



Acetamidrid-induced Cyto- and Genotoxicity in the AR42J Pancreatic Cell Line

AR42J Pankreas Hücre Hattında Asetamidrid ile İndüklenen Sito- ve Genotoksisite

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ABSTRACT

Objectives: Neonicotinoid insecticides, 30% of insecticides marketed worldwide, have selective toxicity on insects through $\alpha 4\beta 2$ nicotinic acetylcholine receptors. Although it is known that acetamidrid exerts toxicity on several organ systems, its toxic effects on the pancreas and its mechanism of action have not been clarified yet. Therefore, in the present study, the cytotoxic and genotoxic potentials of acetamidrid on the AR42J pancreatic cell line were evaluated.

Materials and Methods: The (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) (MTT) assay and comet assay were conducted for the cyto- and genotoxicity evaluations, respectively. Reactive oxygen species (ROS) production was assessed by flow cytometry and glutathione (GSH) levels were determined by ELISA for oxidative damage potential, which is thought to be an underlying mechanism of cyto-/genotoxic effects.

Results: To reveal the dose-response relationship the concentration range of 1-6 mM was selected for the assays. Cell viability decreased in a dose-dependent manner and the inhibitory concentration 50 value was calculated as 12.61 mM by the MTT assay. Acetamidrid induced DNA damage in all concentrations tested in a dose-depending manner. The mean tail intensity values were 3.84 and ≤ 32.96 for the control and exposure groups, respectively. There was no significant difference for ROS production; however, the GSH level was reduced at the highest concentration.

Conclusion: It is thought that the present study will contribute to the literature due to the lack of data on the potential toxic effects of acetamidrid on the pancreas. To better understand acetamidrid toxicity, further studies including a wide range of mechanistic parameters are needed.

Key words: Acetamidrid, AR42J pancreatic cell line, cytotoxicity, genotoxicity, oxidative damage

ÖZ

Amaç: Neonikotinoid insektisidler dünya piyasasındaki insektisidlerin %30'luk kısmını oluşturur ve etkilerini seçici olarak böceklerdeki $\alpha 4\beta 2$ nikotinik asid reseptörünü inhibe ederek gösterirler. Asetamidridin çeşitli organ ve sistemler üzerine toksik etkileri biliniyor olmasına karşın, pankreas üzerindeki etkisi ve etki mekanizması bilinmemektedir. Bu çalışmada, asetamidridin AR42J pankreas hücre hattı üzerinde sitotoksik ve genotoksik etkileri araştırıldı.

Gereç ve Yöntemler: Sitotoksisite ve genotoksisite değerlendirmesi için (3-[4,5-dimetiltiazol-2-il]-2,5 difenil tetrazolyum bromür) (MTT) ve comet analizi gerçekleştirildi. Reaktif oksijen türlerinin (ROS) oluşumu akış sitometresi ile, glutatyon (GSH) düzeyi ise ELISA yöntemi ile belirlenmiştir.

Bulgular: MTT sitotoksisite analizine göre, hücre canlılığının doza bağımlı olarak azaldığı gözlemlendi ve inhibitör konsantrasyon 50 değeri 12,61 mM olarak tespit edildi. Analizlerde doz yanıt ilişkisini gösterebilmek için 1-6 mM doz aralığı seçildi. Asetamidrid DNA hasarını doza bağımlı olarak artırdı ve ortalama kuyruk yoğunluğu değerleri kontrol ve çalışma grupları için sırasıyla 3,84 ve $\leq 32,96$ olarak belirlendi. ROS üretimi açısından gruplar arasında anlamlı bir fark gözlenmemiş, GSH değerinin en yüksek doz grubunda anlamlı olarak çok düştüğü tespit edilmiştir.

Sonuç: Bu çalışma literatürde asetamidridin pankreas üzerine olası toksik etkisine yönelik eksik olan verilere katkı sağlamaktadır. Asetamidridin toksik etkisinin detaylandırılması için daha geniş çaplı mekanizma temelli çalışmalara ihtiyaç bulunmaktadır.

Anahtar kelimeler: Asetamidrid, AR42J pankreas hücre hattı, sitotoksisite, genotoksisite, oksidatif hasar

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INTRODUCTION

Many xenobiotics pose important threats for both human and environmental health. Pesticides, the most common pollutants, are harmful for biological structures via several mechanisms in acute and long-term exposure.¹⁻³ Neonicotinoid pesticides as a new class of insecticides that are commonly used instead of organophosphate, and carbamate pesticides have selectively neurotoxic effects on nicotinic acetylcholine receptor. Neonicotinoid pesticides are highly effective insecticides that can disperse in all parts of plants, plant fluids, and fruits that grow on plants. Recent studies revealed that neonicotinoid pesticides can be associated with several adverse effects including decreased sperm production and function, decreased pregnancy rates, increased embryo death, stillbirth, and premature birth in vertebrate and invertebrate species.⁴⁻⁶

Acetamiprid [(E)-N-[(6-chloro-3-pyridyl) methyl]-N-cyano-N-methylacetamidine] is one of the most commonly used neonicotinoid class insecticides in many countries for crop pests on agricultural products. In general, acetamiprid has been considered a safe insecticide; however, several different adverse health effects may occur after exposure to acetamiprid as well as other neonicotinoids.³ In previous studies, it has been reported that acetamiprid showed teratogenic, mutagenic, and genotoxic effects via induction of oxidative stress. However, the data about its cyto- and genotoxic potentials are contradictory.⁷⁻¹² As is well known, the worldwide rate of diabetes continues to rise, and the major molecular mechanisms underlying diabetes are increased oxidative stress and altered enzyme functions in pancreatic tissue.¹³ Indeed, no association between diabetes risk and neonicotinoid pesticides has been reported. Furthermore, there has been no study on the toxic effects of acetamiprid on the pancreas. Therefore, for the first time, we aimed to investigate the cytotoxic and genotoxic effects of acetamiprid on the AR42J pancreatic cell line and evaluated its oxidative damage potential as an underlying molecular mechanism.

MATERIALS AND METHODS

Chemicals

Acetamiprid, dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), and 2',7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA) were purchased from Sigma Chemical Co. Ltd. (St. Louis, Missouri, USA). The cell culture medium [Roswell Park Memorial Institute (RPMI 1640)] and other chemicals were purchased from Multicell Wisent (Quebec, Canada), while the disposable materials were purchased from Corning (Amsterdam, the Netherlands). All other chemicals at required biological grade were purchased from Merck (New Jersey, USA).

Cell culture and treatments

The AR42J (CRL1492) cell line was obtained from the American Type Culture Collection (ATCC, Virginia, USA), and for all cell applications incubation was carried out according to the manufacturer's instructions. The cells were grown with

1640 cell culture medium including 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin in a humidified incubator in 5% CO₂ at 37 °C. Subculturing was performed every 2-3 days when the cells reached confluence. Prior to exposure cells were seeded into appropriate plasticware and incubated overnight to ensure cell attachment.

Acetamiprid stock solution was prepared by dissolving in 100% DMSO, and stored at -20 °C until the day the assays were conducted. Before the cell treatments acetamiprid was diluted with culture medium to the desired concentrations, and DMSO concentration finalized as 1%. Treatments were performed at a concentration range for 24 h to evaluate dose-dependent effects. All experiments were performed in triplicate on three separate days.

MTT cytotoxicity test

The AR42J cells were placed into 96-well plates (1x10⁴ cells/100 µL cell culture medium/well). The cells were treated with acetamiprid following overnight incubation at the concentration range of 1-50 mM for 24 h. After 5 mg/mL MTT was added to each well, the cells were again kept for 3 h at 37 °C in the dark. Cell culture medium was used as a growth control, 1% DMSO was used as a solvent control, and 10% SDS was used as a positive control. The wells were washed with poly (butylene succinate) (PBS) twice after 3 h. Following the washing step, 100 µL of DMSO was added, followed by incubation for 5 min on an orbital shaker (150 rpm) for evenly dissolved formazan crystals and optical densities were measured using a microplate reader (Biotek, Epoch, Vermont, USA) at 570 nm. The percentage of inhibition of cell viability for each concentration and the inhibitory concentration 50 (IC₅₀) value were determined.

Comet genotoxicity assay

AR42J cells were placed into 6-well plate as 5x10⁵ cells/2 mL cell culture medium/well and kept overnight for incubation. The cells were exposed to acetamiprid at 1, 2, 4, and 6 mM concentrations and 1% DMSO as a negative control for 24 h. After the cells were detached with trypsin-ethylenediaminetetraacetic acid (EDTA) and washed with PBS twice, the viability of cells was evaluated with the trypan blue test, and cell viability was determined as ≥80% for all concentrations. Next 100 µL of single cell suspension was mixed with 100 µL of prewarmed 0.65% low-melting agarose and then layered on microscope slides coated with 1.5% normal-melting point agarose. After lysing for 1 h at 4 °C, the slides were incubated in cold fresh electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13) at 4 °C for 20 min for DNA unwinding. The electrophoresis conditions were 4 °C for 20 min (20 V/300 mA). The slides were neutralized in 0.4 M tris-HCl buffer (pH 7.5). DNA staining was performed with 20 mg/mL ethidium bromide dye and evaluated under a fluorescent microscope (Olympus BX53, Olympus, Tokyo, Japan) at 40x10 magnification by Comet Assay IV, Perceptive software (Suffolk, UK). One hundred cells were counted and scored for each concentration and %TDNA and tail intensity were evaluated.¹⁴

Oxidative stress parameters

A total reactive oxygen species (ROS) assay was performed via (2'-7'-dichlorodihydrofluorescein diacetate) analysis by flow

cytometry. Next 5×10^5 cells/2 mL cell culture medium/well were placed into a 6-well plate. After one day of incubation, the cells were exposed to acetamiprid at 1, 2, 4, and 6 mM concentrations and 1% DMSO as a negative control for 24 h. After 24 h the plates were washed with PBS twice. The cells were incubated with $20 \mu\text{M}$ $\text{H}_2\text{DCF-DA}$ at 37°C for 30 min on a shaker in the dark. They were detached from the plates via trypsinization and resuspended in $150 \mu\text{L}$ of PBS with 1% bovine serum albumin. Fluorescence intensity was measured by FITC channel with excitation 488 nm and emission 530 nm via an ACEA NovoCyte flow cytometer (San Diego, California, USA). The results were shown as median fluorescence intensity.

Glutathione (GSH) levels were determined by [5,50-dithiobis-2-nitrobenzoic acid (DTNB)] reagent method described by Beutler.¹⁵ This method is based on DTNB reduction by free SH groups of GSH to 5-mercapto-2-nitrobenzoate. After treatment with acetamiprid, 1 mL of cell lysates was deproteinated with 1.67 g of metaphosphoric acid, 0.2 g of Na_2EDTA , and 30 g of NaCl solved in distilled water. After that, 2.4 mL of Na_2HPO_4 and 0.3 mL of DTNB were added, followed by centrifugation for 10 min at $3000 \times g$. 5-Thio-2-nitrobenzoic acid formation was measured at 412 nm by spectrophotometer. GSH results were expressed as $\mu\text{mol/g}$ protein.¹⁵

Statistical analysis

The experimental results were analyzed by One-Way ANOVA post hoc Dunnett's t-test and given as mean \pm standard deviation. The level of statistical significance was set as $p \leq 0.05$. All analyses were performed using SPSS version 20.0 (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Cell viability

The MTT assay is one of the most frequently used, simple, and rapid colorimetric cell viability/cytotoxicity assays and yields quantitative data. This assay is based on reduction of water-soluble yellow tetrazolium salt by the mitochondrial succinate dehydrogenase enzyme in metabolically active/live cells dehydrogenase and quantified color intensity of dissolved formazan crystals by spectrophotometer.¹⁶

The cytotoxicity of acetamiprid on the AR42J cell line was evaluated with the MTT assay in the dose range of 1-50 mM after 24 h exposure and the IC_{50} value was determined as 12.61 mM (Figure 1).

Comet assay

The alkaline comet assay is a very common method for measuring DNA damage in a single cell suspension via migration of DNA under electrophoresis conditions. It has been reported that the tail intensity value is the most recommended end point for an alkaline comet assay in a dose-dependent manner.¹⁷

According to the results of the comet assay in the concentration range of 1-6 mM, acetamiprid significantly increased DNA damage in a dose-dependent manner. The mean tail intensity values were significantly increased in all exposure groups compared to the control group (Figure 2).

Oxidative stress parameters

Oxidative damage via ROS plays a key role in different human diseases such as cancer, cardiovascular diseases, diabetes, and neurodegeneration. Dichlorodihydrofluorescein diacetate (DCFH-DA) is a widely used assay that enables direct measurement of the redox state in the cells. This method is very sensitive, easy to use, and cheap and can be used to follow changes in ROS over time.¹⁸

There were no significant differences between the control and exposure groups according to total ROS levels, which were evaluated by $\text{H}_2\text{DCF-DA}$ with a flow cytometer. However, the GSH level was significantly reduced in the 6 mM group compared to the control group. It was observed that 6 mM of acetamiprid dramatically reduced GSH level by 98.07% (Figure 3).

DISCUSSION

Widespread use of acetamiprid in agriculture alone or in combination with other insecticides may cause pesticide spread into the environment and the food chain, resulting in toxicity in humans and animals. An increased risk of pancreatic

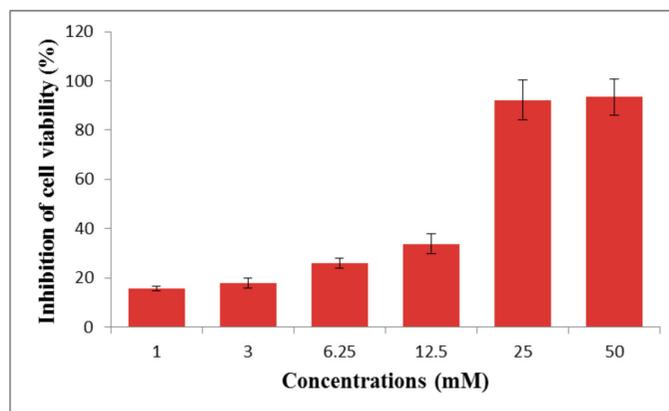


Figure 1. The inhibition of the cell viability values by MTT test in the AR42J cell line

MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide

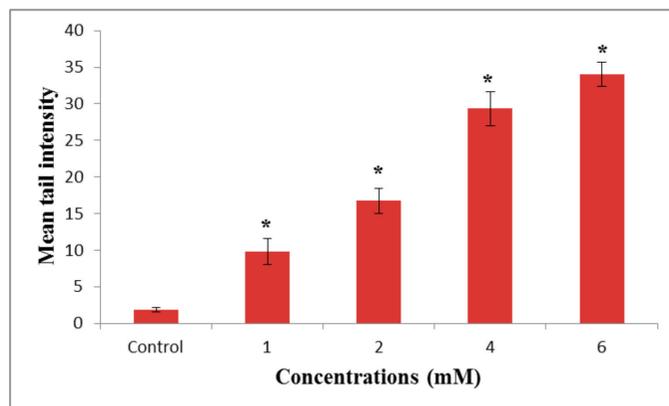


Figure 2. Acetamiprid-induced DNA damage in AR42J cell line at a concentration range of 1-6 mM as observed by comet assay. Representative single cell images clearly indicated that tail intensity was increasing while head of the cell was decreasing in a dose-dependent manner. The error bar represents \pm standard deviation and * $p < 0.05$ compared to other groups

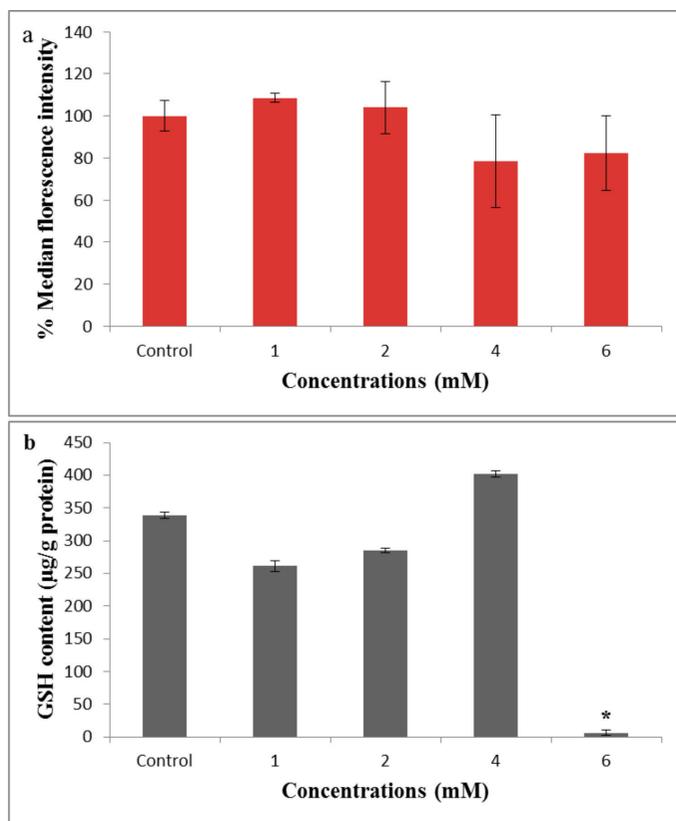


Figure 3. Oxidative damage potential of acetaminophen (1, 2, 4, or 6 mM) evaluated by H_2DCF -DA (a) and GSH (b) assays. The ROS production was expressed as mean fluorescence intensity and GSH levels were expressed as $\mu\text{g/g}$ protein. The error bar represents \pm standard deviation and * $p < 0.05$ compared to other groups

GSH: Glutathione, ROS: Reactive oxygen species, H_2DCF -DA: 2',7'-dichlorodihydrofluorescein diacetate

cancer is found in those in agricultural occupations; however, pesticides' effects on oncogenesis mechanisms have not been extensively evaluated yet.^{19,20} There are limited data about the effects of neonicotinoids on pancreatic tissue; moreover, there are no data about acetaminophen's effects on the pancreas. Khalil et al.¹³ reported that 0.5 and 1.0 mg/kg bw imidacloprid over 60 days disrupted glucose homeostasis in male rats. In treated groups, the GLUT4 mRNA expression level was decreased and pancreatic islets shrinkage and infiltration of round cells, interlobular congestion, and hemorrhage were observed with histopathologic examination, and also decreased insulin expression in the pancreatic β cells was detected in the imidacloprid-treated groups.

To further examine the potential associations between their usage and toxic profiles for neonicotinoid pesticides, we conducted a study on *in vitro* acetaminophen exposure in a pancreatic cell line. Although there are a few studies on acetaminophen's cyto- and genotoxicity potentials in the literature, there is no *in vitro/in vivo* study focusing on the pancreas. It has been reported that the LD_{50} value is the range of 140-417 mg/kg b.w. in rodents and the NOAEL level is 400 ppm in 13 week old mice for acetaminophen.²¹ It has been reported in another study that IC_{50} values of acetaminophen on SH-SY5Y and HepG2

cell lines were 2.16 and 3.61 mM, respectively.²² In our study, the cytotoxicity was evaluated by MTT assay in acetaminophen (1-50 mM) treated AR42J cells after 24 h exposure. The IC_{50} value was calculated as 12.61 mM.

In the same study, significantly increased DNA damage was shown at 500 μM in SH-SY5Y cells.²² In another study, $>50 \mu\text{M}$ acetaminophen significantly increased micronuclei formation and DNA breaks in IMR-90 human lung cells.⁸ Genotoxic effects of acetaminophen on sister chromatid exchange, micronucleus, and chromosomal aberration analysis with 25, 30, 35, and 40 $\mu\text{g/mL}$ doses for 24 and 48 h were shown in human peripheral blood lymphocytes. Micronucleus formation was significantly induced compared to the control group while the proliferation index was decreased.¹² It has been reported that acetaminophen increased micronuclei per cell and chromosomal aberrations in Swiss albino male mice bone marrow depending on concentration with acetaminophen treatment over 60 and 90 days at 4.6 and 2.3 mg/kg/day i.p.²³ According to the results of the comet assay performed with the concentration range of 1-6 mM, acetaminophen significantly induced DNA damage depending on concentration. The different results obtained from several studies can be related to cell types, study duration, and/or method selection.

Oxidative stress mechanisms could underlie the cyto- and genotoxic potentials of neonicotinoid pesticides.^{7,8,12} It has been reported that pesticides may impair the redox balance effects in different cells. However, the mechanisms underlying oxidative stress are still not fully understood.¹⁶ There are several studies about the effects of acetaminophen on oxidative stress parameters in different species such as rodents, bacteria, plants, and fish.²⁴⁻³⁰ In Wistar rat brain tissue 3.14 mg/kg acetaminophen exposure resulted in increased mitochondrial oxidative stress status that was significant. Decreased oxidative stress parameters were GSH level, GSH peroxidase, and catalase activities. Increased parameters determined were malondialdehyde level, GSH s-transferase, and superoxide dismutase activities.³¹ In our study, no significant difference was found between the control and exposure groups according to total ROS levels. However, the GSH levels were significantly different compared to the control at the highest concentration. It was observed that 6 mM acetaminophen dose dramatically reduced GSH level by 98.07%. In earthworms, it has been demonstrated that different concentrations of acetaminophen (0, 0.05, 0.10, 0.25, and 0.50 mg/kg of soil) with different exposure periods (7, 14, 21, and 28 days) increased the ROS levels to varying degrees. Olive tail moment, which indicates DNA damage in the comet assay, increased in a dose-dependent manner, indicating that subchronic acetaminophen exposure might cause oxidative stress and induce DNA damage in earthworms.²⁴

Acetaminophen is classified as an "unlikely" human carcinogen according to EPA guidelines and its target organ toxicity data are not clear yet. Acetaminophen's acute oral toxicity category is "II" for rats and its acute inhalation toxicity category is "III" for rabbits. Its NOAEL value for rats is 12.4/14.6 mg/kg/day (M/F) and its LOAEL value for rat is 50.8/56.0 mg/kg/day (M/F). The chronic carcinogenicity NOAEL value for rats is 7.1/8.8 mg/

kg/day (M/F). According to the EPA acetamiprid is not yet classified as genotoxic.³² A target organ toxicity assessment for further subchronic and chronic *in vivo* studies may clarify the risk caused by acetamiprid for pancreas tissue-based diseases.

CONCLUSION

Under different durations in the pancreatic cell line acetamiprid may affect these oxidative stress parameters significantly. To clarify the oncogenic potential of acetamiprid on pancreatic tissue it is necessary to perform further *in vivo* studies with subchronic or chronic studies with molecular mechanistic observations.

Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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