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Expansion of Mesenchymal Stem Cells under Atmospheric Carbon Dioxide

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Abstract

Stem cells are needed for an increasing number of scientific applications, including both fundamental research and clinical disease treatment. To meet this rising demand, improved expansion methods to generate high quantities of high quality stem cells must be developed. Unfortunately, the bicarbonate buffering system – which relies upon an elevated CO₂ environment – typically used to maintain pH in stem cell cultures introduces several unnecessary limitations in bioreactor systems. In addition to artificially high dissolved CO₂ levels negatively affecting cell growth, but more importantly, the need to sparge CO₂ into the system complicates the ability to control culture parameters. This control is especially important for stem cells, whose behavior and phenotype is highly sensitive to changes in culture conditions such as dissolved oxygen and pH. As a first step, this study developed a buffer to support expansion of mesenchymal stem cells (MSC) under an atmospheric CO₂ environment in static cultures. MSC expanded under atmospheric CO₂ with this buffer achieved equivalent growth rates without adaptation compared to those grown in standard conditions and also maintained a stem cell phenotype, self-renewal properties, and the ability to differentiate into multiple lineages after expansion.

Keywords

Mesenchymal stem cell; Carbon dioxide independent medium; Bicarbonate buffer; Expansion

Introduction

Stem cells offer much potential for scientific applications. In addition to enabling insights into developmental biology, pathology, and toxicology, stem cells also show promise in treating human ailments such as spinal cord injuries, myocardial infarction, Parkinson's, type I diabetes, and sickle cell anemia.^{1–9} Many treatments require large quantities of stem cells, but currently there are no readily available sources that can meet these high demands.^{4,5,10–12} Consequently, *in vitro* expansion methods must be developed to generate

high numbers of stem cells.^{5,7,8,11,13–15} In addition to high quantities, maintaining high quality in stem cell yields is equally important to maximize treatment responses.^{7,14} The stem cell niche—consisting of factors such as oxygen tension, pH, and mechanical forces—influences the behavior of stem cells and dictates whether the stem cells will proliferate, differentiate, or undergo apoptosis.^{3,7,13,15–19} Learning how these various factors regulate stem cell fate *in vivo*, and then constructing systems that can replicate the desired conditions *in vitro* will be crucial.^{3,13} Through development of cost-efficient methods that allow maintenance of optimal culture parameters during *in vitro* expansion, it may be possible to more effectively direct stem cell yields and enhance the clinical success of stem cell-based therapies.^{9,11,13}

Stirred systems—in particular fed-batch, computer-controlled bioreactors—are capably equipped to deliver high quantities of high quality mammalian cells due to operational flexibility.^{4,6,12–15,20} Bioreactors, where CO₂ sparging is used for pH control, support the expansion of mammalian cells in suspension.^{4,21} Expansion of anchorage-dependent cells, such as mesenchymal stem cells (MSC), can also be achieved with the use of microcarriers in stirred bioreactors.^{7–9,22} Through dynamic monitoring and online control of parameters, stirred bioreactors can enable culture conditions to be maintained within a desired range.^{4,6,9,12,15,20} Additionally, bioreactors allow for scalability that is simply not possible with other culture formats, potentially paving the way for delivery of the large numbers of stem cells required for widespread clinical use.^{6,12,15} However, the bicarbonate buffering system used to regulate pH appears to be limiting the potential of stem cell expansion as it can hinder the ability of stirred bioreactors to provide a highly consistent culture pH environment.^{9,21,23} The bicarbonate buffering system relies on the equilibrium between the CO₂ gas sparged into the bioreactor and the dissolved CO₂ (dCO₂) in the medium, which is maintained well above physiological levels and can negatively affect cell growth and behavior.^{21,23–25} This situation is compounded by the production of CO₂ by metabolically active cells.²³ Previous efforts have been undertaken to remove excess dCO₂ from the bioreactor by adjusting the sparging profile; however, the coupling of the CO₂ supply to the oxygen supply complicates both dissolved oxygen and pH control.^{23,26} This mutual dependency leads to recurring cyclic fluctuations of both dissolved oxygen and pH in bioreactors as efforts to regulate one inadvertently affects the other.^{6,9,15,21,25,27,28} Other approaches to remove dCO₂ have modified the size of sparged bubbles, but this can compromise oxygen transfer.²⁶ Thus, methods to independently control dissolved oxygen and pH may enable bioreactor systems to improve stem cell expansion as stem cells are extremely sensitive to fluctuations in both dissolved oxygen and pH.^{3,9,15,17,19}

One simple method to reduce the dependence between dissolved oxygen and pH control in mammalian cell culture is to decrease or eliminate the bicarbonate buffer's role in pH buffering. This would allow atmospheric CO₂ levels to be used in the air/oxygen sparging supplies. Several groups have developed media to support mammalian cell lines under an atmospheric CO₂ environment. Leibovitz (1963) developed the L-15 medium, which uses an amino acid buffer in addition to substituting galactose for glucose, for use in mammalian cell culture.²⁹ Battista and Weiss (1991) developed a medium for Chinese hamster ovary cells and baby hamster kidney (BHK) cells; however, it is not appropriate for undifferentiated stem cell expansion, due to the presence of β -glycerophosphate, which induces osteogenesis.^{30,31} Goudar et al. (2006) developed a 3-(N-morpholino)propanesulfonic acid (MOPS)-histidine buffer for BHK cells; however, the low pH of 6.8 makes it too acidic for stem cells.²³ Swain and Pool (2009) investigated the use of various nonbicarbonate buffers in Tyrode's solution, composed of salt and glucose, for potential use with *in vitro* fertilization, although they did not culture cells in their proposed buffer.³² To our knowledge, attempts to expand stem cells under an atmospheric CO₂ environment have not been conducted.

The overall aim of this study was to develop a buffer system for a stem cell medium that de-emphasized CO₂-dependent pH buffering and to ensure that MSC expanded in this medium under atmospheric CO₂ behaved similarly to those expanded under standard conditions. These initial studies were conducted in static cultures; however, by eliminating the need for elevated CO₂ sparging, large-scale stem cell expansion could potentially be improved by preventing excessive dCO₂ levels, as well as enabling simpler controls to increase batch-to-batch reproducibility. The first goal of this study was to determine whether MSC could be effectively expanded under atmospheric CO₂ levels as this has not yet been investigated. L-15 medium and various combinations of MOPS-4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffered media were examined to select a medium buffer that maintained growth rates and final cell densities comparable to the standard buffer conditions without any prior adaptation period. One MOPS-HEPES buffered medium was selected to address the subsequent goals, where the second goal was to determine whether MSC maintained an undifferentiated state and self-renewal ability when cultured in the MOPS-HEPES buffered medium under atmospheric CO₂ without prior adaptation. Histological staining and stem cell-associated gene expression markers were compared for the undifferentiated MSC cultured under the standard conditions and the MOPS-HEPES buffered medium under atmospheric CO₂. The third goal of the study was to assess the differentiation potential of the MSC expanded under atmospheric CO₂. Histological staining and multiple lineage gene expression markers were compared for differentiated MSC that were previously expanded under the standard conditions or in the MOPS-HEPES buffered medium under atmospheric CO₂. Finally, the fourth goal was to determine whether MSC growth rates and stemness were maintained through multiple passages in the MOPS-HEPES buffer under atmospheric CO₂.

Materials and Methods

MSC expansion

Undifferentiated mouse MSC (Gibco) were maintained as anchorage-dependent monolayers in T75 flasks (Nunc) under 5% CO₂ at 37°C in minimal essential medium- α (MEM- α) (Gibco), which was supplemented with 10% fetal bovine serum (FBS) (Gibco). For our custom compositions developed for atmospheric CO₂ conditions, MOPS and HEPES were added as buffering components, while sodium hydroxide was used to adjust the initial pH. Leibovitz L-15 medium was also supplemented with 10% FBS. For growth rate comparisons, undifferentiated MSC were initially seeded into 6-well plates (Nunc) at 3.8×10^4 cells/well, corresponding to 4.0×10^3 cells/cm². Warmed medium was added to the wells (1.5×10^3 cells/mL). Plates were incubated under 5% CO₂ or atmospheric CO₂ at 37°C. Cell growth in all three media was measured at five time points over 57 h. Five parallel sets of cultures were grown for each condition. One set of cultures was harvested at each time point. Each data point represents distinct independent cultures in triplicate. Cell densities were quantified using the Scepter 2.0 Handheld Automated Cell Counter (Millipore).

MSC differentiation

For the differentiation experiments, undifferentiated MSC were grown for 48 h in T75 flasks at 37°C. The undifferentiated MSC were seeded at 4.0×10^3 cells/cm² in either the standard medium or MOPS-HEPES buffered medium. The standard medium cultures were incubated under 5% CO₂, while the MOPS-HEPES buffered medium cultures were incubated under atmospheric CO₂. The undifferentiated MSC were harvested and used to examine differentiation along the adipogenic, osteogenic, and chondrogenic lineages. The StemPro Differentiation kits (Gibco) were used following the manufacturer protocols. All differentiation cultures were incubated under 5% CO₂ and harvested at 16 days for analysis

by histological staining and gene expression. The MSC differentiation cultures were conducted in four triplicate sets, one set for each of the histological staining and qRT-PCR analysis.

Extended MSC expansion

Undifferentiated MSC were seeded at 4.0×10^3 cells/cm² in either the standard growth medium under 5% CO₂ or the MOPS-HEPES buffered growth medium under atmospheric CO₂. One T75 flask was used to seed parallel three 6-well plates for end-point cell density analysis and a single T75 flask for subsequent passages. MSC were passaged every 60 h for five passages, which corresponds to approximately 75% confluent and a 6.25-fold expansion at the end of each passage.

Histological staining

Osteogenic activity was measured using the Alkaline Phosphatase Detection Kit (Milipore). HCS LipidTox Green neutral lipid stain (Molecular Probes) was used to detect the presence of lipids in the adipogenic cultures. The adipocyte samples were mounted in SlowFade Gold Antifade reagent with DAPI (Molecular Probes), and imaged using an Eclipse Ti Confocal microscope. Alcian Blue (Poly Scientific) was used to stain glycosaminoglycans in the chondrogenic cultures. Chondrogenic and osteogenic samples were mounted using Permount (Fisher Scientific) and imaged using the Nikon AZ 100 Multizoom microscope.

Real time qRT-PCR

Total RNA was extracted from triplicate undifferentiated T75 flask cultures or triplicate differentiated T25 flask cultures. The RNeasy Mini Kit (Qiagen) was used following manufacturer's protocol. cDNA was synthesized by Super-Script III First-Strand Synthesis System for RT-PCR (Invitrogen) with 200 ng total RNA. RT² SYBR[®] Green qPCR Mastermix (SABiosciences) was used for qPCR analysis with MyiQ Optical System Software Version 1.0 (Bio-Rad). Further data analysis was conducted using Real-time PCR Miner software (UC Berkeley). Normalized gene expression values were obtained using the method previously outlined.³³ The primer sequences are listed in Table 1.

Statistical analysis

Statistical analysis of the growth rates, final cell densities, and gene expression data was performed with SAS 9.3 using the general linear model procedure for the final cell densities and gene expression levels ($P < 0.05$) and the regression procedure for the growth rate data ($P < 0.05$).

Results and Discussion

Formulation of a CO₂-independent medium

Initially, the growth rate of undifferentiated MSC was evaluated in MEM- α buffered with various concentrations of MOPS and HEPES under atmospheric CO₂ by endpoint assessment of cell densities. The range of MOPS and HEPES investigated were 12 to 18 mM. The cell densities using MOPS and HEPES were compared to the cell densities obtained from the standard conditions using a common seed culture. Many of the MOPS and HEPES combinations could achieve cell densities equal to that attained in the standard medium under 5% CO₂ (data not shown). The MOPS-HEPES buffered medium selected to be examined further contained 12 mM MOPS, 12 mM HEPES, and 5 mM sodium bicarbonate (NaHCO₃). This formulation was selected to minimize the potential cytotoxic effects associated with HEPES buffer.³⁴ A low level (5 mM) of NaHCO₃ was added to the

MOPS-HEPES buffered medium, because bicarbonate is reported to be an essential cell nutrient for mammalian cells.³⁵

There are several considerations when formulating a buffer for MSC expansion under atmospheric CO₂. Due to the multipotency of MSC, it is crucial that none of the buffering components interfere with the normal behavior of undifferentiated MSC during expansion. For instance, the β -glycerophosphate in Invitrogen's CO₂ independent-medium can induce osteogenesis, and therefore does not promote maintenance of an undifferentiated MSC phenotype.^{30,31} At the same time, the behavior of MSC is highly pH-dependent, so the buffering components must have sufficient buffering capacity within the desired pH range.¹⁷ As Swain and Pool (2009) pointed out, pH buffering must also be accomplished with buffer concentrations that are low enough to avoid cytotoxicity.³² Their work demonstrated that buffers used in combination allow for lower concentrations without compromising buffering capacity, a principle that was used in our work. In short, the buffering components to be substituted for bicarbonate must maintain pH effectively within a precise range while not altering the health or phenotype of the MSC.

Undifferentiated MSC can be effectively expanded under an atmospheric CO₂ environment while retaining stem cell characteristics

To evaluate the growth rates and final cell densities, undifferentiated MSC were first expanded in standard MEM- α medium under 5% CO₂ and this common stock was used to seed subsequent cultures. The undifferentiated MSC were then grown in the standard MEM- α medium and the MOPS-HEPES buffered MEM- α medium under both 5% and atmospheric CO₂ environments without prior adaptation. Additionally, the undifferentiated MSC were grown under atmospheric CO₂ in Leibovitz L-15 medium. The L-15 medium was designed to support CO₂-independent growth of mammalian cells, but is now mainly used to grow specific cancer cell lines and buffer solutions while performing analysis of cells under atmospheric conditions.^{29,36,37} To date, Leibovitz L-15 medium has not been investigated for stem cell expansion. As expected, the cultures grown in the standard MEM- α medium under 5% CO₂ had exponential growth without a lag phase, and the cultures grown in the MEM- α standard medium under atmospheric CO₂ failed to grow (Figure 1A). In contrast, cultures grown in Leibovitz L-15 medium under atmospheric CO₂ had observable growth, albeit at a lower rate. The lower growth rate in the L-15 medium was most likely due to the medium's buffering system and carbon energy source. The L-15 medium relied on high amino acid concentrations to provide buffering capacity. Elevated amino acids are not always problematic, but it has been observed that some amino acids in elevated concentrations reduce growth rates in mammalian cells.^{38,39} Specifically, the L-15 medium contained 225 mM alanine compared to 25 mM in the standard MEM- α medium.²⁹ The L-15 medium also substitutes galactose for glucose. Galactose has been implicated in shifting cell metabolism from a predominantly glycolytic state to one relying more on oxidative phosphorylation in several cell lines.^{40,41} Actively growing cells appear to rely more heavily on glycolysis than oxidative phosphorylation for energy production,^{5,19,42} which means galactose may not be a suitable primary carbon source for maintaining high growth rates. Thus, the lower growth rates associated with MSC in the L-15 medium is hypothesized to be due to the presence of high amino acid levels and the use of galactose instead of glucose.

The undifferentiated MSC cultures grown in the MOPS-HEPES buffered medium under 5% CO₂ grew better than in Leibovitz L-15 medium ($P = 0.05$), but still at a suboptimal rate. The decreased growth rate in the MOPS-HEPES buffered medium under 5% CO₂ can be attributed to the low culture pH. More importantly, the undifferentiated MSC cultures grown in the MOPS-HEPES buffered medium under atmospheric CO₂ had exponential growth

without a lag phase. The observed growth rates for the undifferentiated MSC in standard medium under 5% CO₂ and the MOPS-HEPES buffered MEM- α medium under atmospheric CO₂ were not significantly different ($P > 0.05$) with observed growth rates of 0.033 h⁻¹; which corresponds to a doubling time of 21 h. These results indicate that undifferentiated MSC can be expanded in MOPS-HEPES buffered MEM- α medium under atmospheric CO₂ at an equivalent rate to undifferentiated MSC in standard MEM- α medium under a 5% CO₂ environment, and also achieve final cell densities that are not significantly different.

While growth rates were equivalent for the undifferentiated MSC cultured in the standard MEM- α medium under 5% CO₂ and the MOPS-HEPES buffered MEM- α medium under atmospheric CO₂, equally important is that the MSC maintained an undifferentiated state and self-renewal abilities. The phenotype and multipotency of undifferentiated MSC were assessed for MSC expanded for 48 h in the standard MEM- α medium under 5% CO₂ and the MOPS-HEPES buffered MEM- α medium under atmospheric CO₂. First, undifferentiated MSC were stained for markers of adipogenic, osteogenic, and chondrogenic differentiation after expansion as negative controls. The histological staining of the undifferentiated MSC showed absence of lineage markers (Figure 1B), as expected, suggesting no gross signs of differentiation for both conditions. Only the MSC cultured in the MOPS-HEPES buffered MEM- α medium under atmospheric CO₂ negative control stainings are shown (Figure 1B), as the MSC cultured in the standard MEM- α medium under 5% CO₂ were not significantly different from negative control the images provided in the standard staining protocol. The negative control stainings for the undifferentiated MSC cultured in the MOPS-HEPES buffered MEM- α medium under atmospheric CO₂ can be compared with the positive control stainings shown in Figure 2A, 3A, and 4A. Second, the MSC were analyzed for a gene marker indicative of an undifferentiated stem cell state after expansion. Gene expression levels for Oct4 ($P = 0.75$) (Figure 1C) were not significantly different between undifferentiated MSC cultured in either the standard MEM- α medium under 5% CO₂ and the MOPS-HEPES buffered MEM- α medium under atmospheric CO₂, representing a similar extent of stemness.

These results indicate that undifferentiated MSC can be expanded under atmospheric CO₂ without compromising growth rates or the stem cell phenotype as determined by gene expression markers, and supported by the undifferentiated appear under histological staining. This is important because many MSC-based treatments rely on undifferentiated MSC. Even in applications where the differentiated progeny of MSC are desired, differentiation is typically induced after initial expansion generates a sufficient quantity of undifferentiated MSC.²² These results indicate that MSC can be effectively expanded without prior adaptation to the MOPS-HEPES buffer and atmospheric CO₂ conditions. The lack of an adaptation phase would allow for easier translation to clinical applications.

MSC retain multilineage differentiation potential after prior expansion under atmospheric CO₂

In order to characterize the differentiation potential of MSC cultured under atmospheric CO₂, cells were obtained from T75 flasks that had been cultured for 48 h in either the standard MEM- α medium under 5% CO₂ or the MOPS-HEPES buffered MEM- α medium under atmospheric CO₂. These MSC were then differentiated along the adipogenic, osteogenic, and chondrogenic lineages under 5% CO₂ to assess multipotency. As shown in Figure 2A, staining for lipid vesicle formation indicated a similar extent of adipogenic differentiation between the two conditions. Also, adipogenic gene expression levels for lipoprotein lipase ($P = 0.095$), PPAR γ 2 ($P = 0.86$), and C/EBP α ($P = 0.47$) were not significantly different between the two conditions (Figure 2B). As shown in Figure 3A,

staining for alkaline phosphatase activity indicated a similar extent of osteogenic differentiation between the two conditions. Also, osteogenic gene expression levels for collagen I ($P = 0.89$), osteonectin ($P = 0.088$), and *CBFa1* ($P = 0.99$) were not significantly different between the two conditions (Figure 3B). As shown in Figure 4A, staining for glycosaminoglycan production indicated a similar extent of chondrogenic differentiation between the two conditions. Also, chondrogenic gene expression levels for collagen II ($P = 0.22$), aggrecan ($P = 0.55$), and Sox9 ($P = 0.94$) were not significantly different between the two conditions (Figure 4B). These results demonstrate that the tri-lineage differentiation potential of undifferentiated MSC along the adipogenic, osteogenic, and chondrogenic lineages were not impacted by prior expansion in the MOPS-HEPES buffered medium under atmospheric CO₂.

Undifferentiated MSC retain expansion potential and stem cell characteristics after multiple passages under atmospheric CO₂

While expanding MSC effectively without prior adaptation to the MOPS-HEPES buffer and atmospheric CO₂ enables easier translation to clinical applications, prolonged expansion may be required to generate larger quantities of undifferentiated stem cells. To this end, serial passages of undifferentiated MSC were conducted. Both the standard medium under 5% CO₂ and the MOPS-HEPES buffered medium under atmospheric CO₂ were examined for five passages. To ensure that growth rates were maintained in the exponential phase, the cultures were only expanded to approximately 75% confluent at the 60-h time point representing a potential fold-expansion in each passage of 6.25-fold. The final cell densities for all five passages were not significantly different between MSC expanded in the standard medium under 5% CO₂ and the MOPS-HEPES buffered medium under atmospheric CO₂ ($P > 0.05$) (Figure 5A). Additionally, the final cell densities were not different between the passages ($P > 0.05$). These end-point cell densities correspond to an overall growth rate of 0.031 h⁻¹ over 60 h, which is not statistically different ($P > 0.05$) from the 57 h overall growth rates observed for the passage 1 MSC cultured in the standard medium under 5% CO₂ and the MOPS-HEPES buffered medium under atmospheric CO₂ (Figure 1A). Gene expression levels for Oct4 were not significantly different for the undifferentiated MSC expanded in the standard medium under 5% CO₂ and the MOPS-HEPES buffered medium under atmospheric CO₂ after passage five ($P > 0.05$) (Figure 5B). It has been previously shown that MSC growth rates change *in vitro* after only five passages,⁴³ thus maintenance of growth rate in the MOPS-HEPES buffer under atmospheric CO₂ for this duration would meet current expected outcomes. Additionally, *in vitro* MSC growth rates are a highly sensitive indicator of stemness and were shown to decrease prior to changes in differentiation markers.⁴³ It has been previously shown that Oct4 gene expression levels are highly correlated with a more primitive stem cell-like state capable of extended proliferation, increased colony forming potential, and multilineage differentiation. For example, hypoxia increased MSC stemness as determined by the potential for proliferation, colony formation, and differentiation and coincided with the upregulation of Oct4 and Rex-1 gene expression.⁴⁴ In addition, overexpression of Oct4 and Nanog were shown to enhance stem cell properties in MSC as determined by proliferation and differentiation potential, while knockdown of Oct4 and Nanog decreased proliferation and differentiation potential.⁴⁵ Therefore, the similar Oct4 expression levels and similar growth patterns that were observed indicate similar levels of stemness between the MSC grown in 5% CO₂ and those MSC grown in atmospheric CO₂.

Potential impacts of CO₂ independence on large-scale stem cell expansion in stirred bioreactors

Stirred bioreactors—with unmatched scalability—represent a very promising culture format for delivering high yields of either suspension or anchorage-dependent stem cells with

uniform properties.^{4,7,14,15} The online monitoring and control capabilities allow culture parameters to be adjusted throughout the duration of expansion, which can provide crucial batch-to-batch reproducibility.¹¹ The biopharmaceutical industry has long taken significant advantage of these systems, but stem cell expansion techniques have lagged behind and not yet transitioned into truly large-scale vessels.^{6,10} Many stem cell-based treatments require large quantities of stem cells, and because native stem cells are relatively scarce in adult tissues, sufficient quantities of stem cells must be expanded in a timely manner *in vitro* for use in treatments. Additionally, adult stem cells decline in quantity and quality over time in a person, thus reducing the ability to harvest autologous supplies.¹⁹ These considerations have led some to suggest that establishing stem cell banks may be a more practical solution.⁷ Allogenic stem cell stocks would allow patients access to stem cells on demand, in much the same way that blood banks currently work. These stem cell banks would further necessitate large-scale cultivation at optimal growth rates to produce sufficient quantities of cells. The MSC cultured in the MOPS-HEPES buffer under atmospheric CO₂ were able to maintain high/optimal growth rates for five passages, which would allow for large-scale cultivation.

As stem cell expansion moves toward large-scale, issues similar to those currently encountered in other large-scale mammalian cell cultures will likely be encountered. Many of these problems result from the widespread use of the bicarbonate buffering system to regulate culture pH. In contrast to pH buffer redundancies in the body, the bicarbonate buffering system essentially functions alone in typical mammalian cell culture. In cell culture media, elevated levels of NaHCO₃ are added, which necessitates the use of high CO₂ levels in the gas phase that are significantly higher than physiological levels (See Figure 6).^{23,24} The indirect equilibrium between the CO₂ from the gas phase and the NaHCO₃ added to the medium maintains pH, via H⁺ concentration. Due to the nonpolar nature of CO₂, it can freely diffuse across cell membranes. At high concentrations, it can lower intracellular pH.^{23,24} In addition to affecting growth rates, these elevated dCO₂ levels can also alter the metabolism, productivity, and glycosylation profiles in other mammalian cell lines, although this has not yet been reported in stem cells.^{23,24,26} As cell densities increase in large-scale cultures, additional CO₂ is produced by the actively growing cells, which causes a buildup of dCO₂ and gas phase CO₂ levels.²³ This dCO₂ buildup can negatively impact cells grown in stirred bioreactors, and the effects increase with vessel size.^{23,26} The use of a nonbicarbonate buffer would reduce dCO₂ buildup and potentially improve growth.

In stirred bioreactors, oxygen and CO₂ gases are sparged into the vessel as bubbles, to increase mass transfer between the gas and liquid phases.¹⁴ The breakup of these bubbles at the gas-liquid interface is responsible for the majority of cell death and damage during stirred bioreactor cultures.^{9,16,25} Interestingly, managing the size of these bubbles has been proposed as a solution to the problem of dCO₂ buildup.²⁴⁻²⁶ Larger bubbles are more effective in stripping dCO₂, while smaller bubbles are more effective at delivering oxygen to the culture.²⁶ With both oxygen and CO₂ being supplied via the same sparged gas phase, only one bubble size distribution can be supplied. As vessels are scaled-up, the dCO₂ buildup becomes problematic, such that sparger designs allow for larger bubbles to strip dCO₂ at the expense of adequate oxygen supply.²⁶ Hu et al. (2011) proposed decoupling the oxygen and CO₂ supplies by supplying pure oxygen via microspargers into the bioreactor, and air via standard spargers; however, they were concerned about the added control complexity.²⁶ A nonbicarbonate buffer would allow for a single gas supply (air/oxygen), thus decreasing controller complexity.

The coupling of the oxygen and CO₂ supplies complicates control of culture parameters and allows for pH and dissolved oxygen variability that is unacceptable, particularly for stem cells.^{7,9,13,17,19} Several researchers have indicated that this pH/CO₂/O₂ interdependence complicates bioreactor control and have gone on to quantify the effects of pH, CO₂, or

dissolved oxygen variability on growth or productivity^{21,28}; however, few have suggested replacing the bicarbonate buffer system as a means to improve pH control.²³ Yet it would appear far simpler to use a nonbicarbonate buffer—such as the MOPS-HEPES combination used in this study—that does not rely on elevated dCO₂ levels for pH control. This work was the first to demonstrate that the bicarbonate buffering system and an elevated CO₂ environment are not required for effective expansion of undifferentiated MSC. Elimination of the bicarbonate buffer should lead to improved control of culture conditions and enhanced stem cell expansion in bioreactors.

Conclusions

Stem cells will be used in increasing numbers of scientific applications in the future, ranging from basic research to clinical treatments. To provide stem cells for these applications, new large-scale stem cell expansion methods must be developed. Stirred bioreactors possess many advantages that allow for generation of large quantities of uniform stem cells in a reproducible manner. Unfortunately, the bicarbonate buffer used in mammalian cell culture, and the excessive CO₂ sparging required by that system hinder the ability to optimize process controls and generate stem cell yields of the highest quality. This work developed a MOPS-HEPES buffering system that de-emphasized the role of CO₂ in pH buffering. Undifferentiated MSC were expanded without prior adaptation under an atmospheric CO₂ environment in the presence of the MOPS-HEPES buffer at growth rates equal to those expanded under 5% CO₂ in the presence of the standard bicarbonate buffer. Additionally, the undifferentiated MSC expanded under an atmospheric CO₂ environment retained equivalent differentiation potential along the adipogenic, osteogenic, and chondrogenic lineages compared to undifferentiated MSC previously expanded under 5% CO₂, as determined by histological staining and gene expression markers. Furthermore, serial passaging of MSC in the MOPS-HEPES buffered medium under atmospheric CO₂ did not hinder MSC expansion or decrease stemness gene expression markers as compared to MSC serially passaged under standard conditions. Utilization of CO₂-independent buffers, such as the MOPS-HEPES buffer developed here, has the potential to improve stem cell bioreactor expansion by allowing for tighter control of pH and dissolved oxygen.

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Figure 1. Proliferation of MSC

A: Growth profiles for MSC cultured in a standard medium, a MOPS-HEPES buffered medium under both 5% and atmospheric CO₂ conditions as well as the Leibovitz L-15 medium under atmospheric CO₂. B: Negative control staining for lineage-specific markers in undifferentiated MSCs cultured in MOPS-HEPES buffered medium for 48 h. C: Oct4 normalized gene expression levels for undifferentiated MSCs cultured in the standard medium in 5% CO₂ (Std) and the MOPS-HEPES buffered medium in atmospheric CO₂ (M-H) for 48 h as indicated in panel A. Error bars represent 95% confidence intervals.

Figure 2. Adipogenic differentiation

A: Histological staining of MSC cultured in a standard medium under 5% CO₂ (Std) and in a MOPS-HEPES buffered medium under atmospheric CO₂ (M-H). The stain HCS LipidTox was used to visualize lipids (green) and DAPI was used to visualize the nuclei (blue). B: Lipoprotein lipase, PPAR γ 2, and C/EBP α normalized gene expression levels in adipogenically differentiated MSC. Error bars represent 95% confidence intervals.

Figure 3. Osteogenic differentiation

A: Histological staining of MSC cultured in a standard medium under 5% CO₂ (Std) and in a MOPS-HEPES buffered medium under atmospheric CO₂ (M-H). The stains FRV and Naphthol Phosphate were used to visualize alkaline phosphatase activity in pink. B: Collagen I, Osteonectin, and CBFα1 normalized gene expression levels in osteogenically differentiated MSC. Error bars represent 95% confidence.

Figure 4. Chondrogenic differentiation

A: Histological staining of MSC cultured in a standard medium under 5% CO_2 (Std) and in a MOPS-HEPES buffered medium under atmospheric CO_2 (M-H). The stain Alcian Blue was used to visualize glycosaminoglycans in blue. B: Collagen II, Aggrecan, and SOX9 normalized gene expression levels in chondrogenically differentiated MSC. Error bars represent 95% confidence intervals.

Figure 5. Extended MSC expansion

A: End-point cell density analysis for undifferentiated MSC through five passages cultured in a standard medium under 5% CO₂ (Std) and in a MOPS-HEPES buffered medium under atmospheric CO₂ (M-H). B: Oct4 normalized gene expression for undifferentiated MSC after passage 5. Error bars represent 95% confidence intervals.

Figure 6. Bicarbonate buffer equilibrium

NaHCO₃ salt is commonly added to mammalian cell culture medium to provide buffering in concert with elevated gas phase CO₂, which will equilibrate with dCO₂.

Table 1
Primer Sequences

Gene	Forward Primer Sequence	Reverse Primer Sequence
Oct4	5'-GAA GCC GAC AAC AAT GAG AAC	3'-ACA GAA CCA TAC TCG AAC CAC A
Lipoprotein lipase	5'-AGC CAC CAG GCA GAT AGA GA	3'-CCT TGT TGG AGG AGG TGT GT
PPAR γ	5'-ACA GCC ACA TGA ATG GTG AA	3'-TTA ACT GCC CAG CGC TAG AT
C/EBP α	5'-CTC CCA GAG GAC CAA TGA AA	3'-AAG TCT TAG CCG GAG GAA GC
Collagen I	5'-AGG TGT GTG TCA AGG GGA AG	3'-CTC TCT TCC TGC ACC CAA AG
Osteonectin	5'-TCT GTT GTG TTG GGG ACG TA	3'-GCT TGA GCC ATC ACA CTT GA
CBF α	5'-AAA CTC ACA GCC TCG AAG GA	3'-TGT GGC TCA TTG TTC TCT GC
Collagen II	5'-GAA GGT GCT CAA GGT TCT CG	3'-TTT GTG ACA AGG CCA CAC AT
Aggrecan	5'-CGA GCT ACA GAG GGA ACC TG	3'-CCA GAC TTG GAA CCT GTG GT
Sox9	5'-CCC CAA AAG CAC ACA TTC TT	3'-ACC CGC AGG TAC ACA CTA GG