Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.6, No.2, 45-56, 2019

The Japanese Society of Toxicology

Fundamental Toxicological Sciences

URL : http://www.fundtoxicolsci.org/index_e.html

Original Article

Cytotoxic effects of parathion, paraoxon, and their methylated derivatives on a mouse neuroblastoma cell line NB41A3

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(Received December 13, 2018; Accepted December 24, 2018)

ABSTRACT — Organophosphorus compounds (OPCs) are widely used as pesticides, but often show high toxicity in mammalian cells. To assess the toxic potential of OPCs, we examined the cytotoxicity of paraoxon, methyl-paraoxon, parathion, and methyl-parathion exposures on NB41A3 neuroblastoma mouse cell lines. The LC50s (median lethal concentrations) at 24 hr of exposure were determined from the acute toxicity test including time course experiments. The LC50 values suggest higher toxicity of paraoxon (0.42 mM) compared to parathion (0.66 mM). In addition, the methylated derivatives of both OPCs indicated similar but slightly lower levels of toxicity compared to paraoxon and parathion (0.46 mM for methyl-paraoxon and 0.77 mM for methyl-parathion). However, the results from time course experiments indicated obvious reduction of cell viability for parathion and methyl-parathion as early as 1 hr of exposure, whereas the effects of paraoxon and methyl-paraoxon were not significant before 6 hr of exposure. We also report the most affected biological processes in NB41A3 cells in response to parathion exposure using microarray experiment. Among the statistically overrepresented biological processes are the ones related to neuronal development, apoptosis, cell stress, and cell signaling.

Key words: Paraoxon, Parathion, Neuroblastoma, Cell viability, LC50, Microarray

INTRODUCTION

Organophosphates or organophosphorus compounds (OPCs) are known as the largest group and the most widely used pesticides (Lee *et al*., 2016). OPC was first synthesized in the early 1800's (Singh and Khurana, 2009). Over the years these compounds have been used for protecting crops, livestock, and human health. Due to the high toxicity and their status as chemical warfare agents, more studies are being conducted on various OPCs recently.

OPC poisoning is one of the most common mode of poisoning worldwide, and about 200,000 people die from self-poisoning of OPC-pesticides in the developing countries (Eddleston *et al*., 2008; Lee *et al*., 2016). Furthermore, such pesticides poisoning cases are most likely underreported (Calvert *et al*., 2008; Rohalman *et al*., 2011). In the United States (US) alone, the recent estimates indicate that approximately 33 million pounds of OPC pesticides are applied annually (US EPA, 2011). In the US, there are multiple regulations and safety training requirements under the purview of EPA for the handling and transport of OPCs. However, poisonings with OPC pesticides particularly in the agricultural industry still occur frequently (Rohlman *et al*., 2011).

OPCs can be absorbed by animals and humans through all routes, including inhalation, ingestion, and dermal

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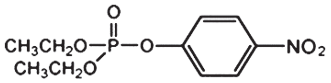
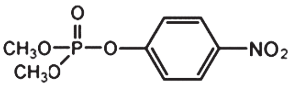
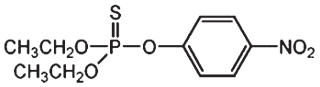
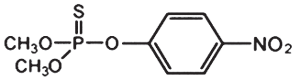
absorption, and cause a number of toxic effects in those organisms (Wright *et al*., 2006; Fawcett *et al*., 2009; Angelini *et al*., 2015). The major effect of OPCs at the molecular levels is the inhibition of acetylcholinesterase (AChE) enzyme, leading to a pathological excess of acetylcholine and to the symptoms of cholinergic excess (Mileson *et al*., 1998; Čolović *et al*., 2013). Neurotransmitters such as acetylcholine are profoundly important in the brain's development, and many OPCs have neurotoxic effects on organisms even under low levels of exposure (Fukuto, 1990; Čolović *et al*., 2013). Muscarinic reactions include bradycardia, salivation and abdominal pain. Stimulation of nicotinic receptors results in hypertension and tachycardia. Furthermore, due to significant neurotoxic effects, various OPCs have been developed as chemical warfare agents (Black and Read, 2013; Delfino *et al*., 2009).

Parathion (*O*,*O*-diethyl *O*-(4-nitrophenyl) phosphorothioate) is a broad spectrum organophosphate pesticide that is classified as a Restricted Use Pesticide (RUP) due to its high toxicity (Meister, 1992; Worthing, 1987). It can be absorbed by all routes of exposure, and human fatalities have been caused by ingestion, dermal adsorption, and inhalation (Hayes and Laws, 1990). As the active metabolite of parathion, paraoxon (*O*,*O*-diethyl *O*-(4-nitrophenyl) phosphate) is also rarely used as an insecticide due to the risk of poisoning to humans and other animals (Neal, 1967).

The objective of this study is to examine and compare the cytotoxicities of parathion and paraoxon as well as their methylated derivatives, methyl-parathion and methyl-paraoxon in mouse NB41A3 neuroblastoma cell line (Table 1). To determine the cytotoxic effects, cell viability was monitored over the time course at different concentrations. In addition, through a microarray experiment we identified the parathion-responsive gene sets in NB41A3 and the most affected biological processes.

Table 1. Organophosphate compounds (OPCs) used in this study.

OPC	Structure
Paraoxon $C_{10}H_{14}NO_6P$	
Methyl-paraoxon $C_8H_{10}NO_6P$	
Parathion $C_{10}H_{14}NO_5PS$	
Methyl-parathion $C_8H_{10}NO_5PS$	

MATERIALS AND METHODS

Cell culture

Mouse NB41A3 cell line (ATCC® CCL-147™, Manassas, VA, USA) was cultured according to the instructions of ATCC in a Nunclon™ Delta Surface 75 cm^2 flask (Cat. No. 16337125). The culture media was F-12K Medium supplemented with 2.5% fetal bovine serum (ATCC® 30-2021), 15% horse serum (ATCC® 30-2041). 100 µL of Penicillium G and streptomycin were added to 15 mL of the fresh media in a 75 cm^2 cell culture flask. Then, 200 µL of the mouse cell line NB41A3 was added to the flask and incubated in the incubator at 37°C with 5% CO_2. After incubating for 48 hr, the cells were visually examined under a microscope. To release the cells from the bottom of the cell culture flask, 3 mL of 0.25% (w/v) Trypsin, 0.53 mM EDTA (ATCC® 30-2001) solution was added and incubated for 5 min at 37°C with 5% CO_2. After incubation, 6 mL of fresh media was added to rinse the back of the flask. Then the whole media containing the cells and trypsin was aspirated from the flask and centrifuged for 10 min at 1000 rpm. Cells collected at the bottom were resuspended homogeneously with the fresh media for further tests.

Cell viability test

Organophosphorous compounds (OPC) used in this study, paraoxon, methyl-paraoxon, parathion, and methyl-parathion, were purchased from Supelco (Bellefonte, PA, USA). The stock solutions of all OPC were prepared in dimethyl sulfoxide (DMSO) and diluted with culture medium to 100 mL volume total, and stored in 4°C (Hong *et al*., 2003). 500 µL of cell suspension was added into each well in a 24-well-plate and incubated under the same condition mentioned above. After 24 hr of incubation, 50 µL of medium containing different concentrations of an OPC were added along with control medium (10% DMSO in F-12K medium) in three control wells. The well-plate was further incubated for 24 hr under the same condition. After 24 hr of exposure, each well was washed three times with fresh medium, then replaced with 500 µL fresh Media. Cells were incubated with additional 50 µL of Cell Counting Kit Solution (CCK Kit-8, Molecular Dojindo Technologies, Kumamoto, Japan) for 3 hr. Then, the absorbance of the cells in each well was measured at 450 nm using Bio-Tek Elx800 plate reader (Winooski, VT, USA), and the absorbances were confirmed by UV-Vis Spectrosco-

py (Cary 100 BIO, Agilent Technology, Santa Clara, CA, USA). Cell viabilities (%) were calculated as

$$\text{Cell Viability\%} = \frac{\text{Asample-Ab}}{\text{Ac-Ab}} \times 100$$

(Patel *et al*., 2009), where A_{sample}=Abs. of test sample, A_b=Abs. of blank, and A_c =Abs. of control sample.

LC50 determination

LC50 (median lethal concentration) values refer to the concentration of a chemical killing 50% of the test organism in a given time (Wang *et al*., 2014). Pilot experiments were carried out to find the minimum concentration for 0% cell viability. Then, a dilution series of that concentration was used to obtain cell viability data at different concentrations (Wang *et al*., 2013, 2014). Those cell viability data were plotted against log transformed OPC concentrations. The regression lines were determined in MS-Excel using the equation for the line the concentration for 50% cell mortality (LC50) was determined.

Time courses detection

According to 24 hr LC50 of each OPC, different concentrations of each OPC were set to test the cell viability of the cell line at different time points including 1 hr, 3 hr, 6 hr, 9 hr, 15 hr, and 24 hr.

Statistics

All data tested were found to conform to assumptions of equal variance and normality, and expressed as mean ± SEM.

Total RNA extraction for microarray experiment

The mouse NB41A3 cell culture for total RNA extraction was carried out in the same way as the cell viability test in 24-well plates. However, to ensure enough RNA yield for microarray experiments, we used multiple wells for each sample and condition (8 to 16 wells per sample). Furthermore, to obtain accurate data on parathion-induced change in gene expression, untreated samples were grown parallel to the parathion-treated samples so that the experimental samples can be compared with the control samples at the same time points. Also, to ensure the same level of confluency of the cells between parathion-treated samples and non-treated samples, we had multiple control samples that were initiated with different numbers of cells and chose the samples with the same level of confluency with the parathion-treated wells.

One day after the cells were dispensed in 24-well plates, control solution or parathion stock solutions were added to its LC50 level as shown above. After 2 hr and 24 hr, cells were lysed with RLT plus buffer (RNeasy Plus, QIAGEN, Hilden, Germany) directly in the wells and processed following manufacturer's instruction. The quantity and quality of the total RNAs were tested by using NanoDrop (Thermo Scientific, Waltham, MA, USA) and gel electrophoresis and by using Agilent RNA Nano Bioanalyzer (Santa Clara, CA, USA). All samples had three biological replicates.

GeneChip Mouse Gene 2.0 ST Array

GeneChip Mouse Gene 2.0 ST Array (Affymetrix, Santa Clara, CA) produces 41,345 probes data that include ~28,000 coding transcripts and ~7,000 non-coding transcripts, among which ~2,000 are long intergenic non-coding transcripts. Only 27,673 probes (out of 41,345) have accession numbers and others do not. Information for probes is available in the supporting files provided by Affymetrix ("Current NetAffx Annotation Files: MoGene-2_0-st-v1 Transcript Cluster Annotations, CSV, Release 36," Jan. 2017; https://www.thermofisher.com/order/catalog/product/902119).

Microarray data analysis

Probe labeling, hybridization, and scanning were carried out at the Integrated Genomics Core at Augusta University (Augusta, GA, USA) using Affymetrix GeneChip™ Mouse Gene 2.0 ST Array. The CEL files were further analyzed at Albany State University using an in-house generated R script. Briefly, signal intensity data in the CEL files were imported and RMA normalized through Bioconductor *Oligo* package (Carvalho and Irizarry, 2010). The signal ratios (treated/untreated) were computed for each probe, and then the averages and the standard deviations were computed for each probe. The Bioconductor package *RankProd* (Hong *et al*., 2006) was used to compute the p-value and the pfp (percentage of false prediction) for each gene. OPC-responsive genes were selected when pfp value was equal to or lower than 0.05. Clustering and visualization were carried out by using *Cluster 3.0* and *TreeView* (Eisen *et al*., 1998). Options selected are: hierarchical clustering > genes/arrays cluster (uncentered correlation) > centroid linkage clustering.

RESULTS

Determination of LC50

To delineate the acute toxicity of OPCs in NB41A3 cells, LC50 values of four compounds were determined. After 24 hr of exposures, paraoxon, methyl-paraoxon, parathion and methyl-parathion induced a dose-depend-

ent decrease in cell viabilities in the NB41A3 cell line (Table 2). According to the nominal exposure concentration and log-transformed calculation, 24 hr LC50 of paraoxon, methyl-paraoxon, parathion, and methyl-parathion were 0.42 mM, 0.46 mM, 0.66 mM, and 0.77 mM, respectively (Table 3; Fig. 1). The LC50 data exhibited a trend of higher LC50 values of parathion compared to paraoxon (0.66 mM vs. 0.42 mM; Table 3; Fig. 1). On the other hand, methylated derivatives of each organophosphate compound (methyl-paraoxon and methyl-parathion) exhibited slightly higher LC50 values compared to ethylated compounds (paraoxon and parathion; Table 3; Fig. 1).

Dynamics of cytotoxicity

To further dissect the cytotoxicity of OPCs over time, the cell viabilities along the time course were determined under different concentrations of OPCs (Fig. 2). Paraoxon did not affect the viability of NB41A3 cells significantly until 3 hr of exposure at concentrations up to 2 mM (Fig. 2AB). Reduction of cell viability was observed starting from 6 hr at 0.25 mM or higher concentrations. Lower dosage (0.125 mM) did not show any effect up to 24 hr. Similar trends were observed in the methyl-paraoxon exposure, but the significant effect was delayed to 9 hours of exposure at 0.5 mM or higher concentrations (Fig. 2CD). Lower dosage (0. 25 mM) did not show any effect on cell viability up to 24 hr. On the other hand, parathion exposure affected cell viability already at 1 hr at concentrations of 0.5 mM or higher (Fig. 2EF). In this range, a linear decrease in cell viability was observed when exposure concentrations were

Table 2. Cell viability (%) after 24 hr of exposure. Values shown are the means ± standard error (n = 3).

Conc. (mM)	Conc. (Log mM)	Paraoxon	Methyl-paraoxon	Parathion	Methyl-parathion
0.125	-0.903	94.67 ± 6.22	84.64 ± 7.89	93.77 ± 2.22	99.25 ± 4.94
0.25	-0.602	66.36 ± 10.88	81.67 ± 11.34	90.85 ± 8.09	92.04 ± 5.06
0.5	-0.301	37.56 ± 12.12	41.35 ± 16.49	58.77 ± 8.31	77.44 ± 1.28
1	0.000	13.07 ± 4.31	19.77 ± 8.91	30.18 ± 7.08	35.03 ± 7.32
2	0.301	7.40 ± 5.51	8.05 ± 5.87	19.61 ± 12.06	16.18 ± 4.49

Table 3. The equations for the trend lines, the correlation coefficients, and LC50 from Fig. 1B.

	Paraoxon	Methyl-paraoxon	Parathion	Methyl-parathion
$y =$	$-75.7x + 21$	$-71.5x + 25.6$	$-69.4x + 37.7$	$-74.1x + 41.7$
$R^2 =$	0.96	0.94	0.95	0.93
LC50 (24 hr)	0.42 mM	0.46 mM	0.66 mM	0.77 mM

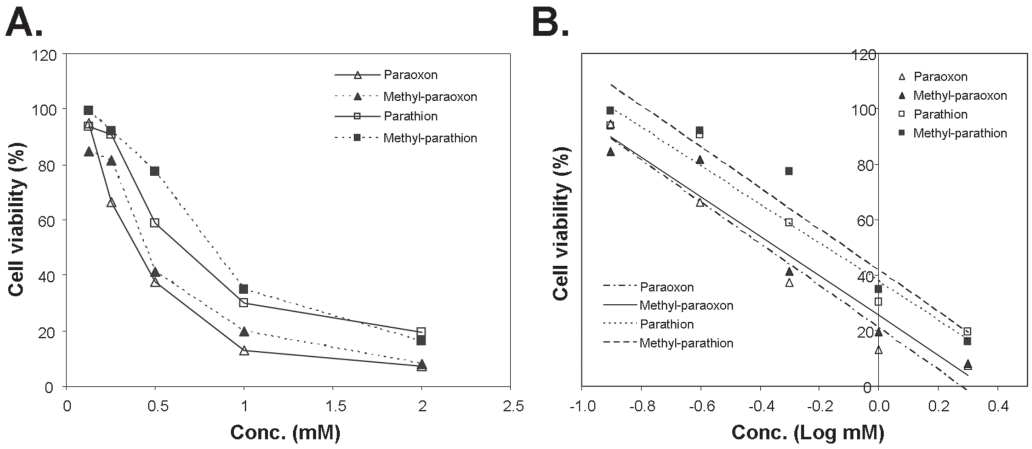


Fig. 1. (A) Cell viability after 24 hr of exposure to different concentrations of organophosphate compounds. (B) Same data as *(A)* is plotted after log transformation of concentration (x-axis). Trend lines were used to find LC50.

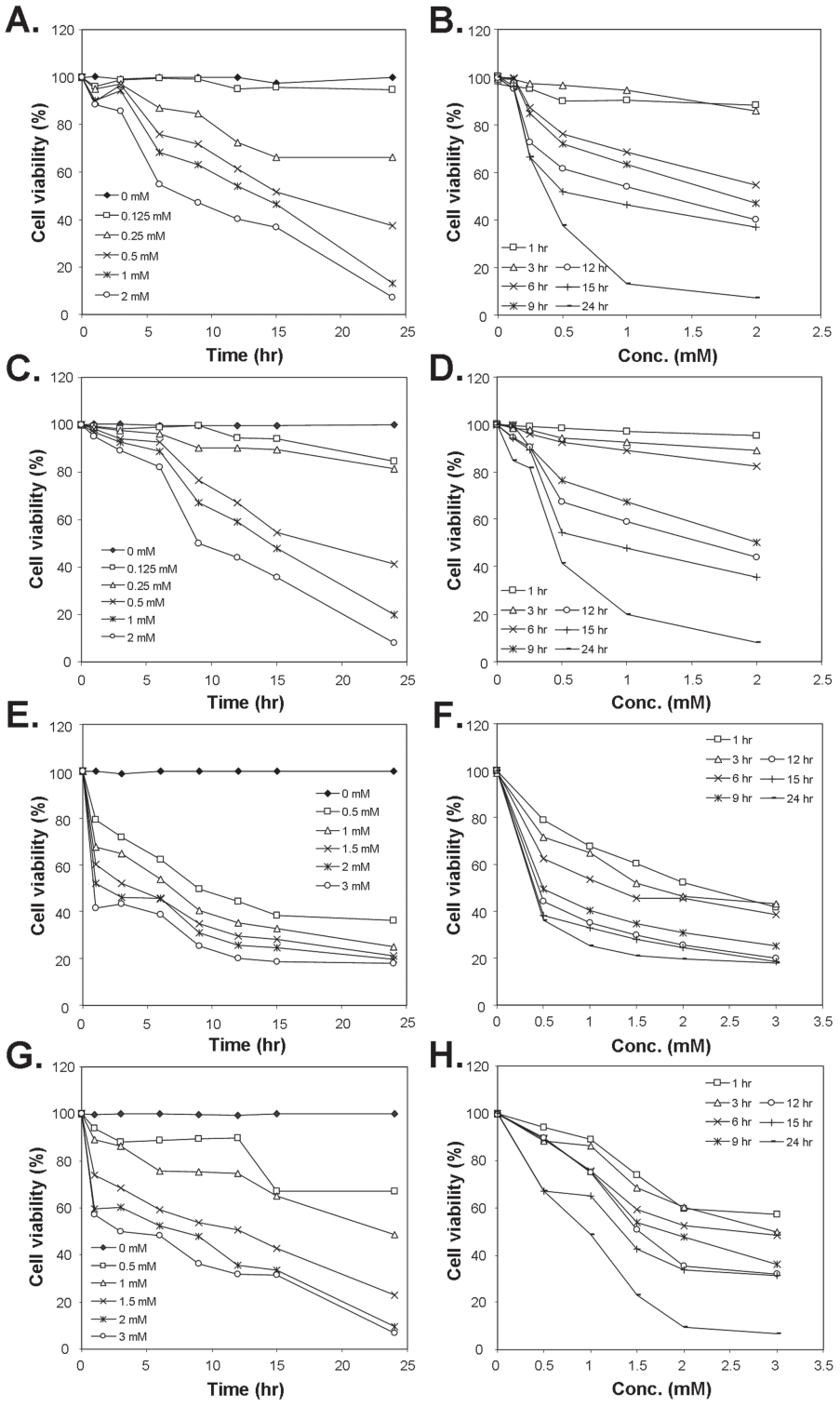


Fig. 2. Cell viability tests along the time course under different concentrations of (A&B) paraoxon (C&D) methyl-paraoxon (E&F) parathion (G&H) methyl-parathion. Cell viability data were plotted either as a function of time (ACEG) or of OPC concentrations (BDFH).

increased. Methyl-parathion also showed the similar pattern (Fig. 2GH), but exposure to 0.5 mM did not exhibit reduction of cell viability up to 12 hr. Overall, this data suggests that parathion affects the cell viability earlier (1 hr) than paraoxon (6 hr). The methylated derivatives of both paraoxon and parathion began to affect the cells at slightly higher concentrations. Both methyl-paraoxon and methyl-parathion started to affect the cell viability at 0.5 mM, but the effect of methyl-parathion was less prominent. In addition, such effects were slightly delayed compared to ethylated compounds (9 hr for methyl-paraoxon and 15 hr for methyl-parathion at 0.5 mM). Except the very fast toxic effect of parathion, these data align well with the LC50 data from 24 hr time point (Table 3; Fig. 1), indicating higher toxicity of paraoxon compared to parathion.

Parathion responsive genes

OPCs can directly or indirectly affect cell physiology that leads to changes in gene expression patterns. To identify genes that respond to parathion, we carried out microarray analysis using mouse NB41A3 cells at early (2 hr) and later (24 hr) stages after the onset of parathion exposure. The concentration used was LC50 determined in this study (Table 3). After the RMA normalization, the average log ratios ($\log_2$ [treated/untreated]) for each probe were computed for the whole 41,345 available probes in the GeneChip Mouse Gene 2.0 ST Array (Affymetrix). Those probes include not only protein coding genes but also many non-coding transcripts (Material and Methods). Identification of responsive probes through the Bioconductor package *RankProd* (pfp $\leq$ 0.05) revealed 128 probes (70 up-regulated; 58 down-regulated) as early responsive genes (2 hr exposure) and 715 probes (487 up-regulated; 228 down-regulated) as late responsive genes (24 hr exposure). Except a few, most of the responsive probes were expressed at a specific time point, i.e. either early (2 hr) or later (24 hr) when statistically significant probes were considered (Fig. 3). Only about 16% of the early responsive probes (21 out of 128) were still responsive at 24 hr time point, and the rest responded transiently. The lists for each category in Fig. 3 are available in Suppl. Data 1.

To identify the most affected biological processes in response to parathion exposure, statistical overrepresentation tests were carried out through a web-based online tool at *Panther Classification System* (Mi *et al*., 2013; http://pantherdb.org/). To this end the microarray probes with a UniGene ID were first identified. Unfortunately, only about a half of the responsive probes has a UniGene ID (Table 4) among the large number of responded probes

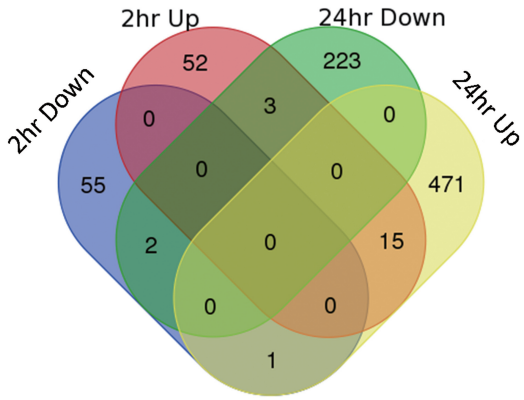


Fig. 3. Venn diagram showing overlaps among the responded probes.Venn diagram was constructed using an online tool at Bioinformatics & Evolutionary Genomics site (http://bioinformatics.psb.ugent.be/webtools/Venn/). Numbers indicate the number of responded probes with statistical significance.

Table 4. Number of probes in each responsive group.

Time Point	Expression Change	With UniGene ID	Without UniGene ID	Total Number
2 hr	Up	45	25	70
	Down	0	58	58
24 hr	Up	270	217	487
	Down	111	117	228
	Sum	426	417	843

(Fig. 3), which reduced the power of the analysis significantly, especially for the data from the 2 hr time point.

A few representative results are shown in Table 5 (the full list is available in Suppl. Data 1). Briefly, the genes up-regulated early (2 hr) are related to signal transduction, gene transcription, cell cycle, and apoptosis. Considering the cell line used in this study (NB41A3, mouse neuroblast cells), it is particularly interesting to find GO terms that are related to developmental processes including *nervous system development* (GO:0007399). On the other hand, *transcriptional regulation*, *endoplasmic reticulum stress/unfolded protein response* (24 hr, up) and *steroid biosynthesis process* (24 hr, down) are prominent in the later stage (Table 5).

Different expression patterns among parathion responsive genes

To dissect the above result further, the expression data for parathion responsive genes from both early and late time points were consolidated. As a result, we obtained 822 non-redundant probes that exhibited statistically

Table 5. Overrepresented gene ontology terms (Complete list available in Suppl. Data 1).

2 hr	24 hr	
Up	Up	Down
nervous system development (GO:0007399)	positive regulation of transcription from RNA polymerase II promoter in response to endoplasmic reticulum stress (GO:1990440)	cholesterol biosynthetic process (GO:0006695)
neural crest cell differentiation (GO:0014033)	negative regulation of transcription, DNA-templated (GO:0045892)	secondary alcohol biosynthetic process (GO:1902653)
glial cell differentiation (GO:0010001)	intrinsic apoptotic signaling pathway in response to endoplasmic reticulum stress (GO:0070059)	sterol biosynthetic process (GO:0016126)
umbilical cord development (GO:0061027)	endoplasmic reticulum unfolded protein response (GO:0030968)	isoprenoid biosynthetic process (GO:0008299)
regulation of auditory receptor cell differentiation (GO:0045607)	negative regulation of fat cell differentiation (GO:0045599)	alcohol biosynthetic process (GO:0046165)
metanephric nephron tubule development (GO:0072234)	regulation of intracellular signal transduction (GO:1902531)	
placenta blood vessel development (GO:0060674)	response to chemical (GO:0042221)	
heart valve development (GO:0003170)		
embryonic morphogenesis (GO:0048598)		
muscle organ development (GO:0007517)		
positive regulation of epithelial cell proliferation (GO:0050679)		
stem cell development (GO:0048864)		
mesenchymal cell differentiation (GO:0048762)		
positive regulation of cell proliferation (GO:0008284)		
positive regulation of apoptotic process (GO:0043065)		
negative regulation of apoptotic process (GO:0043066)		
regulation of cell cycle (GO:0051726)		
negative regulation of ERK1 and ERK2 cascade (GO:0070373)		
negative regulation of MAPK cascade (GO:0043409)		
regulation of protein serine/threonine kinase activity (GO:0071900)		
regulation of Notch signaling pathway (GO:0008593)		
regulation of transcription from RNA polymerase II promoter (GO:0006357)		
response to lipid (GO:0033993)		

significant response to parathion at any of the two time points we tested (Suppl. Data 2). The 822 parathion-responsive probes data were clustered and subdivided into 9 different groups based on their expression patterns (Fig. 4; Suppl. Data 2). The overrepresented GO terms were identified in each of the expression pattern found through clustering (Fig. 4; Groups [*a*] ~ [*i*]). Again, due to limited numbers of probes with a UniGene ID in each group (Table 6), overrepresented GO terms were found only in 5 gene expression groups among 9 different groups (Fig. 5).

The genes transiently up-regulated at 2 hr, but reduced their expression to the background levels at 24 hr, are involved in *transcription*, *cell cycle*, and *apoptosis* as well as various *developmental processes* such as *neurogenesis* (Fig. 5 Group[*b*]). On the other hand, the MAPK cascade-related signal transduction process was also affected transiently at the early stage, but their expression levels did not fully return to the background levels at 24 hr (Fig. 5 Group[*f*]). There are two groups of genes up-regulated at later stage (24 hr). Strictly late responders include *TOR signaling regulators*, suggesting that cell growth regulation process is also affected at this stage (Fig. 5 Group[*i*]), whereas genes up-regulated gradually over time include the ones related to *endoplasmic reticulum unfolded protein response* which may be due to the physiological stress caused by parathion (Fig. 5 Group[*g*]). Lastly, genes related to *lipid biosynthetic processes* such as *cholesterol* and *isoprenoid biosynthetic pathways* were down-regulated at 24 hr but not at 2 hr time points (Fig. 5 Group[*h*]).

Table 6. Number of probes in each expression pattern group.

Group	With UniGene ID	Without UniGene ID	Total Number
a	19	22	41
b	17	12	29
c	0	54	54
d	1	40	41
e	91	56	147
f	20	9	29
g	98	60	158
h	0	7	7
i	167	149	316
Sum	413	409	822

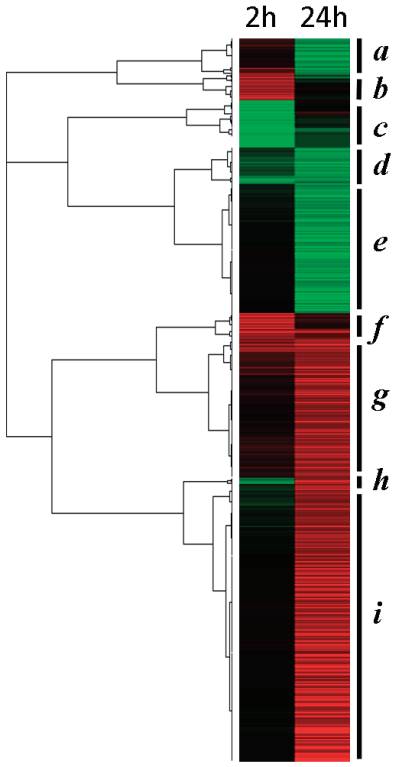


Fig. 4. Hierarchical clustering (Cluster v3.0) of probes data that are statistically significant at least at a single time point (parameters used: Similarity metrics = correlation (uncentered), method = complete linkage).

DISCUSSION

In this study, we report the levels of cytotoxicity of paraoxon and parathion as well as their methylated derivatives, methyl-paraoxon and methyl-parathion, in mouse cell line NB41A3. The potential biological processes affected by parathion exposure were also interrogated through a microarray experiment.

The toxicity of parathion and paraoxon

Our data indicate that the level of 24 hr LC50 was lower for paraoxon (0.42 mM) than the one for parathion (0.66 mM), suggesting that paraoxon is more toxic to NB41A3 cell line than parathion (Table 3). The data also suggest that the toxicity of methylated compounds is similar to ethylated compounds with a slight tendency of lower toxicity. The LC50 values for methyl-paraoxon and methyl-parathion were 0.46 mM and 0.77 mM, respectively (Table 3). However, the time course data indicate that the onset of detectable levels of cytotoxicity occurred faster for parathion and methyl-parathion than for paraoxon and methyl-paraoxon despite their lower toxicity level and higher LC50 (Fig. 2).

There are little LC50/LD50 data about cell lines under paraoxon or methyl-paraoxon. However, studies on its ecological effect showed LC50 values of paraoxon and methyl-paraoxon are both higher than those at Water flea (PAN Pesticides Database; Kegley *et al*., 2016). Stud-

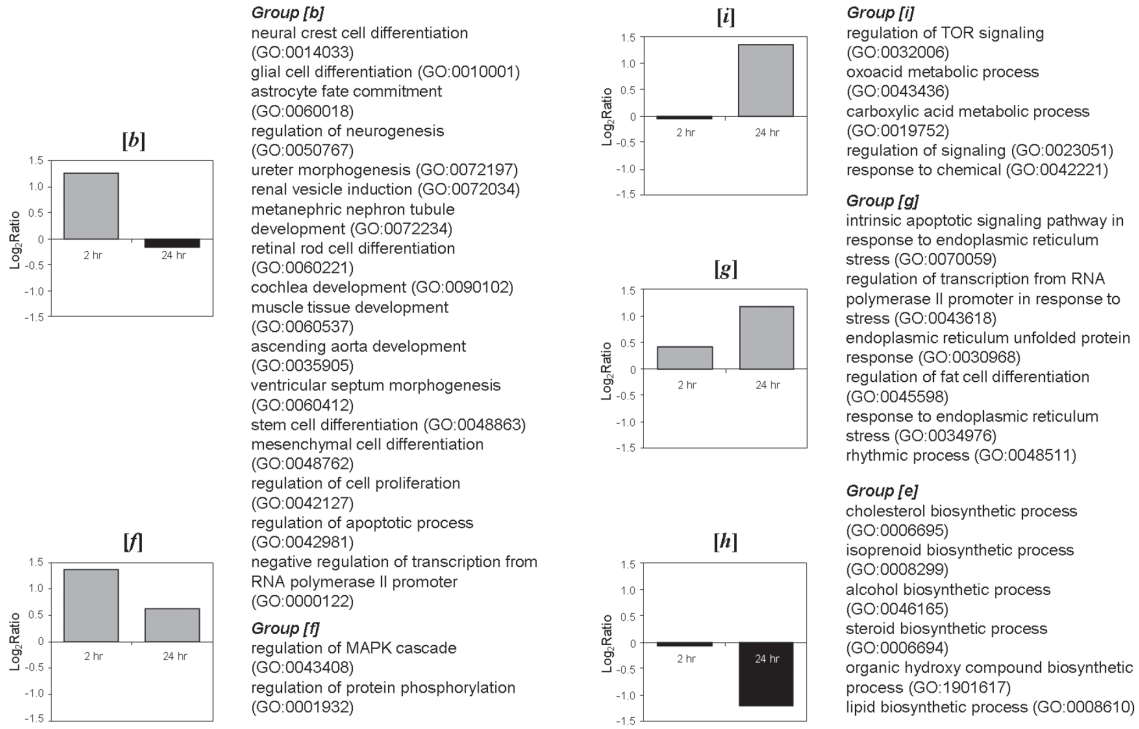


Fig. 5. Some representative GO terms are shown with the average expression patterns in each group from Fig. 4. The overrepresented GO terms were identified by using *Panther Classification System* (Mi *et al*., 2013). Full list is provided in Suppl. Data 2.

ies on paraoxon and methyl-paraoxon have indicated similar levels of toxicity to mouse brain (Kardos and Sultatos, 2000). The 48 hr LD50 values of parathion and methyl-parathion were determined to be 26.20 mM and 23.58 mM for HepG2 liver carcinoma cells, respectively (Edward *et al*., 2013). The LC50 values of parathion and methyl-parathion for NB41A3 in this study showed much lower than those for human HepG2 cells, suggesting that the levels of toxicity may vary in different cell lines (liver carcinoma cells vs. neuroblastoma cells).

Toxicokinetics and toxicodynamics are based on the time including binding of toxicants to the target and the dissociation process (Tennekes and Sánchez-Bayo, 2013). The primary target receptor of OPCs' neurotoxicity is acetylcholinesterase (AChE) enzyme. OPCs bind to AChE by forming covalent bond and phosphorylating. The OPCs binding rate depends on the leaving group, which is replaced by the oxygen of serine in the active site of AChE, and the dissociation of OPCs from the active site is very slow (Čolović *et al*., 2013; Marimuthu *et al*., 2019). Thus, the toxic effects of OPCs are altered by the binding energy of the OPCs-AChE complex. The basic structures of these four OPCs comprise a central phosphorus atom (P), and a nitrophenyl group as leaving group. Paraoxon and its analogue comprise the characteristic phosphoric (P=O) bond, and parathion and its analogue comprise thiophosphoric (P=S) bond. Between paraoxon and parathion, the different levels of toxicity depends on the bond P=O or P=S. The P=O bond in paraoxon and methyl-paraoxon is less polarizable than the P=S bond in parathion and methyl-parathion (Bae *et al*., 2013), and the toxicity of paraoxon/methyl-paraoxon is higher than that of parathion/methyl-parathion, which is consistent with the previous finding that high polarizability and molecular mass could decrease toxicity of OPCs (Can, 2014).

An early stage toxicity of parathion at low concentrations (Fig. 2E) indicates that P=S bond with a diethyl group has a fast binding energy to target receptor. Even methyl-parathion shows a more acute toxicity at lower exposure concentrations compared to paraoxon and methyl-paraoxon. The obvious early stage toxicity of parathion and methyl-parathion indicates P=S bond could accelerate the process of OPC binding to AChE. More-

over, the lower toxicity (high LC50 values) of parathion and methyl-parathion indicates that the binding energy of the group including P=S bond is not very strong, and that the dissociation process of parathion and its analogue from target receptor is faster than paraoxon/methyl-paraoxon.

Gene expression change in response to parathion

Out of 41,345 probes on GeneChip Mouse Gene 2.0 ST Array, 822 probes were responsive to parathion at least either one of the time points we tested (2 hr and/or 24 hr). Among those 822 probes, about 40% of the probes (329) do not have GenBank accession numbers, suggesting that they have never been reported, and 47% of the probes (387) do not have any annotation (not even "*predicted gene*"). Moreover, 50% of the probes (409) do not have an assigned UniGene ID, meaning that they are not conventional genes. Among the microarray probes used in this study, there are small non-coding RNAs (e.g. miRNAs) and long intergenic non-coding RNAs (lincRNAs), which also affect expression of other genes at various levels from chromatin assembly and transcription to translation and post-translational modification (reviewed in Ching *et al*., 2015). Due to lack of information on individual lincRNA on this array, however, it was not possible to link the role of those unknown lincRNA and the organophosphate responses. Further data mining and additional information about those lincRNA will allow us to investigate this in the future.

Nevertheless, using the probes with UniGene ID (413 or 50% of 822) we found some overrepresented Gene Ontology (GO) terms that were responsive to parathion exposure. Considering that organophosphate compounds generally inhibit the AChE enzyme that breaks down the neurotransmitter acetylcholine (De Jong and Benschop, 1988; Taylor, 2001), it is not surprising to find *neurogenesis* related genes in a responsive group. It has been reported that the *development of neurons* was the targeted biological process in developing zebrafish embryo and rat PC12 cells when exposed to chlorpyrifos-oxon (Jacobson *et al*., 2010) and to tris (1,3-dichloro-2-propyl) phosphate (Slotkin *et al*., 2017).

However, this group was overrepresented only among the early responders at 2 hr, but not at 24 hr (Fig. 5). It is not clear if the transient up-regulation of neurogenesis related genes is attributed to their natural response pattern that might be sufficient to trigger other downstream genes related to this process, or to physiological decision made by the cells, switching their response mode from differential development to apoptosis due to a high level of physiological stress. Indeed, genes related to *cell signaling*, *cell cycle*, *apoptosis*, and *endoplasmic reticulum unfolded protein response* are the overrepresented groups in early (2 hr) and in late phases (24 hr) of parathion response (Fig. 5), implying the onset of programmed cell death presumably due to cell stress, such as unfolded protein stress in the endoplasmic reticulum. The unfolded protein response is an adaptive process to restore proper folding of proteins in the secretory pathway (Zhang and Kaufman, 2004). Under the prolonged stress, however, this signaling response can cause cell death. For example, dysfunctions in this pathway can lead to neuronal degeneration such as Alzheimer, Parkinson and Huntington disease and amyotrophic lateral sclerosis (Remondelli and Renna, 2017).

Also, in previous studies, genes related to *cell cycle* and *apoptosis* were targeted by organophosphate pesticide chlorpyrifos in rat PC12 cells (Slotkin and Seidler, 2012), by diazinon in human NT2 cells (Aluigia *et al.*, 2010), and by malathion in N2a neuroblastoma cells (Venkatesan *et al*., 2017).

The biological processes mentioned above may be attributed to other effects of parathion that are not directly related to the inhibition of AChE as in previous reports (Berríos *et al*., 2015; Rush *et al*., 2010; Yousefpour *et al*., 2006). Further study and data mining will be required to investigate and interpret the data. In fact, our study was based on 409 parathion responsive probes on the Affymetrix array. There are 413 other parathion responsive probes that do not have UniGene ID, which include many non-protein coding genes. Information on expression changes of those genes might provide valuable insight on cellular responses to organophosphate compounds in the future.

ACKNOWLEDGMENTS

We thank Dr. Muthusamy Thangaraju at Augusta University for discussion on cell culture. This work was supported by the Department of Defense (DoD) [D01_W911SR-14-2-0001-0007].

Conflict of interest---- The authors declare that there is no conflict of interest.

Suppl. Data 1. All data for the statistically significant 822 probes are listed according to the response groups shown in Fig. 3. Also shown are the complete list of gene ontology (GO) terms (Biological Process) that responded to parathion significantly ($p < 0.05$).

Suppl. Data 2. Complete list of the statistically significant 822 probes are shown along with the probe set data for each group indicated in Fig. 4 and Table 6. Statistically significant gene ontology (GO) terms (Biological Process) for each group are also shown ($p < 0.05$).

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