

# The subchronic effects of acetamipride on the global DNA methylation levels in Sprague-Dawley rat brain and liver

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## ABSTRACT

Acetamidrid, which is a neonicotinoid insecticide, is used to control leafy vegetables, fruiting vegetables, fir seeds, citrus fruits, pome fruits, grapes, cotton and ornamental plants and absorbent insects on flowers. The present study aim to evaluate global DNA methylation and gene expression of DNA methylation related enzymes in liver and brain tissues of male Sprague-Dawley rats after a 90-day subchronic exposure to acetamidrid at low doses of 12.5, 25 and 35 mg/kg body weight (b.w.). Global DNA methylation resulted in a significant decrease in the levels of 5-methylcytosine (5-mC%) at the doses of 25 and 35 mg/kg b.w. in the liver and 35 mg/kg b.w. in the brain compared to the vehicle control group. Consistently, expression of DNA methyltransferase enzymes decreased at doses of 12.5, 25 and 35 mg/kg b.w. in liver and 35 mg/kg b.w. in brain. It has been suggested that non-genotoxic (epigenetic) mechanisms may be involved in the toxicity of acetamidrid and further investigations are needed to elucidate the epigenetic effects of neonicotinoid insecticides.

**Keywords:** Acetamidrid, DNA methylation, Sprague-Dawley rats, liver, brain

## INTRODUCTION

Pesticides are defined as substances or mixtures of substances used to remove, reduce, suppress or degrade harmful organisms in order to increase productivity in agriculture. Pesticides are claimed to be quite useful as a result of their use in appropriate doses and conditions for their intended purpose. However, when they are used incorrectly and in higher than recommended doses, they harm human health as well as increase environmental pollution and affect other living things. Neonicotinoids are a relatively new type of insecticide used to control a variety of pests in agriculture and livestock (Honda et al. 2006). These pesticides are currently preferred to organophosphates and carbamates throughout the world owing to their ability of resistance against day light and higher toxicity to insects than mammals and aquatic organisms because of their affinity to the nicotinic acetylcholine receptors in insects (Kiryama et al. 2003; Tomizawa and Casida, 2003; Casida and Quistad, 2004; Whitacre and Ware, 2004; Ford 2008; Yu 2008). Neonicotinoids are systemic acting insecticides and affect the central nervous system of insects, resulting in paralysis and death. They can also be persistent in the environment. Some neonicotinoids are suspected to be carcinogenic and mutagenic in mice (Dich et al. 1997; Office of Prevention, Pesticides and Toxic Substances 2003; Green et al. 2005). Acetamidrid (N-[(6-chloropyridin-3-yl)methyl]-N'-cyano-N-methylethanimidamide), a neonicotinoid pesticide, was the second insecticide to be manufactured in this group after the launch of imidacloprid and was first marketed in Japan under the brand name Mospilan (Yamamoto and Casida 1999; Ünver and Uysal 2014). It has been reported that acetamidrid is widely used in agriculture in Turkey (Kocaman and Topaktaş 2007). Acetamidrid is a selective agonist of nicotinic acetylcholine receptor in postsynaptic membrane. LD<sub>50</sub> value of acetamidrid is 140-417 mg/kg body weight (b.w.) in different rat strains (Kanungo and Solecki 2011).

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Acetamidrid may lead to oxidative damage by producing reactive oxygen species in target tissues (Yao et al. 2006; Ford et al. 2011; Zhang et al. 2011). Additionally, it has been reported that chronic exposure to acetamidrid caused disturbance of matrix oxidative status, and a loss of mitochondrial membranes integrity in rat brain via generating reactive oxygen species (Gasmi et al. 2016 and 2017). Acetamidrid accumulates in the brain of murine and rats (Devan et al. 2015; Terayama et al. 2016). Besides, it can cause changes in brain functions -such as the break-down of learning ability (Mondal et al. 2014; Mandal et al. 2015). In a few studies, the genotoxic and cytotoxic effects of acetamidrid have been investigated. It has been reported that acetamidrid induced frequency of sister chromatid exchange, chromosomal aberrations, and micronucleus formation in human peripheral lymphocytes (Kocaman and Topaktas 2007), micronucleus formation and DNA damage in Caco-2 cells (Cavas et al. 2012) and micronucleus formation and DNA damage in human lung fibroblast cells (Cavas et al. 2014), while acetamidrid did not increase micronucleus formation in peripheral blood lymphocytes (Muranli et al. 2015). According to these studies results on the mechanisms of action in acetamidrid toxicity have been controversial. It has become crucial for evaluation of possible genotoxic and cytotoxic effects of acetamidrid on living organisms to take place, due to it becoming increasingly widespread in the world.

DNA methylation, one of the most studied non-genotoxic (epigenetic) modifications, plays an important role in cell proliferation and various diseases such as cancer and diabetes (Baylin 1997; Moggs et al. 2004; Jones and Baylin 2007; Kulis and Esteller 2010; Anderson et al. 2012; Bansal and Pinney 2017). DNA methylation contributes to alterations in gene expression of key molecular pathways including global DNA hypomethylation and hypermethylation of CpG islands of tumor suppressor genes (Baylin et al. 1986; Watson and Goodman 2002). There has only been one study that shows alterations of global and gene-specific DNA methylation after acetamidrid exposure in mouse embryonic stem cells (Wang et al. 2019). Therefore, we aimed to investigate the role of DNA methylation changes in acetamidrid toxicity. For this purpose, we determined the global levels DNA methylation and gene expression of related enzymes in liver and brain tissues after subchronic acetamidrid exposure to Sprague-Dawley albino adult rats. This is the first study which analyzed the effects of acetamidrid on global DNA methylation as a key molecular mechanism of epigenetic modulation in rat tissues. Because acetamidrid is a neuro-active insecticide and acts as neurotoxic agent and because the liver is the main target organ for acetamidrid metabolism and elimination, we selected brain and liver tissues for the evaluation of DNA methylation analysis.

## MATERIALS AND METHODS

### Chemicals

Acetamidrid, technical purity 97%, was obtained from a national company (Hektaş Ticaret T.A.Ş. Istanbul, Turkey) and weekly suspended in an aqueous solution of 0.5% methylcellulose (Merck, Darmstadt, Germany) before use. All other supplements were purchased from Wisent Bioproducts (Saint-

JeanBaptiste, QC, Canada) and sterile plastic materials were purchased from Nest Biotechnology (Jiangsu, China). DNA, RNA isolation kits and cDNA synthesis kits were obtained from Roche Life Sciences (Penzberg, Germany). 5-methylcytosine (5-mC) DNA ELISA kit was purchased from Epigentek Research (Farmingdale, NY, USA). Syber green master mix was obtained from Biorline (London, UK) and primers for gene expressions were obtained from Sentromer DNA Technologies (Istanbul, Turkey).

### Animal treatments

In this study male Sprague-Dawley albino adult rats, aged 8-12 weeks and weighing 250-375 g were obtained from Aziz Sancar Institute of Experimental Medicine, Istanbul University. The animals were housed throughout the experiment in polypropylene standard cages in which 4-5 animals were placed. Animals were maintained under controlled conditions of temperature at 22-24°C, normal photoperiod (12-12 h light-dark cycle) and relative humidity of (50±10%). The animals were allowed free access to standard dry pellet diet and tap water ad libitum. The experiments reported here complied with the current laws and regulations of the Turkish Republic on the care and handling of experimental animals and the local ethics committee of experimental animals of Istanbul University (IUHADYEK; 2016/35 and 2016/42).

### Experimental design

The animals were randomly divided into four experimental groups. The substances were administered in the morning (between 09.00 and 11.00 a.m.) to rats who had not fasted:-

Group I (Vehicle control Group): Control rats received intragastrically (i.g.) a vehicle (0.5% methylcellulose) (n=11).

Group II: Acetamidrid at the dose of 12.5 mg/kg body weight (b.w.) acetamidrid (NOAEL) in a vehicle (0.5% methylcellulose) was applied to rats i.g. once a day for 90 days (n=12).

Group III: Acetamidrid at the dose of 25 mg/kg b.w. acetamidrid in a vehicle (0.5% methylcellulose) was applied to rats i.g. once a day for 90 days (n=12).

Group IV: Acetamidrid at the dose of 35 mg/kg b.w. acetamidrid in a vehicle (0.5% methylcellulose) was applied to rats i.g. once a day for 90 days (n=13).

Selection of treatment concentrations in the present study were based on no observable adverse effect level (NOAEL) of acetamidrid (12.4 mg/kg b.w.), and on increased liver weight and centrilobular hepatocyte hypertrophy for the 90 days rat study (EFSA 2016). At the end of the treatments, the rats were sacrificed on the 90<sup>th</sup> day by removing a large volume of blood from the orbital veins under inhalation anesthesia induced by diethyl ether. Brain and kidney samples were dissected, placed in a sufficient amount of phosphate buffered saline (PBS) (1x) and immediately stored at -80°C until analysis. The tissues were homogenized in 0.9% NaCl using a tissue homogenizer (Ultra-Turrax T-18, IKA Werke GmbH&Co., Staufen, Germany) to make up the 10% homogenate (w/v). After that DNA and RNA isola-

tion was carried out from these homogenates (10%) of liver and brain tissues.

**Global DNA methylation analysis**

Genomic DNA was isolated from liver and brain tissue homogenates (10%) using the High Pure PCR Template Preparation kit (Roche Life Sciences, Penzberg, Germany) according to the manufacturer’s instructions. To measure global levels of 5-mC%, 100 ng of DNA samples were applied to MethyFlash™ Methylated DNA Quantification kit (Epigentek, Farmingdale, NY) according to the manufacturer’s instructions as previously described (Karaman and Ozden 2019).

**Gene expression analysis of DNA methyltransferases**

Total RNA was isolated from liver and brain tissues using a High Pure RNA Tissue kit (Roche Life Sciences, Penzberg, Germany). Reverse transcription was performed by Transcriptor First Strand cDNA Synthesis kit (Roche Life Sciences, Penzberg, Germany) from 500 ng of total RNA and the mixture of anchored-oligo(dT) and random hexamer primers. 5 µL of the 1/10 diluted RT-reaction was used as the template in real-time quantitative PCR. Gene expressions of DNA methyltransferases such as *DNMT1*, *DNMT3a*, *DNMT3b*, were measured using Bio-Line SensiFast™ Syber® No-Rox kit (London, UK) on LightCycler® 480 Instrument II (Roche Life Science). Primer sequences and their annealing temperatures of genes are illustrated in Table 1. Evaluations of results for all genes were performed as described previously (Karaman and Ozden 2019).

**Statistical analysis**

Results of 5-mC% levels and gene expression were represented as mean ± standard deviation (SD). Statistical analysis was performed by ANOVA followed by Dunnett’s multiple comparison test using The Statistical Package for the Social Sciences (SPSS) version 21.0 for Windows, statistical program (IBM Corp.; Armonk, NY, USA). P values of less than 0.05 and 0.001 were selected as the levels of significance.

**RESULTS**

**Effects of acetaminophen on the global DNA methylation levels**

Levels of 5-mC% were measured after 12.5, 25 and 35 mg/kg b.w. of acetaminophen treatments for 90 days by Elisa kit. 25

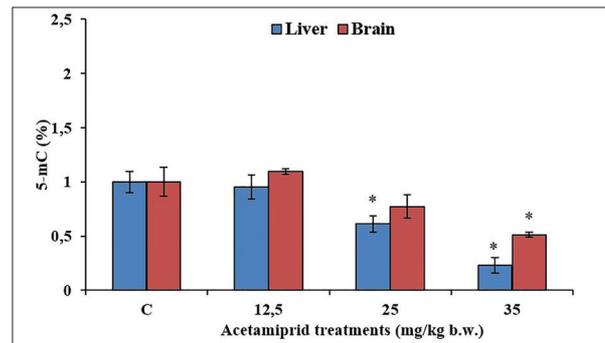
and 35 mg/kg b.w. of acetaminophen treatments resulted in a significant decrease in 5-mC% status (38.7%,  $p < 0.05$  and 77%,  $p < 0.05$ , respectively) in liver comparison with the vehicle control group. 5-mC% levels reduced significantly (48.7%,  $p < 0.05$ ) after 35 mg/kg b.w. of acetaminophen treatments in brain comparison with the vehicle control group (Figure 1).

**Effects of acetaminophen on DNA methyltransferases gene expression levels**

We analysed the gene expressions of DNA methyltransferases (*DNMT1*, *DNMT3a*, *DNMT3b*) to support the global DNA methylation results of acetaminophen in liver and brain. In Figure 2a, our data showed that acetaminophen treatments (12.5, 25 and 35 mg/kg b.w.) significantly decreased expression levels of *DNMT1* ( $\geq 1.96$  fold), *DNMT3a* ( $\geq 2.86$  fold) and *DNMT3b* ( $\geq 1.95$  fold) in comparison with the vehicle control group. In Figure 2b, 35 mg/kg b.w. of acetaminophen significantly decreased expression levels of *DNMT1* (2.44 fold), *DNMT3a* (2.63 fold) and *DNMT3b* (1.92 fold) in brain in comparison with the vehicle control group.

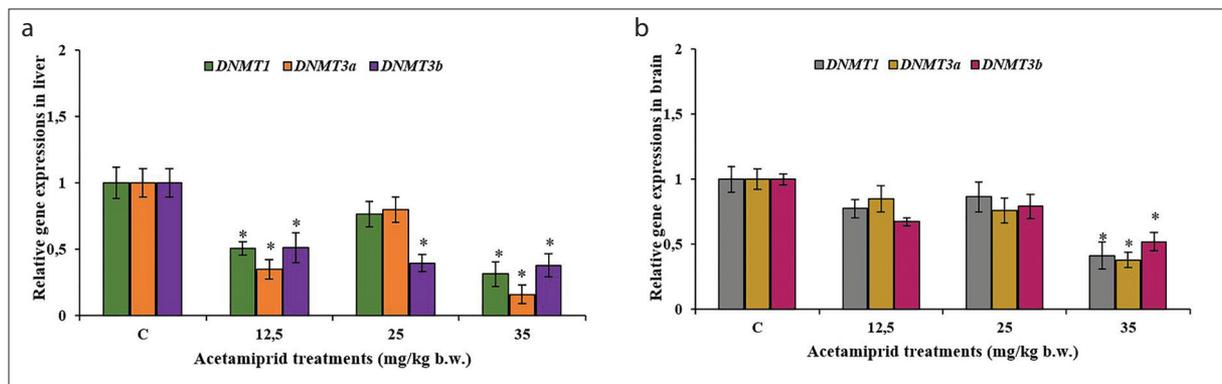
**DISCUSSION**

Due to their low cost and easy application, the use of pesticides have been preferred as a way of combating plant diseases, pests and weeds which cause a loss of significant amounts of the product or product quality at postharvest storage and agricultural production times. Because of unconscious, incorrect applications of pesticides by manufacturers at harvest and



**Figure 1.** Effects of acetaminophen (12.5, 25 and 35 mg/kg b.w.) on levels of 5-mC% in liver and brain tissues of male Sprague-Dawley albino adult rats. Data are presented as mean ± SD. Statistically significant changes are indicated by \* $p < 0.05$ . (one way ANOVA-Dunnett post hoc test.).

Gene	Primer sequence (5'-3')	Tm (°C)	Reference
<i>DNMT1</i>	F: GGTCTGCGCGGGGACAGAC	66	Xu et al. 2015
	R: CCGGCAACATGGCCTCAGGG		
<i>DNMT3a</i>	F: GGTGTGTGTCGAGAAGCTCA	60	Xu et al. 2015
	R: CCAAGGGCCCACTCAATCAT		
<i>DNMT3b</i>	F: GGGCCGCTACCACGTTCCAGG	64	Xu et al. 2015
	R: AGGGCCGTCCTGGCTCAAGT		
<i>β-actin</i>	F: GCGTCCACCCGCGAGTACAA	64	Xu et al. 2015
	R: ACATGCCGGAGCCGTTGTGC		



**Figure 2. a, b.** Effects of acetamiprid (12.5, 25 and 35 mg/kg b.w.) on relative gene expression levels (*DNMT1*, *DNMT3a*, *DNMT3b*) in liver (a) and brain (b) tissues of male Sprague-Dawley albino adult rats. Data are presented as mean $\pm$ SD. Statistically significant changes are indicated by \* $p$ <0.05; \*\* $p$ <0.001. (one way ANOVA-Dunnett post hoc test.).

close to the harvest period, the consumption of pesticides above what is necessary to get higher quality products, high levels of drug residues are found on foodstuffs resulting in toxic effects on humans and the environment. Neonicotinoids which act as agonists on the nicotinic acetylcholine receptors (nAChRs) of insects and mammals, form a commercially important pesticide group used as insecticides with increasing use in recent years (Tomizawa and Casida 2003; Sanyal et al. 2008; Simon-Delso et al. 2015).

Acetamiprid is one of the most widely used neonicotinoids which is distributed throughout the body, especially in the liver, kidney, adrenal and thyroid glands, by reaching a high concentration (EFSA Panel 2013). Chakroun et al. (2016) evaluated the hematological, biochemical, and histopathologic effects of acetamiprid on Wistar rats during 60 days and have observed a significant decrease in body weight gain, hematological parameters and an increase in the relative liver weight. They reported that acetamiprid induced liver toxicity through the increases in the activities of the enzymes including aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and lactate dehydrogenase which are the indicators of hepatocellular damage (Chakroun et al. 2016). It has been reported that acetamiprid inhalation in humans causes headache, dizziness, nausea, vomiting and other symptoms (Chen et al. 2007).

Investigating epigenetic alterations such as DNA methylation could be useful biomarkers for the toxicity assessments of environmental exposures (Baccarelli and Bollati 2009; Collotta et al. 2013; Greally and Jacobs 2013; Maqbool et al. 2016). It was shown that loss of 5-mC residues termed as global DNA hypomethylation is a common feature in the oncogenesis of many tumor tissues, leading to genomic instability (Gama-Sosa et al. 1983). Emerging evidence indicates that environmental chemical exposure such as dichlorodiphenyltrichloroethane (DDT), organochlorine pesticides, methylmercury chloride or polychlorinated biphenyls, causes epigenetic changes via DNA methylation machinery malfunctions with an increase of carcinogenic risk and developing neurodegeneration (Desaulniers et al. 2009; Shutov et al. 2009; Kanthasamy et al. 2012; Collotta et al. 2013). It has been reported that blood levels of persistent organic pollutants which accumulate in adipose tissue were inversely related

with global DNA methylation levels (Collotta et al. 2013). Kim et al. (2010) observed that exposure to organochlorine pesticides caused global DNA hypomethylation in healthy Koreans. As shown in previous studies it has been emphasized that potential role of epigenetic changes serve as markers for environmental chemical exposures and risk assessment. In the risk assessment process, the evaluation of epigenetic alterations in the toxicity of neonicotinoid insecticides is important. Only one study has been performed on the epigenetic alterations in acetamiprid exposure in cell culture (Wang et al. 2019). Therefore, we aimed to investigate global DNA methylation levels in response to acetamiprid exposure in rat liver and brain. We showed that acetamiprid decreased the global DNA methylation levels in liver and brain tissues of rats, and consistently expression levels of the genes regulating DNA methylation *DNMT1*, *DNMT3a*, *DNMT3b* have also decreased. Wang et al. (2019) showed that neonicotinoids induced global DNA methylation, and imidacloprid had greater effects than acetamiprid in embryonic stem cells. Ivkovic et al. (2018) suggested that DNA methylation status was disrupted in acetamiprid treated-zebrafish embryos and they reported acetamiprid induced alterations in the methylation levels of certain genes such as *CYP19A1*, *p53*, *p21* during the early embryonic development of zebrafish.

In conclusion, we showed that global DNA methylation could be associated with acetamiprid toxicity in rat liver and brain tissues. Further studies are needed to better understand the role of epigenetic modifications in the mechanisms of toxicity for acetamiprid and also for other neonicotinoids in the risk assessment processes.

**Ethics Committee Approval:** The experiments reported here complied with the current laws and regulations of the Turkish Republic on the care and handling of experimental animals and the local ethics committee of experimental animals of Istanbul University (IUHADYEK; 2016/35 and 2016/42).

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

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