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A Screening System for Detection of Neurotoxic Potency of Chemicals using *in vitro* Model

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Abstract

Background: The major categories of peripheral nerve injury (neuropathy) are generally neuronopathy, axonopathy and myelinopathy. Numerous chemicals are known to cause neuropathy, and the exact mechanism of neuronal toxicity is not known for the majority of chemicals. It is expected that the elucidation of the underlying mechanism of chemicals-induced peripheral neuropathy may lead to its prevention and treatment. **Objective:** The aim of this study is whether the type of neurotoxicity induced by pravastatin, colchicine, amiodarone, cisplatin, and $CoCl_2$ can be identified by using the *in vitro* model. **Materials and Methods:** The cytotoxicity was performed in Neuro-2a neural cells. The levels of DNA fragmentation and the mRNA and protein levels of Bax and Bcl2 were used as apoptosis markers. To evaluate the axon damage, the extracellular phosphorylated neurofilament heavy chain (pNF-H) was investigated by Western blotting. **Results:** The half of cytotoxic concentration of pravastatin, colchicine, amiodarone, cisplatin and $CoCl_2$ were 19.69 µM, 109.2 nM, 11.89 µM, 5.54 µM and 337.3 µM, respectively. To investigate morphological changes of Neuro-2a exposed to neurotoxic chemicals, Neuro-2a neural cells were incubated with 10 µM pravastatin, 100 nM colchicine, 10 µM amiodarone, 1 µM cisplatin, or 300 µM $CoCl_2$, and evaluated by immunofluorescence assay. Pravastatin, colchicine and cisplatin significantly decreased the axon length. DNA fragmentation and apoptosis-related genes were investigated. Cisplatin significantly increased the degree of fragmentation and the ratio of Bax/Bcl-2. The protein levels of pNF-H in both supernatants and cell lysates were evaluated. The levels of pNF-H in the supernatant of pravastatin-treated, colchicine-treated, and $CoCl_2$-treated cells were higher than in that of vehicle-treated cells. **Conclusion:** The neuronopathy and axonopathy induced by chemicals could be determined by the evaluation of the levels of apoptosis marker and extracellular pNF-H in Neuro-2a neural cells, respectively.

Keywords: Apoptosis, Neuro-2a, Neuropathy, Phosphorylated Neurofilament Heavy Chain, Statin

1. Introduction

Peripheral neuropathy, a result of damage to the nerves outside of the brain and spinal cord (peripheral nerves), frequently causes numbness and pain, in the whole body, particularly extremities. The major categories of peripheral nerve injury (neuropathy) are generally neuronopathy, axonopathy, and myelinopathy[1–4]. Neuronopathy results from damage or death of the neuronal cell body, and both axons and myelin sheaths are secondarily damaged. Axonopathy refers only to the primary degeneration of axons, and secondary effects due to primary axonal destruction lose myelin sheath. In myelinopathy, the myelin sheath is primarily destructed, the axons are relatively preserved. In general, differences exist in the prognosis of patients depending on lesion loci[5]. Therefore, the elucidation of the mechanism of neuropathy may lead to identify approaches for prevention and treatment.

Numerous chemicals are known to cause neuropathy, and the exact mechanism of neuronal toxicity is not known

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for the majority of chemicals[2-6]. However, several studies have reported the neurotoxic mechanism for various chemicals. Hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors (Statin drugs) and colchicine associated with axonopathy[7-9]. Amiodarone induces myelinopathy[10]. Cisplatin induces neuronopathy[11,12]. Cobalt induces the axonal degeneration[13] or neural cell death[14]. In clinical practice, the peripheral neuropathy is relatively caused by chemicals used in the treatment, prevention, or diagnosis of a disease[5,15,16].

Neuronopathy is associated with apoptosis in the cell body of the neuron[2,17]. Apoptosis is a genetically programmed mechanism of cell death that is triggered in response to cellular stress. BCL-2 family proteins, such as Bax and Bcl-2, are well-known as key regulators of apoptosis. Bax is a pro-apoptotic protein that promotes cell death. On the other hand, Bcl-2 protects cells against apoptosis. The ratio of Bax/Bcl-2 is associated with increased apoptotic response and is generally used as a marker of apoptosis in various cell types[18-23]. In neural cells, treatment of cobalt induced protein expression of Bax and the number of apoptotic cells[13]. The late stage of apoptosis is characterized by DNA fragmentation[24].

The phosphorylated neurofilament H (pNF-H) is one of the major proteins of axons[25,26]. The pNF-H is a blood biomarker of traumatic brain injury and release from contused axons[26-28] and peripheral neuropathy[29]. The levels of pNF-H released from neuron assume to be useful in determining axonopathy. However, it is unknown whether pNF-H is released from axon-damaged cells *in vitro*.

Recently, many *in vitro* methods for animal toxicity testing have been developed and validated as an alternative to animal experiments. However, there are not sensitive and specific *in vitro* assays for neurotoxicity. Mouse neuroblastoma Neuro-2a cells exposed to all-*trans* retinoic acid undergo neural differentiation relatively easily[30]. Therefore, the aim of this study is whether the type of neurotoxicity induced by chemicals can be identified by using Neuro-2a neural cells.

2. Materials and Methods

2.1 Reagent

Cisplatin, colchicine, cobalt (II) chloride ($CoCl_2$), and pravastatin sodium salt were obtained from Fujifilm Wako Pure. Chemical Co. (Osaka, Japan). Amiodarone hydrochloride was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

2.2 Cell culture

Neuro-2a cells obtained from JCRB cell bank (Osaka, Japan) were maintained as previously described[30]. Briefly, Neuro-2a cells were grown to 50% confluence in Dulbecco's Modified Eagle Medium, high glucose (12100-046: Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum, 1.5g/L $NaHCO_3$, 100 units/mL penicillin, and 100 µg/mL streptomycin (growth medium) at 37°C in 5% CO_2 and 95% air atmosphere at 100% humidity. Cells were sub-cultured with the growth medium every 2–3 days. To differentiate Neuro2a cells into neural cells, cells were grown to 50% confluence in the growth medium and then cultured in phenol red-free Dulbecco's Modified Eagle Medium, high glucose supplemented with 2% horse serum, 10 µM all-*trans* retinoic acid 1.5g/L $NaHCO_3$, 100 units/mL penicillin, and 100 µg/mL streptomycin (differentiation medium) at 37°C in 5% CO_2 and 95% air atmosphere at 100% humidity. The differentiation medium was replaced at 48-hour intervals, and cells were cultured for 4 days.

2.3 *In vitro* Cytotoxicity

Lactate dehydrogenase (LDH) is a stable cytoplasmic enzyme in cells and is released into the cell culture supernatant during cytoplasmic membrane damage. In this study, the cytotoxic effects of test substances on Neuro-2a neural cells were investigated by using LDH Cytotoxicity Detection Kit (MK401; Takara Bio, Shiga, Japan) as described previously[30]. In brief, Neuro-2a neural cells were incubated with the various concentrations of test substances for 48 hours in a 96-well plate, and eight replicates were prepared for each treatment.

2.4 Immunofluorescence Assay

The axons of Neuro-2a neural cells were morphologically evaluated by immunofluorescence assay using an antibody for pNF-H. In brief, Neuro-2a neural cells were incubated with test substance for 48 hours in 24-well plate, and fixed in 4% paraformaldehyde in PBS for 10 minutes and permeabilized with 0.2% Triton X-100 in PBS for 5 minutes as described previously[31]. Fixed cells were blocked and incubated with mouse monoclonal pNF-H antibody (clone 7H11; Santa Cruz Biotechnology,

Inc., CA, USA) at room temperature for 1 hour, followed by Alexa Fluor 488-conjugated anti-mouse IgG (Thermo Fisher Scientific). After 1 hour, cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Dojindo Laboratory, Kumamoto, Japan). Fluorescent images were captured using a fluorescence microscope (BX53; Olympus, Tokyo, Japan). The length of axons was measured using a software ImageJ (version 1.52p, National Institutes of Health).

2.5 DNA Ladder Assay

DNA ladder assay was performed by the method of Harada *et al.*[32] Neuro-2a neural cells, including floating cells, were lysed with 10mM Tris–HCl, pH 7.5 containing 10mM EDTA and 0.5% Triton X-100, followed by incubation with 1% SDS, 0.2 mg/ml RNaseA (Nippon Gene Co., Ltd, Tokyo, Japan), and 0.2 mg/ml Proteinase K (Qiagen, Valencia, CA, USA). After ethanol precipitation, all samples were run on agarose gel electrophoresis, and then were stained with fluorescent dye (EzFluoroStainDNA; ATTO Corporation, Tokyo, Japan). DNA ladder was detected by a 505-nm excitation light (WSE-5600CyanoView; ATTO Corporation). The degree of fragmentation well was determined by densitometry using ImageJ (version 1.52p).

2.6 Quantitative Real-time Reverse Transcription-polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted and reverse transcribed with PrimeScript reverse transcription Reagent Kit (Takara Bio) from Neuro-2a neural cells in 12-well plates as described previously.[29] The resultant cDNA was subjected to quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) using the following specific primers: *Bax* (forward primer 5'-TGAAGACAGGGGCCTTTTTG-3'andreverseprimer 5'-AATTCGCCGGAGACACTCG-3'), Bcl2 (forward primer 5'-ATGCCTTTGTGGAACTATATGGC-3' and reverse primer 5'-GGTATGCACCCAGAGTGATGC-3'), Actb (forward primer 5'-CAGCCTTCCTTCTTGG GTAT-3' and reverse primer 5'-GCTCAGTAACAGTC CGCCTA-3')[33]. The PCR profiles consisted of denaturation at 95°C for 1 minute, primer-annealing at 55°C for 1 minute, and primer extension at 72°C for 30 seconds. The final primer extension was performed at 72°C for 10 minutes. The PCR in qRT-PCR was performed with SYBR Premix Ex TaqII (Takara Bio) on Thermal Cycler Dice, TP-900 (Takara Bio). Ct values were transformed into relative quantification data by $2^{-\Delta\Delta}$Ct method, and data were normalized to the *Actb*.

2.7 Western Blotting

Neuro-2a neural cells were lysed in radioimmuno precipitation buffer (50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.25% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS) contained ethylenediaminetetraacetic-acid free protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and sonicated. Cell culture supernatants were purified by acetone precipitation. In brief, supernatants were mixed with four-volume of cold (-20°C) acetone and were centrifuged at 15,000 × g for 10 minutes at 4°C. The precipitate were re-suspended in radioimmunoprecipitation buffer. Samples were subjected to SDS-PAGE and analyzed by Western blotting with following primary antibodies: mouse monoclonal anti-Bax antibody (clone 2D2; Santa Cruz Biotechnology), mouse monoclonal anti-Bcl-2 antibody (clone C-2; Santa Cruz Biotechnology), mouse monoclonal anti-β-Actin antibody (clone 7D2C10; Proteintech, Rosemont, IL, USA) and mouse monoclonal anti-pNF-H antibody (clone 7H11). Immunoreactive proteins were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG, anti-mouse IgG, or anti-rat IgG and reacted with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific), followed by detection with a Chemiluminescence detector (WSE-6100 LuminoGraph I; ATTO Corporation). The intensities of immunoreactive proteins were quantified by densitometry using ImageJ (version 1.52p).

2.8 Statistical Analysis

The half-maximal cytotoxic concentration (CC_{50}) values were calculated by fitting to a standard 4-parameter logistic curve using the R (version 3.6.1). Data were statistically compared by Dunnett's multiple comparison tests using the statistical analysis software Pharmaco Basic (version 15.0; Scientist Press Co., Ltd., Tokyo, Japan). Data were expressed as mean ± S.D., and differences were considered statistically significant at a p value of < 0.05 (n = 3).

3. Results

3.1 *In vitro* Cytotoxicity Assay

To determine which the concentrations of test substances have having minimal toxicity against Neuro-2a neural cells, cell viability was analyzed after 48 hours of exposure to different concentrations of five neurotoxic chemicals, pravastatin, colchicine, amiodarone, cisplatin, and $CoCl_2$. Cell viability was decreased in a dose-dependent manner by exposing all chemicals, and the CC_{50} values of pravastatin, colchicine, amiodarone, cisplatin and $CoCl_2$ were 19.69 μM, 109.2 nM, 11.89 μM, 5.54 μM and 337.3 μM, respectively [Table 1].

Table 1. The values of CC_{50} of various chemicals against Neuro-2a neural cells

	CC_{50}
Pravastatin	19.69 μM
Colchicine	109.2 nM
Amiodarone	11.89 μM
Cisplatin	5.54 μM
$CoCl_2$	337.3 μM

3.2 Evaluation of Axon Length

To investigate that morphological changes of Neuro-2a exposed to neurotoxic chemicals, Neuro-2a neural cells were incubated with 10 μM pravastatin, 100 nM colchicine, 10 μM amiodarone, 1 μM cisplatin or 300 μM $CoCl_2$, followed by immunofluorescence assay. Colchicine-treated cells had round shapes [Figure 1a]. Pravastatin, colchicine, and cisplatin significantly decreased the axon length compared to vehicle control [Figure 1b].

3.3 Evaluation of Apoptosis Marker

To determine whether apoptosis occurred, we evaluated the levels of apoptosis markers. DNA fragmentation was detected in cisplatin-treated cells [Figure 2a], and cisplatin significantly increased the degree of fragmentation compared to vehicle control [Figure 2b]. The levels of *Bax* mRNA in cisplatin-treated and $CoCl_2$-treated cells were higher than in vehicle-treated cells [Figure 2c]. On the other hand, there were no statistically significant changes in the levels of *Bcl2* mRNA [Figure 2d]. Cisplatin increased the protein levels of Bax, but not Bcl-2 [Figure 2e]. Bax to Bcl-2 protein ratio in cisplatin-treated and $CoCl_2$-treated cells was significantly increased compared to vehicle-treated cells [Figure 2f].

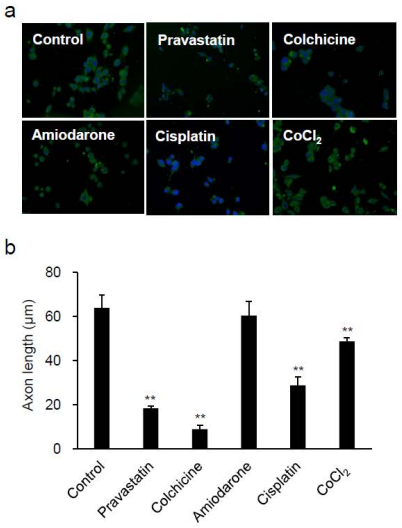


Figure 1. Effects of various chemicals on axon length in Neuro-2a neural cells. Neuro-2a neural cells were incubated with 10 μM pravastatin, 100 nM colchicine, 10 μM amiodarone, 1 μM cisplatin or 300 μM $CoCl_2$ for 48 hours. Fixed cells were visualized with anti-pNF-H antibody and fluorescent-labeled secondary antibody. The nuclei were counterstained with DAPI. **(a)** The image is a representative resultant photograph. **(b)** The average of length at least 50 axons per well was calculated for each well (values represent the mean ± SD; $n = 3$). Statistically significant differences compared with the control are indicated by **, $p < 0.01$ in Dunnett's test.

3.4 Evaluation of Phospho-neurofilament Heavy Chain Release

To investigate the axon damage by neurotoxic chemicals, we evaluated the protein levels of pNF-H in both supernatants and cell lysates. The levels of pNF-H in the supernatant of pravastatin-treated, colchicine-treated, and $CoCl_2$-treated cells were higher than in that of vehicle-treated cells [Figures 3a and 3b]. On the other hand, in cell lysate, the level of pNF-H in pravastatin-treated cells was lower than in vehicle-treated cells [Figure 3c].

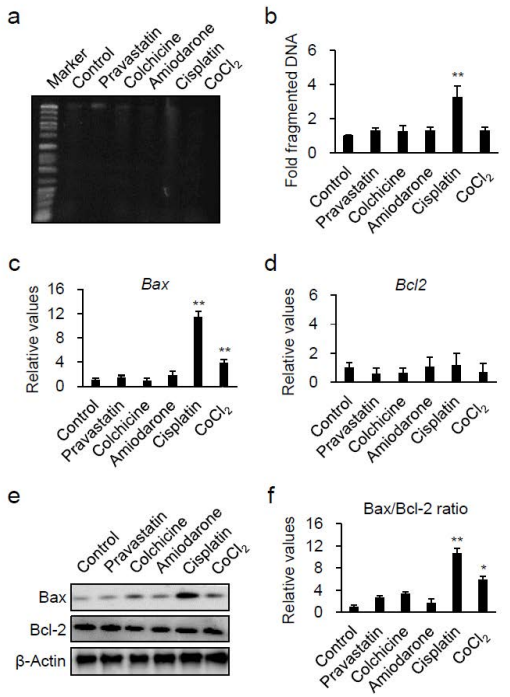


Figure 2. Effects of various chemicals on apoptosis markers in Neuro-2a neural cells. Neuro-2a neural cells were incubated with 10 μM pravastatin, 100 nM colchicine, 10 μM amiodarone, 1 μM cisplatin or 300 μM $CoCl_2$ for 48 hours. DNA ladder assay was performed. **(a)** The image is a representative gel electrophoresis. **(b)** The degree of fragmentation well was determined by densitometry (values represent the mean ± SD; *n* = 3). Neuro-2a neural cells were incubated with 10 μM pravastatin, 100 nM colchicine, 10 μM amiodarone, 1 μM cisplatin or 300 μM $CoCl_2$ for 48 hours. qRT-PCR was performed for *Bax* mRNA **(c)** and *Bcl2* mRNA **(d)** and data were normalized to *Actb* mRNA as the endogenous control (values represent the mean ± SD; *n* = 3). Neuro-2a neural cells were incubated with 10 μM pravastatin, 100 nM colchicine, 10 μM amiodarone, 1 μM cisplatin or 300 μM $CoCl_2$ for 48 hours. Western blotting was performed for Bax protein **(e)** and Bcl-2 protein **(f).** The intensities of immunoreactive proteins were quantified by densitometry and data were normalized to β-Actin protein as the endogenous control (values represent the mean ± SD; *n* = 3). Statistically significant differences compared with the control are indicated by *, $p < 0.05$ and **, $p < 0.01$ in Dunnett's test.

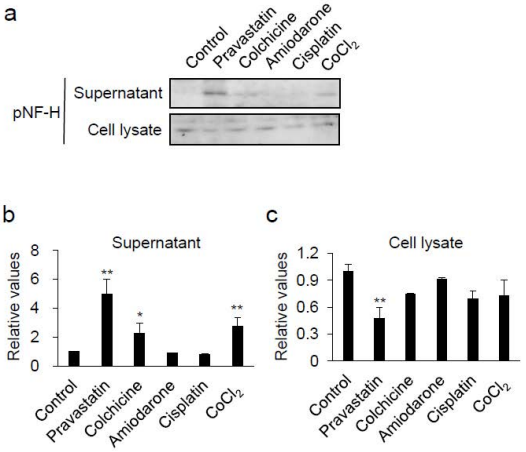


Figure 3. Effects of various chemicals on release of phosphorylated neurofilament heavy chain in Neuro-2a neural cells. Neuro-2a neural cells were incubated with 10 μM pravastatin, 100 nM colchicine, 10 μM amiodarone, 1 μM cisplatin or 300 μM CoCl2 for 48 hours. Western blotting was performed for phosphorylated neurofilament heavy chain (pNF-H) protein in the both supernatant and cell lysate (a). The intensities of pNF-H proteins were quantified by densitometry in the supernatant (b) and cell lysate (c) (values represent the mean ± SD; n =3). Statistically significant differences compared with the control are indicated by *, $p < 0.05$ and **, $p < 0.01$ in Dunnett's test.

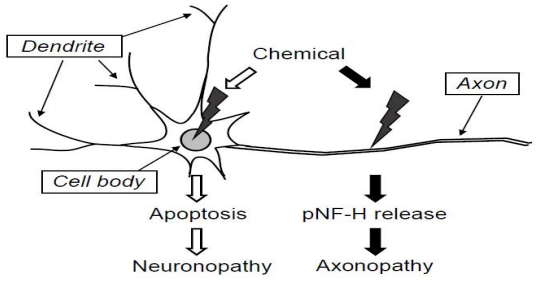


Figure 4. Schematic difference between neuronopathy and axonopathy by neurotoxic chemicals. Neuronopathic chemicals induced the apoptosis marker and axonopathic chemicals induced the extracellular phosphorylated neurofilament heavy chain (pNF-H) in Neuro-2a neural cells.

4. Discussion

Chemicals cause neuropathy by three major pathologic mechanisms causing peripheral neuropathy: axonopathy, neuronopathy, and myelinopathy. There is little potential for recovery in neuronopathy due to loss of function. By contrast, partial or complete recovery can occur in both axonopathy and myelinopathy since peripheral axons can regenerate[5]. Therefore, the elucidation of the mechanism of neuropathy is indispensable to identify potential targets and approaches for prevention and treatment. In this study, the various neurotoxic chemicals were evaluated by using Neuro-2a neural cells. We demonstrated that neuronopathic and axonopathic chemicals were specifically detected by evaluation of both levels of apoptosis marker and pNF-H [Figure 4].

pNF-H is expected for clinical application as a blood biomarker of disorders of axons, because pNF-H is specifically expressed in the axon[28,34]. In contrast, Sumitani *et al.* suggested that serum pNF-H is not suitable as a marker of peripheral neuropathy[35]. In this study, we demonstrated for the first time that axonopathic chemicals, such as pravastatin and colchicine, induced the levels of pNF-H in the culture supernatants, but cisplatin and amiodarone did not affect the levels of pNF-H. In short, the axon damage induced the increase of pNF-H release from Neuro-2a neural cells. These indicate that the extracellular pNF-H levels was useful as a marker of axonopathy *in vitro*.

Human study showed an increased risk of peripheral neuropathy with long-term statin drug use[36,37]. The mechanisms of statin-induced peripheral nerve injury are fully unclear. Due to the intrusive role of these drugs in the production of cholesterol, they might change the function and integrity of cell membranes, in which cholesterol plays a key role. In addition, statins inhibit the synthesis of coenzyme Q10, which is an integral component of the respiratory chain[38,39]. Inhibition of cholesterol synthesis in cell bodies inhibited axonal growth[40]. Atorvastatin causes a marked decrease in blood coenzyme Q_{10} concentration[41]. Thus, it is assumed that statin drugs damage the axons of neuron. In this study, the short length of the axon and the release of pNF-H were observed in the exposure of pravastatin without inducing apoptosis. Another statin drug, atorvastatin, significantly decreased the axon length and increased the levels of extracellular pNF-H compared to vehicle control at the concentration of 10 μM (data not shown). These indicate that our system can detect axonopathic chemicals such as statin drugs.

Colchicine-treated cells had round shapes in Neuro-2a neural cells. Many studies reported that colchicine induced round morphology in various cells by inhibition of microtubule polymerization[42-44]. The neurotoxicity of colchicine appears to be related to the destruction of microtubules. The transport of protein and metal ions was more profoundly blocked by colchicine in the neuron[45,46]. Colchicine has the axonopaphic mechanism different from statin drugs. Thus, our system can detect the potency of various axonopathic chemicals.

Cisplatin decreased the length of axons and increased the apoptosis markers, but had no effects on the release of pNF-H. Apoptosis is associated with the short length of the axon[47]. Several mechanisms contributing to cisplatin-induced neurotoxicity have been suggested cell apoptosis by oxidative stress[48,49]. Anti-oxidants, such as vitamin E and amifostine, has protective effects against cisplatin-induced neuropathy[50-52]. These suggest that cisplatin induces apoptosis and consequently probably leads the decrease in axon length of Neuro-2a neural cells. Therefore, neuronopathy can cause the short length of axons. Thus, these indicate that the evaluation of both pNF-H and apoptosis marker is needed to identify the mechanism of neuronal toxicity at the least.

$CoCl_2$ treatment induced the decrease of axonal length, and an increase of apoptosis markers and extracellular pNF-H. $CoCl_2$ is well known as a hypoxia-inducing chemical to mimic hypoxic conditions[53]. $CoCl_2$ induced cell death in rat neuroblastoma PC12 and Neuro-2a neural cells[13,54]. Several groups have investigated the role of hypoxia in central and peripheral neuropathy. The loss of oxygen to the brain may not completely recover[55]. There is a significant positive correlation between the degree of hypoxemia and the severity of neuropathy[56]. However, the type of neuropathy of $CoCl_2$ was still incompletely understood. This study suggest that $CoCl_2$-mimicked hypoxia may simultaneously damage both axons and cell bodies in Neuro-2a neural cells. Thus, we expect that our system contributes to the elucidation of the mechanism of neuropathic chemicals.

Unfortunately, amiodarone had no effects of axon length, apoptosis markers levels, and pNF-H release; thereby the mechanism of myelinopathy was not determined in our system as expected. Pathogenic mechanisms of amiodarone against neuron is myelin disorder[9]. The myelin is not generated in a single culture

of Neuro-2a neural cells such as this study. To evaluate the myelinopathic potency of chemicals using *in vitro* model exactly, co-culture systems utilizing the nerve cells and Schwann cells are needed[57-59]. However, the our system that can be carried out by easy and simple procedure are advantageous and valuable to identify the neuronopathic or axonopathic chemicals. Therefore, we believe that our system can be useful as the first screening.

5. Conclusion

In summary, we demonstrated that axonopathic chemicals, such as pravastatin and colchicine, induced the release of pNF-H from Neuro-2a cells without inducing apoptosis. On the other hand, neuronopathic chemical, cisplatin, induced the levels of apoptosis marker alone. Further, we found that $CoCl_2$ damaged both cell bodies and axons of Neuro-2a neural cells. Therefore, this study indicates that the evaluation with a combination of apoptosis marker and pNF-H release can distinguish the neuronopathy and axonopathy. Thus, our system supports the elucidation of the neuropathic mechanism of chemicals.

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