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An Overview of Development of Quantitative Neurotoxicity Testing *In vitro*

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Author's contribution

The sole author prepared the manuscript that retrospects their works carried out in his laboratory. Each contribution to those works was shown in the original papers.

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ABSTRACT

In vitro neurotoxicity testing has been hampered by the fact that the brain architecture is complex. However, a series of innovation of neuroculturing broke through the barrier. The establishment of culturing for the primary neuron and the immobilised neuroblastoma cells enables neurotoxicity testing *in vitro*. Following to necrotic cell death, extensive morphological changes as seen during neuronal differentiation was used for the endpoints of neurotoxicity. Two-dimensional imaging techniques facilitated quantitative analyses of toxicity of many neutoxicants. Three-dimensional culturing of neurospheres *in vitro* has been expected to investigate the neurodevelopmental toxicity. The neurosphere assay *in vitro* also improved the sensitivity to estimate the neurotoxicity. The present study highlights an overview about the *in vitro* neurotoxicity testing for rotenone, a dopaminergic pesticide as an environmental toxicant.

Keywords: PC12 cells; NB-1 cells; neural stem cells; neurospheres; rotenone; quantitative neurotoxicity.

ABBREVIATIONS

bFGF: basic fibroblast growth factor; DMEM: Dulbecco's Modified Eagle's Medium; DOHaD: developmental origins of health and disease; E14: embryonic day 14; EGF: epidermal growth factor;

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FBS: fetal bovine serum; GFAP: glial fibrillary acidic protein; MAPS: microtubule-associated proteins; MEM: minimal essential medium; TUNEL: terminal deoxynucleotide transferase-mediated dutp nick-end labeling.

1. INTRODUCTION

In light of the large number of chemicals, there is a demand to develop rapid screening techniques. Historically, PC12 cells have been used for testing neurotoxicity. The PC 12 cell line was derived from rat pheochromocytoma, a tumour arising from chromaffin cells of the adrenal medulla and developed to study cell differentiation [1]. Neuronal differentiation is a complex process that induces both morphological and biochemical changes. The most obvious things are a decrease in cell proliferation, and the emergence of extending processes. During neuronal differentiation, cells also acquire excitability and start to express some chemical coding genes that provide their functional identity. Upon exposure to neuronal stimulation, PC12 cells gradually exit the mitotic cycle and begin to differentiate, developing axonal projections, electrical excitability, and the characteristics of cholinergic and catecholaminergic neurons. Therefore, the PC12 model enables the detection of environmental toxicants.

Following to rat PC12 model cells, human cell lines such as NB-1 and SH-SY5Y were used to investigate potential species-specific differences, rather than non-human cell origins. It was expected to extrapolate human toxicity.

Recent evidence points to important contributions of exposure to environmental neurotoxicants in the marked increase in neurodevelopmental disorders [2-4]. In response to the need for more efficient methods to identify potential developmental neurotoxicants, neurosphere assay has recently been established. Neural stem cells play an essential role in the development of the central nervous system, having self-renewal potency and being multi-potential. In 1992, it was demonstrated that cells from central nervous system of adult and embryonic mice can be isolated and propagated in culture [5]. In the presence of epidermal growth factor, cell agglomerations, termed neurospheres were formed. They proliferate in culture and have the ability to migrate and differentiate into neurons, astrocytes, and oligodendrocytes. Neurosphere culturing is three-dimensional cell systems and there a valuable *in vitro* model that mimics basic processes of brain development. Therefore, neurospheres are a useful tool for testing chemicals for their abilities to interfere with these processed: proliferation, migration, differentiation, and apoptosis.

In this paper, we compare a variety of cell-based neurotoxicity testing with several endpoints using rotenone, a dopaminergic pesticide (Fig.1, ref [6-8]).

2. MATERIALS AND METHODS

2.1 Culture of PC12 Cells

PC12 cells (RCB 0009; RIKEN, Tsukuba, Japan) were grown in Dulbecco's modified Eagle's Medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc., Rockville, MD), 4.5 mg/ml glucose, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. The cells were subcultured (1:3) 2 to 3 times per week. Cell viability was determined by trypan blue exclusion method.

2.2 Culture of NB-1 Cells

Human neurobalstoma NB-1 cells were cultured in 45% RPMI 1640 and 45% Eagle's minimum

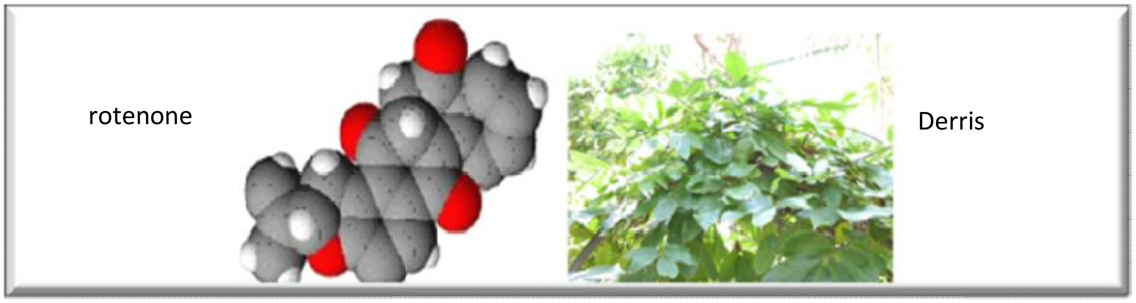

Fig. 1. The structure of rotenone and its derived plant

essential medium containing 10% FBS, sodium pyruvate, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. The cells were subcultured (1:6) once a week. The viable cell number of the NB-1cells was estimated by crystal violet staining. Fixed and dried cells in a plate were rehydrated with distilled water and photographed under a phase-contrast microscope (DMIRB, Leica Microsystems, Tokyo, Japan) equipped with digital camera. The digital images obtained were then analysed using image analysis software by counting the cell number and total neurite length in the image field. The degree of neurite extension is represented as the total length of neuritis in micrometer per cell in randomly chosen phase-contrast microscope fields.

2.3 Culture of Neurosphere

Pregnant Wistar rats at embryonic day 14 (E14) were obtained from Clea (Tokyo, Japan). The animals were maintained in home cages at 22°C with a 12-h light-dark cycle. They received the MF diet (Oriental Yeast Corp., Tokyo, Japan) and distilled water ad libitum. All animal care procedures were in accordance with National Institute for Environmental Studies guidelines. The rats were sacrificed by diethyl ether overdose on E16. The embryos were removed and transferred to a minimal essential medium (MEM; Sigma-Aldrich). Subsequently, the mesencephalons were dissected from the embryos, and were enzymatically digested with 50 U deoxyribonuclease I (Takara Corp., Kyoto, Japan) and 0.8 U papain (Sigma-Aldrich) at 32°C for 12 min. After stirring, the digestion mixture was passed through a 70-µm cell strainer (BD Biosciences). The run-through containing the neural stem cells was centrifuged at 800 x g for 10 min. It was then resuspended in Dulbecco's Modified Eagle's Medium (DMEM) and F12 medium (1:1; Invitrogen, Tokyo, Japan) supplemented with B27 (Invitrogen), 20 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Inc., MN) and 10 ng/ml epidermal growth factor (EGF; Roche Applied Science, Tokyo, Japan), and cultured in uncoated dishes without serum. Fresh culture medium containing EGF and bFGF was added after 3-4 days.

The neurospheres were seeded in an uncoated glass-bottomed dish (D110300; MATSUNAMI, Tokyo, Japan) in the presence of bFGF and EGF for 3 h, allow cells to adhere. The migrating distance of the cells was statistically measured from the edge of the sphere, using National Institute of Health ImageJ 1.38x softwear (public domain software).

TUNEL staining was carried out, following a standard protpcol. The cells were fixed in 4% paraformaldehyde, washed twice with PBS, and permeabilized in 0.5% Triton X-100 for 5 min on ice. TUNEL labelling was done with fluorescein dUTP (Roche Applied Science, Mannheim, Germany) in the presence of terminal deoxynucleotidyl transferase for 1 h at 37°C. Following labelling, the cells were washed with PBS twice and then directly surveyed under a fluorescence microscope. Images were captured using Viewfinder Lite ver.1.0 camera software through DP-50 digital camera (Olympus, Tokyo, Japan). For quantification of TUNEL-labeled cells, every field containing TUNEL-positive signals was photographed at 100x optical magnification. Then, TUNEL-positive cells were counted.

3. RESULTS

3.1 Cell Viability of PC12 Cells Exposed to Rotenone

Rotenone neurotoxicity in PC12 cells was examined by trypan blue exclusion method. PC12 cells were exposed to a variety of concentration of rotenone for 3 days. Following fixing the treated cells, a number of cells were counted. Cell viability was decreased in a semilogarithmic-linear, dose-dependent manner. IC_{50} was about 1 µM (Fig.2).

3.1. 1 Inhibition of neurite outgrowth of NB-1 cells exposed to rotenone

Rotenone neurotoxicity in NB-1 cells was examined as an endpoint of neurite outgrowth. NB-1 cells were seeded on a culture plate and rotenone (0~50 µM) was added for 24h. The treated cells were fixed and the length of neurite outgrowth was measured by imaging analyses. The length was decreased in a semilogarithmic-linear, dose-dependent manner. IC_{50} was about 1 µM (Fig.3).

3.1.2 Neurosphere assay for neuro-developmental toxicity of rotenone

We isolated neural stem cells from E15 rat embryos (Fig.4a), using pooled mesencephalons from 12 fetuses. After 2~3 weeks in culture, neurospheres appeared (Fig. 4b), suggesting self-renewal occurred. Neurospheres of about 200 µm in diameter consisted of about 10^3 cells (Fig. 4c).

To identify neural stem cells, we stained the neurospheres with an anti-nestin antibody, as shown in Fig. 5a. The nestin-positive cells were localized both at the edge and within the spheres. Since neural stem cells are multipotent for neural differentiation, we also immunostained the neurospheres for MAPs, which were located in cells at the edge of the spheres (Fig. 5b). Since on E15, when we isolated the neuronal stem cells, rat embryos are undergoing gliogenesis, we stained the neurospheres with anti-GFAP antibody, which mainly stained cells at the sides of the spheres (Fig. 5c). Our results suggested that heterogeneous cell populations were present in neurospheres, at late embryonic stages.

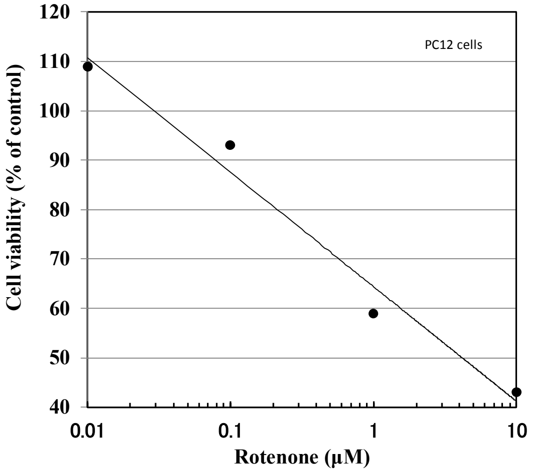

Fig. 2. Rotenone neurotoxicity in rat pheochromocytoma PC12 cells

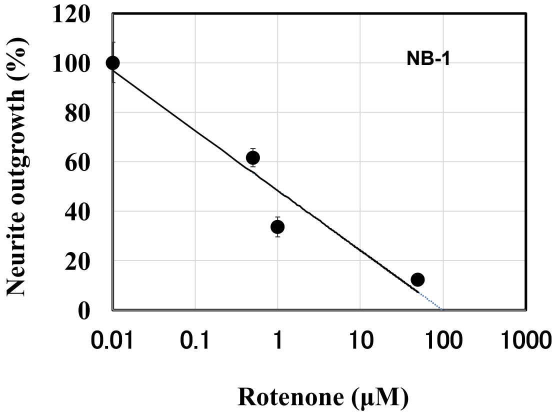

Fig. 3. Neurite outgrowth in rotenone-exposed human neuroblastoma NB1 cells

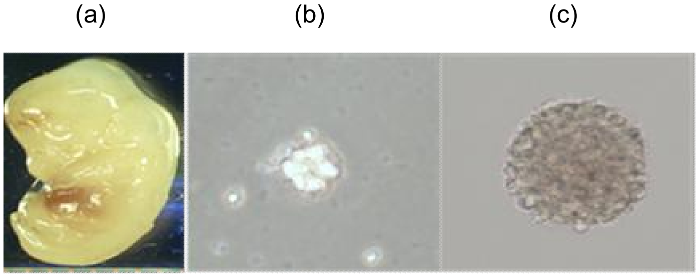
(a) (b) (c)

Fig. 4. (a) An E15 rat fetus showing the mesencephalon. (b) Primary neurospheres after 7 days *in vitro* (c) Primary neurospheres after 2~3 days *in vitro*. Adapted from ref. [9]

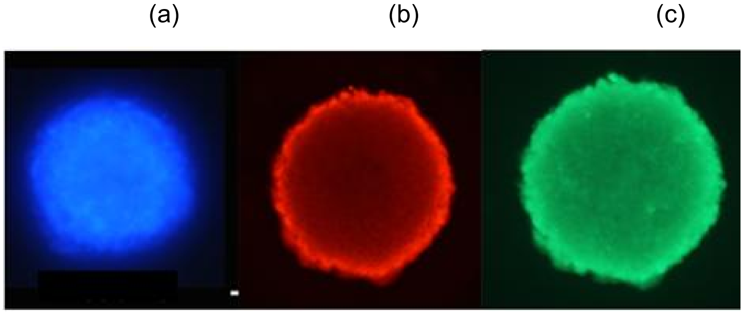

Fig. 5. Identification of cultured mesencephalic neurosphere. Neurospheres were immunostained with (a) anti-nestin antibody, (b) anti-MAPs antibody, or (c) anti-GFAP antibody. Adapted from ref. [9]

During the culture, cells emerged from the plated neurospheres and migrated along the radial axis (Fig. 6 a and b). After 3 h, the plated cells were treated with various concentrations of rotenone (0-10 μM) for 24 h (Figs.7 b-f). The migration distance of the cells was measured from the edge of the neurospheres using NIH ImageJ 1.38x public domain software. Rotenone prevented the cells from migrating from the neurospheres in a linear, dose-dependent manner (Fig. 7g). The half-maximal inhibitory concentration (IC_{50}) was 0.32 μM.

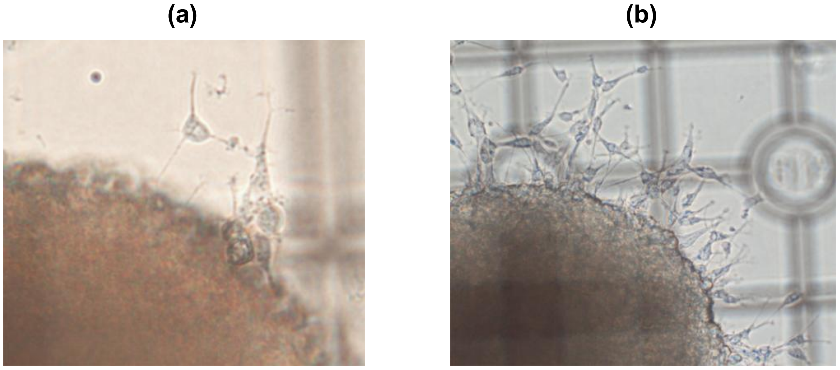

Fig. 6. Neural stem cells radically migrated from neurosphere. (a)The early stage, or (b) the later stage of the culturing

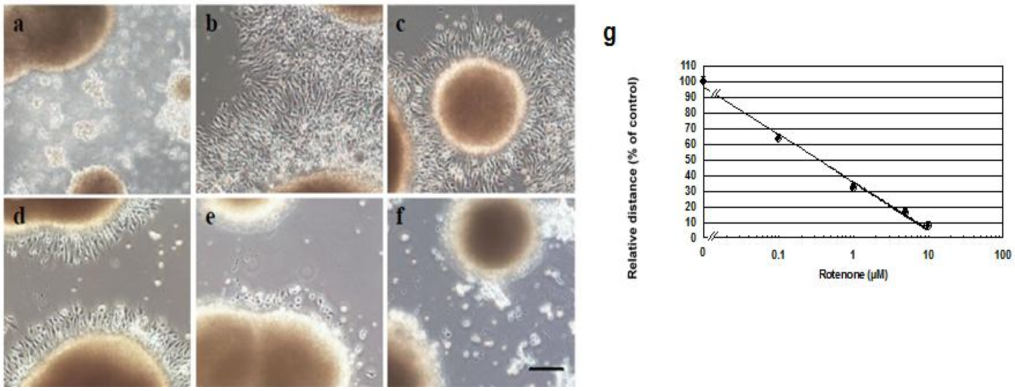

Fig. 7. Rotenone inhibition of cell migration from neurospheres *in vitro* at various concentrations (a) No migrating cells during the initial 3 h, (b) 0 μM, (c) 0.1 μM , (d) 1 μM, (e) 5 μM, (f) 10 μM. The migration distance was quantitatively measured with NIH ImageJ 1.38x software (g). Adapted from ref. [10]

To compare the toxicity of 2 chemicals, neurosphere assay for bisphenol A, an endocrine disruptor, was carried out under the condition where rotenone was done (Fig. 8). The percent inhibition of migration by bisphenol A and rotenone at 1 μM was 35% and 70%, respectively. Thus, the rank order of potency of chemicals was: bisphenol A < rotenone. The value of other endpoints was summarised in Table 1.

4. DISCUSSION

Rotenone is a botanical pesticide [6-8]. Neurotoxic nature of rotenone has been used to produce adult Parkinson model animals due to nigrostriatal dopaminergic lesions [12-14]. Upon the working hypothesis of the developmental origins of health and disease (DOHaD; ref. [15]), it is important to examine the neuro-developmental toxicity of rotenone in

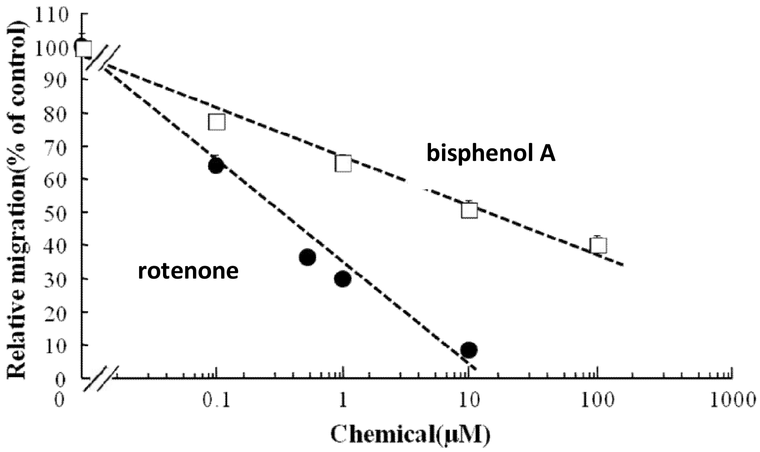

Fig. 8. Comparison of neurotoxicity of 2 chemicals by neurosphere assay *in vitro*. Modified from ref. [11]

Table 1. Multiple endpoints used in chemical neurosphere testing *in vitro* Modified from refs. [10 and 11]

Endpoint	**Chemical**	
	Rotenone	**Bisphenol A**
1. Migration (IC_{50})	0.32 μM	↓
2. Proliferation (IC_{50})	1.9 μM	5.0 μM
3. Apoptosis	↑	ND
4. Log K_{ow}	4.10	3.32

↑:induction; ↓:inhibition; ND: not detected; values of Log K_{ow} were reported in International Chemical Safety Cards (ICSC, Japanese Ver.).

Table 2. Sensitivity to rotenone neurotoxicity in various *in vitro* testing

Source	**Half maximal effects**	**Periods**	**Endpoint**	**Reference**
Rat PC12 cells	1 μM <	24 h	Cell viability	20
	1 μM	72 h	Cell viability	Unpublished data
Human NB-1 cells	1 μM	24 h	Neurite outgrowth	Unpublished data
Human neurosphere	4 μM	24 h	Cell viability	21
Rat E16 neurosphere	0.32 μM	24 h	Migration	10
	1.9 μM	24 h	Proliferation	10
	1.4 μM	24 h	Apoptosis	10

neurosphere assays, comparing other cell lines. The hypothesis suggests that the environmental origin of human sporadic Parkinson disease occur early in life. One possible explanation for this phenomenon is that early exposure to neurotoxic chemicals reduces the number of dopaminergic neurons in the substantia nigra to levels below those needed to sustain normal function during the course of the neuronal attrition associated with aging.

There are many endpoints to evaluate neurotoxicity [Table 2]. Ultimate endpoint for neurotoxicity is cell death. Therefore, neuronal cell viability was used for evaluating its toxicity. Catastrophic cell death by neurotoxins is observed as perturbation of energy producing systems, cellular membrane defects, and increased influx of calcium ions. This is concomitant with necrotic action of the toxin. For more sensitive detection of the toxicity, a new biochemical marker is needed.

About 20 years ago, apoptotic nature of environmental toxins were discovered in several types of cells, including renal and neuronal cell [16,17]. Apoptosis is a programmed form of cell death mediating precisely controlled deletions of ‘unwanted’ cells. This phenomenon is initiated not only by physiological stimuli but also by an extensive array of nonphysiological agents. The characteristics of apoptosis are DNA fragmentation and chromatin condensation, in which are endpoints of toxicants as earlier phase than necrotic phase of toxins.

Development of techniques for Image acquisition enabled to open a new way to evaluate neurotoxicity: neurite outgrowth was also used for endpoint of neurotoxicity [18,19]. The growth of axonal and dendritic processes during brain development is a critical determinant of neural connectivity, and disruption of this process could lead to neuronal dysfunction. Neurite outgrowth can be recapitulated *in vitro* using a variety of cell models. These models have been become valuable tools for investigating the mechanism for known developmental neurotoxicants.

The developing human brain can be more susceptible to injury caused by toxic agents than the brain of an adult. Probably all potential neutoxic compounds would also cause damage to the developing brain and at much lower doses. Indeed, neurodevelopmental disorders in children such as attention deficit disorder or autism have been associated with the exposure to chemicals in the environment during early fetal development [22].

Particularly, it has been suggested that one of possibility of autism-spectrum disorders are initiated in the embryonic neural stem cells [23]. Neural stem cells play an essential role in the development of central nervous system, having self-renewal potency and being multipotential: they are able to differentiate to neurons, astrocytes and oligodendrocytes to form neuronal architecture. Indeed, in the culture of neural stem cells, they form free-floating three-dimensional structures. Therefore, application of neurosphere for neurodevelopmental toxicity testing is reasonable.

Quantitative analyses in this study revealed the linearity in function as migration inhibition versus chemical concentration. Owing to the linearity of the functional relationship between the migration inhibition and the concentration of test chemicals, this approach could be employed as a reliable quantitative assay system, excluding the issue of nonlinearity in low dose of an endocrine disruptor such as bisphenol A [24,25].

5. CONCLUSION

This paper highlights an overview about the neurotoxicity of rotenone, investigating with rat PC12 cell, human NB-1 cells and rat embryonic neural stem cells. Furthermore, quantitative analysis revealed a linear function between the cellular endpoints and the rotenone concentration. This could be employed as a simple and rapid screening for neurotoxicity of environmental chemicals. Particularly, neurosphere assay would be hoped to develop the risk assessment methods for chemicals based on infant physiology.

ETHICAL APPROVAL

As per international standard or university standard written ethical permission has been collected and preserved by the authors.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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