Effects of Halobenzoquinone Water Disinfection By-Products on Human Neural Stem Cells

by

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Abstract

Water disinfection inactivates microbiological pathogens in drinking water but also unintentionally produces disinfection byproducts (DBPs). Epidemiological studies have observed potential correlations between the consumption of chlorinated water with an increased risk of developing bladder cancer and have found inconsistent correlations with adverse reproductive effects. Public health organizations, such as the World Health Organization, United States Environmental Protection Agency and Health Canada, have placed regulations on a small number of DBPs. These DBPs include total trihalomethanes (TTHMs) and five haloacetic acids (HAA5). Halobenzoquinones (HBQs), an emerging class of DBPs, are capable of causing damage to cellular DNA and proteins in T24 and CHO cells. Little is known about the neurotoxicity of DBPs in *in vitro* and *in vivo* systems. Human neural stem cells (hNSCs) are a potentially useful model to test the effects of chemical exposure, such as DBPs, on developmental neurotoxicity. The aim of this thesis is to study the effects of DBP exposure on the differentiation of hNSCs into neurons. Two HBQs, 2,6-dibromobenzoquinone (2,6-DBBQ) and 2,6-dichlorobenzoquinone (2,6-DCBQ), were selected because of their frequent and widespread occurrence in drinking water. To understand the significance of HBQ neurotoxicity, I also included two regulated HAAs, bromoacetic acid (BAA) and chloroacetic acid (CAA), in these studies. First, I used qualitative imaging methods to observe the physical characteristics and changes in protein expression of differentiated and undifferentiated hNSCs. Next, I analyzed the impact of HBQ and HAA exposure on the growth and cell cycle of hNSCs. Finally, I assessed the effects of HBQ and HAA exposure on the differentiation of

hNSCs into neurons and measured axon length. I observed that hNSCs lost the expression of stem cell differentiation markers nestin and Sox2 after five passages. Differentiation was induced at passage three or four in subsequent experiments. Flow cytometry analysis showed that hNSCs exposed to 0.5 μM 2,6-DBBQ and 1 μM 2,6-DCBQ for 96 hours resulted in higher proportions of cells in S-phase. This result suggests cell cycle arrest in the S-phase, the phase at which DNA replication occurs. Additionally, the ratio of mature neurons to immature neurons was lower in the cells exposed to 0.5 μM BAA and 0.5 μM CAA for 12 days compared to negative controls, indicating that the tested DBPs can inhibit the cell maturation process. Overall, these results suggest that hNSCs are an appropriate model to test the *in vitro* developmental neurotoxicity of DBPs. Further research on DBP neurotoxicity will contribute to the understanding of the potential developmental effects of DBPs.

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List of Symbols, Terminology and Abbreviations

%	Percent
°C	Degrees Celsius
®	Registered trademark
×	Times
Ab	Antibody
ADME	Absorption, distribution, metabolism, excretion
ANOVA	One-way analysis of variance
As^{III}	Sodium arsenite
BBB	Blood-brain-barrier
CHO	Chinese hamster ovary
CO_2	Carbon dioxide
confluence	The percentage of culture plate covered with adherent cells
d	Days
DBP	Water disinfection by-product
DCX	Doublecortin
DNA	Deoxyribonucleic acid
DNT	Developmental neurotoxicity
EPA	U. S. Environmental Protection Agency
FACS	Flow cytometry
GSH	Glutathione
HAAs	Haloacetic acids
HANs	Haloacetonitriles
HBQs	Halobenzoquinones
hNSC	Human neural stem cell
IC_{50}	Half maximal inhibitory concentration
L	Litre
LEC	Lowest effective concentration
LOAEL	Lowest observable effect level
M	Molar

μ	Micro-
m	Meter
MCL	Maximum contaminant level
n	Nano-
NOAEL	No observable adverse effect level
OECD	Organization for Economic Cooperation and Development
OH-	Hydroxyl-
P	Passage, subculturing cells by seeding cells onto new plates
PI	Propidium iodide
pKa	Acid dissociation constant
QSAR	Quantitative structure-activity relationships
QSTR	Quantitative structure-toxicity relationships
ROS	Reactive oxygen species
rpm	Rotations per minute
SD	Standard deviation
THMs	Trihalomethanes
™	Trademark
WHO	World Health Organization

Chapter 1: Literature and Objectives

1.1 Overview

Safe drinking water is disinfected to inactivate harmful pathogens found in source water. Chlorine and chloramine are commonly used because they are cost-effective for preventing waterborne diseases [1,2]. In Canada, the majority of drinking water treatment plants use chlorination or chloramination to disinfect drinking water.

An unavoidable consequence of using these disinfection processes is the formation of drinking water disinfection byproducts (DBPs). For assuring complete disinfection, maintaining residual chlorine throughout the drinking water distribution system is current best practice. Free chlorine concentrations in some Canadian drinking water distribution systems range from 0.04 to 2.0 mg/L [3]. DBPs are mainly formed by the reactions between naturally occurring organic material present in source water and free chlorine or chloramine. The most abundant compounds formed include trihalomethanes (THMs) and haloacetic acids (HAAs). New classes of DBPs are continuously being discovered in treated drinking water. Halobenzoquinones (HBQs) are a recently discovered class of DBPs that have been shown to be potentially more cytotoxic than regulated DBPs in quantitative-structure toxicity relationship (QSTR) analysis and *in vitro* cell studies.

Previous epidemiological studies have observed inconsistent correlations between prenatal exposure to DBPs and an increased risk of adverse developmental effects. To date, no effective experimental model is available for studying the mechanisms of developmental effects caused by DBP exposure. The aim of this thesis was to investigate

whether a human neural stem cell line can serve as a model for testing developmental neurotoxicity (DNT) resulting from DBP exposure, with a focus on the new class of HBQ-DBPs. The rationale is that cultured human neural stem cells (hNSCs) have shown a promising, high-throughput model to test the adverse effects of chemicals on developing nervous systems. Endpoints that can be measured by this model include cellular morphology, biochemical markers, neurotransmission and molecular events, which enhance the predictivity of DNT testing.

This introductory chapter consists of four main parts. First, I will provide an overview of current DBP regulations and outline DBP research with regards to occurrence and toxicity. Next, I will discuss the occurrence and toxicity of HBQs. Third, this chapter will summarize different models used for DNT testing and will establish the potential value of using stem cell technologies, such as hNSCs, to test the developmental neurotoxic effects of DBP exposure. Fourth, the objectives of my thesis research are also outlined.

1.2 Water Disinfection By-products (DBPs)

1.2.1 History of Water Disinfection

The establishment of public water systems in the 19th century opened the potential for disease to spread to communities that relied on these water systems. The connection between drinking water and disease was established in 1854 when John Snow linked the Broad Street pump and the cholera epidemic ravaging London [4]. This incident confirmed that illnesses could be spread indirectly by water to people who had not previously come in contact with ill individuals. The popular account is that when Snow

removed the handle of the water pump, the illness was contained. The reality is that the Broad Street pump outbreak was already declining when he removed the pump handle. However, Snow demonstrated by means of epidemiological analysis that showed that sewage contamination of water was strongly correlated with cholera incidence. The connection between water sources and illness emphasized the importance of avoiding the transportation of bacteria and pathogens, while meeting people's need to access essential water resources. Drinking untreated water may result in gastrointestinal illness caused by various pathogens, such as parasites, *Cryptosporidium* or *Giardia*, or bacteria, such as *Escherichia coli* [5,6].

In 1884, scientists discovered that adding chlorine to water would render the water "germ free", and this method of water purification was adopted on a larger scale in areas of Europe [7]. The first incidence of large-scale water disinfection was in 1896 when George W. Fuller applied chlorine and sodium hypochlorite at the Louisville, Kentucky Experimental Station [4]. The first permanent application of chlorine to disinfect drinking water occurred in 1902 in Belgium [4]. Since then, chlorination has been used throughout the world. In the early 1900s, using a combination of chlorine and ammonia received attention because it was thought that the cost of chlorination would be reduced if ammonia was used. The practice of chloramination quickly caught on and was adopted in 1916 by a water treatment plant in Ottawa, ON [8].

The World Health Organization (WHO) recognizes the access to "safe and clean drinking water and sanitation as a human right that is essential for the full enjoyment of life and all human rights" [9,10]. The WHO further defines safe drinking water as "free from microbiological organisms, chemical substances and radiological hazards that

constitute a threat to a person's health". It is imperative for governments to provide safe drinking water to their citizens to ensure that their basic needs are fulfilled.

The responsibility of water disinfection in Canada rests with municipalities; however, the provincial and federal governments regulate municipal water treatment plants. Chlorination and chloramination are the most widely used methods of drinking water disinfection in Canada. In fact, approximately 57,000 of the 63,000 drinking water treatment facilities in Canada and the United States rely on chlorine chemistry as a primary disinfectant [11]. In comparison, around 7% of drinking water treatment facilities disinfect drinking water with ozone and less than 2% use ultra-violet radiation [11]. Combinations of methods are commonly used and different water treatment methods result in the formation of different classes of DBPs [12].

In 2002, the United States Environmental Protection Agency (US EPA) published the results of the Nationwide Disinfection By-Products Occurrence Study [13]. This study analyzed water samples from a diverse selection of geographic regions and different source water quality for priority DBPs. These priority DBPs included iodinated THMs, other halomethanes, a nonregulated haloacid, haloacetonitriles, haloketones, halonitromethanes, haloaldehydes, halogenated furanones, haloamides, and nonhalogenated carbonyls. One important finding from the study was that although the use of alternative disinfectants minimized the formation of the four regulated THMs, other potentially more toxic DBPs were produced and at higher levels than in water treated with chlorine alone. The results of the study revealed the presence of many DBPs in sample waters that deserve our attention.

1.2.2 Epidemiological and Toxicological Studies on DBP Exposure and Adverse Developmental Effects

DBPs have been shown in epidemiological studies to be correlated to various chronic diseases. Previous studies have mostly focused on retrospective data, matching total THM levels with hospital records of bladder cancer [14]. Exposure to DBPs has shown a correlation with an increased incidence in bladder cancer [15,16] and has been correlated with several other adverse health outcomes; including, rectal cancer [16], colon cancer [17] and leukemia [18].

Inconsistent results have been reported across different epidemiological studies, including some studies that have reported associations between DBP exposure and increased risk of adverse developmental outcomes including low birth weight [19,20], birth defects such as cardiovascular or neural tube defects [21,22], spontaneous abortions [23-25] and stillbirths [15,26]. In 2009, Nieuwenhuijsen and colleagues reviewed epidemiological studies that linked exposure of DBPs to adverse reproductive outcomes and found that small fetuses for gestational age was the most consistent correlation between DBP exposure and an adverse endpoint [27].

To test the developmental toxicity of HAAs, Hunter and colleagues investigated the comparative effects of HAAs in whole embryo culture [28,29]. The particular HAAs tested were trichloroacetic acid (TCAA), dichloroacetic acid (DCAA), monochloroacetic acid (CAA), monobromoacetic acid (BAA), dibromoacetic acid (DBAA), tribromoacetic acid (TBAA), fluoroacetic acid (FAA) and difluoroacetic acid (DFAA). The study found that exposure of mouse conceptuses to HAAs affected the development of the neural tube, craniofacial region and heart. Exposure to CAA affected embryonic development at

concentrations as low as 175 μM, while embryos exposed to 5 μM BAA exhibited abnormal neural tube development. The benchmark concentrations required for induction of neural tube defects produced by HAAs are shown below ranked from the most to least potent.

Table 1.1: The minimum concentration of various chemicals necessary to induce neural tube defects in whole embryo culture [29].

Potency Rank	HAA	Concentration (mg/L)	Concentration (μM)
1	BAA	0.7	5
2	CAA	16.5	175
3	DBAA	54.5	250
4	TCAA	>326.8	>2000
5	TBAA	>890.2	>3000
6	DCAA	>757.0	>5871
7	DFAA	>864.3	>9000

Several studies have generally observed that many known teratogens are weak acids [30,31]. Richards *et al.* studied the pKa values of HAAs and found a trend of generally increasing teratogenic potency with increasing pKa value [28]. The precise mechanism of chemical-induced biological change in whole embryo culture has not been elucidated.

In 2015, Narotsky and colleagues evaluated a drinking water mixture of the four regulated THMs and five regulated HAAs in a multigenerational reproductive toxicity assay using Sprague-Dawley rats [32]. The rats were exposed to a realistically proportional mixture of regulated DBPs at 500x, 1000x or 2000x of the US EPA's maximum contaminant levels (MCLs). The researchers found that a mixture of regulated DBPs up to 2000x the MCLs had no adverse effects on various reproductive endpoints,

including fertility, pregnancy maintenance, prenatal survival, postnatal survival, or birth weights. The results of this study suggest that non-regulated DBPs may account for the toxicity observed in epidemiological studies or the epidemiological results may be non-causal correlation. Furthermore, the authors suggested that using rats as an animal model might not be a realistically representative model of developmental neurotoxicity experienced by humans.

Thus far, there have been no epidemiological studies that have focused on the correlation between disease and exposure to non-regulated DBPs; however, it is likely that the toxic effects observed in epidemiological studies, if authentic, are due to the exposure to a combination of regulated and non-regulated classes of DBPs. More research into the cytotoxic, genotoxic and developmental effects of non-regulated DBPs is useful to understand the human health effects of DBP exposure.

The Water Research Foundation and the American Water Works Association commissioned a panel review to evaluate scientific studies about DBPs and bladder cancer risk. The panel established that quantitative risk estimates from toxicological risk assessments of DBPs cannot currently be reconciled with epidemiological studies [33]. Hrudey *et al.* recommend that further studies be conducted with independent populations to improve the understanding of the connection between exposure and disease [33]. Although the inconsistent results are insufficient to prove a causal link between DBP exposure and adverse reproductive outcomes, the pervasiveness of drinking water exposure supports reasonable, precautionary regulations [34]. Out of the more than 600 unique DBPs that have been identified, very few (including four THMs and five HAAs) are regulated by the US EPA.

1.2.3 DBP Regulations

THMs and HAAs are closely monitored by water treatment plants and government agencies due to the described epidemiological studies the the pervasiveness of exposure. The US EPA passed the Safe Water Drinking Act in 1974 to ensure that drinking water is free of contaminants that could adversely affect human health. The US EPA set enforceable MCLs for total THMs and levels of five HAAs. Total THMs (TTHMs) include chloroform, bromoform, bromodichloromethane and dibromochloromethane and the five HAAs (HAA5) included in the guidelines are monochloro-, dichloro-, monobromo-, dibromo- and trichloro acetic acids [35]. The MCL for TTHMs is 0.08 mg/L, while the MCL for HAA5 is 0.06 mg/L [35].

Similarly, Health Canada has also set maximum level guidelines for THMs and tri-HAA levels [36]. The MCL for TTHMs is 0.1 mg/L and 0.08 mg/L for HAA5 [36]. Furthermore, the WHO has established guidelines for MCLs of specific DBPs; including, chloroform, bromoform, dibromochloromethane and bromodichloromethane [37].

To respond to these regulations, several water disinfection facilities switched to alternative disinfection methods, such as chloramination, to reduce the levels of regulated DBPs in drinking water. However, Krasner *et al.* suggest that certain emerging unregulated DBPs, which could be more toxic than regulated DBPs, can be formed when alternative disinfectants are used [12]. This shift in DBPs presents a growing challenge to identify new compounds and characterize the toxicity of emerging classes of DBPs. The importance of identifying and characterizing new DBP classes of toxicological relevance is made more imperative because multiple studies have indicated that regulated DBPs

cannot explain the correlation of DBP exposure with an increased incidence of cancers reported in epidemiological studies.

1.3 Halobenzoquinones (HBQs)

HBQs are a recently discovered class of DBP. Four HBQs, 2,6-dichloro-1,4-benzoquinone (2,6-DCBQ), 2,6-dichloro-3-methyl-1,4-benzoquinone (DCMBQ), 2,3,6-trichloro-1,4-benzoquinone (TriCBQ) and 2,6-dibromo-1,4-benzoquinone (2,6-DBBQ) have all been identified in tap water at nanogram per litre levels [38,39]. Thus, it is important that the toxic effects of this emerging class of DBPs be better understood.

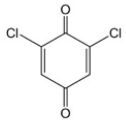
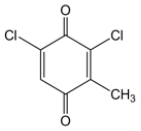
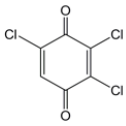
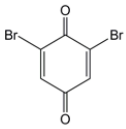
Quantitative structure toxicity relationship (QSTR) analysis has predicted that some halobenzoquinones (HBQs) have a lowest observed adverse effect level (LOEAL) up to 10,000 times lower than regulated DBPs [40]. Compounds or transformation products with similar structures, such as benzoquinones, interact through multiple pathways such as redox reactions, alkylation and interactions with a variety of biologically active molecules, such as DNA and proteins, causing hazardous effects [41]. The International Agency for Research on Cancer lists a total of 12 benzoquinone-like compounds included in both Group 2B ("possibly carcinogenic to humans") and Group 3 ("Not classifiable as to its carcinogenicity to humans") [42].

Toxicological results of structurally similar compounds indicate that HBQs may be potent carcinogens, and recent studies have identified the occurrence and toxicological importance of the presence of HBQs in drinking water [43].

1.3.1 Occurrence of HBQs in Treated Water

Halobenzoquinones (HBQs) were first discovered in drinking water in 2010. A study of nine treatment plants found that four different HBQs, 2,6-DCBQ, DCMBQ, TriCBQ, and 2,6-DBBQ, were found in treated water at the nanogram per litre level [44]. These treatment plants used a variety of disinfection methods; including, chlorination, chloramination, chloramination with chlorination, and ozonation with chloramination. 2,6-DCBQ was the HBQ with the highest occurrence frequency and was found in all treated water samples.

Table 1.2: The concentrations of various HBQs found in finished drinking water by Zhao *et al.* [44]. The structure of each HBQ is also shown.

Halobenzoquinone	Concentration (ng/L)	Structure	Occurrence Frequency
2,6-dichloro-1,4-benzoquinone (2,6-DCBQ)	165.1 ± 9.1		37.5%
2,6-dichloro-3-methyl-1,4-benzoquinone (DCMBQ)	1.3 ± 0.2		18.8%
2,3,6-trichloro-1,4-benzoquinone (TriCBQ)	9.1 ± 0.6		68.8%
2,6-dibromo-1,4-benzoquinone (2,6-DBBQ)	0.5 ± 0.1		Data unavailable

HBQs have also been identified in drinking water in other parts of the world. A study in Hong Kong identified 2,6-DBBQ and other isomers using their mass

spectrometry methods [45]. It is likely that the concentrations of HBQs range in drinking waters around the world [46].

HBQs have also been found in recreational waters treated with chlorine [47]. The concentration of 2,6-DCBQ in ten swimming pools was found to be up to one hundred times higher than their concentrations in the input tap water. TriCBQ and 2,6-DBBQ were present at the nanogram per litre level and a new compound, dibromo-5,6-dimethyl-1,4-benzoquinone, was also identified at the sub-nanogram per litre level. The formation of this compound is likely due to the presence of personal care products or cosmetics, which can react with chlorine [47].

1.3.2 HBQ Cytotoxicity

HBQs have shown to be cytotoxic in cell studies using different cell lines. A recent study on the structure and toxicity relationship of HBQs demonstrates the potential toxicity of HBQs is correlated with the formation of reactive oxygen species (ROS) causing oxidative stress [48].

IC_{50} values of various HBQs have been studied using various technologies in different cell lines. IC_{50} values indicated the concentration of a substance needed to inhibit the biological function measured by the specific technology or assay by 50%. T24 bladder cancer cells have been studied using the neutral red uptake (NRU) assay, the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4- sulfophenyl)-2H-tetrazolium (MTS) assay, and a real-time cell analysis (RTCA) impedance-based assay. The 24 h IC_{50} values for several HBQs in T24 cells were found to be in the μM levels, as found in Table 1.3.

Table 1.3: The IC_{50} values of HBQs in T24 bladder cancer cells determined by various assays [49].

HBQ	IC50, μM		
	NRU Assay	MTS Assay	RTCA
2,6-DBBQ	45.7	142.0	21.4
2,6-DCBQ	11.4	94.5	1.9
DCMBQ	148	110.1	58.7
TriCBQ	113	150.7	95.6

The relative cytotoxicity of HBQs was further studied in Chinese hamster ovary (CHO) cells. Wang *et al.* studied that cytotoxicity of four HBQs at three time points [50]. The results showed that DCMBQ had the lowest IC_{50} value, and therefore the highest cytotoxicity, followed by 2,6-DBBQ, 2,6-DCBQ and TriCBQ in decreasing order of cytotoxicity (Table 1.4). The differences in toxicity between cell lines may be due to different characteristics of the cell lines. Compounds interact differently with cell membrane proteins and intracellular enzymes, which results in different biological responses.

Table 1.4: The IC_{50} values at different time points for four HBQs [50].

Compound	IC_{50} (μM ± SEM)		
	24h	48h	72h
2,6-DCBQ	27.3 ± 1.0	35.5 ± 1.0	41.5 ± 1.3
DCMBQ	11.4 ± 0.5	13.7 ± 0.5	15.9 ± 0.9
TriCBQ	45.5 ± 2.5	63.7 ± 2.1	72.9 ± 3.6
2,6-DBBQ	19.8 ± 1.5	29.2 ± 1.8	35.5 ± 0.7

It is unlikely that the regulated DBPs account for the increased risk of bladder cancer associated with the consumption of treated drinking water; therefore, it is important that the toxicity of new classes of DBPs like the HBQs be compared to the toxicity of the regulated DBPs, such as the THMs and HAAs. According to these

separately conducted studies, the 72 h IC_{50} values of HBQs are up to 100 times and 10 times lower than those of the THMs and HAAs, respectively (Table 1.5).

Table 1.5: 72 h IC_{50} values of regulated DBPs (THMs and HAAs) and HBQs in CHO cells (adapted from [43])

DBP	72 h IC_{50} (μM)
THMs [51]	
Chloroform	9620
Bromodichloromethane	11500
Dibromochloromethane	5360
Bromoform	3960
HAAs [52]	
MCAA	810
DCAA	7300
TCAA	2400
MBAA	100
DBAA	590
HBQs [50]	
2,6-DBBQ	35.5
2,6-DCBQ	41.5
DCMBQ	15.9
TriCBQ	72.9

1.3.3 HBQ Transformation

In the environment, HBQs undergo hydrolysis, redox, photodegradation and nucleophilic reactions, resulting in the formation of various new compounds such as semiquinones, hydroquinones, hydroxyl-quinones and benzenetriols [43]. Identifying transformation mechanisms and products is important in determining toxicity because the transformed products can elicit different toxic effects. Wang *et al.* conducted a study that used high-resolution mass spectrometry and analyzed drinking water systems at different points in the drinking water distribution system [50]. They found that HBQs transformed into halo-hydroxyl-benzoquinones (OH-HBQs). As the distance from the drinking water

treatment plants increased, the concentrations of HBQs decreased while the concentrations of OH-HBQs increased.

Wang *et al.* further analyzed HBQs using RTCA, and 24 h IC_{50} values for the compounds were determined in CHO cells. The IC_{50} values of four HBQs were lower than their four corresponding OH-HBQs analogues [50], indicating that OH-HBQ transformation products are less cytotoxic than their parent compound. These finding are relevant to HBQ toxicity because it is likely that humans are exposed to a mixture of HBQs and OH-HBQs in drinking water. Determining the cytotoxic properties of each compound is important to understand the impact of exposure on human health.

1.3.4 Mechanism of HBQ Toxicity

The mechanisms of HBQ cytotoxicity are complex. HBQs can induce the formation of ROS, resulting in oxidative damage to DNA and proteins in cells. Exposure to HBQs produced increased 8-hydroxydeoxyguanosine (8-OHdG) levels and increased protein carbonylation in T24 cells, indicating oxidative damage to genomic DNA and proteins [49]. Furthermore, the depletion of cellular glutathione (GSH) was found to sensitize cells to HBQs, and extracellular GSH supplementation could reduce HBQ-induced cytotoxicity, emphasizing the role of GSH-mediated and GSH-related enzyme-mediated detoxification of HBQs [48]. These findings are consistent with the reported toxic effects of quinone in organisms, particularly those involved in disrupting protein-handling systems [53].

Characterizing the exposure effects of compounds found in drinking water is important to predict the effect of human consumption of drinking water on human health.

The potential developmental neurotoxicity (DNT) of DBPs has not been well studied. Considering that epidemiological studies on DBP exposure and adverse reproductive effects in exposed populations observed inconsistent findings, research into developmental neurotoxic effects of DBP exposure may provide useful information.

In vivo studies to confirm the toxic effects observed in cytotoxicity studies have not yet been conducted. These studies, in addition to further data regarding the toxicological mechanisms of HBQ exposure, bio-molecular interactions, and human exposure risk, are necessary to better understand the full health risks of HBQ exposure. Moreover, no study has investigated comparative developmental neurotoxic effects of the regulated DBPs and HBQs.

1.4 Developmental Neurotoxicity (DNT)

DNT is defined as adverse effects of substances on the nervous system associated with exposure to foreign compounds or xenobiotics during development [54]. DNT is a facet of reproductive toxicity, as reproductive toxicity encompasses a broader field. To thoroughly test the risk of exposure to different chemicals, it is important to include DNT testing in toxicological risk assessments.

Regulatory guidelines for studies that generate information about DNT have been issued by the Organization for Economic Cooperation and Development (OECD) and many national regulatory agencies [55]. The purpose of the OECD guidelines is to identify chemicals that permanently or reversibly affect the nervous system, to characterize any chemical-induced alterations in the nervous system and to estimate dose levels (points of departure) for regulatory uses. To serve this purpose, appropriate models

to test large numbers of chemicals must be identified. Through an assessment of relevant literature, the developmental neurotoxic potential of DBPs will be evaluated, different models used to test developmental neurotoxicity will be discussed, and studies using hNSCs as a DNT model will be summarized.

1.4.1 Models Used to Test DNT

Various methods have been used to test the DNT of chemicals: *in silico* models, *in vitro* models, and *in vivo* models. These methods are able to model different target organs and different developmental endpoints.

1.4.1.1 *In silico* models

In silico computer simulations are often used as a first step when assessing the toxicity of chemicals. *In silico* models use the physical and chemical characteristics of a new compound, and compare those traits to a characterized compound to estimate toxicity in humans. The models have been successfully used to identify important pathways of toxicity [56]. Predictions are derived from databases that weigh and mathematically quantify characteristics of the chemical through quantitative structure-activity relationships (QSAR) [57]. Although computer models are valuable for predicting the absorption, distribution, metabolism, excretion (ADME) pathways of chemicals, they are only as accurate as the parameters set usually by assumption on computer models. Because every chemical acts in a slightly different way with human cells, comparing the accuracy of *in silico* against a different model's outcomes may add to the weight of evidence supporting a particular mechanism of action.

1.4.1.2 *In vitro* models

In vitro cell studies are widely used in toxicological studies because they are high throughput and can mimic toxic effects in many different tissues. Scientists are able to measure different doses and combinations of different chemicals in *in vitro* systems and observe the effects on the growth of cells. The benchmark dose level is the dose that corresponds to a specific change in an adverse response compared to unexposed groups at the 95% confidence limit [58]. From the results of *in vitro* cell culture studies, scientists can extrapolate an approximation of a concentration that would be toxic in humans. Mouse and rat midbrain *in vitro* cell cultures have been used to study the DNT of various compounds [59].

In vitro models are a useful first step when testing toxicity; however, *in vitro* systems do not reflect *in vivo* ADME of compounds. Important systems interactions and organ functions, such as liver detoxification, are not adequately mimicked in *in vitro* cell cultures. Human systems are far more complex than a collection of cells. In addition, *in silico* and *in vitro* modeling are not yet widely used as a standard for DNT testing since modeling data is not yet sufficient to satisfy regulatory guideline-setting requirements [55].

1.4.1.3 *In vivo* models

Non-mammalian and mammalian *in vivo* models have been used to test the DNT of compounds. These models are able to examine DNT endpoints such as cell proliferation, neuronal precursor differentiation, and maturation, and they can be used to analyze behavior and gene expression [54]. A popular non-mammalian DNT model uses

zebrafish embryos since they are transparent and changes in an embryo's structure can be visually monitored. The zebrafish model provides a biological system in which chemicals can rapidly be screened for neuromodulatory effects on multiple behavioral endpoints. In addition, mice and rats are popular models used to analyze behavioural endpoints such as memory, sensory perception, fear perception and spatial awareness [60]. Researchers can also 1) test the motor function of animals, 2) perform neuropathological analysis using perfusion-fixed tissues, and 3) analyze effects of exposure on offspring [55].

Growing evidence, however, indicates that the toxic effects observed in animal models may not be indicative of the same effects in humans. Hartung and colleagues showed that using a method with 60% inter-species-correlation, two species, and a prevalence of 2.5% hazardous substances, resulted in 63% false positive findings [61]. Interspecies differences can create false positives or false negatives when screening compounds for DNT. Moreover, false positive results are unlikely to be noticed, since most regulatory tests are done only once and toxicological studies are often not reported publicly, which means that the self-corrective mechanisms of science do not reach the study results [56]. Furthermore, animal models are not routinely used because of high costs and the requirement of using a high number of animals [55].

One of the most glaring examples of a false negative is in the screening of the drug, thalidomide [62]. The drug was deemed safe for consumption in animal studies and widely used, despite not being approved by the Food and Drug Administration (FDA). Thalidomide was first used as a sleep aid and was also prescribed for off-label uses to manage morning sickness in pregnant women. The drug interfered with babies' normal development, causing many of them to be born with phocomelia, a rare birth disorder

involving the malformation of limbs. Although animal testing can provide researchers with valuable toxicity information, the results may not be reflective of the same effects in humans.

Because of high costs, there has also been efforts towards limiting the use of animal models, mammalian and non-mammalian; and instead, using alternative models to test toxicity. Traditional animal based paradigms are impractical for screening large numbers of chemicals because of high resource requirements [63]. The report, "Toxicity Testing in the 21st Century: A Vision and a Strategy", by the National Research Council emphasizes experimental *in vitro* approaches for the assessment and characterization of chemical toxicities [64]. These tests are able to determine key initiating molecular events, which cannot be studied *in vivo*. The US EPA adopted this strategy in 2009 and has implemented its ToxCast program to screen the toxicity of chemicals [65].

Most existing data on acute and chronic DNT effects are obtained from animal studies and applied to humans, but few have specifically looked at human DNT [66]. Furthermore, although many public health agencies regulate selected DBP levels in drinking water, many of the DBPs evaluated in toxicology studies do not completely account for the increased incidence of diseases observed in epidemiological studies. The regulated DBPs do not cause bladder cancer or adverse reproductive outcomes in animal studies and the concentrations used in many studies do not reflect the levels present in the environment [32,40]. Further development of *in vitro* models remains to better assess potential health effects of DBPs.

1.5 Human Neural Stem Cells (hNSCs)

Studying the differentiation of stem cells presents a promising model for evaluating potential development effects of environmental chemicals [67]. Mouse embryonic stem cells (mESCs) were first isolated and cultured *in vitro* by Evans and Kaufman in 1981, and in 1998, Thomson *et al*. isolated and characterized human embryonic stem cells (hESCs) [68,69]. Since then, embryonic stem cells (ESCs) have been used in a variety of fields to study tissue regeneration and to better understand normal development and to screen drugs and toxicants [70,71]. ESCs have the ability to differentiate into the three germ layers: the ectoderm, mesoderm and endoderm [69]. The ectoderm is the germ layer that forms the neural epithelium, embryonic ganglia and stratified squamous epithelium.

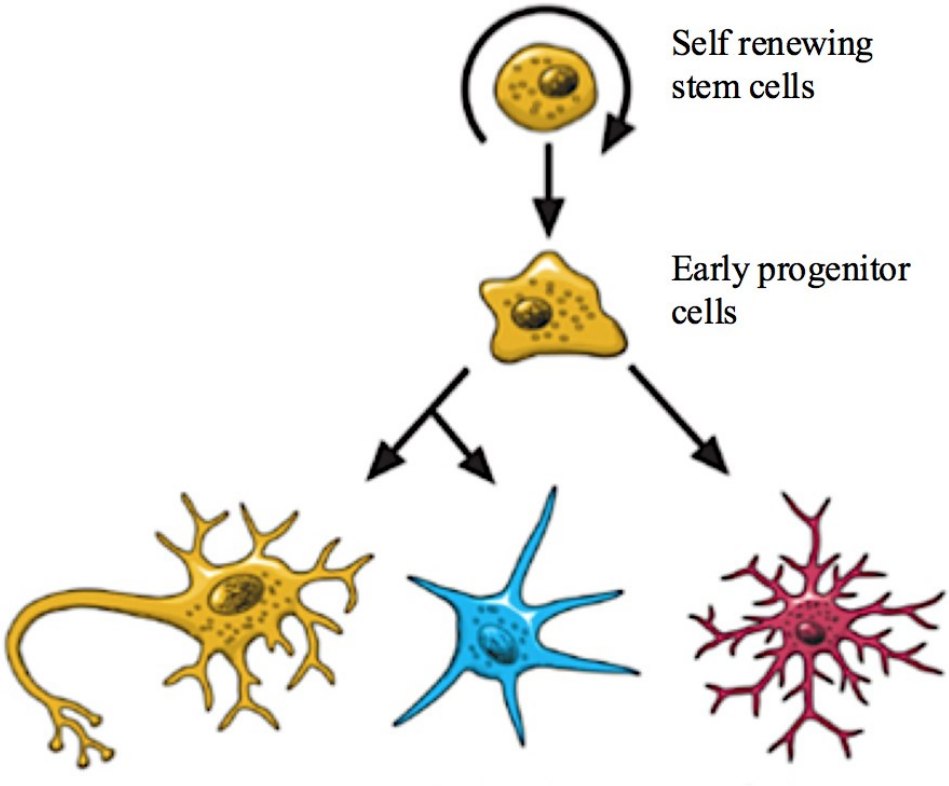


Figure 1.1: The differentiation of neural stem cells in neurons, oligodendrocytes and astrocytes, the three main phenotypes of the nervous system [72].

hNSCs are multipotent cells that generate the main phenotype of the nervous system and *in vitro* cultures can mimic the development of the embryo [73]. This cell line can be derived from embryonic stem cells or induced pluripotent stem cells and are commercially available [74]. hNSCs have the potential to differentiate into the three main phenotypes of neural cells: neurons, oligodendrocytes and astrocytes [74]. hNSCs can be cultured in an undifferentiated proliferative state as free-floating neurospheres or as a monolayer [75]. The cells can be grown as progenitor cells for several generations and differentiate into different populations of neural cells when neural growth factor is removed from media.

Radio *et al*. reviewed *in vitro* models for testing developmental effects of chemicals and emphasized the need to focus on cell cultures of human origin, with emphasis on the emerging area of neural progenitor cells [63]. hNSCs are human-derived and do not incur the same interspecies uncertainties as using animal models or animal cell lines. The advantages of using hNSCs as a model to test DNT include: 1) the cells are human cells, 2) it includes cells at different neurodevelopmental stages, 3) uses simple colourmetric or fluorescence assays, immunostaining, and image analysis, and 4) it is highly sensitive to low doses of neurotoxins, but generally not sensitive to non-neurotoxic compounds [66]. hNSCs have high potential as high throughput tests to screen many chemicals quickly. This is a useful strategy considering the high number of chemicals that are yet to be screened for toxicity [76].

Ryan *et al*. developed an assay with the potential to screen large numbers of chemicals for DNT [77]. This assay focused on neurite outgrowth, one of the mechanisms of neurotoxicity. This endpoint is specific to DNT, compared to other endpoints that are

commonly used in cellular-based assays such as LEC (lowest effective concentration), NOAEL/LOAEL (no observable adverse effect level/lowest observable effect level) or LD_{50}.

Using stem cells to test DNT has many potential benefits; however, the number of toxins examined to date has been small. The value of this model in predicting neural toxicity requires testing of a larger number of chemicals [78].

1.5.1 Applications of hNSCs used to Study DNT

hNSCs have been used in other fields, such as the pharmaceutical industry, and to test other environmental contaminants, including arsenic, mercury and lead.

1.5.1.1 Pharmaceuticals

The pharmaceutical industry has used hNSCs to determine allowable doses of drugs that do not interfere with nervous system development [79]. These studies are especially relevant to mothers who undergo a caesarean section and might have to be put under general anesthetic for the procedure. Animal studies have shown that exposure to general anesthetics can induce widespread neuronal death, but these effects are unconfirmed in human populations. Bai and colleagues and Lei and colleagues used hNSCs to analyze the DNT of general anesthetics and found that the effects were consistent with the *in vivo* animal studies results [80,81]. The researchers recommended maximum allowable doses of anesthetic that did not cause neurotoxic effects in hNSCs to

be used as a starting point for guidelines with regards to general anesthetic administration to pregnant women.

In addition, Cao and colleagues studied the impact of exposure to four antiepileptic drugs (phenobarbital, valproic acid, lamotrigine, carbamazepine) on the risk of DNT [82]. They specifically examined DNA fragmentation, cell viability and cell cycle disruption endpoints. The researchers concluded that this new data shows that modelling neurogenesis *in vitro* using a human stem cell line may be a powerful method to predict risks of DNT *in vivo* with psychotropic drugs.

1.5.1.2 Inorganic Compounds

hNSCs have also been used in studies to determine the DNT of ubiquitous environmental chemicals, particularly heavy metals. Ivanov and colleagues investigated whether sodium arsenite affects signaling pathways that control cell survival, proliferation and neuronal differentiation in hNSCs [83]. The researchers used immunofluorescence analysis and flow cytometry to qualitatively and quantitatively analyze the effects of sodium arsenite exposure. The researchers found that exposure of hNSCs to low doses of sodium arsenite could induce the mitochondrial apoptotic pathway by increasing the expression of MEK-ERK and suppressing the expression of PI3K-AKT. They also found cell cycle length abnormalities in cells exposed to sodium arsenite [83,84]. Changes in gene expression and cell cycle abnormalities are indicators of abnormal neural growth and proliferation.

Using hNSCs, researchers have also analyzed the DNT of methyl mercury (MeHg). MeHg widely exists in the environment and is present in many foods that

bioaccumulate the toxin in its flesh, such as fish. Ceccatelli and colleagues used flow cytometry to analyze the effects of MeHg on hNSCs [85]. The group found that an exposure dose of 3 μM resulted in cell cycle arrest and cell death. Tamm and colleagues investigated the mechanism of apoptosis and found that MeHg induced apoptosis in both models via Bax activation, cytochrome-c translocation, and caspase and calpain activation [86]. Stummann and colleagues also found that hNSCs responded to low doses of MeHg by changing the expression of several neuronal marker genes that are implicated in neuron proliferation [73]. If hNSC differentiation is impeded, it may lead to the abnormal development of the carefully timed process of neural growth during development.

Lead is another environmental contaminant that is found in food and water. Huang and colleagues studied the effects of lead exposure on the differentiation of hNSCs into the three mature neuronal phenotypes [87]. Neurons, astrocytes and oligodendrocytes were separately stained and quantified. The researchers found that lead exposure caused a significant decrease in the number of mature oligodendrocytes with an increase of mature astrocytes; the oligodendrocytes that did appear were malformed. Brain development is a coordinated process of cell proliferation, migration, differentiation, synaptogenesis and apoptosis. Considering the important role of astrocytes during development to guide the normal migration of neurons, abnormal differentiation of astrocytes and increased astrocyte numbers after lead exposure could contribute to abnormal migration of differentiating and maturing neurons.

The above studies show the applicability of using an hNSC model to analyze the DNT of environmental contaminants, which also indicates its suitability to test the DNT

of DBPs. The hNSC model is extremely sensitive to low doses of chemical exposure and has the potential to analyze a multitude of DNT relevant endpoints.

1.6 Hypotheses and Objectives of Thesis

Previous epidemiological and toxicological studies do not explain whether DBPs can adversely affect human neural development. hNSCs have shown to be a useful model for testing chemical neurotoxicity. The objective of this thesis is to study the effects of DBPs on hNSCs with a particular focus on the newly discovered class of DBPs, HBQs. Specifically, this thesis will focus on the following objectives: (1) characterize hNSCs culture for DBP testing, (2) study the effects of HBQs on the viability, cell cycle, and differentiation of hNSCs and (3) assess relative effects of HBQs to regulated DBPs, HAAs.

1.7 Thesis Scope

This thesis contains five chapters. Chapter 2 will describe the design of my experiments and will characterize hNSCs in terms of the effects of passage number and differentiation. The effects of HBQs and HAAs on hNSC viability and cell cycle will be evaluated in Chapter 3. Chapter 4 will then compare the effects of DBPs on neuron differentiation to other regulated DBPs, such as HAAs. Conclusions, limitations of these studies and possible avenues of future research will be discussed in Chapter 5.

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Chapter 2: Characterization of Human Neural Stem Cells

2.1 Introduction

Analyzing the effects of chemicals on stem cells is a promising model to measure the impact of exposure to environmental contaminants on embryonic development [1]. Human embryonic stem cells were first isolated by Thomson *et al.* in 1998 and Zhang *et al.* first isolated neural precursor cells from embryonic cell culture in 2001 [2,3]. Stem cells are a popular tool in fields such as regenerative medicine, but also hold potential to be a valuable model for toxicological screening. Current methods of toxicology testing focus on animal models or primary cell lines. Though studying these models is informative, these methods suffer potential drawbacks from inter-species differences and do not reflect the process of development. Human neural stem cells (hNSCs) allow researchers to test the effects of chemicals on the developing neural system because stem cells are widely represented in the developing nervous system [4]. Thus far, the number of toxins examined using hNSCs has been relatively small [5].

hNSCs are self-renewing cells that are capable of generating the main phenotype of the developing nervous system [6]. The cells have been used to study the effects of pharmaceuticals and various environmental contaminants on the nervous system. Although primary NSCs isolated from rat and mouse models are used because the cells reflect neural development, they are difficult to obtain, lack standardization between cultures, are non-human and expensive. Alternatively, commercially available hNSCs overcome these obstacles. hNSCs derived from H9 embryonic stem cells are particularly useful because the cells are cultured in an undifferentiated state and have the potential to

differentiate into neurons, oligodendrocytes and astrocytes [3]. Researchers are able to direct differentiation into one particular phenotype of interest, or allow differentiation into all three phenotypes in one culture. One of the primary challenges of working with hNSCs as a high throughput model is their slow growth rate, as they have a reported doubling time of 40-50 hours [7].

To validate the use of hNSCs as an appropriate model of developmental neurotoxicity (DNT), it is essential to characterize the expression of undifferentiated and differentiated cell markers to gain appropriate context for further experiments analyzing neuron differentiation and growth. Hence, several characteristics of hNSCs should be assessed: 1) the morphology of differentiated hNSCs, 2) effects of passage number on differentiation, and 3) protein expression changes during differentiation.

Several key proteins are involved in the differentiation of hNSCs. Zimmer *et al.* studied marker expression during neuronal differentiation and found that the neuroectodermal marker, nestin, peaked after 7 days of cell growth, followed by downregulation [8]. Thus, nestin can be used as a marker of undifferentiated stem cells. Sox2 is a neural progenitor marker expressed in the nuclei of stem cells. The expression of Sox2 inhibits neuronal differentiation and results in the maintenance of neural progenitor characteristics [9]. Like nestin, Sox2 can be used as a marker for undifferentiated neurons and the loss of Sox2 expression indicates the lack of ability of a progenitor cell to differentiate. Doublecortin (DCX) is an important protein expressed during corticogenesis and is involved in the migration and differentiation processes of neurons. It is a neuron-specific phosphoprotein, expressed in the cell cytoplasm, and affects the leading processes of migrating neurons and the axons in differentiating

neurons [10]. DCX is present in differentiating neurons, but not neural stem cells. The expression of these markers at different periods of neural development can be used as references for the stage of neuron differentiation.

The objective of this chapter is to assess the potential of using hNSCs as a model of DNT by characterizing the various traits of hNSCs. Cells will be cultured, and differentiation markers will be studied using various methods. As outlined in Chapter 1, hNSCs are a promising tool for evaluating the impacts of environmental contaminants. Hence, before conducting experiments, I will establish the culture conditions and analyze the characteristics of hNSCs and to evaluate properties of neural differentiation.

2.2 Experimental Methods

2.2.1 Safety Considerations

The experiments were performed in a level 2 certified biosafety laboratory following the appropriate procedures for working with biohazards. Cells were cultured in a sterile biosafety cabinet and handled in a sterile environment until fixed in 4% paraformaldehyde (PFA).

2.2.3 Cell Culture

Cryopreserved human embryonic neural stem cells (hNSCs) were obtained from Gibco/Life Technologies (Carlsbad, CA, USA; Cat. No: N7800100). The cells are derived from H9 (WA09) human embryonic stem cells and selectively differentiated into hNSCs before shipment. The cells were cryopreserved in liquid nitrogen. Contents of the cryopreservation tube were thawed in a 37°C water bath. Then the tube contents were

centrifuged for 4 min at 1,200 rpm. The cells were plated in 60 mm x 15 mm dishes or 6-well plates (Corning Incorporated, Corning, NY, USA) coated with 1:1000 CELLstart™ CTS™ Substrate (ThermoFisher Scientific Incorporated, Waltham, MA, USA) and incubated a humidified chamber, at 37°C, and in the presence of room air and 5% CO_2.

hNSC culture was maintained with complete hNSC media containing KnockOut™ D-MEM/F-12 with 2 mM GlutaMAX™-I supplement, 20 ng/mL fibroblast growth factor-basic (bFGF), 20 ng/mL epidermal growth factor (EGF) and 2% StemPro® Neural Supplement (ThermoFisher Scientific). Media was changed 1 d after culture was established, and every 3 d thereafter. Passaging, also known as sub-culturing, allows for multiple plates of cells to be grown from the initial culture. To passage, media and dead cells were aspirated from plates, then live cells were detached using StemPro® Accutase® Cell Dissociation Reagent (Life Technologies). Cells were passaged after plates reached 90% confluency, on average every 6 d. Confluence was estimated through a IV-900 Inverted Microscope (MicroscOptics, Holly, MI, USA) at 20 × magnification. The percent confluency corresponded to the percentage of the bottom of the plate covered by cells. The addition of 3 mL complete hNSC media was added to the plates to stop the dissociation process when all cells were detached. Cells were collected and centrifuged for 4 min at 1,200 rpm and re-suspended in complete hNSC media. The media and cell mixture were then seeded on newly coated plates. An additional 4 mL of complete hNSC media was added to each plate.

Differentiation was induced when hNSCs grew to 75-80% confluence. Complete hNSC media was replaced by changing to differentiation media in cell culture plates. Differentiation media contained 1x Neurobasal Medium, 2mM GlutaMAX™-I

supplement, and 2% B-27® supplement (50×). hNSCs differentiated into neurons for 12 d, with media changes every 3 d. All reagents were obtained from ThermoFisher Scientific.

2.2.4 Morphology of Cultured hNSCs

Cells were seeded onto coated 6-well plates (Corning Incorporated, Corning, NY, USA) and grown for 6 d using complete hNSC media. Approximately 95,000 cells were seeded onto each plate. After cells reached 75-80% confluency, differentiation was induced by replacing complete hNSC media with differentiation media. Cells were allowed to differentiate for 14 d with fresh media changes every 3 d. Cells were imaged using light microscopy to analyze the morphology of differentiated cells. Cells were imaged on day 0, 5, 10 and 14 through a IV-900 Inverted Microscope (MicroscOptics, Holly, MI, USA) at 20 × magnification.

Cells were also imaged for neural stem cell-type specific markers, nestin and Sox2, and nuclear marker, DAPI using fluorescence microscope. The detailed protocol is shown in 2.2.5. A WaveFX spinning disk confocal microscope (Olympus IX-81 motorised microscope base Yokagawa CSU X1 spinning disk confocal scan-head Lenses with Velocity software from Quorum Technologies, Ontario, Canada) was used for immunofluorescence image analysis.

2.2.5 Effect of Passage Number on Differentiation

According to the supplier's instructions, hNSCs are able to retain their proliferation and differentiation potential for at least three passages after thawing. Cells

were analyzed at passage (P) 3, 4, 5, and 6 to determine optimal passage number for further experiments. Cells were stained for neural stem cell-type specific markers, nestin and Sox2, and the nuclear marker, DAPI.

Immunochemical staining was performed using standard protocols. Cells were fixed with 4% PFA (Sigma-Aldrich, St. Louis, MO, USA) in Dulbecco's phosphate buffered solution (DPBS) without $CaCl_2/MgCl_2$ (w/o Ca/Mg) (Gibco, Life Technologies) for 30 min. Fixed cells were then incubated in 200 μL blocking buffer for 30 min. Blocking buffer contains 5% goat serum, 1% bovine serum albumin (BSA), and 0.1% Triton-X, all dissolved in DPBS with Ca/Mg. Blocking buffer (0.4 mL) was added to each sample. Plates were incubated at room temperature. Blocking buffer was removed and cells were incubated with 100 μL primary antibody diluted in 5% serum. Cells were stained for the undifferentiated hNSC marker, nestin, using rabbit polyclonal antibody (Ab) from ThermoFisher Scientific (Cat No. PA5-11887). Cells were stained for the undifferentiated stem cell marker, Sox2, using monoclonal mouse Ab from R&D Systems, Incorporated (Minneapolis, MN, USA). The tubes were incubated at 37°C for 1 h. After incubation, the primary Ab was aspirated and cells were washed three times with DPBS for 5 min on a shaker.

The secondary Abs used were Alexa-Fluor 555 goat anti-mouse IgG and Alexa-Fluor 488 goat anti-rabbit IgG, both from Molecular Probes/Life Technologies (Carlsbad, CA, USA), added to 5% goat serum solution in DPBS with Ca/Mg in 1/1000 concentrations. The samples were incubated in the dark at 37°C for 45 min. After incubation, the secondary Ab was aspirated and cells were washed three times with DPBS for 5 min on a shaker.

Next, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) solution (1:10,000) dissolved in DPBS for 5 min at room temperature. After incubation, cells were washed three times with DPBS for 5 min on a shaker. DBPS (200 μL) was added to each well for sample analysis. The same microscope was used for immunofluorescence image analysis of the differentiation of cells as section 2.2.4 above.

2.2.6 Induction of Differentiation

After cells reached 75-80% confluency, differentiation was induced by replacing complete hNSC media with differentiation media. Cells were allowed to differentiate for 14 d with fresh media changes every 3 d. Cells were imaged using confocal microscopy to analyze immunofluorescence and western blot analysis to look at protein expression of markers.

For the imaging experiments, cells were fixed and stained with nestin as described above (Section 2.1.4). Cells were stained for the differentiated stem cell marker, doublecortin (DCX), using monoclonal mouse Ab from Santa Cruz Biotechnology (SCB; Dallas, TX, USA). The secondary Abs used were as described in Section 2.2.5, while immunofluorescence analysis was also performed as described in Section 2.2.5. The 491 nm emission filter was used to identify nestin and the 561 nm emission filter was used to detect DCX.

Western blots were performed using standard protocols. Samples were collected at 0 d and 14 d after differentiation was induced. First, cells were detached from cell culture plates using StemPro® Accutase® Cell Dissociation Reagent (Life Technologies). Detached cells were centrifuged for 4 min at 1,200 rpm to form a cell

pellet. Cell lysis mixture (50 μL) was then added to each sample. Cell lysis mixture contains 5% 1M Tris HCl, 1x PI Mix, 1% ETDA, 0.25% SDS, 0.3% DNAase I and 1% $MgCl_2$. Samples were incubated at 37°C for 30 min and tubes were shaken every 5 min. After cells were dissolved, samples were centrifuged at 16,000 rpm for 10 min. A standard curve was created using Coomassie Plus™ (Bradford) Assay Kit (ThermoFisher Scientific) to quantify protein concentrations in samples.

Proteins (10 μg) were resolved using 10% SDS-PAGE (Bio-Rad Laboratories Inc., Hercules, CA, USA). The monoclonal Abs used for western blotting included: 1) anti-β-actin (Sigma-Aldrich) and 2) DCX (monoclonal mouse Ab from SCB). The polyclonal Ab used was nestin (rabbit polyclonal Ab from Abcam). The secondary Abs were goat anti-mouse (GAM) and goat anti-rabbit (GAR) horseradish peroxidase (HRP) in 1:10,000 dilutions (ThermoFisher Scientific). Clarity™Western ECL Substrate (Bio-Rad Laboratories Inc.) was used to detect proteins bound to blotting membranes. Signals were detected using ImageQuant™ LAS 4000 (GE Healthcare Life Sciences Limited, Mississauga, ON, Canada). Images were processed using the ImageQuant™ TL software.

2.3 Results and Discussion

2.3.1 Morphology of Cultured hNSCs

The first step to characterize the hNSCs was to image the cultures at different days after differentiation was induced to examine their physical properties. The longer the hNSCs were left to differentiate, the longer the dendrites appeared to grow, as shown in Figure 2.1. At 5 d after differentiation was induced, visible dendrites appeared to form,

extending from the cell bodies of neurons. By 10 d and 14 d after differentiation was induced, the dendrites appeared to grow longer.

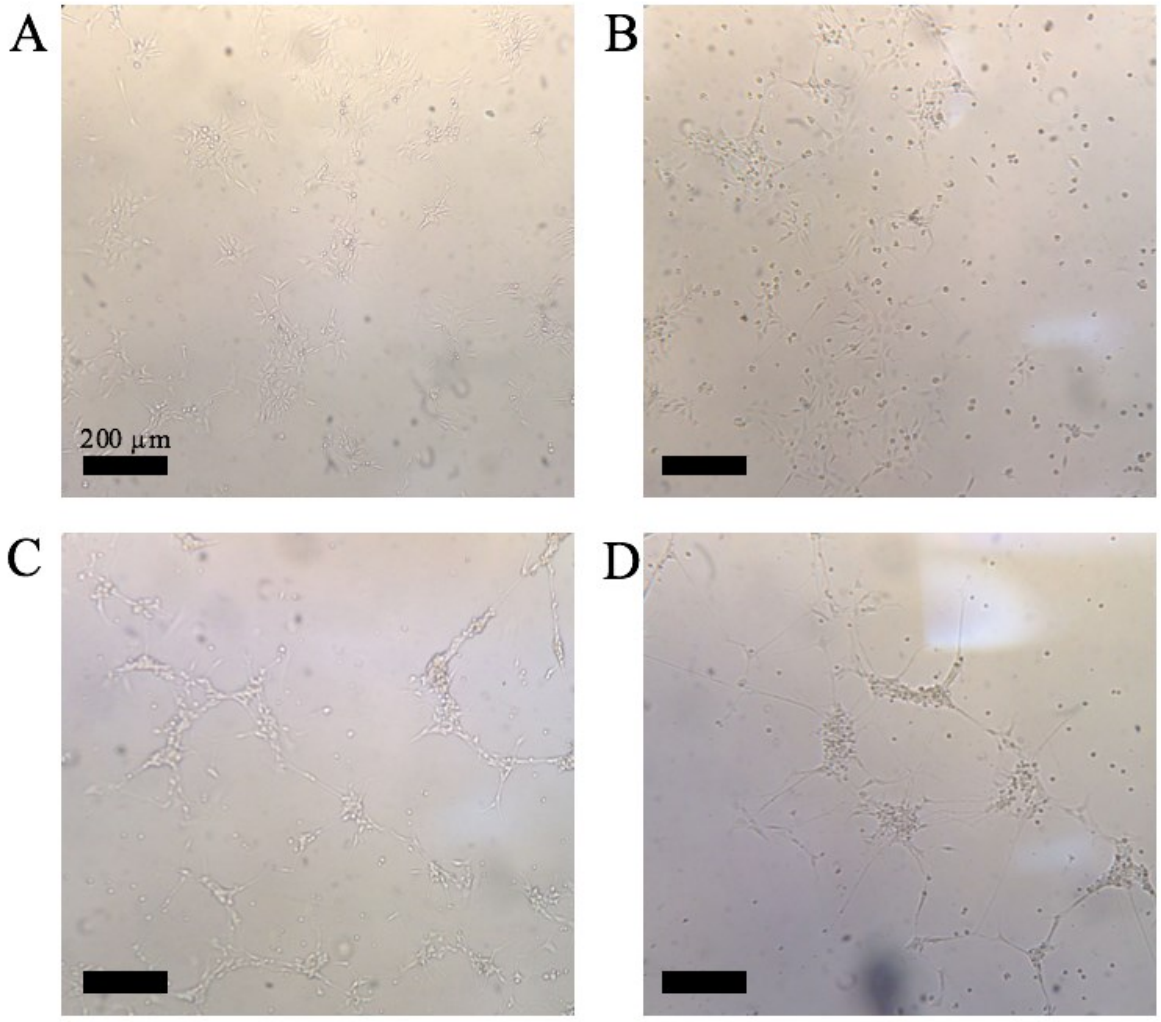

Figure 2.1: Light microscopy image (20×) of hNSCs at 0 d (A), 5 d (B), 10 d (C) and 14 d (D) in differentiation medium. Cells started to form visible dendrites 5 d after differentiation was induced. The longer the hNSCs were left to differentiate, the longer the dendrites grew. Each scale bar represents 200 µm.

At the early stages of differentiation, 0 d (Fig. 2.1A) and 5 d (Fig. 2.1B) after changing the media to differentiation media, cells remain growing in a monolayer and are relatively evenly distributed at the bottom of the cell culture plate. Comparatively, in the

images of cell cultures 10 d (C) and 14 d (D) after the induction of differentiation, cells no longer appear to grow in a monolayer; instead, cells appear to have formed aggregates. These aggregates are likely neurospheres, which naturally form when hNSCs cells are cultured *in vitro*. Neurospheres are a heterogeneous cell population and contain many differentiated cells in addition to progenitor cells [11]. Culturing cells at lower densities may result in the formation of fewer neurospheres in culture, making it easier to identify individual neurons.

Fluorescence microscopy is another method to show the morphology of cultured hNSCs. Confocal microscope was used to image the hNSCs, and characterize two undifferentiated hNSC markers, the cytosolic nestin and nuclear Sox2. As shown in Figure 2.2, Sox2 is stained red, which is co-localized with DAPI (blue) in nuclei, and nestin is shown as green. From the merged image, we can know that these cells are in their undifferentiated status, which means they possess the properties of neural stem cells.

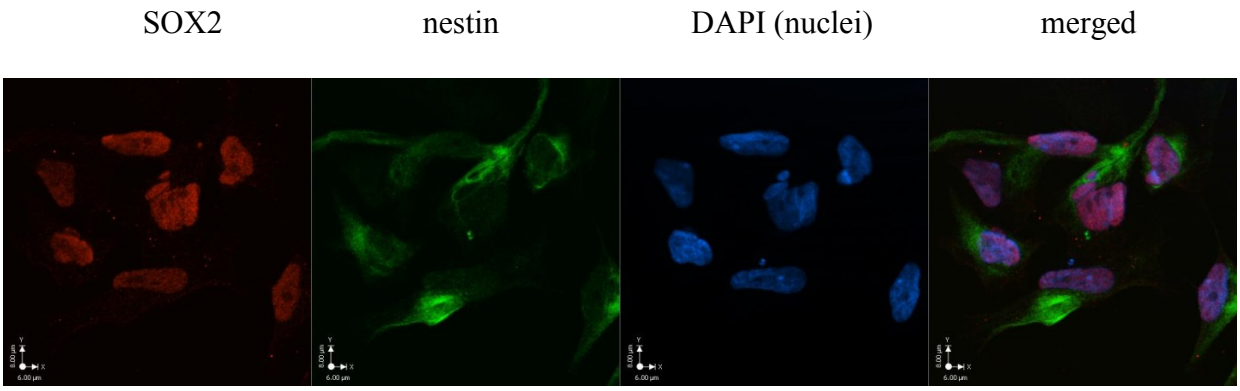

Figure 2.2 Confocal fluorescence image of hNSCs at P3. Cells are stained for the undifferentiated NSC markers: nestin (green) and Sox2 (red). Cell nuclei were counterstained with DAPI (blue). Each scale bar represents 6 μm.

Another interesting aspect of hNSC morphology was that at all time points of analysis after media was switched to differentiation media, there appeared to be cells floating in cell media. These cells are likely either dead cells that have detached from the bottom of the cell culture plates, or they may be viable cells that have detached and may subsequently reattach to the bottom of the plate.

2.3.2 Effect of Passage Number on Differentiation

Figure 2.3 shows the fluorescence images (20×) of hNSCs at P3 (A), P4 (B), P5 (C) and P6 (D) in complete culture medium and stained for the undifferentiated hNSC markers, nestin and Sox2. Approximately 80-90% of the cells stained positive for the undifferentiated hNSC markers, nestin and Sox2, at P3, P4 and P5. At P6, there was low expression of undifferentiated hNSC markers, indicating that there are few remaining hNSCs in the culture and that hNSCs cannot differentiate after P6.

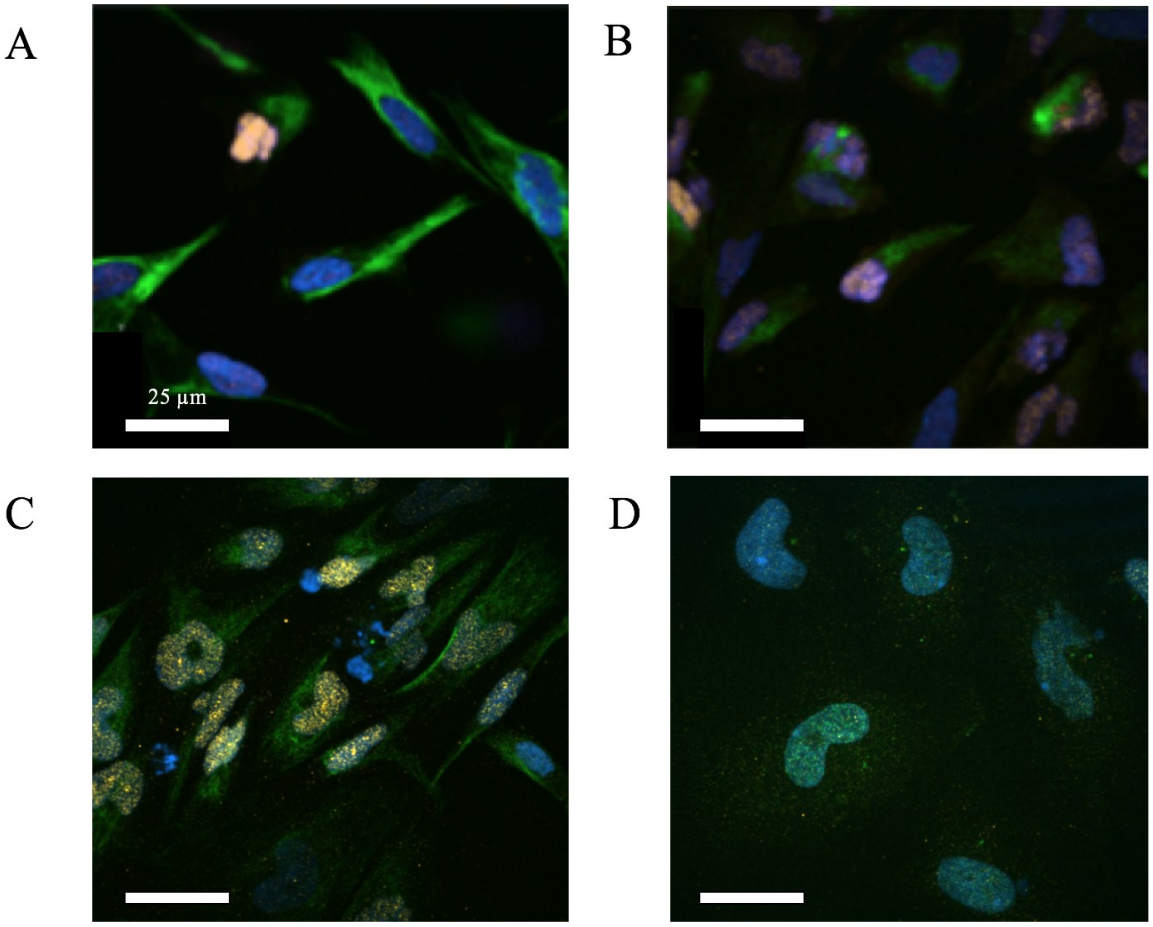

Figure 2.3: Fluorescence image (20×) of hNSCs at P3 (A), P4 (B), P5 (C) and P6 (D) in complete culture medium and stained for the undifferentiated hNSC markers: nestin (green) and Sox2 (yellow). Cell nuclei were counterstained with DAPI (blue). At P6, there are almost no undifferentiated hNSC markers. Each scale bar represents 25 µm.

In Figures 2.3A and 2.3B, nestin (green) is clearly present in the cytoplasm of the cells. From Figure 2.3C, it is clear that these hNSCs express Sox2, found in the nucleus of the cells. Comparatively, the cells in Figure 2.3D do not express nestin or Sox2. The expression of undifferentiated neural progenitor cell markers is an indicator that the hNSC has the potential to differentiate into different neural phenotypes. hNSC cells that do not express these markers may not have the potential to differentiate into neurons,

oligodendrocytes or astrocytes. The results of this experiment show that these hNSCs do not have the potential to differentiate at passage 6. Therefore, it is important to ensure that all subsequent experiments are conducted with hNSCs that have been passaged 5 or fewer times.

Many studies have observed that hNSCs undergo cellular and replicative senescence after being passaged multiple times in culture [12-14]. These observations may be explained two ways [12]. First, inadequate cell culture conditions may pose stress on cells in a phenomenon called cell culture shock. This phenomenon hypothesizes that hNSCs incur irreversible damaging changes through each passage. This damage accumulates and ultimately triggers senescence. The second theory posits that telomere attrition is an intrinsic mechanism that triggers senescence after a certain number of passages. Telomeres shorten after each subsequent generation and cells at a critical telomere length die.

These results confirm that hNSCs can only be passaged a finite number of times before the cells lose the expression of stem cell differentiation markers. In future experiments when differentiation is induced, cells will be differentiated at passage 4 or 5.

2.3.3 Induction of Differentiation

To understand changes in protein expression in undifferentiated and differentiated stem cells, confocal microscopy and western blotting were used to visualize the expression of doublecortin (DCX), a differentiated neuron marker, and nestin, an undifferentiated neuron marker.

Cells were grown in differentiation medium for 14 d. Figure 2.3 shows hNSCs at day 0 (A) and day 14 (B) after differentiation was induced. At day 0, the hNSCs express both nestin and the nuclear marker, DAPI, but do not express DCX. Comparatively, on day 14, the hNSCs express all three: DCX, nestin, and DAPI. During the 14 days of differentiation, the amount of DCX expressed by hNSCs increased, while the hNSCs maintained the expression of nestin and DAPI. Thus, DCX can be used as a marker of differentiation since it is expressed in cells that have been allowed to differentiate compared to the undifferentiated stem cells.

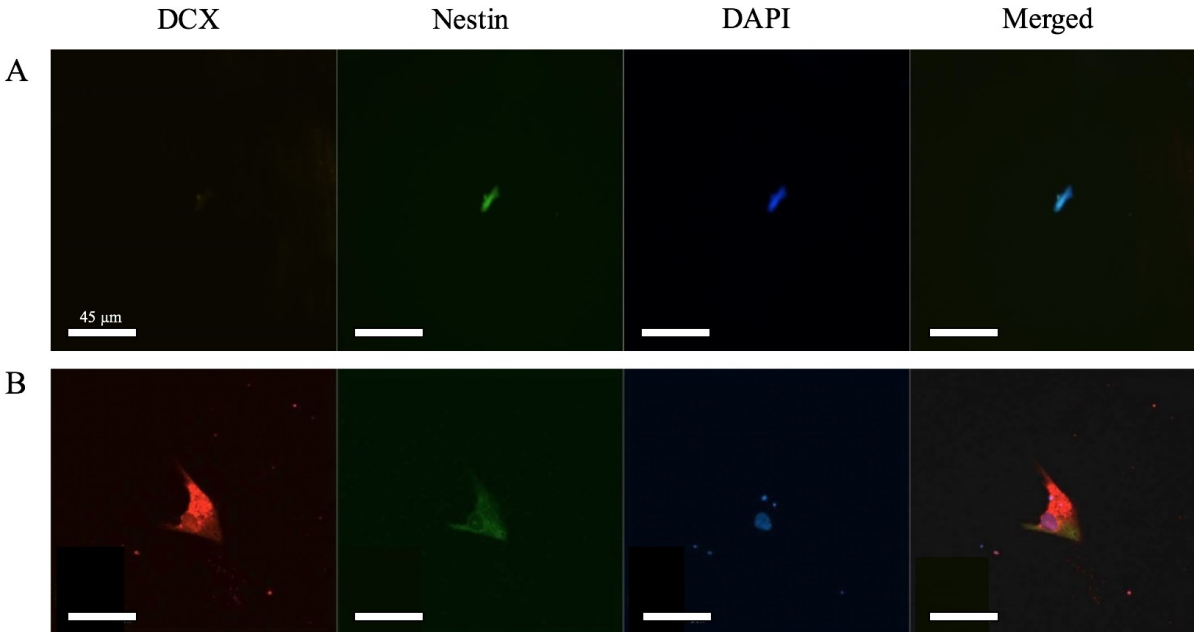

Figure 2.4: Confocal microscopy images of hNSCs at day 0 (A) before differentiation and day 14 (B) after differentiation was induced. Images were taken at 20 × magnification. The four frames show the same cell with the DCX (red), nestin (green) and DAPI (blue) channels separated. Each scale bar represents 45 µm.

The results observed through confocal microscopy were verified using western blot analyses of total protein extracts before and after the induction of differentiation. Total β-actin levels were consistent between the differentiated and undifferentiated

groups and were used as a control. A distinguishing factor of differentiation was the expression of DCX in cells that had been exposed to differentiation media for 14 d compared to the undifferentiated group. Both 0 d and 14 d groups expressed nestin, but only the day 14 group expressed DCX. To measure protein content, I used the Bradford assay and the data may not be reliable because of the presence of SDS in the cell lysates. The BCA assay would be a more appropriate assay to use for measuring protein content for this experiment.

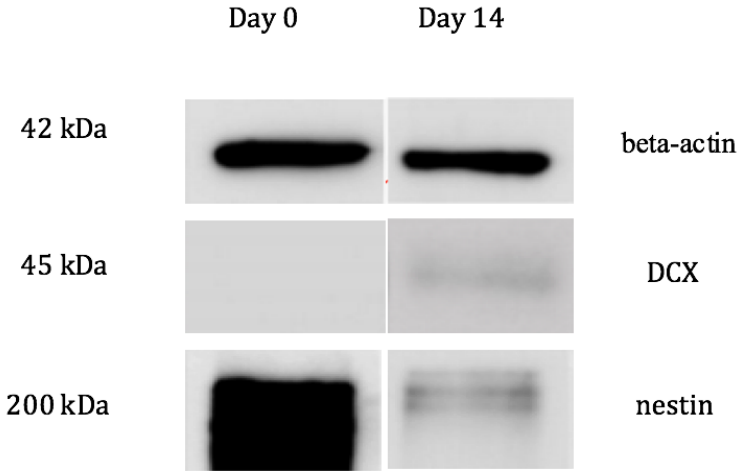

Figure 2.5: Proteins were detected using western blot analyses on day 0 and day 14 after differentiation was induced. β-actin was used as a control. DCX is expressed in day 14 cells, but not in undifferentiated (day 0) cells. Nestin is expressed in both day 0 and day 14 cells. Images are from the same blot from non-neighboring columns.

These two experiments confirm that nestin is an appropriate marker to use to indicate the presence of undifferentiated stem cells, and DCX is an appropriate marker for differentiated cells. Hence, these protein markers will be used as a reference for the different stages of differentiation of hNSCs. Changes in these protein markers after DBP exposure will allow us to assess the DNT effects of regulated and nonregulated DBPs.

Key characteristics that are important for models to be used for high-throughput toxicity testing is the ability to test many chemicals in a time-efficient manner. hNSCs grow extremely slowly compared to other cell lines and require being plated at high densities. Furthermore, I found that it was difficult to grow a sufficient number of cells to provide sufficient power for my analysis. As a result, I grew my cells in 60 mm dishes, which limited the number of experiments I could conduct because of limited incubator space. Because of these two factors, it is difficult to quickly screen chemicals for the effect on developmental toxicity using the protocols employed in my experiments. These limitations can be overcome by scaling-up the number of cells grown.

2.4 Summary

In summary, experiments were conducted to characterize the properties of hNSCs to inform the design of further experiments. I imaged the differentiation of hNSCs over a 14 d period and observed dendrite growth and the formation of neurospheres in cell culture. I also determined that cells could not differentiate beyond passage 5. In addition, I used qualitative imaging methods to measure protein expression of undifferentiated and differentiated cells. Undifferentiated cells express nestin and do not express DCX. Comparatively, differentiated hNSCs express both nestin and DCX, indicating that DCX can be used as an indicator of mature neurons. Overall, hNSCs are an appropriate model for evaluating the potential DNT effects of DBP exposure; however, long doubling times and high density culture requirements are two issues that must be overcome for researchers to use hNSCs as a high-throughput toxicity testing technology.

2.5 References

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Chapter 3: Effects of DBP Exposure on Human Neural Stem Cells

3.1 Introduction

In previous studies that focused on DBP exposure in *in vitro* cell cultures, DBP exposure has been shown to be cytotoxic and can adversely affect the cell cycle. Several studies have shown the negative effects of DBP exposure on cell viability [1,2]. To meet the objectives of my thesis, testing cell viability at different concentrations of DBP exposure is important to ensure that exposure concentrations used in subsequent experiments are appropriate to observe the sub-lethal effects of DBP exposure in hNSCs. Out of the identified HBQs, Zhao *et al*. found the highest levels of 2,6-DCBQ levels in drinking water at 165.1 ng/L levels [3]. Wang *et al*. found that the IC_{50} levels after 48 h in CHO cells was 35.5 μM [4]. The 2,6-DCBQ concentrations that humans are exposed to in drinking water is over 30,000 times lower the IC_{50} values in CHO cells. The goal of my exposure experiments will be to expose the hNSCs to sub-lethal concentrations of DBPs to focus on the non-lethal cellular effects of exposure, which can model chronic, low-dose DBPs effects.

Studies conducted on hNSCs have shown cell cycle alterations as a result of exposure to environmental contaminants such as mercury and arsenic [5,6]. DNA damage in cells has been highly associated with cell cycle modulation effects. Exposure to compounds that damage DNA affects pathways that regulate cell cycle arrest and apoptosis [7]. In addition, studies examining DBP exposure to different cell lines have shown that DBP exposure alters the normal cell cycle of cells, such as CHO cells [8]. One study showed that haloacetonitrile (HANs) exposure results in cells with abnormal

numbers of chromosomes. Furthermore, exposure to HAAs have been shown to alter transcriptome profiles associated with genes regulating cell cycle and apoptosis in human small intestine epithelial cells [9].

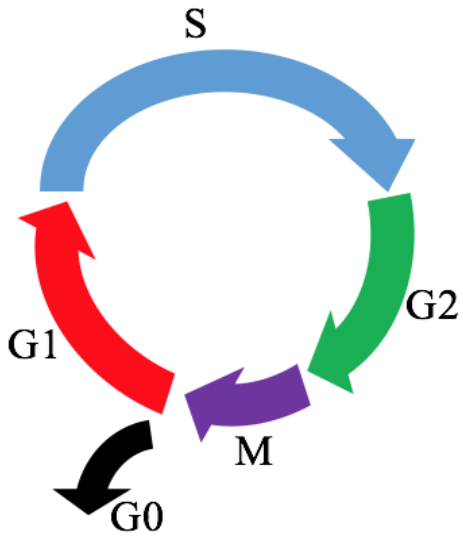

Figure 3.1: Diagram of cell cycle phases. Cell replication consists of four main phases: G0/G1, S, G2 and M phase. G0/G1 phase is the resting and growth phase of cells, S-phase is where DNA replication occurs, G2 is the second growth phase, and M-phase is when the cell divides.

Eukaryotic cell division is typically divided into two distinct phases: mitotic (M) phase and interphase (G0/G1, S, and G2) [10]. The cell grows throughout interphase, which includes G1, S and G2. During S-phase, DNA replication occurs. There are checkpoints at each cell phase to ensure that cells are capable of undergoing replication. During M-phase, cell growth stops and the cell divides into two daughter cells.

Cell cycle analysis is conducted using propidium iodide (PI) staining, a chemical that intercalates with DNA and fluoresces PI fluorescence intensity proportional to the DNA content of cells. Flow cytometric analysis is used to produce a frequency histogram

that counts the number of cells by DNA content. Hence, cells in G0/G1 will has one set of DNA content, while cells in G2/M have two sets of DNA content. Cells in S-phase will have between one and two sets of DNA content due to the replication occurring during this phase.

The effects of HBQs on cell cycle will be compared to that of a regulated class of DBPs, haloacetic acids (HAAs). The cytotoxicity of HAAs have been more widely studied than other classes of DBPs. Plewa and colleagues compared the cytotoxicity of HAAs in CHO cells to mutagenicity of HAAs in *S. typhimurium* [1]. The cytotoxicity and mutagenicity studies ranked the four HAAs in the same order: BAA > DBAA > CAA > DCAA. Pals *et al.* examined the biological mechanisms of HAA toxicity [11]. They found that HAAs inhibit glyceraldehyde-3-phosphate dehydrogenase (GAPDH) activity in a concentration-dependent manner with BAA being more cytotoxic than CAA. The results showed the rate of inhibition of GAPDH and the toxic potency of the monoHAAs are highly correlated with their alkylating potential and the propensity of the halogen leaving group.

Although regulated DBPs show toxic effects in cell studies, an animal study conducted by Narotsy *et al.* indicates that non-regulated DBPs may account for the toxicity observed in epidemiological studies [12]. I will also use sodium arsenite as a positive control because arsenite exposure has been shown to induce cell cycle effects [5].

The first part of this chapter will focus on testing the cell viability of hNSCs after exposed to DBPs and selecting concentrations that result in over 80% cell viability after 24 h exposure. Then, I will analyze the cell cycle effects of DBP exposure on hNSCs.

Since some DBPs have been shown to be genotoxic [11,13], we hypothesize that the cell cycle of hNSCs will be affected by DBP exposure. Thus, the objective of this chapter is to study the effects of DBP exposure in hNSCs to determine if HBQ or HAA exposure alters normal cell cycles.

3.2 Experimental Methods

3.2.1 Reagents

Stock solutions of the DBPs used in these experiments were prepared in methanol (HPLC grade, Fisher Scientific, Ottawa, ON, Canada) to ensure the stability of the DBPs. 2,6-dibromo-1,4-benzoquinone (2,6-DBBQ) was purchased from Indofine Chemical Company (Hillsborough, NJ), and 2,3,6-trichloro-1,4-benzoquinone (TriCBQ) was synthesized by Shanghai Acana Pharmatech (Shanghai, China). 2,6-dichloro-1,4-benzoquinone (2,6-DCBQ), bromoacetic acid (BAA) and chloroacetic acid (CAA) were purchased from Sigma-Aldrich (St. Louis, MO). 2,6-DBBQ, 2,6-DCBQ and TriCBQ were stored as 50 mM stock solutions while BAA and CAA were stored as 100 mM stock solutions. Stock solutions were stored at -20°C at 30 µl volumes in 200 µl tubes.

3.2.2 Cell Culture

Cells were cultured according to the same procedures as section 2.2.3 in Chapter 2 of this thesis.

3.2.3 Cell Viability Analysis

Cells were seeded onto coated 6-well plates (Corning Incorporated, Corning, NY, USA) and grown for 6 d using complete hNSC media. After cells reached 75-80% confluency, differentiation was induced by replacing complete hNSC media with differentiation media. Cells were allowed to differentiate for 14 d with fresh media changes every 3 d. Cells were imaged using light microscopy to analyze the morphology of differentiated cells. Cells were imaged on day 0, 5, 10 and 14 through a IV-900 Inverted Microscope (MicroscOptics, Holly, MI, USA) at 20 × magnification. Qualitative observations on cell density and growth were recorded.

For further cell viability studies, after the establishment of hNSC culture, cells were exposed to differentiation cell culture media (negative control), sodium arsenite (As^{III}; 0.5 μM), 2,6-DBBQ (0.1, 0.5 μM), 2,6-DCBQ (0.1, 0.5 μM), BAA (0.1, 0.5 μM), or CAA (0.1, 0.5 μM). Exposure to As^{III} was used as a positive control for adverse effects on cell viability.

Cell viability was assessed in two ways. First, cell viability was assessed using the trypan blue exclusion assay visualized with an IV-900 Inverted Microscope (MicroscOptics, Holly, MI, USA) with 10 × magnification. Cells were detached from plates using StemPro® Accutase® Cell Dissociation Reagent (Life Technologies). After dissociated cells were collected, plates were washed with phosphate buffered saline (PBS; Gibco, Life Technologies) twice. Detached cells were then centrifuged at 1,200 rpm for 4 min and re-suspended in 1 mL PBS. Then, 0.1 mL of 0.4% trypan blue solution was added to the 1 mL suspension of cells, and 10 μL of the suspension was loaded onto a hemocytometer. The proportion of live cells and the number of total cells were counted,

and viability was calculated relative to the control group. Trypan blue cell viability experiments were conducted in a single sample. Cell viability was calculated via the following equation:

$$\%\ viable\ cells = \left[1 - \left(\frac{number\ of\ blue\ cells}{number\ of\ total\ cells}\right)\right] \times 100$$

In the second cell viability experiment, detached cells were centrifuged for 4 min at 1,200 rpm, re-suspended in 1 mL PBS, and stained using 1 µL of LIVE/DEAD® Fixable Violet Dead Cell Stain (405 nm excitation, ThermoFisher) for 30 min on ice. Cells were then fixed with 4% PFA in PBS for 30 min and analyzed using an LSRFortessa X-20 flow cytometer (BD Biosciences, San Jose, CA, USA) with accompanying BD FACSDiva software to preview and record data. FlowJo LLC data analysis software (Ashland, OR, USA) was used to analyze the FACS data. FACS analysis was performed in triplicate.

In both sets of these experiments, the relative cell viability between days 9-12 (the day of the final media change to the day of analysis) was measured. It is important to note that the cell viability measurements are used to compare the treatment groups to each other and not to be used as a measure of the total cell viability of the entire differentiation process. Results of cell viability experiments are displayed as the percent viability compared to the negative control.

3.2.4 Cell Cycle Analysis

Cells were exposed to complete hNSC cell culture media (negative control), As^{III} (0.5 µM), 2,6-DBBQ (0.5, 1 µM), 2,6-DCBQ (0.5, 1 µM), BAA (0.5, 1 µM), or CAA

(0.5, 1 μM). Exposure to As^{III} was used as a positive control for negative effects on cell viability. hNSCs were exposed to DBPs for 4 d, with a media change on day 3. Treatment groups were prepared in triplicate.

Cells were detached from the matrix on the plates with StemPro® Accutase® Cell Dissociation Reagent (Life Technologies). Detached cells were centrifuged for 4 min at 1,200 rpm, re-suspended in 1mL Dulbecco's phosphate buffered solution (DPBS; Gibco, Life Technologies), and stained using 1μL LIVE/DEAD® Fixable Violet Dead Cell Stain Kit, for 405 nm excitation (ThermoFisher Technologies Inc.). After incubating cells on ice for 30 min, cells were centrifuged at 1,200 rpm for 10 min and fixed by re-suspending cells in 600 μL DPBS before adding 1.4 mL chilled 100% ethanol to create a final concentration of 70% ethanol in 2 mL.

After fixing, cells were centrifuged for 10 min at 1,300 rpm and re-suspended in 0.5 mL of FxCycle™ PI/RNase Staining Solution (ThermoFisher Technologies). Samples were incubated for 30 min at room temperature, protected from light, and analyzed using flow cytometry with the LSRFortessa X-20 flow cytometer (BD Biosciences, San Jose, CA, USA) and BD FACSDiva software (BD Biosciences) to preview and record data. Live cells were gated to ensure that only the cell cycles of live cells were analyzed for DNA content. Samples were run in triplicate until the number of gate events reached 100,000. FlowJo LLC (Ashland, OR, USA) data analysis software was used to analyze the FACS data. The cell cycle analysis function in FlowJo was used to create histograms and to separate peaks showing different phases of the cell cycle.

3.2.5 Statistical Analysis

All data is presented as means ± standard deviation (SD) unless otherwise noted. All measurements were conducted in triplicate for each treatment group and controls. Statistical analyses were performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). One-way analysis of variance (ANOVA) was used for multiple comparisons among treatment and control groups. Differences were considered statistically significant at $P < 0.05$.

3.3 Results and Discussion

3.3.1 Effects of DBP Exposure on Cell Viability

This experiment is necessary to guide further experiments using sub-lethal doses of DBPs. First, I exposed cell cultures to a large range of DBP concentrations to observe the qualitative effects of DBP exposure on the differentiation of hNSCs. Figure 3.2 shows the results of 2,6-DBBQ exposure on hNSCs as an example. Then, two quantitative methods were used to measure cell viability: the trypan blue exclusion assay and flow cytometry using a live-dead cell dye.

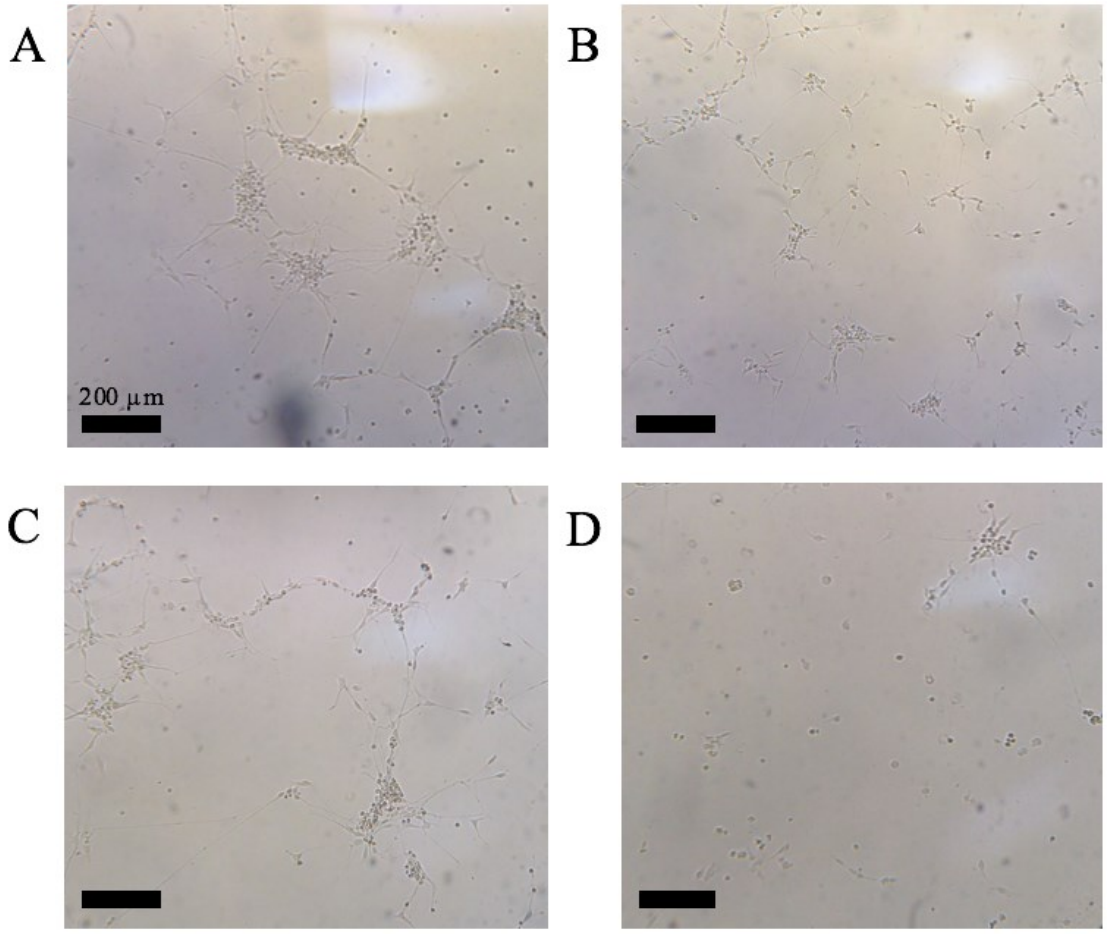

Figure 3.2: Light image (10×) of hNSCs at 14 d after media was changed to differentiation medium. The exposure groups were no treatment/control (A), 0.5 μM 2,6-DBBQ (B), 1 μM 2,6-DBBQ (C) and 10 μM 2,6-DBBQ (D) in differentiation medium. Each scale bar represents 200 μm.

Light microscopy images in Figure 3.2 show hNSC cultures exposed to three concentrations of 2,6-DBBQ for 14 d after the induction of differentiation. In the negative control group (A), cells form aggregates and many viable dendrites extend from the clusters of cell bodies. The 0.5 μM 2,6-DBBQ (B) and the 1 μM 2,6-DBBQ (C) treatment groups also have a comparable density of cells compared to the control and visible dendrites. Comparatively, the 10 μM 2,6-DBBQ treatment group has a very low density

of cells attached to the bottom of the plate and there are more detached cells floating in cell media. Cells that undergo apoptosis detach from the bottom of the cell culture dish. This effect may explain the lower density of cells observed in these images since detached cells are aspirated from the culture dish during media changes. Based on the qualitative analysis of the light microscopy images of the hNSCs exposed to the DBPs under examination in this study, I will use DBP concentrations lower than 1 μM to ensure high viability in all treatment groups in further experiments.

The trypan blue exclusion assay and flow cytometry experiments were then used to quantify the decrease in cell viability at day 9 and day 12 after differentiation was induced. The total decrease in cell viability over 12 d of exposure could not be determined since all dead cells are aspirated during media changes. In adherent cultures of hNSCs, only live cells remain adhered to the bottoms of plates, while both dead and live cells may be floating in the cell culture media. During media changes, the cell media containing both dead cells and some live cells are aspirated. At day 12, the media collected contains live cells and cells that had died between day 9 and day 12.

Cell viability was studied using the trypan blue exclusion assay. In the negative control sample, the cell viability was low, 46.8%. This number confirms the observations made in Chapter 2 where many cells were observed floating in culture. These observations indicate the hNSCs are a fragile cell line and are extremely sensitive when grown in culture. All exposure groups decreased the cell viability of hNSCs in comparison to the control group with the exception of the 0.1 μM BAA exposure group.

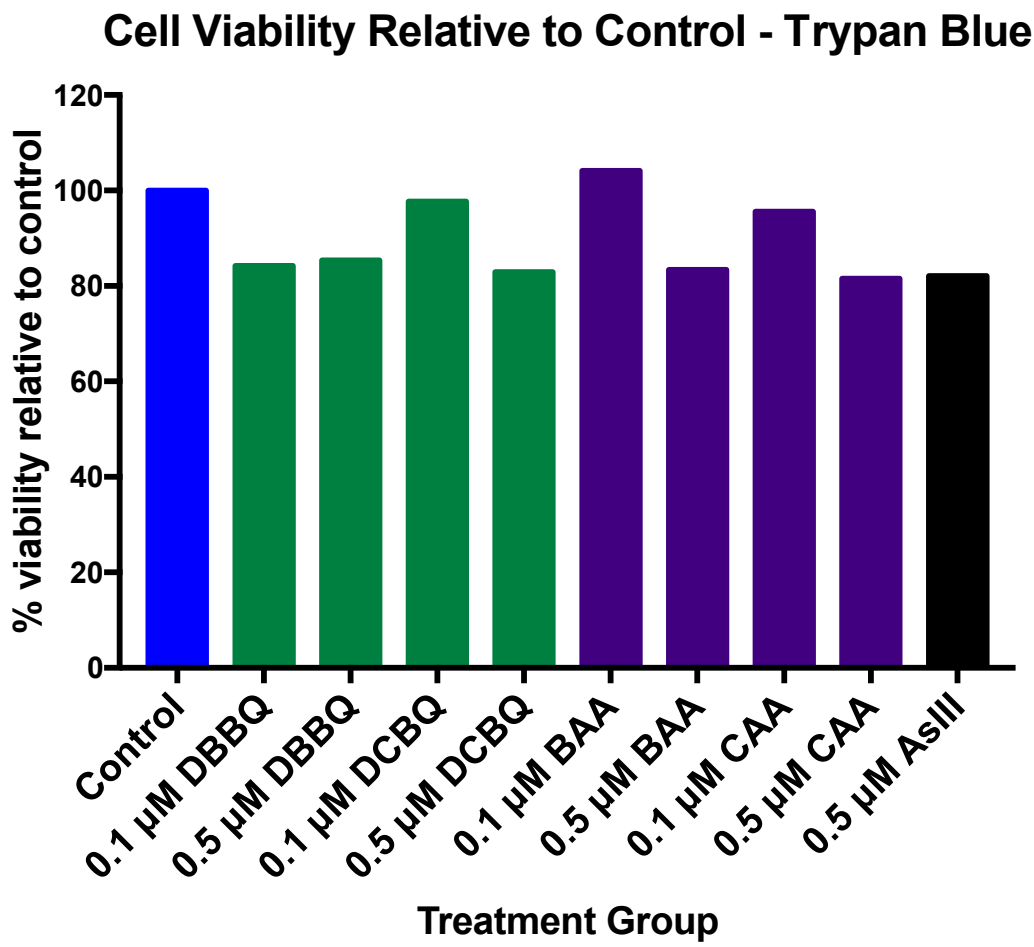

Figure 3.3: Cell viability was confirmed using the trypan blue exclusion assay to determine the relative cell viability of treatment groups compared to the control. No exposure group resulted in a greater than 20% decrease in cell viability relative to untreated control.

Using the trypan blue exclusion assay to determine cell viability is a labour-intensive process that requires each cell to be counted, making it difficult to study the cell viability of multiple treatment groups in triplicate. Since this experiment only treated one well with each treatment group, significance could not be determined. Thus, alternative methods to study cell viability are recommended for hNSCs when studying many treatment groups to determine significance. Alternative methods could include using MTT or neutral red uptake assays to determine cell viability. I have selected to use flow

cytometry methods as a secondary method to assess the effects of HBQs and HAAs on hNSC cell viability, as flow cytometry can be used to assess the viability of both adherent and suspended cells.

Flow cytometry (FACS) analysis to study the effects of DBP treatment on hNSC cell viability used a commercially available LIVE/DEAD® Fixable Violet Dead Cell Stain Kit. The stain is impermeable to the cell membrane when the cell is alive, creating a peak at a lower signal. If a cell is dead, the dye easily crosses the cell membrane and is retained, providing a stronger signal to the flow cytometer. Hence, in a flow cytometry histogram, the left peak will represent the number of live cells in the sample and the right peak will reflect the number of dead cells. Figure 3.4 shows an example of a flow cytometry histogram created during data analysis using FlowJo software.

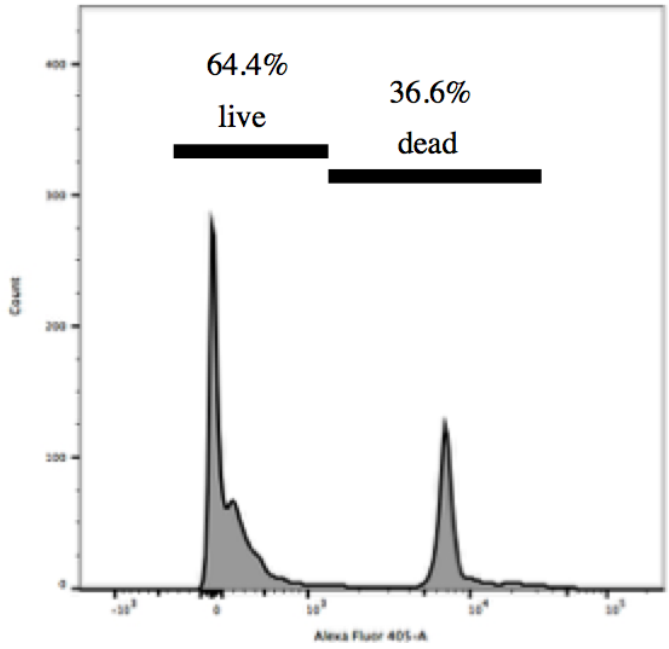

Figure 3.4: A flow cytometry histogram of cells stained with LIVE/DEAD® Fixable Violet Dead Cell Stain Kit, showing two distinct peaks. The peak on the lower intensity (left) represents the cells that are alive and the peak that gave a higher fluorescence signal (right) reflects the proportion of dead cells in the sample.

Figure 3.5 shows the flow cytometry results of HBQ and HAA exposure on hNSCs. Here, it is clear that the concentrations of DBPs selected for this experiment only resulted in a statistically significant decrease in cell viability for the 0.5 μM BAA treatment group. For the other treatment groups, the average cell viability of the groups was lower, compared to the control, but not statistically significant. Thus, the FACS analysis confirmed the results observed in the trypan blue staining experiment. Interestingly, the concentration of 2,6-DBBQ that the cells were exposed to did not seem to make a difference in cell viability, whereas the higher concentration of the other DBPs resulted in lower cell viability compared to the lower concentration of the same DBP. It is not known why this occurred, but we suspect that different dose kinetics may occur at different concentrations. Nevertheless, a decrease in cell viability was observed for all tested DBPs, and these results informed the concentrations used in subsequent experiments to ensure that sub-lethal concentrations used.

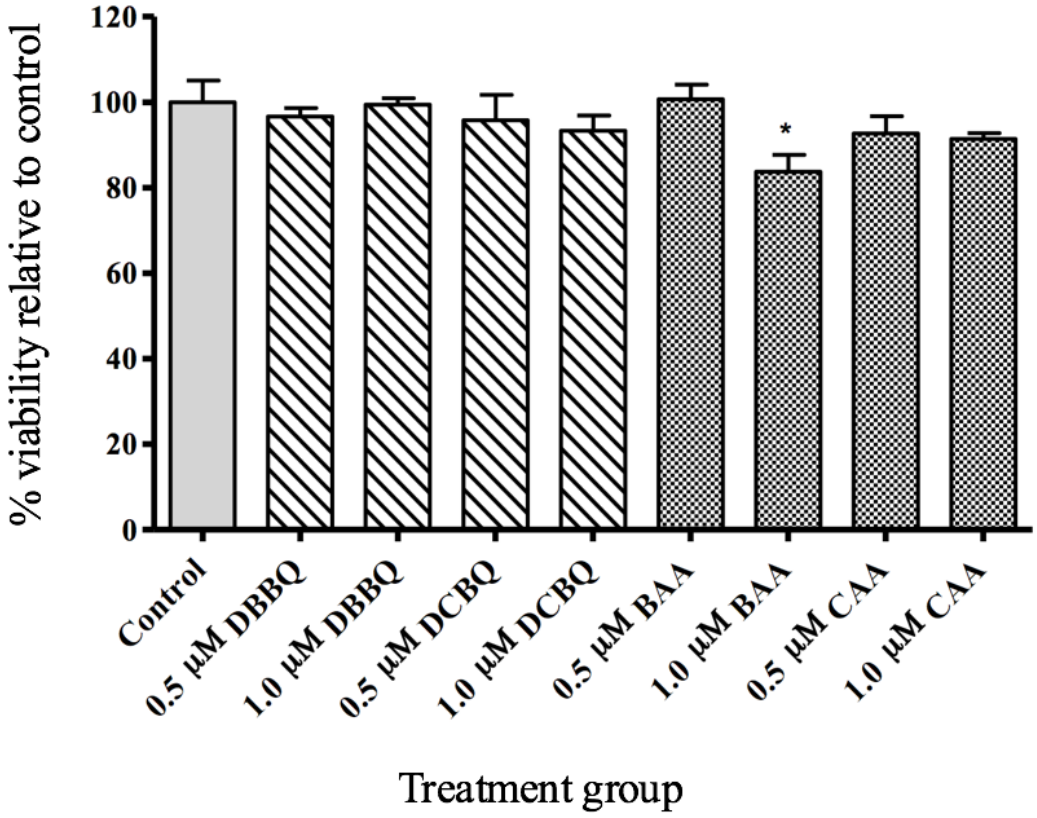

Figure 3.5: Cell viability of each exposure group compared to the control using FACS analysis of cells stained with LIVE/DEAD® Fixable Violet Dead Cell Stain. The concentrations used were low to ensure that relative cell viability exceeded 80% compared to the control group. N = 3, *$P < 0.05$.

3.3.2 Effects of DBP Exposure on Cell Cycle

Cells were cultured and exposed to different concentrations of DBPs and to control groups to determine the effects of DBP exposure on the cell cycle of hNSCs. After 4 d exposure, cells were collected and analyzed using flow cytometry. Figure 3.6 shows the study design for this experiment and the structures of the DBPs in the exposure groups. All groups were grown, treated and analyzed according to the same timeline.

The concentrations used in the cell cycle experiments (0.5 μM and 1 μM) were slightly higher than the concentrations used in the first cell viability experiment (0.1 μM and 0.5 μM). Higher concentrations were necessary for these experiments as the time of exposure was 4 d compared to the 12 d of exposure examined in the viability experiments. Higher concentrations were used to ensure that cell cycle effects on the hNSCs could be observed. Because the reported doubling time of the hNSCs from the manufacturer is around 40-50 hours, an endpoint of 96 hours was chosen to ensure that all cells had completed at least one full cell cycle at the time of analysis.

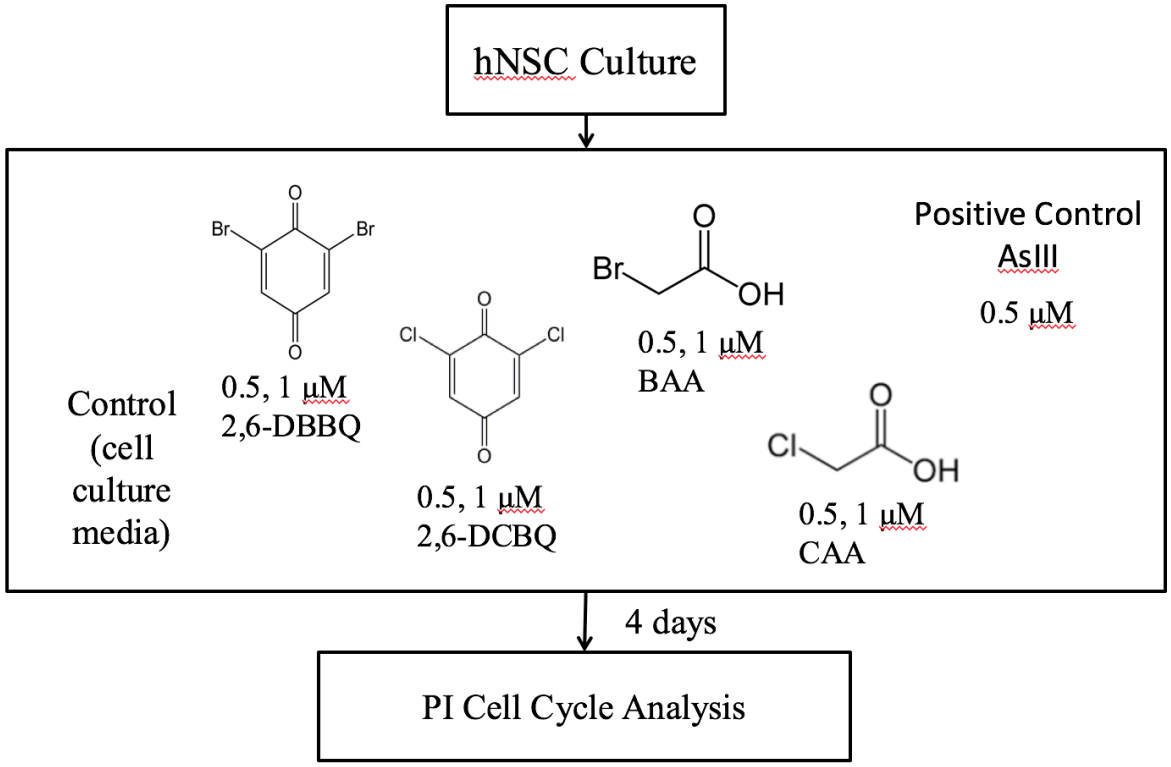

Figure 3.6: The study design for cell cycle analysis of hNSCs exposed to DBPs. A total of ten groups were analyzed using flow cytometry.

Using FlowJo software, I was able to create cell cycle histograms for each analyzed sample, as seen in Figure 3.7. During analysis, the main peaks were aligned so

that the G0/G1 peak was at 100K and the G2 peak was aligned at 200K to reflect relative DNA content in cells. Cells that appeared below the G0/G1 peak (<G0/G1) are apoptotic cells that the live/dead cell dye did not select out, and could thus be excluded from the analysis. Cells appearing above the G2 peak are likely clumps of cells, as they have a higher DNA content.

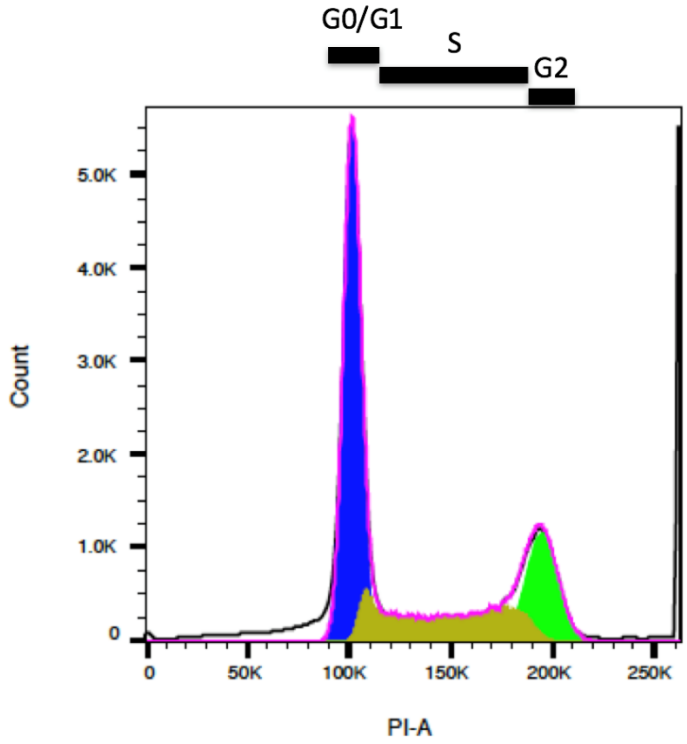

Figure 3.7: Histogram of cell cycle produced using FlowJo software. The histogram shows the cell population peaks at different phases of the cell cycle, shown in the bars above. The software analysis shows the G0/G1 peak, S-phase, and G2 peak.

Triplicate groups were averaged and the results of the cell cycle analysis are shown in Figure 3.8. The control group had the highest proportion of cells in G0/G1 phase out of all of the groups. Compared to the control group, there was a statistically significant decrease in the proportion of cells in G0/G1 phase in the 0.5 μM 2,6-DBBQ, 1

μM 2,6-DBBQ, 0.5 μM 2,6-DCBQ, 1 μM 2,6-DCBQ, 0.5 μM BAA and 1 μM BAA treatment groups. The 0.5 μM 2,6-DBBQ, 1 μM 2,6-DCBQ and 0.5 μM As^{III} positive control groups also had statistically significant increases in the proportion of cells in S-phase. Furthermore, the 1 μM BAA, 0.5 μM CAA and 1 μM CAA treatment groups had a statistically significant decrease in the number of cells in G2 phase.

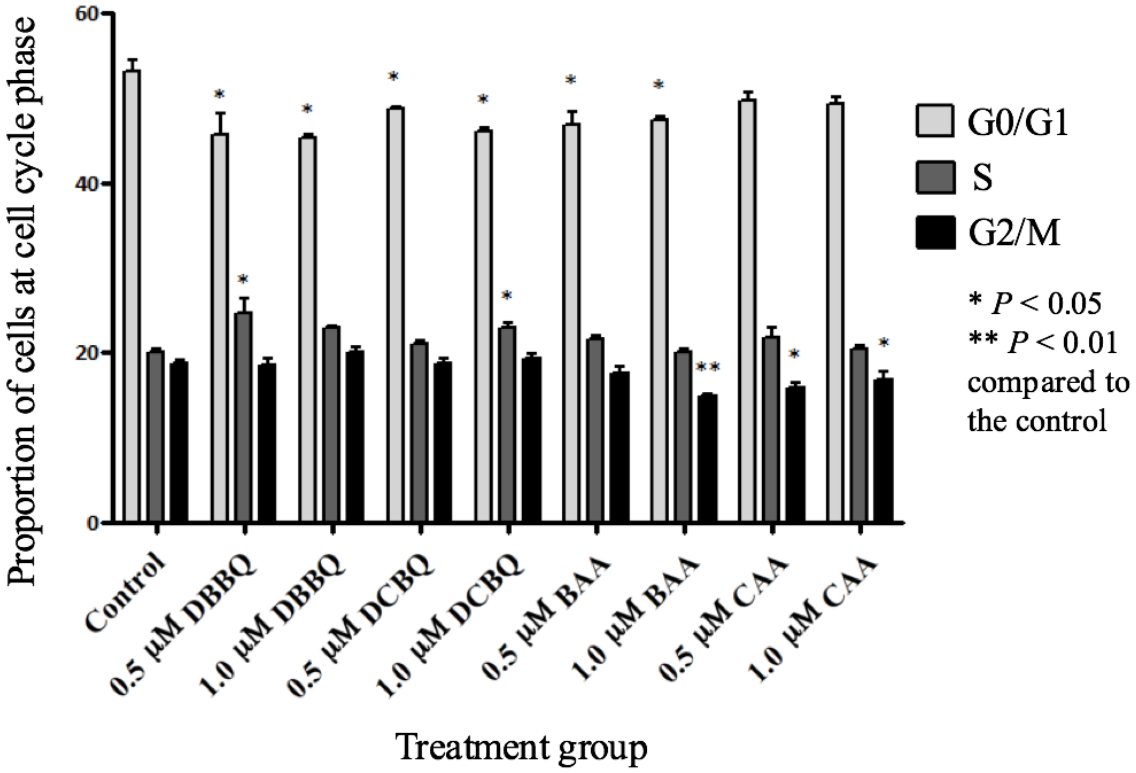

Figure 3.8: The percentage of hNSCs at each stage of the cell cycle after 96 hours of exposure to DBPs. The percentage of cells in each treatment group does not add up to 100% since some cells fell in the <G0/G1 or >G2 regions of the flow cytometry histogram. * *P*<0.05 and ** *P*<0.01 compared to the control.

Increases in a phase during the cell cycle indicates cell cycle arrest at that phase. Since there was a statistically significant increase in the number of cells at S-phase in the 0.5 μM 2,6-DBBQ, 1 μM 2,6-DCBQ and 0.5 μM As^{III} treatment groups, it is likely that

2,6-DBBQ, 2,6-DCBQ and As^{III} induce cell cycle arrest at S-phase. There was no statistically significant change in the proportion of cells in S-phase for the HAA treatment groups.

In some groups, there was a statistically significant decrease in the proportion of cells in one phase, without a corresponding increase in the number of cells observed in another phase of the cell cycle. One possible explanation is that it is possible that there was an increase of cells in one or both of the other phases that were not statistically significant. Or, that there were more cells at either <G0/G1 or >G2 regions on the histogram. Both of these explanations may account for decreases in one phase without corresponding increases in other phases. More research is needed to elucidate these findings.

Previous studies have not analyzed the effects of HBQ exposure on the cell cycle of cell lines, however, studies have looked at the effects of HAA exposure. In human small intestine epithelial cells, exposure to HAAs resulted in altered transcriptome profiles of genes responding to DNA damage or genes regulating cell cycle or apoptosis [9]. The alteration of these genes may lead to cell cycle arrest. The researchers hypothesized that oxidative stress was one of the main mechanisms of HAA toxicity and that DNA lesions induced by HAAs require extended times for DNA repair and repressed cell division. During S-phase, there are intra-S-phase DNA damage checkpoints to delay cell cycle progression and to repair defects [14]. Damage of cellular DNA during replication may explain why cell cycle arrest at S-phase was observed in hNSCs. This mechanism may explain the observed cell cycle arrest in S-phase caused by HBQ

exposure because studies have shown that HBQ exposure results in the formation of reactive oxygen species and causes oxidative DNA damage in cells [2,13,15].

Additionally, my results showed an increase in the percentage of cells at S-phase in the 0.5 μM 2,6-DBBQ treatment group, but not for the 1 μM 2,6-DBBQ treatment group. The same trends were seen in the cell viability experiments. This similarity may be caused by different dose kinetics at different concentrations of exposure, but more research has to be done to draw conclusions. Another reason for this result may be due to a combination of the high variation in the stage of differentiation of hNSCs and that the difference between concentrations is not substantially different between 0.5 μM and 1 μM in 2,6-DBBQ, in particular. Conducting cell cycle analysis at a greater number of exposure concentrations may provide more insight on how DBP dose affects the cell cycle of hNSCs.

3.4 Summary

This chapter analyzed the effects of DBP exposure on cultures of hNSCs. I found that 10 μM 2,6-DBBQ resulted in a low number of cells that remained adherent to cell culture plates. In addition, exposure to concentrations between 0.1 μM and 0.5 μM of selected HBQs and HAAs did not decrease cell viability by more than 20%. These concentrations were selected to be used in further experiments. Cell cycle analysis of the effects of DBP exposure on hNSCs showed a decrease in the proportion of cells in G0/G1 phase of the cell cycle. At 0.5 μM 2,6-DBBQ and 1 μM of 2,6-DCBQ, cell cycle arrest was observed in S-phase. Cell cycle arrest in S-phase was also observed in the positive

control of 0.5 μM As^{III}. There was no concentration-dependent effect on the cell cycle of hNSCs observed in these experiments.

3.5 References

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Chapter 4: Effects of DBP Exposure on hNSC Differentiation

4.1 Introduction

Brain development is a coordinated process of differentiation, cell proliferation, migration, synaptogenesis and apoptosis [1]. Exposure to compounds that disrupt the timing of these processes and events can have impacts on future development. Several key proteins are involved in the differentiation process of human neural stem cells (hNSCs). Studying the differentiation of neural stem cells into neurons can provide insight into one of these important processes.

While neural stem cells mature into differentiated neurons, expression of specific proteins changes during the differentiation process. The functions of nestin and doublecortin (DCX) were presented in Chapter 2 of this thesis. A characteristic feature of undifferentiated hNSCs as early progenitor cells is the cytoplasmic expression of nestin. As hNSCs differentiate, there is the gradual disappearance of nestin expression and the gradual increase of DCX expression [2]. Nestin has been found to correlate with proliferating progenitor cells of many regions of the central nervous system [3]. The gain and loss of nestin expression may have consequences on the differentiation potential of hNSCs. DCX is also expressed in the cytoplasm. DCX is involved in the migration process of neurons and the elongation process of differentiation neurons [4]. Tracking the expression of these proteins during differentiation and recording aberrations in cells exposed to environmental contaminants can be an indicator of developmental neurotoxicity (DNT).

Exposure to trivalent inorganic arsenic has been shown to upregulate nestin expression and downregulate DCX expression in hNSCs that have been exposed to μM concentrations for ten days [5]. The researchers found a decreasing ratio of DCX to nestin as treatment concentrations of sodium arsenite increased. Studies have also found that exposure to methyl mercury suppresses the expression of neuronal precursor markers [6].

Culturing cells in a monolayer allows researchers to look at assay endpoints such as neurite outgrowth, neurite number and length [7]. As cells mature, processes will extend from the cell bodies of cells and increase in length and complexity [8]. First, neurites grow rapidly in length, then after the formation of the axon, neurites elongate and become dendrites. In more mature cultures, neurite branching can also be quantified as an indicator of neuron growth. Neuron length and rate of growth can be used as an indicator of the relative health of neurons.

The objective of this chapter is to analyze the expression of the differentiation markers nestin and DCX in hNSCs exposed to DBPs and to analyze the neuron length of cultures exposed to DBPs. Changes in the expression of these differentiation markers may indicate that exposure to DBPs affects some key cellular pathways.

4.1 Experimental Methods

4.1.1 Reagents

The reagents used in this section were obtained by the same suppliers as in section 3.1.1 in Chapter 3 of this thesis.

4.2.2 Cell Culture

Cells were cultured according to the same procedures as section 2.2.3 of this thesis. Please refer to Chapter 2 for the full details.

4.2.3 Differentiation Marker Expression Analysis

4.2.3.1 Confocal Microscopy

After cells reached 75-80% confluency, differentiation was induced by replacing complete hNSC media with differentiation media. Cells were allowed to differentiate for 14 d with fresh media changes every 3 d. Cells were imaged using confocal microscopy to analyze immunofluorescence and western blot analysis to look at protein expression of markers.

For the imaging experiments, cells were fixed and stained with nestin as described above (Section 2.1.4). Cells were stained for the differentiated stem cell marker, doublecortin (DCX), using monoclonal mouse antibody (Ab) from Santa Cruz Biotechnology (SCB; Dallas, TX, USA). The secondary Abs used were as described in Section 2.1.4, and immunofluorescence analysis was also performed according to the same procedure described in Section 2.1.4. The emission filters used were the same as described in section 2.2.6.

4.2.3.2 Flow Cytometry

Cells were first stained with a cell viability cell dye and fixed as follows. Cell culture media was pipetted from the plates into numbered 15 mL tubes. Then, cells were detached from plates using StemPro® Accutase® Cell Dissociation Reagent (Life

Technologies). After dissociated cells were collected, plates were washed with phosphate buffered solution (PBS; Gibco, Life Technologies) twice. Detached cells were centrifuged for 4 min at 1,200 rpm, re-suspended in 1 mL Dulbecco's phosphate buffered saline (DPBS; Gibco, Life Technologies), and stained using 1μL LIVE/DEAD® Fixable Violet Dead Cell Stain Kit, for 405 nm excitation (ThermoFisher Technologies Inc.). Next, a 4% paraformaldehyde solution was made by adding 8 mL of PBS into each 15 mL tube containing 2 mL of 20% PFA. The tubes were incubated in a 37°C water bath until the solution had dissolved. Cells were then centrifuged at 1,200 rpm for 4 min and re-suspended in 1 mL PBS. Re-suspended cells were transferred to 1.5 mL tubes and centrifuged at 1,200 rpm for 4 min. 1 mL of 4% PFA solution was added to each tube and the tubes were incubated at room temperature for 30 min. After incubation, tubes were centrifuged at 1,200 rpm for 4 min, PFA solution aspirated, and cell pellet was re-suspended in 1 mL DPBS and centrifuged again.

Fixed cells were then incubated in blocking buffer for 30 min. Blocking buffer contained 5% goat serum, 1% bovine serum albumin (BSA), and 0.1% Triton-X, all dissolved in DPBS with Ca/Mg. 0.4 mL blocking buffer was added to each sample. Tubes were incubated at room temperature. After incubation, samples were centrifuged at 1,300 rpm for 10 min.

Blocking buffer was removed and cells were incubated with primary Ab diluted in 5% serum. 5% goat serum was dissolved in DPBS with Ca/Mg and rabbit anti-nestin Ab (1/200 dilution) and mouse-anti-DCX (1/400 dilution) solution was made. 0.2 mL of primary Ab solution was added to each sample. The tubes were incubated at 37°C for 1 h.

After incubation, cells were centrifuged at 1,300 rpm for 10 min, washed with 1 mL DBPS with Ca/Mg and centrifuged again at 1,300 rpm for 10 min.

Next, cells were incubated with fluorescence-labeled secondary Abs. Alexa-Fluor® 488 Goat Anti-Rabbit IgG Ab and Alexa-Fluor® 647 Goat Anti-Mouse IgG Ab (both from Life Technologies) were added to 5% goat serum solution in DPBS with Ca/Mg in 1/1000 concentrations. 0.2 mL secondary Ab solution was added to each sample. The samples were incubated in the dark at 37°C for 45 min. After incubation, the tubes were centrifuged at 1,300 rpm for 10 min and washed with 1 mL DPBS with Ca/Mg. Cells were centrifuged again and re-suspended in 0.5 mL DPBS with Ca/Mg for analysis.

Cells were analyzed using flow cytometry with an LSRFortessa X-20 flow cytometer (BD Biosciences, San Jose, CA, USA) and accompanying BD FACSDiva software to preview and record data. First, the forward scatter and side scatter of the samples were plotted and gated, then the proportion of live cells were gated so that nestin and DCX readings were only taken of the live cells. Nestin was analyzed on the 488 nm wavelength channel and DCX was analyzed using the 647 nm wavelength channel. FlowJo LLC (Ashland, OR, USA) data analysis software was used to analyze the FACS data. FACS analysis was performed in triplicate.

4.2.4 Neuron Length Analysis

For neuron length analysis after the establishment of hNSC culture, cells were exposed to differentiation cell culture media (negative control), 2,6-DBBQ (0.1, 1, 10 μM), 2,6-DCBQ (0.1, 1, 10 μM), or TriCBQ (0.1, 1, 10 μM). Cells were left to

differentiate for 12 d with media changes every 3 d. At 10 d, 20 pictures were taken per group through the lens of a IV-900 Inverted Microscope (MicroscOptics, Holly, MI, USA) at 20 × magnification.

The pictures were blinded, randomized and then analyzed through the Simple Neurite Tracer program on image analysis software: Fiji (ImageJ, Madison, WI, USA) [9]. To use Simple Neurite Tracer, the colour channels were split in red, blue and green and the green channel was selected for analysis. The field of view for the microscope was determined to be 1.9 mm. Neurons were individually traced in each picture and a list of neuron lengths was created. Then, lengths were exported from Fiji to Microsoft Excel for analysis. A total of 325 to 544 neurons were measured per group.

4.2.5 Statistical Analysis

Data was analyzed with the same procedure as Chapter 3 of this thesis. Please refer to section 3.2.5 for full details on how statistical analysis was conducted.

4.3 Results and Discussion

4.3.1 Effects of DBP Exposure on Expression of hNSC Differentiation Markers

Cultured hNSCs were exposed to different concentrations of DBPs in differentiation media to assess the effects of DBP exposure on hNSC differentiation markers. Cells were exposed to these concentrations for 12 d and were then collected for flow cytometry analysis. Figure 4.1 shows the study design for this experiment and the structures of the DBPs used. All exposure groups were grown in parallel and analyzed on the same days. The concentrations chosen for protein expression analysis were the same

concentrations tested in Chapter 3 of this thesis to ensure that the cell viability of cultures were maintained at higher than 80% viability compared to the control. The purpose for selecting these concentrations was, again, to measure the sub-lethal effects of DBP exposure.

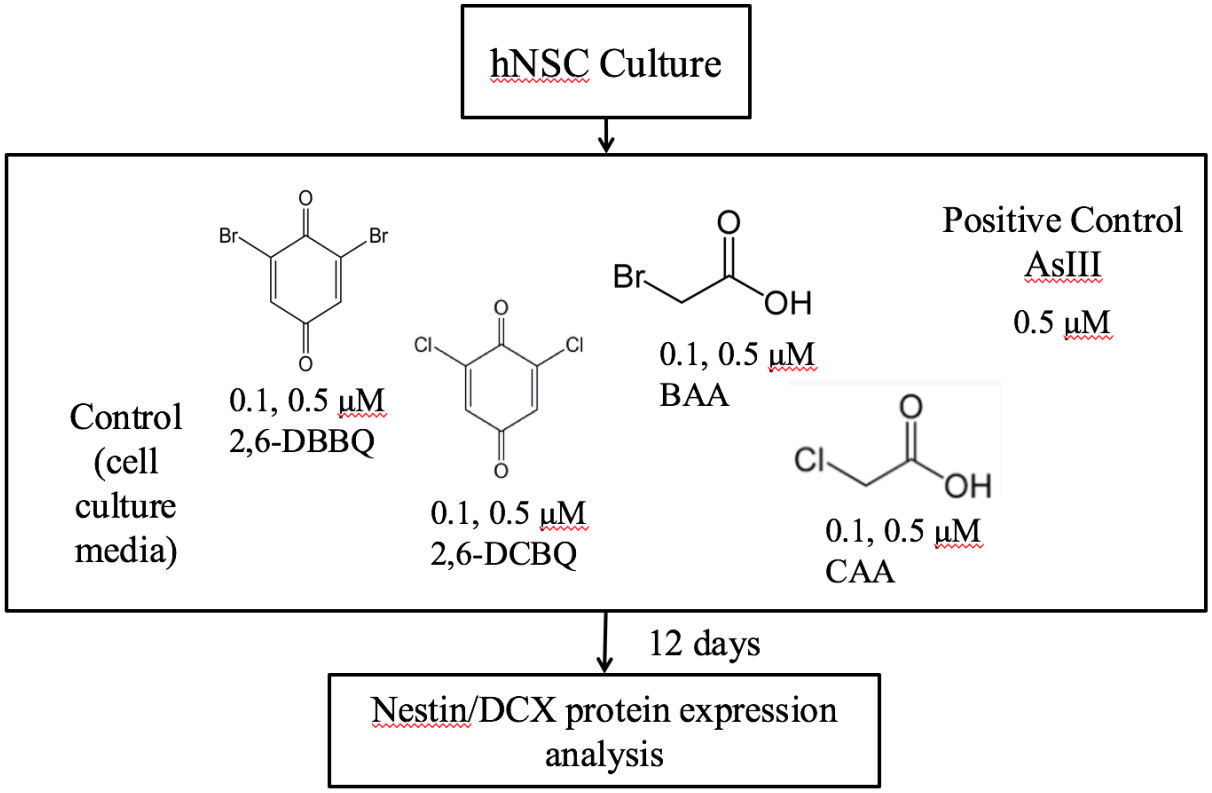

Figure 4.1: The study design for protein expression analysis of hNSCs exposed to DBPs. A total of ten groups were analyzed using flow cytometry.

hNSCs were also exposed to different concentrations of DBPs and analyzed for DCX and nestin using confocal microscopy. Qualitative changes were observed in the cell culture images for groups that were exposed to DBPs in comparison to the untreated control cells (Figure 4.2).

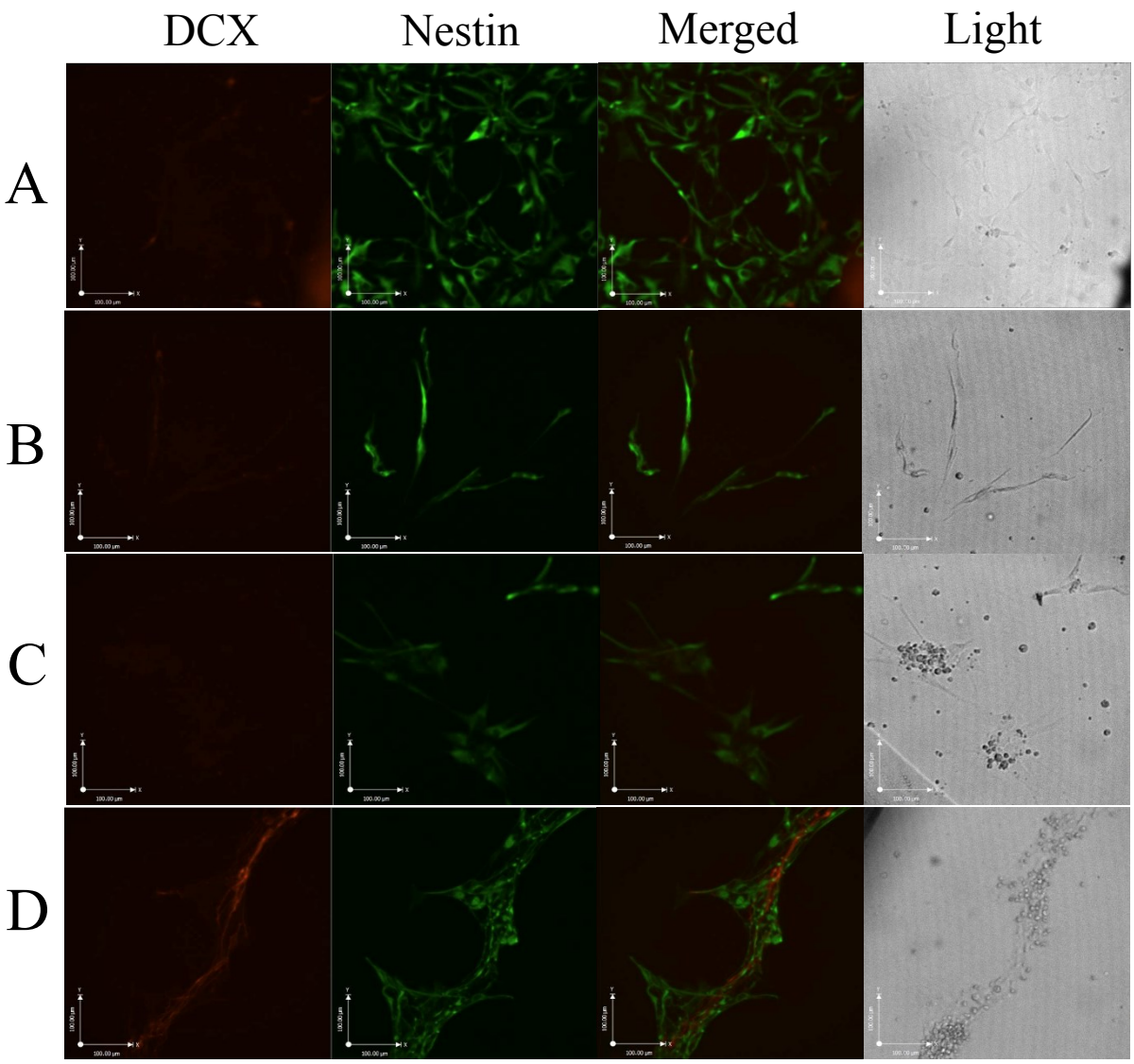

Figure 4.2: Confocal microscopy images of hNSCs treated with (A) cell culture media (negative control), (B) 0.5 μM As^{III} (positive control), (C) 0.5 μM 2,6-DBBQ, and (D) 0.5 μM 2,6-DCBQ. Qualitative differences between treatment groups are clearly observed. Each scale bar represents 100 μm.

In all groups, DCX and nestin were both expressed. In the image of the negative control (Fig 4.2A), cells are disbursed in a monolayer and attached to the bottom of the plate. Comparatively, cells exposed to the positive control (Fig 4.2B) appeared to be fewer in number populating the bottom of the plate. This observation was also consistent

with the HBQ-treated groups (Fig 4.2C and D) where the cells were less evenly dispersed at the bottom of the plate.

Compared to the negative control, cells that were treated with 0.5 μM 2,6-DBBQ (Fig 4.2C) or 0.5 μM 2,6-DCBQ (Fig 4.2D) formed clumps of cells, likely formed when cells detached from the bottom of the plate and reattached to the plate. The formation of these neurospheres was observed in HBQ-treated groups. These images indicate that exposure to HBQs may cause cell detachment. This is consistent with the study of Ivanov *et al.*, who observed both adherent and floating cells in culture plates of hNSCs exposed to As^{III}, analyzed using confocal microscopy [5]. They found that plates treated with higher concentrations of As^{III} had fewer cells attached to the plate and more cells floating in culture media. Ivanov *et al.* considered cells that were floating in the cell culture medium as dead cells in viability calculations.

Both nestin and DCX are expressed by hNSCs. Using FlowJo software, I was able to create histograms of the FACS results showing nestin and DCX expression, based on intensity, in hNSCs. It is important to note that while both nestin and DCX were expressed by cells, there are no clear peaks of expression, meaning that expression in hNSCs is variable. These two proteins are not proteins that are either expressed or not. Instead, they are expressed at different levels in hNSCs, resulting in different signal intensities. Figure 4.3 shows a representation of nestin and DCX expression in one of the control group cells. The pattern of expression was consistent among all exposure groups.

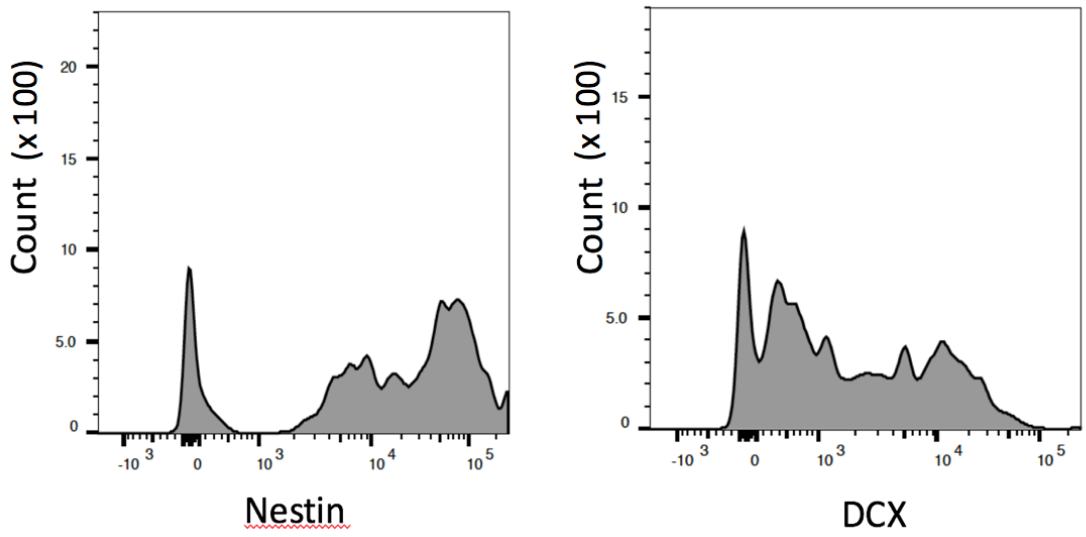

Figure 4.3: Histograms of nestin and DCX expression produced using FlowJo software. These expression histograms show that were are no clear peaks of protein expression levels; instead, cells express nestin and DCX in variable intensities.

The nestin and DCX expression in hNSCs were plotted in a contour plot, showing the expression of nestin and DCX levels in cells of each sample. The x-axis of the graph shows the nestin expression, while the y-axis of the plot shows the DCX expression of cells within the sample. From this plot, I was able to identify three areas of the plot that had a high density of cells. I gated these populations into three groups: cells that were negative for both DCX and nestin, mature cells and immature cells. The negative population are likely cell debris in the sample. Mature cells are cells that have higher DCX expression relative to nestin expression, while immature cells have greater nestin expression relative to DCX expression.

The three populations can be seen in Figure 4.4 where the contour plot indicates that there is a higher density of cells in particular regions of the plot. These three populations were consistently found in all samples at the same expression frequencies. I

was able to identify the populations on the graphs produced by every treatment sample, and gate the population of mature, immature, and negative expression cells.

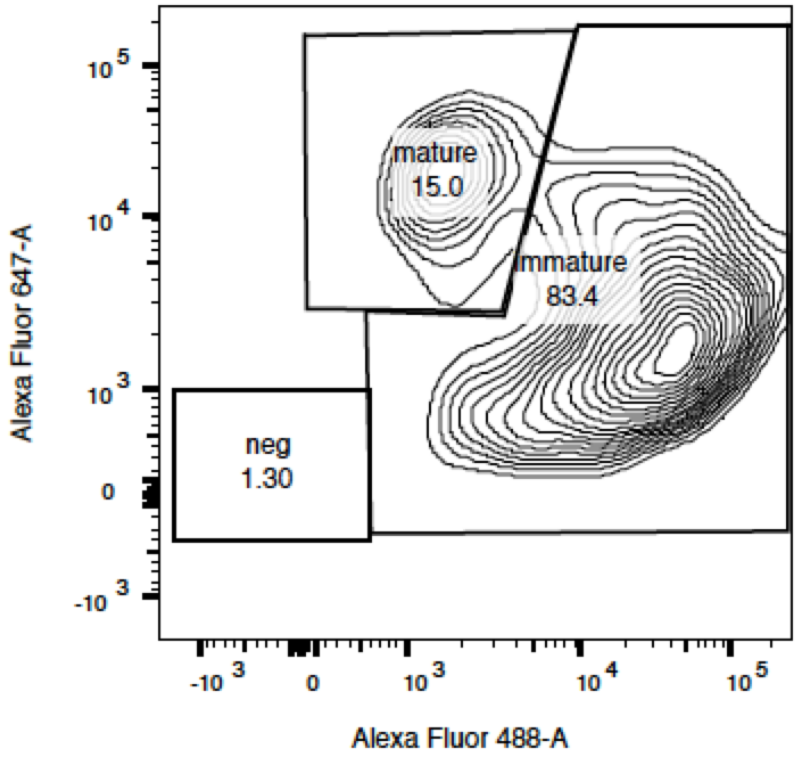

Figure 4.4: Graph showing the three populations of cells found in the flow cytometry analysis samples. The x-axis of the graph shows the nestin expression of cells and the y-axis shows the DCX expression. Cells that express higher DCX levels relative to nestin expression are grouped as mature cells, while cells that express higher levels of nestin relative to DCX are classified as immature cells. The negative group that does not express nestin or DCX were not considered for analysis and are most likely cell debris.

Next, I used the data on the cell populations to create a mature to immature cell ratio for each treatment group. Since each treatment group was run in triplicate, the ratio of each group was averaged and standard deviations were calculated. The population of

cells that were negative for DCX and nestin were disregarded in this analysis. The results of the mature to immature cell ratio calculations can be found in Figure 4.5.

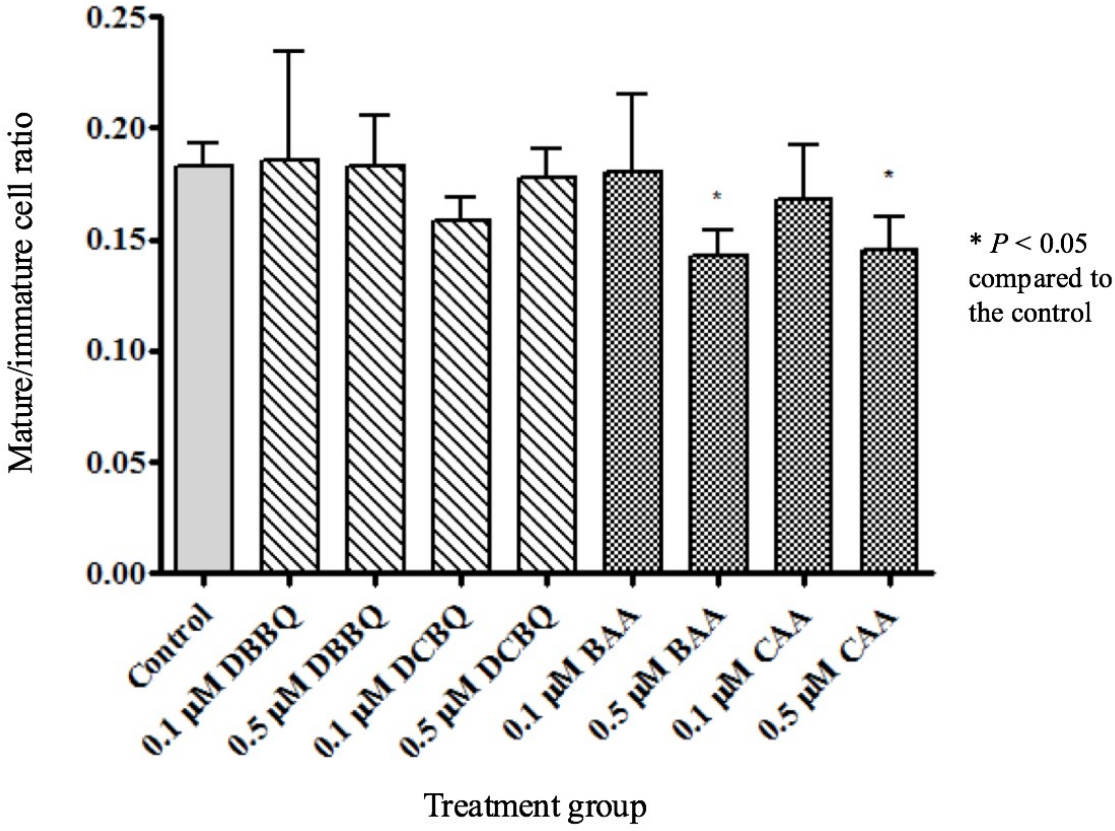

Figure 4.5: The ratio of mature cells to immature cells in hNSCs exposed to DBPs. The blue bar represents the negative control and the black bar shows the positive control (sodium arsenite, As^{III}). The green bars are HBQs and the purple bars are HAAs.

Compared to the control group, there was a statistically significant decrease of the mature to immature cell ratio in the 0.5 μM BAA and 0.5 μM CAA treatment groups. Although the ratio in the positive control group was lower than the negative control, the difference was not statistically significant.

Interestingly, a statistically significant decrease of the stage of differentiation in the 0.1 μM 2,6-DCBQ treatment group was also observed that was not present in the 0.5 μM 2,6-DCBQ treatment group. The reason for this result may be due to the high variation in the stage of differentiation observed in hNSC culture. Furthermore, the difference between 0.1 μM and 0.5 μM 2,6-DCBQ may not have a substantially different effect on cells. Conducting this experiment with a greater range of exposure concentrations may provide greater insight into the effect of different concentrations of DBPs on hNSCs.

Thus far, there have been no studies conducted measuring the expression of differentiation markers in hNSC cultures exposed to DBPs. However, Ivanov *et al.* analyzed the expression of nestin and DCX in hNSCs exposed to sodium arsenite [5]. The researchers used confocal microscopy to compare the expression of nestin and DCX in images. They found that the DCX to nestin ratio of negative control cells was an average of 1.45, while the DCX to nestin ratios of 2 μM and 4 μM sodium arsenite were 0.275 and 0.15, respectively. These results indicate that the proportion of cells expressing nestin increased in the exposure groups, while the proportion of cells expressing DCX decreased when cells were exposed to sodium arsenite. However, my results did not show that 0.5 μM of sodium arsenite had an effect on the mature to immature neuron ratio. This result may be explained by the lower concentration used in my experiment compared to the concentrations used by Ivanov *et al.* [5]. I used 0.5 μM sodium arsenite in my experiments so the concentration of that control was comparable to the DBP concentrations used. Under my experimental conditions, 0.5 μM sodium arsenite did not produce a statistically significant effect.

Furthermore, studies focusing on nestin expression show that nestin phosphorylation is a result of cdc2 kinase, an enzyme that is part of the M-phase promoting factor complex [10]. This complex is a key molecule that regulates the transition from the G2-phase to the M-phase of the cell cycle. If there is increased cdc2 kinase activity, which results in the increased nestin expression observed in DBP-treated cells, the transfer from G2 to M-phase in cells would also be promoted. This enzyme activity would explain why cell cycle arrest was not observed in Chapter 3, when I analyzed the effects of DBP exposure on cell cycle.

Furthermore, a limitation to my experiment may be the low seeding density of cells. I initially seeded my cells at 1.0 x 10^5 cells/well. The comparatively low density is a result of the difficulty to culture a sufficient number of cells to include all the treatment groups. Culturing hNSCs on a larger scale may increase the seeding density in future experiments.

The reason for the large standard deviation in this experiment may be due to the cells in the culture being present at different stages of differentiation. I conducted neuron length analysis to confirm this hypothesis.

4.3.2 Effects of DBP Exposure on Neuron Length

After the induction of neural differentiation, hNSCs begin to grow larger and form axons and dendrites. At early stages of differentiation, hNSCs that have been directed to differentiate into neurons start forming a main axon that extends from the cell body. At later stages, dendrites begin to extend from the axons. I measured axon length of neurons 14 d after the induction of differentiation. At this point, only axons had begun to form

and I observed few dendrites branching from main axons. Measurement of neuron length and rate of growth can be used as an indicator of neuron health [8]. Figure 4.6 shows an example of how neuron length was measured.

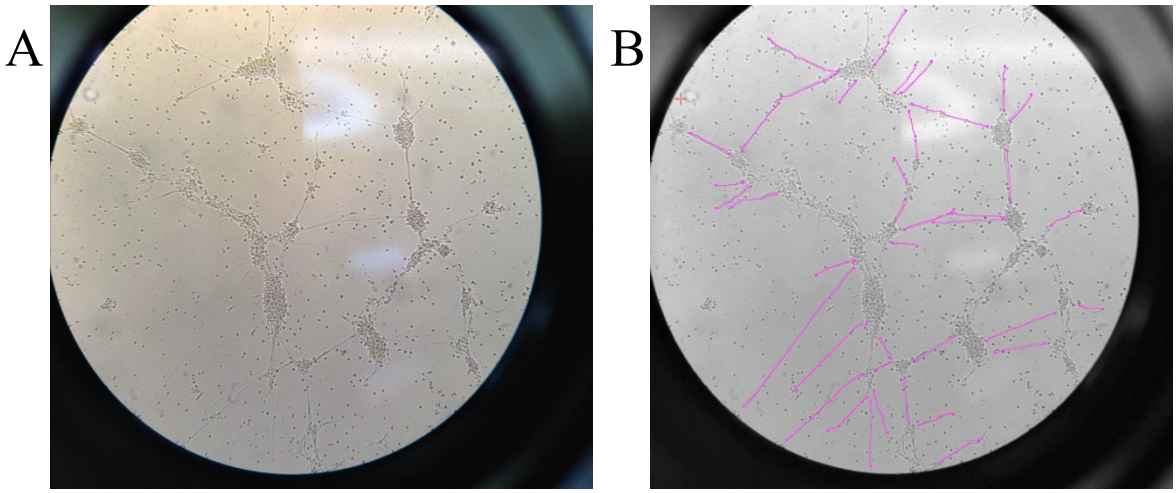

Figure 4.6: Neuron length measurements using the Simple Neurite Tracer program. (A) Image of differentiated neuron culture taken through the lens of a light microscope at 20 × magnification 14 days after differentiation was induced. (B) The same image after the colour channels have been split and the neurons have been traced.

Ten treatment groups were included in the neuron length analysis. The treatment groups were: negative control (differentiation cell culture media), 2,6-DBBQ (0.1, 1, 10 μM), 2,6-DCBQ (0.1, 1, 10 μM), and TriCBQ (0.1, 1, 10 μM). The average neuron lengths are displayed in Table 4.1.

Table 4.1: The average neuron length (mm ± SD) of cells grown in media containing different concentrations of HBQs.

Treatment Group	N	Average Length	SD
Negative Control	444	0.14	0.06
2,6-DBBQ			
0.1 μM	526	0.14	0.06
1 μM	325	0.13	0.05
10 μM	544	0.14	0.05
2,6-DCBQ			
0.1 μM	401	0.15	0.07
1 μM	386	0.13	0.05
10 μM	530	0.14	0.05
TriCBQ			
0.1 μM	382	0.15	0.07
1 μM	502	0.13	0.05
10 μM	542	0.14	0.06

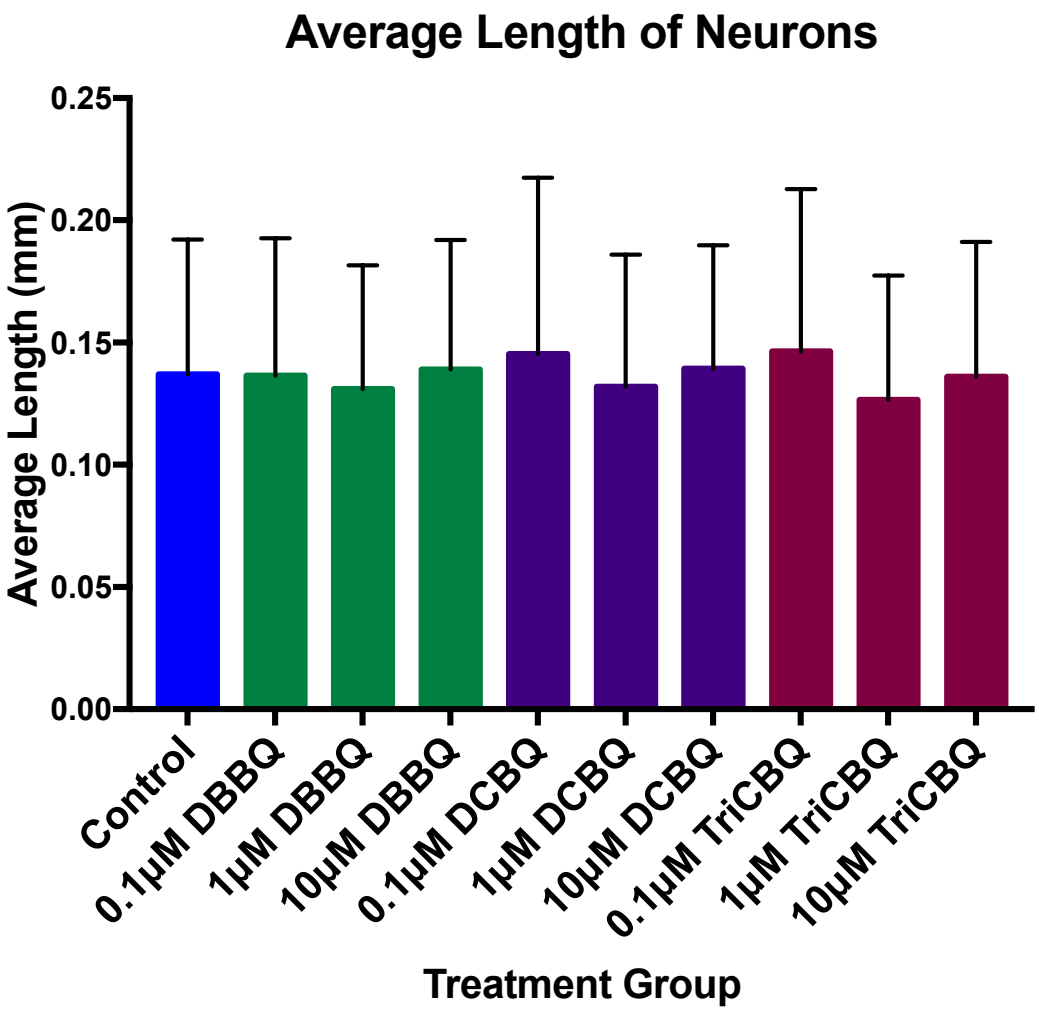

Figure 4.7: The average neuron length (mm ± SD) of cells grown in media containing different concentrations of HBQs. There is no statistically significant difference between the neuron lengths between any of the treatment groups.

Neuron length analysis showed a large variation between the lengths of neurons within each plate for each treatment group at day 10 after differentiation was induced. The standard deviation for neuron length of all groups was very high. For example, neuron lengths in the control group ranged from 0.048 mm to 0.41 mm. This large variation indicates that the measured neurons are at a different stage of growth. While some neurons may start differentiating immediately after hNSC media is switched to

differentiation media, other neurons may start growing later. It is possible that measuring neurons at a different time point would be a more appropriate measure of neuron health and is a future avenue of research.

Since all neurons were counted manually, there is potentially a source of human error in identifying all neurons that need to be counted. On some plates, it was difficult to count individual neurons because there was a high level of overlap. Furthermore, when cells collected into neurospheres, it was difficult to decipher exactly where the axon or dendrite began, as axons were measured from the neuron's cell body to the end of the axon or branching dendrite. Depending on the picture, some cell bodies were easier to identify than others. In the future, it may be easier to measure neuron length using images from fluorescence microscopy with nuclear and cytoplasm stains compared to the images taken with a light microscope that I used. Furthermore, using automated software to quantify neuron length would achieve more consistent results because the software could identify cell nuclei and reduce human error.

As mentioned above, through my neuron length analysis, I observed that there was a large range in the length of neurons, indicating that the cells may be in different stages of differentiation. The range of differences in nestin and DCX expression as differentiation markers helps support this observation. The large differences within each culture plate may also be, in part, due to the endpoint chosen for analysis. Performing the procedure at day 14 or later after differentiation may result in different observations.

4.4 Summary

Protein expression analysis of nestin and DCX in hNSCs shows that both proteins are expressed in differentiating hNSCs in a range of levels. In addition, when nestin expression and DCX expression are plotted in the same graph, two populations of differentiating hNSCs can be identified: relatively more mature and immature neurons. When the mature and immature cell population ratios are compared, my analysis shows that hNSCs exposed to DBPs show a decrease in the mature to immature cell ratio in 0.5 μM BAA and 0.5 μM CAA exposure groups. There was no concentration dependent effect of the ratio of hNSCs in HBQs, but the higher exposure concentration of HAA had a statistically significant effect on the mature to immature cell ratio when the lower concentration did not (0.5 μM vs. 1 μM). In addition, measuring neuron length is not a reliable indicator of neuron health at 10 days after differentiation is induced due to the high variation in neuron length at that time point.

4.5 References

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Chapter 5: Summary, Limitations and Future Work

5.1 Summary

The current work addresses the questions of whether halobenzoquinone (HBQ) and haloacetic acid (HAA) water disinfection by-products (DBPs) can adversely affect human neural stem cells (hNSC). DBPs are unintentionally produced during the water disinfection process and have been associated with an increased risk of bladder cancer in epidemiological studies [1]. Epidemiological studies of DBP exposure and adverse reproductive outcomes have inconsistent findings [2]. The objective of this thesis is to study the effects of emerging DBPs on human stem cell differentiation, contributing to the understanding of the developmental effects of DBPs.

Before studying the effects of DBP exposure, I characterized the hNSC culture, including the morphology of hNSCs and the expression of differentiation markers. I used qualitative imaging methods to assess the physical characteristics of hNSCs and to measure protein expression of undifferentiated and differentiated hNSCs. Undifferentiated hNSCs express nestin and do not express DCX. Comparatively, differentiated neurons express both nestin and DCX, confirming that DCX can be used as an indicator of mature neurons and the presence of nestin without the expression of DCX can be used as a marker of undifferentiated cells.

Next, I studied the effects of HBQ and HAA exposure on cell viability and the cell cycle of hNSCs. I evaluated DBP exposure concentrations in hNSC culture to ensure at least 80% cell viability in further experiments using both the trypan blue exclusion assay and an improved flow cytometry assay that could measure the viability of both

adherent and suspended hNSCs. Furthermore, I found that exposure to HBQs results in cell cycle arrest at S-phase in cells exposed for 96 h. I also observed that exposure to HBQs and HAAs decreased the proportion of cells in G0/G1 phase.

Flow cytometry was also used to examine the effects of DBP exposure on hNSC differentiation by examining nestin and DCX expression in all exposure groups by classifying cells into mature and immature cell populations. hNSCs exposed to DBPs show a decrease in the mature to immature cell ratio in the 0.5 μM BAA and 0.5 μM CAA exposure groups. In both cell cycle analysis and differentiation marker expression analysis, there was no concentration dependent effect on cell cycle arrest or change in protein expression. Finally, I found that dendrite length is not a reliable indicator of neuron health at 10 days after differentiation is induced due to the high variation in neuron length at that time point.

5.2 Limitations of Research

The DBP exposure concentrations used in these experiments exceeded the concentrations of DBPs found in drinking water. The objective of *in vitro* experiments is to provide a starting place to characterize the human health effects of exposure to environmental compounds. In DBP research specifically, *in vitro* experiments are conducted to reconcile exposure results with the effects of long-term exposure observed in epidemiological studies. In order to induce observable effects in cell culture, often higher exposure concentrations are used in place of low environmental exposure levels. The effects of long term exposure to low dose DBPs on human neurotoxicity require

further research. The findings of this study only provide indication of *in vitro* neurotoxicity.

In cell cycle and differentiation marker expression analysis, I exposed hNSC cultures to 0.1 μM and 0.5 μM of DBPs. Converted to μg/L units, 0.1 μM is equivalent of 26.5 μg/L of 2,6-DBBQ, 17.7 μg/L of 2,6-DCBQ, 13.9 μg/L of BAA and 9.5 μg/L of CAA. In assessments of treated water, HBQs were identified in much lower concentrations: 165.1±9.1 ng/L for 2,6-DCBQ and 0.5±0.1 ng/L for 2,6-DBBQ [3]. HAAs are found in drinking water at higher levels, ranging from <0.5 μg/L to 1230 μg/L [4]. Since the experimental exposure concentrations to DBPs differ from exposure concentrations in drinking water, the effects observed in laboratory experiments are incongruent with predicted results that are observed in humans. However, it is important to study the effects of DBPs on neurotoxicity using such *in vitro* models, as a starting point for understanding the toxicity of DBPs.

Furthermore, these experiments exposed cells to constant concentrations of DBPs whereas human exposure to DBPs fluctuates greatly depending on the season. Health Canada reports high fluctuations in DBP levels from season to season. Total trihalomethanes (TTHMs) are often used as a correlate to total DBP levels in drinking water. An assessment conducted in 1993 reports that the winter mean for TTHM content is 6.8 to 33.4 μg/L, compared to the summer mean at 31.2 to 66.7 μg/L [5]. Fluctuating water quality likely poses challenges in characterizing individual short term exposure since it is difficult to track DBP content that corresponds to the particular window of pregnancy and these levels that may be relevant to causing an adverse reproductive outcome [6].

5.3 Questions for Future Work

The results of my thesis studies represent a small section of an important and expanding field of DBP research. Further research on neurotoxicity of DBPs using hNSCs as a model is required. As noted in the discussion sections of previous chapters, it would be useful to conduct cell viability, cell cycle, and expression of differentiation marker experiments with a greater range of concentrations. In my experiments, the cells would detach from the plate, form neurospheres suspended in media, and re-attach at the bottom of the plate, resulting in inconsistent readings. Finding appropriate techniques to measure a wider range of exposure concentrations will allow future researchers to establish IC_{50} values for DBPs in this cell line. Furthermore, researchers would be able to determine the dose effect of DBPs on hNSC culture.

In addition, further research can be conducted to select optimal time points at which to analyze hNSCs for developmental neurotoxicity. As differentiation markers increase and decrease at various points during growth, research into the time point best suited to measure the differences in protein expression would be a useful tool. Ivanov *et al.* exposed cells to sodium arsenite for 48 h in apoptosis experiments and 10 d in differentiation marker experiments [7]. Comparatively, Stummann *et al.* allowed differentiation to occur for 42 d in methyl mercury exposure studies [8]. Selecting an appropriate time point may depend on the exposure substance and the specific cell markers, but testing out various days to conduct analysis may provide a more definitive picture of changes to hNSC differentiation.

Furthermore, the developmental effect of a greater number of DBPs should be tested in hNSCs. In my thesis studies, I focused on testing two classes of DBPs based on

their potential toxicity found in other studies. Many *in vitro* and animal studies have found that regulated DBPs may not account for the full toxic potential of DBPs found in treated water [9]. In total, there are over 480 established and novel DBPs, and additional DBPs are continuously being discovered [10]. In particular, the comparative toxicity of HBQ isomers on hNSCs should be studied. Specific HBQ isomers, such as 2,5-DCBQ and 2,5-DBBQ, may induce a greater toxic effect on these cells than other isomers (2,6-DCBQ and 2,6-DBBQ). The differential toxicity may be due to the isomer structures interacting with different cell functions resulting in the generation of reactive oxygen species (ROS). Studies looking at specific DBPs will allow researchers to gain insight on the comparative toxicity of the many classes of DBPs found in drinking water.

5.4 Conclusions

It is important to look at *in vitro* DBP research within the context of the entire field of DBP research, which includes toxicological analysis, epidemiological research, and analytical chemistry in addition to many other disciplines. My thesis studies observed adverse effects of DBP exposure on hNSCs, a model used by toxicologists and pharmacologists to test the developmentally toxic effects of environmental contaminants and pharmaceuticals. I found that exposure to the DBPs studied adversely affects hNSC cell cycles by causing cell cycle arrest at S-phase and impedes the normal differentiation and maturation processes of cells. The observation of these adverse effects indicates the needs for further research in this area as a precautionary measure because of the pervasiveness of drinking water exposure. Information on neurotoxicity of DBPs may help future consideration of DBP control policy decisions by public health organizations.

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