Using an In Vitro Model of Hypoxic-Ischemic Encephalopathy to Study Stem Cell Therapy in the Developing Brain

Alan D. Salgado, Candace Rossignol, Dr. Brent Reynolds, and Dr. Michael Weiss

College of Medicine, University of Florida

Hypoxic-ischemic encephalopathy (HIE) can result in long-term neurological disabilities and even death in newborns. Stem cell therapy provides a potential treatment for neonatal brain damage after HIE as stem cells have the ability to migrate to areas of injury and differentiate into multiple phenotypes. The objectives of this study were to establish an in vitro model of HIE and examine the application of this model to understand the optimal timing of transplants, the number of transplants needed, the amount of transplanted cells necessary to rescue injured endogenous cells, and the mechanism of protection. Hypoxia chambers perfused with 5% CO₂ gas combined with glucose free artificial cerebrospinal fluid were used to replicate HIE, while the lactate dehydrogenase (LDH) assay measured cytotoxicity. After exposure to hypoxic gas for 24 hours, cell death increased (as measured by absorbance) in P1 multipotent astrocytic stem cells (MASCs) at 6, 12, and 24 hours and in E14 neuroblasts at 24 hours, as compared to controls (p < 0.05). The degree of injury was characterized into mild, moderate, and severe categories. This in vitro model of HIE can be used to assess the application of stem cell therapy in future in vitro and in vivo studies.

INTRODUCTION

Hypoxic-ischemic encephalopathy (HIE), or a lack of adequate oxygen and glucose supply to the entire brain, can result in chronic neurological disabilities and even acute mortality in newborns (Shalak & Perlman, 2004; Vannucci & Hagberg, 2004). HIE occurs in approximately 2 to 4 of 1,000 full-term births and has an incidence approaching 60% in low birth weight, premature newborns (Pimentel-Coelho & Mendez-Otero, 2010; Vannucci & Hagberg, 2004). The brain injury that develops after HIE is an evolving process, beginning with a hypoxic-ischemic insult and ending in the death of neuronal tissue. The initial insult triggers multiple cellular and molecular cascades resulting in a toxic environment characteristic of oxidative stress, cell death, inflammation, and endogenous repair (Ferriero, 2004). Most treatments, although unsuccessful, have taken a neuroprotective approach in reducing ischemic injury in effort to salvage short- and long-term neurodevelopmental outcomes (Shalak & Perlman, 2004). Due to the poor outcomes of HIE and the lack of success in developing neuroprotective strategies, stem cell therapy may provide an innovative approach to prevent further brain damage and rebuild circuitry by providing diffusible (i.e. neurotrophins) and non-diffusible factors (i.e. cell-to-cell contact guidance) that can facilitate proliferation, migration, and differentiation of neurons (Burns, Verfaillie, & Low, 2009). Thus, studies conducted in an in vitro model of HIE can provide insight into the effectiveness of stem cells in rebuilding neural circuitry in the damaged brain, allowing investigators to narrow the clinical variables for a well-designed animal model.

Although neonatal HIE remains a significant cause of neurological disability early in life, therapeutic interventions such as hypothermia have been able to slow down and impede delayed injury at an early developmental stage (Shankaran et al., 2005). Animal models have revealed the neonatal brain’s capacity to respond to injury, demonstrating characteristics of resistance and vulnerability depending on the circumstance (Yang & Levison, 2006). The endogenous response that takes place after injury, however, is inadequate in fully repairing the damaged tissue after HIE. In addition, the delayed oxidative damage and inflammation that takes place in the neonatal brain post-reperfusion present a detrimental environment for potential exogenous transplants.

Recent evidence has provided valuable insight into the possibility of transplanting progenitor/stem cells into the area of tissue damage in an animal model of HIE (Zheng et al., 2006). A better understanding of the migration and differentiation of progenitor/stem cells is imperative to investigate the possibility of using these cells to impede injury, augment the endogenous response, and rebuild lost and damaged neural connections. To this end, our lab has focused on building an in vitro model of neonatal HIE (i.e. oxygen-glucose deprivation model) in effort to study the potential of using stem cell therapy in the event of neonatal HIE. We hypothesized two stem cell lines, multipotent astrocytic stem cells (MASCs) and neuroblasts, will incur a rise in cell death (LDH) after being exposed to hypoxic gas over the course of 24 hours and stages of injury (mild, moderate, and severe) can be differentiated. By using this model, multiple variables, such as the composition and dose of transplants, the optimal timing of transplantation, and the mechanism of protection, can be controlled and
examined leading to a well-designed in vivo experiment. Future in vivo experiments will verify the power of the in vitro model in predicting in vivo results. The potential if a parallel is proven is powerful since performing experiments utilizing many variables in an animal model is logistically prohibitive.

**METHOD**

**Multipotent Astrocytic Stem Cells (MASCs)**

The method for generating MASCs has been previously described (Laywell, Rakic, Kukekov, Holland & Steindler, 2000; Zheng et al., 2006). Monolayers were generated in which 95-100% of the cells expressed the astrocytes marker GFAP; none expressed the neuronal marker β-III tubulin; a small percentage expressed the microglial specific surface protein CD11b. MASCs were dissected from subventricular zones (SVZ) of homozygous green fluorescence protein (GFP) transgenic neonatal mice between postnatal day 1 and 6. Tissue was incubated in 0.25% trypsin/EDTA and dissociated into a single cell suspension. Cells were pelleted and washed three times in medium before plated in culture flasks containing N2 medium supplemented with 5% fetal bovine serum (FBS). MASCs used for oxygen-glucose deprivation experiments were passed 1-3 times before collected via trypsinization and counted with a hemocytometer. Cells were counted and approximately two million cells were dispensed per well into a sterile 6 well culture plate.

**Neural Progenitor Cells/Neuroblasts**

Neural progenitor cells were harvested and microdissected from the ganglionic eminence of embryonic day 14 (E14) mice via the neurosphere assay (Azari, Sharir,fun, Rahman, Ansari & Reynolds, 2011). After neurospheres were harvested from the ganglionic eminence, they were plated at 200,000 cells per milliliter in complete neural stem cell (NSC) medium with 20 ng/mL of epidermal growth factor (EGF). After 5-7 days in incubation (37°C and 5% CO₂), the expanded neurospheres were isolated through the neuroblast assay. Neuroblasts were generated through this assay, and purified through fluorescence-activated cell sorting (FACS). Prior to experiments, neuroblasts were collected and counted with a hemocytometer. Two million neuroblasts were dispensed per well into a sterile 6 well culture plate.

**Experimental Procedure**

Experiments were conducted within a day of counting and plating the cells (both MASCs and neuroblasts) in a 6 well culture plate. To replicate oxygen-glucose deprivation in an in vitro system, artificial cerebrospinal fluid (CSF) without glucose was prepared to wash and plate the cells in. Artificial cerebrospinal fluid consisted of 122.0 mM NaCl, 3.3 mM KCl, 1.2 mM CaCl₂, 0.4 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, and 10.0 mg/L phenol red and was adjusted to a pH of 7.4. 5% CO₂ balanced with nitrogen gas was used to replicate HIE in vitro in both conditions, while 10% H₂ 5% CO₂, balanced with nitrogen gas was used to examine a difference in the effects of gases on MASCs. Two experimental (MASC and neuroblast cultures) and control conditions were examined.

Artificial CSF was warmed to 37°C in a water bath. After warming, artificial CSF was aerated with both gases (5% CO₂, balanced with nitrogen and 10% H₂ 5% CO₂, balanced with nitrogen) for 20 minutes. After 20 minutes, each condition was washed three times with the appropriate purged artificial CSF. The control time point was collected from each condition. Each hypoxia chamber was purged with the appropriate gas for 15 minutes to remove residual oxygen. After 15 minutes, each chamber was sealed and placed in the incubator. After 10 minutes in the incubator each chamber was purged, releasing a small amount of gas to prevent pressure buildup. After 1, 2, 3, 4, 5, 6, 12, and 24 hours of incubation, the MASC culture plates were removed and 0.125 mL of each sample was collected and placed in a 1.5 mL tube. After 1, 2, 3, 4, 5, 6, and 24 hours of incubation, the neuroblast culture plates were removed and 0.125 mL of each sample was collected and dispensed in a 1.5 mL tube. After each sample was collected, the cell culture plates were purged once again with the appropriate gas for 20 minutes, placed in incubation, and purged after 10 minutes. After each time point was collected, all samples were prepared for the cytotoxicity detection kit (LDH assay).

**Data and Statistical Analysis**

To measure cell death, the LDH assay was used to measure the amount of LDH released into the extracellular environment. LDH is a stable cytoplasmic enzyme present in all cells, thus LDH activity can easily be measured in culture supernatants by a single measurement at one time point (Aras, Hartnett & Aizenmann, 2008). After samples were collected, the samples were prepared to run through a spectrophotometric microplate reader. The absorbance (optical density) of each sample was measured. In addition, the absorbance measurements were studied using a one-way analysis of variance (ANOVA) and a Tukey-Kramer multiple comparisons test. Statistical significance was determined if the p value was less than 0.05. In assessment of LDH, the absorbance of all samples taken at each time point was averaged for both cell cultures.

**RESULTS**

**MASCs**

The LDH levels were higher in both of the experimental plates (5% CO₂, balanced w/ nitrogen and 10% H₂/5%
CO₃, balanced w/ nitrogen) at 6, 12, and 24 hours compared to the control plates (p < 0.05). Table 1 shows the mean optical density (OD) of all samples, as well as the time points of collection and the standard error, while figure 1 shows the changes in absorbance over time. The bolded value and asterisk at 6 hours of injury represents statistical significance (p < 0.05) while the bolded values and asterisks at 12 and 24 hours represent an extremely statistical significance (p < 0.0001) according to ANOVA and the Tukey-Kramer multiple comparisons test.

There was a small difference (0.001) between the mean OD at 6 hours of 5% CO₂, balanced with nitrogen gas exposed MASCs and 10% H₂/5% CO₂, balanced with nitrogen gas exposed MASCs, while there was no difference between the mean OD at 12 and 24 hours of both gas exposed cultures. While there was an incremental increase in the mean OD of control samples up until 24 hours, there was only an incremental increase in the mean OD of both gases between the 6 and 12 hour time points, while the mean OD at 24 hours remained the same (OD= 2.909).

Table 1. In Vitro HIE Model Using MASCs

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Control</th>
<th>5% CO₂,balanced w/ nitrogen</th>
<th>10% H₂/5% CO₂,balanced w/ nitrogen</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Mean OD</td>
<td>Standard error</td>
<td>Mean OD</td>
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<tr>
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<td>0.04375</td>
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<td>0.1018</td>
<td>0.09017</td>
</tr>
<tr>
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<td>0.1048</td>
<td>0.1235</td>
</tr>
<tr>
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<td>0.1139</td>
<td>0.1293</td>
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<td>0.05433</td>
</tr>
<tr>
<td>5</td>
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<td>0.09267</td>
</tr>
<tr>
<td>6</td>
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<td>0.1783</td>
<td>*1.546</td>
</tr>
<tr>
<td>12</td>
<td>1.293</td>
<td>0.1746</td>
<td>**2.909</td>
</tr>
<tr>
<td>24</td>
<td>1.688</td>
<td>0.1774</td>
<td>**2.909</td>
</tr>
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</table>

* values that were statistically significant and had a p-value less than 0.05
** values that were extremely statically significant and had a p-value less than 0.0001

Figure 1. Increase of absorbance (cytotoxicity) over time in MASC cultures after the exposure to two different gases, 5% CO₂ and 10% H₂.
Neuroblasts/Neural Progenitor Cells

The LDH levels were higher in the experimental plates (5% CO₂, balanced with nitrogen) at 24 hours of gas exposure compared to all other control plates (p < 0.0001). The neuroblast mean OD at 24 hours was 1.174, whereas the MASC mean OD reached 2.909 at 12 hours, and remained at 2.909 at the 24 hour mark. Table 2 shows the mean of all collected samples, as well as the time points of collection and the standard error, while figure 2 shows the absorbance over time for neuroblast cultures, with standard error bars. The bolded values and asterisks at 24 hours show extremely statistical significance (p < 0.0001) according to ANOVA and the Tukey-Kramer multiple comparisons test.

<table>
<thead>
<tr>
<th>Time (hours)</th>
<th>Control Mean OD</th>
<th>Standard error</th>
<th>5% CO₂-balanced w/ nitrogen Mean OD</th>
<th>Standard error</th>
</tr>
</thead>
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<tr>
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<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>24</td>
<td>0.6105</td>
<td>0.1006</td>
<td>**1.174</td>
<td>0.07726</td>
</tr>
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</table>

Figure 2. Increase of absorbance (cytotoxicity) over time in neuroblast cultures after the exposure to 5% CO₂ gas.

Stages of Injury

Stages of injury were characterized after examining the degrees of injury in the absorbance data. The three stages of injury assigned were mild injury, moderate injury, and severe injury. In the MASC cultures, there was a minimal amount of cell debris after 6 hours of exposure to both gases. 0 to 6 hours of gas exposure was therefore characterized as mild injury. The amount of cell debris peaked at 12 hours of gas exposure. Moderate injury was characterized to occur between 6 and 10 hours, while severe injury was characterized to occur after 10 hours of gas exposure. Figures 3(a) and 3(b) illustrate the rise in absorbance over time in both MASC and neuroblast...
cultures. In addition, the shaded regions correlate to the degree of the injury incurred.

![Graph](a)

![Graph](b)

**Figures 3.** Stages of injury were characterized according to the changes in absorbance over time. Figure 3(a) represents stages of injury characterized for MASC cultures, while Figure 3(b) represents stages of injury characterized for neuroblast cultures. Mild, moderate, or severe injury categories were assigned based on the changes of absorbance as a function of time.

**DISCUSSION**

Using an *in vitro* model of HIE may lead to a better understanding of how to optimize stem cell therapy in the neonatal brain. The potential of using stem/progenitor cells to target HIE through this model may provide further insight into enhancing future tedious *in vivo* studies by providing a high throughput system which can optimize variables (timing of transplant, dosing, dosing frequency). We have shown that an *in vitro* model of oxygen-glucose deprivation can be used to examine the rise of cell debris over time. Stages of injury were characterized to draw a
parallel to the clinical tool used by clinicians in diagnosing the severity of HIE injury (Sarnat & Sarnat, 1976). Although a true in vitro model of HIE entails the use of primary cortical cultures with this experimental setup, our findings provide insight into eventually using these cultures to replicate HIE injury in future studies.

As evident in the literature, stem cell therapy has been examined in animal models of human neurological diseases, and studies are currently investigating stem cell therapeutic approaches in animal models of HIE (Lindvall, Kokaia & Martinez-Serrano, 2004; Zheng et al., 2006). Our model of oxygen-glucose deprivation demonstrates a rise in cell debris after a distinct period of time in both MASCs and neuroblasts. As expected, there was a difference in the amount of cell debris in both cultures. More specifically, the MASC cultures reached what seemed to be a plateau of cell death as there was no difference in the absorbance between 12 and 24 hours of gas exposure (OD= 2.909), whereas the cell death in neuroblast cultures continued to rise through 24 hours of gas exposure. Both cultures were harvested from the mouse brain; however MASCs were derived from the SVZ of postnatal day 1 to 6 mice, and neuroblasts were derived from the ganglionic eminence of E14 mice. Previous reports have demonstrated that neurons are more vulnerable than glial cells to ischemia-induced injury; however the difference we found in LDH values must be attributed to the difference in the developmental stage of the mouse brain that was harvested, as we suggest neuronal precursors at an earlier stage of development are more resistant to a hypoxic-ischemic environment than MASCs (Wang, Shum & Wang, 2002). For the purpose of using these cell lines to replace damaged cells in this in vitro model, neuroblasts demonstrated a more robust response to hypoxic-ischemic conditions. Yet, the long-term viability of neuroblasts remains unknown.

In characterizing stages of injury, MASC cultures had a 6-hour window of cell death, where the rise of LDH started after 6 hours of gas exposure (5% CO$_2$ balanced with nitrogen and 10% H$_2$/5% CO$_2$, balanced with nitrogen) and stopped at 12 hours of gas exposure. Neuroblasts proved to be more resistant to hypoxic-ischemic conditions, and only increased to an OD of 1.174 after 24 hours of gas exposure. We speculated that the rise of LDH in both control cultures over time were attributed to the glucose free artificial CSF.

We expected MASC cultures to be more vulnerable to 10% H$_2$/5% CO$_2$ gas as compared to hypoxic-ischemic conditions. However, this was not the case. For LDH samples collected at 6 and 12 hours both conditions resulted in the same OD. The purpose of using both gases on MASCs was to determine a difference in injury, if one existed. Future studies conducted in both MASCs and other cultures may confirm this observation. Moreover, the exposure of neuroblasts to hypoxic-ischemic conditions resulted in a rise in LDH, yet, unlike MASCs, there was no plateau in cell debris. Neuroblasts must be exposed to hypoxic-ischemic conditions for a longer period of time.

Ultimately, we have established an in vitro model of HIE. With this model, we have assigned stages of injury that can be further used in studies examining stem cells’ effect at each level of injury. The ultimate goal of this study was to develop an in vitro system to achieve a greater understanding of the potential of using stem cell therapy in the neonatal brain after hypoxic-ischemic injury. With this system, we can eventually establish a well-designed in vivo experiment from understanding how stem cells can be used in this model. Building a parallel between an in vitro and in vivo model is powerful because performing experiments utilizing many variables in an animal model is logistically prohibitive.

Future studies should examine the effects of hypoxic-ischemic conditions on primary cortical cultures derived from a postnatal animal brain. In particular, cell cultures must be examined in two conditions: glucose-free artificial CSF and artificial CSF. These conditions will further show the difference glucose has on cells while exposed to hypoxia. In addition, stages of injury must be established for the purposes of examining differences in future stem cell candidates. The most effective concentration and time point in maximizing cell survival in this model must be assessed using stem cell transplant candidates, such as MASCs and neural stem/progenitor cells. Stages of injury will allow us to examine the differences in maximizing cell survival in the in vitro model and adjust the time points of stages of injury, if necessary.

From this study, we have established that MASCs are robust to a hypoxic-ischemic environment for a distinct amount of time. With this information, future studies can use MASCs to transplant into an in vitro model of HIE. These studies must also involve a comparison between isolated populations of transplants, as a mixture of cell phenotypes in each injection was a limitation in the Zheng et al. (2006) transplant model of HIE. These studies can potentially introduce trophic factors, such as brain-derived neurotrophic factor (BDNF) and bone morphogenetic protein 4 (BMP4) to study the potential of rebuilding neural connections that have been lost.

Another future implication is to examine multiple approaches in addressing neonatal HIE. As hypothermia has been proven to be effective in treating mild to moderate HIE in neonates, a study has demonstrated that hypoxic preconditioning in an in vitro model of oxygen-glucose deprivation results in ischemic tolerance in primary cortical neurons of embryonic rats (Bruer, et al., 1997; Zheng et al., 2006). Slowing down hypoxic-ischemic injury via hypothermia and enhancing the rebuilding process via stem cell therapy may result in partially restoring neurodevelopmental outcomes in the long-term.
REFERENCES


