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IN VITRO TOXIC EFFECTS OF AZOXYSTROBIN ON HUMAN NEUROBLASTOMA CELL LINE

AZOXYSTROBİN'İN İNSAN NÖROBLASTOMA HÜCRE HATTI ÜZERİNDEKİ *İN VİTRO* TOKSİK ETKİLERİ

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ABSTRACT

Objective: Exposure to agricultural chemicals is associated with health issues such as autism, brain aging, and neurodegenerative diseases. Azoxystrobin (AZS), a strobilurin-derived fungicide, is a commonly used chemical. Although studies have demonstrated that AZS induces toxic effects on various tissues, there is a lack of clear understanding of the mechanisms underlying its neurotoxic effects. The mechanisms linking pesticide exposure to these health outcomes might be oxidative stress and apoptosis. Therefore, we conducted this study to investigate oxidative stress and cell death induction in human neuroblastoma SH-SY5Y cells, a widely established *in vitro* model for neurotoxicity experiments, after exposure to AZS.

Materials and Methods: SH-SY5Y cells were exposed to different concentrations of AZS for 24 h. Cytotoxicity was evaluated using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide assay, the induction of reactive oxygen species (ROS) was determined using a fluorescent dye, and cell apoptosis was detected using the FITC Annexin V assay.

Results: The half-maximal inhibitory concentration value of AZS was 44.87 µM. The levels of ROS (at least 1.5-fold; p≤0.05) and apoptosis (at least 5-fold; p≤0.05) increased in a dose-dependent manner at AZS exposure concentrations of 6.25-25 µM.

Conclusion: AZS-induced neurotoxic effects may be a consequence of ROS generation and ROS-induced apoptosis. However, the mechanisms underlying AZS-induced neurotoxicity should be evaluated by further studies to gain a deeper understanding of its toxic capacity and conduct evaluations of occupational and environmental risks.

Keywords: Azoxystrobin, apoptosis, oxidative stress, neurotoxicity

ÖZ

Amaç: Çalışmalar, tarım ilaçlarına maruziyetin otizm, beyin yaşlanması ve nörodejeneratif hastalıklar gibi sağlık sorunları ile ilişkili olabileceğini bildirmektedir. Yaygın kullanılan tarım ilaçlarından biri de strobilurin türevi bir fungusit olan azoksistrobin (AZS)'dir. Araştırmalar, çeşitli dokularda AZS'nin toksik etkilerini gösterse de nörotoksik etkilerin altında yatan temel mekanizmalar halen tam olarak anlaşılamamıştır. Tarım ilaçlarına maruziyetin sebep olduğu sağlık sorunlarının oksidatif stres ve apoptoz ile ilişkili olabieceği bildirilmektedir. Bu nedenle, nörotoksisite değerlendirmelerinde yaygın kullanılan bir *in vitro* model olan insan nöroblastoma (SH-SY5Y) hücrelerinde AZS'nin oksidatif hasar oluşturma ve hücre ölümünü indükleme etkisinin değerlendirilmesi amaçlanmıştır.

Gereç ve Yöntem: SH-SY5Y hücreleri 24 saat boyunca AZS'nin farklı konsantrasyonlarına maruz bırakılmıştır. MTT testi ile sitotoksisite değerlendirilirken spesifik floresan boya ile reaktif oksijen türlerinin (ROS) oluşumu ve FITC Annexin V testi ile de hücresel apoptoz seviyeleri incelenmiştir.

Bulgular: AZS'nin IC_{50} değeri 44.87 µM olarak bulunmuştur. ROS (en az 1,5 kat; p≤0,05) ve apoptoz (en az 5 kat; p≤0,05) seviyeleri 6.25-25 µM AZS maruziyet aralığında doza bağlı artmıştır.

Sonuç: Bulgularımız, AZS'nin neden olduğu nörotoksik etkilerin ROS üretimi ve ROS ile indüklenen apoptozun bir sonucu olabileceğini düşündürmektedir. Ancak, AZS'nin toksik etki potansiyelinin daha iyi anlaşılması, mesleki ve çevresel risk faktörlerinin değerlendirilmesi, AZS kaynaklı nörotoksisiteye neden olan temel mekanizmaların aydınlatılması için ileri araştırmalara ihtiyaç duyulduğu da göz ardı edilmemelidir.

Anahtar Kelimeler: Azoksistobin, apoptoz, oksidatif stres, nörotoksisite

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INTRODUCTION

Fungicides are globally used to protect crops and plants from fungal diseases originating in the soil and to prevent spoilage after harvest. They are essential for ensuring food safety, maintaining healthy plant growth, increasing agricultural yields, and maintaining the quality of produce. The adverse effects of fungicides on the environment and human health have been reported in association with both occupational and consumer risks (1). The use of fungicides in agriculture poses a potential health threat because they can spread from the field and present a risk to the local people and children in addition to agricultural laborers due to contact with air, soil, and water. Exposure to these environmental chemicals can cause reproductive dysfunction, developmental toxicity, hepatotoxicity, and neurotoxicity (2-9).

Strobilurins, a class of fungicides derived from the fungus *Strobilurus tenacellus*, have gained considerable attention in the agricultural industry (8, 10). Strobilurin fungicides work by binding to cytochrome b1 in fungal mitochondrial membranes, inhibiting mitochondrial respiration and disrupting the electron transport chain. This results in the inhibition of fungal growth and control of disease (8).

AZS is a strobilurin-based, systemic fungicide with a broad spectrum of application for fruit and vegetable crops for the postharvest treatment of agricultural products. The fact that AZS is applied postharvest implies that the time between fungicide application and consumption is relatively short compared with that for other active ingredients used during cultivation. Studies have demonstrated that postharvest fungicides can contribute to > 95% of the total human toxicity effects (5-7, 11-13). Recently, AZS has been incorporated into certain types of mold-resistant wallboard to prevent fungal growth (14). Over the past decade, AZS has gained popularity as a patented ingredient in mold-resistant building materials such as wallboard. These products were formally registered with the EPA (United States Environmental Protection Agency) in 2004 (15). Human exposure to AZS, other than through dietary ingestion of products containing residues, may be due to the recent use of AZS in mold-resistant building materials, including wallboard, which can eventually accumulate in house dust and potentially be inhaled or come into contact with the skin. This raises concerns regarding the possibility of AZS exposure indoors (14).

The widespread use of AZS has led to the accumulation of residues in the environment, including air, water, and soil, posing a risk to nontarget organisms such as aquatic organisms, animals, and humans. AZS has the ability to endure in soil for more than 300 days and reach its highest concentration approximately 30 days after application, posing a potential risk of contamination to nearby ecosystems (8). The European Food Safety Authority (EFSA) report on pesticide residues in food indicated the presence of residues on honey and beekeeping products (16). Soydan et al. found AZS to be the most frequently detected fungicide in the range of 0.011-0.758 mg/kg in fruit samples collected between 2012 and 2016 in Turkey, commonly being found in dried apricot and fig, grapes, strawberries, and peach (16, 17). Cooper et al. reported that AZS was the most commonly detected strobilurin (93% of dust samples) and was found at the highest concentrations, ranging from < MDL (method detection limit) to 10.587 ng/g, in house dust samples from homes in North Carolina during 2014-2016 (14).

AZS exerts its mechanism of action by acting as a typical mitochondrial complex III inhibitor by binding to ubiquinone Qo site and disrupting mitochondrial function by inhibiting electron transport, preventing adenosine triphosphate production and promoting neuronal cell death in fungi (18, 19). AZS was found to be cytotoxic to human esophageal cancer cells through the mitochondrial pathway and cause mitochondrial dysfunction (20). Mitochondrial dysfunction is associated with degenerative diseases of the nervous system, including Parkinson's and Alzheimer's diseases. The potential neurotoxicity of AZS raises concerns regarding its impact on human health and the development of neurological disorders. Despite the potential risks, there has been limited research on its neurotoxic effects (5, 8, 21), and the mechanisms underlying the neurotoxic effects of AZS are not completely understood. Therefore, to address the existing knowledge gap regarding AZS-induced neurotoxicity, we investigated the potential neurotoxicity of AZS in SH-SY5Y cells, a widely established *in vitro* model of neurodegeneration (22).

MATERIAL and METHODS

Chemicals

AZS (PESTANAL®, CAS # 131860–33–8) was purchased from Sigma-Aldrich (MO, USA), FITC Annexin V Apoptosis Detection Kit with PI was purchased from BioLegend (CA, USA), and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide), 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA), and other chemicals were obtained from Sigma-Aldrich (MO, USA). Cell culture supplements were purchased from Multicell Wisent (QC, Canada).

Cell Culture and Treatment Conditions

The SH-SY5Y cell line (CRL-2266, American Type Culture Collection, Virginia, USA) was grown in Dulbecco's Modified Eagle Medium F-12 supplemented with fetal bovine serum (10%) and penicillin/streptomycin (100 U/100 µg/mL). Cells (1×10^6/mL) were exposed to AZS. The working solutions of AZS were prepared at concentrations of 3.125, 6.25, 12.5, and 25 µM (in dimethyl sulfoxide [DMSO]). The solvent and positive controls were DMSO (1%) and Triton X-100 (1%), respectively.

Evaluation of Cytotoxicity

The effect of AZS on cell viability was evaluated using the MTT assay. Cells (1×10^5/100 µL cell culture medium/well) were incubated overnight and then treated with AZS at concentrations of 50-400 µM for 24 h. Then, the cells were incubated with MTT for 3 h at 37°C in the dark, after which the medium was removed, and DMSO (100 µL) was added to each well. Optical densities were measured at 590 nm using a microplate spectrophotometer system (Biotech, Epoch, Vermont, USA).

The half-maximal inhibitory concentration (IC_{50}) was then calculated using the formula from the logarithmic curve of the inhibition concentration graph.

Total Reactive Oxygen Species Assay

The 2',7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) dye was used to evaluate the production of cellular ROS (23). After 24 h of exposure, the cells were trypsinized and rinsed with phosphate-buffered saline, followed by incubation with H_2DCF-DA dye (20 mM) for 30 min. The intensity of ROS-dependent fluorescence after rinsing was detected using an ACEA NovoCyte flow cytometer (California, USA). Data were analyzed using the Novoexpress software (ACEA, CA, USA). The median fluorescence intensity (MFI) was used to quantify changes in cellular ROS generation, which was obtained by dividing the MFI of the amount tested by the MFI of the control and then multiplying the result by 100. The MFI values of all treatment groups were normalized to the control group.

Cell Apoptosis Analysis

Early and late apoptotic and necrotic cells were identified using the FITC Annexin V Apoptosis Detection Kit with PI according to the manufacturer's guideline. After trypsinization and washing steps, the cells were incubated with Annexin V and PI dyes for 15 min at room temperature. The fluorescence intensities of 10,000 gated events per sample were measured at 488 nm on the ACEA NovoCyte flow cytometer (California, USA). Results were expressed as the percentage of the total number of cells from the quadrants.

Statistical Analysis

Statistical analysis was conducted using Graphpad Prism 9 (GraphPad Software Inc, CA, USA). Statistical differences were evaluated using one-way ANOVA followed by the Tukey test. Results were expressed as mean ± standard deviation (SD), and $p<0.05$ was considered significant. All experiments were performed multiple times for reproducibility.

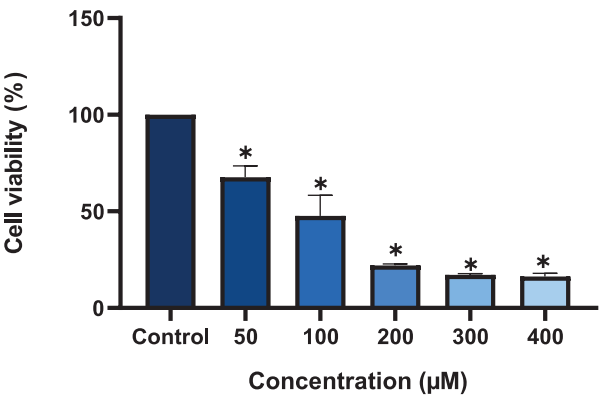


Figure 1: Effects of AZS (50-400 μM) on cell viability evaluated using the MTT assay in SH-SY5Y cells after 24 h of treatment. Data are expressed as mean ± SD. *p<0.05 indicates groups with significant differences from the control.

RESULTS

The cytotoxic effects of AZS on SH-SY5Y cells were evaluated using the MTT assay, which revealed a dose-dependent reduction in cell viability at concentration of up to 200 μM. At 300 and 400 μM, ≥ 95% cells died. The IC_{50} value of AZS was 44.87 μM (Figure 1).

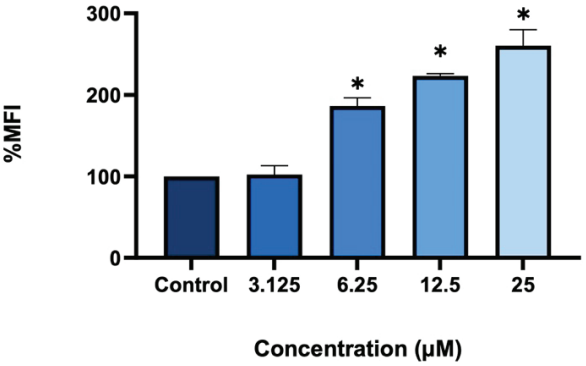


Figure 2: Effects of AZS (3.125-25 μM) on total ROS levels in SH-SY5Y cells after 24 h of treatment. Data are expressed as mean ± SD. *p<0.05 indicates groups with significant differences from the control.

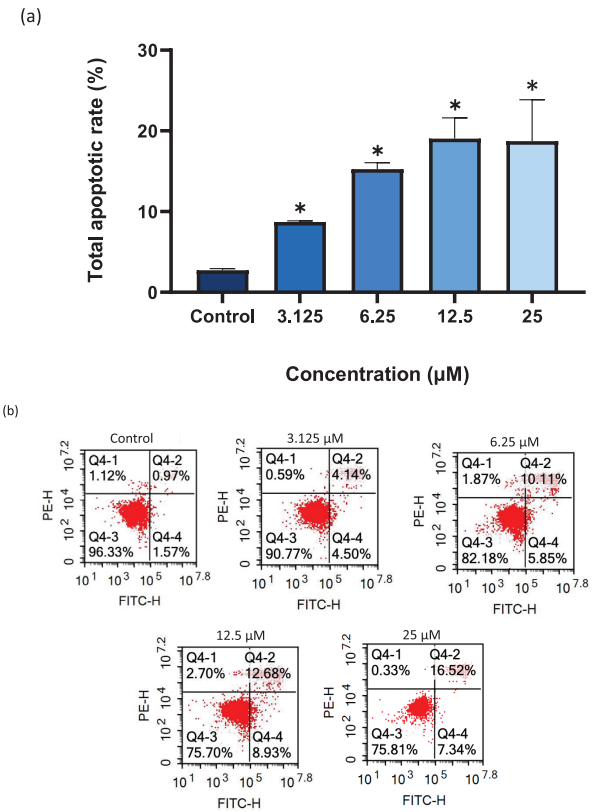


Figure 3: Effects of AZS (3.125-25 μM) on total apoptotic rate (a) and early and late apoptotic effects of AZS (b) measured using flow cytometer in SH-SY5Y cells after 24 h of treatment. Data are expressed as mean ± SD. *p<0.05 indicates groups with significant differences from the control. FITC-H: fluorescein isothiocyanate, PE-H: R-phycoerythrin.

Based on the results of the MTT assay, we decided to investigate concentrations less than the IC_{30} value for subsequent experiments. We observed that ROS levels increased significantly (at least 1.5-fold; p≤0.05) compared with that in the control cells after exposure to AZS at concentrations of 6.25-25 µM for 24 h (Figure 2).

The results of the Annexin V assay revealed that AZS exposure led to ROS-induced apoptosis in SH-SY5Y cells. The apoptosis levels increased significantly (at least 5-fold; p≤0.05) after AZS exposure at concentrations of 6.25-25 µM for 24 h (Figure 3a). At the highest concentration, late apoptosis levels increased up to 16.52%, and early apoptosis levels increased up to 8.93% (Figure 3b).

DISCUSSION

Numerous *in vitro* and *in vivo* studies have demonstrated that pesticides from various categories can exert toxic effects associated with oxidative stress caused by ROS generation. High levels of ROS production can cause damage to cellular components, potentially resulting in cell death (24). Furthermore, ROS can be highly reactive and harmful because they have the ability to alter the structure and function of cellular macromolecules, resulting in behavioral abnormalities, cytotoxicity, and even apoptosis formation (25). The neurotoxic effects of agricultural fungicides can result in neuronal cell damage through oxidative damage, mitochondrial dysfunction, neuroinflammation, and metabolic disorders. Although the ecotoxicological effects of certain strobilurin fungicides are well documented (4), the associated risks to animals and humans have not been investigated in detail. To address this knowledge gap, we explored the pathways that contribute to neurotoxicity in the SH-SY5Y cell line.

Our results demonstrated that AZS significantly reduced cell viability within 24 h of exposure, with an IC_{50} value of 44.87 µM. In a recent study that investigated neurotoxicity via the mitochondrial pathway in differentiated SH-SY5Y cells, the cells were differentiated and exposed to AZS at concentrations of 25-200 µM for up to 48 h to elucidate the mitochondrial mechanisms causing neurotoxicity. It was found that exposure to AZS at 25-200 µM resulted in reduced cell viability but not cytotoxicity after 48 h in differentiated SH-SY5Y cells (26).

In another study, the developing mouse brain was exposed to AZS both acutely and chronically (24 h and 7 days, respectively) to elucidate the neurotoxic effects (5). It was found that AZS was cytotoxic to the primary cortical neuron culture with an IC_{50} value of 30 µM after 24 h of exposure. The lower IC_{50} value was explained by the fact that primary cells are more sensitive than immortalized cells. Another study reported that AZS accumulated in the cerebral cortex of mice during embryonic and early postnatal stages at a concentration of approximately 10 nM after chronic exposure and caused cell death in cortical neurons cultured *in vitro* (21). Furthermore, Shi et al. found that AZS exhibited inhibitory effects on the proliferation of human esophageal squamous cell carcinoma KYSE150 cells with an IC_{50} value of 2.42 µg/mL after 48 h of treatment (20). Chen et al. also demonstrated that AZS inhibited cell viability in human oral squamous cell carcinoma CAL27 and SCC15 cells with IC_{50} values of 4.4 and 7.82 µg/mL, respectively (27).

Damage to mitochondria could cause excessive production of ROS in cells, leading to oxidative stress in organisms. An essential component in the production of ROS is mitochondrial complex III (28). Takahashi et al. investigated the levels of apoptosis and cell cycle arrest induced by AZS in the p53-negative human myelogenous leukemia cell line HL-60RG and p53-positive human T-cell leukemia cell line MOLT-4F (12). They reported AZS-induced cell death, but the mechanisms underlying the damage differed between the cell lines. Chen et al. demonstrated that treatment with 5 µg/mL AZS significantly suppressed the function of mitochondrial complex III and increased the levels of both mitochondrial and intracellular ROS in CAL27 cells, suggesting that AZS causes ROS accumulation by preventing mitochondrial complex III from functioning (27).

In the present study, AZS caused a significant increase in cellular ROS levels at concentrations of ≥ 6.25 µM. Similar to our results, Kang et al. demonstrated that 5 µM AZS caused ROS induction in primary cortical neuronal cultures in developing mouse embryos (5). However, Nguyen et al. indicated that there was no increase in ROS levels with AZS exposure up to 200 µM in differentiated SH-SY5Y cells (8). Cao et al. demonstrated that AZS increased the levels of ROS and MDA and triggered the activities of SOD and CAT in larval and adult zebrafish at a concentration of 0.20 mg/L (3). Furthermore, Wei et al. showed that AZS-induced oxidative stress in *Chironomus dilutus* by altering the levels of H_2O_2 and MDA in addition to the induction of SOD and CAT enzymes at a concentration of more than 0.07 ± 0.01 mg/L, which is almost similar to the environmentally relevant concentration for aquatic environments throughout the world (29).

Mitochondria are extremely important organelles involved in the generation of apoptosis in the cell. Elevated ROS levels trigger apoptotic pathways mediated by mitochondria, resulting in cellular death (30). Our results indicated that excessive cellular ROS generation induced the formation of apoptosis in SH-SY5Y cells. After exposure to AZS at concentrations of 6.25-25 µM, the apoptosis levels significantly increased compared with those in the control group, even at the lower AZS concentration of 6.25 µM. Similar to our results, Nguyen et al. demonstrated increased caspase-3/7 activity after exposure to AZS (50 µM) and trifloxystrobin (25 µM) in differentiated SH-SY5Y cells at 48 h, indicating autophagic apoptosis (8). Kang et al. claimed that AZS activated the intrinsic mitochondrial apoptosis pathway related to Bcl2/Bax and cleaved caspase 3, resulting in cell death (5). Neuronal cells were rescued from reduced viability after the addition of the ROS scavenger NAC in combination with AZS (5). Chen et al. suggested that treatment with AZS at 5 µg/mL for 48 h induced apoptosis of esophageal squamous cell carcinoma cells CAL27 and SCC15 (31). Another previous study showed that 0.20 mg/L AZS-induced an increase in p53,

bax, and apaf 1 levels in the zebrafish liver, which clearly confirmed the occurrence of apoptosis (3).

CONCLUSION

High levels of ROS can trigger mitochondrial-mediated apoptotic pathways, resulting in cell death (32). Our findings suggest that the AZS-induced neurotoxic effect is a consequence of ROS generation and ROS-induced apoptosis. Nonetheless, the mechanisms underlying AZS-induced neurotoxicity must be further investigated to determine the environmental and occupational risks as well as gain a better understanding of its potential toxicity.

Ethics Committee Approval: Since the study was an in vitro study, ethics committee approval was not required.

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study- A.B., G.Ö.; Data Acquisition- A.B.; Data Analysis/Interpretation- A.B., G.Ö.; Drafting Manuscript- A.B.; Critical Revision of Manuscript- A.B., G.Ö.; Final Approval and Accountability- A.B., G.Ö.; Material and Technical Support- G.Ö.; Supervision- G.Ö.

Conflict of Interest: The authors have no conflict of interest to declare.

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