ACETAMIPRID INDUCED CYTO-AND GENOTOXICITY IN AR42J PANCREATIC CELL LINE

ASETAMİPRİDİN AR42J PANKREAS HÜCRE DİZİSİNDE SİTO-VE GENOTOKSİSİTEYİ İNDÜKLEMESİ

Kısa Başlık: Asetamipridin Sito-ve Genotoksik Etkisi

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ABBREVIATIONS

Acetamiprid, (E)-N-[(6-chloro-3- pyridyl)methyl]-N-cyano-N-methylacetamidine;DCFDA, 2'-7'-dichlorodihydrofluorescein diacetate; DMSO, dimethyl sulfoxide; DNA, deoxyribonucleic acid; DTNB, 5,50-dithiobis-2-nitrobenzoic acid;FBS, foetal bovine serum; GSH, glutathione; IC_{50}, inhibitory concentration 50; MTT, (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide);nAChR, α4p2 nicotinic acetylcholine receptors; ODs, optical densities; ROS, reactive oxygene species; RPMI 1640, Roswell Park Memorial Institute 16

ABSTRACT

Introduction: Neonicotinoid insecticides, 30% of insecticides marketing in the world, have selective toxicity on insects through α4p2 nicotinic acetylcholine receptors (nAChR). Although, it is known that acetamiprid exerts its toxicity on several organ systems; toxic effects on pancreas as well as mechanism of action have not been clarified yet. Therefore; in the present study, cytotoxic and genotoxic potential of acetamiprid on AR42J pancreatic cell line were evaluated.

Methods: MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay and comet assay were condeucted for cyto- and genotoxicity evaluations, respectively. Reactive oxygene species (ROS) production was assessed by flowcytometry and glutathione (GSH) levels were determined by ELISA assay for oxidative damage potential which is thought as an underlying mechanism of cyto-genotoxic effects.

Results: To reveal dose-response relationship concentration range of 1-6 mM was selected for the assays.Cell viability was decreased in a dose-dependent manner and inhibitory concentration 50 (IC_{50})value wascalculated as 12.61 mM by MTT assay. Acetamipridinduced DNA damage in all concentrations tested in a dose-depending manner. The mean tail intensity

values were 3.84 and ≤32.96 for control and exposure groups, respectively.There was no significant difference for ROS production; however, GSH level was reduced at the highest concentration.

Discussion and Concluson: It is believed that the present study will contribute to the literature due to the lack of data on the potential toxic effects of acetamipridon pancreas.To better understanding for acetamiprid toxicity further studies including a wide range of mechanistic parametersare needed.

Keywords: Acetamiprid; AR42J pancreatic cell line; cytotoxicity; genotoxicity; oxidative damage.

ÖZET

Giriş ve Amaç: Neonikotinoid insektisidler dünya piyasasındaki insektisidlerin %30'luk kısmını oluşturur ve etkilerini seçici olarak böceklerdeki α4p2 nikotinik asid reseptörü (nAChR)'nü inhibe ederek gösterirler. Asetamipridin çeşitli organ ve sistemler üzerine toksik etkileri biliniyor olmasına karşın, pankreas üzerindeki etkisi ve etki mekanizması bilinmemektedir. Bu çalışmada, asetamipridin AR42j pankreas hücre hattı üzerinde sitotoksik ve genotoksik etkisi araştırıldı.

Metod ve Yöntemler: Sitotoksisite ve genotoksisite değerlendirmesi için MTT (3-[4,5-dimetiltiyazol-2-il]-2,5 difenil tetrazolyum bromür) ve comet analizi gerçekleştirildi. Reaktif oksijen ürünleri oluşumu (ROS) değerledirmesi akış sitometresi ile ve GSH analizi Eliza yöntemi ile gerçekleştirildi.

Bulgular: MTT sitotoksisite analizine gore, hücre canlılığının doza bağımlı olarak azaldığı gözlendi ve inhibitor konsantrasyon 50 (IC50) değeri 12.61 mM olarak tespit edildi. Analizlerde doz yanıt ilişkisini gösterebilmek için 1-6 mM doz aralığı seçildi. Asetamiprid DNA hasarını doza bağımlı olarak artırdı ve ortalama kuyruk yoğunluğu değerleri tüm gruplar

için 3.84 ve ≤32.96 arasında olarak belirlendi. ROS üretimi açısından gruplar arasında nalamlı bir fark gözlenmez iken, GSH değerinin en yüksek doz grubunda anlamlı olarak çok düştüğü tespit edildi.

Tartışma ve Sonuç: Bu çalışma literatürde asetamipridin pancreas üzerine olası toksik etkisine yönelik eksik olan verilere katkı sağlamaktadır. Asetamipridin toksik etkisinin detaylandırılması için daha geniş çapta mekanisik çalışmalara ihtiyaç bulunmaktadır.

Anahtar Kelimeler: Asetamiprid; AR42J pancreas hücre hattı; sitotoksisite; genotoksisite; oksidatif hasar.

INTRODUCTION

Many xenobiotics are important threatening factors for human health and environment. Pesticides, the most common pollutants, are harmful for biological structures via several mechanisms in their acute and long term exposure [1-3]. Neonicotinoid pesticides as a new class of insecticides which are commonly used instead of organophosphate and carbamate pesticides have selectively neurotoxic effects on nicotinic acetylcholine receptor (nAChRs). Neonicotinoid pesticides are highly effective insecticides which can disperse all part of plants, plant fluids and fruits grown on plants. Recent studies revealed that neonicotinoid pesticides can be associated with several adverse effects including decreased sperm production and function, 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 widely used neonicotinoid insecticides in many countries against to crop pests on agricultural products such as cotton, tobacco, potato, tomato, and nuts. In general, acetamiprid have been considered as a safe insecticide; however, adverse effects including headaches, nausea, dizziness, vomiting, and other symptoms may occur after exposure to acetamiprid as well the other neonicotinoids [3]. In few 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 is contradictory [7-12].As it is well known, worldwide rate of diabetes have risen every passing day, and the major molecular mechanisms lying under diabetes are increased oxidative stress and altered enzyme functions in pancreatic tissue [13].Indeed, any association between diabetes risk and neonicotinoid pesticides has not been reported. Furthermore, there was no study on toxic effects of acetamiprid on pancreas. Therefore, as the first time, we aimed to investigate cyto- and

genotoxic effects of acetamiprid on AR42J pancreatic cell line and evaluated the oxidative damage potential as an underlying molecular mechanism.

MATERIAL AND METHODS

Chemicals

Acetamiprid, Dimethyl sulfoxide (DMSO), sodium dodecyl sulphate (SDS), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) and 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA) were obtained from Sigma Chemical Co. Ltd. (St. Louis, Missouri, USA).Cell culture medium (RPMI 1640) and other supplements purchased from Multicell Wisent (Quebec, Canada); and sterile plastic wares were purchased from Corning (Amsterdam, The Netherlands). All other chemicals at required biological grade were obtained from Merck (New Jersey, USA).

Cell Culture and Treatments

AR42J (CRL1492) cell line was purchased from the American Type Culture Collection (ATCC, Virginia, USA), and cells were maintained as manufacturer's instructions. The cells were grown with Roswell Park Memorial Institute (RPMI) 1640 cell culture medium containing 10% of foetal bovine serum (FBS), 100 U of penicillin/mL, and 100 mg of streptomycin/mL in a humidified incubator supplied with 5% of CO_2 at 37°C. Sub-culturing was performed in every 2-3 day when the cells reached co-fluency. Prior to exposures cells were seeded into appropriate plastic-ware and incubated for overnight to ensure cell attachment.

Acetamiprid stock solution was prepared by dissolving in 100% of dimethyl sulfoxide (DMSO), and stored in -20°C until the day of assays conducted. Before the cell treatments acetamprid was diluted with cell culture medium to desired concentrations; and final DMSO concentration was 1%. Treatments were done at a concentration range for 24h to evaluate

dose-dependent effects. All experiments were performed in triplicates in three separate days. acetamiprid.

MTT Cytotoxicity Assay

The AR42J cells were seeded into 96-well plates ($1x10^4$ cells/100 µL cell culture medium/well). After overnight incubation cells were treated with acetamiprid at the concentration range of 1-50mM for 24h. Then, 5 mg/mL of MTT was added into each well and cells were incubated for further 3h at 37°C in the dark. Cell culture medium, 1% DMSO and 10% SDS were used as growth control, solvent control and positive control, respectively. The medium was removed after 3h, and the wells were washed with PBS for twice. Following the washing step, 100 µL of DMSO was added, incubated 5min on an orbital shaker (150 rpm)for evenly dissolved formazan crystals and optical densities (ODs) were measured at 570 nm using a microplate reader (Biotek, Epoch, Vermont, USA). Percentage of inhibition of cell viability was calculated by ODs for each concentration and IC_{50} value was determined.

Comet Genotoxicity Assay

The alkaline comet assay was performed according to Singh *et al.* (1988) with minor modifications [14]. AR42J cells were seeded at $5x10^5$ cells/2 mL cell culture medium/well into a 6-well plate and incubated for overnight. Then, cells were treated with acetamipridat 1, 2, 4 and 6 mM concentrations and 1% DMSO as a negative control for 24h. After cells were detached with trypsin-EDTA and washed with PBS twice, the viability of cells was checked with trypan blue dye exclusion; and cell viability was ≥80% in all concentrations. Briefly, 100 µL of single cell suspension was gently and evenly mixed with 100 µL pre-warmed low-melting point agarose (0.65% in PBS), layered on conventionalmicroscope slides pre-coated with normal-melting point agarose (1.5% in distilled water) and covered with cover slip. After slides were lysed for 1h at 4°C, DNA was unwinded for 20 min in cold-fresh electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH 13) at 4°C and electrophoresis was performed at 4°C

for 20 min (20 V/300 mA).. Then, the slides were neutralized with 0.4 M tris-HCl buffer (pH 7.5) 3 times for 5 min. DNA was stained with ethidium bromide (20 mg/mL) just before slide examination under a fluorescent microscope (Olympus BX53, Olympus, Tokyo, Japan) at 400 (40x10) magnification by using an automated image analysis system (Comet Assay IV, Perceptive Instruments, Suffolk, UK). A total of 100 cells were scored per concentration and DNA damage to individual cells was expressed as a percentage of DNA in the comet tail (%TDNA, tail intensity).

Oxidative StressPrameters

Total reactive oxygen species (ROS) assay was performed byDCFDA (2'-7'-dichlorodihydrofluorescein diacetate) assay by flow-cytometry (Schupp et al., 2008). Briefly, the cells were seeded at $5x10^5$cells/2 mL cell culture medium/well into a 6-well plate. After overnight incubation, the cells were treated with acetamprid at 1, 2, 4 and 6 mM concentrations and 1% DMSO as a negative control for 24h. Then, the exposure medium was discarded; the plates were washed with PBS twice, and incubated at 37°C for 30 min in PBS containing 20 µM H2DCF-DA on an orbital shaker in the dark. Cells were detached with trypsin-EDTA, washed into ice-cold PBS for two times, and re-suspended in 150 µl PBS with %1 bovine serum albumin (BSA). ROS dependent fluorescence intensity was measured in FITC channel (excitation at 488 nm; emission at 530 nm) on a ACEA NovoCyte flow cytometer (San Diego, California, USA). The results were expressed as the median fluorescence intensity.

GSH levels were determined in the cell homogenates using DTNB (5,50-dithiobis-2-nitrobenzoic acid) reagent method of Beutler (1975). This method based on DTNB reduction via free SH groups of GSH to 5-mercapto-2-nitrobenzoate.After treatment with acetamiprid, one millilitre of cell lysates were de-proteinated by addition of a solution containing 1.67 g metaphosphoric acid, 0.2 g Na_2EDTA and 30 g NaCl in distilled water. 2.4 ml Na_2HPO_4 and

0.3 ml DTNB were added to supernatants and centrifuged at 10 min 3000 g/min and the formation of 5-thio-2-nitrobenzoic acid measured spectrophotometrically at 412 nm against reagent controls at 25°C. GSH results were expressed as µmol per g protein using a standard calibration curve using standard calibration curve [15].

Statistical Analysis

Data were analysed by one-way ANOVA Posthoc Dunnett t-test and expressed as mean±SD. The level of statistical significance was set at $p \leq 0.05$. All analyses were performed using the statistical package SPSS version 20.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

RESULTS

Cell viability

The MTT assay is one of the most used,simple and rapid colorimetric cell viability/cytotoxicity assayyields quantitative data. MTT assay is based on reduction of water-soluble yellow tetrazolium saltby the mitochondrial succinate dehydrogenase enzymein metabolically active/live cells dehydrogenase and quantified colour intensity of dissolved formazan crystals with spectrophotometer [16].

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

Figure 1

Comet Assay

Alkaline comet assay (single cell gel electrophoresis) is a widely used method for measuring the amount of damaged DNA 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 alkaline comet assay in dose dependent manners and comparable behaves [17].

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

Figure 2

Oxidative Stress Parameters

Oxidative damage via ROS play key role in different human diseases as cancer, cardiovascular diseases, diabetes, neurodegeneration etc. Dichlorodihydrofluorescein diacetate (DCFH-DA)is widely used assay which enable to directly measure redox state in the cells. This method is very sensitive, cheap and lead to easy follow ROS changes in the cell over time [18].

There were no significant differences between control and exposure groups according to total ROS levels which evaluated by H2DCF-DA with flowcytometer. However, the GSH levelwas significantly reducedin 6mM group compared to control group. It has been observed that 6 mM of acetamiprid dramatically reduced GSH level as 98.07 % **(Figure 3).**

Figure 3

DISCUSSION

Widespread use of acetamiprid in agriculture alone or in combination with other insecticides may cause that pesticide entry into the food chain, which in turn leads toxicity in human and animals. Andreotti *et al.* (2009) reported in their cohort study that, the increased risk of pancreatic cancer is in accordance with agricultural occupations; however, pesticides' effects on oncogenesis mechanisms have not been extensively evaluated yet [17]. There is limited data about effects of neonicotinoids on pancreatic tissue; moreover, there is no data about

acetamiprid effects on pancreas. Khalil *et al.* (2017) reported that 0.5 and 1.0mg/kg bw imidaclopride for 60days disrupt glucose homeostasis in male rats. In treated groups, the GLUT4 mRNA expression level was decreased and, infiltrational regions, remarkable shrinkage of pancreatic islets and round cell infiltration, interlobular congestion, and haemorrhage were observed with histopathologic examination, and also decrease in the expression of insulin in the pancreatic β cells and reduction in the expression of insulin were detected in imidaclopride treated groups [13]To further examine the potential associations between their usage and toxic profiles for neonicotinoid pesticides, we conducted a study on in-vitro acetamiprid exposure in pancreatic cell line. Although there are a few studies on acetamiprid's cyto- and genotoxicity potentials in the literature, there is no in vitro/in vivo study focused on pancreas. It has been reported that LD_{50} value is the range of 140-417 mg/kg b.w. in rodents, and NOAEL level is 400 ppm in 13 week mice for acetamiprid [21]. Şenyıldız et al. (2018) reported IC_{50} values of acetamiprid on SH-SY5Y and HepG2 cell lines were 2.16 and 3.61 mM, respectively [22]. In our study, the cytotoxicity was determined by MTT assay in the acetamiprid (1-50 mM) treated AR42J cells after 24h exposure. IC_{50} value was calculated as 12.61 mM.

Şenyıldız et al. (2018) also showed significantly increased DNA damage at 500 µM in SH-SY5Y cells [22]. Çavaş et al. (2014), reported that >50 µM acetamiprid significantly increased micronuclei formation and DNA breaks in IMR-90 human lung cells. Kocaman and Topaktaş (2007) showed genotoxic effects of acetamiprid on sister chromatid exchange, micronucleus and chromosomal aberration analysis with 25, 30, 35, and 40 µg/ml doses for 24 and 48h in human peripheral blood lymphocytes. Micronucleus formation was significantly induced compared to control group while proliferation index was decreased [12]. Bagri and Jain (2018) reported acetamiprid increased micronuclei per cell and chromosomal aberrations in Swiss albino male mice bone marrow with depending on concentration with acetamiprid treatment

during 60 and 90 days at 4.6 and 2.3 mg/kg/day i.p [23]. According to the results of comet assay performed with concentration range of 1-6 mM, acetamiprid 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 cyto- and genotoxic potentials of neonicotinoid pesticides [7,8,12]. It has been reported that, pesticides may have impair the redox balance effects in the different cells. However, the mechanisms underlying oxidative stress still not fully understood [16]. Thereare several studies about the effects of acetamiprid on oxidative stress parameters in different species such as rodents, bacteria, plants, and fish [24-30]. Gasmi*et al.* (2017) demonstrated in Wistar rat brain tissue 3.14 mg/kg acetamiprid exposure results with increased mitochondrial oxidative stress status which are significant. Decreased oxidative stress parameters were glutathione (GSH) level, glutathione pyroxidase (GPx) and catalase (CAT) activities. Increased parameters were determined as MDA level, glutathione s-transferase (GST) and superoxide dismutase (SOD) activities [31]. In our study, there was no significant difference between control and exposure groups according to total ROS levels. However, the GSH levels were significantly different compared to control in the highest concentration. It has been observed that 6 mM acetamiprid dose dramatically reduced GSH level as 98.07%. In earthworms, it has been demonstrated that various concentrations of acetamiprid (0, 0.05, 0.10, 0.25 and 0.50 mg/kg of soil) respectively after 7, 14, 21 and 28 days, the ROS level increased in varying degrees in the most of exposure concentrations. Olive Tail Moment (OTM) which indicates DNA damage in comet assay increased with dose dependent manner which concluded sub-chronic exposure of acetamiprid might cause oxidative stress and DNA damage in earthworms [24].

Acetamiprid classified as “unlikely” human carcinogen according to EPA guidelines and its target organ toxicity data are not clear yet. Acetamiprid’s acute oral toxicity category is “II”

for rat and acute inhalation toxicity category is “III” for rabbit. NOAEL value for rat is 12.4/14.6 mg/kg/day (M/F) and LOAEL value for rat is 50.8/56.0 mg/kg/day (M/F). Chronic carcinogenicity NOAEL value for rat is 7.1/8.8 mg/kg/day (M/F). According to EPA acetamiprid is not yet classified as genotoxic [32]. Target organ toxicity assessment for sub-chronic and chronic in-vivo further studies may clarify risk of acetamiprid for pancreas tissue based diseases.

Under different time durations in pancreatic cell line acetamipridmay affect these oxidative stress parameters significantly. To clarify whether oncogenic potential of acetamiprid on pancreatic tissue it is needed to further in vivo studies with sub-chronic or chronic studies with molecular mechanistic observations.

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FIGURE LEGENDS

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

Figure 2: Acetamprid 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 decreasing in a dose-dependent manner. The error bar represents ±standard deviation and*p <0.05 compared to other groups.

Figure 3: Oxidative damage potential of acetamiprid (1, 2, 4 or 6 mM) evaluated by H2DCF-DA and GSH assays. The ROS production was expressed as mean fluorescence intensity and GSH levels were expressed as µg/g protein. The error bar represents ±standard deviation and *p<0.05 compared to other groups.

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