Effects of halobenzoquinone and haloacetic acid water disinfection byproducts on human neural stem cells

Katherine Z. Fu1,⁎⁎, Jinhua Li1,⁎⁎, Sai Vemula1, Birget Moe1,2,* Xing-Fang Li1,⁎

1. Division of Analytical & Environmental Toxicology, Department of Laboratory Medicine & Pathology, Faculty of Medicine & Dentistry, University of Alberta, Edmonton, Alberta T6G 2G3, Canada
2. Alberta Centre for Toxicology, Department of Physiology & Pharmacology, Faculty of Medicine, University of Calgary, Calgary, Alberta T2N 4N1, Canada

ARTICLE INFO

Article history:
Received 25 November 2016
Revised 8 February 2017
Accepted 9 February 2017
Available online 20 February 2017

Keywords:
Disinfection byproducts
Human neural stem cells
Differentiation
Halobenzoquinones
Haloacetic acids
Cell cycle arrest

ABSTRACT

Human neural stem cells (hNSCs) are a useful tool to assess the developmental effects of various environmental contaminants; however, the application of hNSCs to evaluate water disinfection byproducts (DBPs) is scarce. Comprehensive toxicological results are essential to the prioritization of DBPs for further testing and regulation. Therefore, this study examines the effects of DBPs on the proliferation and differentiation of hNSCs. Prior to DBP treatment, characteristic protein markers of hNSCs from passages 3 to 6 were carefully examined and it was determined that hNSCs passaged 3 or 4 times maintained stem cell characteristics and can be used for DBP analysis. Two regulated DBPs, monobromoacetic acid (BAA) and monochloroacetic acid (CAA), and two emerging DBPs, 2,6-dibromo-1,4-benzoquinone (2,6-DBBQ) and 2,6-dichloro-1,4-benzoquinone (2,6-DCBQ), were chosen for hNSC treatment. Both 2,6-DBBQ and 2,6-DCBQ induced cell cycle arrest at S-phase at concentrations up to 1 μmol/L. Comparatively, BAA and CAA at 0.5 μmol/L affected neural differentiation. These results suggest DBP-dependent effects on hNSC proliferation and differentiation. The DBP-induced cell cycle arrest and inhibition of normal hNSC differentiation demonstrate the need to assess the developmental neurotoxicity of DBPs. © 2017 The Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences.

Introduction

The human nervous system is particularly sensitive to toxic chemicals at early stages of development. Environmental chemicals are potentially more harmful to developing brains than adult brains due, in part, to the increased vulnerability during neuron growth and development during the early stages of life (Rice and Barone, 2000). Disruption of development may occur in different ways, including altered commitment of neural stem cells, proliferation of stem cells or neuronal progenitor cells, cell migration, neuron growth, apoptosis, neuronal communication, myelination, or development of the blood-brain-barrier (BBB) (Coecke et al., 2007). The adverse effects during exposure can manifest at any point after exposure. Much evidence has shown that the disruption of these neurodevelopmental pathways has been directly linked to neurodevelopmental diseases from environmental exposures (Grandjean and Landrigan, 2014; De Felice et al., 2015).
The effects of drinking water disinfection byproducts (DBPs) on human neural development are unclear. Epidemiological studies have shown inconsistent correlations between a mother’s exposure to treated water and adverse developmental effects in fetuses (Nieuwenhuijsen et al., 2009). However, associations between DBP exposure and an increased risk of adverse developmental outcomes, including stillbirth, spontaneous abortion, birth defects, and low birth weight, have been reported (Colman et al., 2011; Levallois et al., 2012; Grazuleviciene et al., 2013; Smith et al., 2016). Recently, a multigenerational in vivo experiment was conducted in rats, showing that exposure to a mixture of trihalomethanes (THMs) and haloacetic acids (HAAs) up to 2000 times regulated levels had no adverse effects on various reproductive endpoints (Narotsky et al., 2015). It was also proposed that using rats as an animal model might not be a representative model of developmental neurotoxicity experienced by humans. Thus, these results suggest two possible explanations: either (1) methods currently under use for assessment of human developmental neurotoxicity are not sensitive enough to detect DBP-induced effects, or (2) regulated DBPs are not the DBPs responsible for the observed effects.

Human stem cells are quickly becoming an established model in the field of drug discovery and development (Liu et al., 2013; Ko and Gelb, 2014). They are also a promising new model for evaluating the potential developmental effects of environmental chemicals (Liu et al., 2013; Mori and Hara, 2013). Many studies have used stem cell technology to characterize the toxicity of various environmental contaminants, such as bisphenol-A and tetrachloro-1,4-benzoquinone (Yin et al., 2015; Li et al., 2015, 2017). Stem cell models have the potential to proliferate into cells of all three germ layers (Thomson et al., 1998). In particular, human neural stem cells (hNSCs) are able to differentiate into all three phenotypes of the developing nervous system: neurons, oligodendrocytes, and astrocytes (Zhang et al., 2001). hNSCs can be grown as progenitor cells for several generations and can differentiate into different populations of neural cells when neural growth factor is removed from media. Thus, researchers can test cells at different neurodevelopmental stages, providing a distinct advantage over traditional cell-based models used for developmental neurotoxicity assessment. Furthermore, hNSCs can be analyzed by a variety of methods (colorimetric/fluorometric assays, immunostaining, image analysis) and are highly sensitive to low doses of toxins (Buzanska et al., 2009; Breier et al., 2010; Mori and Hara, 2013).

Halobenzoquinones (HBQs) are an emerging class of DBPs identified in drinking water in 2010 (Qin et al., 2010; Zhao et al., 2010). Specifically, 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 been identified in tap water at nanogram per liter levels (Zhao et al., 2010). In addition to tap water, HBQs have also been identified in chlorinated recreational waters, presenting additional routes of human exposure (Wang et al., 2013). The toxicological significance of HBQs have been evaluated on the recommendation of quantitative structure toxicity relationship (QSTR) analyses that predicted HBQs have the potential to be up to 1000 times more toxic than regulated DBPs (Bull et al., 2006). Indeed, HBQs were found to increase both 8-hydroxydeoxyguanosine (8-OHdG) and protein carbonylation levels in T24 cells, indicating oxidative damage to genomic DNA and proteins (Du et al., 2013). 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 (Li et al., 2014). These findings are consistent with the reported toxic effects of quinone in organisms, particularly those involved in disrupting protein-handling systems (Xiong et al., 2014). However, the developmental effects of HBQs have yet to be examined.

Previous epidemiological and toxicological studies have shown that DBPs have the potential to affect neural development. hNSCs present a model which mimics the development of the human nervous system. The objective of our study is to examine the effects of regulated and non-regulated DBPs on hNSC proliferation and differentiation. Prior to DBP testing, we will first characterize the undifferentiated and differentiated hNSC cell populations for assessment of stem cell proliferation and differentiation. We have selected two regulated DBPs, monobromoacetic acid (BAA) and monochloroacetic acid (CAA), and two emerging DBPs, 2,6-DBBQ and 2,6-DCBQ, for analysis. BAA and CAA are HAA-DBPs, which are nearly ubiquitous in treated water. BAA and CAA have been demonstrated to be both cytotoxic and genotoxic (Plewa et al., 2010), and although they have been found to be developmental neurotoxins in vitro (Hunter et al., 1996), the in vivo and epidemiological evidence surrounding HAAs is unclear (Narotsky et al., 2015; Smith et al., 2016). Because comparative toxicity analysis of 2,6-DBBQ, 2,6-DCBQ, BAA, and CAA has revealed that these classes of DBPs preferentially influence different biological pathways (Procházkova et al., 2015), these two classes of DBPs may also have different effects on neurodevelopment.

1. Materials and methods

1.1. Chemicals

2,6-DBBQ was purchased from Indofine Chemical Company (Hillsborough, NJ, USA). 2,6-DCBQ, BAA, and CAA were purchased from Sigma-Aldrich (Oakville, ON, Canada). Table S1 in Appendix A presents a list of the tested DBPs and their chemical structure.

1.2. Cell culture

Cryopreserved embryonic hNSCs were obtained from Gibco/Life Technologies (Carlsbad, CA, USA; Cat. No.: N7800100). Cell culture was established and maintained according to the manufacturer’s instructions and incubated in a humidified chamber at 37°C and 5% CO2. Undifferentiated hNSC culture was maintained with complete hNSC media containing KnockOut™ D-MEM/F-12 basal medium with 2 mmol/L 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, Waltham, MA, USA). Media was refreshed 1 day after thawing...
and every 3 days thereafter according to the manufacturer’s instruction. Cells were passaged at 90% confluency, approximately 6 days after cell culture was established. For differentiation, hNSCs were seeded into 6-well plates (Corning Incorporated, Corning, NY, USA) coated with CTS™ CELLstart™ Substrate (ThermoFisher Scientific) at a density of 9.5 × 10^4 cells per well. Cells were then grown in complete hNSC media for 3 days. When cells reached 75%–80% confluence, complete hNSC media was replaced by differentiation media to induce differentiation. Differentiation media contained Neurobasal® medium, 2 mmol/L GlutaMAX™-1 supplement, and 2% B-27® supplement (50×) hNSCs containing fresh differentiation media were returned to the incubator to differentiate into neurons over the next 12 days with fresh media changes every 3 days. To collect cells for passaging or further experimentation, cells were detached using StemPro® Accutase® cell dissociation reagent (Life Technologies) and pelleted via centrifugation at 1200 r/min for 4 min.

1.3. **Immunohistochemistry analysis**

1.3.1. Effect of passage number on neural stem cell markers

Undifferentiated hNSCs grown in complete hNSC media were collected at passage numbers 3, 4, 5, and 6 as previously described (Section 1.2) to confirm the proliferation potential of hNSCs at each passage. Cells were fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich) in Dulbecco’s phosphate buffered saline (DPBS) without CaCl2/MgCl2 (Gibco, Life Technologies). Fixed cells were then incubated in blocking buffer containing 5% goat serum, 1% bovine serum albumin (BSA), and 0.1% Triton-X, all dissolved in DPBS with CaCl2/MgCl2, for 30 min. Cells were stained for the undifferentiated stem cell markers, nestin and Sox2, using polyclonal rabbit antibody (Ab) (Abcam, Toronto, ON, Canada) and pelleted and stained with LIVE/DEAD® Fixable Violet Dead Cell Stain Kit (ThermoFisher Scientific) at a density of 9.5 × 10^4 cells per well. Cells were then grown in complete hNSC media for 3 days. When cells reached 75%–80% confluence, complete hNSC media was replaced by differentiation media to induce differentiation. Differentiation media contained Neurobasal® medium, 2 mmol/L GlutaMAX™-1 supplement, and 2% B-27® supplement (50×) hNSCs containing fresh differentiation media were returned to the incubator to differentiate into neurons over the next 12 days with fresh media changes every 3 days. To collect cells for passaging or further experimentation, cells were detached using StemPro® Accutase® cell dissociation reagent (Life Technologies) and pelleted via centrifugation at 1200 r/min for 4 min.

1.3.2. Expression of neural stem cell markers during differentiation

To examine the change in expression of neural stem cell markers during differentiation, cells were collected at 0 days and 12 days after differentiation was induced. Cells were then fixed and stained with nestin and DAPI as described above (Section 1.3.1). During nestin staining, cells were also stained for the differentiated stem cell marker, doublecortin (DCX), using monoclonal mouse Ab (Santa Cruz Biotechnology, Dallas, TX, USA). The secondary Abs used were Alexa-Fluor 488 goat anti-rabbit IgG (Molecular Probes) and Alexa-Fluor 488 goat anti-rabbit IgG (Molecular Probes) Cells were incubated with the secondary Abs and DAPI and imaged via confocal microscopy as described above (Section 1.3.1). The 491 nm emission filter was used to identify nestin and the 561 nm emission filter was used to detect DCX.

1.3.3. Western blotting

Cells were collected at 0 days and 14 days after differentiation was induced. Proteins were resolved with 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Bio-Rad Laboratories, Hercules, CA, USA). The monoclonal Abs used for western blotting included: 1) anti-β-actin (Sigma-Aldrich) and 2) DCX (monoclonal mouse Ab; Santa Cruz Biotechnology). The polyclonal Ab used was nestin (Section 1.3.1). The secondary Abs were goat anti-mouse and goat anti-rabbit horseradish peroxidase in 1:10,000 dilutions (ThermoFisher Scientific). Clarity™ Western ECL Substrate (Bio-Rad Laboratories) was used to detect proteins bound to blotting membranes. Signals were detected using an ImageQuant™ LAS 4000 (GE Healthcare Life Sciences Limited, Mississauga, ON, Canada). Images were processed using the accompanying ImageQuant™ TL software.

1.3.4. The ratio of DCX to nestin in differentiating hNSCs exposed to HBQs and HAAs

hNSCs were exposed to differentiation media (negative control), 2,6-DBBQ (0.1, 0.5 μmol/L), 2,6-DCBQ (0.1, 0.5 μmol/L), BAA (0.1, 0.5 μmol/L), or CAA (0.1, 0.5 μmol/L) for 12 days. Cells were collected and stained using the LIVE/DEAD® Fixable Violet Dead Cell Stain Kit (ThermoFisher Scientific) according to the manufacturer’s instructions for 405 nm excitation. Cells were then fixed and stained for nestin and DCX as previously described (Sections 1.3.1 and 1.3.2) and analyzed with an LSRFortessa X-20 flow cytometer (BD Biosciences, Mississauga, ON, Canada) with accompanying BD FACSDiva software to preview and record data. The proportion of live cells was gated so that nestin and DCX readings were only measured in live cells. Flow cytometry analysis was performed in triplicate with 10,000 gate events for each sample.

1.4. **Morphological assessment of differentiating hNSCs**

hNSCs grown in differentiation media were imaged using light microscopy at 0, 5, 10, and 14 days growth to observe morphological changes during differentiation. An IV-900 Inverted Microscope (MicroscOptics, Holly, MI, USA) at 20× magnification was used for imaging. Differentiating neurons were traced using the Simple Neurite Tracer program on the image analysis software Fiji (Image, Madison, WI, USA). To use Simple Neurite Tracer, the color channels were split in red, blue, and green, and the green channel was selected for analysis for optimal visual clarity. Neurites were individually traced to show the elongation process.

1.5. **Cell viability and cell cycle analysis of undifferentiated hNSCs exposed to HBQs and HAAs**

Undifferentiated hNSCs were exposed to complete hNSC media (negative control), 2,6-DBBQ (0.1-1 μmol/L), 2,6-DCBQ (0.1-1 μmol/L), BAA (0.1-1 μmol/L), or CAA (0.1-1 μmol/L) for 96 hr. Cells were detached and stained with LIVE/DEAD® Fixable Violet Dead Cell
Stain Kit according to the manufacturer’s instructions for 405 nm excitation. Cells were then fixed using 70% chilled ethanol (Commercial Alcohols, Brampton, ON, Canada). Prior to analysis, cells for cell cycle analysis were re-suspended in FxCycle™ PI/RNase Staining Solution (ThermoFisher Scientific). Samples were analyzed using flow cytometry with an LSRFortessa X-20 flow cytometer and its accompanying BD FACSDiva software to record data and assess cell viability. Live cells were gated to ensure that only live cells were analyzed. Samples were run in triplicate and each sample was run until the number of gate events reached 100,000. FlowJo LLC (Ashland, OR, USA) data analysis software was used to analyze the cell cycle flow cytometry data. The cell cycle analysis function in FlowJo was used to create histograms and to separate peaks showing different phases of the cell cycle.

1.6. Statistical analysis

All data is presented as mean ± 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) with a Dunnett’s post-test was used for multiple comparisons among equimolar treatment and control groups. Differences were considered statistically significant at $P < 0.05$.

2. Results and discussion

2.1. Characterization of undifferentiated and differentiating cells in the hNSC model

To confirm the proliferation of hNSCs and their differentiation into neurons using exogenous growth factors, we first characterized the 1) undifferentiated and 2) differentiating cell populations in our selected hNSC model. As shown in Fig. 1, cells grown in hNSC media will continue to propagate as undifferentiated hNSCs. Switching from hNSC media to differentiation media will induce the directed differentiation of hNSCs into neurons. Thus, through our hNSC model, we are able to examine two distinct pathways in neuronal development: proliferation and differentiation.

2.1.1. Undifferentiated hNSCs

Many studies have observed that hNSCs undergo cellular and replicative senescence after multiple passages in culture, losing the ability to differentiate (Ostenfeld et al., 2000; Bai et al., 2004; Villa et al., 2004). Assessing the characteristics of undifferentiated hNSCs at different passage numbers will ensure the cells used in our experiments possess the capacity to differentiate into different neural phenotypes. Thus, we examined the expression of two key proteins, nestin and Sox2, in hNSCs after passage numbers 3 to 6 (P3–P6). Nestin, a neuroectodermal marker, has been observed to peak after seven days of cell growth, followed by downregulation, during neuronal differentiation (Zimmer et al., 2011). The neural progenitor marker, Sox2, has been found to inhibit neuronal differentiation, resulting in the maintenance of neural progenitor characteristics (Graham et al., 2003). Thus, the expression of nestin and Sox2 are characteristic features of undifferentiated hNSCs (Ivanov and Hei, 2013).

Fig. 2 shows fluorescence images of nestin (green) and Sox2 (yellow) expression in hNSCs after P3–P6. Cells were counterstained with the DNA-binding compound, DAPI (blue), to indicate cell nuclei. Nestin (green) is clearly expressed in the cytoplasm of the cells from P3–P5, while Sox2 (yellow) is clearly detected in the nucleus of the cells from P3–P5 (Figs. 2a–c). Comparatively, the cells of P6 (Fig. 2d) do not appear to express nestin or Sox2, suggesting these cells have lost stem cell characteristics. The loss of stem cell characteristics in hNSCs...
after multiple passages in culture has been observed in a previous study (Villa et al., 2004). From these results (Fig. 2), it is clear that hNSCs passed less than six times express undifferentiated neural progenitor cell markers, indicating they have the potential to differentiate into different neural phenotypes. Therefore, only hNSCs from P3–P4 were used in all further undifferentiated hNSC experiments to ensure that the hNSCs examined maintained stem cell characteristics.

2.1.2. Differentiating hNSCs

The addition of differentiation media to our hNSC cultures induces the differentiation of hNSCs to neurons over time. The differentiation media contains exogenous growth factors which direct differentiation into neurons as the primary neural phenotype. To confirm the presence of differentiating hNSCs in our hNSC model, we assessed both the morphological and protein characteristics of hNSCs grown in differentiation media over 12 days growth.

2.1.2.1. Morphological assessment. The morphology of neurons are characterized by the cell body, or soma, and the physical extensions from the cell body, the neurites, which can be axons or dendrites (Pannese, 2015). Fig. 3 shows the elongation of neurites as hNSCs are grown in differentiation media, demonstrating the morphological changes undergone by the hNSCs during the process of differentiation. Cells were imaged at 0 days (Fig. 3a), 5 days (Fig. 3b), 10 days (Fig. 3c), and 14 days (Fig. 3d) growth in differentiation media. At 5 days growth, neurites begin to form and, as the differentiation process continues from 10 days to 14 days growth, the neurites clearly extend further from the cell body. Interestingly, compared to cells at day 0, on day 14, cells no longer appear to be growing as a monolayer, and aggregates of cells can be observed. These aggregates are likely neurospheres, which naturally form when hNSCs are cultured in vitro. Neurospheres are a heterogeneous cell population and contain many differentiated cells in addition to progenitor cells (Lanza and Atala, 2013). These morphologic characteristics confirm the differentiation of hNSCs into neurons over time in our hNSC model.

2.1.2.2. Expression of differentiation markers. DCX is a neuron-specific phosphoprotein expressed during corticogenesis and is involved in the migration and differentiation processes of neurons (Francis et al., 1999). As DCX is not present in neural stem cells, DCX is a key marker of differentiated neurons. To further confirm the differentiation of hNSCs into neurons in our hNSC model, we used confocal microscopy and western blotting to examine the change in expression of undifferentiated (nestin) and differentiated

Fig. 2 – Confocal microscopy images (20×) of hNSCs at (a) P3, (b) P4, (c) P5, and (d) P6 in complete hNSC medium and stained for the undifferentiated hNSC markers: nestin (green) and Sox2 (yellow). Cell nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (blue). At P6, most cells no longer express undifferentiated hNSC markers.
Fig. 3 – Light microscopy images of hNSCs (a) 0 days, (b) 5 days, (c) 10 days, and (d) 14 days after differentiation was induced. Elongating neurites were traced using Simple Neurite Tracer. As differentiation progressed, neurites extended further from the cell bodies of the hNSCs.

Fig. 4 – Confocal microscopy images of hNSCs (a) 0 days and (b) 12 days after differentiation was induced. Images were taken at 20× magnification. The four frames show the same cell with the doublecortin (DCX) (red), nestin (green), and 4',6-diamidino-2-phenylindole (DAPI) (blue) channels separated. (c) Proteins were detected using western blot analyses 0 days and 12 days after differentiation was induced. DAPI and β-actin were used as controls in each assay, respectively, and are expressed in cells at both time points. DCX is expressed in cells grown for 12 days, but not in undifferentiated (0 days) cells. Nestin is expressed in cells both 0 days and 12 days after differentiation was induced.
(DCX) protein markers in hNSCs over 12 days growth in differentiation media. Fig. 4 shows confocal images of hNSCs 0 days (Fig. 4a) and 12 days (Fig. 4b) after differentiation was induced. At 0 days growth (Fig. 4a), the hNSCs express both nestin (green) and the nuclear marker DAPI (blue), but do not express DCX (red). Comparatively, after 12 days growth (Fig. 4b), the hNSCs express all three markers: DCX, nestin, and DAPI.

Fig. 4c shows the results of western blot analysis with the original gel images included in Fig. S1 of Appendix A. Total β-actin levels were measured as an internal control and were consistent between the undifferentiated (0 days) and differentiated (12 days) hNSCs. DCX was only expressed in differentiated hNSCs (12 days), consistent with our confocal results (Fig. 4a–b). Similarly, nestin was expressed in hNSCs at both 0 days and 12 days growth (Fig. 4c), although it appears that nestin expression decreases over time, as reported in other studies (Zimmer et al., 2011). Both our confocal imaging and western blot analysis results confirm that hNSCs grown in differentiation media differentiate into neurons over 12 days growth, as confirmed with the increasing presence of DCX over time. Thus, DCX and nestin expression over time can be used as neural markers to assess the progression of hNSC differentiation.

Having characterized the undifferentiated and differentiating hNSC populations within our selected hNSC model, the effects of HBQs and HAAs on the 1) proliferation and 2) differentiation of hNSCs can be examined.

2.2. Effects of HBQs and HAAs on undifferentiated hNSCs

Because DBPs are present in drinking water at low μg/L and ng/L concentrations, it is important to evaluate sub-lethal effects of DBP exposure in in vitro assays to more accurately assess the potential human health risks associated with chronic, low-dose DBP exposures. To ensure our selected treatment ranges for the HBQs and HAAs used in our experiments would not be acutely cytotoxic, we first evaluated the cell viability of hNSCs after 96 hr of HBQ or HAA exposure.

Fig. 5 shows the effects of HBQ and HAA exposure on hNSC cell viability determined using flow cytometry. Here, it is clear that the hNSCs treated with DBP concentrations of 0.1 and 0.5 μmol/L maintain cell viability greater than 80%. There was a statistically significant decrease in cell viability observed in the 0.5 μmol/L BAA and CAA treatment groups; however, cell viability remained higher than 80%. Because the selected treatment concentrations decreased cell viability by less than 20%, these concentrations were used in subsequent experiments to ensure that the effects of HBQs and HAAs at non-acutely cytotoxic exposure concentrations were examined. Furthermore, all data analysis was performed across equimolar treatment groups to ensure an equal basis of comparison of any observed toxic effects induced by the two DBP classes under examination.

To assess the effects of HBQs and HAAs on the normal proliferation of undifferentiated hNSCs, we performed cell cycle analysis using flow cytometry. Alterations to the cell cycle can indicate various types of cellular damage, including damage to DNA or other molecular and structural damages (Elledge, 1996; Zhou and Elledge, 2000). Thus, cell cycle analysis is a broad, but sensitive indicator of adverse cellular effects. The results of cell cycle analysis of hNSCs exposed to HBQs or HAAs for 96 hr are found in Fig. 6. Here, a statistically significant decrease in the proportion of cells in the G0/G1 phase was observed in both of the 2,6-DBBBQ treatment groups and in the 1 μmol/L treatment groups of 2,6-DCBBQ, BAA, and CAA, as compared to the untreated control group. A statistically significant decrease in the proportion of cells in G2/M phase was also observed in the 1 μmol/L BAA and 0.5 μmol/L CAA treatment groups. More importantly, a statistically significant increase in the proportion of cells in S-phase was induced by both 0.5 μmol/L and 1 μmol/L 2,6-DBBBQ and 1 μmol/L 2,6-DCBBQ. This is an interesting find, as increases in a phase during the cell cycle indicate cell cycle arrest at that phase. Since there was a statistically significant increase in the number of cells at S-phase in these treatment groups, it is likely that 2,6-DBBBQ and 2,6-DCBBQ induce cell cycle arrest at S-phase. This is consistent with the known mechanisms of HBQ-induced cytotoxicity, as studies have shown that HBQ exposure results in the formation of reactive oxygen species and causes oxidative DNA damage in cells (Du et al., 2013; Li et al., 2014, 2016). 2,6-DCBBQ exposure in Chinese hamster ovary (CHO) cells was also found to increase expression of the p53 protein (Li et al., 2016), a well-known activator of cell cycle arrest (Levine, 1997). Because there are also intra-S-phase DNA damage checkpoints to delay cell cycle progression and repair defects (Bartek et al., 2004), damage of cellular DNA during replication may explain why cell cycle arrest at S-phase was observed in HBQ-treated hNSCs.

While no previous studies have analyzed the effects of HBQ exposure on the cell cycle, studies have examined these effects after HAA exposure. In human small intestine epithelial cells, exposure to non-cytotoxic concentrations of BAA, CAA, or monoiodoacetic acid (IAA) resulted in altered transcriptome profiles of genes responding to DNA damage and oxidative stress, as well as genes regulating cell cycle and apoptosis (Attene-Ramos et al., 2010). The researchers hypothesized that oxidative stress was one of the mechanisms for the observed HAA-induced genotoxicity, as DNA lesions induced by HAAs would require extended time for DNA repair and repressed cell division. In the same cell line, 60 μmol/L BAA was also shown to modulate genes associated with the cellular response to DNA double strand breaks and to cell cycle regulation (Muellner et al., 2010). Hence, it is interesting that HAA-induced cell cycle arrest was not observed in our results. However, differences between the cell lines examined may account for these inconsistent observations. Cell cycle effects of other DBP classes have been reported previously. The haloacetonitriles (HANs) were found to induce cell cycle effects at non-cytotoxic exposure concentrations in CHO cells, although the HAN-induced effects were much more prominent than the HBQ-induced effects reported here (Komaki et al., 2014). N-nitrosodiphenylamine (NDPhA), a nitrosamine DBP, was also found to induce G0/G1 arrest in CHO cells at 50% inhibitory concentrations (Boyd et al., 2008).

2.3. Effects of HBQs and HAAs on hNSC differentiation

The differentiation process is a critical time in the lifespan of a stem cell, as it determines the identity of the mature
Fig. 5 – Cell viability of each exposure group compared to the control after 96 hr halobenzoquinone (HBQ) or haloacetic acid (HAA) exposure determined using flow cytometry analysis. The concentrations were selected so that relative cell viability exceeded 80% in comparison to the control group to ensure that treatment concentrations were not acutely cytotoxic; * $P < 0.05$ in comparison to the control.

Fig. 6 – Percentage of hNSCs at each stage of the cell cycle after 96 hr exposure to HBQs or HAAs; * $P < 0.05$ and ** $P < 0.01$ in comparison to the control.
cell. Chemicals that affect this sensitive process can greatly influence the normal development of the human nervous system. As determined in Section 2.1, both nestin and DCX are expressed by hNSCs, with DCX a marker specific to differentiated hNSCs. The expression of these markers at different periods of neural development can be used as references to indicate the stage of neuron differentiation. Thus, changes in the expression of these differentiation markers after DBP exposure may indicate DBP-induced effects. To examine the effects of HBQs and HAAs on hNSC differentiation, we will measure the ratio of DCX to nestin in hNSCs after 12 days exposure using flow cytometry. To illustrate how this data was collected, Fig. 7a shows an intensity histogram of nestin and DCX expression in hNSCs after 12 days growth in differentiation media. Here, 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, as they are most likely cell debris. The pattern of expression and subsequent population gating was consistent among all exposure groups. The data from the intensity histograms was then used to create mature to immature cell ratios for each treatment group, as found in Fig. 7b.

Compared to the control group, there was a statistically significant decrease in the ratio of mature to immature cells in the $0.5 \mu\text{mol}/\text{L}$ BAA and $0.5 \mu\text{mol}/\text{L}$ CAA treatment groups (Fig. 7b). The decrease in the ratio of mature to immature neurons indicates that BAA and CAA exposure affects the differentiation process of hNSCs, as fewer hNSCs were able to fully mature in culture, increasing the relative number of immature neurons present. While these effects were observed in the HAA treatment groups, no effects were observed in the HBQ treatment groups. Because HAAs are a highly prevalent class of DBPs in treated drinking water (Roberts et al., 2002; Krasner et al., 2006, 2016), these differentiation results are a significant finding of our study. The HAA treatment dose of $0.5 \mu\text{mol}/\text{L}$ used in our experiment is equivalent to 69 $\mu\text{g}/\text{L}$ of BAA and 47 $\mu\text{g}/\text{L}$ of CAA. Thus, the tested concentrations are on the same order of magnitude as the maximum containment levels (MCLs) set by various government regulatory agencies for total HAAs, including the United States Environmental Protection Agency value of 60 $\mu\text{g}/\text{L}$ (USEPA, 2006) and the Health Canada value of 80 $\mu\text{g}/\text{L}$ (HC, 2014). However, it is important to note that fetal exposure to DBPs is not fully understood; thus, it is unknown whether exposure concentrations found in treated water would translate to fetal exposures. More research is needed to understand exposure dynamics of DBPs in pregnant women. Furthermore, epidemiological studies have found no consistent association between HAA-DBP consumption and developmental effects in humans (Smith et al., 2016). However, mouse whole embryo exposures have reported adverse neural effects induced by HAAs, including effects on the development of the neural tube, craniofacial region, and heart (Hunter et al., 1996). It was found that exposure to CAA affected embryonic development at concentrations as low as $175 \mu\text{mol}/\text{L}$, while embryos exposed to $6 \mu\text{mol}/\text{L}$ BAA exhibited abnormal neural tube development as well as rotational, pharyngeal arch, and heart defects.

Both our cell cycle analysis and differentiation results reveal that the hNSC model used in this study was capable of evaluating two important pathways in neural development:

![Fig. 7](image.png)

**Fig. 7** (a) Intensity histogram of DCX and nestin expression in untreated hNSCs, determined using flow cytometry analysis, showing the three distinct populations of cells (mature, immature, and negative) found in culture following 12 days growth in differentiation media. (b) The ratio of mature cells to immature cells present in the culture of differentiating hNSCs after 12 days exposure to HBQs or HAAs; * $P < 0.05$ in comparison to the control. Neg: negative.
the 1) proliferation and 2) differentiation of stem cells. This is significant, as in vitro methods that can assess multiple endpoints provide an important advantage for predicting toxicity. On the basis of total organic halogen analysis, it is estimated that identified DBPs only account for 30% of predicted halo-DBPs, meaning the majority of DBPs have yet to be identified (Krasner et al., 2006). As new DBPs are identified, the need for high-throughput in vitro assays capable of predicting the toxicity of DBPs and mixtures of DBPs becomes critical, particularly for sensitive endpoints such as the development of the nervous system. Thus, our hNSC method may provide an important tool for the future assessment of DBPs when used concurrently with other testing methods, improving assessment of potential DBP health risks.

### 3. Conclusions

The developmental neurotoxicity of DBPs is poorly understood, as there is a lack of both data and methods to evaluate developmental effects. We found that the two classes of DBPs examined in this study may more strongly influence different endpoints during neural development. The HBQs (2,6-DBBQ and 2,6-DCBQ) were found to influence the proliferation of hNSCs by causing S-phase cell cycle arrest, while the HAAs (BAA and CAA) were found to affect the differentiation of hNSCs by preventing the maturation of neurons. Hence, the hNSC model was able to differentiate between the toxicity pathways of different classes of DBPs. However, more research is needed to assess the effects of a larger range of exposure concentrations, as this study was limited to two concentrations. This is particularly important for the HAAs examined in this study because their effects were assessed at the same order of magnitude they are regulated in treated drinking water. Furthermore, more research is needed to evaluate the dynamics of fetal exposure to DBPs in order to provide greater insight into the observed effects of the tested concentrations of these DBPs in hNSCs. Nevertheless, the results of this study demonstrate that hNSCs are a promising model through which researchers can analyze the developmental effects of DBPs.

### Acknowledgments

This work was supported by funding from the Natural Sciences and Engineering Research Council (NSERC) of Canada, Alberta Innovates-Energy and Environmental Solutions, and Alberta Health. The authors would also like to thank the University of Alberta Faculty of Medicine and Dentistry Imaging and Flow Cytometry Cores for their assistance.

### Appendix A. Supplementary data

Supplementary data to this article can be found online at [http://dx.doi.org/10.1016/j.jes.2017.02.006](http://dx.doi.org/10.1016/j.jes.2017.02.006).

### REFERENCES


