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Weaning and Adaptation of SH-SY5Y Cells to Low Glucose Media for Manganese Exposure Studies

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Abstract

SH-SY5Y cells are a dopaminergic cell line commonly used as a model for neurodegenerative diseases. Parkinson's disease is characterized by a loss of dopamine signaling as a result of dopaminergic cell death within the substantia nigra. Manganism is an idiopathic Parkinsonism-like disease caused by an accumulation of manganese in cells of the basal ganglia and presents symptoms similar to Parkinson's. Extracellular dopamine levels are decreased in SH-SY5Y cells exposed to manganese. Several potential mechanisms may explain the effects of manganese on dopamine levels, including changes in dopamine metabolism. Growth of SH-SY5Y cells in high glucose Dulbecco's Modified Eagles Medium (DMEM) prevented us from discerning the levels of other dopamine metabolites using high performance liquid chromatography (HPLC) with electrochemical detection. The objective of this experiment was to determine if SH-SY5Y cells could be weaned off of DMEM high glucose media and adapted to DMEM low glucose or EMEM/Ham's F12 media, without altering cell viability. A sequential method was developed to adapt cells. The number of days between passages was recorded, and prior to each cell passage, a trypan blue exclusion assay was conducted to measure cell viability. SH-SY5Y cells were successfully adapted to EMEM/Ham's F12 media, whereas cell proliferation was hindered when grown in DMEM low glucose media. Cells grown in DMEM low-glucose media appeared to lose their morphology and detach from the growth surface. SH-SY5Y cells grown in EMEM/Ham's F12 media appeared larger in size compared to cells grown in DMEM high-glucose media. Ongoing studies are quantitating differences in cell size and examining the HPLC profile for EMEM/Ham's F12 media. If dopamine metabolite levels can be discerned, EMEM/Ham's F12 media may allow further analysis of dopamine metabolism in SH-SY5Y cells following manganese exposure for use as a model of neurodegenerative diseases.

Keywords: SH-SY5Y, dopaminergic, glucose

1. Introduction

Manganism, or manganese poisoning, is caused by an accumulation of this essential trace element in dopaminergic cells of the basal ganglia from prolonged atmospheric exposure, ingestion, and inhalation at concentrations higher than the suggested 2 mg/day[1-6]. Most cases of manganism are related to occupations where manganese exposure is common, such as welding, as well as ingestion after well water contamination[1,3,7-8]. It is considered a Parkinsonism-like disease because it presents similar, but not identical, symptoms to Parkinson's disease, including limb tremor, bradykinesia, and rigidity[4,7-9]. While a relationship between manganese accumulation and neurotoxicity has been recognized, its biochemical effects on dopaminergic cells are not well understood[1,3,5,10]. It is hypothesized that manganese accumulation in dopamine-producing cells may cause oxidative stress and metabolic dysfunction that leads to disruptions in neurotransmitter signaling and cell death in the basal ganglia[1,3-5]. Some studies have shown support for this notion, including observations of deregulation of glutamate homeostasis in GABAminergic cells of the basal ganglia[1,5,11]. Similarly, previous studies in our laboratory and others have exposed dopaminergic cell lines

to manganese and have observed a significant decrease in the concentration of extracellular levels of dopamine and decreased cell viability[9,12-15].

The SH-SY5Y cell line is an immortalized sub-line derived from the SK-N-SH line, which originated from cells from a bone marrow biopsy of a patient with neuroblastoma[16-19]. While cancer cell lines like this are most commonly used in cancer genetics and tumor formation studies, they are also used to study neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease, and Huntingdon's disease[16]. In designing an experiment using a cell line, it is important to weigh the pros and cons of its use and evaluate the conditions necessary to culture the cells. The value of the SH-SY5Y cell line as a model of neurodegeneration has been speculated and reviews for and against their use have been published[16,19]. The most notable support for the use of this cell line as an *in vitro* model in neurological studies include shared characteristics with dopaminergic neurons, such as the ability to synthesize dopamine[18,20], the expression of the transporter for the uptake of dopamine native to the central nervous system[21], and the presence of neuroprotective dopamine agonists also found *in vivo*[19]. SH-SY5Y cells are also able to differentiate into a more pronounced dopaminergic neuronal phenotype when exposed to different agents[16,19]. The drawbacks for this cell line include relatively low levels of expression of dopamine receptors and dopamine synthetic enzymes as compared to *in vivo* levels[19]. Agents that differentiate SH-SY5Y cells have some neuroprotective characteristics, making the cells less sensitive to neurotoxins than primary mesencephalic neurons, which is not ideal for studying the neurotoxic effects that contribute to Parkinson's disease[22]. While the SH-SY5Y cell line may not be ideal for all *in vitro* neurodegeneration studies, it is suitable to investigate the *in vitro* effects of manganese on dopamine metabolism and dopaminergic cell viability because of their dopaminergic characteristics and ability to proliferate over long periods of time. However, the experimental design should include a careful evaluation of the cell growth conditions to maintain and treat the cell line as these may impact the conclusions drawn from the study.

For example, this study arose to overcome technical challenges encountered when attempts were made to measure extracellular levels of dopamine metabolites in response to manganese treatment of SH-SY5Y cells grown in a high glucose Dulbecco's Modified Eagles Medium (DMEM) containing 4 mM L-glutamine and supplemented with fetal bovine serum and penicillin/streptomycin. Although the extracellular levels of several monoamine neurotransmitters such as dopamine, serotonin and 5-hydroxyindoleacetic acid were detected and measured using HPLC with electrochemical detection[13], other metabolites of interest were not detectable due to a large, unknown HPLC chromatogram peak that displayed a retention time off of the column similar to the expected retention time of the metabolites of interest (unpublished data). The large, unknown HPLC chromatogram peak was not attributable to the fetal bovine serum or penicillin/streptomycin solution (unpublished data). This led us to review the choice of media used within the study and to investigate the components found in the media in order to identify alternative growth media to culture the SH-SY5Y cell line. Due to the costs associated with purchasing new cells, we also sought an alternate method to transition cells between media types.

In this study, we transitioned the SH-SY5Y cell line between media with different glucose concentrations and other media components, like L-glutamine and other amino acids, and measured the effect of the transition on cell viability. Most established protocols for weaning and adapting cell lines were designed to transition cells from a serum-containing medium to a serum-free medium. After comparing several suggested protocols for cell adaptation[23-25], a sequential adaptation method, instead of a direct transition, was selected. A decrease in cell viability was not observed by the end of the adaptation process. However, cell proliferation or health of the cells grown in a DMEM low glucose media formulation appeared to decrease over time. In contrast, the cells appeared to proliferate and grow similarly in a 1:1 mixture of EMEM/Ham's F12 media compared to the DMEM high glucose media, making this a possible alternative growth media to culture the SH-SY5Y cell line. The results of this study also show the potential for using sequential adaptation methods to transition cell lines between media with differing formulations. Although not always necessary, this process may be preferred over buying and growing new cells in a different medium when funding is limited or when a primary cell source limits the availability of new cells.

2. Methodology

2.1 Literature Search

A literature search was conducted in PubMed to determine the most common media formulations used to culture the SH-SY5Y cell line in May 2014. Three different search criteria were used to identify free, full-text primary research articles: (SH-SY5Y[Title]) AND "cell culture"[Title/Abstract], (SH-SY5Y[Title]) AND glucose[Title/Abstract], and

(SH-SY5Y[Title]) AND dopamine[Title/Abstract]. Each article was reviewed for information regarding the media type and growth conditions used to maintain SH-SY5Y cells in culture.

2.2 Cell Culture

SH-SY5Y (CRL-2266™) cells were purchased from the American Type Culture Collection (ATCC®). Cells were grown in culture using three different media types: DMEM high glucose with 25 mM of D-glucose and 4 mM of L-glutamine (Fisher Scientific, SH30022), DMEM low glucose with 5 mM of D-glucose and 4 mM of L-glutamine (Fisher Scientific, SH30021), and Eagle's Modified Essential Medium (EMEM) (ATCC®, 30-2003™) mixed 1:1 with Ham's F12 medium (Lonza, 12-615) with a final concentration of 7.7 mM of D-glucose and 1.5 mM of L-glutamine. All media were supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were grown in T25 tissue-culture treated flasks coated with rat collagen I and maintained at 5% CO_2 at 37° C. Cells were fed every 2-4 days, and passaged when cells reached 80-90% confluence by trypsinization (0.25% trypsin/0.53 mM EDTA in Hanks Balanced Salt Solution without calcium and magnesium). Cells were plated at a density of 0.5 x 10^6 viable cells at each passage for each set of conditions defined below.

2.2.1 weaning and adapting SH-SY5Y cells

The process for weaning and adapting the SH-SY5Y cell line between different media types was modeled after those suggested by Corning[24] and Life Technologies[25] to transition cells from serum-containing media to serum-free media. Two independent experiments were completed. In experiment one, cells were 1) maintained in 100% DMEM high glucose throughout the experiment, 2) weaned and adapted from 100% DMEM high glucose to 100% DMEM low glucose or 3) weaned and adapted from 100% DMEM high glucose to 100% EMEM/Ham's F12. In experiment two, cells were 1) maintained in 100% DMEM high glucose throughout the experiment, 2) weaned and adapted from 100% DMEM high glucose to 100% EMEM/Ham's F12 or 3) maintained in 100% EMEM/Ham's F12 throughout the experiment.

SH-SY5Y cells in 100% DMEM high glucose media were allowed to grow until they reached 80-90% confluence; this was defined as passage one (p1). These cells were then passaged and transferred to a mixture of high glucose and low glucose media as illustrated in Figure 1. The following ratios of media were used over a period of eight passages: 100% (DMEM high glucose media):0% (low glucose media), 75% (DMEM high glucose media):25% (low glucose media), 50% (DMEM high glucose media):50% (low glucose media), 25% (DMEM high glucose media):75% (low glucose media), 0% (DMEM high glucose media):100% (low glucose media). Upon reaching 100% low glucose media, cells were passaged a total of three times before being considered fully adapted to the new media. As a control, cells were maintained in 100% DMEM high glucose media throughout both experiments. In the second experiment, cells maintained in 100% EMEM/Ham's F12 media were used an additional control.

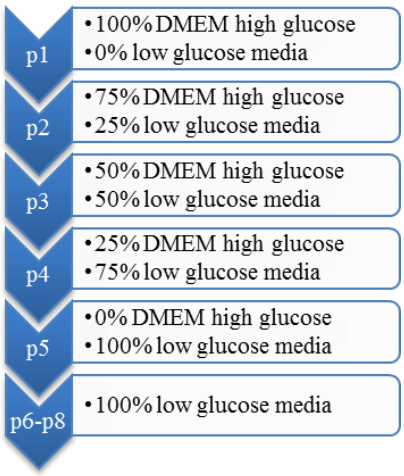

Figure 1: Sequential adaptation method used to wean and adapt SH-SY5Y cells from DMEM high glucose media to low glucose-containing media. (p) refers to the passage of cells.

2.3 Cell Viability Analysis

Prior to each passage, cells were photographed at a total magnification of 100X, and the number of days between passages was recorded. Cells were collected by trypsinization followed by centrifugation. Cells were resuspended in the same ratio of media from which they were removed from. Ten microliters of each cell resuspension was mixed 1:1 with trypan blue. Cell viability was measured using the trypan blue exclusion assay with a TC20™ automated cell counter. The total number of cells, the number of viable cells, and the percent cell viability was recorded for each sample. Each sample was counted twice and averaged; 0.5×10^6 cells from the resuspension were added to the next ratio of media appropriate for each sample and plated. A two-sample Student's t-test was used to compare the average percent cell viability between groups where n = 2.

3. Results and Discussion

A brief review of the literature was conducted to determine the methods most commonly used to culture SH-SY5Y cells. In total, 33 free, full-text primary research articles were found in PubMed using three different search criteria. The methods to culture and use SH-SY5Y cells for experimentation were reviewed. From these papers, the most common media types utilized were DMEM and a 1:1 mixture of DMEM with Ham's F12. At least three of the 33 research articles specifically used DMEM high glucose media, defined as a concentration of 25 mM D-glucose. The concentration of D-glucose in the media used in the remaining research articles varied or was not defined. Where more information could be located for DMEM used in each study, the most common concentration of D-glucose was 17.5 mM, which is still a relatively high concentration compared to normal levels found in human blood (5 mM)[28]. Only one of the 33 research articles was found to use DMEM containing 5 mM D-glucose. No search results using the defined criteria revealed the use of EMEM alone or a 1:1 mixture of EMEM with Ham's F12 (7.7 mM D-glucose) though the SH-SY5Y cell line was originally developed from the cell line SK-N-SH in EMEM media[17,18], and ATCC®, the most commonly used vendor to purchase the SH-SY5Y cell line in our review, recommends the use of a 1:1 mixture of EMEM/Ham's F12 supplemented with 10% fetal bovine serum[29]. In addition to differences in the concentration of D-glucose, other components in the media differed considerably across the research articles examined. For example, a consistently noted difference between media types was the L-glutamine concentration. In some cases no L-glutamine was present, whereas other studies used between 2 – 4 mM L-glutamine. This difference could be important for studies exposing SH-SY5Y cells to manganese due to the effects manganese has on glutamate homeostasis[1,5,11]. The concentrations of other amino acids and vitamins were also found to differ between DMEM and EMEM[30-33]. Nearly two-thirds (21 out of 33) of the research articles reviewed grew the SH-SY5Y cells in media supplemented with 10% fetal bovine serum or fetal calf serum along with penicillin/streptomycin. Although this review of the literature lent some credibility to the media choice and cell culture methods used in our previous studies, they also revealed the variability in methods used to maintain the same cell line, SH-SY5Y, across multiple laboratories for similar or diverse purposes.

Based on our review of the media formulations used by others and recommended by the vendor, we chose to identify and select two new media formulations containing a lower concentration of glucose to grow SH-SY5Y cells and compare their cell viability to cells grown continuously in DMEM high glucose media. A low glucose media was chosen because human *in vivo* blood glucose concentrations are estimated to be 5.5 mM and concentrations approaching 10 mM are considered pre-diabetic to diabetic levels[28]. Because cell viability is also commonly measured in response to various agents, including manganese, the concentration of glucose present in the media may impact cell viability. After a review of available options, DMEM low glucose media was chosen because it most closely matched the media formulation of DMEM high glucose media except for the concentration of D-glucose, which was 5.5 mM rather than 25 mM, respectively. The second media formulation selected for further study was the 1:1 mixture of EMEM/Ham's F12 media recommended by ATCC®, which contained a total concentration of D-glucose of approximately 7.7 mM. This mixture was chosen because of its lower concentration of D-glucose as well as lower concentrations of other media components such as L-glutamine and other amino acids. L-glutamine levels in the DMEM media were 4 mM and in EMEM/Ham's F12 medium was approximately 1.5 mM. Table 1 summarizes the concentration of each component present in all of the media types used in this study[30-33].

In most cases, a laboratory would purchase a cell line and place them directly in a desired media. However, in some situations this is difficult due to the cost of the cell line or when there is a limited source of cells such as the use of primary cells. For this study, the SH-SY5Y cell line was grown in three different media types. SH-SY5Y cells were available in the lab, but grown in DMEM high glucose media, and the cost to purchase three new, separate

vials of cells was not possible, so alternative methods to grow SH-SY5Y cells in different media were explored. Although no examples were found to transfer a cell line from one media type to another, many examples explained how to transition cells from a serum-containing media to a serum-free media[23-25]. The most commonly used method to transition cells in this way is the sequential adaptation method[23-25].

The sequential adaptation method was chosen and used in an attempt to wean and adapt SH-SY5Y cells from DMEM high glucose media to either DMEM low glucose media or a 1:1 mixture of EMEM/Ham's F12 media. Over a period of eight passages, the original, high glucose medium was sequentially reduced from 100% to 0%, while the percentage of new, low glucose medium increased from 0% to 100% (Figure 1). Cells were not considered fully adapted until grown in 100% of the new, low glucose media for a total of three passages. SH-SY5Y cells grown continuously in DMEM high glucose media were included as a control. When the weaning and adaptation experiment was repeated, SH-SY5Y cells were also grown continuously in a 1:1 mixture of EMEM/Ham's F12 as a second control.

Table 1: Media Formulations Used in Weaning and Adaptation Study with SH-SY5Y Cells[30-33]

Components of Liquid Media	DMEM High Glucose	DMEM Low Glucose	EMEM	Ham's F12
Supplier	Fisher Scientific	Fisher Scientific	ATCC	Fisher Scientific
Vendor	HyClone	HyClone	ATCC	Lonza
Catalog Number	SH30022	SH30021	30-2003	12-615
Inorganic Salts				
Calcium Chloride - $CaCl_2$	0.2 g/L	0.2 g/L	0.2 g/L	0.0333 g/L
Cupric Sulfate Pentahydrate	0.0 g/L	0.0 g/L	0.0 g/L	0.0000025 g/L
Ferric Nitrate - $9H_2O$	0.0001 g/L	0.0001 g/L	0.0 g/L	0.0 g/L
Ferric Sulfate - $FeSO_4$-$7H_2O$	0.0 g/L	0.0 g/L	0.0 g/L	0.00083 g/L
Magnesium Chloride	0.0 g/L	0.0 g/L	0.0 g/L	0.05719 g/L
Magnesium Sulfate - $MgSO_4$	0.098 g/L	0.098 g/L	0.098 g/L	0.0 g/L
Potassium Chloride - KCl	0.4 g/L	0.4 g/L	0.4 g/L	0.224 g/L
Sodium Chloride - NaCl	6.4 g/L	6.4 g/L	6.8 g/L	7.599 g/L
Sodium Phosphate Monobasic H_2O - NaH_2PO_4-H_2O	0.125 g/L	0.125 g/L	0.14 g/L	0.0 g/L
Sodium Phosphate Dibasic, Anhydrous	0.0 g/L	0.0 g/L	0.0 g/L	0.142 g/L
Zinc Sulfate Heptahydrate	0.0 g/L	0.0 g/L	0.0 g/L	0.00086 g/L
Amino Acids				
L-Alanine	0.0 g/L	0.0 g/L	0.0089 g/L	0.00891 g/L
L-Arginine-HCl	0.084 g/L	0.084 g/L	0.1264 g/L	0.211 g/L
L-Asparagine-H_2O	0.0 g/L	0.0 g/L	0.015 g/L	0.01501 g/L
L-Aspartic Acid	0.0 g/L	0.0 g/L	0.0133 g/L	0.01331 g/L
L-Cysteine-HCl-H_2O	0.0 g/L	0.0 g/L	0.0 g/L	0.03512 g/L
L-Cysteine-2HCL	0.06257 g/L	0.06257 g/L	0.0312 g/L	0.0 g/L
L-Glutamic Acid	0.0 g/L	0.0 g/L	0.0147 g/L	0.01471 g/L
L-Glutamine	0.584 g/L	0.584 g/L	0.292 g/L	0.146 g/L
Glycine	0.03 g/L	0.03 g/L	0.0075 g/L	0.00751 g/L
L-Histidine-HCl-H_2O	0.042 g/L	0.042 g/L	0.0419 g/L	0.02096 g/L
L-Isoleucine	0.1048 g/L	0.1048 g/L	0.0525 g/L	0.00394 g/L
L-Leucine	0.1048 g/L	0.1048 g/L	0.0525 g/L	0.01312 g/L
L-Lysine-HCl	0.1462 g/L	0.1462 g/L	0.0725 g/L	0.03654 g/L
L-Methionine	0.03 g/L	0.03 g/L	0.015 g/L	0.00448 g/L
L-Phenylalanine	0.066 g/L	0.066 g/L	0.0325 g/L	0.00496 g/L
L-Proline	0.0 g/L	0.0 g/L	0.0115 g/L	0.03453 g/L
L-Serine	0.042 g/L	0.042 g/L	0.0105 g/L	0.01051 g/L
L-Threonine	0.0952 g/L	0.0952 g/L	0.0476 g/L	0.01191 g/L
L-Tryptophan	0.016 g/L	0.016 g/L	0.01 g/L	0.00204 g/L
L-Tyrosine-2Na-$2H_2O$	0.10379 g/L	0.10379 g/L	0.0519 g/L	0.0 g/L
L-Valine	0.0936 g/L	0.0936 g/L	0.0468 g/L	0.01171 g/L
Vitamins				
D-Biotin	0.0 g/L	0.0 g/L	0.0 g/L	0.000007 g/L
Choline Chloride	0.004 g/L	0.004 g/L	0.001 g/L	0.01396 g/L
Cyanocobalamin	0.0 g/L	0.0 g/L	0.0 g/L	0.00136 g/L
Folic Acid	0.004 g/L	0.004 g/L	0.001 g/L	0.00132 g/L
I-Inositol	0.0 g/L	0.0 g/L	0.0 g/L	0.018 g/L
DL-Alpha-Lipoic Acid (Thioctic Acid)	0.0 g/L	0.0 g/L	0.0 g/L	0.00021 g/L
Myo-Inositol	0.007 g/L	0.007 g/L	0.002 g/L	0.0 g/L
Nicotinamide (Niacinamide)	0.004 g/L	0.004 g/L	0.001 g/L	0.000037 g/L
D-Na Pantothenic Acid	0.0 g/L	0.0 g/L	0.0 g/L	0.0 g/L
Pyridoxine-HCl	0.0 g/L	0.004 g/L	0.001 g/L	0.000062 g/L
Riboflavin	0.0004 g/L	0.0004 g/L	0.0001 g/L	0.000038 g/L
Thiamine-HCl	0.004 g/L	0.004 g/L	0.001 g/L	0.00034 g/L
Thymidine	0.0 g/L	0.0 g/L	0.0 g/L	0.00073 g/L
D-Ca Pantothenate	0.004 g/L	0.004 g/L	0.001 g/L	0.00024 g/L
Pyridoxal HCl	0.004 g/L	0.0 g/L	0.0 g/L	0.0 g/L
Other				
D-glucose	4.5 g/L	1 g/L	1 g/L	1.802 g/L
Hypoxanthine Disodium Salt	0.0 g/L	0.0 g/L	0.0 g/L	0.00546 g/L
Linoleic Acid Methyl Ester	0.0 g/L	0.0 g/L	0.0 g/L	0.000088 g/L
Phenol Red, Sodium Salt	0.0159 g/L	0.0159 g/L	0.01 g/L	0.001242 g/L
Putrescine Dihydrochloride	0.0 g/L	0.0 g/L	0.0 g/L	0.00016 g/L
Sodium Pyruvate	0.0 g/L	0.11 g/L	0.11 g/L	0.11 g/L
L-Tyrosine Diodium Salt, Dihydrate	0.0 g/L	0.0 g/L	0.0 g/L	0.007842 g/L
HEPES	0.0 g/L	0.0 g/L	0.0 g/L	0.0 g/L
Sodium bicarbonate	3.7 g/L	3.7 g/L	1.5 g/L	1.176 g/L

Prior to each cell passage, cells were photographed (Figure 2) to document overall cell morphology. Normal cell growth was seen throughout the eight passages in the DMEM high glucose control, the EMEM/Ham's F12 control, and both DMEM high glucose to EMEM/Ham's F12 transitions. As seen in the photographs for these cells (Figure 2B&C), the cells were flat and attached to the growth surface. The cells in the DMEM high glucose to DMEM low glucose transition, however, detached from the growth surface and were seen floating in the medium and were round and shiny, all indicative of poor cell health (Figure 2A). This difference in morphology contributed to the decision to eliminate the DMEM low glucose media as an option for future studies after the first experiment. A potential difference in size was also noted between the cells grown in DMEM high glucose and those grown in or transitioned to EMEM/Ham's F12. Future studies may be designed to measure cells grown in each medium to quantify this difference.

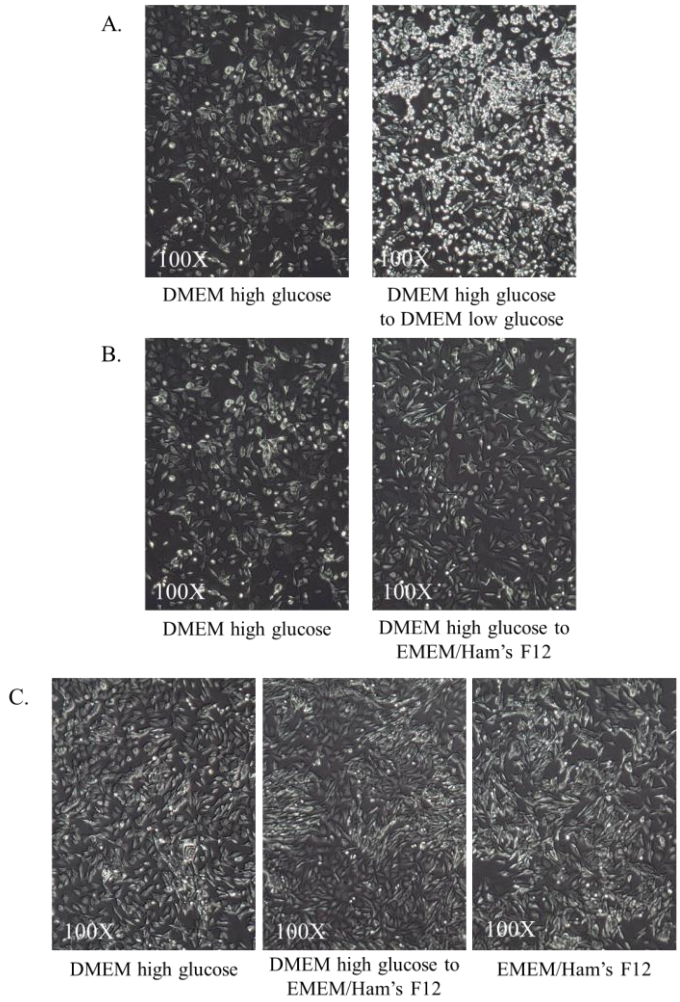

Figure 2: Comparative pictures of cell morphology for A) cells grown only in DMEM high glucose vs. cells transitioned from DMEM high glucose to DMEM low glucose in the first experiment at p8, B) cells grown only in DMEM high glucose vs. cells transitioned from DMEM high glucose to EMEM/Ham's F12 in the first experiment at p8, and C) cells grown only in DMEM high glucose and cells only grown in EMEM/Ham's F12 vs. cells transitioned from DMEM high glucose to EMEM/Ham's F12 in the second experiment at p5.

At each passage, cell viability was determined using a trypan blue exclusion assay to assess cell health during weaning and adaptation. Over eight passages in two independent experiments, the percent cell viability ranged from 91% - 99% for SH-SY5Y cells maintained in the original, DMEM high glucose media. The average percent cell viability at passage 8 (p8) for these control cells was 96.5% ± 2.8%, n = 2 (Figure 3A&B). When SH-SY5Y cells were weaned and adapted to the DMEM low glucose media in the first experiment, the percent cell viability ranged from 87% - 100%, and the average percent cell viability at p8 was 94.5%, n = 1 (Figure 3A). Similar results were observed for SH-SY5Y cells that were weaned and adapted to the 1:1 mixture of EMEM/Ham's F12 media. The

percent cell viability ranged from 88% - 95% over eight passages in two independent experiments, and the average percent cell viability at p8 was 92.5% ± 8.5%, n = 2 (Figure 3B). Based on a two-sample Student's t-test, the percent cell viability did not differ significantly between cells grown in DMEM high glucose media at p8 compared to cells that were weaned and adapted after eight passages to EMEM/Ham's F12 (Figure 3B). A statistical analysis was not completed for the trial where cells were weaned from DMEM high glucose to DMEM low glucose because this procedure was not replicated in the second experiment due to the qualitative analysis of the cells.

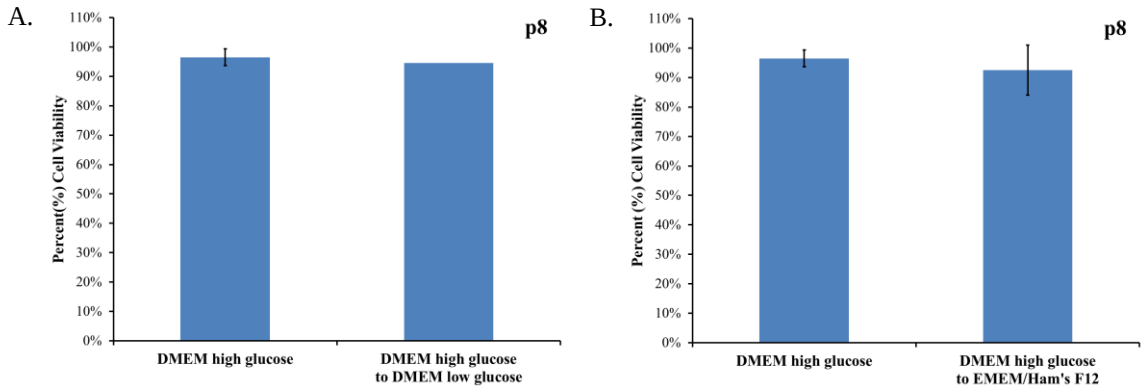

Figure 3: SH-SY5Y percent cell viability at p8 following weaning and adaptation method from DMEM high glucose media to A) DMEM low glucose media or B) a 1:1 mixture of EMEM/Ham's F12 media. The percent cell viability did not differ significantly between groups in (B) based on a two-sample Student's t-test ($p > 0.05$).

The cell viability results suggest that SH-SY5Y cells could be grown in any of the three alternative media formulations used in this study without a significant loss in cell viability. However, the number of days between each passage was also recorded. Because SH-SY5Y cells were plated at the same density following each passage, the number of days between passages revealed possible differences in the rate of proliferation or loss of cells between passages when cells were transitioned to different media types. The number of days between passages ranged between 3-9 days for cells grown in DMEM high glucose media and 3-9 days for cells that were weaned and adapted to EMEM/Ham's F12 media. SH-SY5Y cells that were weaned and adapted to DMEM low glucose media began taking longer to reach 80-90% confluence and numerous cells were observed to begin floating between changes in media at the higher passage numbers (Figure 2A). For example, from p6 to p7, these cells required 10 days and from p7 to p8 they required 16 days to reach a similar level of confluence observed in other groups in less time. Due to the decreased ability for these cells to maintain a consistent number of days between passages, it was concluded that this medium may not be optimal to maintain SH-SY5Y cells in culture for longer periods of time. When the weaning and adaptation experiment was repeated, the DMEM low glucose media was eliminated due to the inability of the cells to reach 80-90% confluence over a relatively consistent amount of time and observed changes in cell morphology.

To begin the second trial, two, new vials of SH-SY5Y cells were purchased from ATCC®. Upon arrival, one vial was plated in DMEM high glucose media and the second vial was plated in a 1:1 mixture of EMEM/Ham's F12 media. Cells were again weaned and adapted from DMEM high glucose media to the 1:1 mixture of EMEM/Ham's F12 media using the sequential adaptation method over a total of eight cell passages. Cells maintained in DMEM high glucose media or a 1:1 mixture of EMEM/Ham's F12 media were used as controls. SH-SY5Y percent cell viability ranged from 92% - 98% in cells maintained in a 1:1 mixture of EMEM/Ham's F12 media throughout the experiment. The average percent viability at p8 for these control cells was 96.5%, n = 1. Notably, the average percent viability of cells maintained in DMEM high glucose media compared to EMEM/Ham's F12 media was nearly identical at p8 and appeared to be similar to cells weaned between DMEM high glucose and EMEM/Ham's F12 media (Figure 4).

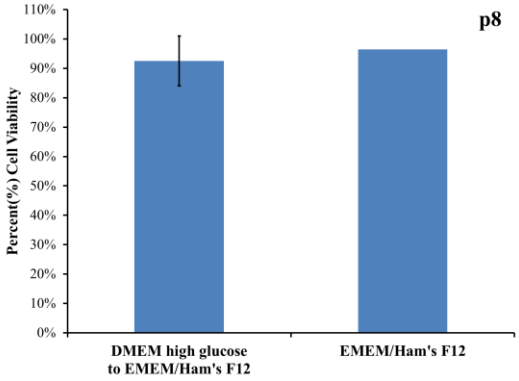

Figure 4: SH-SY5Y percent cell viability at p8 when weaned and adapted from DMEM high glucose media to a 1:1 mixture of EMEM/Ham's F12 compared to cell viability at p8 when grown continuously in a 1:1 mixture of EMEM/Ham's F12 media upon receipt from ATCC®.

These results demonstrate that the sequential adaptation method is a valid tool to wean and adapt the SH-SY5Y cell line from one media type to alternative media types with different formulations. SH-SY5Y cell viability did not appear to be compromised when cells were adapted to EMEM/Ham's F12 media compared to cells grown in EMEM/Ham's F12 media from original thawing. SH-SY5Y cells grown in EMEM/Ham's F12 media displayed similar percent cell viability after eight cell passages compared to cells grown in DMEM high glucose media. Thus, either DMEM high glucose media or EMEM/Ham's F12 media appear to be able to maintain a viable population of SH-SY5Y cells in culture.

4. Conclusions

The SH-SY5Y cell line is used as an *in vitro* model for a wide variety of neurodegenerative diseases, neurotoxicology, cancer genetics and tumor growth, and more[16,19]. As the purpose of each experiment utilizing this cell line differs, the culture conditions may also vary to complement the study at hand because the conditions may have an effect on the outcome or conclusions made. Previous experiments in our laboratory studying the effects of manganese exposure on the concentrations of different dopamine metabolites in extracellular media were examples of how a commonly used growth medium can produce inconclusive results. As seen in the summary of results from the literature search, there are several suitable media available and used for the SH-SY5Y cell line, but it is important to consider how the medium and other reagents used for cell maintenance may affect the endpoint of the study when designing an experiment.

By completing studies to determine how SH-SY5Y cells would respond to each medium, it was possible to eliminate those not suited for future experiments related to manganese-induced changes in dopamine metabolism. At the conclusion of this study, the 1:1 mixture of EMEM/Ham's F12 was chosen as the most suitable medium for future studies involving the exposure of SH-SY5Y cells to manganese as an *in vitro* model of manganism. Several factors supported this choice including 1) EMEM/Ham's F12 media is the suggested media for growth of the SH-SY5Y cell line from the vendor and 2) there was no difference in average percent cell viability between cells grown in the EMEM/Ham's F12 adapted group and cells grown in the DMEM high glucose control group at p8. Furthermore, preliminary experiments to examine the HPLC chromatogram profile of EMEM/Ham's F12 media further support its potential use in manganese-exposure studies to study dopamine metabolism (Sohl, B. et al; unpublished data). The results of this study also demonstrate the validity of the sequential adaptation method to wean and adapt a cell line between different media types. Other researchers looking to optimize cell culture conditions to grow a cell line for use as an *in vitro* model to study disease or other cellular processes may find this method both valuable and feasible.

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