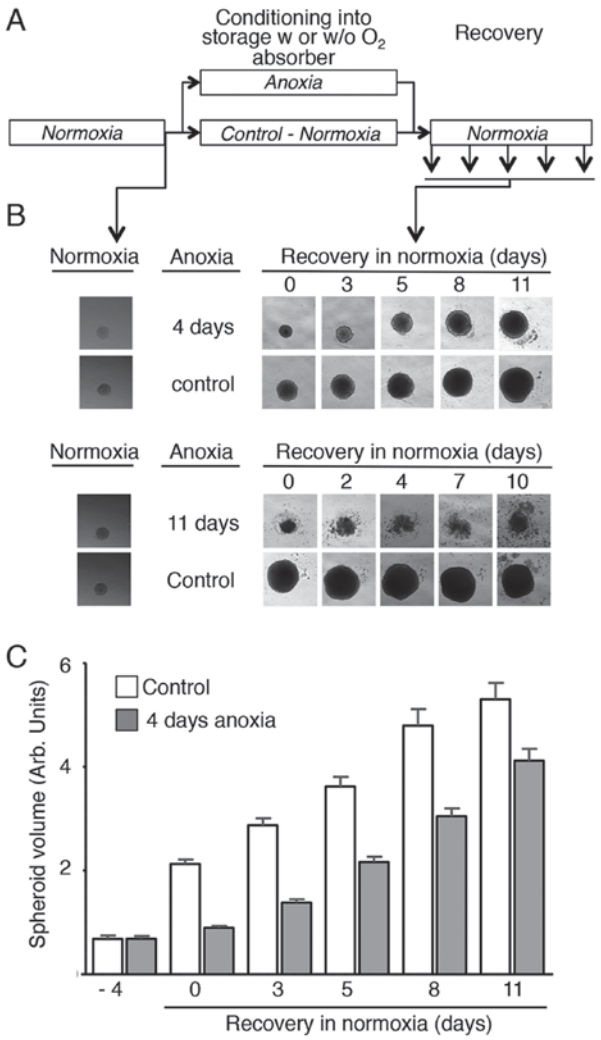

Figure 2. Reversible growth arrest after absorber-induced anoxia for 4 days at 37˚C. (A) Schematic representation of the experiments. Spheroids were prepared and grown initially in normoxia, then stored at 37˚C in the presence or absence (control) of an oxygen absorber to generate hypoxia for 4 or 11 days. Then, spheroids were allowed to recover at 37˚C in normoxia. (B) Representative micrographs illustrating the effect of anoxia at 37˚C for 4 and 11 days on spheroid growth. (C) Spheroid volume determination before (-4), after 4 days of anoxia at 37˚ (0) and during recovery. Data are the mean (± SD) of two independent experiments with one plate of spheroid (i.e., 60 spheroids) for each time point.

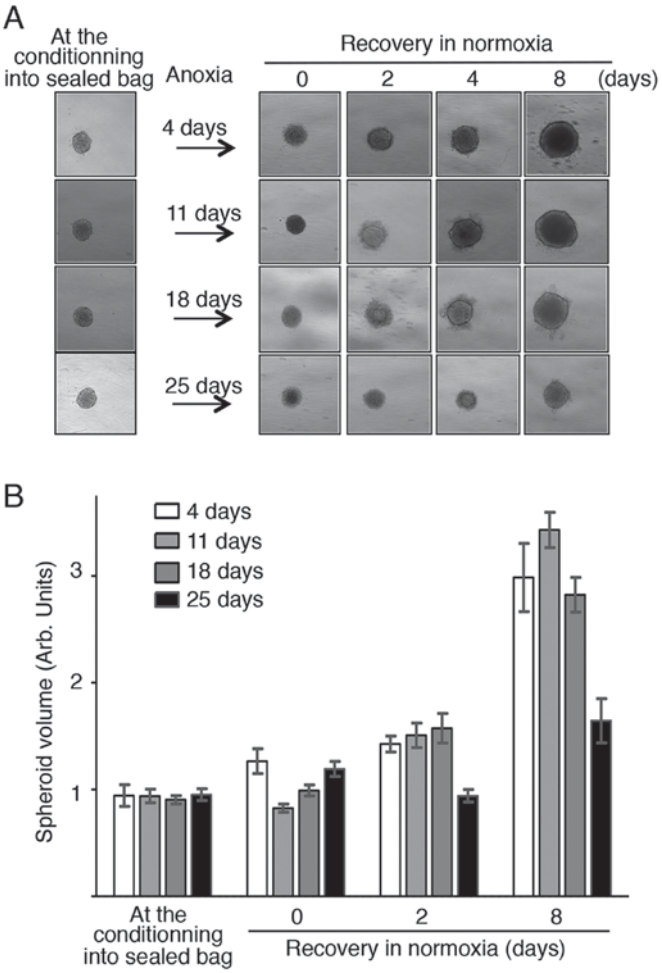

Figure 3. Reversible growth arrest in oxygen absorber-induced anoxia at 4˚C. (A) Representative micrographs illustrating the effect of anoxia at 4˚C for 4, 11, 18 and 25 days on spheroid growth and during recovery at 37˚C in normoxia. (B) Spheroid volume determination before (at packaging), after anoxic storage at 4˚C for 4, 11, 18 and 25 days (0) and during recovery. Data are the mean (± SD) of 50-60 spheroids per determination.

but not for 11 days (Fig. 2B). Indeed, spheroids kept in anoxic conditions for 11 days, rapidly dissociated and did not resume growth upon return to normoxia (Fig. 2B, lower panels). Quantification of the spheroid volume (60 spheroids/condition for each time point) confirmed that growth of spheroids stored at 37˚C in anoxia for 4 days was totally inhibited during the storage period, but proliferation resumed once back to normoxia, with a slope similar to controls (Fig. 2C).

Together, these observations indicate that when placed at 37˚C in an anoxic environment obtained with an ATCO Biosystem 96P oxygen absorber, spheroid proliferation is arrested. Back to normoxia, proliferation resumes in the same way as for control spheroids.

Spheroid growth is reversibly arrested by storage in oxygen absorber-induced anoxia at 4˚C up to 18 days. Although it may be of interest to slow down or stop microtissue proliferation at 37˚C for a short period, the storage and transport of such biological material would be more easily performed in standardized refrigerated conditions. Therefore, the ability of spheroids to resume proliferation after storage in oxygen absorber-induced anoxia at 4˚C was assessed using

the same experimental set-up (Fig. 2A). Plates were kept in anoxia, or not (controls), at 4˚C for 4, 11, 18 and 25 days, before returning to normoxia at 37˚C for recovery. Similarly to the results obtained at 37˚C, oxygen absorber-induced anoxia led to growth arrest without loss of structure integrity or structure changes (Fig. 3A). Growth was resumed after return to normoxia, although it was less fast in spheroids stored in anoxia at 4˚C for 18 and 25 days compared with up to 11 days. On the other hand, spheroids stored at 4˚C without oxygen absorber could not resume growth after return to normal culture conditions (data not shown). Quantification of the spheroid volume (50-60 spheroids/condition per time point) at different time points after return to normoxia (Fig. 3B) confirmed growth resumption after storage at 4˚C for up to 18 days.

These results indicate that storage of spheroids at 4˚C in an anoxic environment obtained with an ATCO Biosystem 96P oxygen absorber leads to fully reversible growth arrest (up to 18 days of anoxia), which is technically very easy to achieve.

Spheroid storage in oxygen absorber-induced anoxia does not modify the response to etoposide. To definitively confirm that oxygen absorber-induced anoxia may represent a major advance for spheroid storage, spheroid preservation was evaluated by assessing their response to etoposide, a DNA polymerase inhibitor currently used in the clinic for cancer treatment. To this aim, spheroids stored with the oxygen absorber at 4˚C for 4, 7 or 14 days were allowed to recover in normoxia for 24 h before incubation with increasing concentrations of etoposide for 72 h. Growth inhibition caused by etoposide cytotoxic effect was comparable in stored spheroids and in controls (spheroids of similar diameter at the time of treatment grown in normoxia), with similar IC_{50} values (Table I). Thus, storage of spheroids in anoxia at 4˚C for 4, 7 or 14 days does not modify their response to a reference genotoxic agent.

In this study, we investigate whether anoxia generated by using ATCO Biosystem 96 P oxygen absorbers represents a valid method for the storage and shipment of 3D tumor spheroids. We found that by simply packing a 96-well plate in a heat-sealed plastic bag that contains an oxygen absorber, total anoxia can be generated in about 2 h. Moreover, we show that in conditions of total anoxia, spheroid growth is fully stopped and that proliferation can be resumed after up to 4 days of storage in anoxia at 37˚C.

Mammalian cells are in principle unable to survive in hypoxic conditions because the unbalance between the decreasing ATP supply and the demand to ensure homeostasis progressively leads to mitochondria dysfunction and cell death. However, adaptive molecular responses allow a hypometabolic response that transiently prevents cell death (16). Recent work has shown that in acidic conditions, hypoxia can promote tumor cell survival by preserving the ATP level (17). Furthermore, a study on pancreatic islet conservation prior to transplantation demonstrated that storage at low temperatures prevent cell damage associated with hypoxia and may improve transplantation efficiency (18). In line with these reports, here we found that lowering the temperature to 4˚C offers the possibility to fully resume spheroid growth after up to 18 days of anoxia. Although further work is needed to validate this storage method in other cancer cell lines our results already open a real and major opportunity for spheroid storage, functional preservation and shipment.

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