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RESEARCH ARTICLE

Neurotoxins Induced Toxicogenomic Patterns on Human Induced Pluripotent Stem Cell based Microphysiological System

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ABSTRACT

The traditional testing requirements for both adult and developmental neurotoxicity evaluations are based on in vivo animal models while the neurotoxic risks associated with molecules or vaccines is mainly determined by neurobehavioral and neuropathological effects in the experimental model chosen. The poor correlation between preclinical in vitro or in vivo data (non-human) with the real time clinical effects leading to severe progressive adverse events is a major concern in general. The employed bioinformatics search tools helped us to short list the affected common genes in neurotoxicity induced by viral, bacterial infections and cytokine storms. Here, we used our group characterized human induced Pluripotent Stem Cell (hiPSC) system developed as an in vitro microphysiological model to record phenotype and genotype perturbations when treated with selected known representative neurotoxins like TEA, Tetanus toxin, MSG, Dopamine, Bungarotoxin etc. The objective was to assess the application qualification of the novel in vitro model that yields human relevant readouts. The recorded phenotype perturbations were barcoded with SOD, BAX, HDAC1, TNFalpha, MAPK14 like gene expressions in generating in vitro patterns to correlate the human functional toxicogenomics information. We showed hiPSC system to be phenotypically responsive and genotypically reactive when treated with neurotoxins. Out of 7 gene expression data sets generated, SOD and BAX were recorded to be downregulated at all the micro-conditions created in the hiPSC system while HDAC was consistently upregulated except in Dopamine treated system. The bioinformatics analysis performed on the selected genes gave insight into their roles in disease specific signalling pathways like JAK-STAT, TNF, Neurotrophin etc. We report configured hiPSC system suitability as an in vitro human surrogate platform/model in generating toxicogenomics signatures to support prediction on the test material in any assay system developed on this well characterized microphysiological base.

Keywords: Human microphysiological system, neurotoxicity, hiPSC, phenomics, in vitro prediction mode

Introduction

The toxins that target the nervous system either by impairing the functions or the microenvironment of the human nervous system are termed as neurotoxins. Neurotoxicity in the human body occurs when the exposure to toxic agents alters the normal activity of the nervous system either permanently or temporarily [1,2] while the toxic agents can be chemical or biological. The traditional testing strategies for both adult and developmental neurotoxicity evaluations are based on in vivo animal models and the neurotoxic potential, risks associated of molecules or vaccines is mainly determined by neurobehavioral and neuropathological effects [3-6]. In vitro studies are generously considered complementary to animal tests because they provide an understanding of the molecular/cellular mechanisms involved in neuropathology. Whether it is in vivo or in vitro assay system, human relevant data will take precedence if made available surrounding the test material. The complexity of nervous system and multiple functions of the individual cell types, the limited knowledge of the biochemical processes involved in neurotoxicity are some of the factors to state that no single test can yield the desired and meaningful readouts [7,8].

Various types of in vitro approaches have been practised to evaluate the neurotoxic effects of the molecules/vaccines recruiting cell lines [7,9] like SH-SY5Y, PC12 and C6 in the methods. The several related end points of neurotoxicity have been assessed such as cell viability [10,11], interference with neurite outgrowth [12], or disruption of mitochondria function [13]etc. Although these cell lines have been in use for ages, either in differentiated or undifferentiated forms, the use of the transformed cell lines comes with limitations in measuring mutations, heterogeneity in cellular configuration, lack of human relevant baseline genotype, signal pathways etc. Primary cultures derived from different parts of the brain region such as hippocampus, cortex, striatum, or cerebellum from rat model system [12,14,15] has been in preclinical use for testing neurotoxicity, but the restriction with respect to the human relevance remained the same. The 2D or 3D cultures present significant challenges including obtaining and maintaining the cultures, dearth of enough biological material to harvest in preparing large scale culture systems, compared to cell lines.

Pluripotent stem cell based and derived from embryonic stem cell lines (mouse and human) represent a compelling model system to study early neural development and has also been employed to screen toxicants, defining toxicity pathways [16-19]. Importantly, animal models may not be good testing models for drug toxicity as any chemical may be toxic to an animal but may not be toxic to human and vis a vis (shanks) is as well a reality. Hence working on human surrogate cell and tissue based microphysiological models make the assay system more relevant and need no extrapolation to human species. Access to human induced pluripotent stem cell derived cardiomyocytes, hepatocytes and neuronal lineages provides powerful context

supporting not only toxicity testing assay systems but also important tools in drug discovery process [20-22]). Thus, integration of advanced novel assay methodologies yielding insights into the cellular, molecular and proteomics in the microphysiology is a promising approach to be integrated in the workflow.

In this study, we use human induced Pluripotent Stem Cell (hiPSC) system developed from abundantly available biological discard as an in vitro microphysiological model to record phenomics, perturbations when treated with selected neurotoxins. Previously, we have shown that hiPSC in vitro system generated from human cord tissue, expressed neuronal progenitor cell gene markers and were able to differentiate towards neuronal lineage with minimal in vitro induction [23]. Here, we used the same characterized system in the experiments to generate human toxicogenomic signature patterns upon treatment with selected neurotoxic agents. This study relies upon the theory that cellular phenotypes are the downstream effects of interactions between genes, environment, disease, molecular mechanism and chance. The primary question that has been answered through this report is to check if the treatment of drugs/toxins/agents induce morphological perturbations in the hiPSC based microphysiological system barcoding the targeted changes in gene expressions. The analysis and correlation of multilayered data between gene expression and large collection of heterogenous phenotypic possibilities opens up a totally new subject on phenomics for pharma and biopharmaceutical safety and efficacy assessments.

Methods

Preparing the Microphysiological System

Primary and progenitor cells were harvested from discarded human cord tissue in a cell reactor. An in vitro system was prepared by configuring passage 3 cultures as per the protocol described previously [23].

Neurotoxins Selection Method

Nine compounds (9) with known toxic mechanisms were shortlisted to treat the configured cells and record the perturbations caused. For each toxin selected, two (2) sub-cytotoxic concentrations were applied and with each concentration, the cells were treated for two different time intervals resulting in total of four (4) treatments per toxin. The rationale for the chosen concentration ranges was (1) to test minimal required concentration that induces cytotoxicity/toxicity and (2) to include the known in vivo/in vitro median inhibitory concentration value-IC50 values into the tested concentration range. Details of toxins and concentrations have been listed in Table 1.

Table 1

Sl. No.	Neurotoxin	Catalogue No.	Company	Concentrations used	Ref
1	TEA	T2265-100G	Sigma	5mM and 50mM	[24]
2	TT	T3194	Sigma	40nM and 80nM	[59]
3	CT	C5238	Sigma	600nM and 1.2uM	[25]
4	JWH-018	S-035	Suplco	1um and 10um	[27]
5	NH4OH	1336-21-6		0.1mM and 5mM	[28]
6	Methanol		Qualigen	0.1% and 2.5%	
7	MSG	23229	SRL	10mM and 100mM	[29]
8	DOPA	45462	SRl	100uM and 250uM	[30]
9	BT	203980	Millipore	200pM and 1nM	[31]

Tetraethylammonium (TEA): *Is a non-selective K+ channel inhibitor and has been widely employed as a molecular probe to investigate the structure of enzymes and the characteristics of the regulatory pathways. TEA induces apoptosis and the mechanism is by inhibition of Bcl-2 and Bax expression, increased intracellular ROS production, up-regulation of p53 and p21 protein expression, and arrest of cells in the G1 phase [24].*

Tetanus toxin (TT): *A potent neurotoxin generated by Clostridium tetani as a single polypeptide chain. It is a near homolog of the vast family of botulinum neurotoxins (150 kDa). It mainly blocks the neurotransmitter release thereby causing neuroparalytic diseases.*

Cholera toxin (CT): *A 36-amino acid basic peptide from the venom of the scorpion Leiurus quinquestriatus [25]. It blocks the chloride channels which helps in the regulation of basic physiological and cellular functions such as maintaining pH, proliferation, differentiation etc [26].*

JWH-018: *Is an agonist, synthetic cannabinoid. Cell cultures treated with this toxin significantly decreases the cell viability and increases the ROS production. Also, this compound induces caspase-9 activation, which causes apoptosis in the cells [27].*

Ammonium hydroxide (NH_4OH): *Ammonia is one of primary products of cell metabolic wastes and is involved in inhibition of cell growth and metabolism. It has been known previously that ammonium ions enter cells through diffusion and induces oxidative stress thereby inhibiting cell growth [28].*

Methanol: *Is also known as methyl alcohol and metabolises to formic acid (formate) in the cell; Formate is a toxic by-product as it inhibits mitochondrial cytochrome c oxidase, metabolic acidosis and a variety of other metabolic disturbances.*

MonoSoduim glutamate (MSG): *Is the most abundant excitatory neurotransmitter in the brain and is necessary for proper brain functioning* [29]*. It causes apoptotic neuronal cell death at high concentrations and apoptotic cell death was associated with regulation of genes such as Bcl-2, Bax, and/or caspase-3 and mitochondrial cytochrome c.*

Dopamine (DOPA): *In addition to being a neurotransmitter, DOPA also has neurotoxic effects under some pathological circumstances, such as age-related neurodegeneration. Neurotoxicity induced by DOPA is mainly because of oxidative metabolism. In vitro assays revealed that DOPA and 6-OH-DOPA, it's metabolic by-product, can cause apoptosis linked to ROS in a number of cell types* [30]*.*

Bungarotoxin (BT): *Is a family of three-finger neurotoxic proteins that are present in the venom of kraits. The beta subunit of bungarotoxin causes cell death by an intracellular signalling cascade that included an abrupt increase in free Ca2+, an accumulation of reactive oxygen species (ROS), membrane lipid peroxidation, and, eventually, apoptosis* [31]*.*

Treatment with toxins

The cells (0.25 mil cells) seeded per well in a 6-well plate and were treated with agents -like TEA, MSG, JWH-108, DOPA, BT, TT, CT, methanol and NH4OH at the mentioned concentrations (Table 1) and incubated for 3hr and 12hr at 37^0C in CO2 incubator.

Treatment with biologic agents

Cells were seeded as mentioned above and incubated overnight with 50ul of inoculum: *Staphylococcus, Salmonella, Candida, E.coli, Saccharomyces,* and *Pseudomonas*. 12hr after incubation, cells were rinsed in 1X PBS and images were captured under the microscope. The cells were infected with 0.1moi 2019-nCoV virus for 3hr in serum free media and later replaced with complete medium for 24hr for imaging.

Phase contrast microscopy

Live cells were profiled under inverted phase contrast microscope at 20X magnification.

RNA isolation followed by q-RTPCR

Total RNA from the treated cells was extracted using the RNeasy (Qiagen) method. The final RNA concentration and purity were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Thermo Fisher Scientific). First strand cDNA was generated using Prime Script 1st Strand cDNA kit (Takara) according to manufacturer's instructions. Real time PCR was performed using CFX96 PCR system with SYBR Premix Ex Taq II kit (Takara). The primers used for qRT-PCR analysis are listed in Table 2.

Table 2 – Primer table

Sl. No.	Gene	5` Forward Primer	5` Reverse Primer	Size of the amplicon
1	GAPDH	ATCACCATCTTCCAGGAGCGA	TTCTCCATGGTGGTGAAGACG	101
2	Actin	GCGGCATCCACGAAACTAC	TGATCTCCTTCTGCATCCTGTC	138
3	SOD2	CAAAGGGGAGTTGCTGGAAG	CCTGATTTGGACAAGCAGCA	164
4	BAX	TCAGGATGCGTCCACCAAGAAG	TGTGTCCACGGCGGCAATCATC	103
5	TNF	CTCTTCTGCCTGCTGCACTTTG	ATGGGCTACAGGCTTGTCACTC	135
6	APP	CCTTCTCGTTCCTGACAAGTGC	GGCAGCAACATGCCGTAGTCAT	141
7	HSPA	GACTGCCATGCTTTTGTCCA	CGCAAGCAATTAAGACCAGC	156
8	HDAC1	GGAAATCTATCGCCCTCACA	AACAGGCCATCGAATACTGG	168
9	MAPK14	GAGCGTTACCAGAACCTGTCTC	AGTAACCGCAGTTCTCTGTAGGT	141

Results

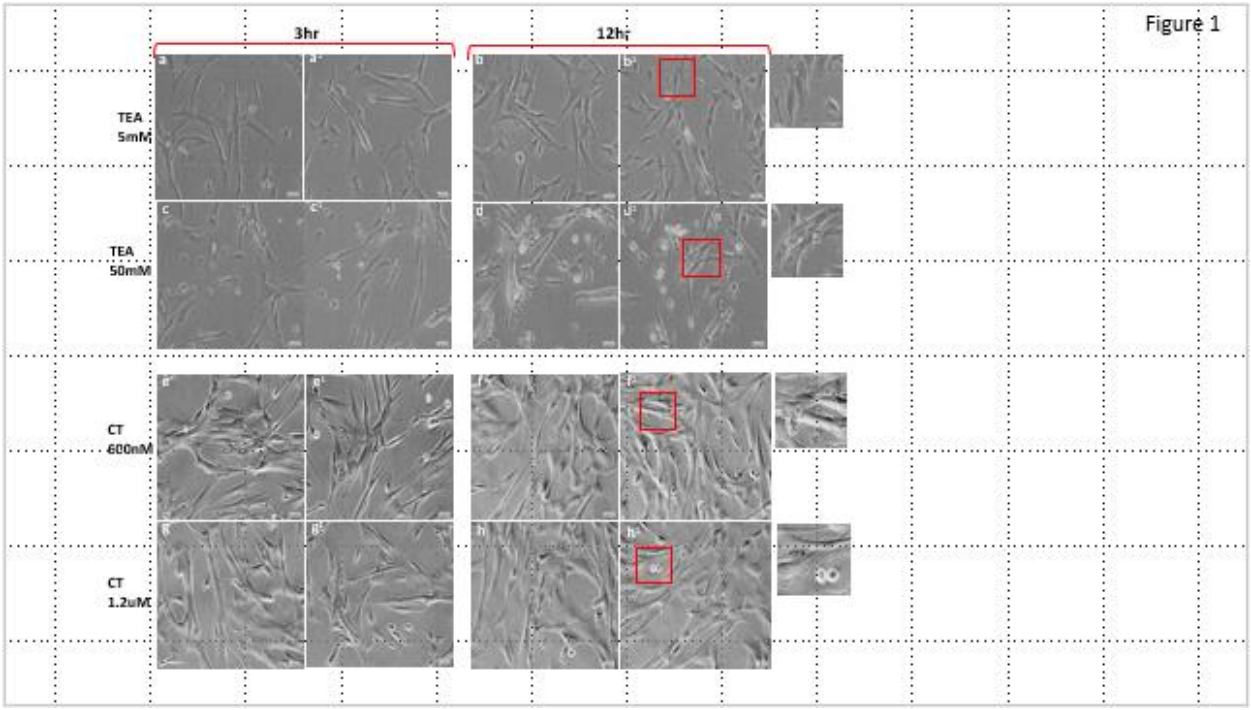
Phenotypically responsive Microphysiological System

The cell platform resident configuration is pluripotent in nature and was not subjected to any genetic manipulation. This system has been used to understand the morphological perturbations elicited upon exposure to neurotoxic agents.

As shown in the figure 1, the morphological changes were apparent with longer duration of the incubation time as well with higher concentrations of the agents. The various morphological topographies including necrotic cell death, apoptotic cell death was recorded as observed. For example, treatment with TEA, where it is known to induce apoptotic cell death (Figure 1a-d[1]), the 3hr treatment with 50mM concentration showed subtle phenotypes as reduction in cell size, lack of cellular processes and vacuole formation were documented. In case of 12 hr incubation, it showed substantial phenotype changes such as cell death. Similarly, 10mM treatment for 12hr showed visible phenotype corresponding to the apoptotic cell death while 3hr treatment showed not so evident phenotype. CT-1.2uM treatment for longer duration caused overall change in the appearance with lack of nucleus in many cells. Also, cell margins appeared to be thin as if ready for detachment compared to the controls (Figure1e-h[1]). NH4OH is a well known toxic and inhibitory molecule for mammalian cell cultures for several years [32]. It causes necrosis of tissues through disruption of cell membrane lipids, a process called saponification. In our study, the cells were treated with NH4OH in suboptimal concentrations (0.1mM and 5mM) to visualize the early morphological changes of necrotic cell death (Figure 11-l[1]) [32]. In general, majority of the treated cells were granulated revealing the cell membrane destruction that led to loss in original spindle morphology. MSG, is a known major excitatory neurotransmitter in the mammalian central nervous system activating the glutamate receptors leading to cell death via excitotoxicity [33]. As published

previously, the cells treated with MSG (10mM and 100mM) showed morphological features as elongated cells with prominent nucleus and granulated surroundings [34]. Further treatment of cells with BT and TT (known toxins for snake and *Clostridium tetani* respectively) as shown in Figure 1u-ab[1], we noticed cells exhibited typical aggregation phenotype which is a characteristic feature of necrotic cell death [35]. As both the toxins function mostly at the neuromuscular junctions and their role as neurotoxin reveals the importance of receptors in the biology of toxin induced phenotypes. DOPA is a precursor for a neurotransmitter dopamine and is known to induce toxicity in neuronal and non-neuronal cells through apoptosis. As shown in the figure 1ag-aj[1], treatment of cells with DOPA led to the reduction in cell and nucleus size, a hallmark feature of apoptotic cell death [36]. JWH-018, is an agonist to cannabinoid receptors, while on cells in this study caused qualitative reduction in cell size and cell death(Figure 1ac-af[1]).

Additionally, our cell system was found to be responsive to biological agents like bacteria, yeast and virus. As shown in figure 1ak-ak[1], corona virus, a known neurotropic agent treated cells exhibited lack of cellular processes and elongated phenotype. The other biological agents caused cell death like phenotype (Figure 1ak-aq).



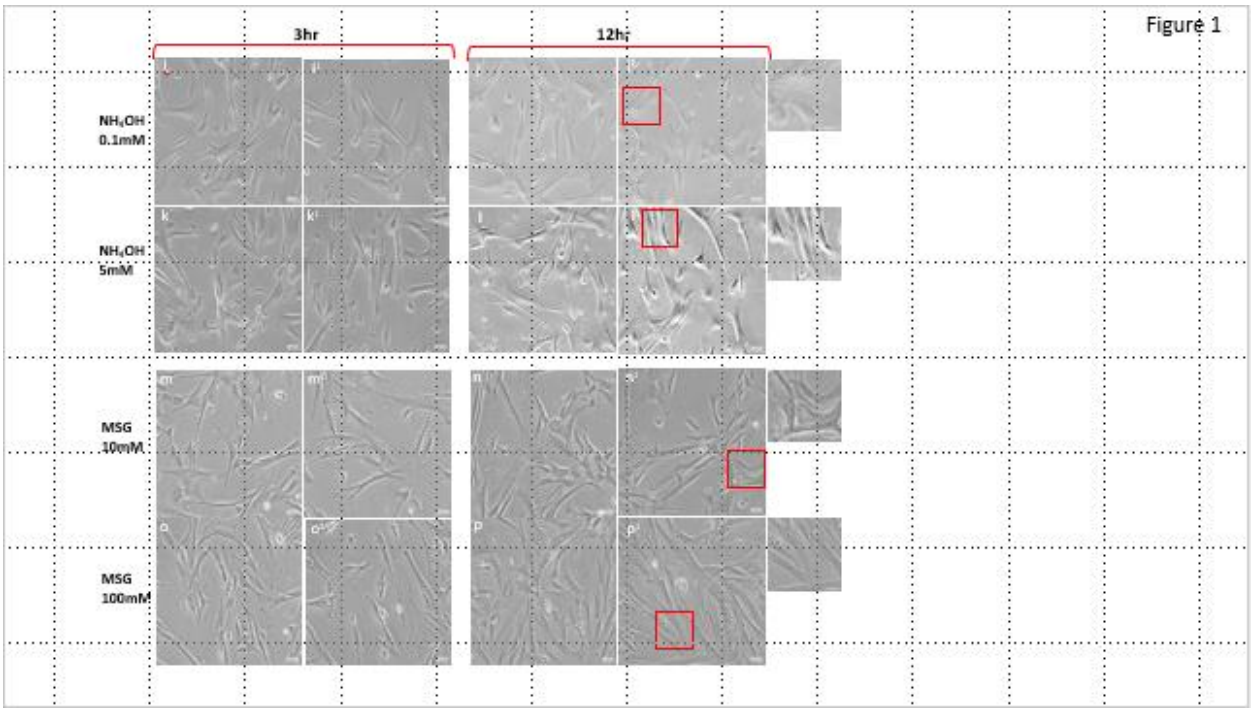


Figure 1
3hr
12hr
NH4OH
0.1mM
NH4OH
5mM
MSG
10mM
MSG
100mM

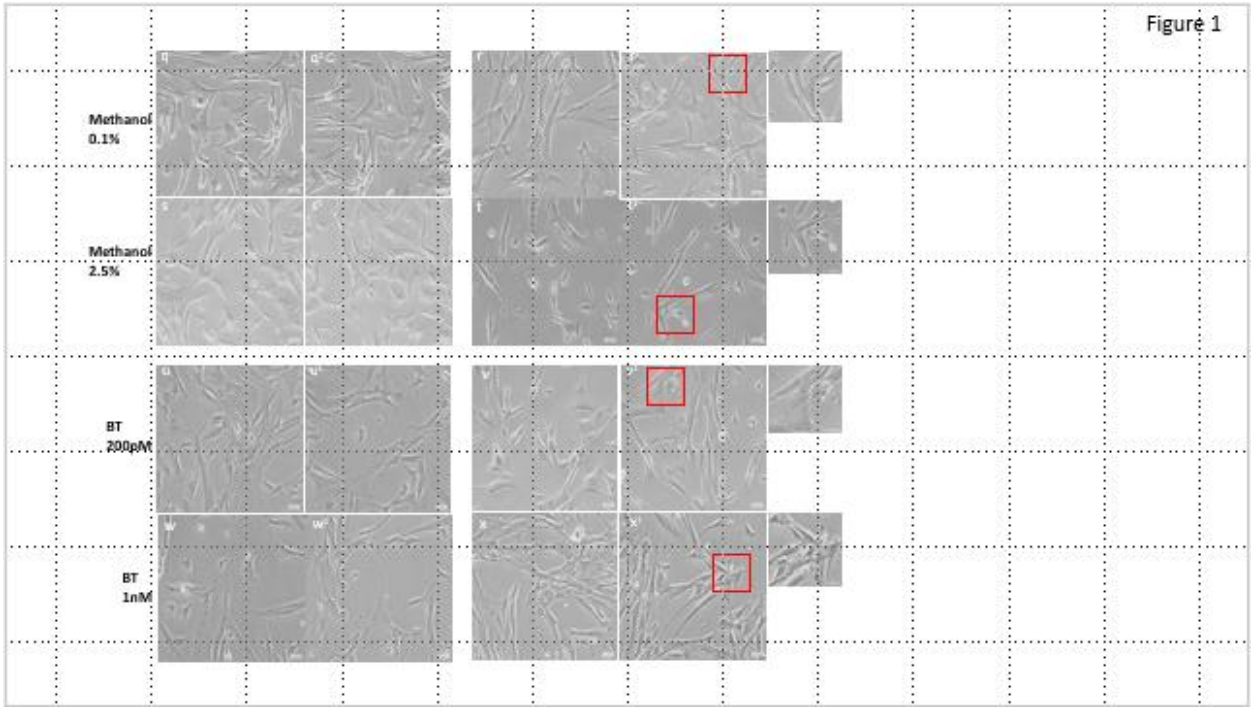


Figure 1
Methanol
0.1%
Methanol
2.5%
BT
200pM
BT
1nM

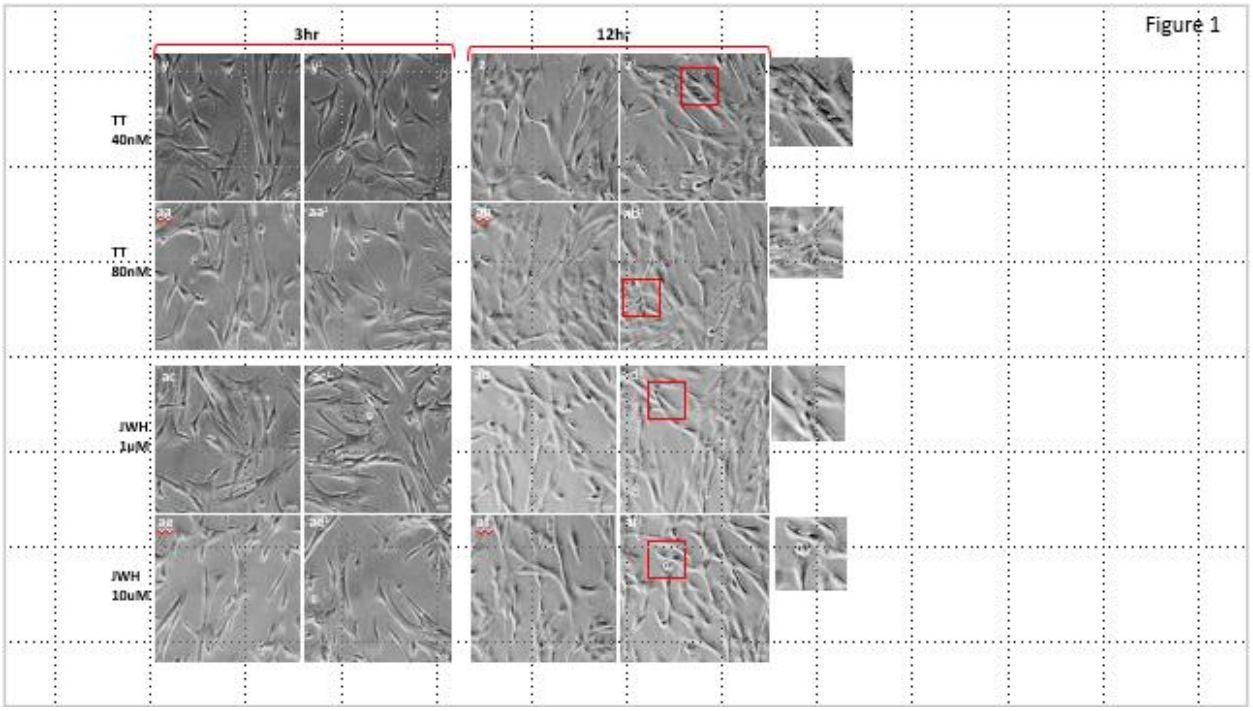


Figure 1
3hr
12hr
TT
40nM
TT
80nM
JWH
1uM
JWH
10uM

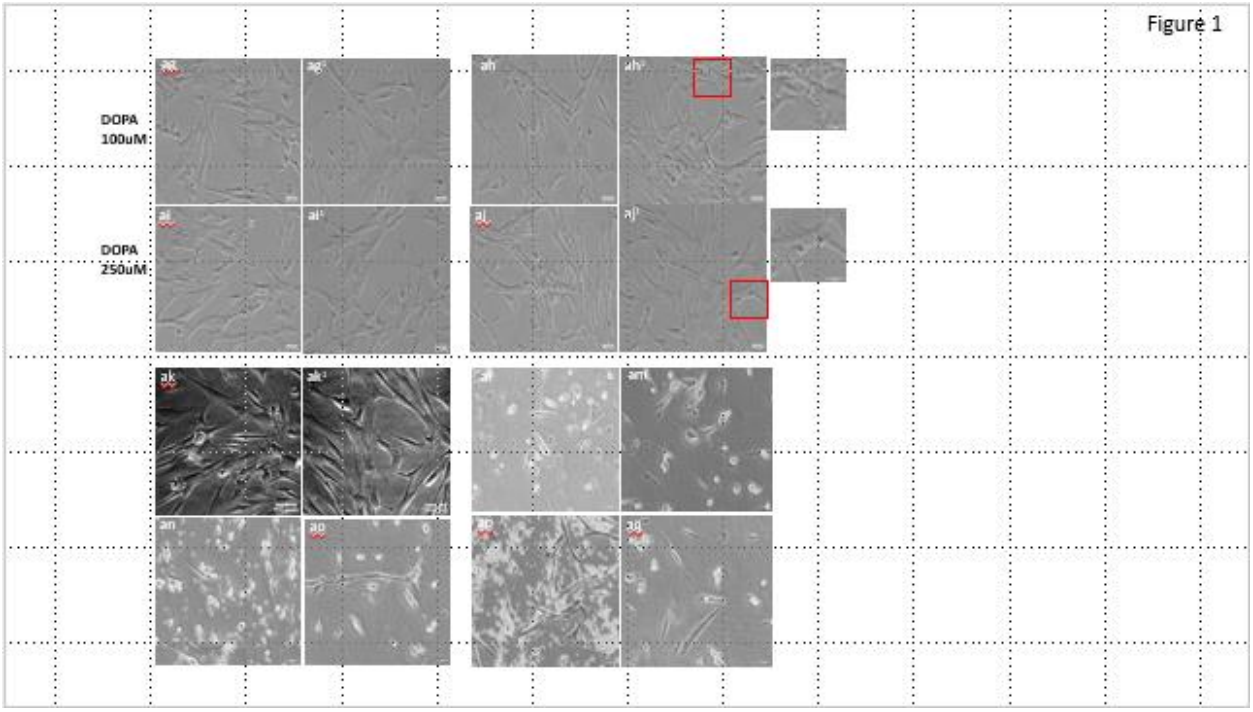


Figure 1
DOPA
100uM
DOPA
250uM

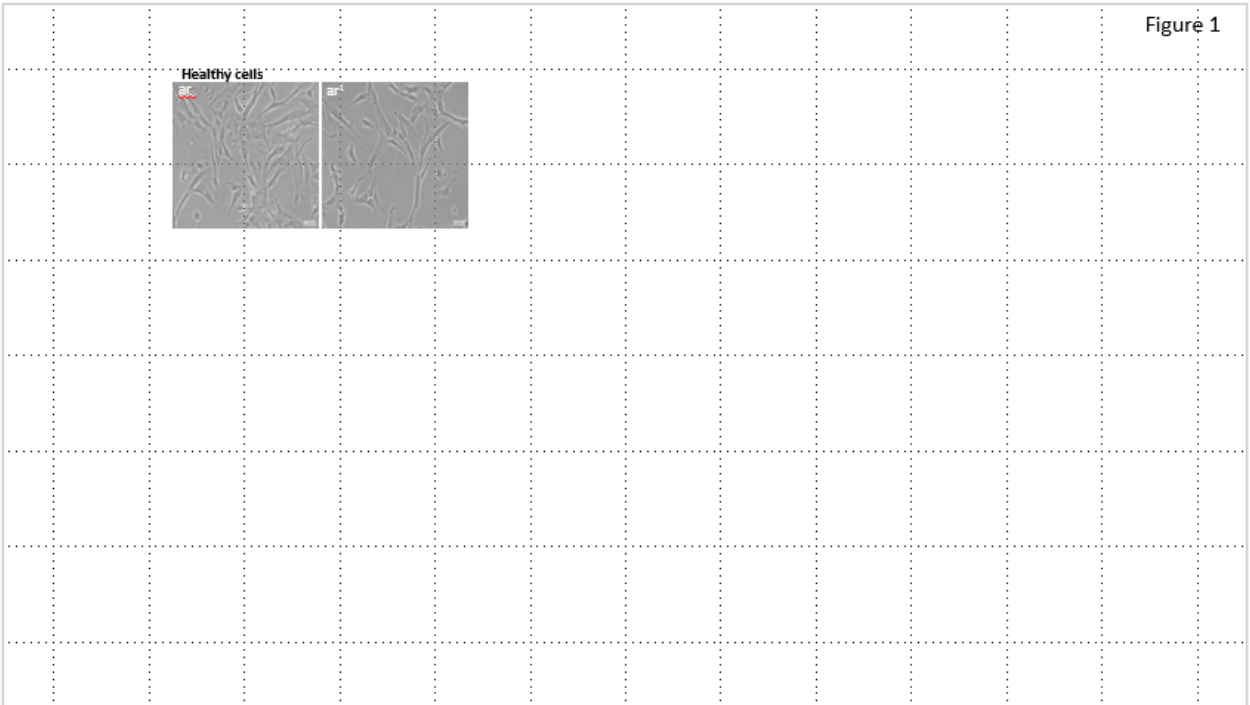


Figure 1 (figures):

Morphological features exhibited by human microphysiological system under the spell of toxic agents.

Human induced pluripotent stem cells exist in different shapes and sizes and all the cells possess visible nucleus in them. Presence of granulated cytoplasm, number of extensions, change from spindle shape are all characteristic features to be classified as cells in shock or affected or abnormal cells, represented in the insets.

These cells were cultured and treated with different concentrations of toxins at two different time periods:3hr and 12hr as stated in the figure. For each perturbation duplicate images were shown. a-d) TEA treatment induces apoptosis; there is increased cell death in case of higher concentrations and longer exposure time, e-h) cells treated with CT at 12hr incubation appeared to have caused stress as absence of nucleus is noticed, i-l) cells treated with NH_4OH lead to cell death and is evident by the presence of circular cells, m-p) cells treated with MSG that induces apoptosis and cells showed lack of nucleus and less number of vacuoles in them, q-t) cells treated with methanol caused reduction in the cellular processes, u-x) treatment with BT induces cell death by showing accumulation of cells, y-ab) cells treated with TT induced stress in the cells as they lack nucleus which is evident in 12hr treatment time, ac-af) cells treated with JWH causes distortion in the shape of cells and they lack nucleus, ag-aj) cells treated with DOPA induces accumulation of cells thereby causing the cell death, ak) cells infected with 2019-nCoV virus at 24hr post infection, al-aq) cells infected with Staphylococcus, Salmonella, Candida, E.coli, Saccharomyces, Pseudomonas respectively and imaged at 24hr, ar) control cells.

Toxicogenomics patterns observed on treated Microphysiological System

We have searched the open access bioinformatics data bases (genome data bank, ensemble) to select genes that are crucial for specific pathways by using the search terms like human neurotoxicity, cytokine storm, bacterial infection, viral infection. Three different data bases were used for generating the list of genes that are crucial for each term. For generation of proteins list, nextprot was probed along with pubmed.

We identified a total of 299 genes with documented role in neurotoxicity; 624 genes in cytokine storm; 955 genes in bacterial infection and 1041 genes in viral infection. This list was further screened to classify unique genes and common genes. As shown in the figure 2, the total number of common genes expressed in all the 4 search terms were 94, which was further subjected to gene ontology pathway analysis to generate the common pathways associated with the search queries (Figure 3).

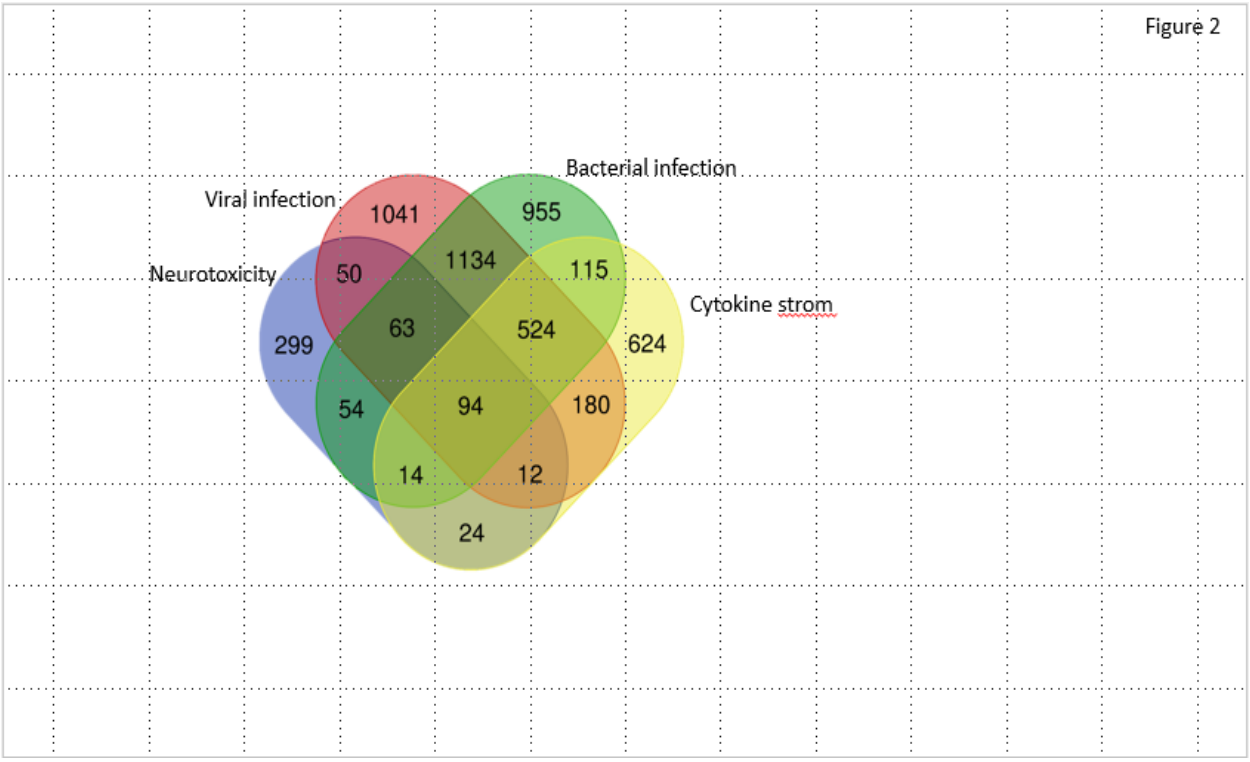


Figure 2:

Identification of common genes and stream specific unique genes.

Venn diagram shows the number in each group and the overlap of the up or down regulated-differentially expressed genes among the four categories.

Functional annotation analysis of the key 94 genes was performed using PANTHER GENE ONTOLOGY software and DAVID (Figure 3).

In panther gene ontology, GO molecular function revealed maximum percentage of genes classified as ATP dependent pathway

(GO:0140657) while in biological process category, the cellular process (GO:0009987) and biological regulation (GO:0065007) have maximum hits.

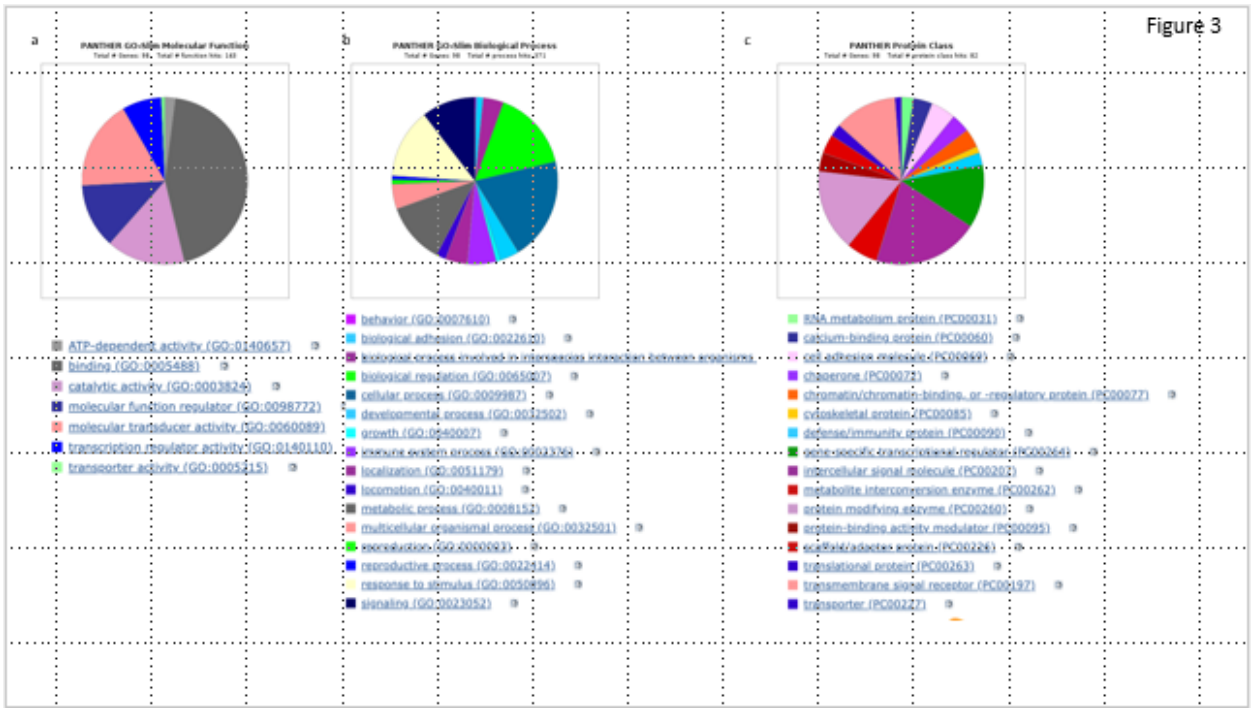


Figure 3:

Protein analysis through evolutionary relationships - Gene Ontology annotation analysis.

The distribution of GO terms categorization based on PANTHER GO Slim on 94 common genes for each annotation. a) molecular function term- basic activities of a gene product at the molecular level with 143 hits b) biological process with 371 hits and c) protein class with 82 hits.

Network analysis that provided information about molecular and cellular interactions of proteins within the network represent genes (nodes) and their functional co-relationships (edges). The 94 common genes given as input data to PPI interaction used Cytoscape 3.9.0 database and plugin Network Analyser, Y files to generate 1494 interactions with confidence score 0.4, average degree nodes of 31.7. Among the genes, TNFa turned out to be a super hub gene having highest node degree in the network with second highest BC value (Supplementary file sheet 3).

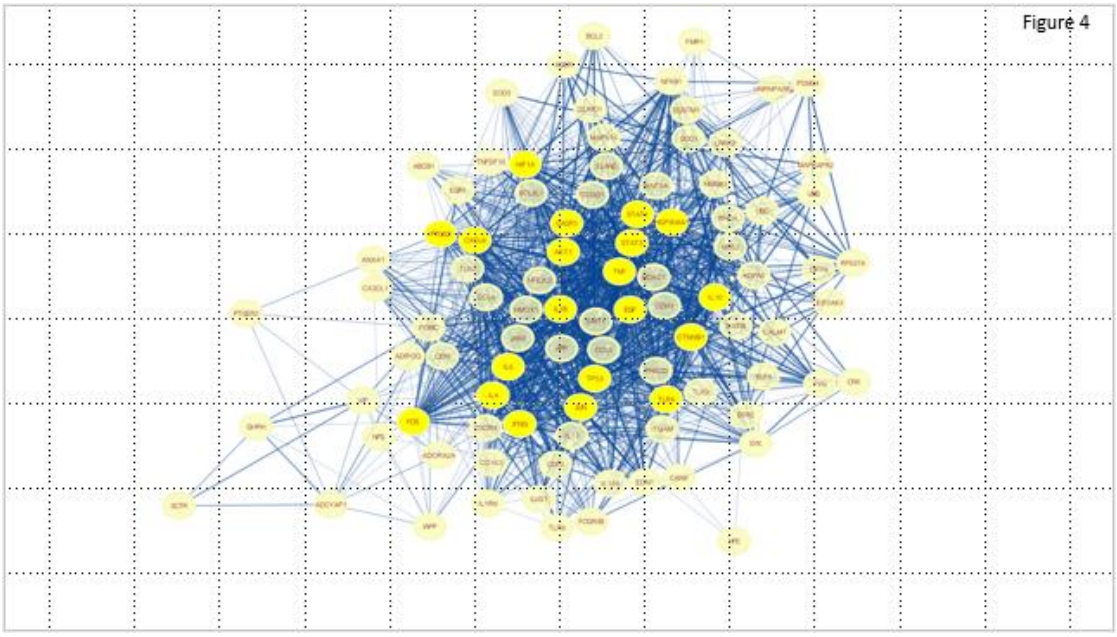
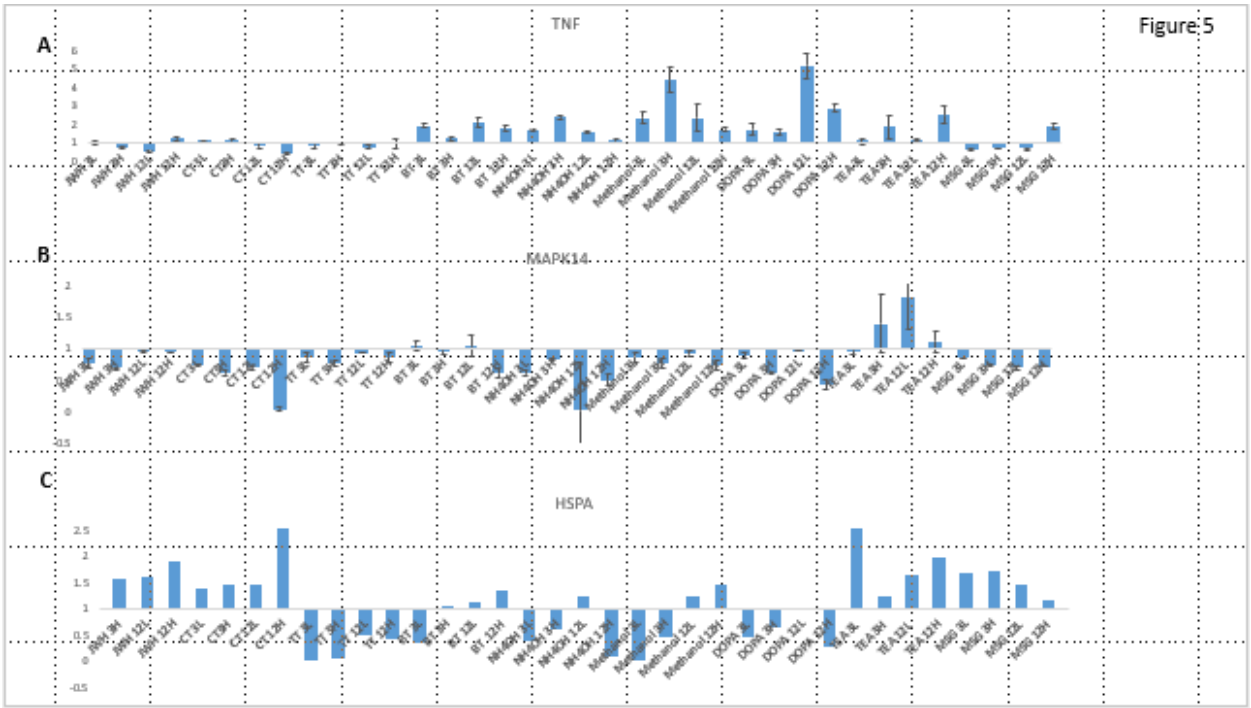
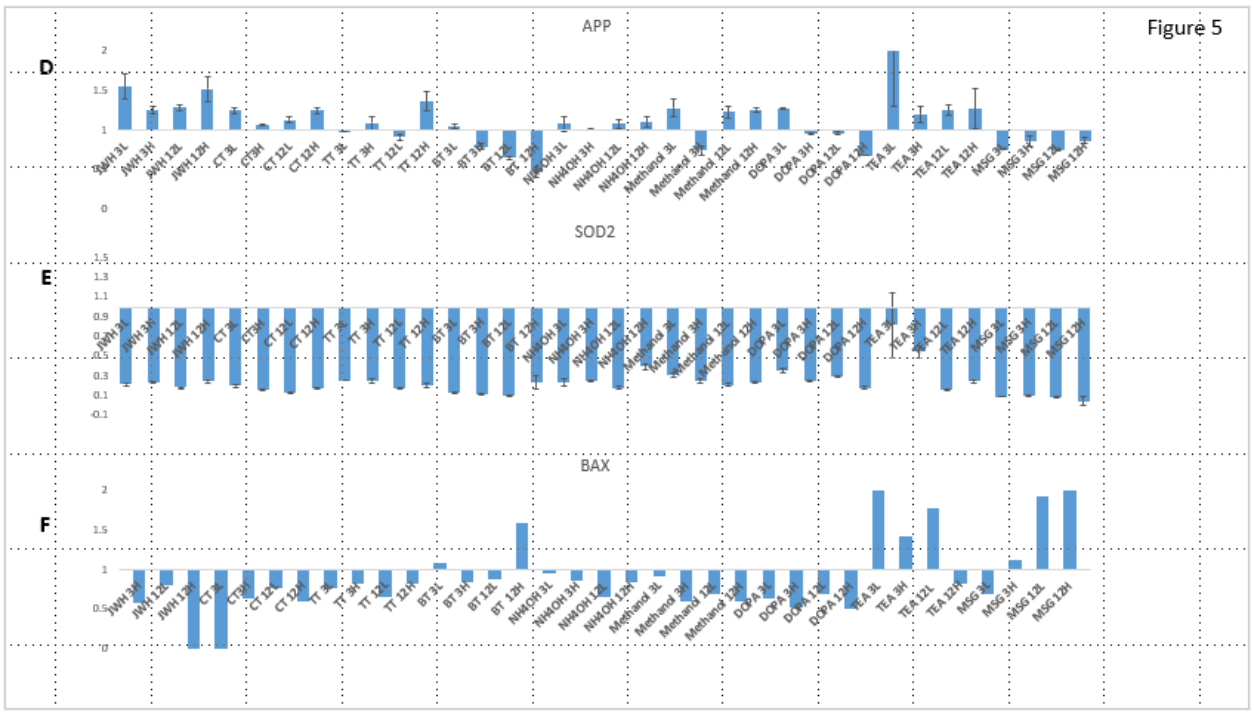


Figure 4:

Protein-Protein Interactions of common genes.

PPI network of common genes visualized by Cytoscape. Each node represents a protein and edges connecting the circles indicate protein-protein interactions, with thickness of the edges representing confidence of the bonds. The network includes 1494 edges between 94 nodes with confidence score of 0.4 as the analysis parameter. The nodes with yellow colour represent the key genes in the network with node degree >50.





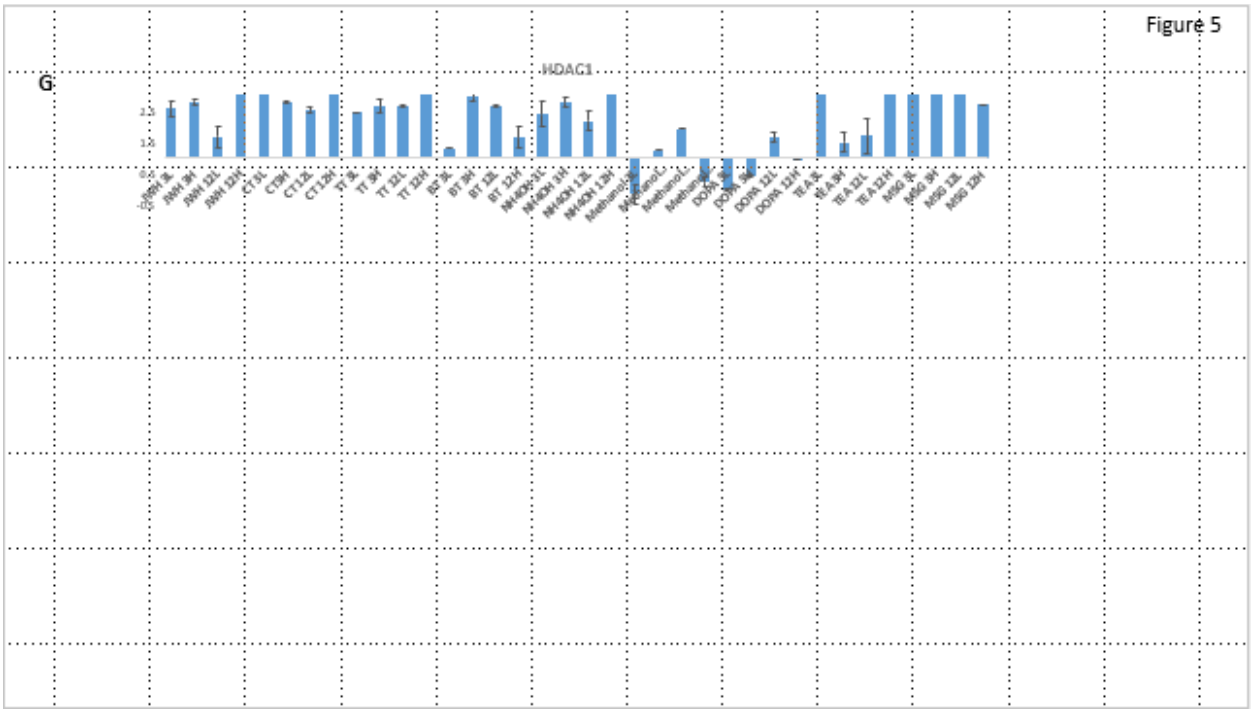


Figure 5 (figures): Gene expression analysis on treated cell system.

The cells were treated with the mentioned toxins at two different concentrations for 3hr and for 12hr. Cells were harvested after the incubation period and analyzed for relative changes in mRNA levels of the indicated genes using quantitative RT-PCR. Untreated cells served as negative control; normalized to the median of housekeeping genes GAPDH and B-actin. The level of transcript in each group was compared to that of untreated cells and is expressed as a relative fold change. For each condition n=2 was considered. Bar graphs show mean +/-SEM.

Gene expression changes in Microphysiological System upon toxin treatments

In order to correlate and barcode phenomics, perturbations recorded of the cells treated with different toxins, gene expression studies were performed. Among the list of common genes generated, 7 genes were selected based on the functionality: TNF, MAPK14, SOD2, HDAC1, HSPA, APP, BAX. All these gene expressions were tested on the toxin/agent treated MS units. As shown in the figure 5, TNFa expression was reduced in case of toxins like JWH018, TT and CT whereas in other toxins, it was found to be increased. MAPK14, SOD2, BAX expression was decreased with most of the toxins with few exceptions. HDAC expression was consistently increased with toxins used with few exceptions (Methanol and DOPA treatment). AAP expression was found to be gradually increasing with toxin treatment except BT, DOPA, MSG; HSPA expression also was shown to be increased in case of JWH018 and CT except TEA, MSG. In TT treated cells, HSPA reduced expression was documented while TEA and MSG treatment, induced HSPA expression. These data suggest that the phenotypic changes data captured in the cell model with toxin treatment can be meaningfully correlated to variable gene expression patterns.

Discussion

Advantages with Microphysiological System recruited in the study

The phenotypically responsive and genotypically reactive, characterized cellular system is a competent in vitro human surrogate model recapitulating known hallmark events (cellular and molecular) that are actually taking place in the native context and can be efficiently exploited to screen variety of pharma and biopharmaceutical candidates [37-39].

Cell configuration offers a scenario mimicking developmental phase of the nervous system which is a dynamic process requiring coordinated expression of the molecular and cellular events in a time and phase dependent manner which is largely lost in any isolated primary or transformed cell line-based models employed.

Cellular functions can be understood by measuring the cell perturbations quantified. Cell morphological phenotypes including shape, size, intensity, and sub cellular compartments have been shown to play an important role in conveying the pathological messages. Our work along with the published work [40] suggest that morphological phenotypes exhibited by the cells in 2D cultures show heterogenous signatures encrypted with phenomics. Several studies have demonstrated that changes in cell morphology can be used to understand the mechanism of action of drug molecules [41-43] and to predict the functions. Correlation studies between landmark gene expressions and context dependent cell morphology alterations after exposure to chemical or biological insults substantiate our observations on the model [44-46].

Frequently, in vitro experiments involving perturbations created by chemicals, multiple concentrations of the agent were considered to establish the dogma of increased sensitivity in 2D cultures [19,47]. In this report, the cell system treated with selected toxins (chemical and biological) at two (2) different concentrations and two (2) different time intervals responded in a pattern that could be considered as benchmark signals in building a prediction model for detecting human neurotoxicity signals. Although we observed agent dependent cellular effects and associated gene expression signatures that is hard to disentangle, these are apparent quantifiable phenomic changes.

Mechanistic importance and morphological perturbations

CT treatment on the glioma cells causes blockage of Cl channel, and it causes retraction of the membrane protrusions [48]. On similar grounds, CT treatment caused reduction in cell and nuclear size on our microphysiological system. The effects noticed with NH4OH treatment were most probably due to the increase in pH in the intracellular compartments.[32]. Treatment with BT reduced the viability and cell proliferations significantly in glioma cells and A172 cell lines through inhibition of nicotinic acetylcholine receptor, nAchR channel [49]. The biological agents as shown previously decreased macrophage migration and induced proinflammatory cytokine production [50,51] supporting some of our observations in this report as described in the results section that our cell system too has induced viral-host phenotypes like cytotoxicity and cytokine production.

Discussion on selected genes in predicting toxicity

Superoxide dismutase gene SOD/SOD2 was repressed during diverse differentiation protocols showing an expression pattern similar to Nanog gene. Also, SOD2 gene regulation was achieved by modulating the expression of Oct4 and Nanog in ESCs by shRNAs and downregulation of any of them reduced SOD2 expression [52]. In similar fashion, we documented reduced SOD2 gene expression in toxin treated cell system.

Heat shock proteins (Hsps) are expressed during development of the nervous system in a temporally and spatially controlled pattern that does not appear to be linked to activation of heat shock transcription factors. The distinct patterns of Hsp expression suggested that they perform unique roles during development and neuronal differentiation [53]. Studies indicate that these proteins can inhibit programmed cell death, regulate cytoskeletal dynamics during neurite outgrowth, axon pathfinding and under toxin treatment, as well as interact with and regulate intracellular signalling molecules that are involved in neuronal differentiation.

Overall, through their involvement in these various processes, expression of Hsps favours neuronal differentiation and survival and, as such, Hsps are emerging as important regulators of the delicate balance between cell death and survival/differentiation during

development of the nervous system [54,55]. Toxin treated human microphysiological model as well showed distinct Hsp expression pattern hinting at the right milieu, configuration of the cell system as a platform in the prediction model.

APP has been shown to regulate various aspects of neurogenesis and its metabolites might play a role in neuronal differentiation [56]. Elevated levels of TNF-α have been associated with various neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [57] with it's documented critical proinflammatory role in regulating neuroinflammation. Lastly, deletion of HDAC1 by viral infection of neural stem cells was found to be sufficient to compromise neuronal differentiation *in vitro* [58]. Toxin treated human microphysiological model did yield patterns of APP, TNF- α and HDAC1 demonstrating its responsiveness correlating with phenomics.

Continuing with the discussion on phenomics, our approach involved bioinformatics queries that included both the cause and the consequences of the pathophysiology involving viral, bacterial infection induced inflammation and release of cytokines, neurotoxicity induced various cell death pathways. The network-based analysis helped in prioritising the key gene network from the available sources in PPI network identifying proteins that could be biomarkers in toxicogenomic studies. We believe this is the first study to probe the PPI network with query based gene list and seek the contributions of functional proteins. (supplementary file sheet 3). This set of analyses resulted in 94 genes that are known to play significant role in regulating stress induced by viral infection, bacterial infection, cytokine storm and neurotoxicity while the experimental evidence also confirmed the differential regulation of these gene candidates on the microphysiological system based human surrogate model. By broadening the list of genes and the list of toxins to treat the model, volumes of data (phenotype, corresponding functional genotype, proteomics) can be generated complemented with neural networks to strengthen the prediction model in detecting human neurotoxicity signals as a New Approach Methodology.

Conclusions

To the best of our knowledge this is the first time any demonstration of phenomic effects of toxins on Human Microphysiological System is reported. We have shown that the platform is phenotypically responsive and genotypically reactive serving any New Approach Methodology based assay systems to be developed to test predict test agent's safety and efficacy like concerns.

TRANS-MSC is the trade name given to the human microphysiological model used in this article, by Transcell Oncologics that has a Science Exchange registered vertical www.transtoxbio.com with all new league of human surrogate in vitro micro physiological platforms used as tools in preclinical research and testing.

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Author Contributions

SD and VD conceived the study. VD executed the in vitro experimentation, design, analysis and drafting of the manuscript. PB played important role in generation of human microphysiological model, in vitro work; SKD provided support to VD and LG backed the technical operations in conducting lab work. VD and SD analysed the data, contributing to manuscript submission.

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Conflict of Interest

The authors have no relevant financial or non-financial interests to disclose.

Ethical Approval

The study was performed in line with Ethics committee approval handling biological discards for in vitro experimentation.

Consent to Participate

Informed consent was obtained from the donors to collect their biological discards utilized in the study.

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