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10 ABSTRACT

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In vitro neurotoxicity testing has been hampered by the fact that the brain architecture is complex. However, a series of innovation of neuroculturing broked through the barrier. The establishment of culturing for the primary neuron and the immobilized 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 neutoxicicants. 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. In this paper, I will retrospectively take an overview about the in vitro neurotoxicity testing for rotenone, a dopaminergic pesticide as an environmental toxicant.

An Overview of Development of Quantitative

Neurotoxicity Testing In Vitro

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

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1. INTRODUCTION

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19 In light of the large number of chemicals, there is a demand to develop rapid screening 20 techniques. Historically, PC12 cells have been used for testing neurotoxicity. The PC 12 21 cell line was derived from rat pheochromocytoma, a tumor arising from chromaffin cells of the adrenal medulla and developed to study cell differentiation [1]. Neuronal differentiation is 22 a complex process that induces both morphological and biochemical changes. The most 23 obvious things are a decrease in cell proliferation, and the emergence of extending 24 25 processes. During neuronal differentiation, cells also acquire excitability and start to express 26 some chemical coding genes that provide their functional identity. Upon exposure to 27 neuronal stimulation, PC12 cells gradually exit the mitotic cycle and begin to differentiate, 28 developing axonal projections, electrical excitability, and the characteristics of cholinergic 29 and catecholaminergic neurons. Therefore, the PC12 model enables the detection of 30 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 central nervous system, having self-renewal potency and being multi39 potential. In 1992, it was demonstrated that cells from central nervous system of adult and 40 embryonic mice can be isolated and propagated in culture [5]. In the presence of epidermal 41 growth factor, cell agglomerations, termed neurospheres were formed. They proliferate in 42 culture and have the ability to migrate and differentiate into neurons, astrocytes, and 43 oligodendrocytes. Neurosphere culturing is three-dimensional cell systems and there a 44 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 45 46 processed: proliferation, migration, differentiation, and apoptosis.

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

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Figure 1. The structure of rotenone and its derived plant



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52 2. MATERIAL AND METHODS

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54 2.1 Culture of PC12 cells

55 PC12 cells (RCB 0009; RIKEN, Tsukuba, Japan) were grown in Dulbecco's modified 56 Eagle's Medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS; Life 57 Technologies, Inc., Rockville, MD), 4.5 mg/ml glucose, penicillin (100 U/ml), and 58 streptomycin (100 μ g/ml) in a humidified atmosphere of 95% air, 5% CO₂ at 37 °C. The cells 59 were subcultured (1:3) 2 to 3 times per week. Cell viability was determined by trypan blue 60 exclusion method.

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62 2.2 Culture of NB-1 cells

63 Human neurobalstoma NB-1 cells were cultured in 45% RPMI 1640 and 45% 64 Eagle's minimum essential medium containing 10% FBS (Life Technologies, Inc.), sodium pyruvate, penicillin (100 U/ml), and streptomycin (100 µg/ml) in a humidified atmosphere of 65 95% air, 5% CO2 at 37 °C. The cells were subcultured (1:6) once a week. The viable cell 66 number of the NB-1cells was estimated by crystal violet staining. Fixed and dried cells in a 67 plate were rehydrated with distilled water and photographed under a phase-contrast 68 microscope (DMIRB, Leica Microsystems, Tokyo, Japan) equipped with digital camera. The 69 70 digital images obtained were then analyzed using image analysis software by counting the 71 cell number and total neurite length in the image field. The degree of neurite extension is 72 represented as the total length of neuritis in micrometer per cell in randomly chosen phase-73 contrast microscope fields.

74 2.3 Culture of neurosphere

75 Pregnant Wistar rats at embryonic day 14 (E14) were obtained from Clea (Tokyo, 76 Japan). The animals were maintained in home cages at 22°C with a 12-h light-dark cycle. 77 They received the MF diet (Oriental Yeast Corp., Tokyo, Japan) and distilled water ad 78 libitum. All animal care procedures were in accordance with National Institute for 79 Environmental Studies guidelines. The rats were sacrificed by diethyl ether overdose on 80 E16. The embryos were removed and transferred to minimal essential medium (MEM; 81 Sigma-Aldrich). Subsequently, the mesencephalons were dissected from the embryos, and 82 were enzymatically digested with 50 U deoxyribonuclease I (Takara Corp., Kyoto, Japan) 83 and 0.8 U papain (Sigma-Aldrich) at 32°C for 12 min. After stirring, the digestion mixture 84 was passed through a 70-µm cell strainer (BD Biosciences). The run-through containing the 85 neural stem cells was centrifuged at 800 x g for 10 min. It was then resuspended in 86 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; 87 88 R&D Systems, Inc., MN) and 10 ng/ml epidermal growth factor (EGF; Roche Applied 89 Science, Tokyo, Japan), and cultured in uncoated dishes without serum. Fresh culture medium containing EGF and bFGF was added after 3-4 days. 90

91 The neurospheres were seeded in an uncoated glass-bottomed dish (D110300; 92 MATSUNAMI, Tokyo, Japan) in the presence of bFGF and EGF for 3 h, allow cells to 93 adhere. The migrating distance of the cells was statistically measured from the edge of the 94 sphere, using National Institute of Health ImageJ 1.38x softwear (public domain software).

95 TUNEL staining was carried out, as described previously [26, 27]. The cells were fixed in 4% 96 paraformaldehyde, washed twice with PBS, and permeabilized in 0.5% Triton X-100 for 5 97 min on ice. TUNEL labelling was done with fluorescein dUTP (Roche Applied Science, 98 Mannheim, Germany) in the presence of terminal deoxynucleotidyl transferase for 1 h at 99 37°C. Following labelling, the cells were washed with PBS twice and then directly surveyed 100 under a fluorescence microscope. Images were captured using Viewfinder Lite ver.1.0 101 camera software through DP-50 digital camera (Olympus, Tokyo, Japan). For quantification 102 of TUNEL-labeled cells, every field containing TUNEL-positive signals was photographed at 103 100x optical magnification. Then, TUNEL-positive cells were counted.

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105 3. RESULTS

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107 3.1. Cell viability of PC12 cells exposed to rotenone

108 Rotenone neurotoxicity in PC12 cells was examined by trypan blue exclusion method. 109 PC12 cells were exposed to variety of concentration of rotenone for 3 days. Following fixing 110 the treated cells, a number of cells were counted. Cell viability was decreased in a 111 semilogarithmic-linear, dose-dependent manner. IC_{50} was about 1 μ M (Fig.2).

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Figure 2. Rotenone neurotoxicity in rat pheochromocytoma PC12 cells.

UNDER PEER REVIEW



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120 3.1. 1. Inhibition of neurite outgrowth of NB-1 cells exposed to rotenone

121 Rotenone neurotoxicity in NB-1 cells was examined as an endpoint of neurite 122 outgrowth. NB-1 cells were seeded on a culture plate and rotenone ($0 \sim 50 \mu$ M) was 123 added for 24h. The treated cells were fixed and the length of neurite outgrowth was 124 measured by imaging analyses. The length was decreased in a semilogarithmic-125 linear, dose-dependent manner. IC₅₀ was about 1 μ M (Fig.3).

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



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130 3.1.2. Neurosphere assay for neurodevelopmental toxicity of rotenone

UNDER PEER REVIEW

131 We isolated neural stem cells from E15 rat embryos (Fig.4a), using pooled mesencephalons 132 from 12 fetuses. After 2~3 weeks in culture, neurospheres appeared (Fig. 4c), suggesting 133 self-renewal occurred. Neurospheres of about 200 μ m in diameter consisted of about 10³

134 cells.

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Figure 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].



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Figure 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].



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151 To identify neural stem cells, we stained the neurospheres with an anti-nestin antibody, as shown in Figure 5a. The nestin-positive cells were localized both at the edge and within the 152 153 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 154 155 spheres (Fig.5b). Since on E15, when we isolated the neuronal stem cells, rat embryos are 156 undergoing gliogenesis, we stained the neurospheres with anti-GFAP antibody, which mainly 157 stained cells at the sides of the spheres (Fig.5c). Our results suggested that heterogeneous 158 cell populations were present in neurospheres, at late embryonic stages.

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159 During the culture, cells emerged from the plated neurospheres and migrated along the 160 radial axis (Fig. 6 a and b). After 3 h, the plated cells were treated with various 161 concentrations of rotenone (0-10 μ M) for 24 h (Figs.7 b-f). The migration distance of the 162 cells was measured from the edge of the neurospheres using NIH ImageJ 1.38x public 163 domain software. Rotenone prevented the cells from migrating from the neurospheres in a 164 linear, dose-dependent manner (Fig. 7g). The half-maximal inhibitory concentration (IC₅₀) 165 was 0.32 μ M.



186Figure 7. Rotenone inhibition of cell migration from neurospheres in vitro at187various concentrations . (a) No migrating cells during the initial 3h, (b) 0 μ M, (c) 0.1 μ M,188(d) 1 μ M, (e) 5 μ M, (f) 10 μ M. The migration distance was quantitatively measured with NIH189ImageJ 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 did (Fig.8). The percent inhibition of migration by bisphenol A and rotenone at 1 µM was 35% and 70%, respectively.

UNDER PEER REVIEW

- 211 Thus, the rank order of potency of chemicals was: bisphenol A< rotenone. The value of
- 212 other endpoints was summarized in Table 1.



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



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 Table 1. Multiple endpoints used in chemical neurosphere testing In Vitro

 Modified from refs. 10 and 11.

	Chemical			
Endpoint	Rotenone	Bisphenol A		
 Migration (IC₅₀) Proliferation (IC₅₀) Apoptosis Log K_{ow} 	0.32 µM 1.9 µM ↑ 4.10	↓ 5.0 µM ND 3.32		

1222 ↑:induction; ↓:inhibition; ND: not detected; values of Log K_{ow} were reported in International
 223 Chemical Safety Cards (ICSC, Japanese Ver.).

Table 2. Sensitivity to rotenone neurotoxicity in various In Vitro testing

Source	Half maximal effects	Period s	Endpoint	Reference
Rat PC12 cells	1 µM <	24h	Cell viability	24
	1 µM	72h	Cell viability	Unpublished data
Human NB-1 cells	1 µM	24h	Neurite outgrowth	Unpublished data

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Human neurosphere	4 µM	24h	Cell viability	25
Rat E16 neurosphere	0.32 µM	24h	Migration	10
	1.9 µM	24h	Proliferation	10
	1.4 µM	24h	Apoptosis	10

227 4. DISCUSSION

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229 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]. 230 Upon the working hypothesis of the developmental origins of health and disease (DOHaD; 231 ref.15), it is important to examine the neurodevelopmental toxicity of rotenone in 232 233 neurosphere assays, comparing other cell lines. The hypothesis suggests that the 234 environmental origins of human sporadic Parkinson disease occur early in life. One possible 235 explanation for this phenomenon is that early exposure to neurotoxic chemicals reduces the 236 number of dopaminergic neurons in the substantia nigra to levels below those needed to 237 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 [20].

Particularly, it has been suggested that one of possibility of autism-spectrum disorders are initiated in the embryonic neural stem cells [21]. Neural stem cells play an essential role in the development of central nervous system, having self-renewal potency and being 266 multipotential: they are able to differentiate to neurons, astrocytes and oligodendrocytes to 267 form neuronal architecture. Indeed, in the culture of neural stem cells, they form free-268 floating three-dimensional structures. Therefore, application of neurosphere for 269 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 [22,23].

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5. CONCLUSION

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In this paper, I take an overview about 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.

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