Influence of the Hypercapnic Tumor Microenvironment on the Viability of Hela Cells Screened by a CO₂-Gradient-Generating Device

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ABSTRACT: Carbon dioxide (CO₂) levels outside of the physiological range are frequently encountered in the tumor microenvironment and laparoscopic pneumoperitoneum during clinical cancer therapy. Controversies exist regarding the biological effects of hypercapnia on tumor proliferation and metastasis concerning time frame, CO₂ concentration, and cell type. Traditional control of gaseous microenvironments for cell growth is conducted using culture chambers that allow for a single gas concentration at a time. In the present paper, Hela cells were studied for their response to varying levels of CO₂ in an aerogel-based gas gradient-generating apparatus capable of delivering a stable and quantitative linear CO₂ profile in spatial and temporal domains. Cells cultured in the standard 96-well plate sandwiched in between the device were interfaced with the gas gradient generator, and the cells in each row were exposed to a known level of CO₂ accordingly. Both the ratiometric pH indicator and theoretical modeling have confirmed the efficient mass transport of CO₂ through the air-permeable aerogel monolith in a short period of time. Tumor cell behaviors in various hypercapnic microenvironments with gradient CO₂ concentrations ranging from 12 to 89% were determined in terms of viability, morphology, and mitochondrial metabolism under acute exposure for 3 h and over a longer cultivation period for up to 72 h. A significant reduction in cell viability was noticed with increasing CO₂ concentration and incubation time, which was closely associated with intracellular acidification and elevated cellular level of reactive oxygen species. Our modular device demonstrated full adaptability to the standard culture systems and high-throughput instruments, which provide the potential for simultaneously screening the responses of cells under tunable gaseous microenvironments.

INTRODUCTION

As a primary byproduct of oxidative metabolism, carbon dioxide (CO₂) is an important physiological gas, of which transport and elimination become clinically crucial in regulating physiologic pH and maintaining homeostasis. The precise control of CO₂ concentration is critical for maintaining the in vitro mammalian cell or tissue culture. Most incubator chambers matched the CO₂ concentration with physiological conditions at a set point, typically 5%, to maintain a constant pH of 7.2−7.5. Aside from its physiological significance, in standard medical practice, such as laparoscopic pneumoperitoneum, endoscopy, and arthroscopy, CO₂ is also frequently used as an insufflation gas to expand body cavities to provide a better visual field of the surgical area for treating cancer, thus increasing the chance of tumor cell exposure to increased CO₂ concentration. Pathophysiological high levels of CO₂ have been reported to be closely associated with the tumor microenvironment. Earlier research had shown that excess CO₂ production is responsible for the acidification of tumor tissues and increased pCO₂ values (59−84 mmHg) have been found in solid tumors. However, the biological effects of elevated CO₂ levels on cancer cells remained controversial. For example, an earlier in vitro study demonstrated that when CO₂ concentration changes from 5 to 20%, it could enhance the invasive capacity of colon cancer cells, and a 10% CO₂ environment would lead to an increasing colony formation and proliferation rate in both MIA-PaCa-2 and Panc-1 cell lines than the 5% CO₂ culture condition. On the contrary, other studies have mentioned that chronic hypercarbia would lead to decreased cell growth, or incubation with high CO₂ concentration alone has no effect on cancer cells. Studies have also suggested that the hypercapnic tumor microenvironment may confer chemo-resistance to lung
cancer cells by reprogramming mitochondrial metabolism in vitro.7,9 Concerns also exist that pneumoperitoneum with the introduction of 100% CO2 in abdominal insufflation may be associated with tumor cell migration and invasion.10 It is worth mentioning that CO2 concentration and exposure time duration varied in these available reports, leading to controversial findings regarding the effects of hypercapnia on cells. Clearly, the dose-dependent influence of CO2 on modulating cellular behaviors remains a further study in the spatial and temporal domains.

Conventional methods for controlling CO2 levels in the cell culture environment are normally conducted using incubator chambers where one single gas concentration is supplied at a time. As the traditional approach can be extremely time and labor consuming, the improvement in throughput for rendering different CO2 concentrations simultaneously is highly favorable, which can benefit experimental investigations elucidating the cellular response to various gaseous micro-environments. Recently, versatile microfluidic devices have been developed with high-throughput and precise spatial control of gas concentration generation.11 In one example, on-chip control of pCO2 was achieved in a microfluidic cell culture system by flowing pre-equilibrated aqueous solutions through fabricated channels across the device.12 Taking advantage of the gas permeability of the polydimethylsiloxane (PDMS) material, PDMS-based gas diffusion has been involved in the microfluidic device for modulating the gas microenvironment for cell growth.13 An ultra-thin glass sheet
filter with micropores has previously been fabricated, which can control gas concentration by the distance from the holes in the film and thus improve the gas and nutrient permeability to the cell culture. However, besides the sophisticated architectural design and complicated fabrication process or the involvement of additional chemical reagents with potential cytotoxicity, these microfluidic devices hardly fit with existing instruments for high-throughput studies, such as the liquid handling system or the plate readers.

Eddington and co-workers recently developed a 3D-printed gas delivery device that adapts to the standard 96-well plate. In their design, a linear oxygen profile was produced by mixing two gases through distribution networks, and O2 of 12 quantized concentrations was equally delivered to the wells of each column. Without the need for specialized gas mixing network fabrication, we previously developed a modular device that can generate gradient CO2 at tunable concentrations for the full-factorial evaluation of the effects of multiple interacting environmental stressors on microalgae. Based on the efficient mass transport of CO2 through an air-permeable aerogel monolith, the assembled device can support the generation of the CO2 profile of a variety of gradients in a short period of time. Herein, we adopted this gas-gradient-generating apparatus for mammalian tumor cell culture and investigated the cellular response to varying levels of CO2 existing in the hypercapnic tumor microenvironment. Hela cells were seeded in the standard 96-well plate, and each row of cells was exposed to a distinct CO2 concentration in the device. The change of cell viability in response to the hypercapnic microenvironment under acute exposure or over a longer cultivation period was determined accordingly by screening multiple CO2 conditions in a single experimental setup.

## RESULTS

### CO2 Gradient Generation

Figure 1 shows the schematic and fabricated modular device consisting of top layering sheets of laser-cut acrylic with a pair of source-sink gas perfusion channels, a commercial 96-well plate, an air-permeable aerogel monolith, and a bottom plate. The individual picture of each component of the assembled device is provided in Figure S1.

Prior to the experiment, CO2 diffusion through the aerogel to the 96-well plate was numerically simulated by the finite element modeling using COMSOL Multiphysics. The boundary conditions used for COMSOL simulations are shown in Figure 2a. COMSOL simulations of the gas gradient generator show the equilibration of a spatial linear CO2 distribution, which was transversal to the gas flow direction (Figure 2b). As we have previously examined the time required for CO2 to reach diffusion equilibrium in the aerogel and its dependence on the gas transport properties of porous media (e.g., porosity and pore size), it took about 20 min in the current experimental setting to achieve a stable distributed CO2 profile (Figure 2c).

As the solution acidification upon CO2 absorption can be visualized by the color change of the dissolved pH indicating dye in the 96-well plate before and after CO2 gradient exposure over time. The color change from green to yellow-orange indicated the corresponding decrease of pH. (b) Color change in the 96-well plate loaded with cell culture medium containing phenol red. The color changed from pinkish-purple to orange-yellow with the decrease of pH.

![Figure 3](image-url)

Figure 3. (a) Color change of the dissolved pH indicating dye in the 96-well plate before and after CO2 gradient exposure over time. The color change from green to yellow-orange indicated the corresponding decrease of pH. (b) Color change in the 96-well plate loaded with cell culture medium containing phenol red. The color changed from pinkish-purple to orange-yellow with the decrease of pH.

![Figure 4](image-url)

Figure 4. (a) Extracellular pH measured in the supernatant medium in the 96-well plate from row A to row H and (b) time-dependent changes of intracellular pH in Hela cells after exposure to various CO2 concentrations. The dashed line indicates a pH value of 7.67 measured at 5% CO2.

As the solution acidification upon CO2 absorption can be visualized by the color change of the dissolved pH indicating components, the 96-well plate loaded with visible pH indicators was inserted into the device to characterize the established CO2 gradients. As shown in Figure 3a, the color change over time from green (pH ≈ 7) to yellow-orange (pH < 4.5), therefore, demonstrated that the spatial CO2 gradients could diffuse through the aerogel and subsequently result in gradient CO2 concentrations across the width of 96-well plate. The relation between color changes and pH values was previously determined by titration (data not shown). In the present study, we chose the full range of the gas concentration
of CO2 from 0.04% (0.01 mol/m3) to 98% (40.07 mol/m3) to demonstrate the utility of the device. The simulated and experimental results suggested that, with equal flow rates, 0.04% CO2 and 98% CO2 gas streams that entered the device could generate 8 discrete CO2 concentrations ranging from 12 to 89% starting from row A through row H with an interval of 11% in the 96-well plate. Wells within each row were remarkably consistent, as demonstrated by a low average standard deviation.

**Extracellular and Intracellular pH.** Phenol red, also known as phenolsulfonphthalein, has been used as a pH indicator dye in cell culture media, which exhibits a gradual transition from orange-yellow to pinkish-purple over a pH range of 6.2 − 8.2.\(^{18}\) Figure 3b demonstrates that a gradient change in color of cell culture media from row A to row H in the 96-well plate matched with the increasing concentration of dissolved CO2 over time. In good agreement with simulation results, the direct visualization of the color change in pH indicating solution and phenol-red-containing cell culture medium also showed that the gradient diffusion of CO2 in the device reached equilibrium in about 20 min. The gradient profile of extracellular pH was further verified in the bar graph shown in Figure 4a when plotting the pH value as a function of the position from row A to row H in the 96-well plate, as indicated in Figure 3. Experiments using the cell-permeant pH-sensitive fluorometric dye 2',7'-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) showed a gradual decreasing intracellular pH in Hela cells alone with increasing CO2 levels. Additionally, cumulative total amounts of CO2 evolved in lower pH as the exposure time extended from 3 to 24 h. Typically, after 24 h of exposure to the hypercapnia microenvironment, the intracellular pH value decreased from 5.46 at 12% CO2 to 4.91 at 89% CO2 (Figure 4b). Clearly, Hela cells were acidified in a dose- and time-dependent manner, and the intracellular pH significantly reduced as the extracellular pH decreased.

**Cellular Reactive Oxygen Species Production and O2 Consumption in Response to Elevated CO2 Levels.** Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assays were performed to determine the effects of elevated CO2 levels on Hela cells in terms of reactive oxygen species (ROS) production. As shown in Figure 5a, the level of cellular ROS significantly increased after 3 and 24 h of exposure in high CO2 conditions. These results are consistent with previous studies clearly demonstrating that exposing cells to extracellular acidosis could induce ROS formation.\(^{19,20}\) Meanwhile, a higher O2 consumption rate was noticed in high CO2-exposed samples (Figure 5b), pointing to an alteration in cell metabolism coexisting with low intracellular pH and enhanced ROS production in elevated CO2 conditions.

**Cell Viability in Response to Elevated CO2 Levels.** The impact of elevated CO2 levels on cell viability was analyzed using the MTT assay. As shown in Figure 6, the influence of high CO2 concentrations on cell growth demonstrated time and dose dependence. We confirmed a negative correlation between the cell viability and CO2 concentration by fitting a linear regression model to estimate the main effects of CO2 on cell growth (Figure 6a). Compared with the standard condition for normal cell culture (5% CO2), high CO2 concentrations significantly decreased the cell viability. Figure 6b shows an obvious decrease in cell viability along with the length of time exposure in the presence of elevated CO2 levels. Under the acute exposure within 3 h, elevated CO2 levels have not yet resulted in a significant change in cell viability. On the
contrary, chronic exposure to high CO₂ concentrations up to 72 h could lead to significantly decreased cell viability in Hela cells. The viability of cells grown under 89% CO₂ conditions drastically declined from 85.58 to 29.97% from 3 h to 72 h of exposure treatment, suggesting a cumulative effect of elevated CO₂ levels. Thanks to the convenience of our device for simultaneously providing multiple CO₂ concentrations at a time, cell growth in response to the hypercapnia microenvironment was also observed with its dependence on the CO₂ gradients. In the chronic treatment with longer cultivation time, more viable cells were found in the lower CO₂ conditions as compared to the higher CO₂ group (p < 0.05), and the lowest cell viability was always found in the groups exposed to the highest CO₂ condition (89%).

Morphological Changes in Hela Cells upon Exposure to CO₂ of Varying Levels. Figure 7 depicts representative bright-field images of Hela cells that have been exposed to various CO₂ conditions. It can be clearly observed that Hela cells in the control group were attached to the wall of the well plate, demonstrating typical leaf-like morphology. During the entire culture period, all cells were in good condition reaching 95% confluence at 72 h. However, the cells started to present triangular morphology without parapodium upon CO₂ exposure for 3 h. Afterward, some cells started to shrink, turned into spherical shapes, and split into smaller forms. The detached cells were observed in the culture medium suspension after 24 h upon high CO₂ treatment. The shrinkage and detachment of cells were aggravated with increasing incubation time and CO₂ concentration, as the most significant morphological alteration and cell detachment were observed in the cells treated with 89% CO₂ for 72 h.

At the end of each set time point after treatments in the device, the Hela cells were fixed and subjected to crystal violet staining for viability assessment. As crystal violet stains the cell nuclei a deep purple color by binding to DNA and proteins in cells, the stained cells directly inferred remaining attached cells. As shown in Figure 8, the crystal violet-stained results revealed a similar trend of viable cells as identified by MTT assay and cell morphological observation, demonstrating noticeable toxicities of the hypercapnic microenvironment. Hela cells remained viable under the normal culture condition (5% CO₂), while they displayed a significant reduction in cell adhesion and declined colony formation with increasing CO₂ concentration and incubation time.

**DISCUSSION**

Our experimental setup offered various CO₂ concentrations involving those applied in the pathophysiological hypercapnic microenvironment (12% CO₂) and those applied in clinical pneumoperitoneum (∼100% CO₂). Compared to other air-permeable materials such as PDMS, the diffusion of CO₂ across the aerogel could efficiently create a linear gas gradient profile in a short period of time to which the cells in the 96-well plate may be exposed. The observed CO₂ concentration profiles agreed well with the simulated results of linear gradients, allowing for precisely assigning a known level of CO₂ with a specific position. With the ease of integrating with standard cell culture well plates, our system provides a convenient way to control various gaseous microenvironments for cell growth or versatile chemistry measurement science. By simply adjusting the concentrations or the contents of the feed gases within the device, tunable gas gradients with known concentrations can be achieved for broad physiological or pathophysiological conditions.

The pH and CO₂ levels in the tumor microenvironment are two key variables that have major impacts on metabolic routes in cell growth. Based on our results, upon exposure to elevated CO₂ conditions, the excess CO₂ content not only resulted in the acidification of the extracellular microenvironment but also led to the decrease of the intracellular pH. Previous evidence in the literature has suggested that the intracellular acidosis induced by elevated CO₂ conditions could increase cellular ROS levels by triggering ROS generation, inhibiting ROS removal, or a combination of the two. Indeed, we have found significantly increased cellular...
ROS in Hela cells after exposure to high CO2 conditions as reflective of DCF fluorescence.

Regardless of its role in influencing extracellular and intracellular pH, CO2 participates in a variety of enzymatic reactions as a substrate and product and affects cellular functions.27 Several studies have previously suggested that elevated pCO2 conditions and subsequent accumulation of HCO3− could react increasingly with hydrogen peroxide, a key enzyme of glycolysis, to form the peroxymonocarbonate ion (HCO3−), which is an extremely ROS.23 The oxidative stress would be aggravated by the overproduction of ROS and consequently induce apoptosis and necrosis. The hypercapnic microenvironment is thus proven to be linked to cell death induction by decreasing intracellular pH and increasing cellular ROS levels side by side.

Moreover, as hypoxia and hypercapnia are often concurrent microenvironmental features during certain pathophysiological states,24 O2 consumption has often demonstrated an intimate relationship with CO2 concentrations. In our study, we have noticed a significantly enhanced O2 consumption rate in Hela cells after exposure to high CO2 conditions, and the intracellular hypoxia exacerbated with the increase of the CO2 concentration and incubation time. Recent works have proposed that the intracellular acidic conditions extant in hypercapnia would reduce the hypoxia-inducible factor 1−α (HIF−α) protein stabilization by facilitating the lysosomal degradation.25 As HIF−α is considered as the master transcriptional regulator of cellular and developmental response to hypoxia,26 the adaptive hypoxic response could thus be suppressed when high CO2 concentration exists. As intracellular acidosis may also deprive cells nutritionally as well, tumor growth may be inhibited along with diminished cell defensive response to the hypoxic stimuli. Taken together, although the mechanism by which elevated CO2 concentrations affect cellular functions still awaits final elucidation, hypercapnia appears to impair tumor cell proliferation at least in part by causing mitochondrial metabolism dysfunction or exerting suppressive effects on the HIF pathway, thereby inducing more oxidative stress.

In a previous study, Vohwinkel et al. reported that the chronic exposure of alveolar epithelial cells (AS49) to elevated CO2 levels for 3 days led to a slower proliferation rate of 25−30%, manifested by decreased ATP production associated with mitochondrial dysfunction.9 In their study, various CO2 levels were achieved by equilibrating the medium overnight in a humidified chamber. Zhu et al. have investigated the effects of elevated pCO2 levels on the growth of the Chinese hamster ovary (CHO) cells, and they found that an increase in the pCO2 level from 60 to 100 mmHg did not affect cell growth significantly, while the highest pCO2 level (150 mmHg) could suppress cell growth by about 9%.27 On the contrary, Nevler et al. reported an increased colony formation and proliferation rate in both MIA-PaCa-2 and Panc-1 cell lines, which were subjected to a 10% CO2 environment as compared to the 5% CO2 controls for up to 5 days.7 In another study, Hela cells were cultured with 8 and 16 mmHg of 100% CO2 stimulating the CO2 pneumoperitoneum environment in vitro for 1, 2, 3, and 4 h. In the 100% CO2 setting, the cells increased the ability of proliferation after a short time of inhibition and reduced the ability of invasion, migration, and adhesion accordingly.28 In our present study, thanks to the wide-ranged CO2 levels offered by our platform for observing the chronic response of cells in time course, to our interest, we have found that the hypercapnic microenvironment influenced cell proliferation in a time-dependent manner and also depended on CO2 concentrations. This finding suggests the importance of controlling the time frame and CO2 dosage in determining the effects of hypercapnia on cells, which could at least partially explain controversies that existed in the abovementioned studies.

### CONCLUSIONS

In the present study, we adopted a modular device for headspace delivery of multiple CO2 gradients to Hela cells in a standard 96-well plate based on efficient mass transport through an air-permeable aerogel. The cellular response to various elevated CO2 levels was simultaneously investigated in time course with increased throughput. Cell viability linearly decreased with the acidification of the microenvironment upon exposure to high CO2 concentrations, which was paralleled by increased levels of ROS production and exacerbated hypoxia conditions. The assembled device provides a convenient way to generate a full range of CO2 concentrations for screening cellular behavior or chemical reaction and is amenable to simulate different gaseous microenvironments in vitro by simply changing the feed gas concentration or composition, while still easily integrating with standard 96-well plates and high-throughput instruments.

### EXPERIMENTAL SECTION

**Device Design and Fabrication.** The design of the aerogel-based gas-gradient-generating apparatus was mainly followed by our previous study, where the experimental system was adopted for the full-factorial evaluation of the effects of microorganisms in response to multiple interacting environmental stressors.29,30 As shown in Figure 1, the device consisted of an aerogel monolith and a standard 96-well plate sandwiched between an acrylic layer with source and sink channels and an acrylic backplate. The acrylic plates were designed in AutoCAD and fabricated using laser engraving for the top layer and laser cutting for the bottom plate. The nanoporosity of aerogels allows equilibrium gas profiles dominated by diffusion via mass transport when the source and sink channels were supplied by two distinct feed gases. The well plates loaded with cell cultures were interfaced with the aerogel monolith by applying clamping force using 1/4-20 machine screws and wing nuts. The edges of the aerogel were sealed with polyethylene terephthalate tape to avoid gas leakage.

**COMSOL Theoretical Modeling.** The CO2 concentration gradients generated in the aerogel layer and the 96-well plate were numerically simulated using finite element analysis modeling software COMSOL Multiphysics in accordance with the geometry (15.2 cm × 8.8 cm × 1.0 cm) of the designed device. Figure 2a shows the boundary conditions of applied CO2 concentrations used for COMSOL simulations.

**System Setup and CO2 Gradient Validation.** Two gas streams, 98% CO2 (40.07 mol/m3)/0% O2 and 0.04% CO2 (0.01 mol/m3)/21% O2, both balanced with nitrogen, were delivered into the source and sink channel, respectively, through the inlets. All gas inputs were maintained at constant pressure using pressure regulators, and the gas flow meters were placed at the inlet and outlet of gas-flowing channels to prevent leakage. Prior to delivery to the device, the gas was hydrated by bubbling through sterilized deionized water to...
prevent evaporation. The CO₂ gradient generated in the device was confirmed with pH indicator tests using mixed bromothymol blue solution and methyl red solution. The ratiometric pH-sensitive dye solution was loaded 100 μL per well in the 96-well plate to directly visualize CO₂ of varying levels in terms of pH changes.

**Cell Culture and CO₂ Exposure.** Prior to the CO₂ exposure experiments, Hela cells were maintained in DMEM (Boster Biological Technology Co., Ltd.) containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 3.7 g/L sodium bicarbonate, 2 mM L-glutamine, and 4.5 g/L D-glucose in a humidified incubator (Thermo Fisher) at 37 °C and 5% (v/v) CO₂. The cells in the logarithmic growth phase were harvested and seeded in the 96-well plate at a density of 4 × 10⁵ cells/well and incubated for 24 h for attachment. The cells grown in the 96-well plate were inserted into the CO₂ gradient generating device and placed in the Live Cell Imaging System (OkoLab) with a constant temperature of 37 °C and 5% CO₂. The CO₂ gradient generating device and placed in the Live Cell Imaging System (OkoLab) with a constant temperature of 37 °C and 5% CO₂. Afterward, the medium was replaced with 100 μL of medium containing 0.5 mg/mL MTT and cells were incubated for 4 h at 37 °C and 5% CO₂. Afterward, the medium was replaced with 100 μL of DMSO to dissolve the formazan crystals, and the optical density (OD) of the solution was obtained by measuring the absorbance at 492 nm using a microplate reader (Molecular Devices). The cell viability was calculated as (OD_{control} − background)/(OD_{treated} − background) × 100%. The cells cultured at 37 °C in the 5% CO₂ incubator were set as the control. The data were obtained from three independent assays.

**Morphological Observation of Cells.** The morphological changes in Hela cells at different culture conditions were recorded using an inverted phase-contrast microscope (Leica). After 3, 24, 48, and 72 h of incubation, bright-field images were captured. Cells without any treatment were used as controls. Crystal violet stain assay (Beyotime Biotechnology, Shanghai, China) was carried out according to the manufacturers’ instructions. Cells were seeded in 96-well plates at 4 × 10⁴/mL for overnight incubation. After exposure to various CO₂ conditions in the device for a certain period of time, the culture medium was removed and the cells were fixed with 4% paraformaldehyde at room temperature for 20 min and then stained with 0.05% crystal violet for 15 min. Following washing with PBS three times, images of cells in each well were captured.

**Statistical Analysis.** Data are expressed as means ± standard errors of the means (SEM). Significant differences were statistically analyzed using Student’s t-test or ANOVA, as appropriate. If the results of the ANOVA were significant, the Tukey-Kramer test was used as a post hoc for multiple comparisons. A p-value < 0.05 was considered to indicate a statistically significant difference.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c04422.

Cross-sectional schematic diagram of the assembled device and top and bottom view of the individual components of the multilayer device (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes
The authors declare no competing financial interest.

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