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Can Microgravity Alter the Ability of the Brain to Self-Repair?

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Abstract

The effects of spaceflight on the human body are largely unknown. Research suggests that spaceflight results in a decline of cognitive ability, although the reasons behind it are not known. The purpose of our study was to test the hypothesis that simulated microgravity would impair the function of neural stem cells. Neural stem cells function to produce the different cell types in the brain, in addition to more stem cells. To test our hypothesis, we isolated and cultured cells *in vitro* from embryonic chicken brains in neurosphere assays with growth factors. Neurosphere assays test for stem cells via their function –the ability to produce more stem cells (to self-renew) and the different cell types in the brain. We assessed the number of neurospheres and, using antibodies specific to each cell type, their ability to produce the three main types of neural cells in the brain, neurons, astrocytes and oligodendrocytes. Confocal laser-scanning microscopy was used to capture images of the fluorescent labels attached to each cell type. Initial experiments were performed under normal gravity, whereas future experiments will be under simulated microgravity. We hope to discover whether or not simulated microgravity has an effect on the function of neural stem cells.

Introduction

Although the effects of spaceflight on the human body are largely unknown, previous research has shown that microgravity results in a decrease in the ability of stem cells to regenerate new cells and repair tissues (Blaber et. al, 2015). When astronauts return from space after experiencing microgravity for an extended period of time, astronauts show many deficits, including a decline in cognitive ability. A possible explanation is that the gravity on Earth is required for the proper development of the cells in the nervous system, including neurons. New neurons are produced from specialized cells termed stem cells. Nervous system stem cells can produce all of the different types of nervous system cells including neurons and glia. To test our explanation, one could compare the production of new neurons from nervous system tissue in simulated microgravity vs normal gravity.

Before comparing the effects of gravity on stem cells, an assay for culturing stem cells and allowing them to differentiate into mature neural cells needs to be established. A unique characteristic of stem cells is their ability to self-renew and create more stem cells (Van der Kooy and Weiss, 2000). When mouse neural cells are cultured in a dish, neural stem cells divide to form clusters of cells termed neurospheres. When plated at the appropriate density, each neurosphere originates from a single cell. Stem cells also have the ability to differentiate into mature neural cells. The three main cell types in the brain are neurons, astrocytes, and oligodendrocytes. Neurons function to transmit information, while astrocytes and oligodendrocytes are glial cells that are thought to support and protect the delicate neurons (Louis and Reynolds, 2013), however, recent evidence suggests that their roles are more complex. The process of transitioning from a stem cell to a mature neural cell, especially neurons, is important for proper brain development.

A neurosphere assay has already been developed for neural stem cells in mice (Reynolds and Weiss, 1992). However, a reliable neurosphere assay using neural tissue isolated from embryonic chicks has yet to be established. Here we sought to develop a neurosphere assay using brain tissue isolated from embryonic chicks. Our first tests determined the stage of embryonic development that would best produce neurospheres. Brain cells from embryonic chicks at different stages of development, were dissociated into single cells and plated at varying concentrations to test for neurosphere production. In this assay, it is important to show that the individual neurospheres are produced from a single cell ie. that they were clonal. We used statistical analysis to test the clonality of our neurospheres. After formation, the neurospheres were differentiated to determine the different types of mature brain cells they could produce. Antibodies and fluorescent tags that are specific to each cell type were used to determine which cell types were produced after differentiation of the cells found in the neurospheres.

After establishing our neurosphere assay for embryonic chick tissue, we can then use this assay to test how changes in gravity affect the development of neurons. We hypothesized that simulated microgravity compared to normal gravity, would impair the function of neural stem cells to produce (or regenerate) new neurons.

Identifying neural stem cells is a key area of research. However, delineating the combination of factors that can dictate the path of differentiation of neural stem cells, say to produce new neurons, is required prior for using stem cells for therapeutic medicine. Regenerative medicine using stem cells can help patients who have suffered from a central nervous system trauma by replacing the damaged cells (Louis and Reynolds, 2013).

Materials and Methods

Isolation of Chicken Brain Cells

Fertilized chicken eggs were incubated

at 37°C for 2.5 days (embryonic day 2.5). The brain tissue of the embryos was isolated and placed in 1x Ringers. Accutase enzyme (10 uL) was added to the isolated brain tissue, that had been resuspended in Neurocult medium (see below). The mixture was incubated at 37°C and 5% CO_2 for 5 minutes. The tissue was triturated to dissociate forming a single cell suspension. Viable cells were counted using a hemocytometer using trypan blue exclusion.

Culture of Brain Cells in the Neurosphere Assay

Single cells were plated into non-adherent dishes at varying concentrations (50,000, 100,000, 300,000, and 500,000 cells per 35 mm dish) with Neurocult medium and growth factors. Neurocult medium consisted of Neurocult medium with 1x proliferation supplement, 2mM L-glutamine, 100 ug/mL penicillin, and 100 ug/mL streptomycin (each from Stem Cell Technologies), with fibroblast growth factor (Sigma, 25 ng/mL) and epidermal growth factor (Sigma, 25 ng/mL). After 10 days of incubation at 37°C and 5% CO_2, the number of neurospheres was counted under a light microscope.

Differentiation of Neurospheres

After 11 days in culture, neurospheres were transferred to glass coverslips coated with collagen (5 ug/cm^2) and laminin (2 ug/cm^2) in differentiation medium. Differentiation medium consisted of Neurocult medium (Neurocult, 1x proliferation supplement, 2mM L-glutamine, 100 ug/mL penicillin, and 100 ug/mL streptomycin) plus 10% fetal calf serum. The cultures were incubated for 10 days at 37°C and 5% CO_2 prior to assessment of cell types with light microscopy via using cell morphology and fluorescent imaging using antibody staining. SPOT software was used to image the differentiated cells with light microscopy.

Antibody Staining

Antibody staining using immunofluorescence was done to determine the identity of the differentiated cells. Antibodies specific for glia (S100β;1 in 400; Sigma) and neurons (TUJ1;1 in 500; Covance) were used to visualize and quantitate the cell types present. DAPI (0.5 ug/ml) was used to visualize cell nuclei, to determine the total cell numbers in a field of view. Slides were washed twice for 5 minutes each in PBS, and then permeabilized in 0.1% Triton X-100/PBS/4% bovine serum for 40 minutes. The primary antibodies were incubated overnight at 4°C. The secondary antibodies were added in PBS/2% bovine serum for 45 minutes at room temperature. Slides were washed in PBS for 5 minutes twice and then incubated for 10 minutes in DAPI and washed in PBS for 5 minutes. The coverslips and slides were mounted with Prolong Gold (Invitrogen). An Olympus Fluoview FV1000 confocal microscope with FV10-ASW software was used for imaging the cells showing fluorescent staining with antibodies. All images were compiled using Adobe Photoshop 7.0

Results

Developing an *In Vitro* Neurosphere Assay with Embryonic Chick Tissue

The main goal of our studies were to test the effects of microgravity on the production and function of neural stem cells, as assayed by the growth and differentiation of neurospheres. Prior to comparing the effects of microgravity to the effects of normal gravity on neurospheres, an *in vitro* assay for growing neurospheres from chick tissue needed to be established. The stage of development of the embryonic chick was an important factor to test regarding how well the tissue would grow neurospheres in culture. To determine which developmental stage would best grow neurospheres and hence should be used for our *in vitro* assays, we tested brain cells from embryonic chicks that were incubated for 2 or 2.5 days (E2 or E2.5) post fertilization. Cell morphology and the linear relationship between cell input and the number of neurospheres produced, were used to decide

which developmental stage would be used for future experiments. Embryonic day 2.5 resulted in larger and more robust neurospheres (Fig. 1 A-D) than E2 (data not shown) indicating healthier cell growth. After 11 days of culture for the E2.5 brain cells, some cells derived from neurospheres, had begun to spontaneously differentiate into neurons and glia, as indicated by their morphology under the light microscope (data not shown). This is another indication of healthy cells in culture.

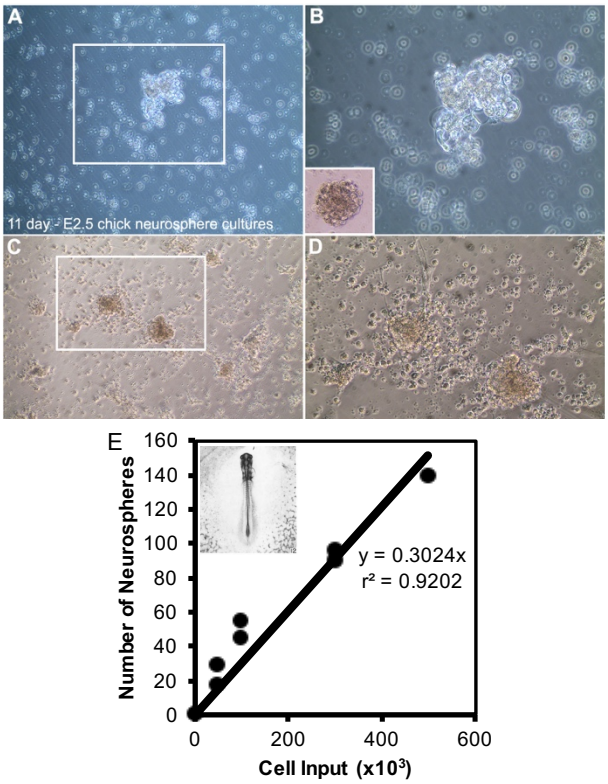

Figure 1. Neurosphere cultures from the E2.5 chick brain. Brain tissue was isolated and plated at 50,000 to 500,000 cells per 35 mm dish in Neurocult medium with growth factors. Each cell input was made in duplicate; each dot represents a single dish. A control without cells determined that the medium was not contaminated. Cultures were incubated at 37°C and 5% CO_2. On Day 11, the number of neurospheres in each dish was counted. A,B) Cultures contained healthy, robust neurospheres, some of which were symmetrical (inset). C,D) Some neurospheres adhered to the dish and began to differentiate, as indicated by cells with processes (arrow). E) Graph showing cell input vs the number of neurospheres, indicating a linear relationship; as the cell number increases so does the neurosphere number (r^2 = 0.9202). Inset shows chick at HH stage 12; the stage from which the brain cells were isolated.

The relationship between cell input and the number of neurospheres grown was assessed to indicate if individual neurospheres had developed from the division of single cells. For E2.5 cells, as the number of input cells increased, so did the number of neurospheres produced (Fig. 1E). This relationship had a good correlation coefficient (r^2=0.9202) providing strength for our result. In contrast, E2 cells did not show a strong correlation (data not shown) and we decided to use E2.5 brain tissue for all future experiments.

Immunofluorescence for Differentiated Cells

Antibody staining using immunofluorescence was done on E2.5 neurosphere cells after 10 days of differentiation on substrate-coated glass coverslips, in Neurocult medium with fetal calf serum rather than growth factors. Immunofluorescence with particular antibodies was done to determine the identity of the cell types formed in culture. Prior to antibody staining, many cell clusters were observed. As indicated by their distinctive morphologies, some of the cells were thought to be neurons, while others looked more like glia (Fig. 2 A-D).

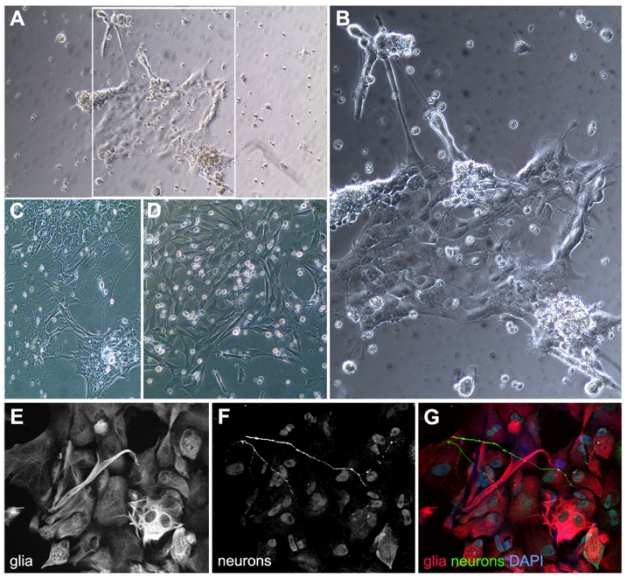

Figure 2. Differentiation of Cells from E2.5 Neurospheres. After 11 days in culture,

neurospheres were transferred onto glass coverslips coated with collagen and laminin substrates, to facilitate adhesion, and grown for an additional 10 days in medium with fetal calf serum, rather than defined growth factors. Cell identification was tested using antibodies specific for glia and neurons. A-D) Bright field images of differentiated cells after 10 days in culture, show different cell morphologies. White box in A) is magnified in B). E-G) Antibody staining using immunofluorescence. Independent data in grey is shown for E) glia and F) neurons, showing the signal emitted from only the red or green channels, respectively, for the antibodies seen together in (G). G) Glial cells are labeled with S100β (red) and neurons with TUJ1 (green). Nucleic acids are labeled with DAPI (blue). Glial cells were also confirmed using GFAP antibodies (data not shown). Most cells in culture are glia, with few neurons.

However, antibody staining was needed to confirm their cell identities. Glial cells were labeled with the antibody S100β (red), a calcium-binding marker, while neurons were detected with the antibody TUJ1 (green), that binds to intermediate filament proteins. To detect cell nuclei, nucleic acids were stained with the chemical DAPI (blue). The results of the antibody staining are shown in Figure 2 (E-G). The antibody staining showed that a majority of the cells were glial cells with only a few neurons present. We confirmed these results using an independent antibody GFAP (glial fibrillary acidic protein), for the detection of glia.

Discussion

Determining the Developmental Stage for the *In Vitro* Assay

To establish our neurosphere assay, we needed to determine the appropriate developmental stage for the isolation and culture of embryonic chick brain tissue. Based on the production and morphology of neurospheres, together with the linear relationship between cell input and the number of neurospheres grown, embryonic day 2.5 was chosen for our assays. Incubating the eggs for 2.5 day rather than 2 days, resulted in healthier and consistent numbers of neurospheres, that directly correlated to the cell input (Fig. 1). This developmental stage will be used for all future experiments.

Immunofluorescence for Identifying Differentiated Cells

Prior to antibody staining to determine the identity of each cell type, the differentiated cells were viewed using bright field microscopy, to visualize cell morphology. It appeared as though there were many more neurons growing and adhering to the glass cover slips than indicated by our antibody staining results. We think it is likely that the staining process and numerous washes were too harsh for the delicate neurons and their processes to remain adherent, and hence were not quantified by immunostaining. This would explain why we saw neurons using the light microscope that were not detected after fluorescent antibody staining. In contrast, glial cells are not as delicate as neurons and were not readily washed away during staining. Glia may also produce adhesion molecules that help them to maintain their grip on the substrates that we provided on our glass coverslips. For future experiments in order to quantitate the true number of neurons produced in culture, the immunofluorescence antibody staining process will have to be modified to be gentler on the neurons.

Comparing Normal Gravity to Microgravity

More experiments with embryonic chicks at embryonic day 2.5 will need to be done to assess the reproducibility of our neurosphere assay. Future experiments will compare the quantity of neurospheres grown in normal gravity compared to simulated microgravity, in addition to the cell types produced after differentiation. Quantitating the number of neurospheres and neurons and glia produced after differentiation for each gravity condition, will determine how microgravity affects neural stem cells and their function. One possible experiment for testing this would be to place the dissociated brain cells in microgravity prior to plating them in a neurosphere assay. Another possible

experiment would be to plate the single cells and allow them to develop into neurospheres before putting them in microgravity. Understanding how gravity affects the development of neurons is important for understanding how microgravity may affect astronauts following space flight and have implications for how to use stem cells in regenerative medicine.

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