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ORIGINAL ARTICLE

Flusilazole Induced Cytotoxicity and Inhibition of Neuronal Growth in Differentiated SH-SY5Y Neuroblastoma Cells by All-Trans-Retinoic Acid (Atra)

All-Trans-Retinoik Asit (Atra) ile Farklılaştırılmış SH-SY5Y Nöroblastoma Hücrelerinde Flusilazole Bağlı Sitotoksisite ve Nöronal Büyüme İnhibisyonu

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ABSTRACT

Objectives: Flusilazole (FLUS) is a broad-spectrum organosilicon triazole fungicide used for protecting economically important cereals and orchard fruits. Considering the exposure route of pesticides, pesticide contamination of food is inevitable. Furthermore, excessive exposure to pesticides causes health problems in both target and non-target organisms. It was aimed to evaluate the effects of the triazole fungicide FLUS on cytotoxicity and neurite extension in differentiated SH-SY5Y neuroblastoma cells.

Materials and Methods: The SH-SY5Y cells were differentiated into mature neurons using 10-μM all-trans-retinoic acid (RA) treatment for 7 days. Then the differentiated SH-SY5Y cells were treated with 50, 100 and 200 μM FLUS for 24 h. Afterwards, cell viability assays were performed including crystal violet, neutral red cell viability, and lactate dehydrogenase leakage assays. The morphological examinations were performed and neurite lenghts of the cells were measured in all experimental groups.

Results: FLUS treatment induced cytotoxicity in SH-SY5Y cells differentiated with RA. Significant decreases in cell viability percentages were observed. Furthermore, neurite lengths were negatively affected by the treatment of FLUS at the highest concentration.

Conclusion: FLUS is a fungicide widely used in agriculture to protect crops from fungal diseases. However, the intensive use of these compounds causes a potential risk to human and environmental health. According to the results of the study, it can be concluded that high concentrations of FLUS cause neurotoxicity by causing neural cell death and adverse effects on neurite outgrowth in differentiated SH-SY5Y cells. FLUS exposure can cause neuronal degeneration in mammals.

Key words: Flusilazole, cytotoxicity, SH-SY5Y cell differentiation, neurite growth

ÖZ

Amaç: Flusilazol (FLUS) ekonomik açıdan önemli tahıl ve tahıl ürünlerini ve meyve bahçelerini korumak için kullanılan geniş spektrumlu bir organosilikonlu triazol fungisittir. Pestisitlerin maruziyet yolları dikkate alındığında besin yoluyla pestisit maruziyeti kaçınılmaz olmaktadır. Ayrıca pestisitlere aşırı derecede maruz kalmak hedef ve hedef olmayan organizmalarda sağlık sorunlarına neden olmaktadır. Çalışmanın amacı, bir triazol fungisit olan FLUS'nin farklılaştırılmış SH-SY5Y nöroblastoma hücrelerinde sitotoksisite ve nörit uzaması üzerine etkilerinin değerlendirilmesidir.

Gereç ve Yöntemler: SH-SY5Y hücreleri 10 μM all-trans retinoik asit (RA) ile 7 gün boyunca olgun nöronlara farklılaştırılmıştır. Farklılaştırılmış SH-SY5Y hücreleri 50, 100 ve 200 μM FLUS ile 24 saat boyunca muamele edilmiştir. Kristal viyole, nötral kırmızısı canlılık testleri ile laktat dehidrogenaz salım testlerini kapsayan hücre canlılık testleri yapılmıştır. Ek olarak, morfolojik incelemeler yapılmış ve hücrelerin nörit uzunlukları ölçülmüştür.

Bulgular: FLUS uygulaması RA ile farklılaştırılan SH-SY5Y hücrelerinde sitotoksisiteyi indüklemiştir. Hücre canlılık yüzdelerinde dikkate değer düşüşler gözlenmiştir. Ayrıca en yüksek konsantrasyondaki FLUS uygulaması ile nörit uzunlukları olumsuz yönde etkilenmiştir.

Sonuç: FLUS tarımda ekinleri fungal hastalıklardan koruma amacıyla yaygın olarak kullanılan bir fungisittir. Fakat bu bileşiklerin yoğun kullanımı insan ve çevre sağlığı açısından potansiyel risk oluşturmaktadır. Çalışma sonuçlarına göre, yüksek konsantrasyonlarda FLUS'nin farklılaştırılmış SH-SY5Y hücrelerinde nöral hücre ölümüne ve nörit büyümesinde olumsuz etkilere neden olarak nörotoksisiteye yol açtığı sonucu çıkarılabilir. FLUS maruziyeti memelilerde nöronal dejenerasyona neden olabilir.

Anahtar kelimeler: Flusilazol, sitotoksisite, SH-SY5Y hücre farklılaşması, nörit büyümesi

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INTRODUCTION

The use of pesticides in agriculture enhances the production quality of food and feeds. However, it causes several health problems due to environmental and food contaminations. Among these pesticides, triazole fungicides are commonly used in agriculture for preventing fungal infections in fruits, cereals, and vegetables and have pharmacological uses for human and animal health.[1,2] Consequently, flusilazole (FLUS) is a broad-spectrum organosilicon triazole fungicide that is used for protecting economically important cereals and orchard fruits.[3,4] Its action mechanism can be attributed to the inhibition of the lanosterol 14 alpha-demethylase (CYP51) enzyme that plays a key role in sterol biosynthesis in fungus.[5] The CYP51 inhibition causes a reduction in the synthesis of ergosterol, which is the basic element of the fungal cell wall and prevents fungal cell growth. Consequently, the cell growth inhibition results in fungal cell death. As CYP51 enzyme exists in human, studies have reported that CYP51 inhibition caused by triazole fungicides can adversely affect mammalian cells.[6] Previous *in vitro* and *in vivo* studies asserted that triazole fungicides have adverse effects on mammalian steroidogenesis and may induce developmental toxicity such as craniofacial malformations.[7,8] One study revealed that the estimated acceptable daily intake of FLUS is about 0-0.007 mg/kg bw for humans.[9] However, considering the exposure route of pesticides, pesticide contamination of food is inevitable and excessive exposure to pesticides can cause health problems on both target and non-target organisms. Although previous studies have already focused on cytotoxicity and oxidative stress of FLUS on dopaminergic PC12 cells,[6] it is believed that the present study is the first to reveal the effects of FLUS on neurite growth and to compare cytotoxicity assays on differentiated SH-SY5Y cells.

The human neuroblastoma cell line SH-SY5Y cells are commonly used in neurotoxicity and neurodegenerative disease models.[10] *In vitro* models of SH-SY5Y can be differentiated into mature dopaminergic neuron-like phenotype via the induction of retinoic acid (RA),[11] which is known to regulate the cell cycle.[12] This differentiation of the neuronal cells in experimental studies could provide homogenous neuronal cells.[11] Additionally, studies have shown that RA-induced differentiation can elevate the susceptibility of SH-SY5Y cells against neurotoxins and protective agents.[13] Furthermore, differentiated SH-SY5Y cells are known to serve as a good model for studying experimental Parkinson's disease model.[13] The increased incidence of neurological diseases in recent years has necessitated the increase in experimental studies evaluating the relationship between chemicals and diseases. As exposure to environmental contaminants is inevitable, revealing the potential neurotoxicity effects of commonly used fungicides is important. In the present study, it is aimed to evaluate the effects of FLUS on neurite extension in differentiated SH-SY5Y neuroblastoma cells and to compare three commonly used cytotoxicity tests.

MATERIALS AND METHODS

Cell culture conditions and differentiation of SH-SY5Y cells

SH-SY5Y human neuroblastoma cells purchased from American Type Culture Collection (ATCC® CRL-2266™, ATCC, VA, USA), which was cultured with Dulbecco's minimum essential medium/nutrient mixture F-12 (DMEM/F-12) (Cegrogen Biotech GmbH, Germany) supplemented by 10% fetal bovine serum (FBS) (Cegrogen Biotech GmbH, Germany) and 1% penicillin-streptomycin antibiotic mixture at 37°C with 5% CO_2 in a humidified incubator. The culture medium was renewed every 3 days and subcultured the cells by detaching them with trypsin ethylenediaminetetraacetic acid (0.05%) in Dulbecco's phosphate-buffered saline (Cegrogen Biotech GmbH, Germany). SH-SY5Y cells then was incubated for 48 h for attachment. Finally, the culture medium was replaced with differentiation medium (DMEM supplemented with 3% FBS and 10 µM all-trans-RA) for 7 days until treatment in the dark at 37°C with 5% CO_2 in a humidified incubator.

Treatment of FLUS

First, FLUS was dissolved (PESTANAL®, analytical standard, Merck KGaA, Darmstadt, Germany) in dimethyl sulfoxide (Applichem, Darmstadt, Germany) to prepare 0.1 g/mL stock solution. Then the working concentrations were prepared by diluting the stock solution with cell culture medium. Next, the differentiated SH-SY5Y cells were incubated with 0-500 µM FLUS concentrations and performed crystal violet cell viability assay to calculate the IC_{50}, which was found at 182.42 µM. The cell viability (15%) at high concentrations (500 µM) was found very toxic. The working concentrations were based on the calculated IC_{50} value for further analyses. It is defined low and middle doses as those with slightly toxic concentrations lower than the IC_{50} and for high dose selection while high doses are those with concentrations higher than the IC_{50} value. Finally, 50-, 100-, and 200-µM FLUS concentrations were selected for further analyses.

Crystal violet cell viability assay

First, the differentiated SY-SY5Y cells were seeded at 1×10^4 cells/well into a 96-well culture plate and incubated them for 24 h for cell attachment. Then the cells were treated with 0, 50, 100, and 200 µM FLUS at 37°C and 5% CO_2 in a humidified incubator for 24 h after which we performed a crystal violet cell viability assay.[14] Briefly, the culture medium was discarded with FLUS and mixed the cells with 4% neutral buffered formalin for 1 h. After removing the fixative, the cells was stained with 0.1% crystal violet solution for 30 min on a shaker at room temperature. The cells then were washed with distilled water several times to remove excess crystal violet dye. The crystal violet dye was extracted in the cells using 10% acetic acid solution until the dye was dissolved and then measured the absorbance at 595 nm wavelength using a microplate spectrophotometer (BIO-TEK µQuant, BIO-TEK Instruments, Inc., USA). Finally, the cell viability was calculated based on the 100% viability of untreated cells.

Neutral red uptake assay

Similarly, the differentiated SH-SY5Y cells were seeded into a 96-well culture plate at a density of 1×10^4 cells/well and allowed them to attach onto the surface and grow for 24 h. Afterwards, the cells were treated with FLUS concentrations (0, 50, 100, 200 µM) and were incubated at 37°C and 5% CO_2 in a humidified

incubator for 24 h. A neutral red uptake assay was performed to determine the cell viability. Briefly, the differentiated SH-SY5Y cells were incubated with 40 µg/mL neutral red dye containing the culture media for 4 h at 37°C and 5% CO_2 in a humidified incubator. Then the culture medium was discarded, the cells were washed twice with phosphate buffer saline twice, and extracted the dye using a neutral red desorb solution (1% glacial acetic acid, 50% ethanol in distilled water) for 20-45 min on a shaker at room temperature. Afterwards, the absorbance was measured at 540 nm wavelength for 1 h using a microplate spectrophotometer (BIO-TEK µQuant, BIO-TEK Instruments, Inc., USA). Finally, the cell viability was calculated based on the 100% viability of untreated cells.

Lactate dehydrogenase (LDH) leakage assay

LDH is a stable cytosolic enzyme that is released from cells when cell membrane damage occurs. Consequently, LDH leakage assay is commonly used to determine cell membrane damage. We performed an LDH assay using a commercial kit (Biovision, K313-500, USA). Briefly, the differentiated SY-SY5Y cells were seeded at a density of 2×10^4 cells/well into a 96-well culture plate and incubated them at 37°C and 5% CO_2 in a humidified incubator for 24 h. Then the culture medium was discarded and the cells were treated with FLUS concentrations (0, 50, 100, and 200 µM) for 24 h. At the end of the incubation time, the cells were centrifuged at 600 g for 10 min using a plate rotor (5810R Centrifuge, Ependorf AG, Hamburg, Germany). Afterwards, I incubated 10 µL sample with 100 µL LDH reaction solution for 30 min in the dark at room temperature. This assay utilizes the enzymatic coupling reaction in which LDH oxidized lactate to generate nicotinamide adenine dinucleotide and where the water-soluble tetrazolium salt present in the reaction solution generates yellow to amber color. The generated color intensity directly correlates with the damaged cell amount. Finally, at the end of the incubation time, we measured the absorbance at 450 nm wavelength using a microplate spectrophotometer (BIO-TEK µQuant, BIO-TEK Instruments, Inc., USA). The percentage of the cytotoxicity was calculated based on the 100% viability of untreated cells.

Cell morphology analysis

The cell mofphology analysis was performed by capturing micrographs of undifferentiated or differentiated treated cells using an inverted microscope (Olympus CKX 41 inverted microscope, CellSence Imaging Software, Olympus, Japan). Each experiment was performed by measuring the neurite lengths of undifferentiated or differentiated neurons using an Image J Package with Neuron J.[15,16]

Statistical analysis

The statistical analyses were performed using the package program of SPSS for Windows and calculated the IC_{50} value through probit analysis. In order to determine the significant differences between FLUS treatment and non-treatment groups, ANOVA test was performed with 95% confidence interval followed by Tukey post-hoc test. The data were expressed as mean ± standard error. All experiments were performed in independent triplicates.

RESULTS

Morphological features of undifferentiated and differentiated SH-SY5Y cells

The SH-SY5Y cells were differentiated by treating them with 10 µM RA for 7 days. Figure 1 demonstrates morphological features of undifferentiated and differentiated cells. Undifferentiated cells can be distinguished by the presence of clustered round cells and short neurites (Figure 1A). On the other hand, morphologically differentiated SH-SY5Y cells are characterized by extensive neurites as well as pyramid-shaped cells (Figure 1B). To better distinguish the differences, the neurite lengths of undifferentiated and differentiated SH-SY5Y cells were measured, as shown in Figure 1C. While undifferentiated cells showed shorter neurites, neurites were longer in cells that underwent 7-day RA-induced differentiation. Differentiated SH-SY5Y cells were treated with different FLUS concentrations. Figure 2 demonstrates the morphological effects of FLUS on RA-induced differentiated SH-SY5Y cells. Accordingly, results showed that FLUS treatment caused remarkable morphological changes in differentiated SH-SY5Y cells, including apoptotic cell death, which is characterized by round and small nuclei and a decrease in neurite length (Figure 2). Apoptotic cells increased in FLUS treatment groups, and neurite length decreased in response to the highest FLUS concentration, which was statistically significant compared with those in the non-treatment group.

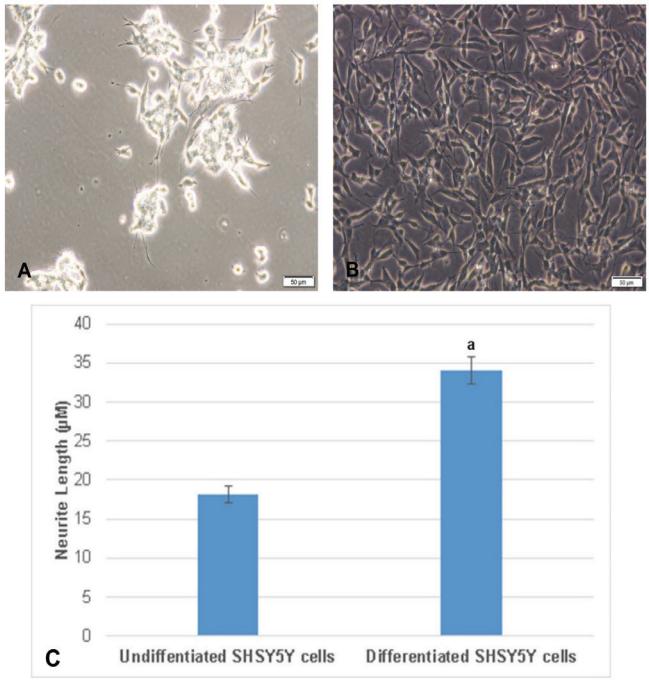

Figure 1. A) Undifferentiated SH-SY5Y cells with clustered, round-shaped cell body and short neurites; B) RA-induced differentiated SH-SY5Y cells with pyramidal-shaped cell body and extended neurites; C) neurite lengths of undifferentiated and differentiated SH-SY5Y cells

RA: Retinoic acid

Cell viability assay results

The cytotoxic effects of FLUS was evaluated by performing different cytotoxicity assays (i.e., crystal violet, LDH, and neutral red cell viability assays). Crystal violet assay revealed an IC_{50} value of 182.42 µM, which was the basis of our working concentrations. As demonstrated in Figure 3, the decrease in cell viability was statistically significant in 100- and 200-µM FLUS treatment groups as compared with that in the control and 50-µM FLUS treatment groups. Meanwhile, as shown in Figure 4, LDH leakage assay results revealed that occurrences of cell death increased in response to increasing FLUS concentrations. At higher concentrations, FLUS increased the percentage of cell death viability, which was statistically significant in the 100- and 200-µM FLUS treatment groups when compared with that in the non-treatment and 50-µM FLUS treatment groups. On the other hand, according to neutral red cell viability assay results (Figure 5), remarkable decreases in cell viability were statistically significant in all treatment groups. Significant decreases were found between non-treatment and FLUS treatment groups. Additionally, the cell viability in the 200-µM FLUS treatment group was significantly different from that in the 50- and 100-µM FLUS treatment groups.

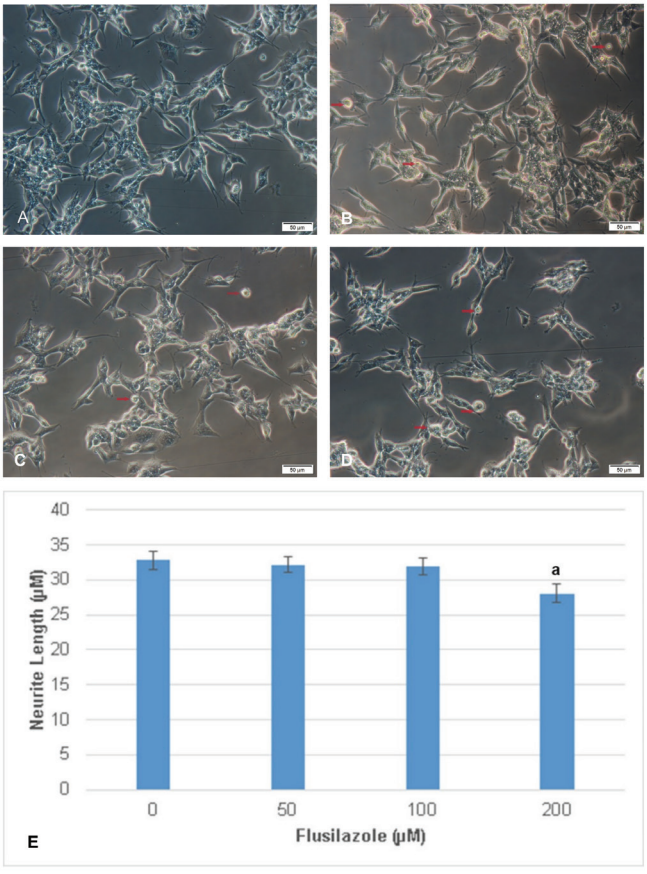

Figure 2. Morphological micrographs of differentiated SH-SY5Y cells in the FLUS treatment groups: A) Control group; B) 50-µM FLUS treatment group; C) 100-µM FLUS treatment group; D) 200-µM FLUS treatment group; (→) apoptotic cell

FLUS: Flusilazole

DISCUSSION

Triazole fungicides have been used globally in various areas including agriculture and medicine. However, excessive use of fungicides could leave harmful residues in the environment and cause risks in human health via consumption of contaminated food and water.[17] For example, studies have shown that triazole

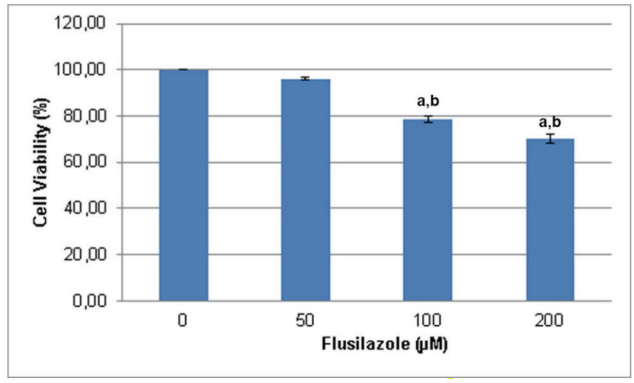

Figure 3. Crystal violet cell viability assay results (%). aSignificantly different from control group. bSignificantly different from 50-µM FLUS group

FLUS: Flusilazole

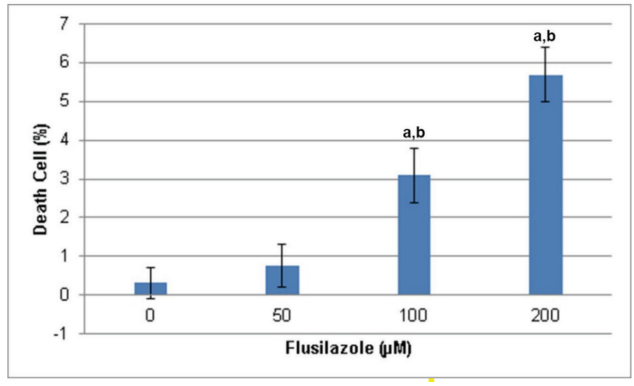

Figure 4. LDH leakage assay results, cell death (%). [a]Significantly different from control group. [b]Significantly different from 50-µM FLUS group

LDH: Lactate dehydrogenase, FLUS: Flusilazole

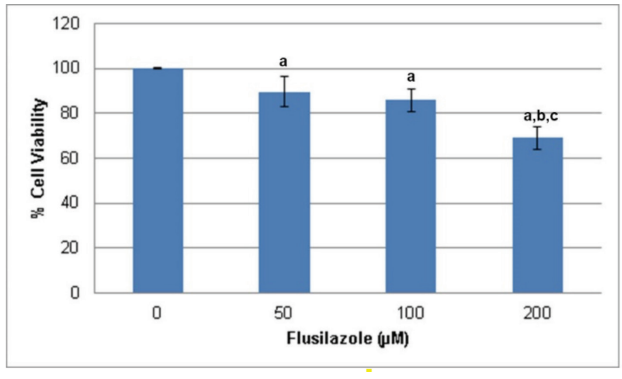

Figure 5. Neutral red cell viability assay results. [a]Significantly different from control group. [b]Significantly different from 50-µM FLUS group. [c]Significantly different from 100-µM FLUS group

FLUS: Flusilazole

fungicides, which are used to inhibit fungal CYP51 and increase fungal cell wall permeability to kill fungi,[6,7] cause harmful effects on the nervous system via neuropathological defects in the murine brain as well as neuropathological lesions in the peripheral nervous system.[18] Other studies also reported several adverse effects of azole fungicides such as birth defects, craniofacial malformations, and inhibition of steroidogenesis in mammals.[5,19,20] Despite the wide range of risks in human health caused by triazole fungicides, studies about the effects of FLUS on human health are very limited.[6,7] Because of the limited information on the effects of FLUS on differentiated neurons, this study aimed to elucidate the toxicity mechanism of FLUS on neurite outgrowth and to compare cytotoxicity tests using FLUS treatment.

In the field of neuroscience, *in vitro* models functionally resembling neurons are lacking, especially for neurodegenerative diseases including Alzheimer's disease. Although cell lines such as SH-SY5Y cells are often used, these cells lack mature neuronal functions and morphology, have ceased cell division, and express specific markers.[21] However, SH-SY5Y cells could be differentiated into mature neuron, and differentiated neuron cells could serve as homogenous cell types, as demonstrated in previous studies where SH-SY5Y cells were differentiated into mature neuron-like cells by RA induction on a 7-day treatment in dark conditions, and differentiated cells morphologically changed as characterized by extensive neurites outgrowth.[22,23] Hence, the present study aimed to investigate the cytotoxic mechanisms of FLUS on differentiated SH-SY5Y cells. The action mechanism of RA was characterized by the activation of transcription via binding to non-steroid nuclear hormone receptors. Additionally, RA was shown to induce the Wnt signaling pathway and play a role in the regulation of the neurotrophin receptor gene transcription.[13] In this study, it was observed that morphologically differentiated SH-SY5Y cells had typical neurons with extensive neurites and pyramid-shaped cell bodies. On the other hand, undifferentiated cells tend to form clusters and had round-shaped cell bodies.[11] According to these results, I can say that the morphological differentiation of SH-SY5Y cells was successful.

In vitro cytotoxicity assays can be useful in determining toxicity levels in humans in toxicological studies. Cytotoxicity assays differ in the types of assays as well as results depending on the test principle.[24] It is important to carry out multiple markers to exert reversible or irreversible effects of toxic substances.[25] In this study, three cytotoxicity assays were performed based on different test principles for differentiated SH-SY5Y cells to evaluate the mechanism of toxicity. The crystal violet assay was selected to evaluate cell viability because of its reliable and quick method and because crystal violet specifically binds to DNA to reveal the cell viability. Neutral red uptake assay as performed to evaluate the percentage of cell viability in terms of lysosomal activity and the LDH leakage assay to evaluate necrotic cell death.

To evaluate the viability of differentiated SH-SY5Y cells, we performed crystal violet cell viability assay, which is a basic method for determining cell viability by staining with crystal violet dye that binds to DNA and proteins. This method depends on staining attached viable cells because dead cells lose their absorption and the amount of crystal violet dye is reduced.[26] Results from this assay showed that FLUS treatment caused the decrease in differentiated SH-SY5Y cells in a dose-dependent manner. A previous study with 9.5-day-old rat embryos *in vitro* reported that triazole treatment altered hindbrain development and caused cranial nerve anomalies. The same study also found that FLUS and other triazole derivatives were teratogenic.[20] The present study has shown that FLUS can induce cell death at higher concentrations, consistent with previous studies.

Results showed that the release of LDH, an intracellular enzyme found in the inner compartment of the cell, was significantly increased at higher concentrations in the FLUS treatment groups. Consequently, LDH leakage assay is described as a cytotoxicity marker that reveals cell membrane damage as irreversible cell death.[27] As necrotic cells have permeable cell membrane, leakage of LDH into the culture media as a result of membrane damage is considered a hallmark of necrotic cell death.[28] Hence, in the present study the LDH leakage assay was used to determine the necrotic cell death percentage. A previous study treated zebra fish liver cell line with triazole fungicides and evaluated the effects using LDH leakage assay. That study revealed remarkable increases in LDH leakage at higher concentrations of FLUS.[29] Similarly, the results of the present study were consistent with the previously reported study as is another previous study that also used triazole fungicide to induce necrotic cell death in L929 cells.[30] The present study revealed that FLUS could adversely affect human neuronal cells as membrane damage occurred in differentiated SH-SY5Y cells after FLUS treatment, which eventually induced necrotic cell death.

The neutral red uptake assay was performed to determine cytotoxicity. The principle of this assay is based on the uptake of neutral red dye (a water-soluble dye) into the lysosomes, which processes energy required for cells.[31] The neutral red uptake depends on pH gradients during the ATP production process. As the net charge of the dye is zero, neutral red dye could enter the cell. However, the interior of the lysosomes has a lower pH than the cytoplasm via proton gradient. Because of the pH gradient, the neutral red dye keeps the cytoplasm positively charged so that the dye could retain inside the lysosomes. Conversely, neutral red dye could not be retained in the lysosomes in case of cell death or pH gradient changes. Studies reported that this assay is highly sensitive to cell viability quantification.[32] When plasma or lysosomal membranes are damaged by chemicals, the ability of the endocytosis, which requires energy for the process, to uptake the neutral red dye decreases. According to neutral red uptake assay results in this study, high concentrations of FLUS in SH-SY5Y cells adversely affected cell viability. These results indicate that FLUS may cause lysosomal damage especially at higher concentrations. All cytotoxicity test results demonstrated a downward trend in cell viability. Despite some variations, it seemed that the most

sensitive test for FLUS in differentiated SH-SH5Y cells was the crystal violet assay.

Environmental neurotoxins including pesticides and fungicides have been reported to play a crucial role in the development of neurodegenerative disorders.[33-35] In differentiated neurons, changes in morphology or neurite growth could be a sign of neurodegenerative diseases. To date, several cell lines including SH-SY5Y have been used to study the effects of chemicals on neurite growth.[36] Consequently, SH-SY5Y cells differentiated via RA induction could serve as a good *in vitro* model for revealing neurotoxicity mechanisms[37] as differentiated SH-SY5Y cells were reported to have the longest neurite measurement after treatment with 10 µM RA for 3 days.[38] In the present study, differentiated SH-SY5Y cells had longer neurites than undifferentiated cells. Neurite growth induced by RA treatment were >1.9 fold compared with that in undifferentiated cells. Previous *in vitro* studies involving organophosphates reported a reduction in neurite outgrowth in N2a mouse neuroblastoma and C6 glioma cells,[39] We observed that FLUS treatment caused the inhibition in neurite outgrowth of differentiated SH-SY5Y cells.

CONCLUSION

As mentioned earlier, FLUS is widely used as a fungicide in agriculture for preventing fungal diseases. However, intensive usage of these compounds has potential health risks for both humans and the environment. The present study has demonstrated that FLUS caused neurotoxicity at higher concentrations resulting in neuronal cell death and adversely affecting neurite growth in differentiated neurons, indicating that FLUS could cause neuronal degeneration in mammals. The results in this study may serve as preliminary data for further studies to elucidate the mechanism of action.

Conflict of interest: No conflict of interest was declared by the author. The author are solely responsible for the content and writing of this paper.

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