Developmental Toxicity Testing of Gaseous Chemicals with a Human Pluripotent Stem Cell-Based Assay

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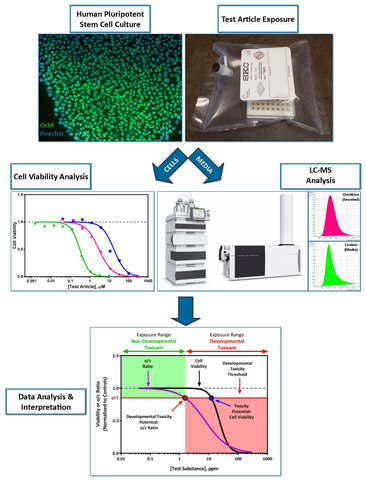
Abstract/Introduction

- Innovative *in vitro* toxicity screening assays aimed at reducing or replacing the use of animals in chemical safety testing are critical to meet the safety requirements across industries. Multiple publications have demonstrated the applicability of various *in vitro* assays for evaluating the developmental toxicity potential of non-volatile chemicals; however, very little information is available regarding application of these assays to volatile chemicals (e.g., gases).
- devTOX^{qP} is an *in vitro* human pluripotent stem cell (hPSC)-based assay that predicts the developmental toxicity potential of chemicals based on changes in ornithine and cystine metabolism. devTOX^{qP} is highly concordant (~85%) with human and animal developmental toxicity outcomes across diverse chemotypes.
- The objective of this study was to modify the devTOX^{qP} hPSC culture methods to test volatile and gaseous chemicals using Tedlar bags and evaluate the potential of chemicals tested in this system to induce a metabolic response in hPSCs. The applicability of the Tedlar bag culture methods was evaluated with four gaseous chemicals that have known developmental toxicity outcomes *in vivo* (HFC-236fa, HFC-245fa, acetaldehyde, and hexafluoroacetone).

Methods

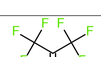
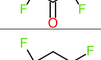
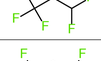
Study Design

Evaluate hPSC Growth and Metabolism during Tedlar Bag Culture vs. Standard Incubator Culture → Demonstrate hPSC Response to Treatment is Equivalent between Tedlar Bag and Standard Incubator Culture Conditions → Evaluate hPSC Response to Gaseous/Volatile Chemicals with Known *In Vivo* Developmental Toxicity Effects



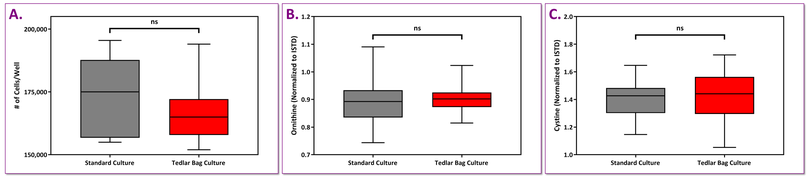
- Human induced pluripotent stem (iPS) cells (Cell Line: HYR0103, ATCC) were maintained in the undifferentiated state in mTeSR1 (StemCell Technologies) on Matrigel (Corning). Cells were plated in 96-well plates and allowed to attach overnight.
- On the day of treatment initiation, culture media was replaced, and the plate was sealed in an airtight Tedlar bag. Air was evacuated and appropriate volumes of test substance and a mixture of 5% CO_2/air were added. Cells were exposed to test substance for 48 hours, with media and test substance replacement each day.
- Spent media from the last 24-hour treatment period was collected and filtered to remove molecules >3 KDa.
- Cell viability was assessed after sample collection using the CellTiter-Fluor Cell Viability Assay (Promega).
- Samples were analyzed with UPLC-ESI-TOF-MS to determine ornithine (ORN) and cystine (CYSS) levels.
- Non-linear dose-response curves analysis for the o/c ratio, ornithine and cystine response and cell viability were fit with GraphPad Prism.
- The developmental toxicity potential (dTP, o/c ratio) and toxicity potential (TP, cell viability) concentrations were predicted from the respective dose-response curves using the iPS cell developmental toxicity threshold (dTT, 0.85).

Chemicals Tested

Chemical	CAS	Molecular Weight	Boiling Point	Structure	*In Vivo* Developmental Effects
Acetaldehyde	75-07-0	44	21°C		Craniofacial, Limb, CNS, Embryo/ Fetal Death, Growth Restriction (1)
Hexafluoroacetone	684-16-2	166	-28°C		Craniofacial, Embryo/Fetal Death, Growth Restriction (2)
HFC-245fa	460-73-1	134	15°C		None
HFC-236fa	690-39-1	152	-1.1°C		None

Results

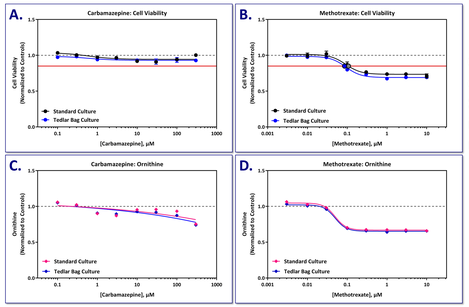
Culturing Human iPS Cells in a Tedlar Bag does Not Impact Growth Rate and Metabolism



Box-plot diagrams showing the number of cells/well (**A**), ornithine levels (**B**), and cystine levels (**C**) for human iPS cells cultured in the inner 60 wells of a 96-well plate under standard incubator (37°C, 5% CO_2, >95% humidity) compared to Tedlar bag conditions (bag filled with 5% CO_2 and cultured in incubator). ISTD: internal standard.

- The number of cells per well, level of ornithine secretion, and cystine consumed were not statistically significantly different between culture conditions (two-way ANOVA, p>0.05), indicating that human iPS cells cultured in Tedlar bags containing normal incubator air behave similarly to cells cultured under standard incubator conditions.

Cell Viability and Ornithine Response following Chemical Exposure are Not Altered by Culture Conditions

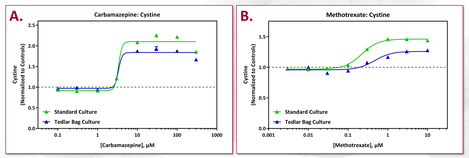


Cell viability and ornithine response following exposure to carbamazepine (**A, C**) and methotrexate (**B, D**) for human iPS cells cultured under standard incubator conditions or in Tedlar bags containing normal incubator air.

- The four-parameter log-logistic nonlinear dose-response curves were not significantly different between culture conditions (extra sum-of-squares F test, p>0.05).

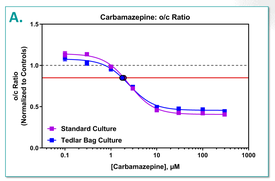
Magnitude of Cystine Response following Chemical Exposure is Decreased under Tedlar Bag Culture Conditions

Cystine response following exposure to carbamazepine (**A**) and methotrexate (**B**) for human iPS cells cultured under standard incubator conditions or in Tedlar bags containing normal incubator air.



- The cystine dose-response curve Hill Slope and IC_{50} values were not statistically significantly different between culture conditions for carbamazepine (p>0.05).
- For methotrexate, a statistically significant difference was observed for the dose-response curve IC_{50} values (p=0.0006).

Concentration where Developmental Toxicity Potential is Observed does Not Differ between Culture Conditions

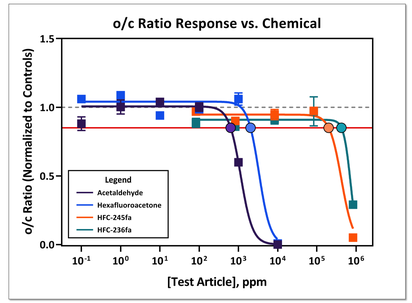


Compound	Standard Culture dTP (µM)	Tedlar Bag Culture dTP (µM)
Carbamazepine	1.9	1.8
Methotrexate	0.06	0.07

o/c Ratio response following exposure to carbamazepine (**A**) and methotrexate (**B**) for human iPS cells cultured under standard incubator conditions or in Tedlar bags containing normal incubator air.

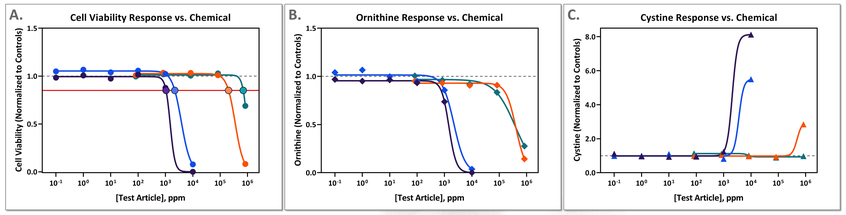
- The o/c ratio response in human iPS cells following exposure to carbamazepine and methotrexate was equivalent between culture conditions, indicating that culturing human iPS cells in Tedlar bags containing normal incubator air does not impact their response to treatment.
  - ◊ The interpolated developmental toxicity potential concentrations (dTP) did not differ between culture conditions.
  - ◊ The Hill slope and IC_{50} vales from the four-parameter log-logistic nonlinear model were not statistically significantly different between culture conditions (extra sum-of-squares F test, p>0.05).

devTOX^{qP} Accurately Separates the Developmental Toxicity Potential of Gaseous Chemicals



Chemical	dTP (ppm)	TP (ppm)
Acetaldehyde	634	1,049
Hexafluoroacetone	2,033	2,196
HFC-245fa	199,177	202,654
HFC-236fa	419,984	695,480

- Acetaldehyde and hexafluoroacetone, which have been shown to cause developmental toxicity in rodents, decreased the o/c ratio at exposures 3 orders of magnitude lower than HFC-245fa and HFC-236fa.



Cell viability (**A**), ornithine (**B**), and cystine (**C**) response following exposure to acetaldehyde, hexafluoroacetone, HFC-245fa, and HFC-236fa.

- Acetaldehyde and hexafluoroacetone, caused a decrease in cell viability and ornithine and an increase in cystine at <10,000 ppm.
- In contrast, HFC-245fa and HFC-236fa did not impact cell viability or human iPS cell metabolism at exposure concentrations that are typically considered limit concentrations in rodent studies (50,000 ppm). Human iPS cell viability and metabolism was only impacted at the highest exposure concentration tested in this assay (833,333 ppm), which is expected to yield plasma concentrations that are orders of magnitude higher than relevant human exposures.

Conclusions

- The devTOX^{qP} *in vitro* hPSC assay cell culture methods were modified to test volatile chemicals that cannot be diluted and tested as a solution (e.g., gases). These new methods were used to predict the developmental toxicity potential of four chemicals with known *in vivo* effects.
- Human iPS cells can be cultured in Tedlar bags containing normal incubator air with minimal impact their growth rate and metabolism.
- Human iPS cells exposed to reference chemicals while cultured Tedlar bags containing normal incubator air respond similarly to human iPS cells cultured under standard incubator conditions.
  - ◊ Tedlar bag culture slightly decreases the magnitude of the cystine response; however, the concentrations where a response was observed was not impacted.
- Chemicals producing developmental toxicity in animals (acetaldehyde and hexafluoroacetone) were successfully discriminated from chemicals that did not produce developmental toxicity in animals (HFC-236fa and HFC-245fa) on the basis of disrupted iPS cell metabolism.
- These results provide a human-relevant endpoint that could inform chemical prioritization and risk assessment of gaseous and volatile chemicals.

Funding

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References

1. Hazardous Substances Data Bank [Internet]. Bethesda (MD): National Library of Medicine (US). Acetaldehyde; Hazardous Substances Databank Number: 230. [Last Revision Date 2015 Oct 19; cited 2019 Jun 6]. Available from: http://toxnet.nlm.nih.gov/cgi-bin/sis/search2/r?dbs+hsdb:@term+@DOCNO+230.
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