

## In-vitro Investigation of Imidaclopride effects on AChE, LDH and GSH levels in L-929 Fibroblast Cell Line

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### Abstract

**INTRODUCTION:** There are several type of pesticides to control pests and several new type of pesticides come into use that could less toxic compared to old ones. Pesticide induced oxidative stress which is one of the main mechanism of toxicity, mostly focused research area in the last decade. There are several different studies in the literature on whether pesticide exposure induce oxidative stress parameters mediated toxicity... Pesticide induced oxidative stress level depends on biochemical feature of mammalian systems. Imidacloprid is a neonicotinoid pesticide which widely use that considered safe, however it has been reported in different studies that imidacloprid may cause changes oxidative stress parameters.

**METHODS:** Thus, in this study we aimed to investigate dose and time dependent imidaclopride effects on AChE, LDH and GSH levels in L-929 fibroblast cell line. 1-500 µg imidaclopride dose range effects on acetylcholinesterase, glutathione and lactat dehydrogenase were investigated in this study.

**RESULTS:** LDH levels were significantly increased dose dependently in 250 and 500 ng imidaclopride compared to control group. GSH levels non-significantly decreased dose dependently and GSH levels were decreased in 500 ng imidaclopride group compared to control. There were no significant difference between groups for acetylcholinesterase levels.

**DISCUSSION AND CONCLUSION:** These results indicated that high doses imifoclopride may induce oxidative stress parameters in fibroblast cells.

**Key Words:** Imidaclopride, L-929 cell line, Oxidative stres, AChE

### Özet

**GİRİŞ VE AMAÇ:** Çok çeşitlilikte pestisit türleri zararlıları kontrol etmek amacı ile bulunmakta ve pek çok yeni tür pestisitte daha az toksik olması amaçlanarak kullanıma girmektedir. Toksikite mekanizmalarında önemli rol alan pestisit uyarımlı oksidatif stress, son on yılda en çok odaklanılan araştırma alanı olarak karşımıza çıkmaktadır. Literatürde pestisitlerin oksidatif stres aracılık toksisiteyi uyarıp uyarmadığına yönelik çok sayıda çalışma bulunmaktadır. Pestisit nedenli oksidatif stres seviyesi, memeli sistemlerin biyokimyasal özelliklerine bağlıdır. İmidacloprid güvenilir olduğu varsayılan bir neonicotinoid türü pestisittir, ancak farklı çalışmalarda imidaclopridin oksidatif stres parametrelerini değiştirdiği bildirilmiştir.

**YÖNTEM VE GEREÇLER:** Bu çalışmada doz ve süre bağımlı olarak imidaclopridin L-929 fibroblast hücrelerinde AChE, LDH ve GSH parametreleri üzerine etkisini inceledik. 1-500 µg imidacloprid dozlarının asetilkolinesteraz, glutatyon ve laktat dehidrojenaz üzerine etkileri araştırıldı.

**BULGULAR:** 250 ve 500 ng imidaclopridin LDH seviyelerini, kontrol grubuna kıyasla anlamlı olarak artırdığı tespit edildi. GSH seviyeleri dozdan bağımsız olarak 500ng imidacloprid dozunda kontrol grubuna kıyasla anlamlı olarak azaldığı tespit edildi. Asetilkolinesteraz seviyeleri arasında anlamlı bir fark gözlenmedi.

**TARTIŞMA VE SONUÇ:** Bu sonuçlara göre yüksek doz imidacloprid maruziyeti fibroblast hücrelerinde oksidatif stres parametrelerini uyarabileceği gözlemlenmiştir.

### Abbreviations:

AChE Acetylcholinesterase

DMEM Dulbecco's Modified Eagle's Media

DMSO Dimethyl sulfoxide

ELISA Enzyme-Linked ImmunoSorbent Assay

GSH Glutathione

LD50 Lethal dose 50

LDH Lactate Dehydrogenase

**Kısa Başlık:** Imidaclopride Induced Oxidative Stress

## 1. Introduction

Pesticides are mixtures of substances used to prevent, control or reduce harmful organisms. Pesticides include active substances and filling material that facilitate usage and increase effect of active substances. Commercially available drugs are manufactured and launch to market with its mixed form with filling material. In Turkey 2500 tons pesticides used every year (1-3).

It has been speculated that, neonicotinoid pesticides exhibit much lower toxic effects on mammals than insects, and therefore replace organophosphates and carbamate insecticides. Neonicotinoid pesticides are at increased risk for humans due to often used not only in agricultural applications but also in the removal of domestic pests. Neonicotinoid pesticides show their selective toxic effects on insects via nicotinic acetylcholine receptors. Imidaclopride which is a member of a new neuroactive neonicotinoid insecticide class, is commonly used insecticide worldwide and also in Turkey. It has been reported that 120 country use commonly imidaclopride since 1990 that was first introduced commercially. It has been reported that, imidaclopride's oral LD50 values for rats 380–650 mg/kg of body weight and for mice 130–170 mg/kg of body weight, respectively. Under aerobic conditions, imidaclopride is environmental persistent chemical with its 3-year half-life which increase risk. (4-7).

Pesticide-induced oxidative stress has been the focus of toxicological research over the last decade as a possible mechanism of toxicity. Several studies have been conducted to determine whether oxidative stress in humans or animals is caused by various agents in this group and is related to their toxic effects (8,9). It has been reported in different studies that imidaclopride induce oxidative stress parameters imbalance in different organisms in-vitro and in-vivo (10-17), however there are no study on imidaclopride's oxidative imbalance effects on fibroblast tissue in the literature.

In this study we aimed to investigate in-vitro effects of imidaclopride on AChE, LDH and GASH parameters in L929 fibroblast cell line.

## 2. Materials-Methods

**Chemicals:** Dulbecco's Modified Eagle's Media (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), penicilin streptomycin solution, and trypsin-EDTA solution were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Imidaclopride was purchased from Acetylcholinesterase Assay Kit (Fluorometric Red, ab138873), Lactate Dehydrogenase (LDH) Assay Kit (Fluorometric, ab197000), and Glutathione Assay Kit (Fluorometric, ab65322) were purchased from Abcam, (Cambridge, UK) L929 cell line (CRL-6364) was purchased from the American Type Culture Collection (ATCC, VA, USA). Imidaclopride-based herbicide (GORTCA FS 600) was purchased from Safa Tarım LTD, Turkey and contained pure imidaclopride (CAS Number: 138261-41-3, Product code: 0210-207) was used in the experiments.

**Cell Culture and Treatments:** All experiments on L929 were performed within 20 passages. The cells were grown with DMEM containing 10% of FBS and 1% penicilin-streptomycin-amphotericin B in a humidified incubator supplied with 5% of CO<sub>2</sub> at 37 °C. Before conducting the treatments, the cells were cultured for 24 h to ensure attachment.

The L-929 cells were incubated with imidaclopride which were dissolved in 100% of dimethyl sulfoxide (DMSO) and diluted with medium to the desired concentrations as 500µg, 250µg, 125µg, 50µg, 25µg, 5µg and 1µg. Studies reported that cytotoxicity is observed at concentrations higher than 500 ug / ml. In present study high concentration of imidacloprid determined as 500. (18). Vehicle control cells received equal volume of DMSO (0.5%) as the treatments for 24 h. LDH, GSH and AChE assays were performed after 24 h exposure.

**LDH and GSH Parameters:** Lactate dehydrogenase (LDH) is a cytosolic enzyme and the measurement of its leakage to the extracellular matrix due to cell membrane damage is an indicator of cell membrane integrity loss and oxidative stress (19).

For this purpose, the L-929 cells were plated in 96-well plates at  $1 \times 10^4$  cells/well and grown for 24 h. Following cell treatments, the LDH release in supernatants due to membrane damage was quantified using a rat LDH ELISA kit (Cat. Number: E-EL-R2547) in accordance with the manufacturer's protocol. 100  $\mu$ l standard or sample were added to each well then incubate 90 minutes at 37°C. After removed the liquid, 100  $\mu$ l Biotinylated Detection Ab added and incubated 1 hour at 37°C. Following aspiration and washing 3 times, 100  $\mu$ l HRP Conjugate added and incubated 15 minutes at 37°C. Following aspiration and washing 5 times, 90  $\mu$ l Substrate Reagent added and incubated 15 minutes at 37°C. When 50  $\mu$ l Stop solution added, it read at 450nm using micro-plate reader (Biotek Epoch, USA).

GSH is an important molecule for the cellular antioxidant system and under oxidative stress conditions GSH level decreases (Robaczewska J et al., 2016). The L-929 cells were cultured in 25 cm<sup>2</sup> flasks to determine GSH levels. Following cell imidaclopride exposure,  $1 \times 10^6$  cells were harvested in 1 mL of PBS and were homogenized by sonication, and the GSH content of L-929 cells was determined using the GSH (Glutathione) ELISA kit (Cat. Number: E-EL-0026) to assay human GSH following the manufacturer's procedure and referred as  $\mu$ mol/gprotein. 50  $\mu$ l standard or sample were added well then immediately added 50  $\mu$ l Biotinylated Detection Ab to each and incubate 45 minutes at 37°C. Following aspiration and washing 3 times, 100  $\mu$ l HRP Conjugate added and incubated 30 minutes at 37°C. Following aspiration and washing 5 times, 90  $\mu$ l Substrate Reagent added and incubated 15 minutes at 37°C. When 50  $\mu$ l Stop solution added, it read at 450nm using micro-plate reader (Biotek Epoch, USA).

**Acetylcholinesterase Assay:** The AChE is strikingly distributed in cell-substrate interface of radiated and migrating fibroblasts (Morphoregulation by acetylcholinesterase in fibroblasts and astrocytes.) and it helps to understand the nervous system work properly. The L-929 cells were cultured in 25 cm<sup>2</sup> flasks to determine Acetylcholinesterase levels. Following cell imidaclopride exposure,  $1 \times 10^6$  cells were harvested in 1 mL of PBS and were homogenized by sonication, and the Acetylcholinesterase content of L-929 cells was determined using the Acetylcholinesterase Assay Kit (Colorimetric, Cat. number: ab138871) 50  $\mu$ l of acetylcholinesterase reaction mixture added into each well of acetylcholinesterase standard, blank control, and test samples to make the total acetylcholinesterase assay volume of 100  $\mu$ l/well then incubated the reaction for 30 minutes at room temperature. Increased fluorescence monitored absorbance OD at 140 nm with micro-plate reader (Biotek Epoch, USA).

**Statistical Analysis:** All the experiments were performed as three replicates and the results were presented as the mean  $\pm$  standard deviation. The statistical comparisons were evaluated using the One-way ANOVA analysis of variance followed by the Tukey's test for post hoc analysis, and the statistical significance was set at  $p < 0.05$  (SPSS, version 21.0, USA).

### 3. Results

LDH results of imidaclopride exposure on L929 cells were shown in Figure 1. We observed that LDH levels were increased dose dependently. 250 and 500 ng imidaclopride increased LDH levels statistically significant compared to control group. GSH results of imidaclopride exposure on L929 cells were shown in Figure 2. GSH levels non-significantly decreased dose dependently and GSH levels were decreased in 500 ng imidaclopride group compared to control. AChE results of imidaclopride exposure on L929 cells were shown in Figure 3. There were no significant difference between groups, however AChE level nonsignificant decreased in 125 ng group.

### 4. Discussion

Neonicotinoids as a worldwide popular pesticides that usage of them have increased since 2000. neonicotinoids show their effects on insects via cellular nAChR mechanism (20,21). In recent years, neonicotinoids takes pyrethroid, organophosphorus and carbamate insecticides places in the usage (22). It is believed that neonicotinoids are safe pesticide group for non-target species, however there are several recent study in the literature about neonicotinoids different toxic effects on non-target organisms (23,24).

)Stable cytoplasmic enzyme lactate dehydrogenase (LDH) is an important biomarker for oxidative stress, apoptosis, necrosis, and other forms of cellular damage, expressed in all cells and rapidly released when the plasma membrane is damaged. In a study conducted in 2018, Kumar et al., showed that LDH level increased with cellular damage in accordance with our study (25, 26). Abu Zaid et al., reported that imidaclopride exposure in Rock pigeon (*Columba livia domestica*) resulted with significant increase of plasma LDH levels in high dose (6 mg/kg) and median dose (3 mg/kg) imidaclopride groups, however there were no significant increase in low dose (2 mg/kg) group compared to control group. Plasma AChEs enzyme activities in all imidaclopride dose groups significantly increased compared to control group (27). Lonare M et al., demonstrated that 28 days, 45 and 90 mg/kg body weight oral exposure of imidaclopride significantly decreased AChE level in rats erythrocytes. In addition, Brain AChE activity in rats of imidaclopride treatment groups dose dependant compared to control group. Also they demonstrated that LDH levels increased and GSH levels decreased significantly imidaclopride treated groups in different tissues of rats (28). Tian et al., showed that imidaclopride exposure in Chinese rare minnows brain increased GSH and the AChE activities. This results indicate that imidaclopride have no effect on Chinese rare minnows (11). Topal et al, demonstrated that imidaclopride have neurotoxic effects through AChE inhibition, and induce oxidative stress in rainbow trout brain tissue (29). It has been reported that imidaclopride exposure significantly decreased AChE levels in plasma and brain of 10 and 20 mg/kg treated female rats (30). In another study 24 hours 20 mg/kg imidaclopride administration decreased AChE activity approximately 22% in brain and 28% in red blood cells (31).

Thus, studies about imidaclopride effects on AChE, LDH and GSH Express controversial results which may related with different study conditions as species variety, exposure time variety, in-vitro and in vivo conditions and different doses. There are studies that include oxidative stress parameters inducing effect of imidaclopride with different species in the literature, however underlying mechanism is still not elucidated yet. Further studies needed to clarify toxic effects of neonicotinoids specially imidaclopride in different species and in different tissues.

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**Figure 1:**

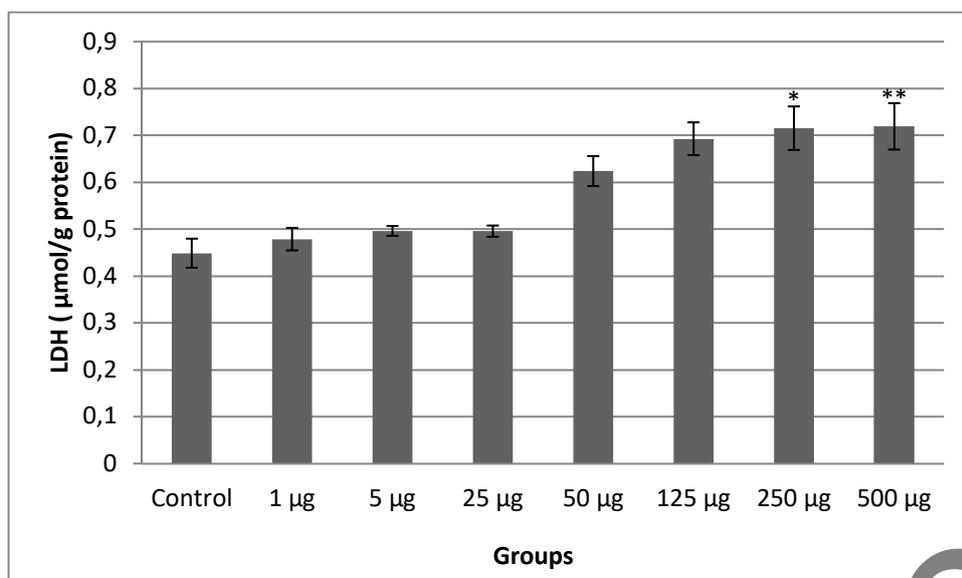


Figure Legend:

Figure 1. LDH level in L-929 cells with imidaclopride exposure.

Figure 2:

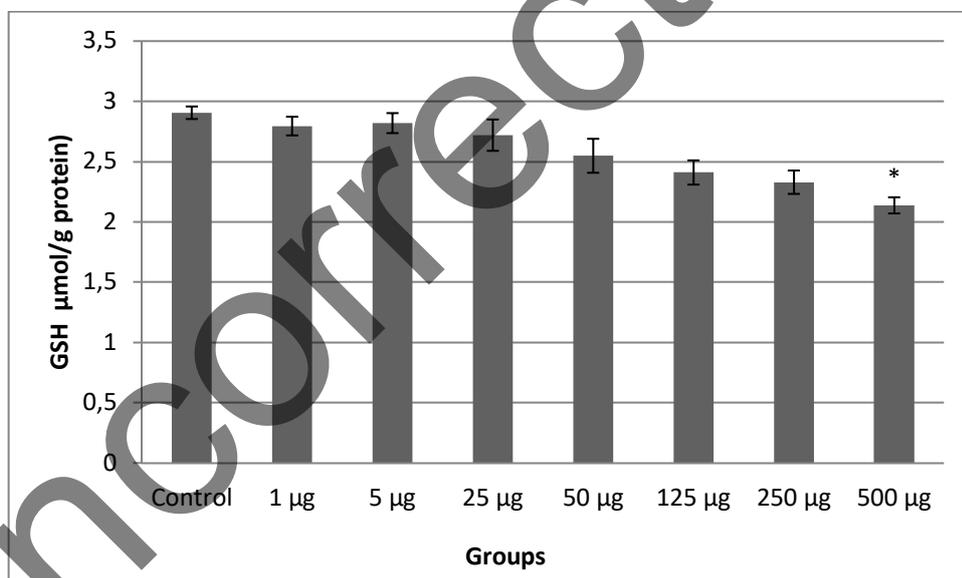


Figure Legend:

Figure 2. GSH level in L-929 cells with imidaclopride exposure.

Figure 3:

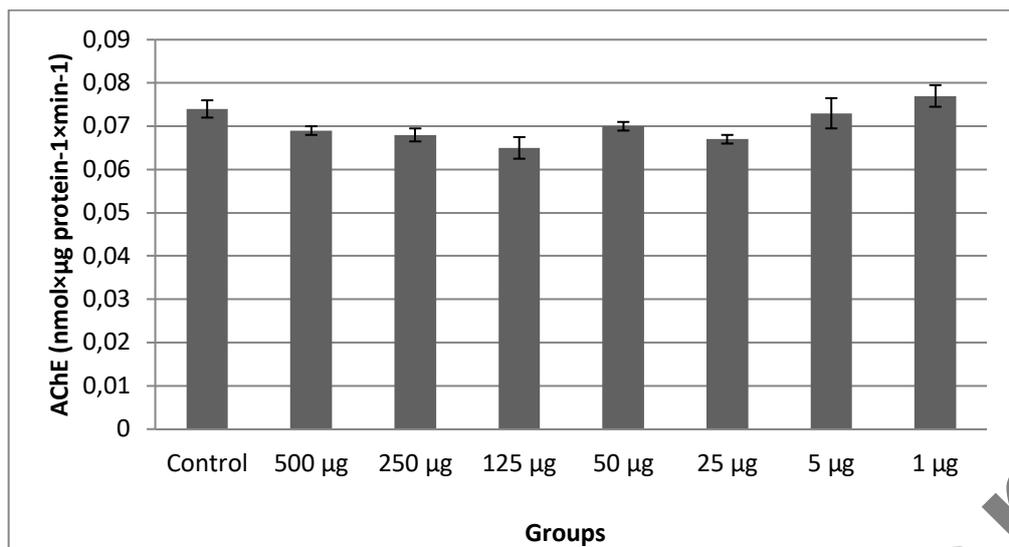


Figure Legend:

Figure 3. AChE level in L-929 cells with imidaclopride exposure.

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