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Bioprocessing Aspects of Neural Stem Cell Production in Bioreactors

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Neurodegenerative diseases such as Parkinson's disease and multiple sclerosis, along with injuries such as stroke affect millions of individuals worldwide and costs healthcare systems billions of dollars each year in North America alone.[37,26] The diseases result from the death of specific cell types within the central nervous system. Current treatment efforts have focused primarily on alleviating symptoms using pharmaceuticals. However, recent advances in our understanding of these conditions, coupled with advances in biology, genomics, transplantation, and biochemical engineering are making cell therapy (the transplantation of viable cells to replace dead cells) more attractive as a potential avenue of treatment.[4,10,17,32] Neural stem cell (NSC) transplantation represents one such treatment.

Neural stem cells are primitive multipotent cells that are defined as having the ability to proliferate and self-renew. They can also differentiate into neurons, astrocytes, and oligodendrocytes, which are all of the major cell phenotypes found in the central nervous system (CNS). NSCs can be derived directly from the adult or fetal CNS, or indirectly from embryonic stem (ES) cells. Regardless of the source, one defining

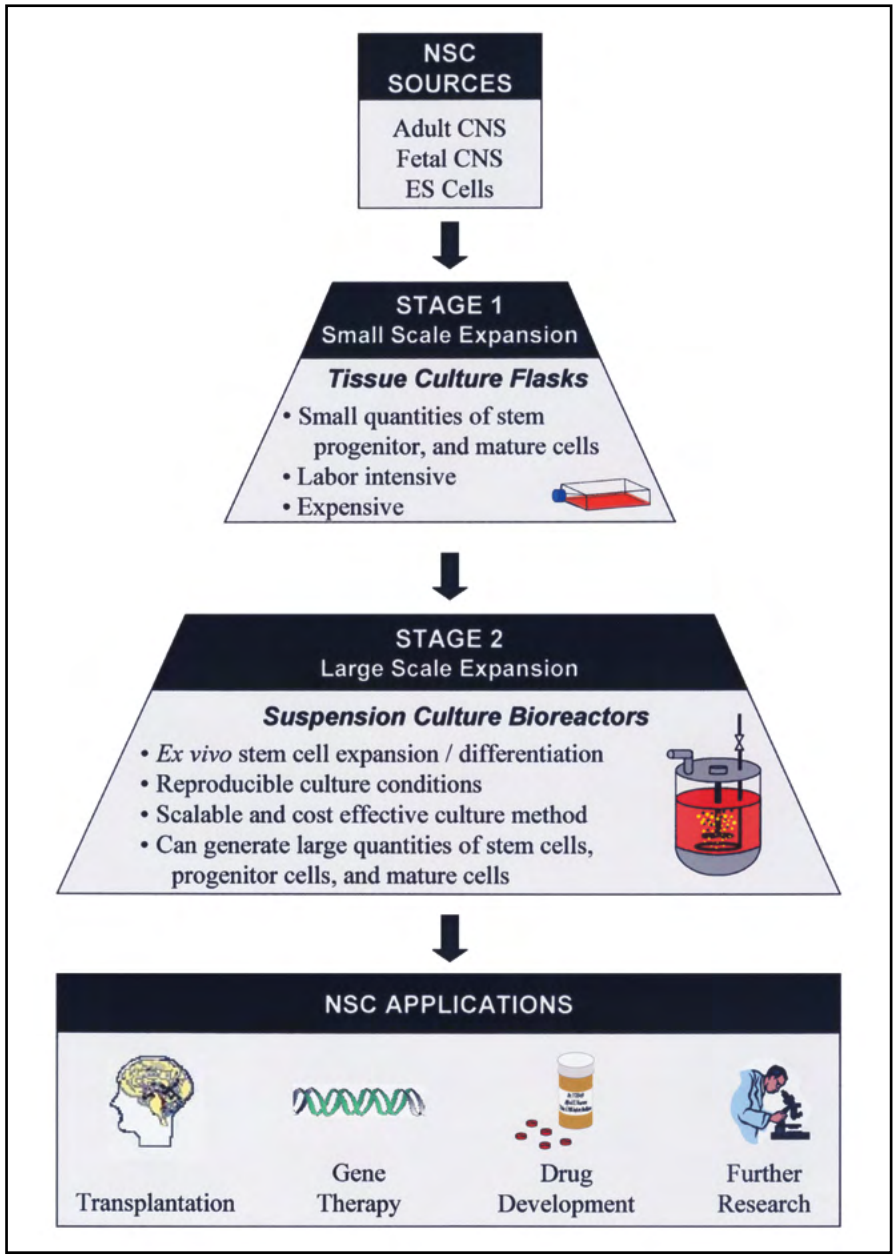

Figure 1. Sources, scale-up methods, and potential applications of neural stem cells.

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characteristic of these cells when placed in culture is that they divide and form clusters of cells termed neurospheres or aggregates.

The ability of neural stem cells to produce all of the primary cell types in the CNS has resulted in an exponential increase in the amount of NSC research being conducted. Much of this research is focused on finding cures for neural diseases, disorders, and injuries. Due to the staggering number of individuals who could benefit from NSC-related therapies, the projected demand for these cells in the near future is immense, and as such, it is imperative to develop methods for producing large quantities of NSCs in an efficient, quality-controlled manner (Fig. 1). This article will examine potential NSC applications, and will discuss important considerations when scaling-up a stem cell production system.

Applications of Neural Stem Cells

The most obvious and desirable application of neural stem cells is in a clinical setting to reverse currently incurable and debilitating neural conditions. However, as summarized in Figure 1, NSCs have several other important applications including gene therapy, further basic biological research, and new neural drug development and drug testing.

Neural Stem Cell Transplantation. The most recent attempts to block the progression of neurodegenerative disorders and reverse their effects have involved the transplantation of fetal tissue.[38] Although the clinical use of fetal tissue for transplantation has produced some degree of success, there are several issues that must be addressed. First, there are moral concerns associated with using fetal tissue that could present a serious obstacle for this type of treatment. Second, even though 70–80% of the transplanted cells are viable, only 5–10% of the tissue survives at the implant site.[2] Thus, to provide enough cells, each treatment requires several fetuses. For example, in order to treat the more than half a million individuals in North America afflicted with Parkinson's disease, millions of aborted fetuses would be necessary for this one disease alone. Obviously, this is not an option for ethical reasons. Third, the cost of isolating fetal tissue and subsequent surgical transplantation is enormous, and could inhibit this form of treatment. And finally, since the fetal tissue is not derived from a single source, it is not well characterized. Typically, the cells that are used come from elective abortions, and not spontaneous abortions, as the latter have a higher incidence of chromosomal abnormalities. Moreover, they could contain microbial contaminants or cancerous cells.[2]

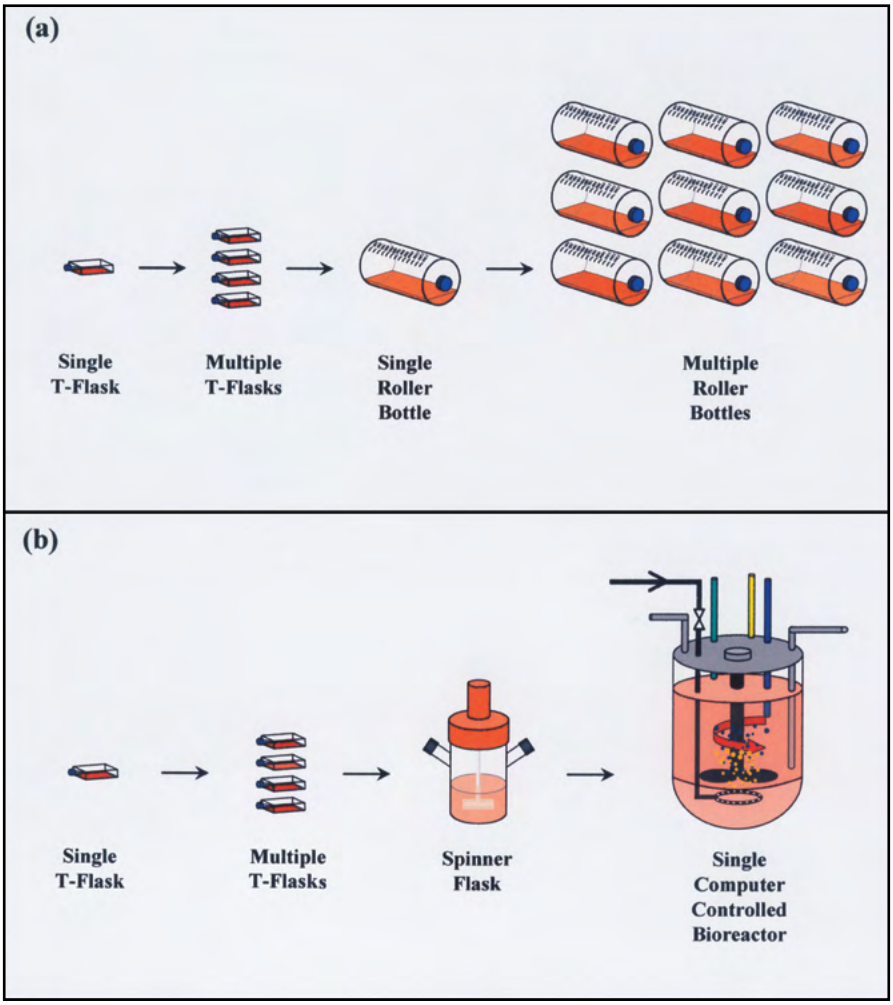

Figure 2. A schematic showing two different approaches to scaling-up the production of neural stem cells. (a) The generation of large numbers of neural stem cells could be achieved by serially subculturing cells in an increasing number of small, static tissue culture flasks followed by roller bottles, or (b) cell production could be scaled-up from tissue culture flasks to small bioreactors (spinner flasks) and then to large computer controlled bioreactors. One large bioreactor could replace the large number of roller bottles used in the inefficient approach depicted in (a).

Although conventional fetal tissue transplantation has several disadvantages, the concept of transplanting neural tissue into the CNS to replace dead or dying cells is still viable. What is needed is a more reliable and better defined source of cells. Neural cells cultured *in vitro* in controlled bioreactors could provide this source. Many studies have demonstrated that neural stem cells grown in culture and then transplanted into animal models of neurodegenerative disease can migrate, proliferate, and integrate into host tissue.[35,22] These cells have been shown to alleviate many of the functional deficits associated with neural disease by repopulating regions of absent or dead cells.

Hamang and colleagues transplanted fetal EGF responsive stem cells into a myelin deficient rat.[14] The stem cell progeny were found to differentiate preferentially into oligodendrocytes in

response to the nonmyelinated CNS, and subsequently produce myelin. Svendsen and colleagues transplanted progenitor cells isolated from the developing human CNS into rats with unilateral dopaminergic lesions.[45] After 20 weeks, differentiated graft-derived neurons appeared close to the transplantation site, and human astrocytes were found to have migrated throughout the striatum. In a few of the animals, a significant number of these new neurons were dopaminergic, and exhibited the ability to reverse rotational deficits associated with the lesions.

Freshly isolated human NSCs have been successfully transplanted into the brains of immune deficient neonatal mice, and human NSCs grown *in vitro* for one year have been shown to migrate and integrate into the developing rat brain.[47,5] Bromodeoxyuridine labeled human NSCs have also been transplanted into the developing non-human primate brain where they were found to integrate into both the mature cerebral cortex and subventricular zone.[28] In addition to the reports discussed here, transplantation studies are routinely used to determine if cells cultured *in vitro* can survive, integrate, and function *in vivo*. The cells are typically labeled with a marker such as GFP, and functionality is assessed by anatomical, electrophysiological, and/or behavioral studies.

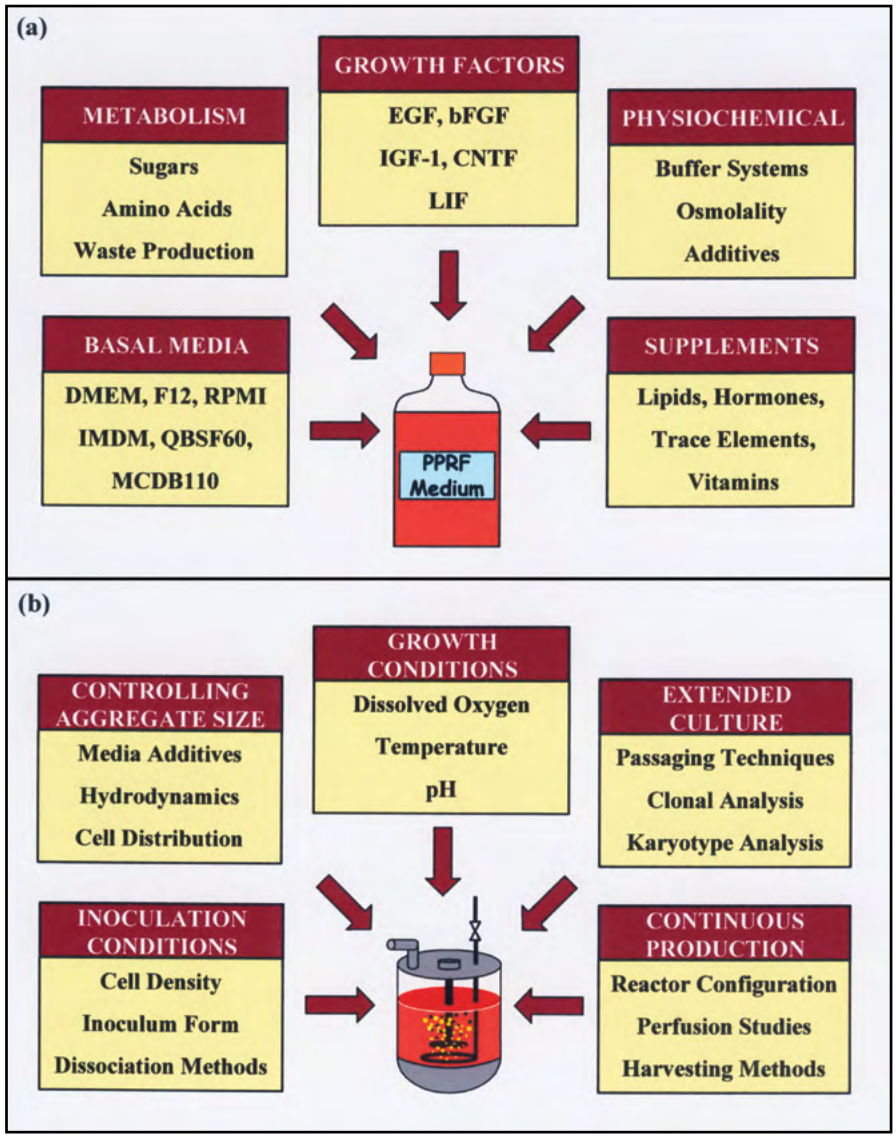

Figure 3. Developing a scaled-up cell production system is a complex task with many important considerations. These considerations can be divided into (a) those that involve the development of a cell expansion medium, and (b) those that involve bioreactor protocols. As shown, many important studies must be conducted in order to develop a scaled-up neural stem cell production system in suspension culture bioreactors.

Other Applications

The main objective of current NSC research is to develop strategies to directly replace lost cells in the CNS with new cells that function identically. However, neural stem cells have other important applications in addition to direct cell replacement, as shown in Figure 1. The first of these is further biological research. Although many studies have been published regarding NSCs, extensive research remains to be conducted in order to study and generate effective methods to tailor the proliferation, migration, and differentiation of neural stem cells. One difficulty in this area is comparing studies from different research laboratories because cells from different sources vary in their behavior. One possible cell production scenario might entail neural stem cells generated in a single large-scale facility, and then distributed to laboratories throughout the world so research laboratories could have uniform cells. In order to address the heterogeneity between cell lines, this facility could expand cell populations procured from several different sources.

In addition, neural stem cells could be used to test novel therapeutic drugs. Currently, animal models are extensively used to test the efficacy and potential hazards of new medicines. Although animal models have been proven to be acceptable, the effects of drugs on animal cells is not identical to the effects on human cells. Indeed, it is often the case that the production and disbursement of particular drugs have been halted due to unforeseen side effects that did not occur in animals. Cultured neural stem cells and their progeny would allow researchers to directly observe the impact of new drugs on human neural cells, thereby enabling the production of safer and more effective medicines. In addition, it may be possible to isolate bioactive products of therapeutic interest from neural stem cells.

A problem with indirectly deliver-

ing therapeutic agents to the CNS is the blood brain barrier. This barrier tightly regulates the passage of compounds into the CNS. Thus, in order to ensure that adequate quantities of a drug are being supplied to the CNS, it is often necessary to produce drugs that have a long half life, and to prescribe high doses of those drugs. To solve this problem, neural stem cells could be used as drug delivery vehicles to supply specific doses of certain therapeutics directly into the CNS. Cells could be genetically manipulated to produce neurotrophic factors, neurotransmitters, and other active compounds, and then transplanted into desired regions within the CNS. In this way it would be possible to supplement chemicals that are produced at sub-effective levels, or to replace chemicals that are not present due to cell death or genetic mutation.

For example, Kordower and colleagues isolated EGF responsive stem cells from transgenic mice.[25] These cells were genetically modified to express human nerve growth factor (hNGF) under the direction of the GFAP (glial fibrillary acidic protein) promoter. These GFAP-hNGF cells were then transplanted by intrastriatal injection into a rat model of Huntington's disease. The study found that the cellular delivery of hNGF by genetically modified stem cells prevented the degeneration of striatal tissue otherwise destined to die. The ability of transgene-expressing NSCs to act as therapeutic vehicles in the treatment of brain disorders is further enhanced by their extraordinary ability to migrate large distances.[9] A study in 2000 by Aboody and colleagues showed that when genetically-modified neural stem cells were implanted into intracranial gliomas in adult rodents, the NSCs distributed themselves extensively throughout the tumour bed, and migrated uniquely in juxtaposition to expanding tumour cells while continuing to express a foreign gene.[1] When they were implanted intracranially at some distance from the tumor, the cells migrated through normal tissue and again targeted the tumor cells. In addition to drug delivery, neural stem cells could also be genetically manipulated to direct their own differentiation, or the differentiation of surrounding precursor cells toward certain desired phenotypes (e.g. dopaminergic neurons).

And finally, neural stem cells grown *in vitro* could be used to further understand aspects of neural development. Specifically, studies could be conducted to understand underlying mechanisms that control the lineage and ultimate fate of different groups of neural precursor cells, including the identification of mediators and inductive factors in neurogenesis. These cells could also be used to create models of neurodegeneration to gain further knowledge about the causes and progression of neural disorders. Moreover, the cells could also be used in the rapidly emerging field of genomics to understand how genetic programs within cells change due to differentiation, and how they are affected by autocrine and paracrine factors.

Scale-Up of Neural Stem Cell Production

For biological research applications, it is often sufficient to be able to grow cells in small, uncontrolled, static culture vessels such as tissue culture flasks. However, in order to generate large quantities of cells for industrial or clinical applications, methods must be developed to scale-up their production in a controlled environment with standard operating procedures.[21]

For example, it has been estimated that the symptoms associated with Parkinson's disease become evident once 60–80% of the approximately 1 x 10^6 dopamine producing cells in the substantia nigra region of the brain die.[17] In order to reverse the symptoms, it will be necessary to replace these dead cells. We estimate that at least 10^9 neural stem cells would have to be generated in a bioreactor in order to perform a single transplantation considering that: only a small percentage of neurons derived from NSCs can produce dopamine; a very large percentage of cells implanted in the brain do not survive the surgical procedure; and there are several cell processing steps between harvesting cells from the bioreactor and surgically implanting them into a patient. A very large number of cells would be required to treat the millions of people afflicted with Parkinson's disease worldwide. Considering that neural stem cells have applications far beyond the treatment of just this one disease, it is very clear that there is a critical need to develop a scaled-up NSC production system.

Figure 2 shows two potential methods for producing large quantities of cells. Figure 2a shows that although it is possible to generate large quantities of cells by serially expanding NSC populations in tissue culture flasks and roller bottles, the process would require a huge number of vessels within a short period of time. Vessels would either have to be handled separately by qualified individuals, or robotic facilities would have to be built. This method of generating large quantities of cells would be labor-intensive and inefficient, and thus, could be cost-prohibitive. An efficient, more practical approach, as shown in Figure 2b, would be to scale-up the production of cells from tissue culture flasks to small bioreactors (spinner flasks), and finally to larger computer-controlled bioreactors.[41] A single 5L bioreactor could easily be controlled by a commercially available computer and one individual, but could hold the same culture volume as dozens of roller bottles or one thousand small tissue culture flasks.

There are several advantages to expanding neural stem cell populations in bioreactors. First, the cells could be grown efficiently in large quantities which would alleviate current and future supply problems for transplantation, and would reduce overall costs. Second, the moral concerns about harvesting large quantities of fetal tissue would be significantly reduced. Third, because a large quantity of cells could be produced from a single source, the cells would be well characterized. By setting up appropriate screening protocols, it could be ensured that the cells are non-transformed, non-tumorigenic, and free of adventitious agents.

In this article, we describe studies conducted in our laboratory that examined the behavior of neural stem cells in both tissue culture flasks and small bioreactors to determine if they could be efficiently generated in larger-scale

bioreactors. Though scaling up neural stem cell production is desirable, growing cells in large bioreactors is a complex task. We have divided this scale-up into two primary sections: developing a cell culture medium, and developing bioreactor protocols.

Developing a Cell Culture Medium. First and foremost, an optimized medium has to be developed. The liquid environment in which a stem cell is grown influences its behavior and can cause a stem cell to proliferate or remain quiescent, to retain its primitive characteristics or differentiate, to live or die. An ideal neural stem cell medium would allow NSCs to proliferate over extended periods of time without losing any of their stem cell characteristics. The development of a medium can be an exhaustive task. Crucial studies that must be completed include investigating the effects of various basal media, cytokines, carbon sources, hormones, amino acids, and trace elements (Fig. 3a). Extensive studies in our laboratory have resulted in the development of a new neural stem cell medium (PPRF-m4 medium) for both murine and human neural stem cells. The behavior of NSCs in this medium will be described later in this article.

Developing Bioreactor Protocols. Once an optimized medium has been developed, it can be used to generate scale-up protocols. Scale-up protocols refer to a set of optimized methods that allow large quantities of cells to be generated in a reproducible manner. For example, it is very important to ensure that certain dynamic operating parameters within a bioreactor remain relatively constant over time, such as pH and dissolved oxygen concentration. Large production systems are invariably computer-controlled, and as such, operate within defined limits. Thus, it is necessary to determine the sensitivity of cells to fluctuations in culture parameters, and design a production system accordingly. A large number of studies are necessary to develop scale-up protocols, as described in Figure 3b. The studies presented here will investigate the effects of inoculation density, pH, osmolality, oxygen concentration, and cell storage. In the following sections, we will begin with a brief literature review of the effects of each parameter on mammalian cells *in vitro*.

Inoculation Density. The requirement of a minimum inoculation density has been shown to exist for mammalian cell cultures including normal diploid fibroblasts, hybridoma cells, and hematopoietic cells.[16,29,52] Researchers postulate that the reason for this is because mammalian cells produce various soluble factors in order to survive *in vitro*, and that the concentration of soluble factors has to reach a critical limit for proliferation. Rubin reported that the minimum seeding density of primary chick embryo cells could be decreased by adding conditioned medium that presumably contained necessary factors.[36] We conducted studies in our laboratory to determine if neural stem cells also required a minimum inoculation density.

pH. The issue of pH maintenance in cell culture media is important because many biochemical reactions are very sensitive to changes in the hydrogen ion concentration. Many of these same biochemical reactions produce or consume hydrogen. Thus, because of this constant flux in the pH, it is necessary to add buffers to media in order to maintain pH within an optimum range. One of the first buffers to be used in cell culture systems was sodium bicarbonate ($NaHCO_3$) with an exogenous source of CO_2. However, one problem with using $NaHCO_3$ alone is that the optimal buffering range of carbonic acid at 37° C is lower ($pk_{a1} \approx 6.2$) or higher ($pk_{a2} \approx 10.2$) than the physiological range of 7.0–7.4.[48] In order to remedy this, Good and colleagues developed zwitterionic N-substituted aminosulfonic acids.[11] (A zwitterion is an ion that has both a positive and a negative charge on the same species.) Of all the substances developed by Good and colleagues, the most successful has been N-hydroxyethylpiperazine-N'-ethanesulfonic acid, more commonly known as Hepes. At 20° C, this buffer has been shown to have a pk_{a1} of 3.0, and a pk_{a2} of 7.55 which makes it useful in the 7.0 to 8.0 pH range.[49]

In cell cultures, the presence of some buffers are known to be toxic to certain cell lines and non-toxic buffers may become detrimental at elevated concentrations.[6] Thus, the type and concentration of buffer must be optimized for each different cell type. Neural stem cell culture medium contains both sodium bicarbonate and Hepes, and their ratio dictates the pH setpoint.

Osmolality. A wide range of acceptable osmolality values (260 mOsm/kg – 320 mOsm/kg) has been reported for cultured mammalian cells.[7] These values are similar to the measured osmolality of human plasma which is 290 mOsm/kg, and the osmolality of murine plasma which is 310 mOsm/kg.[7] In general, when designing a new medium it is better to make it slightly hypotonic to make allowances for water evaporation during the culture period. Determining an acceptable osmolality range for the culture of mammalian neural stem cells is important when attempting to scale-up their production. Adjusting other parameters can adversely affect osmolality. For example, it has been found that in agitated bioreactors, shear can be detrimental to the health of the cells, causing them to be torn apart.[27] Furthermore, sparging necessary gases such as oxygen into a bioreactor can result in the production of foam, which is known to kill cells.[7] These conditions are often alleviated through the addition of viscosity-increasing chemicals and antifoaming agents, thereby reducing shear and foam damage. However, these additives also have a direct effect on the osmolality of the medium, and osmolality effects may harm the cells more than the other physical conditions within the bioreactor. Thus, it is important to determine a safe osmolality range for the culture of neural stem cells.

Oxygen Concentration. Studies have shown that cultures vary in their oxygen requirements. For example, it has been found that the size of tumor spheroids was limited by the amount of oxygen in the culture, suggesting that low levels of oxygen adversely affect tumor cell proliferation.[8] However, studies on hematopoietic stem cells revealed that reducing the oxygen tension enhanced their proliferation in long-term cultures.[23,24] A study published by Shingo *et al* suggests that neural

stem cells also respond to changes in oxygen levels.[44] They reported that neurogenesis is upregulated under hypoxic conditions both *in vitro* and *in vivo*, and that this upregulation is mediated by erythropoietin (EPO) which acts as an autocrine/paracrine factor.

Mammalian neural stem cells grow as aggregates of cells *in vitro* (called neurospheres). Oxygen in the bulk medium diffuses into these spheres in order to supply all of the cells within. However, even though the oxygen concentration in bulk medium may be adequate for the survival of cells at the surface of these spheres, it is possible that beyond a critical sphere diameter, the cells at the center of the aggregate do not receive adequate amounts of oxygen. Though evidence suggests that NSC activity may be upregulated under low O_2 conditions, low levels of O_2 in larger aggregates or in a scaled-up culture system may actually result in greater cell death.[44,19] Thus, determining the sensitivity of neural stem cells to different concentrations of oxygen is important.

Cell Storage. It is essential to maintain a cryopreserved stock of young cells, especially in a clinical setting where the quantity of procured cells from a given source is limited, and thus, must be protected in a safe environment until required. Neural progenitors derived from murine embryonic stem cells have been cryopreserved using 10% DMSO. They retain their multipotency and ability to generate functional neurons, however, the viability after thawing was only 52%.[13] In another study, human fetal CNS progenitor cells were cryopreserved with post-thaw viabilities between 70–95%, and the ability of these cells to form neurons was not hampered by two freeze-thaw cycles.[50] In our paper, additional data on cryopreservation of murine neural stem cells is presented.

Methods and Materials

Cell Lines

The work presented here was conducted using both mouse and human neural stem cell lines.

Murine Cell Lines. Several murine neural stem cell lines (W1, W2, W3, W10, and W16) were used to conduct the experiments described here. The cells were obtained as a gift from the laboratory of Professor Sam Weiss at the University of Calgary, Alberta, Canada. A murine GFP expressing NSC line was provided by Professor Mendez, Dalhousie University, Halifax, Canada. Primary cultures of murine cells were derived from the striatal region of the forebrain of Embryonic Day 14 (E14) CD1 albino mice as outlined by Reynolds and Weiss.[33] Cells were obtained as a primary culture in NM medium.[34] A cell bank was developed by isolating the cells from the primary culture, placing them in NM medium containing 10% DMSO, and freezing them in 1.0 mL aliquots. When needed, the aliquots were thawed and placed into PPRF-m4 medium for two days, after which the cells were passaged into fresh PPRF-m4 medium (passage level 1).

Human Cell Line. Human neural stem cells (hNSCs) were obtained as a gift from Professor Clive Svendsen at the Waisman Center, University of Wisconsin. The cells were derived from the cortex of an eight to 14 week human post-mortem fetus, and named M006 cortex cells. After procurement, the M006 cortex cells were grown in a medium containing EGF, bFGF, and heparin for four weeks. The cells were fed every three to four days by removing spent medium and replacing it with fresh medium. They were passaged every seven to 14 days on average using a McIlwain tissue chopper to section the neurospheres. During the fifth passage, the cells were frozen in serum-free freezing medium (Sigma C-6295). The medium composition as well as a more detailed description of the passaging protocols can be found in Svendsen *et al.*[46] M006 neurospheres were supplied to our laboratory in cryovials on dry ice. After receipt, the cells were stored in liquid nitrogen until cultured.

Medium Preparation

Medium development started with NM medium, a fully disclosed formulation that is widely reported in the literature.[33,34] Using this medium as a starting point, many studies were conducted in our laboratory and eventually resulted in the development of a new medium called PPRF-m4.[43] The basic culture medium was composed of the basal media DMEM (12100-046) and F12 (21700-075) from Invitrogen in a 1:1 mixture. Supplements to the basal media mixture included 5 mM HEPES (H-9136), 0.6% glucose (G-7021), 1.73 g/L sodium bicarbonate (S-5761), 2.0 mM glutamine (25030-016), 0.023 g/L insulin (I-5500), 20 nM progesterone (P-6149), 9.0 mg/L putrescine (P-7505), 0.025 g/L transferrin (T-2252), 30 nM sodium selenite (S-9133), and 20 µg/L EGF (100-15). All concentrations represent final concentrations in the medium. A proprietary supplement mixture developed in our laboratory was also added to the medium. All of the supplements were obtained from Sigma with the exception of glutamine (Invitrogen), and EGF (Peprotech Inc.). The medium was vacuum filtered through a 0.22 µm bottle-top filter (Falcon 7105) prior to use.

Cell Culture Equipment

Tissue culture flasks were obtained from Life Technologies (Gaithersburg, MD). Nunc 75-cm^2 Easyflasks (156499) were used for the maintenance of cell stocks.

Cell Counts and Viabilities

All cell-handling procedures, unless otherwise noted, were performed in a sterile laminar flow hood to prevent contamination by bacteria, fungus, or mycoplasma. Cell density was determined using a hemocytometer, and viability was determined using the standard trypan blue exclusion test (Sigma Chemicals, T8154). Duplicate cell counts were performed by mechanically dissociating spherical aggregates and then taking a sample. The sample was diluted in Ca^{2+} and Mg^{2+} free phosphate-buffered saline (PBS) (0.2 g/L KCl, 0.2g/L KH_2PO_4, 8.0 g/L NaCl, 2.16 g/L $Na_2HPO_4 \cdot 7H_2O$, all from Sigma), and then stained with 0.1% trypan blue.

Cell Passaging

Stationary Culture. Murine cells in stationary culture were expanded in 25 cm^2 tissue culture flasks (T-flasks) and passaged every four days. Cells were harvested by pipetting the T-flask contents into a 15 mL centrifuge tube.

The cell suspension was centrifuged for 10 minutes at 1,000 rpm (173g), and most of the supernatant was removed. Approximately 200 µL (including the cell pellet) was left in the 15 mL centrifuge tube. A homogeneous single cell suspension was obtained for inoculum by mechanically dissociating (triturating) the pellet that remained with a 200 µL pipette (VWR brand tips, 53509-000). The pipette was set at 30 µL less than the volume of the pellet, and the cells were triturated 30 times. While triturating, the tip was held pressed against the bottom of the centrifuge tube at an angle, and the sides of the centrifuge tube were periodically rinsed with the cell suspension to dislodge any attached spheres. Care was taken not to form bubbles in the cell suspension. After the spheres had been dissociated, a small sample was taken for counting, and then the cells were inoculated at 0.75 x 10^5 cells/mL into 5.0 mL of pre-incubated PPRF-m4 medium in 25 cm^2 T-flasks. The T-flasks were placed in a humidified incubator maintained at 37° C and 5% CO_2. Cells were passaged four days later.

Suspension Culture. All suspension culture experiments were performed in 125 mL bioreactors (spinner flasks) from Corning (Corning Glass, NY). Prior to use, the inner surface of the flasks and the outer surface of the magnetic stir bars were siliconized with Sigmacote (Sigma, St. Louis, MO) to prevent the cells from adhering. The flasks were agitated by placing them on Thermolyne Cell-Gro slow-speed magnetic stirrers (Thermolyne, Iowa) set at a speed of 100 rpm.

The bioreactor contents were passaged by pipetting four 10 mL aliquots from the spinner flask, and placing each aliquot in a separate 15 mL centrifuge tube. The aliquots were then centrifuged for 10 minutes at 1000 rpm (173g), and most of the supernatant was subsequently removed. Approximately 200 µL (including cell pellet) was left in each 15-mL centrifuge tube. A homogeneous single cell suspension was obtained in each tube by mechanically dissociating the contents. The contents of the four centrifuge tubes were then consolidated into a single 15 mL tube, and a small sample was taken for counting. The cells were inoculated at 0.75 x 10^5 cells/mL into 100 mL of pre-incubated PPRF-m4 medium in a 125 mL spinner flask. All spinner flasks were placed in a 37° C incubator with a water-saturated atmosphere containing 5% CO_2, and the cells were passaged every four days.

Oxygen Uptake Studies

Studies involving oxygen uptake kinetics were conducted in a modified spinner flask. The flask had been truncated, and the top was replaced with a large rubber stopper that supported an impeller and contained several ports. Medium and cells were placed in the flask leaving no headspace. A calibrated oxygen probe was then used to measure the concentration of oxygen in the medium over time. Because the dissolved oxygen in the medium is the only source of oxygen for the respiring cells, the rate of oxygen depletion from the medium would be equal to the rate of oxygen uptake by the cells. This relationship can be described mathematically as an unsteady-state oxygen mass balance:

$$\frac{dC_{O_2}}{dt} = (-q_{O_2})\,(X) \qquad \text{Eqn. 1}$$

The dissolved oxygen concentration (DO) in the medium was measured by a calibrated probe as a percentage of the maximum oxygen saturation in the medium. The value of C_{O_2} was determined from the DO readings using the relationship:

$$C_{O_2} = \left(\frac{DO}{DO_{span}}\right)(C^*_{O_2}) \qquad \text{Eqn. 2}$$

Substituting Eqn. 2 into 1 and simplifying:

$$\frac{d(DO)}{dt} = (-q_{O_2})\,(X)\left(\frac{DO_{span}}{C^*_{O_2}}\right) \qquad \text{Eqn. 3}$$

The value of $C^*_{O_2}$ was determined using Henry's Law:

$$p = Hx \qquad \text{Eqn. 4}$$

The proportionality constant H has a value of 5.182 x 10^4 atm for oxygen dissolved in water.[31] As the source of oxygen in these experiments was air at atmospheric pressure, the partial pressure of oxygen has a value of 0.21 atm. Using these values, and assuming that the molality of the medium is the same as water (55.6 mol/L), the value of $C^*_{O_2}$ was calculated to be 2.25 x 10^{-4} mol/L.

Cryopreservation of Cells

Cell Freezing. The cells to be frozen were aseptically isolated during their exponential growth phase and placed in 15 mL centrifuge tubes. The samples were centrifuged for 10 minutes at 1,000 rpm. After removing as much supernatant as possible, the pellet was resuspended in 1.0 mL of cryoprotective medium composed of 10% DMSO in normal medium. Resuspension was achieved by passing the pellet through a 200 µL pipette several times. It should be noted that the resuspension was done gently because the objective was to separate the pellet into spheres and not single cells. Each sample was quickly transferred to a 1.2 mL cryovial (Nalgene, 5000-0012), and then the cryovials were placed in a freezing container (Nalgene, 5100-0001). The freezing container had been previously prepared by filling it with 250 mL of isopropanol (before adding the sponge) at room temperature. The freezing jar was placed in a -80° C freezer overnight. The cryovials were quickly removed from the freezing jar and placed in canes for subsequent storage in a 35 L liquid nitrogen dewar. This method results in an acceptable sample cooling rate of -10° C/min.[14]

Thawing of Cryopreserved Cells. Cryopreserved cells should be thawed as rapidly as possible to minimize the exposure of thawed cells to DMSO which is believed to affect cell viability. After removal from the dewar, the cryovials were immediately placed in a Styrofoam box containing liquid N_2, and the box was transferred to a biosafety cabinet. The cryovials were removed from the

liquid nitrogen, and the caps on the vials were loosened (not removed) to exhaust any nitrogen that may have seeped into improperly sealed vials. The vial caps were then tightened, and the cryovials were placed in a 37° C water bath (Haake, Model L D8) and gently agitated until the samples had thawed. After removal from the water bath, the exterior surface of each cryovial was washed with 70% ethanol to ensure sterility. Using a 2 mL pipette, the contents of each cryovial were placed in a 15 mL centrifuge tube containing 10 mL of PPRF-m4 medium. Each cryovial was subsequently rinsed with 1 mL of medium, which was added to the centrifuge tube. The samples were centrifuged for 10 minutes at 1000 rpm. As much supernatant as possible was removed from each centrifuge tube, and the pellets were gently resuspended in 2.0 mL of PPRF-m4 medium. Each sample was used to seed a culture vessel that had been preincubated with PPRF-m4 medium for 24 hours at 37° C and 5% CO_2. After two days, the cells were subcultured.

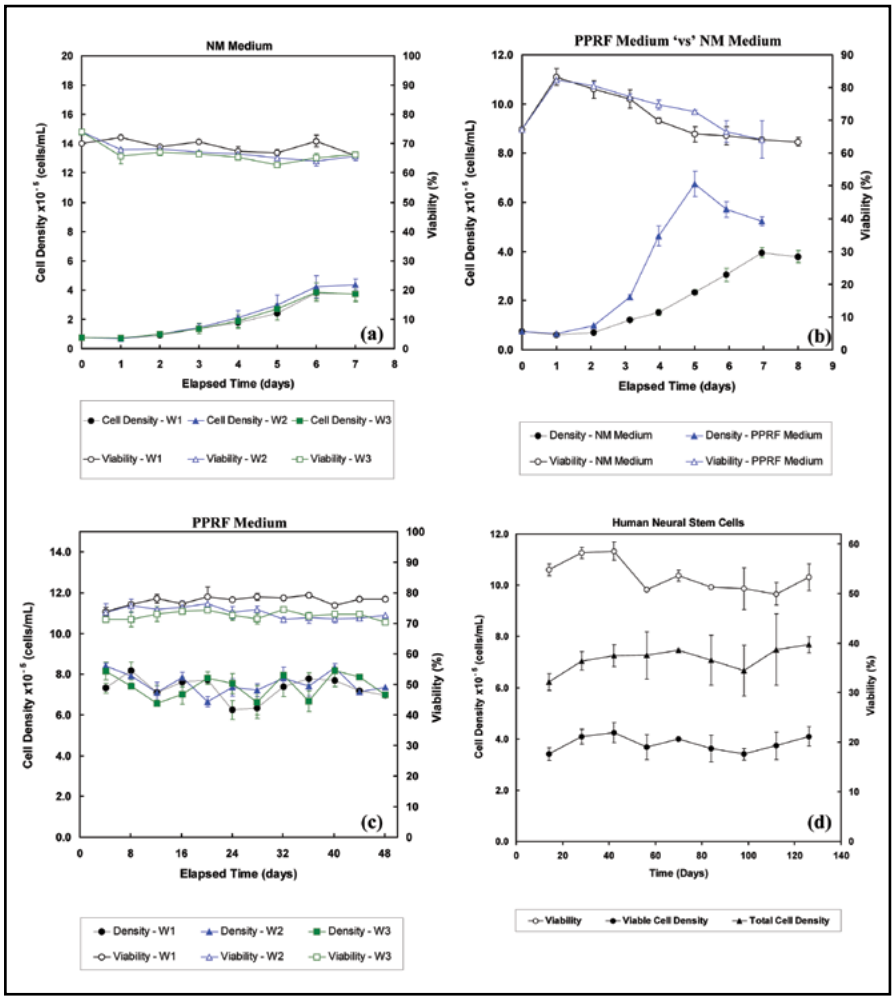

Figure 4. A new medium developed in our laboratory has the capacity to expand both murine and human neural stem cells. (a) Viable cell density and viability for cell lines W1, W2, and W3 expanded in NM medium. The cells were inoculated at 75,000 cells/mL into T-25 flasks containing 5 mL of NM medium. The flasks were then incubated at 37° C in a humidified incubator containing 5% CO_2. (b) The cell density and viability of murine neural stem cell line W3 inoculated into NM medium and PPRF-m4 medium. (c) The cell density and viability of cell lines W1, W2, and W3 serially passaged in PPRF-m4. The cells were inoculated at 75,000 cells/mL into T-25 flasks containing 5 mL of medium. The flasks were then incubated at 37° C in a humidified incubator containing 5% CO_2, and passaged every four days. (d) The viable cell density, total cell density, and viability of human neural stem cells (cell line M006) being serially passaged in PPRF-m4. The cells were inoculated at 200,000 cells/mL into T-25 flasks containing 5 mL of PPRF-m4 medium. The flasks were then placed in a 37° C incubator with a humidified atmosphere containing 5% CO_2. The cultures were fed 2 mL of fresh medium on days four, eight, and 12, and an equal volume of spent medium was removed. After 14 days, the human cells were passaged into fresh PPRF-m4 medium.

Immunocytochemistry. The protocols used to stain neurons, astrocytes, and oligodendrocytes have been previously described elsewhere.[43]

Clonal and Population Analyses. The protocols used to conduct clonal and population analyses have been previously described elsewhere.[43] In general, these assays utilize the formation of a neurosphere from a single cell as the hallmark of a neural stem cell to determine the frequency of stem cells within a given neurosphere or culture sample.

Results and Discussion

Development of a New Medium

Due to the exponential increase of NSC research, several different growth media have been reported in the literature for NSC expansion. The most commonly used murine medium in the literature has been NM medium. The growth of three murine NSC cell lines in NM medium is shown in Figure 4a. The maximum cell density for each cell line was approximately 5.3 times greater than the inoculation density of 7.5 x 10^4 cells/mL, and occurred six days post-inoculation. Using this medium as a starting point, we conducted a large number of experiments en route to the development of a new medium. Our efforts included evaluating different commercially available basal media, and determining the effects of sugars, amino acids, trace elements, vitamins, hormones, and different combinations of growth factors (results not shown).[43] This resulted in the development of a new medium called PPRF-m4 medium.

As shown in Figure 4b, this new med-ium resulted in significantly greater cell expansion, and relatively high viabilities compared to NM medium. There was significantly less cell debris present in the cultures containing PPRF-m4 medium. In addition, this medium could maintain the expansion of murine NSCs procured from different sources for an extended period of time (Fig. 4c), with no loss of the capacity

to self-renew as determined by clonal and population analyses (results not shown), and no loss of multipotentiality as determined by immunofluorescence (results not shown). Photomicrographs showing neural stem cell aggregates in PPRF-m4 (day 4 post-inoculation) are shown in Figure 5. Figures 5a and 5b show NSCs that were not genetically modified, and Figures 5c and 5d show that a GFP transgenic cell line was also capable of growing in suspension by forming neurospheres.

It should be noted that the cost of medium is an important consideration when developing a scaled-up process. The cost of PPRF-m4 medium was 40% less than the cost of NM medium. Moreover, although developed for murine cells, PPRF-m4 also proved able to support the serial expansion of fetal human neural stem cells (Fig. 4d). All subsequent experiments were performed using the new PPRF-m4 formulation.

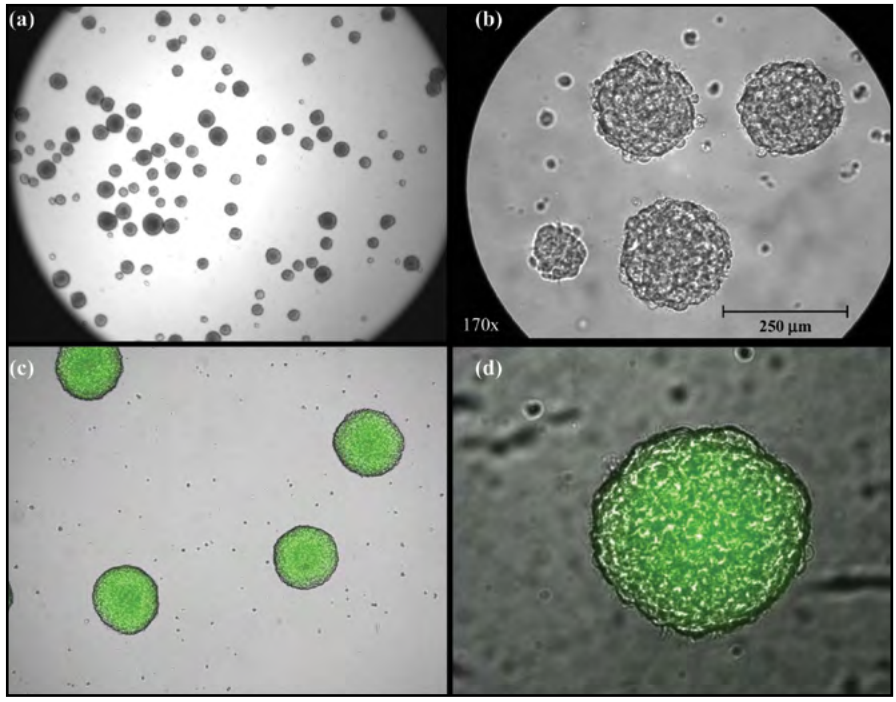

Figure 5. When grown in PPRF-m4 Medium, neural stem cells form clusters of cells called aggregates (neurospheres). Photomicrographs showing (a) murine neural stem cell aggregates after being cultured for four days in suspension culture bioreactors, and (b) a close-up view of murine neural stem cell aggregates after being cultured for four days in suspension culture bioreactors. (c) GFP labeled murine neural stem cell aggregates after being cultured for four days in suspension culture bioreactors, and (d) a single GFP labeled murine neural stem cell aggregate generated after four days in a suspension culture bioreactor. This aggregate is 110 µm in diameter, and contains approximately 1,000 cells.

Development of Bioreactor Protocols

Inoculation Density. A study was conducted to establish a seeding threshold for murine NSCs, and to investigate how inoculation density impacts subsequent proliferation rates in both stationary (T-flasks) and suspension culture (bioreactors).

Tissue Culture Flasks. Figure 6a shows the results of seeding T-flasks between 1,000 and 300,000 cells/mL, and measuring the cell density after four days in culture. Up to a seeding density of 100,000 cells/mL, the general trend was that the greater the initial cell density, the greater the final cell density. However, at 300,000 cells/mL, the average final cell density was actually lower than that observed when seeding with 100,000 cells/mL. We also determined an inverse relationship between the inoculation density and the final viability, which was not unexpected because cultures inoculated at low density and resulting in a low final density, were not subjected to the adverse environmental conditions experienced by cells in high-density cultures.

The inoculation density also had a significant impact on the measured multiplication ratio (MR = Final viable cell density / Initial viable cell density) of the cultures as shown in Figure 6b. In general, the lower the inoculation density, the higher the achieved multiplication ratio after four days in culture. At an inoculation density of 10,000 cells/mL, the MR value was approximately 10.5, whereas the MR at 100,000 cells/mL was approximately eight, which was 24% lower. At a seeding density of 300,000 cells/mL, the MR had a low value of 2.3, and many of the cells were found to have attached to the surface of the T-flask. This corresponded to a doubling time of 71.4 hours, approximately 275% longer than at 10,000 cells/mL. This agrees well with similar trends previously reported for murine NSCs by Kallos and Behie.[19]

At low cell densities, cells do not experience limitations in nutrients or oxygen. Furthermore, they are not subject to detrimental levels of toxic metabolic by-products such as lactate and ammonia. It is interesting to note that decreased expansion was not observed between cultures inoculated at 1,000 cells/mL and 10,000 cells/mL. In fact, the average MR value at 10,000 cells/mL was approximately 5% higher than at 1,000 cells/mL. It is possible that increased cell-cell contact under conditions where nutrient limitations and waste levels were negligible resulted in this slight increase in the MR value. Cell-cell contact has been found to be essential in the expansion of human neural stem cells.[46] It is also possible that the cells produce autocrine or paracrine growth factors which result in cell division. Under the prevailing culture conditions, these factors would have accumulated to significant levels at a faster rate in those cultures initiated with a higher seeding density. At cultures inoculated at very high cell densities (greater than 10^5 cells/mL), other detrimental aspects of the culture (such as diffusional limitations within the aggregates) may have negated their positive effects, thereby resulting in low MR values.

Bioreactors. The effect of varying inoculum levels in batch suspension culture was investigated using 125 mL bioreactors. The bioreactors were inoculated with a dissociated single cell suspension at concentrations of 1,000 cells/mL, 75,000 cells/mL, and 300,000 cells/mL. The resulting growth curves are shown in Figure 6c. Cell proliferation was evident at all three inoculation levels. The highest maximum cell density was attained in the culture inoculated at 300,000 cells/mL. It is interesting to note that the average maximum cell density of 1.15 x 10^6 cells/mL in this culture occurred three days following inoculation. The cell density on day four was 8.0 x 10^5 cells/mL, which is very similar to the density of 7.0 x 10^5 cells/mL achieved four days after inoculation in T-flasks. This suggests that the cell density may also have peaked in T-flasks on day three, but since the T-flasks were not counted until day four, the maximum cell density was missed. If that is the case, then the cell proliferation data in Figures 6a and 6b underestimate what actually occurred.

The maximum cell density at an inoculum level of 75,000 cells/mL occurred five days post inoculation. Initially, the magnitudes of the cell density values in the culture inoculated with 1,000 cells/mL were small relative to the other cultures. However, by day 10, the cultures achieved a maximum average cell density of 6.2 x 10^5 cells/mL. This corresponds to a multiplication ratio of 620, which dwarfs the MR values of 10.5 (day five) and 3.9 (day three) achieved at inoculum levels of 75,000 cells/mL and 300,000 cells/mL, respectively (Fig. 6d). Although the greatest MR value was observed in the culture inoculated at the lowest density, the greatest exponential phase growth rate (μ_{max}) was observed at an inoculation density of 75,000 cells/mL (Fig. 6c). The growth rate of 0.0334 h^{-1} was significantly greater than the growth rates of 0.0291 h^{-1} and 0.0215 h^{-1} observed at the low and high inoculum level cultures respectively. Based on this data, there does not appear to be a correlation between inoculation level, and maximum specific growth rate. The viability data corresponding to the growth curves in Figure 6c showed similar trends to other growth curves of murine NSCs (data not shown).[40]

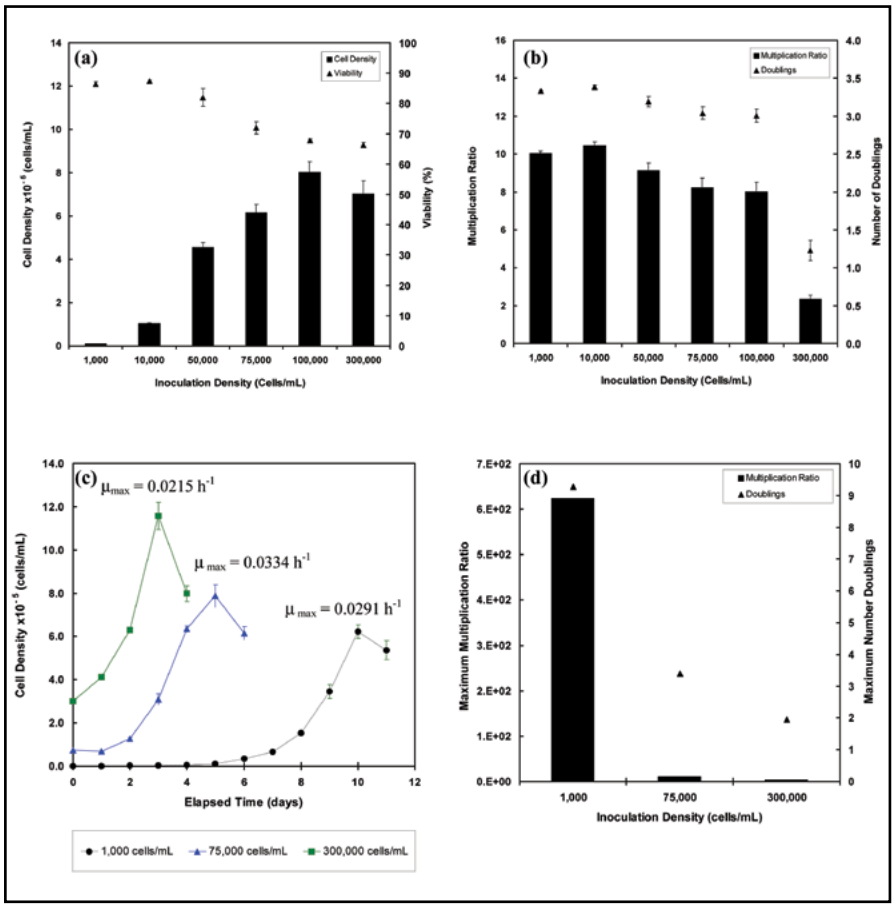

Figure 6. Inoculation density is known to have a significant effect in certain mammalian cell cultures. (a) Effect of inoculation density on measured cell density and viability in stationary cultures of murine neural stem cells. Single cells were inoculated at various densities into T-25 flasks containing 5 mL of PPRF-m4 medium. The flasks were then placed in a 37° C incubator with a humidified atmosphere containing 5% CO_2 for four days. (b) Effect of inoculation density on the multiplication ratio and cumulative number of doublings for the results shown in (a). (c) The effect of inoculation density on the subsequent measured cell density in suspension culture. Single cells were inoculated at various densities into 125 mL spinner flasks containing 100 mL of PPRF-m4 medium. The flasks were then placed in a 37° C incubator with a humidified atmosphere containing 5% CO_2. The agitation rate was controlled at 100 rpm using a magnetic stir plate. The spinner flasks were sampled daily. (d) The effect of inoculation density on the maximum measured multiplication ratio and number of doublings in suspension culture for the results shown in (c).

Medium pH

We have previously reported pH effects on neural stem cells in culture.[39,19] Briefly, murine NSC expansion was found to be affected by initial culture pH, with optimum growth occurring between pH values of 7.3 and 7.7. When either sodium bicarbonate or Hepes was excluded from the medium, no proliferation was observed, indicating that these two buffers act in concert to provide the proper conditions for NSC expansion. The greatest proliferation was observed in those cultures that had an initial pH of approximately 7.5 (as measured in 5% CO_2 at 37° C). This pH is at the upper end of the physiological pH range (7.0–7.5). These NSCs were found to produce relatively small quantities of acidic metabolic by-products during culture (indicating adequate oxygenation) that lowered the pH to 7.3 during the exponential phase of growth.

Medium Osmolality

Osmolality is a measure of the number of particles in a given volume of solution. Mammalian neural stem cells are known to be sensitive to changes

in osmolality.[42] We have previously reported that osmolality levels above 360 mOsm/kg can cause a sharp decrease in NSC proliferation in PPRF medium, and can cause significant cell death beyond 450 mOsm/kg in a proprietary medium.[42,19]

In the study by Sen and colleagues, the osmolality of PPRF-m4 medium was manipulated by adding NaCl. A 1.0 g/L increase in the concentration of NaCl resulted in a 32 mOsm/kg increase in the osmolality. No significant effect was observed at osmolality values up to 360 mOsm/kg (relative to 320 mOsm/kg).[42] However, increasing osmolality to 380 mOsm/kg and beyond was detrimental to the cells. The measured cell density at 380 mOsm/kg was significantly lower than in the control medium, and this decline became more evident as the osmolality increased to 420 mOsm/kg. Prior to conducting this experiment, it had been hypothesized that at high osmolalities, the cells would shrivel and die due to a net outward movement of water that would result in low viability. However, though the cells did appear to be smaller in size than the cells in the control culture, and not as bright and spherical, many were still alive. These cell populations were comprised primarily of single cells, and cells in small aggregates, and thus may not have experienced the high shear that is known to kill up to 30% of cells in large aggregates (such as those in the control culture) during mechanical dissociation.[20] Moreover, under normal osmolality conditions, dead cells may remain intact through the dissociation process, whereas at high osmolalities, they may not be as robust, and thus break apart. As broken cells cannot be accounted for using trypan blue analysis, the measured viability would be artificially high. Based on these results, it was decided to maintain the osmolality of the neural stem cell environment in bioreactors below 360 mOsm/kg.

Oxygen Effects

The experiments involving oxygen effects were completed in two parts. First, the oxygen consumption rate, also known as the Oxygen Uptake Rate (OUR), was determined for exponentially growing neural stem cells in PPRF-m4 medium. Second, the effects of oxygen concentration on cell proliferation and culture viability were investigated.

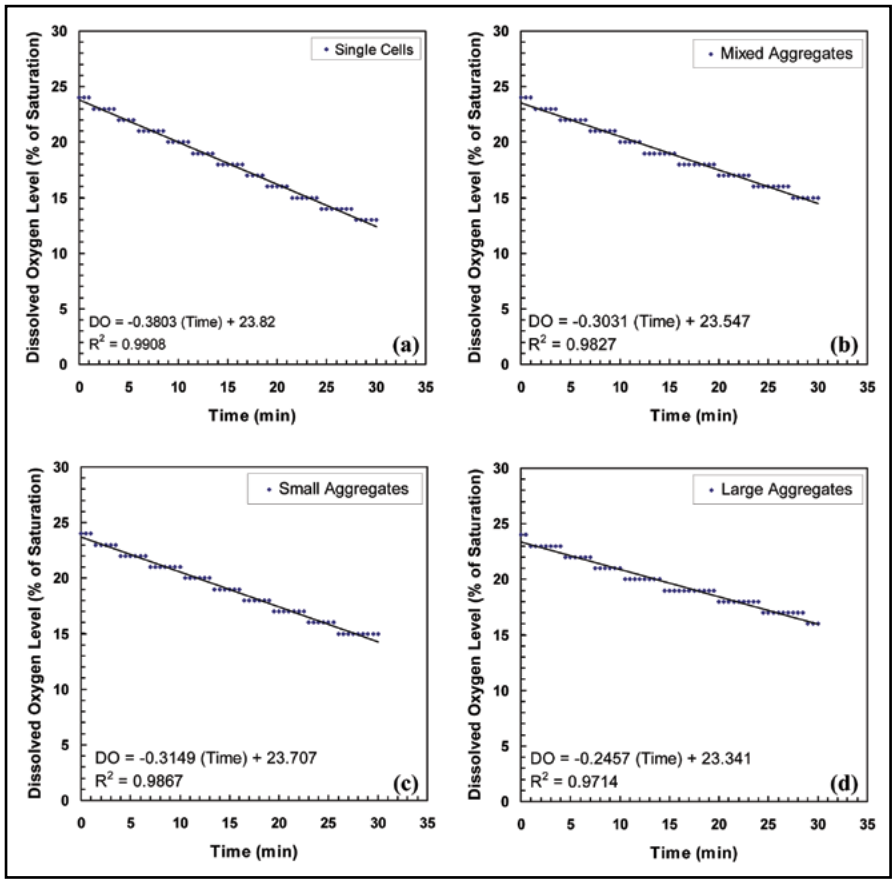

Figure 7. Dissolved oxygen (DO) levels as a function of time for neural stem cells in PPRF-m4 medium. Oxygen depletion in the medium was measured for (a) single cells, (b) mixed size aggregates, (c) small aggregates, and (d) large aggregates.

Oxygen Uptake Kinetics. The cells used to conduct the study were all aggregates isolated from day three (exponential phase) spinner flask cultures (cell line W3, passage 12). OUR values were determined for the cells under four different conditions. In Condition 1, the aggregates were centrifuged, mechanically dissociated, and inoculated into the modified spinner flask as single cells. In Condition 2, aggregates isolated from a spinner flask culture were allowed to settle for five minutes in centrifuge tubes. The spent medium was removed, and the aggregates (mixed sizes) were resuspended in 30 mL of fresh PPRF-m4 medium prior to being inoculated into the modified spinner flask. Conditions 3 and 4 were conducted to determine if the OUR for large and small aggregates are different. In both conditions, the contents of a day three spinner flask culture (100 mL) were allowed to gravity sediment for one minute. This created an environment containing a gradient of aggregate sizes in the culture. The top 20 mL of the culture was discarded. The middle 40 mL of the culture contained small aggregates. The bottom 40 mL of culture contained large aggregates. In Condition 3, the middle 40 mL were carefully removed and placed in a centrifuge tube and allowed to gravity sediment for three minutes to ensure that all of the small aggregates had settled. The spent medium was then removed, and replaced with 30 mL of fresh medium prior to inoculating the modified spinner flask with small aggregates for OUR determination. Condition 4 was identical to Condition 3 except that it involved the bottom 40 mL of the culture, and the aggregates were allowed to settle for one minute in the centrifuge tube to ensure

that only large aggregates were used. After all of the OUR measurements were recorded, the aggregates were recovered and the cell densities in the modified spinner flask were determined. These cell densities were used in Eqn. 3.

The value of d(DO)/dt in Eqn. 3 was determined by calculating the slope of the line generated when the measured DO concentration was plotted as a function of time. The graphs in Figure 7 show that the rate of oxygen depletion from the medium was different in each of the four conditions tested. The greatest rate of depletion occurred for the single cells (Condition 1), and the lowest rate occurred for the large aggregates (Condition 4). Using the values derived from these graphs, along with the other parameters calculated above, it was possible to determine the oxygen consumption rate (q_{O_2}) for each of the four conditions from Eqn. 3. The cell densities, d(DO)/dt values, and calculated q_{O_2} values are given in Table 1. The calculated consumption values agree well with literature values for mammalian cells that range from 1.39×10^{-17} mol O_2/cell•s to 2.8×10^{-16} mol O_2/cell•s (reviewed in Kallos, 1999) and measured values for NSCs in other media (3.06×10^{-17} mol O_2/cell•s).[19]

Table 1. The measured cell density, cell viability, oxygen uptake rate, and specific oxygen consumption rate for mechanically dissociated single cells, small aggregates, large aggregates, and a mixture of small and large aggregates together. Also given is the specific oxygen consumption rate for a mixture of small and large (3–4 day old) NSC aggregates in a proprietary medium as reported by Kallos and Behie.[20]

Condition	Cell Density (cells/mL)	Viability (%)	d(DO)/dt (%DO/min)	q_{O_2} (mol O_2/cell•s)
1 – Single Cells	7.5×10^4	63.1	-0.3803	1.8×10^{-16}
2 – Mixed Aggregates	7.8×10^4	69.5	-0.3031	1.5×10^{-16}
3 – Small Aggregates	5.7×10^4	71.1	-0.3149	2.1×10^{-16}
4 – Large Aggregates	1.3×10^5	68.1	-0.2457	6.9×10^{-17}
Kallos and Behie[20]	–	–	–	3.1×10^{-17}

Oxygen consumption values can be an important indicator of culture health, especially in aggregate cell cultures. Single cells do not generally experience oxygen limitations when they have adequate access to oxygen in the bulk medium, thus their consumption levels are high. However, as aggregates form and start to grow in size, the cells within rely on the diffusion of oxygen into the aggregate from the bulk medium. When aggregates are small, no diffusional limitations occur. However, as aggregate size exceeds a critical diameter, cells at the center of the aggregate may start to experience O_2 deprivation. In these cultures, the average specific oxygen consumption rate would tend to decrease.

The results obtained in this study show that the oxygen consumption rates for single cells and small aggregates are at the high end of the range published in the literature, indicating no limitations. However, the oxygen consumption rate for the large aggregates (Condition 4) was significantly lower than for single cells and small aggregates. This indicates that cells within large NSC aggregates may have been experiencing O_2 deprivation. Therefore, a method should be found to limit aggregate size below a critical diameter, or to efficiently increase O_2 levels in the bulk medium. We have previously reported that neurosphere diameter can be controlled before reaching a dimension limit for which necrosis would be expected to occur in suspension bioreactors.[40] The consumption rate for the mixed aggregates (Condition 2) was in-between the values for the small and large aggregate Conditions.

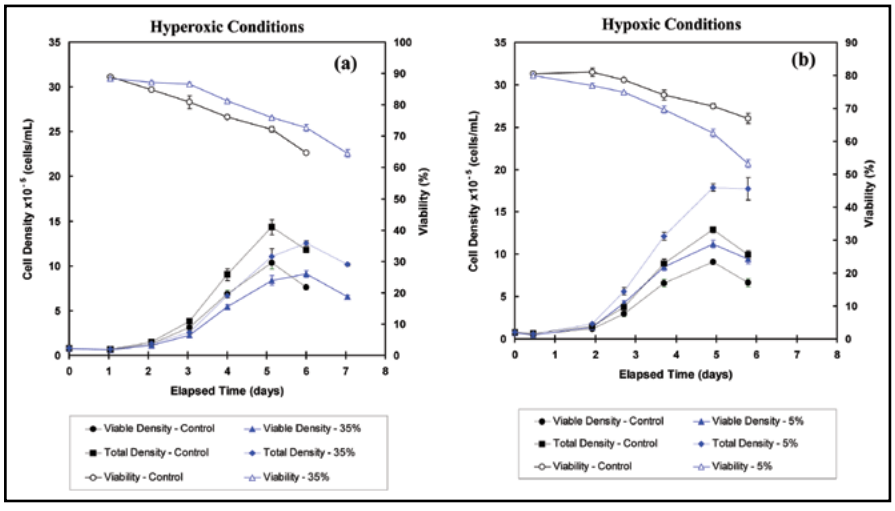

Figure 8. Oxygen levels in culture were found to have an impact of neural stem cell expansion. (a) The effect of 35% oxygen (hyperoxic conditions) on cell proliferation and culture viability in spinner flasks as compared to cells grown in a control culture with 21% oxygen (from air) (normoxic), and (b) the effect of 5% oxygen (hypoxic conditions) on cell proliferation and culture viability in spinner flasks as compared to cells grown in a control culture with 21% oxygen (from air). The peak average viable cell density (day five) in (b) was statistically higher than in the control culture. In both (a) and (b), single cells were inoculated into 125 mL spinner flasks containing 100 mL of PPRF-m4 medium. The flasks were placed in a 37° C incubator with a humidified atmosphere containing 5% CO_2. The agitation rate was controlled at 100 rpm using a magnetic stir plate. The spinner flasks were sampled daily.

Effect of Oxygen Concentration. We undertook a study to determine the effect of oxygen concentration on neural stem cell cultures evaluating oxygen concentrations of 5% (hypoxic), 21% (air – control culture) and 35% (hyperoxic). We produced special gas blends to conduct these studies: the hypoxic gas blend was a mixture of 5% O_2, 5% CO_2, and 90% N_2; and the hyperoxic gas blend contained 35% O_2, 5% CO_2, and 60%

N_2. A flow-through system was devised in order to continuously supply these gases directly into the headspace in the spinner flasks. The spinner flasks were always operated with one of the caps cracked open to maintain atmospheric pressure. Due to potential flammability, special caution was observed while working with the hyperoxic gas mixture.

The cell density and viability in the hyperoxic culture are shown in Figure 8a. Based on the OUR data, we hypothesized that increasing the oxygen concentration in the culture would result in higher viabilities, as well as higher proliferation rates. Although higher viability values were observed relative to the control culture, the proliferation rate was actually lower. The exponential phase specific growth rate was 0.0308 h^{-1}, whereas in the control culture it was 0.0346 h^{-1}. Also, the peak cell density of 9.1 x 10^5 cells/mL occurred slightly later than the control culture peak density of 1.0 x 10^6 cells/mL.

We investigated the effect of hypoxic conditions because high oxygen levels resulted in a decreased rate of cell proliferation. It had previously been demonstrated that low oxygen (i.e. 5% O_2) results in lower viabilities of murine NSCs grown in stationary culture, and much lower (40% decrease from control) proliferation and viabilities in suspension culture.[19] This was also accompanied by large increases in the amount of lactate produced by the NSCs.

The results of reducing the oxygen concentration in culture to 5% O_2 from 21% O_2 (control) are shown in Figure 8b. Lowering the oxygen concentration resulted in a greater amount of proliferation of NSCs. The exponential phase specific growth rate in the hypoxic culture was 0.0378 h^{-1}, and 0.0331 h^{-1} in the control culture. The day five peak cell density in the hypoxic culture was approximately 23% higher than the peak density of 9.1 x 10^5 cells/mL in the control culture. Furthermore, as expected, a significantly lower viability was evident in the hypoxic culture relative to the control culture starting at day two. This suggests that the hypoxic conditions did not adversely affect the cells until aggregate growth was well under way. Furthermore, the actual amount of proliferation that occurred in the culture cannot be appreciated simply by considering the viable cell density. As shown in Figure 8b, the total cell density, which takes into account the measured viability when determining the amount of proliferation that occurred, was much greater under hypoxic conditions than in the control culture.

The different levels of oxygen in the hypoxic, hyperoxic, and control cultures resulted in noticeable physical changes within the aggregates. The aggregates were stained with 0.1% trypan blue to visualize membrane-compromised (dead) cells, and then rinsed with PBS to remove any excess stain. When visualized under the microscope, the aggregates in the control culture were found to contain stained cells at the center of the aggregate (Fig. 9a). The aggregates were not sectioned. Because they were translucent, the stained cells were visible by simply observing them through a microscope. Under hypoxic conditions, the staining within the aggregates appeared to be more intense than in the control. However, when aggregates were isolated from the hyperoxic cultures and subsequently stained, the staining intensity was significantly lower (Fig. 9b). These observations suggest that limitations in oxygen are at least partially responsible for the death of cells in the center of aggregates that have grown beyond a critical diameter.

Increased proliferation under hypoxic conditions, as we observed, is not surprising. Living organisms have evolved adaptive processes designed to promote tissue protection and regeneration when exposed to oxygen deficient conditions.[3] An example of this is the production of erythropoietin (EPO) to promote the proliferation and differentiation of erythroid progenitor cells. The EPO receptor has also been discovered colocalized with nestin in murine E14 neural stem cells, and a recent study has shown that EPO can promote the production of neuronal progenitor cells *in vitro*.[44] Thus, it is possible that hypoxic conditions promote the expansion of neural cells in suspension culture. However, in future studies, it will be important to determine the exact composition of that population. Although the total concentration of cells increased under oxygen deficient conditions, the proportion of neural stem cells may have decreased. Furthermore, it will be necessary to conduct oxygen concentration studies over extended periods of time to determine the effect of long-term exposure to hypoxic conditions. Until those studies have been completed, it would be prudent to continue using air as an oxygen source in culture.

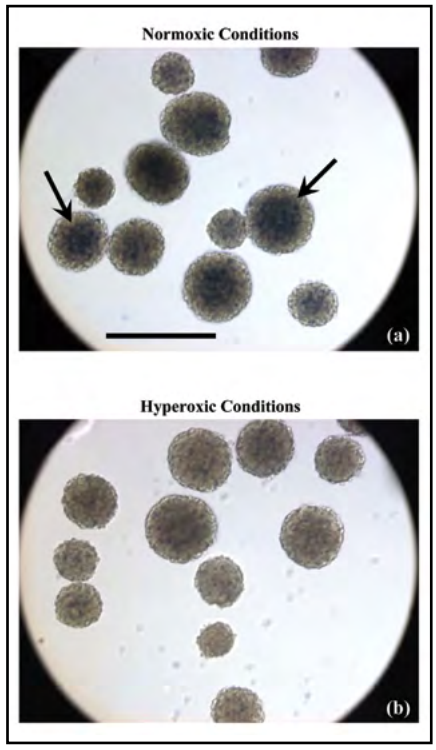

Figure 9. Photomicrographs showing the effect of (a) 21% O_2 (normoxic condition) and (b) 35% O_2 (hyperoxic condition) on the morphology of NSC aggregates. The aggregates were stained with 0.1% trypan blue and then washed in PBS. The proportion of stained dead cells in the aggregates appear to decrease at higher oxygen levels, suggesting that oxygen limitations are at least partially responsible for the death of cells within neural stem cell aggregates. The scale bar in (a) represents 250 µm. The arrows in (a) are pointing to examples of stained dead cells in the center of the aggregates.

Cryopreservation Issues

This section will examine cryopreservation issues as they pertain to neural stem cells. Specifically, the effect of different cryopreservation methods, the

Table 2. The six methods evaluated for neural stem cell cryopreservation.

Method	Cryopreservation Medium	Cryopreservation Form
1	PPRF-m4	Aggregates
2	PPRF-m4	Single Cells
3	PPRF-m4 + 10% DMSO	Aggregates
4	PPRF-m4 + 10% DMSO	Single Cells
5	PPRF-m4 + 10% Glycerol	Aggregates
6	PPRF-m4 + 10% Glycerol	Single Cells

effect of repeated freezing and thawing, and the effect of long-term preservation will be discussed.

Cryopreservation Method. Often, when mammalian stem cells are frozen and then thawed, a significant percentage of the cells die. Thus, we evaluated the cryopreservation techniques being used for the cryostorage of neural stem cells. Freezing neural stem cells as aggregates in growth medium containing 10% dimethyl sulfoxide (DMSO) is common practice.[51] As DMSO is known to be toxic, we decided to investigate if DMSO is actually necessary, and to determine if it could be replaced with glycerol. Glycerol has been widely used in the cryopreservation of embryos.[15,30] In conjunction with this study, we decided to investigate if freezing single cells versus aggregates made any significant difference in cell survival following cryopreservation.

This study was conducted using W16 cells at a passage level of eight. The cells were isolated from a spinner flask culture three days after inoculation, and thus were in the middle of the exponential growth phase. Six different methods were investigated as described in Table 2. In methods 1 and 2, cells were frozen as single cells and aggregates respectively in unmodified PPRF-m4 medium. In methods 3 and 4, cells were again frozen as either single cells or aggregates respectively, but in this case, PPRF-m4 was modified with 10% DMSO. Methods 5 and 6 were identical to methods 3 and 4 except that glycerol was used instead of DMSO. The protocol was devised so that each sample had an identical number of cells prior to cryopreservation. The cell density and viability of the cells were measured prior to cryopreservation to determine a benchmark against which the cells could be measured after being thawed.

The results of this study are presented in Figure 10a. Prior to cryopreservation, the samples that were subsequently frozen were determined to have an average cell density of 9.65 x 10^5 cells/mL, and an average viability of 71%. All samples were thawed quickly in a 37° C water bath, and then analyzed. It should be noted that samples that were frozen as single cells were mechanically dissociated prior to being thawed. However, samples that were frozen as aggregates were mechanically dissociated after being thawed. In this way, all of the samples being analyzed were subject to only a single episode of trituration during this experiment. Using methods 1 and 2 (no cryoprotectant) resulted in cell densities and viabilities that were dismal relative to pre-freezing values. The viability values were below ten percent, and only three to four percent of the cells survived the procedure. This indicates that PPRF-m4 alone is not a suitable freezing medium. Using methods 3 and 4 with both PPRF-m4 and 10% DMSO, the results were much better. Approximately 73% of the cells frozen as aggregates survived, although the viability of the sample did drop 22%. The samples that were frozen as single cells did not do as well as the aggregates. Approximately 60% of the single cells survived the cryopreservation. The viability in this case dropped to 43%.

When comparing methods 3 and 4 to 1 and 2, it is evident that DMSO has the ability to protect cells to some degree during cryopreservation. It is also clear that a significantly greater number of cells survived when frozen as aggregates. It is possible that the clustering of cells formed a niche that provided some degree of protection for the cells located within the aggregate. Using methods 5 and 6 (glycerol cryoprotectant), the cells, regardless of form, did not fare very well. Whereas the PPRF-m4 + 10% glycerol provided some degree of protection when compared to PPRF-m4 medium alone, cell survival was poor in comparison to cells cryopreserved in PPRF-m4 + 10% DMSO. It should be noted, however, that cell survival was again observed to be better in aggregates than as single cells. Overall, this study indicates that freezing the cells does result in some degree of cell death. However, relative to other methods tested, the current protocol for neural stem cell cryopreservation (10% DMSO) should be maintained.

Effect of Repeated Cryopreservation. We undertook a study to determine if repeated freezing and thawing has an adverse effect on neural stem cells. W10 cells frozen at a passage level of one were thawed out as per the protocol described in the Methods and Materials section, and placed in fresh PPRF-m4 medium to recover. After two days of recovery, the cells were isolated, and inoculated in fresh medium at 75,000 cells/mL. After four days in culture, the cell density and viability were measured, and the cells were refrozen thus concluding cycle one of the experiment. These refrozen cells were subjected to the same procedure four more times for a total of five cycles. The effect of repeatedly freezing and thawing the same cells is shown in Figure 10b. We expected that if repeatedly freezing and thawing had an effect on the cells, it would appear as a definite trend. However, we found that although the measured cell density does change, it does not do so in any definitive pattern. Based on the results from this experiment, it may be concluded that repeatedly freezing and thawing neural stem cell aggregates in PPRF-m4 + 10% DMSO does not have any greater effect on the cells than simply freezing and thawing the cells once.

Effect of Long-Term Cryopreservation. We also investigated the effect of extended cryopreservation on murine neural stem cells frozen as aggregates

in PPRF-m4 + 10% DMSO. NSC W10 cell samples frozen at a passage level of two were thawed out every six months for a total of two years. Prior to freezing, cell samples were analyzed, and cell density and viability measurements were recorded. After being thawed, the samples were allowed to recover for two days in fresh medium prior to being inoculated into a new culture at 75,000 cells/mL. After four days in culture, the cells were harvested, and the cell density and viability were measured. The results from this study are displayed in Figure 10c. The measured cell densities at 0, 12, and 24 months were comparable to the cell density of 9 x 10^5 cells/mL determined prior to freezing. However, when thawed out at six and 18 months, the measured cell densities were slightly lower (6.4 x 10^5 and 6.7 x 10^5 cells/mL, respectively). The range of viability values was small (71 to 76.4%) and no different than the viability of 74% measured prior to freezing. Visually, with the exception of the number of cells, no differences were noted between the cultures. This study suggests that cells can be cryopreserved in PPRF-m4 medium containing 10% DMSO for at least two years without suffering any adverse effects, other than those associated with a single freeze-thaw cycle.

Conclusions

We have examined the effects of different environmental conditions and cryopreservation protocols on murine neural stem cells with the aim of developing a bioprocess for NSC expansion. Inoculation density was found to affect subsequent proliferation. As expected, lower inoculation levels resulted in lower final cell densities. However, seeding density was found to be inversely related to the multiplication ratio and viability. This was especially evident in spinner flask cultures. We investigated other environmental factors including osmolality and oxygen levels. Osmolality was found to be a concern only above 360 mOsm/kg. Studies of oxygen uptake rates found that the average cellular consumption of oxygen was lower in large aggregates compared to small aggregates or single cells indicating a possible oxygen deficiency in large aggregates. Growth curves generated at different oxygen levels indicated that lower O_2 levels (5%) slightly increased the rate of proliferation, whereas higher levels (35%) caused a slight decrease in the proliferation rate. However, at 35% O_2, necrotic centers in large aggregates were less apparent, suggesting that reduced oxygen tension plays a role in the death of cells within aggregates. Finally, cryopreservation studies found that DMSO is a better cryoprotectant than glycerol, and that aggregates appear to survive cryopreservation better than single cells. Also, repeated freeze-thaw cycles, and cryopreservation for up to two years did not appear to have adverse effects on cells.

These results suggest that neural stem cells are very responsive to their environment, and as such, when a scaled-up bioprocess for the efficient and reproducible generation of neural stem cells is established, it is extremely important to maintain optimal culture conditions to maximize proliferation.

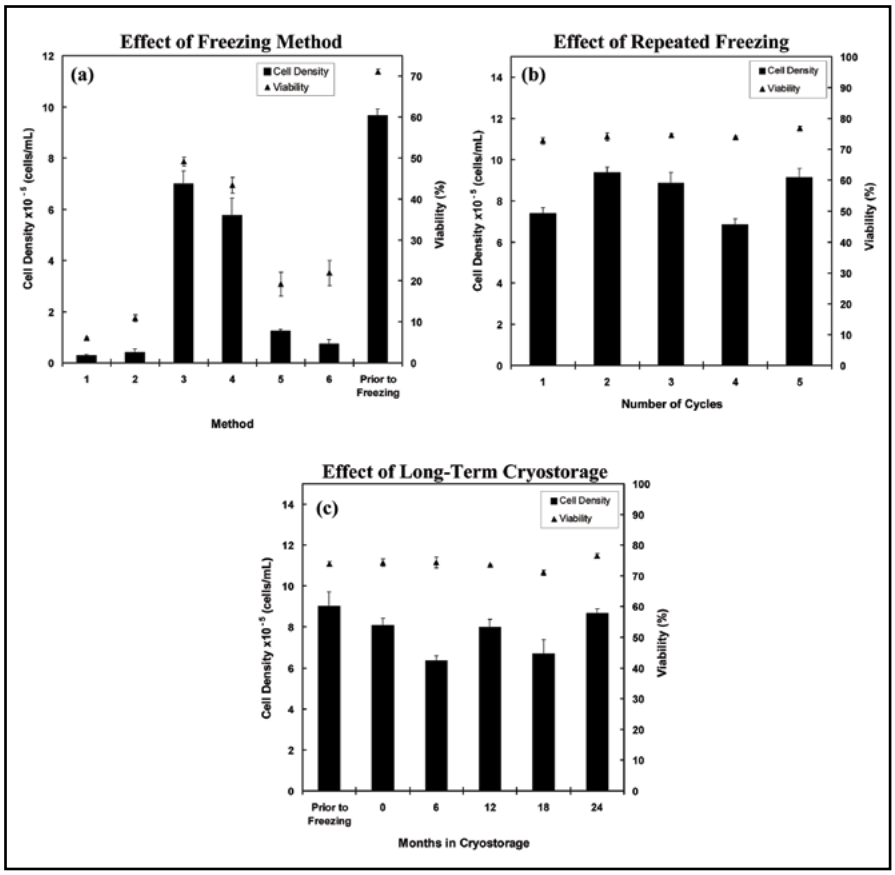

Figure 10. Following cell expansion, it may be necessary to cryostore cells for a period of time prior to being used in a certain application. Thus, developing effective cryopreservation methods, as well as determining the effect of cryopreservation of the cells is necessary. a) The effect of cryopreserving murine neural stem cells using six different methods. Methods one through six are outlined in Table 2. The cell density and viability were measured prior to cryopreservation to act as a baseline against which the methods could be evaluated. The cells remained cryopreserved for nine days before being thawed. The highest post-thaw cell densities were obtained using methods three and four. In each of these cases, the viable cell densities were significantly lower than the value prior to freezing. (b) The effect of repeatedly thawing, culturing, and then freezing the same cells (method 3). In this case, cells from the W10 cell line that had been frozen at a passage level of one were used to initiate the experiment. The cells were subjected to a total of five thaw-culture-freeze cycles. (c) The effect of extended cryopreservation on cell proliferation and culture viability (method 3). Cells from the W10 cell line were initially frozen at a passage level of two. Prior to freezing, the cell density and viability of the cells were measured. The frozen cell samples were thawed out and cultured at six month intervals for a period of 24 months.

NOMENCLATURE

C_{O_2}	Concentration of Dissolved Oxygen (mol O_2/L)
t	Time (h)
q_{O_2}	Oxygen Consumption Rate (mol O_2/cell•h)
X	Viable Cell Density (cells/L)
DO	Dissolved Oxygen reading (% of saturation)
DOspan	Maximum DO reading (%) = 100%
$C^*_{O_2}$	Equilibrium DO Concentration (mol O_2/L)
p	Partial pressure of O_2 in air (atm)
x	Mole fraction of O_2 in the medium (mol O_2/mol medium)
H	Proportionality constant (atm)

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