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Investigation of the toxicity of acetamiprid in SH-SY5Y neural cells

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Abstract

Aim: Acetamiprid (ACE) is one of the most widely used neonicotinoids globally to protect crops from insects. In this study aimed to investigate the potential neurotoxic activity of acetamiprid on human neuroblastoma cell line SH-SY5Y cells.

Materials and Methods: MTT and Muse analysis were performed to examine the effect on cell viability. Increasing doses of ACE were administered for 24 hours in SHSY5 neuroblastoma cell lines. NOS1, NOS2, NOS3; caspase-3 for assessment of apoptosis; Ki67 immunocytochemical staining was performed to evaluate proliferation, and relative mRNA values of these markers were measured by qRT-PCR analysis method to evaluate the efficacy of ACE on oxidative stress in neuroblastoma cell lines

Results: The IC50 value for ACE 24 hours was found to be 21.35 mM. In SHSY5 cells, the immunoreactivity of NOS1, NOS3, and caspase-3 markers in the ACE applied group increased statistically significantly compared to the control group; Ki67 immunoreactivity also decreased ($p < 0.05$). qRT-PCR results were consistent with immunocytochemical findings, and relative mRNA values increased in ACE groups compared to the control group. Ki67 relative mRNA values decreased compared to the control group.

Conclusion: In our study, it was found that ACE suppressed proliferation in SH-SY5Y cells, induced apoptosis, and caused cell toxicity by increasing oxidative stress.

Introduction

Neonicotinoids are a class of pesticides usually used to control pests. With the increasing use of pesticides in agriculture, neonicotinoids are found in most vegetables and fruits [1, 2]. Neonicotinoids account for more than a quarter of insecticides on the market. Acetamiprid, a widely used neonicotinoid, is associated with neurological symptoms, and a need to understand its molecular mechanisms has arisen. Imidacloprid (IMI), a neonicotinoid insecticide. Neonicotinoids are one of most widely used pesticides targeting nicotinic acetylcholine receptors (nAChRs) of insects. In a study, it was shown that acetamiprid (ACE) and IMI cause neural excitation in the cerebellar cells of rats in which nAChRs play a role [3]. These ligand-gated ion channel receptors play diverse roles in many mammalian brains [4, 5]. The effects of neonicotinoids on the mammalian central nervous system are of concern.

Neonicotinoid is a term describing new compounds derived from nicotine, and acetamiprid is in this group. It acts as an agonist of nicotinic acetylcholine receptors, and since these receptors have different types in insects and mammals, they are considered to have low mammalian toxicity [6]. However, some cases of poisoning have still been reported. Oral contact results more toxic effects than dermal and inhalation contact. Vomiting, diarrhea, increased secretion, drowsiness, disorientation, cyanosis may develop after oral administration. Treatment is symptomatic and supportive [7, 8].

It is stated that the amount and detection rate of neonicotinoids in samples of human origin are increasing every year [9]. It is stated that the quantity rate of neonicotinoids in samples of human are increasing. Although it has been stated that neonicotinoids cause negative neurological outcomes in humans, experimental studies on neonicotinoid risks are far from sufficient [10]. In vitro and in vivo studies have been performed in rats and mice to evaluate the effects of neonicotinoids. However, there are

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differences in the amino acid sequence of nAChR subunits between rodents and humans [11, 12].

The dopaminergic neuronal cell line SH-SY5Y from human neuroblastoma has been employed as an in vitro model for neurotoxicity studies [13, 14]. This study aimed to evaluate the potential neurotoxicity effect of ACE on human neuroblastoma cell line SH-SY5Y cells through apoptosis and oxidative stress parameters.

Materials and Methods

Cell culture

Neuroblastoma cell line SH_SY5 was obtained from ATCC (Manassas, VA, USA). Cancer cells were grown in DMEM/F12 medium appended with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine and antibiotics. Cells were cultivate at 37°C in an incubator with a 5% CO2 atmosphere and 95% humidity. Cells were passaged using 0.25% trypsin/EDTA and sowed in fresh medium [15]. In addition, cells were regularly monitored for mycoplasma contamination.

MTT analyses

After trypsinization with 0.25% trypsin+EDTA, the collected cells were centrifuged at 1200 rpm and 4 °C for 5 minutes. Then approximately 104 cells seeded overnight in 96 well plates. ACE was dissolved in medium SHSY5 cells, were treated with 1, 2,5, 5 10, 25, and 50 mM ACE for 24 hours, and cell viability was determined by 3-3-(4,5-D-methylthiazole-2-yl)-2,5- diphenyltetrazolium bromide, thiazolyl blue (MTT) test at 550 nm with a microplate reader (BioTek; Winooski, VT) method, in vitro [16]. Assay was repeated 3 times for statistical analysis.

Muse analyses

The apoptotic cell distribution was determined using the MUSE Annexin V & Dead Cell Kit (Merck MCH100105) according to the manufacturer's instructions [17]. Briefly, after treatment with IC50 concentration of ACE for 24 hours all cells were collected and diluted with PBS containing 1% bovine serum albumin (BSA) as a dilution buffer to a concentration of 5x105cells/ml. Cells were then analyzed using the flow cytometry based Muse™ Cell Analyzer instrument. As a marker of early and late apoptotic processes, annexin-V/7-aminoactinomycin D (7-AAD) staining was used [18]. The apoptotic ratio was determined by the identification of four populations: (i) nonapoptotic cells, not undergoing detectable apoptosis: Annexin V (−) and 7-AAD (−); (ii) early apoptotic cells, Annexin V (+) and 7-AAD (−); (iii) late apoptotic cells, Annexin V (+) and 7-AAD (+); (iv) cells that have died through nonapoptotic pathway: Annexin V (−) and 7-AAD (+). The samples were determined by the Muse Cell Analyzer. The measurements were performed in triplicate and repeated three times.

Immunocytochemistry staining

Cells were divided into two groups as control group and ACE-treated group. The cells in the control group did not add anything to the media. Cells in the control group were under standard culture conditions; ACE-treated groups were incubated with ACE for 24 h at calculated IC50 values. After the incubation, cells were fixed with 4% paraformaldehyde and washed with PBS. Samples washed with PBS and each were incubated with 3% H2O2 (Merck, USA) at room temperature for to inhibit endogenous peroxidase activity. After the cells were incubated with blocking solution (Invitrogen, 859043) at room temperature, the blocking solution was withdrawn without washing, and the primary antibodies NOS1 (Bioorbyt, orb251481), NOS2 (Bioss, bs-2072R), NOS3 (Bioss, bs-13074R) , Ki67 (Bioss, bs-23105R), and caspase-3 (Bioss, bs-0081R) at a dilution of 1:100 were applied and incubated overnight at 4 ° C. The next day, antibodies were removed by washing thrice with PBS, and biotinylated secondary antibody was added and incubated. After each, streptavidin was incubated with hydrogen peroxidase (Invitrogen, 859043) at room temperature. After washing again with PBS, samples that had been incubated with Diaminobenzidine (DAB) (Scytek, AEM080) for 5 min were washed with distilled water to determine the visibility of the immunocytochemical reaction. Samples stained with Mayer's hematoxylin for 2–3 min were washed in distilled water and then covered with a covering medium (Spring Bioscience-DMM125). Quantitative results of immunohistochemical staining were performed using free Fiji by Patera et al. It was done as described. After the color deconvolution is applied to the microphotographs belonging to the groups, DAB, the average gray value was calculated from the image. Optical Density; Optical density was calculated using the formula log (maximum gray density / average gray density). Data are presented as optical density ± standard deviation.

RNA extraction and quantitative reverse transcription PCR

Total RNA isolation was performed using PureLink RNA Mini Kit (Invitrogen, Cat. No: 12183018A) according to the kit protocol. Qualitative and quantitative analyzes of the obtained RNAs were performed spectrophotometrically using the MaestroNano (MaestroGen, USA) device. Isolated RNA samples were converted to complementary DNA (cDNA) using the High-Capacity RNA-to-cDNA kit (Cat. no: 4368814; Invitrogen Corp., Carlsbad, CA, USA). Forward and reverse primer sets of Caspase 3, NOS1, NOS2, NOS3 and Ki67 genes were purchased from Sentegen Biotech, Ankara, Turkey (Table 1). SYBR Green PCR Master Mix (Cat. no: 4344463; ThermoFisher Scienti c, Inc., Waltham, MA, USA) was used to determine change in gene expressions and the reactions were assessed using the StepOnePlus Real-Time PCR System. GAPDH was used as a reference gene, and the relative gene expression differences were calculated using the comparative threshold cycle (2-ΔΔCt) method. The primers of the genes used in the qPCR analysis are shown in Table 1.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 7 software. All the data were presented as mean ± standard deviation (SD). The Student t test was used

Table 1. Sequences of primers for RT-PCR.

Target Genes	Forward Primer	Reverse Primer
Caspase 3	5'CCTCAGAGAGACATTCATGG3'	5'GCAGTAGTCGCCTCTGAAGA3'
Ki-67	5'CTGCCTCAGATGGCTCAAAGA3'	5'GAAGACTTCGGTTCCCTGTAAC3'
NOS1 nNOS	5'CATCAGGCACCCCAAGTT3'	5'CAGCAGCATGTTGGACACA3'
NOS2 iNOS	5'CTTTGCCACGGACGAGAC3'	5'TCATTGTACTCTGAGGGCTGAC3'
NOS3 eNOS	5'GACCCTCACCGCTACAACAT3'	5'CTGGCCTTCTGCTCATTTTC3'
GAPDH	5'GGAGAGTGTTTCCTCGTCCC3'	5'ATGAAGGGGTCGTTGATGGC3'

Table 2. Relative mRNA values

	Control		ACE	
	Mean	Std	Mean	Std
Caspase 3	0.03523	0.00150	0.04153	0.00131
Ki67	0.03520	0.00093	0.01910	0.00111
NOS1	0.02081	0.00096	0.04202	0.00170
NOS2	0.02686	0.00131	0.02867	0.00077
NOS3	0.02955	0.00078	0.04660	0.00099

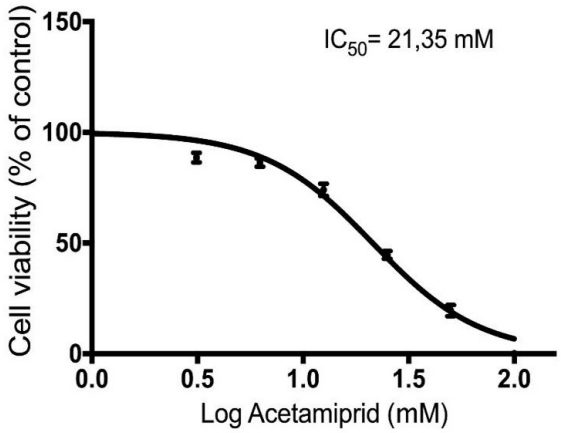
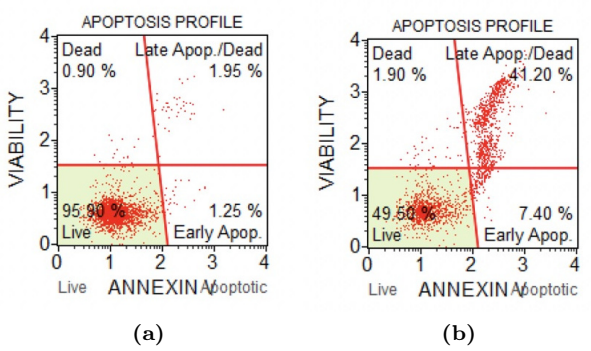


Figure 1. The IC50 value for ACE 24 hours



(a) (b)

Figure 2. Total amount of apoptosis a) control group b) ACE treated group

to evaluate differences between the groups. $P < 0.05$ were considered statistically significant.

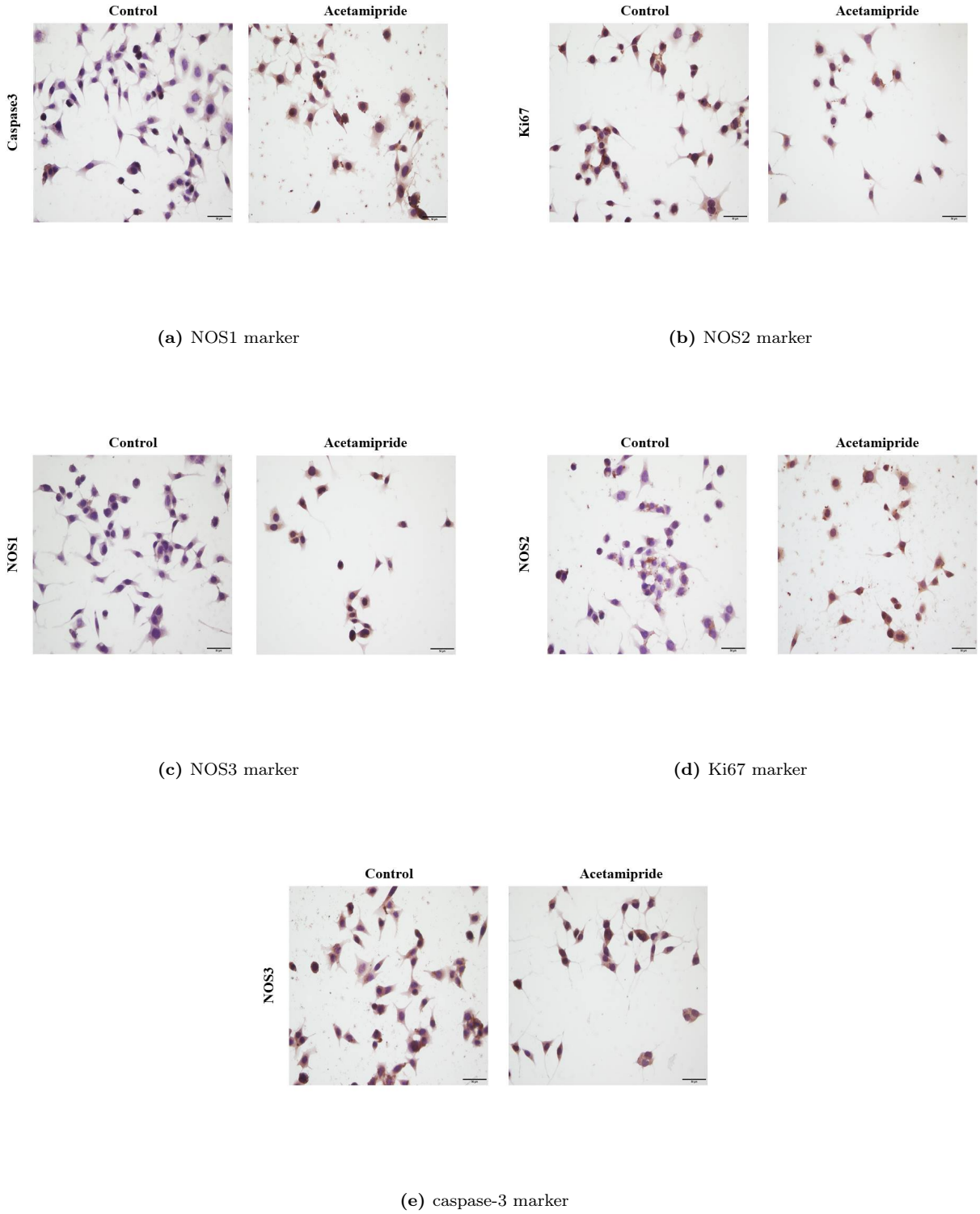
Results

MTT and MUSE analysis were performed to examine the effect on cell viability. Increasing doses of ACE were administered for 24 hours in SHSY5 neuroblastoma cell lines. It was found that increasing doses of ACE concentration decreased cell line viability in direct proportion. The IC50 value for ACE 24 hours was found to be 21.35 mM (Figure 1).

Total apoptosis was found to be 3.2% (1.25% early apoptosis and 1.95% late apoptosis) in the control group. In the ACE treated group, total apoptosis was found to be 48.60% (7.40% early apoptosis and 41.20% late apoptosis) (Figure 2).

NOS1, NOS2, NOS3; caspase-3 for assessment of apoptosis; Ki67 immunocytochemical staining was performed to evaluate proliferation, and relative mRNA values of these markers were measured by qRT-PCR analysis method to evaluate the efficacy of ACE on oxidative stress in neuroblastoma cell lines. In SHSY5 cells, the immunoreactivity of NOS1, NOS3, and caspase-3 markers in the ACE-treated group increased statistically significantly compared to the control group; Ki67 immunoreactivity was also found to be decreased ($p < 0.05$) (Figure 3-4).

Although there was an increase in NOS2, no statistically significant difference was found ($p > 0.05$). qRT-PCR results were consistent with immunocytochemical findings, and relative mRNA values increased in ACE groups compared to the control group. Ki67 relative mRNA values decreased compared to the control group. The mean and standard deviation values of the OD and relative mRNA of the findings are summarized in tables (Table 2).



(a) NOS1 marker

(b) NOS2 marker

(c) NOS3 marker

(d) Ki67 marker

(e) caspase-3 marker

Figure 3. (a) In SHSY5 cells, the immunoreactivity of NOS1 marker (b) NOS2 marker (c) NOS3 marker (d) Ki67 marker and (e) caspase-3 marker.

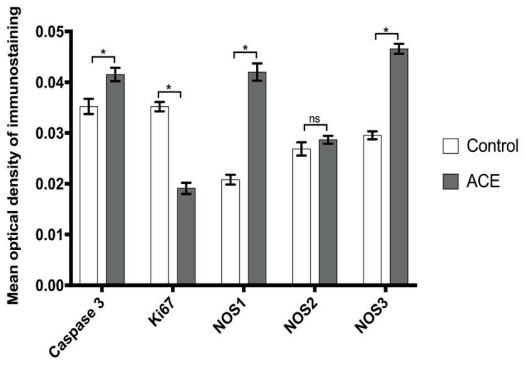


Figure 4. In SHSY5 cells, the immunoreactivity of NOS1, NOS2, NOS3, Ki67 and caspase-3 markers.

Discussion

As nicotinic acetylcholine receptor agonists, neonicotinoids are widely used insecticides introduced to the global market with increasing importance [19]. Neonicotinoids are thought to have a selective binding affinity in the central nervous system of insects and have low toxicity to vertebrate species due to poor penetration of the mammalian blood-brain barrier [6, 20]. However, the high water solubility of neonicotinoids causes accumulation in soil or water [1, 21]. ACE is one of the most widely used neonicotinoids used globally to protect crops from insects [20]. It belongs to a new class of insecticides that reach a high concentration in the body, such as liver, kidney, adrenal, and thyroid glands [22].

The hepatotoxic, reproductive and developmental toxic effects of ACE have been well studied in in vivo animal studies [3, 23]. It has been reported that ACE can cause various changes in brain functions in rats and neurological symptoms in humans [24, 25]. Since inhalation is one of the main routes of exposure for neonicotinoids such as ACE, they can be absorbed in the lung, and then ACE and its metabolites can cross the blood-brain barrier and reach the brain [26]. Therefore, there is increasing evidence for the neurotoxic effects of ACE, and few studies have been conducted on neonicotinoid-induced neurotoxicity in men. Therefore, our study aimed to evaluate the potential neurotoxicity effect of ACE on SH-SY5Y cells through apoptosis and oxidative stress parameters. The dopaminergic neuronal cell line SH-SY5Y from human neuroblastoma has been employed as an in vitro model for neurotoxicity studies [13, 14].

In our study, ACE decreased cell viability depending on the dose, and the IC50 value was calculated as 21.35 mM. In some studies in the literature, the IC50 value of ACE was found to be 2.16 and 6.68 mM in SH-SY5Y and SK-N-SH cells, respectively [27, 28]. On the other hand, the IC50 values of ACE were observed to be less than 1 mM in different cell lines such as human lung fibroblast IMR-90, human placental trophoblast HTR-8/SVneo, and rat adrenal gland pheochromocytoma [29, 30]. The IC50 values and cytotoxic effects of pharmacological agents may vary depending on the cell line used and the method of administration [31-33]. In the light of these data, it can be argued that human nerve cells are more resistant to the cytotoxic effects of ACE.

It has been shown that ACE can cause oxidative damage in various bacteria and disrupt the oxidative state and loss of mitochondrial membrane integrity in the rat reproductive system and brain [34, 35]. Our study found that the immunoreactivity of NOS1 and NOS3 markers in the ACE-treated group in SHSY5 cells increased statistically significantly compared to the control group ($p < 00.5$). This result in our study showed that ACE induced oxidative stress by increasing NOS1 and NOS3 in SH-SY5Y cells. Although there was an increase in NOS2, no statistically significant results were found in immunostaining.

Many studies in the literature have reported the induction of apoptosis in ACE-mediated toxicity [30, 34]. In these studies, Bcl-2 overexpression was also observed in SH-SY5Y cells after exposure to ACE. In our study, it was determined that ACE induced apoptosis by regulating the caspase-3 gene. In addition, in our study, it was observed that the immunoreactivity of Ki67 markers in the ACE-treated group decreased compared to the control group. Ki67 relative mRNA values were decreased compared to the control group. Ki-67 is an important nonhistone nuclear protein used in the determination of cell proliferation index. These results in our study showed that acetamiprid suppressed cell proliferation.

As a result, in our study, acetamiprid suppresses proliferation in SH-SY5Y cells, induces apoptosis, and causes cell toxicity by increasing oxidative stress. We think that larger studies are needed to investigate the toxic effects and mechanisms of acetamiprid in neural cells. In addition, although acetamiprid is thought to be a safe option for controlling harmful agricultural insects, these results show that its widespread use should be strictly controlled.

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