Evaluation of Developmental Neurotoxicity of Toxic Elements Using Human Embryonic Stem Cell-Derived Neural Stem Cells as in vitro Models

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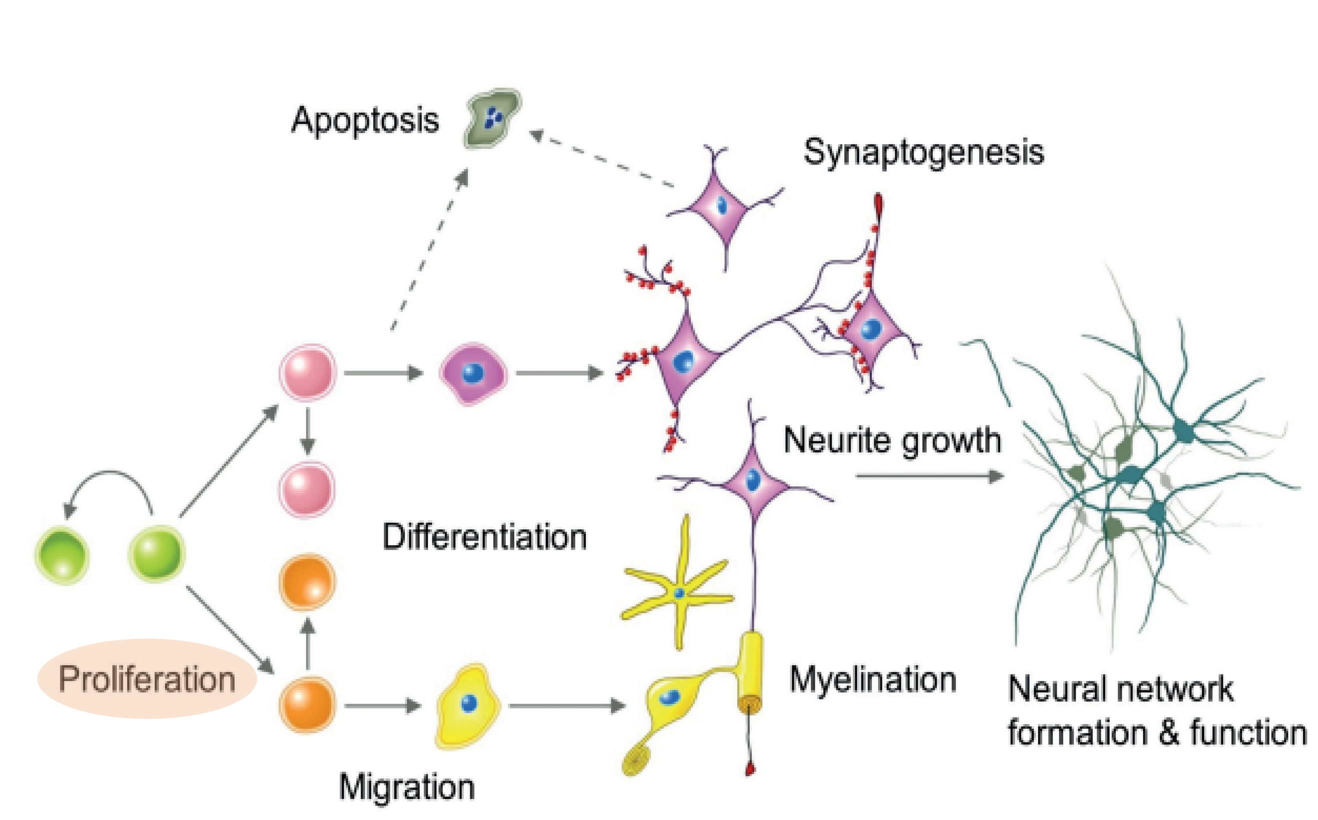
Abstract

Exposure to toxic elements found in foods consumed by infants and young children may cause developmental neurotoxicity (DNT). Due to the high cost and long duration of traditional animal-based testing methods, only a small fraction of chemicals that humans are exposed to have been assessed for DNT activity, thus creating a big knowledge gap that urgently needs to be addressed. Human pluripotent stem cell-derived neural stem cells (NSCs) provide attractive cell sources to study early neurological development and neurotoxicity. In the current study, two human neural stem cell lines, NSC-H9 and NSC-H14, derived from human embryonic stem (ES) cell lines WA09 and WA14, respectively, were characterized and used to evaluate the developmental neurotoxicity of the four most common toxic elements (lead, arsenic, mercury, and cadmium) involved in the foods consumed by infants and children. The BrdU incorporation, CellTiter-Glo cell viability, and Caspase-3/7 activity assays were utilized to evaluate the effects of lead acetate, sodium arsenate, methylmercury chloride, and cadmium chloride on NSC proliferation, viability, and cell apoptosis. The BrdU incorporation assay showed that all four toxic element compounds inhibited NSC cell proliferation in a dose-dependent manner at various concentrations (0.3 nM-100 μM). The IC50 values for lead acetate, sodium arsenate, methylmercury chloride, and cadmium chloride were comparable between NSC-H9 and NSC-H14 cells, after 48 hours of exposure. Specifically, lead acetate had an IC50 value of 5.66 μM in NSC-H9 and 71.59 μM in NSC-H14 cells; sodium arsenate had an IC50 value of 0.37 μM in NSC-H9 and 0.25 μM in NSC-H14 cells; methylmercury chloride had an IC50 value of 0.39 μM in NSC-H9 and 0.70 μM in NSC-H14 cells; and cadmium chloride had an IC50 value of 6.10 μM in NSC-H9 and 21.80 μM in NSC-H14 cells. Both NSC-H9 and NSC-H14 cell viability decreased when exposed to the toxic element compounds, with IC50 values of 93.61 μM, 0.46 μM, 0.78 μM, and 6.24 μM for lead acetate, sodium arsenate, methylmercury chloride, and cadmium chloride, respectively, after 48 hours of exposure in NSC-H9 cells. The IC50 values in NSC-H14 cells were similar to those in NSC-H9 cells. Exposure to the toxic element compounds for 48 hours resulted in weak to moderate stimulatory effects on caspase-3/7 activity. Collectively, the current study demonstrated that human ES cell-derived NSCs represent useful *in vitro* models for assessing early developmental neurotoxicity.

Materials and Methods

Key Neurodevelopmental Processes



- Aschner et al. ALTEX. 2017

Results

Table 1. Test compounds.

Compound	Vehicle	Source	Product	Chemical Formula	Purity
Lead acetate	DMSO	Sigma	Lead(II) acetate trihydrate	$Pb(CH_3COO)_2 \cdot 3H_2O$	99.999%
Sodium arsenate	DMSO	Sigma	Sodium arsenate dibasic heptahydrate	$Na_2HAsO_4 \cdot 7H_2O$	≥98.0%
Methylmercury chloride	H_2O	Fisher Scientific	Methylmercury(II) chloride	CH_3HgCl	NA
Cadmium chloride	DMSO	Sigma	Cadmium chloride	$CdCl_2$	99.99%
D-Sorbitol	DMSO	Sigma	D-Sorbitol	$C_6H_{14}O_6$	≥98%
Cytosine arabinoside	DMSO	Sigma	Cytosine β-D-arabinofuranoside	$C_9H_{13}N_3O_5$	≥90%

Table 2. Human ES cell-derived NSCs.

Cell line	Cell Type	Gender	Karyotype	Culture Platform	Culture Protocol	Passage	NIH Approval
NSC-H9	NSC	Female	Normal XX	NSC Medium/ Matrigel™	WiCell Neural Stem Cell Protocol	P19+5	NIHhESC-10-0062
NSC-H14	NSC	Male	Normal XY	NSC Medium/ Matrigel™	WiCell Neural Stem Cell Protocol	P21+5	NIHhESC-10-0064

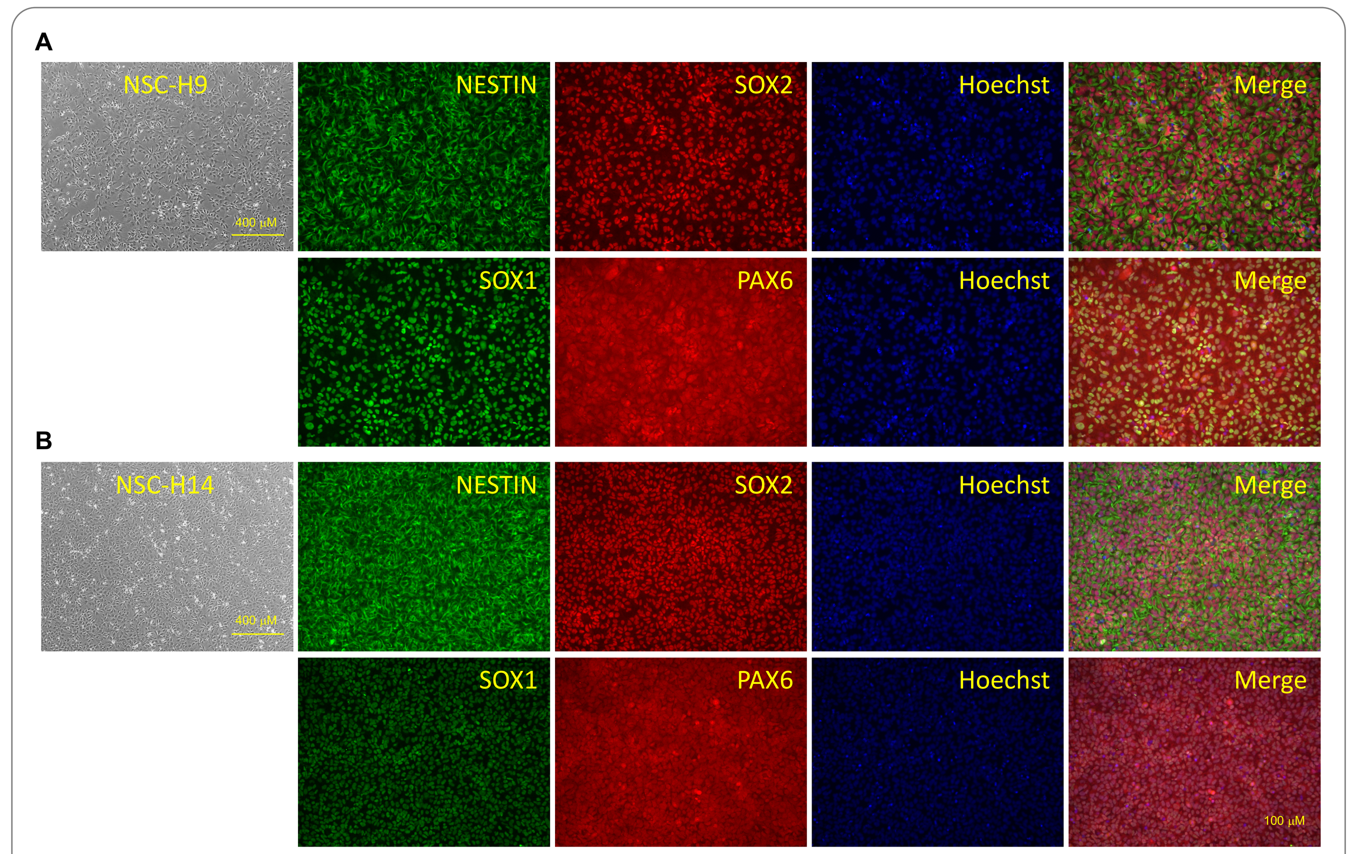


Figure 1. Bright-field cell images and Immunofluorescence staining of protein markers of NESTIN, SOX2, SOX1, and PAX6 in NSC-H9 (**A**) and NSC-H14 (**B**).

Table 3. Chemical concentrations tested *in vitro* in relation to concentrations found in human samples.

Toxic Elements	Concentrations found in human samples	Tested chemicals	Chemical formula	Concentrations tested *in vitro* (μM)
Lead (Pb)	Cord blood: Range 1.09–11.41 μg/L ➔0.0053–0.055 μM Children blood: Range 17.1–100 μg/L ➔ 0.083–0.483 μM	Lead acetate trihydrate	$Pb(CH_3COO)_2 \cdot 3H_2O$	100, 20, 4, 0.8, 0.16, 0.032, 0.0064, 0.0013, 0.0003
Arsenic (As)	Cord blood: 3.5 ± 7.6 ug/L ➔ 0.047 μM Children blood: Range 0.9–26.1 ug/L ➔ 0.012 μM–0.348 μM	Sodium arsenate dibasic heptahydrate	$Na_2HAsO_4 \cdot 7H_2O$	100, 20, 4, 0.8, 0.16, 0.032, 0.0064, 0.0013, 0.0003
Mercury (Hg)	Cord blood: Range 0.70–35 μg/L ➔ 0.0035–0.174 μM Children blood: Range 1.46–6.81 μg/L ➔ 0.0073–0.034 μM	Methylmercury chloride	CH_3HgCl	10, 2, 0.4, 0.08, 0.016, 0.0032, 0.0006, 0.0001, 0.00003
Cadmium (Cd)	Cord blood: Range 0.4–1 ug/L ➔ 0.0036–0.0089 μM Children blood: Range 2.2–17 μg/L➔0.020–0.151 μM	Cadmium Chloride	$CdCl_2$	100, 20, 4, 0.8, 0.16, 0.032, 0.0064, 0.0013, 0.0003

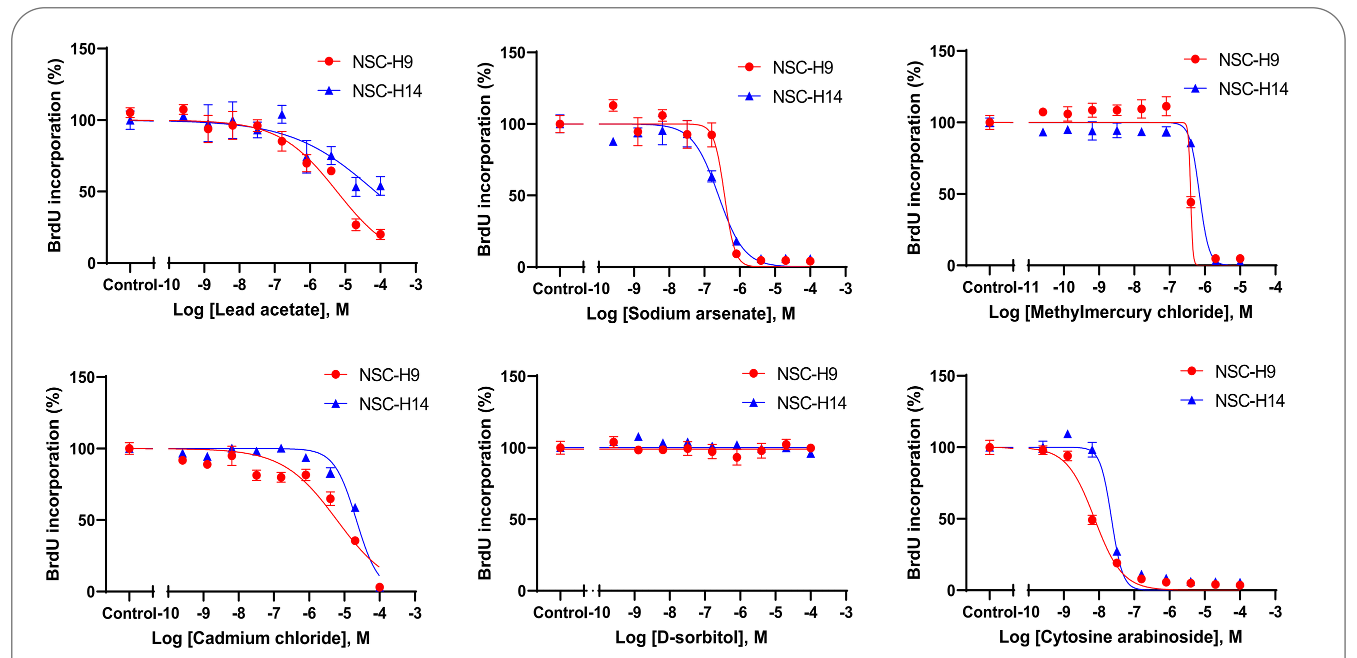


Fig 2. Effects of toxic element compounds on human NSC cell proliferation. NSC-H9 and NSC-H14 cells were exposed to a range of concentrations of toxic element compounds for 48 hours, and the effects of the compounds on cell proliferation were measured using the BrdU incorporation assay. The data shown represent the mean ± standard error of six independent experiments.

Table 4. IC5, IC10, IC20, IC50 comparison: effects of toxic element compounds on NSC cell proliferation.

NSC-H9

Compound	IC5 (μM) (very low toxic)	IC10 (μM) (low toxic)	IC20 (μM) (moderately toxic)	IC50 (μM) (highly toxic)	IC20/10 (μM) (non-cytotoxic)
Lead acetate	0.028	0.11	0.49	5.66	0.049
Sodium arsenate	0.13	0.16	0.21	0.37	0.021
Methylmercury chloride	0.25	0.28	0.32	0.39	0.032
Cadmium chloride	-	-	-	6.10	-
D-sorbitol	-	-	-	-	-
Cytosine arabinoside	0.00062	0.0011	0.0021	0.0075	0.00021

NSC-H14

Compound	IC5 (μM) (very low toxic)	IC10 (μM) (low toxic)	IC20 (μM) (moderately toxic)	IC50 (μM) (highly toxic)	IC20/10 (μM) (non-cytotoxic)
Lead acetate	0.042	0.11	0.32	71.59	0.032
Sodium arsenate	0.037	0.060	0.10	0.25	0.010
Methylmercury chloride	0.34	0.40	0.48	0.70	0.048
Cadmium chloride	3.49	9.88	30.52	21.80	3.05
D-sorbitol	-	-	-	-	-
Cytosine arabinoside	0.0060	0.0080	0.011	0.023	0.0011

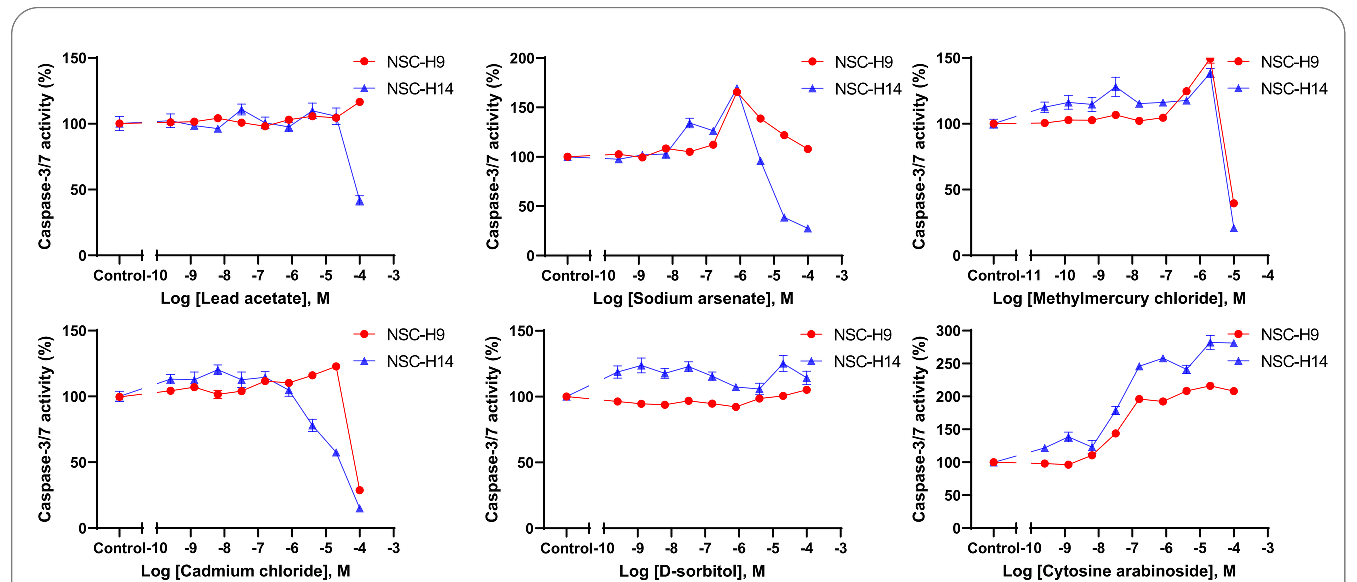


Fig 4. Effects of toxic element compounds on Caspase-3/7 activity in human NSCs. NSC-H9 and NSC-H14 cells were treated with various concentrations of toxic element compounds for 48 hours. Caspase-3/7 activity was measured using the Caspase-Glo 3/7 Assay. The data shown represent the mean ± standard error of six independent experiments.

Table 6. Maximum Caspase-3/7 activity induced by toxic element compounds in human NSCs.

Cell line	Lead acetate	Sodium arsenate	Methylmercury chloride	Cadmium chloride	Cytosine arabinoside
NSC-H9	116.4±4.6%***	165.6±5.0%***	149.7±8.3%***	123.0±4.7%***	216.3±5.8%***
NSC-H14	110.8±10.3%	169.6±4.1%***	138.6±8.4%***	120.4±9.0%**	282.0±25.9%***

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to the untreated control group.

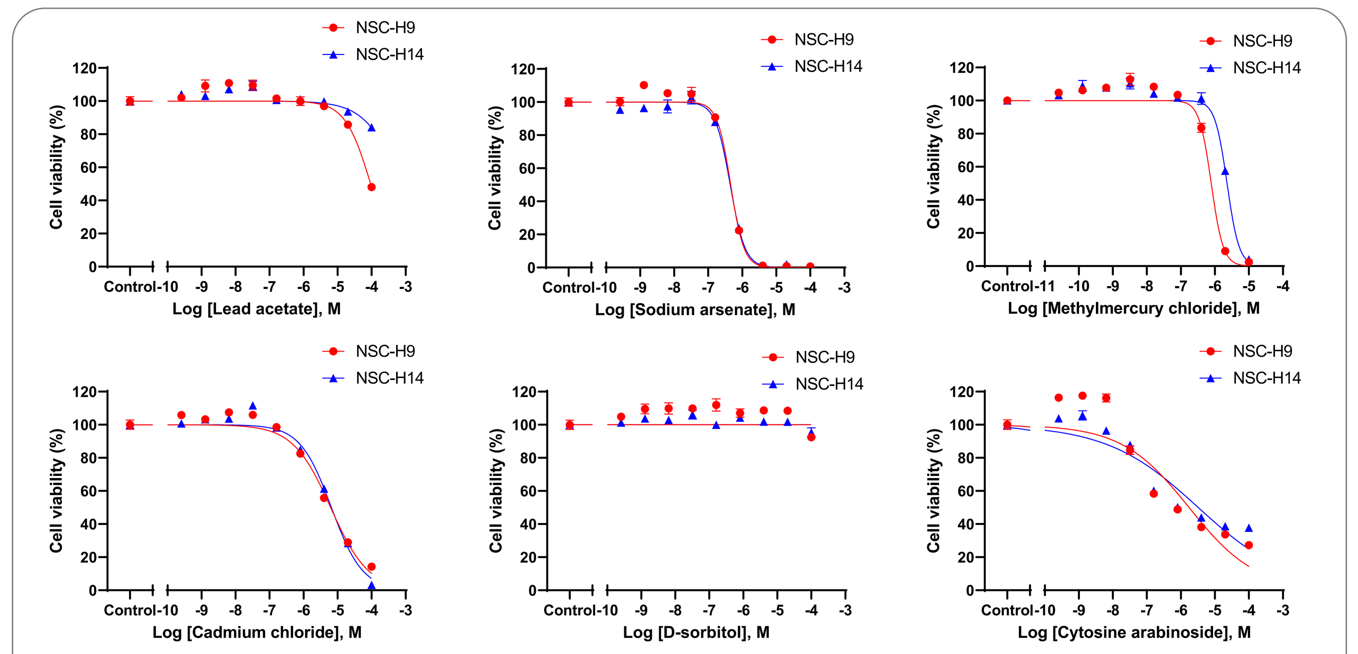


Fig 3. Effects of toxic element compounds on cell viability of human NSC-H9 and NSC-H14. Cells were treated with various concentrations of toxic element compounds for 48 hours, and the impact of the compounds on cell viability was determined using the CellTiter-Glo 2.0 cell viability assay. The data shown represent the mean ± standard error of six independent experiments.

Table 5. IC50 comparison: effects of toxic element compounds on NSC cell proliferation and cell viability.

Compound	NSC-H9		NSC-H14	
	Cell Proliferation (IC50, μM)	Cell Viability (IC50, μM)	Cell Proliferation (IC50, μM)	Cell Viability (IC50, μM)
Lead acetate	5.66	93.61	71.59	753.90
Sodium arsenate	0.37	0.46	0.25	0.44
Methylmercury chloride	0.39	0.78	0.70	2.28
Cadmium chloride	6.10	6.24	21.80	6.57
D-sorbitol	-	-	-	-
Cytosine arabinoside	0.0075	1.74	0.023	3.23

Conclusion

- NSC-H9 and NSC-H14 cells displayed typical human neural stem cell morphology and expressed key protein markers, including NESTIN, SOX1, SOX2, and PAX6.
- The proliferation of NSC-H9 and NSC-H14 cells was found to be dose-dependently inhibited when exposed to lead acetate, sodium arsenate, methylmercury chloride, and cadmium chloride, with varying IC50 values for each compound. Sodium arsenate was observed to be the most potent, with an IC50 of 0.37 μM, followed by methylmercury chloride (IC50=0.39 μM), lead acetate (IC50=5.66 μM), and cadmium chloride (IC50=6.10 μM), in NSC-H9 cells after 48 hours of exposure. Similar IC50 values were observed in NSC-H14 cells.
- Lead acetate, sodium arsenate, methylmercury chloride, and cadmium chloride caused a dose-dependent reduction in cell viability in both NSC-H9 and NSC-H14 cells. Sodium arsenate was found to be the most potent with an IC50 of 0.46 μM, followed by methylmercury chloride (IC50=0.78 μM), cadmium chloride (IC50=6.24 μM), and lead acetate (IC50=93.61 μM) in NSC-H9 cells after 48 hours of exposure. Comparable IC50 values were observed in NSC-H14 cells.
- All four compounds had weak to moderate stimulatory effects on caspase-3/7 activity after 48 hours of exposure, but high concentrations led to a dramatic decrease in activity due to their cytotoxic effects on cells.
- Human ES cell-derived NSCs are useful in vitro models for assessing the early developmental neurotoxicity of chemical compounds.

Regulatory Impact – This work supports the US FDA in developing potential predictive stem cell-based screening assays for hazard assessment of chemicals of concern in its regulated products.