sciendo

DOI: 10.2478/fv-2024-0004

FOLIA VETERINARIA, 68, 1: 31—36, 2024

DNA DAMAGE DETECTION AFTER PESTICIDE EXPOSURE

Sabína Baloďanská, Martina Galdíková, Simona Koleničová, Beáta Holečková, Viera Schwarzbacherová, Jana Halušková

Department of Biology and Physiology, University of Veterinary Medicine and Pharmacy in Kosice, Komenského 73 041 81, Košice
Slovakia

martina.galdikova@uvlf.sk

ABSTRACT

Thiacloprid is an insecticide of the neonicotinoid family that acts on the insect's nAChR receptor and causes its paralysis and subsequent death. The genotoxicity of the insecticide Calypso® 480 SC (with the active substance thiacloprid) to human peripheral lymphocytes was examined *in vitro* by comet analysis and phosphorylated H2AX, where we detected DNA breaks. To detect DNA damage in comet analysis, we used thiacloprid-based insecticide in three different concentrations (60, 240, and 480 µg.ml^{-1}) during 2 h of exposure. We recorded a statistically significant percentage of damage at a concentration of 240 and 480 µg.ml^{-1}. We monitored the toxicity of thiacloprid using γH2AX foci, and we did not observe a statistically significant number of foci compared to the negative control.

Key words: comet assay; DNA damage; neonicotinoids; thiacloprid; H2AX

INTRODUCTION

Neonicotinoids are one of the most important groups of insecticides chemically related to nicotine [10]. They are among the newly emerging groups of pesticides that are used for the protection of plants (crops, fruits, and vegetables), livestock, and domestic animals. The mechanism of action is that by their selective action on the nicotinic acetylcholine receptor (nACHR) in the central nervous system (CNS) of insects, they cause convulsions, paralysis, and subsequently death [9]. Their popularity is due to their unique biological properties such as broad spectral activity, low application doses, excellent absorption and translocation in plants, a novel mode of action, and a favourable safety profile [12].

Despite their positive properties, neonicotinoids have a systemic effect, long persistence, and are highly soluble in water, ultimately leading to the possibility of environmental contamination and toxicity to non-target organisms [1, 13, 18]. Contamination of the environment occurs in a variety of ways, including dust generated during seed pickling, runoff into watercourses, untargeted uptake of the pesticide by the plant through the roots, or deposition of dust on the leaves. Persistent pesticide concentrations in non-target plants, watercourses, and soils are variable and can be affected by the regularity of application [3].

Several recent studies have demonstrated the alarmingly harmful effects of neonicotinoid insecticides on human and animal health. Due to their confirmed toxic effects on bees in particular, in 2018 the EU banned three neonico-

tinoids in particular: clothianidin, imidacloprid, and thiamethoxam for external use. The toxic burden of insecticides has increased dramatically in recent years as neonicotinoids have rapidly replaced older chemical groups in most countries [6, 8].

In our study we focused on the toxicological effects of thiacloprid, one of the eight neonicotinoids, at different concentrations. We used two different genotoxic methods, comet assay, and H2AX phosphorylation assay, to detect DNA damage. Comet assay monitored DNA migration of cells in agarose under simultaneous application of an electric field to detect DNA breaks in human lymphocytes after thiacloprid treatment, whereas H2AX phosphorylation depended on the cellular response to DNA damage to generate yH2AX foci, which we considered to be markers of double-strand breaks (DSBs).

MATERIALS AND METHODS

Detection of double-strand breaks by alkaline comet analysis

Peripheral blood was collected from the vena cubiti of a young woman using a sterile syringe containing heparin solution (2000 IU per 10 cm^3). Human peripheral blood lymphocytes were isolated using Histopaque 1077 separation medium and subsequently incubated in a culture medium consisting of RPMI 1640 containing L-glutamine and HEPES 15 $\mu mol.l^{-1}$ (GE Healthcare Hyclone Lab, Utah, USA), 15 % foetal calf serum (BoFeS, Sigma, ChemicalCo. St. Louis, MO, USA), antibiotics (100 $U.ml^{-1}$ penicillin, 0.1 $mg.ml^{-1}$ streptomycin and 0.25 $\mu g.ml^{-1}$ amphotericin) and mitogen phytohemagglutinin (PHA, 180 $\mu g.ml^{-1}$, Welcome, Dartford, England). The insecticide was added to the cultures for 2 h and dissolved in distilled water and diluted at concentrations of 60, 240 and 480 $\mu g.ml^{-1}$. Hydrogen peroxide (H_2O_2, Mikrochem, SR, 250 $\mu mol.l^{-1}$) was used as a positive control (5 min). The resulting samples were viewed at 20× magnification under a fluorescence microscope (Nikon ECLIPSE Ni-U, Texas Red filter) when 50–100 random cells were recorded. The amount of damaged DNA was evaluated using CASPLab software. Statistical analysis was performed using a simple analysis of variance (ANOVA, Student's t-test), which was used to evaluate the % DNA breaks when comparing the exposed groups and controls.

Detection of double-strand breaks using yH2AX foci

Thiacloprid was added to the culture medium (at a concentration of 480 $\mu g.ml^{-1}$) and doxorubicin 1 $\mu mol.l^{-1}$ was used as a positive control. For detection of histone γH2AX, we used the primary antibody Anti-gamma H2A.X (phosphorus S139, ab26350) and the secondary antibody Goat Anti-Mouse IgG H&L (Alexa Fluor® 488, ab150113). Finally, we stained the nuclei with DAPI+VECTASHIELD. The yH2AX foci were evaluated by fluorescence microscopy using a NIKON Eclipse microscope equipped with a dedicated Progress® camera and a METAFER automated analysis module.

RESULTS

The results of our analysis of DNA damage by alkaline comet assay in human peripheral blood lymphocytes after 2 h exposure to the neonicotinoid insecticide thiacloprid (Calypso® 480 SC) at concentrations of 60, 240 and 480 $\mu g.ml^{-1}$ are shown in Figure 1.

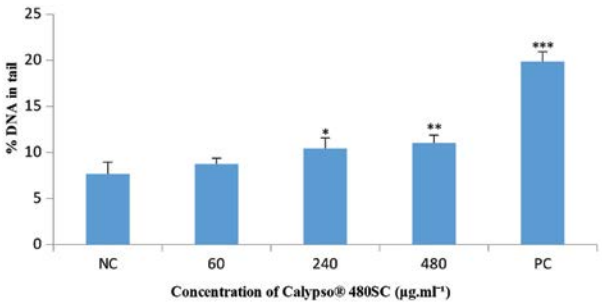


Fig. 1. Percentage of DNA damage in the tail after 2 h exposure of human lymphocytes to thiacloprid

NC (negative control); distilled water PC (positive control): H_2O_2 (250 µmol. l^{-1})

* P < 0.05; ** P < 0.01; ***P < 0.001

Statistically significant lymphocyte DNA damage after exposure to the commercial preparation Calypso® 480 SC with thiacloprid as an active ingredient was observed at concentrations of 240 and 480 $\mu g.ml^{-1}$ ($P < 0.05$; $P < 0.01$).

A graphical representation of lymphocyte viability is shown in Figure 2. Viability was evaluated by microscopy, where we counted the number of dead and live cells using a Bürker chamber. Human peripheral blood lymphocytes had lower survival and apoptosis occurred at increasing concentrations.

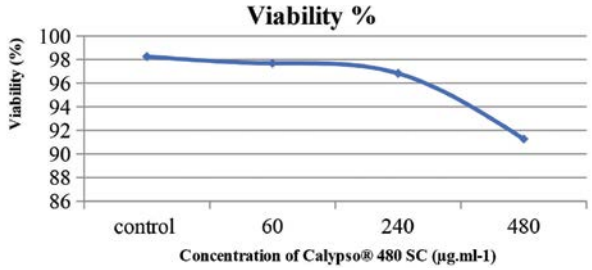


Fig. 2. Cell viability – the ability of cells to survive after exposure to Calypso® 480 SC
Viability of humane peripheral blood lymphocytes determined by the alkaline comet assay.

When determining the viability, we found that at a concentration of 60 µg.ml^{-1} the cells showed 97.69 % viability and there was an average of 3 dead cells per square. At a concentration of 240 µg.ml^{-1}, the viability was 97.69 % and there was an average of 4 dead cells per square. At our highest concentration of thiacloprid in Calypso® 480 SC 480 µg.ml^{-1} the viability was 91.27 % and the number of dead cells was on average 5 per square.

Visualization was performed using a fluorescence microscope (Nikon ECLIPSE Ni-U) equipped with a special (CCD) camera and connected to a computer. With the help of the photos, we were able to visually assess the damage of the comets shown in Figure 3 where we can observe significantly with increasing concentration more and more comets that contain a tail, formed due to the DSB and in this case an important marker of damage to our cells.

DNA damage was investigated after exposure of lymphocytes to thiacloprid. The cells were treated with the insecticide for 2 h. The positive control was H_2O_2 (5 min). a) Negative control; b) 60 µg.ml^{-1}; c) 240 µg.ml^{-1}; d) 480 µg.ml^{-1}; e) positive control H_2O_2.

In the second part of our experiment, we addressed the detection of double-strand breaks (DSBs) using the yH2AX histone. Our assay aimed to detect the ability of yH2AX to form foci after 4 h exposure to thiacloprid at a concentration of 480 µg.ml^{-1} (not shown). In our experiment, we concluded that lymphocytes did not induce statistically significant foci of phosphorylated H2AX histone, which could confirm the DBS caused by the insecticide thiacloprid.

DISCUSSION

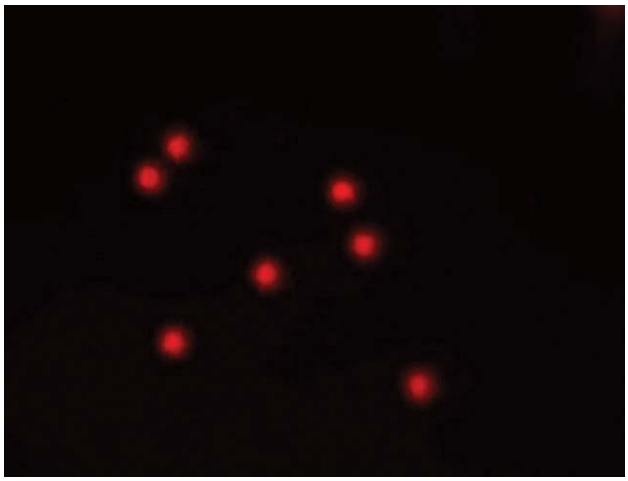
The possible genotoxic effects of the insecticide Calypso® 480 SC with the active substance thiacloprid were evaluated in human lymphocytes by an alkaline version of the comet assay. Our results showed that Calypso® 480 SC can induce significant levels of DNA damage in lymphocyte cells, but only at the higher concentrations tested ($P < 0.05$; $P < 0.01$). Our findings were consistent with those of previous studies, at different concentrations and exposure times, with the authors using bovine or human lymphocytes.

C a l d e r o n – S e g u r a et al. [4] observed significantly increased comet percentages and tail lengths using alkaline comet assay after exposure of human peripheral lymphocytes to the insecticidal products Calypso® 480SC (thiacloprid), Poncho (clothianidin), Gaucho (imidacloprid) and Jade (prothioconazole, tebuconazole). The authors observed an increased percentage of damage in a dose-dependent manner for all pesticides tested. They also found that Jade showed the highest cytotoxicity and genotoxicity compared to the other insecticides tested.

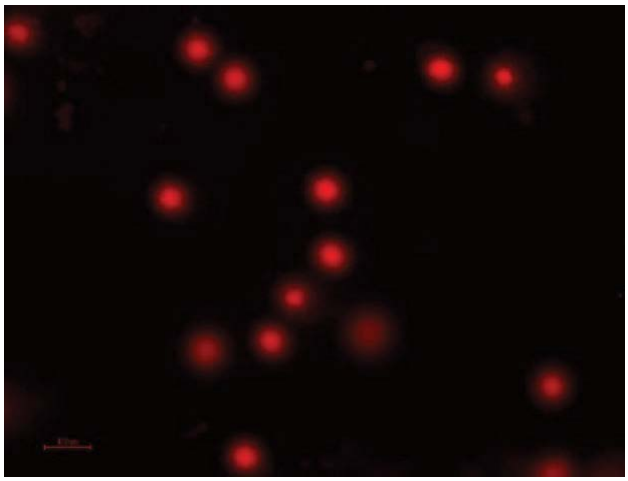
F e n g et al. [7] investigated the effects of thiacloprid on the earthworm *Eisenia fetida*, where the worms were exposed to 1 and 3 mg.kg^{-1} of the insecticide for 7, 14, and 28 days. The results showed that the activities of enzymes such as glutathione-S-transferase (GST), carboxylesterase (CarE), superoxide dismutase (SOD), catalase (CAT), and peroxidase (POD) were inhibited after thiacloprid exposure at one or more sampling times and then increased during recovery compared to control. The activity of these enzymes is often used as an important marker of increased ROS production [2], which is thought to be the primary source of DNA damage following pesticide exposure [5, 16]. In their study, they also confirmed significant DNA damage in the comet assay, which increased with thiacloprid exposure time and then decreased with recovery time.

Genotoxic effects have also been confirmed for other neonicotinoids, such as acetamiprid, which was tested on *Eisenia fetida* [11], where changes were noted in the comet tail after sub-chronic exposure at concentrations of 0.05–0.50 mg.kg^{-1}. Similar to thiacloprid, increased concentrations of ROS and changes in the antioxidant enzymes CAT and GST were observed.

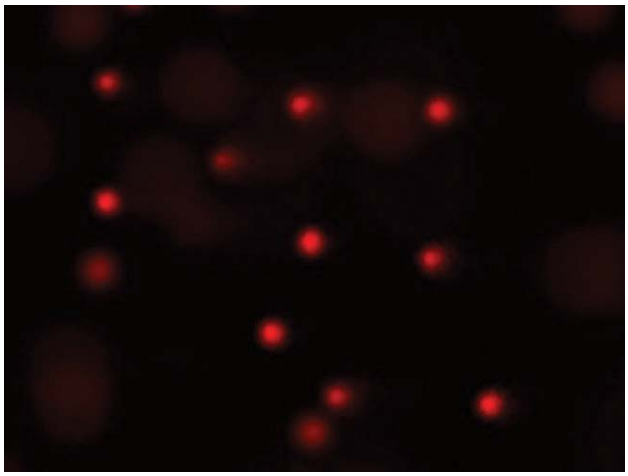
S c h w a r z b a c h e r o v á et al. [17] investigated the genotoxic effects of a thiacloprid-based insecticide



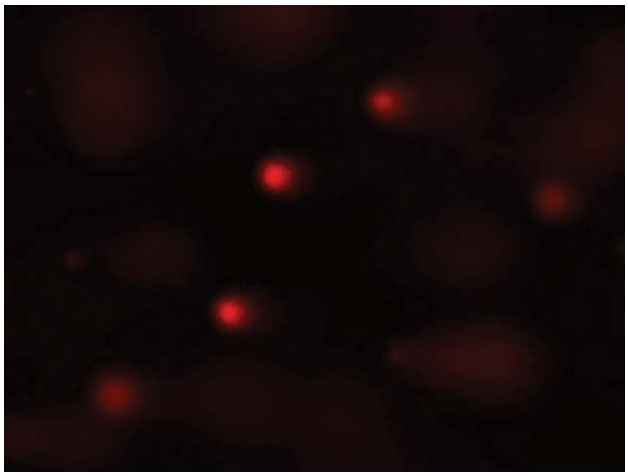
a



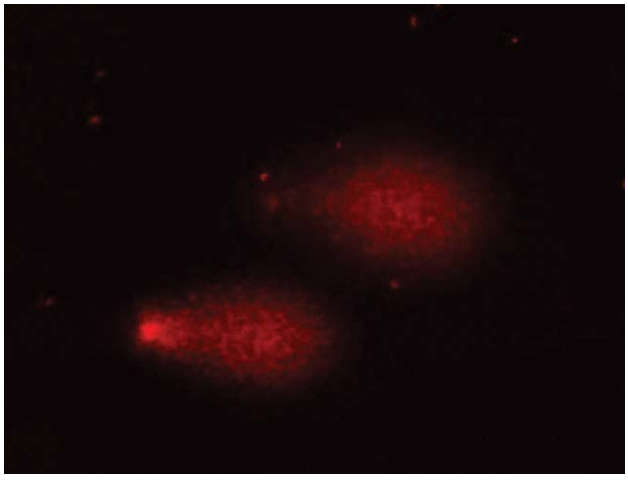
b



c



d



e

Fig. 3. Visualization of comets using a fluorescence microscope (Nikon ECLIPSE Ni-U)

on bovine peripheral lymphocytes using concentrations (10, 30, 60, 120, 240 μg.ml^{-1}) and exposure time (4 h) in their experiment. They used several genotoxicity assays in their study namely: neutral comet assay, cytotoxicity assay with acridine orange/ethidium bromide staining, TUNEL assay, 53BP1 and p53 immunostaining, apoptosis assay, micronucleus assay, and oxidative stress parameters. They found that decreased cell viability and proliferation, 53BP1-mediated cell cycle arrest in the G1/G0 phase, and induction of apoptosis, which was accompanied by increased concentrations of mitochondrial superoxide and carbonylated proteins, were observed after exposure of cells to thiacloprid. Furthermore, oxidant-based DNA damage and DNA damage response (DDR) were observed, specifically micronucleus formation, DNA double-strand breaks, and mild accumulation of p53-binding protein (53BP1) foci.

In the second part of our experiment, we detected foci of yH2AX in peripheral human lymphocytes after 4 h exposure to thiacloprid (480 μg.ml^{-1}), where we did not observe increased foci formation compared to the negative control. Samková et al. [14] also investigated pure thiacloprid at the same concentration on human peripheral lymphocytes, where, similarly to our case, they concluded that lymphocytes did not contain statistically significant foci of phosphorylated H2AX. In addition to yH2AX, they also evaluated chromosomal and chromatid aberrations after thiacloprid exposure. They observed statistically significant increases at concentrations of 240 and 480 μg.ml^{-1} and found that chromosomal and chromatid damage were among the most common aberrations.

Sekeroglu et al. [15] investigated the effects of the insecticide clothianidin on human bronchial epithelial cells (BEAS-2B). They detected the genotoxicity of clothianidin using yH2AX, 53BP1 loci, and by comet assay. Three different concentrations (0.15, 0.3, and 0.6 mmol. l^{-1}) and three different exposure times (24, 72, and 120 h) were used for the analysis. They found that clothianidin exhibited direct toxic effects on BEAS-2B cells and their viability decreased with increasing insecticide concentration, which is comparable to the cell viability observed after exposure to our test insecticide thiacloprid. In the comet assay, tail intensity (%) showed a significant increase at each clothianidin concentration and at each exposure time except at the lowest concentration (0.15 mmol. l^{-1}) for 24 h. Foci of yH2AX and 53BP1 were observed at all concentrations and exposure times, and there were even significantly increased numbers of foci in the recovery groups when cells had time to repair.

CONCLUSIONS

In our study, we were able to record the percentage of DNA in the tail after exposure to Calypso® 480 SC with the active ingredient thiacloprid at higher concentrations (240 and 480 μg.ml^{-1}) which has been confirmed by other studies. Our second test, which evaluated the ability of thiacloprid to generate foci of phosphorylated yH2AX using the automated program Metafer, came out negative compared to the control. Although exposure to Calypso® 480 SC induced formation of yH2AX foci, these increases were not significant ($P > 0.05$). The use of only one genotoxicity test does not provide sufficient information to determine the reliability of pesticides. Therefore, various biomarkers were used to assess the genotoxicity of pesticides. Detection of yH2AX is a suitable alternative, in addition to other assays such as comet assays, to further test the genotoxic effect of various substances.

Considering that neonicotinoids are used to treat crops with which various non-target organisms, such as bees, as well as humans, come into contact, it is important that the toxicity of these substances are continuously investigated in the future. Comet assay and histone yH2AX detection certainly have their place in testing, not only pesticides, but also many other potentially toxic substances that could induce DNA damage.

ACKNOWLEDGEMENT

This study was supported by the grants VEGA 1/0166/21 and KEGA 008UVLF-4/2023.

REFERENCES

1. **Berheim, E. H., Jenks, J. A., Lundgren, J. G., Michel, E. S., Grove, D., Jensen, W. F., 2019:** Effects of neonicotinoid insecticides on physiology and reproductive characteristics of captive female and fawn white-tailed deer. *Sci. Rep.,* 9, 1, 4534. DOI: 10.1038/s41598-019-40994-9.

2. **Bernard, F., Brulle, F., Dumez, S., Lemiere, S., Platel, A., Nesslany, F., et al., 2015:** Antioxidant responses of annelids, Brassicaceae and Fabaceae to pollutants: A review. *Ecotoxicol. Environ. Saf.*, 114, 273–303. DOI: 10.1016/j.ecoenv.2014.04.024.
3. **Bonmatin, J. M., Giorgio, C., Girolami, V., Goulson, D., Kreutzweiser, D. P., Krupke, C., et al., 2015:** Environmental fate and exposure; neonicotinoids and fipronil. *Environ. Sci.*, 22, 1, 35–67. DOI: 10.1007/s11356-014-3332-7.
4. **Calderón-Segura, M. E., Gómez-Arroyo, S., Villalobos-Pietrini, R., Martínez-Valenzuela, C., Carbajal-López, Y., Cortés-Eslava, J., et al., 2012:** Evaluation of genotoxic and cytotoxic effects in human peripheral blood lymphocytes exposed in vitro to neonicotinoid insecticides news. *J. Toxicol.*, 2012, 612647. DOI: 10.1155/2012/612647.
5. **Dong-Jun, F., Ping, L., Jian, S., Sai-Yang, Z., Han-Zhong, X., 2019:** Mechanisms of synergistic neurotoxicity induced by two high risk pesticide residues – chlorpyrifos and carbofuran via oxidative stress. *Toxicol. In Vitro*, 54, 338–344. DOI: 10.1016/j.tiv.2018.10.016.
6. **Douglas, M. R., Sponsler, D. B., Lonsdorf, E. V., Grozinger, C. M., 2020:** County-level analysis reveals a rapidly shifting landscape of insecticide hazard to honey bees (*Apis mellifera*) on US farmland. *Sci. Rep.*, 10, 1, 797. DOI: 10.1038/s41598-019-57225-w.
7. **Feng, L., Zhang, L., Zhang, Y., Zhang, P., Jiang, H., 2015:** Inhibition and recovery of biomarkers of earthworm *Eisenia fetida* after exposure to thiacloprid. *Environ. Sci. Pollut. Res.*, *22*, 9475–9482.
8. **Coulson, D., Thompson, J., Coombs, A., 2018:** Rapid rise in toxic load for bees revealed by analysis of pesticide use in Great Britain. *Peer*, 6, e5255–e5255. DOI: 10.7717/peerj.5255.
9. **Han, W., Tian, Y., Shen, X., 2018:** Human exposure to neonicotinoid insecticides and the evaluation of their potential toxicity: An overview. *Chemosphere*, 192, 59–65. DOI: 10.1016/j.chemosphere.2017.10.149.
10. **Jeschke, P., Nauen, R., Schindler, M., Elbert, A., 2011**: Overview of the status and global strategy for neonicotinoids. *J. Agric. Food Chem.*, 59, 7, 2897–2908. DOI: 10.1021/jf101303g.
11. **Li, B., Xia, X., Wang, J., Zhu, L., Wang, G., 2018:** Evaluation of acetamiprid-induced genotoxic and oxidative responses in *Eisenia fetida*. *Ecotoxicology. Environ. Saff.*, 161, 610–615. DOI: 10.1016/j.ecoenv.2018.06.022.
12. **Maienfisch, P., Huerlimann, H., Rindlisbacher, A., Gsell, L., Dettwiler, H., Haettenschwiler, J., et al., 2001:** The discovery of thiamethoxam: a second-generation neonicotinoid. *Pest Manag. Sci.*, 57, 2, 165–176. DOI: 10.1002/1526-4998(200102)57:2<165::AID-PS289>3.0.CO; 2-G .
13. **Morrissey, C. A., Mineau, P., Devries, J. H., Sanchez-Bayo, F., Liess, M., 2015:** Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: A review. *Environ. Int.*, 74, 291–303. DOI: 10.1016/j.envint.2014.10.024.
14. **Samková, G., Galdíková, M., Schwarzbacherová, V., Kolesnikova, S., 2019:** Detection of chromosomal breaks induced by thiacloprid in human lymphocytes and detection of double-strand breaks based on γH2AX histone phosphorylation. *Folia Veterinaria*, 63, 4, 33–37.
15. **Şekeroğlu, Z. A., Şekeroğlu, V., Aydın, B., Yedier, S. K., İlkun, E., 2020:** Clothianidin induces DNA damage and oxidative stress in bronchial epithelial cells. *Environ. Mol. Mutagen,* 61, 6, 647–655. DOI: 10.1002/em.22376.
16. **Schwarzbacherová, V., Wnuk, M., Lewinska, A., Potocki, L., Zebrowski, J., Koziorowski, M., et al., 2017:** Evaluation of cytotoxic and genotoxic activity of fungicide formulation Tango® Super in bovine lymphocytes. *Environ. Pollut.,* 220 (Part A), 255–263. DOI: 10.1016/j.envpol.2016.09.057.
17. **Schwarzbacherová, V., Wnuk, M., Deregowska, A., Holečková, B., Lewinska, A., 2019:** *In vitro* exposure to thiacloprid-based insecticide formulation promotes oxidative stress, apoptosis and genetic instability in bovine lymphocytes. *Toxicol. In Vitro*, 61, 104654. DOI: 10.1016/j.tiv.2019.104654.
18. **Wood, T. J., Goulson, D., 2017:** The environmental risks of neonicotinoid pesticides: A review of the evidence post 2013. *Environ. Sci. Pollut. Res. Int.*, 24, 21, 17285–17325. DOI: 10.1002/tox.21790. DOI: 10.1007/s11356-017-9240-x.

Received December 8, 2023
Accepted February 14, 2024