Low doses of imidacloprid induce disruption of intercellular adhesion and initiate proinflammatory changes in Caco-2 cells

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Imidacloprid is the most widely used pesticide of the neonicotinoid class. Neonicotinoid toxicities against various insects are well known. Nevertheless, there are rising evidences that neonicotinoids exert cytotoxic effects on different non-target organisms including mammals, fish, birds etc. Besides, depending on pesticide application, the exposed plants absorb some part of used neonicotinoids and their residues are detected in agricultural products worldwide. Thus, the continuous consumption of fruits and vegetables contaminated with neonicotinoids is a high risk factor for humans despite the low doses. Intestine epithelial cells are the first targets of the neonicotinoid cytotoxicity in humans because of its direct way of administration. The epithelial cells provide the barrier function of the intestinal system via specialized intercellular adhesion. The effects of imidacloprid on the intestine barrier function and inflammatory cytokines production are still unknown. In the present study, we exposed the human Caucasian colon adenocarcinoma (Caco-2) epithelial cells to low doses (0.10–0.75 µg/mL) of imidacloprid in order to assess the expression of tight and adherens junctions proteins, occludin and E-cadherin, and production of proinflammatory cytokine TNF α and iNOS. Imidacloprid induced dose-dependent decline in both occludin and E-cadherin levels. By contrast, TNF- α and iNOS contents were upregulated in imidacloprid-exposed Caco-2 cells. Decrease in tight and adherens junctions proteins indicates that the barrier function of intestine epithelial cells could be damaged by imidacloprid administration. In addition, TNF- α and iNOS upregulation indicates that imidacloprid is potent to activate proinflammatory response in enterocytes. Thus, imidacloprid can affect intestine barrier function through the increase of proinflammatory cytokine production and decrease in adhesiveness of enterocytes. The further assessment of the role of adhesion proteins and inflammatory cytokines in neonicotinoid pesticide cytotoxicity as it affects enterocyte barrier function is required to highlight the risk factor of use of neonicotinoids.

Keywords: neonicotinoids; TNF- α ; iNOS; occludin; E-cadherin; intestine barrier function.

Introduction

Imidacloprid is a chloronicotinyl compound and belongs to the neonicotinoid pesticide family designed in the late 1990s. Imidacloprid is the first commercial neonicotinoid insecticide, which was developed and introduced by Bayer Crop Science for the agricultural market (Tomlin, 1997). During the last few decades, neonicotinoid pesticides have been considerably used worldwide due to their strong toxicity to insects. Neonicotinoids have selective synaptic specificity targeting the neuronal nicotinic acetylcholine receptors (nAChR) as the agonists of high affinity. These receptors are responsible for the control of ligand-gated ion channels and for rapid neurotransmission (Tomizawa & Yamamoto, 1993). Research has revealed that neonicotinoids exhibit the highest selective toxicity for insects in comparison with mammals, due to the unique structure of insect nAChR receptors and higher affinity with them (Liu & Casida, 1993). Research has shown that the affinity varies depending on the differences in both the subtypes and binding sites of nAChR (Tomizawa & Casida, 1993; Lansdell & Millar, 2000; Okazawa et al., 2000; Tomizawa et al., 2000). Nevertheless, since the invention of neonicotinoids, there has been published a number of the reports on its toxicity in mammals (Schulz-Jander et al., 2002; Schulz-Jander & Casida, 2002). Moreover, different kinds of toxicity were detected not only for neonicotinoids, but also for several neonicotinoids metabolites which exert affinity for nAChR (Chao

& Casida, 1997; Latli et al., 1999; Tomizawa et al., 2000). For instance, some metabolites of imidacloprid, which are the desnitro-imidacloprid and nitromethylene analogues, were considered as high-level toxic agents following intraperitoneal injection (Chao & Casida 1997).

Actually, the treatment of plants with neonicotinoid pesticides is accompanied by a little direct absorbance through the plant surface. The main part of applied neonicotinoid substances enters the soil environment and ground waters. It makes neonicotinoids acceptable to the plant roots (Struger et al., 2017; Hladik et al., 2018). Due to their stability and aqueous solubility, the residues of neonicotinoids are detected in agricultural products worldwide (Anderson et al., 2015; Struger et al., 2017; Thunnissen et al., 2020). Recently, imidacloprid was considered as one of the most frequently found neonicotinoids in surface waters. For instance, every three out of four samples from several areas of southwestern Ontario had higher imidacloprid content than permitted by the Canadian guidelines (Struger et al., 2017). Several studies reported that imidacloprid concentrations in both soil and surface waters are extremely variable and could account for milligrams per litre as consequences of accidents (Morrissey et al., 2015; Thunnissen et al., 2020). Taking into account that neonicotinoids make up about 30% of all pesticides in the market and penetrate the plants, both neonicotinoids and their metabolites can be present in different agricultural products. Therefore, the continuous consumption of fruits and vegetables contaminated with neonicotinoids is

a high risk factor for humans despite low doses. Furthermore, the permanent increase in neonicotinoid insecticides application in agriculture could result in growth of their accumulation in fruits and vegetables (Wu et al., 2020). Therefore, the intestinal system could be an important target of neonicotinoid toxicity. The intestine digests food and performs intake of nutritional compounds, which manifests its first vital function. Moreover, another important function of the intestine system is the barrier function, maintained by specialized adhesion of intestine epithelial cells. These cells form a tight monolayer and are affected by infectious agents and various toxic chemicals consumed over life. Thus, unique barrier mechanisms of intestine epithelial cells may be the first target of neonicotinoid cytotoxicity in humans.

Studies were conducted to define the immunosuppressive potential of dietary exposure to imidacloprid on both innate and adaptive immune responses in piglets (Hernandez et al., 2018). Recently, Yang et al. (2020) reported that subchronic exposure to imidacloprid can disrupt the gut barrier function and could be potentially toxic to mammals, including humans. Latest studies revealed that neonicotinoids could affect cell viability in the human Caucasian colon adenocarcinoma (Caco-2) epithelial cell model and disrupt monolayer cell integrity (Shi et al., 2021). Thus, pesticides can cause the disturbance in the intestine barrier by disrupting the intercellular junction. Moreover, histopathological and proinflammatory changes were detected in common carp exposed to imidacloprid (Özdemir et al., 2017). Luo et al. (2021) reported that imidacloprid exposure may induce intestinal histological injury and oxidative stress in the gut of zebrafish. Several *in vitro* studies have shown a high rate of neonicotinoid absorbance by human intestine epithelial cells (Brunet et al., 2008; Shi et al., 2021). The disturbance in the gut barrier function was demonstrated after subchronic exposure to imidacloprid in mice (Yang et al., 2020). It should be noted that chronic exposure of the honeybee to imidacloprid or its metabolites is considered as ten thousand times more toxic than acute exposure (Suchail et al., 2001). It also may significantly affect other non-target invertebrates (Stanley & Preetha, 2016; Kozak et al., 2020). Therefore, chronic exposure to low doses of neonicotinoids should be considered as a risk factor for non-target organisms even despite the absence of valid *in vitro* and *in vivo* studies.

In spite of recent progress in studies of neonicotinoids toxicity, the molecular mechanisms of their cytotoxic effect in mammals are still poorly understood. Furthermore, the intestine toxicity of neonicotinoids in mammals has remained a relevant issue of biology, health science and toxicology over the recent years. One of most studied molecular mechanisms, which causes cytotoxicity, and functional decline, is oxidative stress generation. This type of the abnormality was demonstrated in various organisms exposed to neonicotinoids (Hong et al., 2020; Luo et al., 2021). However, pesticide-caused redox imbalance is not specific to the harmful effect of neonicotinoids. Reactive oxygen species (ROS) generation was confirmed to be the commonest unspecific disturbance caused by a large number of toxic compounds. On the other hand, oxidative stress can interact with individual signalling pathways, which may be more closely related to the detrimental effect and specificity of outer stimuli. In the nervous system, for example, the glial fibrillary acidic protein (GFAP) is known as a neurotoxicity biomarker (Shiyntum et al., 2017; Abdel Salam, 2021) which is obviously connected with the ROS production (Gasso et al., 2020a; Fernandes & Özcelik, 2021). On the other hand, the inflammation is an important part of innate defence against infectious agents as well as xenobiotic toxicity. Thus, the combined abnormalities in redox imbalance, proinflammatory changes and the modulation of tissue-specific protein expression could be a promising tool for searching for adequate molecular biomarkers of the neonicotinoid cytotoxicity. Oxidative stress is able to stimulate proinflammatory changes in most cell types. However, the cells form biological barriers, including the gut-blood barrier, which are extremely susceptible to damaging factors and potent to respond to the production of inflammatory cytokines (Banks et al., 2015). A similar response is accompanied by upregulation in the expression and release of both proinflammatory and regulatory cytokines to activate the defence systems. On the other hand, overproduction of proinflammatory cytokines can induce a number of deleterious effects, including decrease in cell viability and specific barrier function. Among the large cytokine family of tumour necrosis factors (TNF) the TNF- α and inducible oxide nitrogen

synthase (iNOS) are the most widely used as biomarkers of the inflammatory imbalance in different cell types (Shen et al., 2021). Both aforementioned cytokines are confirmed to be the initiators of cellular response to different kinds of injuries (Banks et al., 2015).

The barrier function of the intestinal system is mediated by specialized epithelial cells of the gut. These polarized cells develop close contacts between each other through tight and adherens junctions, which are characteristic for epithelial cells. The tight and adherens junctions are formed by specialized proteins occludin and E-cadherin respectively (Hartsock & Nelson, 2008). The adhesive contacts of epitheliocytes provide the strong bindings between contiguous cells and maintain intestinal barrier function. The intestine barrier function could be disrupted by the influence of different toxicants. The effect of neonicotinoids on the intestinal barrier function remains still unknown. Taking into account that intestine epithelial cells are able to absorb neonicotinoids, we hypothesized that imidacloprid could affect the vital barrier function of these cells, perhaps through the disruption of intercellular adhesion and proinflammatory changes. Therefore, the integrative detection of intercellular adhesion and proinflammatory cytokines production could be a promising tool to elucidate the cytotoxic effect of the contaminants as well as the molecular mechanisms of neonicotinoids' toxicity in relation to the barrier function of the intestinal system. In order to understand the mechanisms of cytotoxic effect of neonicotinoids, we used the Caco-2 cell culture exposed chronically to imidacloprid to study the expression of proinflammatory cytokine iNOS known as an inflammatory initiator produced by epithelial cells.

The objective of our work is to study the expression of TNF- α , iNOS, occludin and E-cadherin as the molecular markers of inflammatory changes and intercellular adhesion integrity in Caco-2 intestine cells exposed to imidacloprid.

Materials and methods

The Caco-2 cells were purchased from American Type Culture Collection (HTB-37TM, ATCC, Manassas, VA). All chemicals except imidacloprid were obtained from Sigma-Aldrich. Proliferation of cell culture of Caco-2 cells was carried out in 75-cm² flasks in Dulbecco's modified eagle medium and Hank's F₁₂ medium (1:1) (DMEM/F₁₂), high glucose, 4500 mg/dL; HyClone, Invitrogen Company, USA) supplemented with 10% foetal bovine serum (FBS), 1% non-essential amino acids, 1% sodium pyruvate, 1% glutamine, and 1% streptomycin/penicillin. The cell culture was incubated in a humidified atmosphere in 37 °C and 5% carbon dioxide. The medium was replaced every 48 h. When the cells achieved approximately 90% confluence, the receding for a next passage was carried out using 0.25% trypsin-EDTA (0.25% trypsin, 0.02% EDTA). A minimum of 18 passages were performed before the start of experiment. After the last passage, the cells (7×10^5 cells/well) were placed in 6 cm Petri dishes and cultured to 100% confluence.

Imidacloprid ((EZ)-1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine) was purchased from Bayer (Bayer Inc., Canada). The cells at complete confluence were put into 6 cm Petri dishes, incubated and growth-arrested with 0.2% FBS containing medium 12 hours before the experiment. The stock solution of imidacloprid was prepared by dissolving it in DMEM at 100 μ g/mL immediately before exposure. To study the protein expression of Caco-2 cells (7×10^5 cells/well), we inoculated them into 6 cm Petri dishes and cultivated in the medium containing 10% FBS at 37 °C in a humidified atmosphere with 5% CO₂ to 100% confluence. The medium was changed before the treatment. The Caco-2 cells at complete confluence were exposed to imidacloprid doses of 0.10, 0.25 and 0.75 μ g/mL for 4 days to implement the *in vitro* model of chronic exposure to neonicotinoids. The conditioned media were collected individually from every Petri dish and concentrated to detect the secretory factors, including TNF- α and iNOS. After the exposure, the cells were washed with phosphate saline buffer (PBS) and collected by scratching without trypsinization.

The estimation of imidacloprid cytotoxicity on intestine epithelial cells was performed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Every procedure was carried out according to the manufacturer's recommendations. Briefly, cells inoculated in 96-well plates were grown for 24–48 h in the medium containing 10%

FBS and incubated in 37 °C in humidified atmosphere with 5% CO₂ to form monolayer with 100% confluence. The medium was changed before the treatment. The cells were exposed to 0.10, 0.25 and 0.75 µg/mL doses of imidacloprid and then incubated in 37 °C for 96 h. After the exposure, the cells were washed with phosphate saline buffer (PBS). After rinsing, the cells were exposed to the solution contained 20 µL MTT reagent and 180 µL PBS. The incubation with MTT reagent was carried out for 4 h in 37 °C in humidified atmosphere with 5% CO₂. After incubation, MTT solution was removed and each well was filled with 180 µL DMSO for 10 min followed incubation. The absorbance level was measured at 570 nm length wave in the presence of 20 µL Sorensen's buffer in a 96-well plate Multiskan reader (Thermo-Scientific, Hudson, NH, USA). The obtained data are presented as the percentage of control value.

The production of intracellular forms of ROS was determined with 2',7-dichlorofluorescein diacetate (DCFHDA). Control and exposed cells were washed with PBS, treated with 10 µM DCFHDA and incubated 30 min in 37 °C. The measuring of ROS levels was performed with a SpectraMax Gemini EM spectrofluorometer with 485 nm wavelength excitation and 530 nm wavelength emission.

After 96 h treatment, the control Caco-2 cells and cells exposed to imidacloprid were washed in Petri dishes with cold PBS and harvested by scrapping without trypsinization. The collected cells were centrifuged and lysed in RIPA buffer containing proteinase and phosphatase inhibitor cocktail. The Caco-2 cell proteins were extracted during 60 min in 4 °C. After lysis, the cell extracts were centrifuged at 40,000 g for 20 min. The content of total protein in supernatants was measured with a spectrophotometer according to the modified Bradford's method using BSA as

the standard (Markwell et al., 1978). The supernatant of each protein extract was mixed with Laemmli sample buffer containing 0.1 M of dithiothreitol in 1:1 ratio and boiled for 5 min. Fixed with Laemmli buffer protein, the samples were frozen and stored in -80 °C before the start of the western blot analysis.

The proteins were separated with polyacrilamide gel (PAG) electrophoresis using 5–20% gradient of acrylamide and then the proteins were transferred from gel onto polyvinylidene fluoride (PVDF) membrane with application of the electric field (10 V/cm). After the transfer, PVDF membrane was washed and blocked in 1% bovine serum albumin (BSA) in PBS-Tween-20 solution. Blocked membrane was probed overnight at 4 °C for primary antibodies anti-TNF-alpha (1:2000, Abcam, ab205587), anti-iNOS (1:3000, Abcam, ab205529), anti-occludin (1:2000, Abcam, ab31721), anti-E-cadherin (1:2000, Abcam, ab40772) and anti-GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as a loading control (1:2500, Santa Cruz, sc-365062) antibodies. After washing, the membrane was incubated with according secondary anti-rabbit or anti-mouse IgG antibodies conjugated with horseradish peroxidase (1:20000, Abcam, ab6721). Immunostaining was performed using luminol-hydrogen peroxide solution by the enhanced chemiluminescence method with the use of X-ray films (Konica Minolta, Japan).

Densitometric analysis of the immunostained polypeptide zones was performed using TotalLab TL120 software (USA). The intensity value obtained by scanning every individual band was normalized to the intensity in respect to corresponding GAPDH band. Every track on the scanned image was corrected to the background level, which corresponded to nonreactive area on the X-ray film.

Fig. 1. Caco-2 cell viability (a), relative ROS level (b), occludin (c) and E-cadherin (d) contents in cells exposed to imidacloprid ($\bar{x} \pm SE$; $n = 5$)

We used the arithmetic mean ($\bar{x}$) and standard error (SE) to describe the quantitative traits. Statistical comparisons of the data were performed using one-way analysis of variance (ANOVA) with the StatView 5.0 (SAS Institute Inc., the USA). For multiple comparisons, the differences were calculated taking into account the Bonferroni correction. The graphs were developed in GraphPad Prism 9 (GraphPad Software, the USA). P values less than 0.05 were accepted as statistically significant.

Results

In order to detect the effects of imidacloprid on cell viability in the realistic intestine epithelial cell model, the completely confluent Caco-2 cells were exposed to 0.10, 0.25 and 0.75 $\mu\text{g/mL}$ imidacloprid for 96 h. The decrease in cell viability was observed in the Caco-2 cells treated with 0.25 and 0.75 $\mu\text{g/mL}$ imidacloprid. These doses reduced cell viability by 81% ($P < 0.05$) and 72% ($P < 0.01$) respectively. The exposure to the dose of 0.10 $\mu\text{g/mL}$ induced non-statistical decline in Caco-2 cell viability (Fig. 1a).

Similarly to the cell viability, dose-dependent imidacloprid effect was observed for the ROS level upregulation in the Caco-2 cells exposed to 0.25 and 0.75 $\mu\text{g/mL}$ doses. It should be mentioned that 0.10 $\mu\text{g/mL}$ dose had no effect on the ROS production (Fig. 1b).

Taking into account that tight junction and adherent junction proteins are the main structures responsible for intestine barrier function, we evaluated the expression of their biomarkers such as occludin and E-cadherin respectively. The results we obtain suggest that both occludin (Fig. 1c) and E-cadherin (Fig. 1d) were downregulated in exposed Caco-2 cells. Their statistically significant decrease was observed in the cell groups exposed to 0.25 and 0.75 $\mu\text{g/mL}$ imidacloprid. On the other hand, we found no changes in occludin and E-cadherin contents in the cell group exposed to 0.10 $\mu\text{g/mL}$ imidacloprid.

To assess the individual susceptibility of tight junction and adherent junction proteins to deleterious effect of imidacloprid cytotoxicity, we used comparative analysis of western blot results to evaluate the relative changes in occludin and E-cadherin contents (Fig. 2).

Fig. 2. The results of occludin, E-cadherin, TNF- α , iNOS and GAPDH western blot analysis

The comparative analysis of the decline in the contents of tight junction and adherens junction E-cadherin demonstrated no statistically significant differences (Fig. 3). On the other hand, we found the similar tendencies for exposures to both 0.25 and 0.75 $\mu\text{g/mL}$ doses, expressed in 5% lower E-cadherin level compared with the occludin content under the influence of the same imidacloprid dose. Probably, the aforementioned doses of imidacloprid may induce slightly more significant effect on the E-cadherin expression as compared with the occludin one.

Since TNF- α is recognized as a biomarker of proinflammatory response against various stimuli, including environmental ecotoxins, we detected the expression of this cytokine in the conditioned medium after

imidacloprid exposure. Applied doses of imidacloprid induced statistically significant dose-dependent increase in TNF- α content in the cells exposed to 0.25 and 0.75 $\mu\text{g/mL}$ doses of imidacloprid (Fig. 4).

Fig. 3. The comparative analysis of the decrease in tight junction occludin content and adherens junction E-cadherin content: 0.25 occ, 0.75 occ – occludin content in cells exposed to 0.25 and 0.75 $\mu\text{g/mL}$ of imidacloprid respectively, 0.25 E-c, 0.75 E-c – E-cadherin content in cells exposed to 0.25 and 0.75 $\mu\text{g/mL}$ of imidacloprid respectively ($\bar{x} \pm \text{SE}$; $n = 5$)

Fig. 4. Relative TNF- α content in control (0.00) and exposed to imidacloprid cells ($\bar{x} \pm \text{SE}$; $n = 5$)

Fig. 5. Relative iNOS content in control and exposed to imidacloprid cells ($\bar{x} \pm \text{SE}$; $n = 5$)

The detection of the other biomarker of inflammatory response, iNOS, was carried out to clarify the additional pathways of proinflammatory response initiation in epithelial cells of the intestine. iNOS has been recognized as a calcium-insensitive NOS isoform, which is involved in the cellular response to different innate immune signals as well as toxic factors. The detection of iNOS expression in the Caco-2 cells demonstrated increase in the groups of all exposed cells (Fig. 5). Nevertheless, pronounced statistically significant dose-dependent iNOS changes were observed in the cells exposed to 0.25 and 0.75 µg/mL doses of imidacloprid.

The obtained results revealed that exposure to low doses of imidacloprid may induce intestine epithelial Caco-2 cell reactivity accompanied by significant TNF-α and iNOS upregulations.

Discussion

The mechanisms of neonicotinoids toxicity in respect to non-targeted organisms. The global use of neonicotinoids in agriculture generates the risk factors of its toxicity to non-targeted organisms including humans. Several reports of *in vitro* studies have shown that the absorption of different neonicotinoids by human intestinal epithelium varies significantly. The application of transport inhibitors panel was used to determine the mechanisms of neonicotinoids intestinal epithelium cell absorbance (Brunet et al., 2008). Brunet and co-authors demonstrated the complex ways of permeability of these pesticides, involving both inward and outward active transporters in their accumulation in the intestine system (Brunet et al., 2004). Nevertheless, a high absorption potential of the similar chemical structures acetamiprid and imidacloprid was detected, which could be a cause of its deleterious effect on the intestine. Moreover, several metabolites of imidacloprid were identified in the exposed cells of mammals as well as ultrahigh agonistic effect on the mammalian nicotinic receptor (Tomizawa & Casida, 2003). The affinity of neonicotinoids for vertebrate nAChR may be mediated by structural similarity with well known nAChR agonist epibatidine synthesized in the skin of the Ecuadorean frog (*Epipedobates tricolor* Boulenger, 1899) (Spande et al., 1992). In spite of declared anti-insect prevailing toxicity, the toxic effect of neonicotinoid metabolites was demonstrated in different non-target organisms (Huang et al., 2021; Shi et al., 2021; Zhang et al., 2021). Recently, imidacloprid-caused gut toxicity was demonstrated in adult zebrafish (Luo et al., 2021). The toxicity of imidacloprid was suggested for crabs and piglets (Hernandez et al., 2018; Hong et al., 2020). Many different effects of neonicotinoids were found in amphibian larvae (Gasso et al., 2020b). Since neonicotinoids exert affinity for nAChR, the most important targets of its toxicity are the cells that are highly expressing nAChR. Neuronal and muscular cells express this integral membrane protein as prevailing acetylcholine neuromediator receptor. Thus, the abovementioned cells are the main targets of the neonicotinoid toxicity. However, barrier function of intestine epithelial cells is controlled by many factors, including enteric neural system signalling. Taking into account that the enteric neural system is heavily involved in the intestine barrier function, the detrimental effect of chronic neonicotinoid exposure could be mediated by both direct effect on intestine enterocytes and indirect effect on intestine neurons.

Despite the widespread global application of neonicotinoids, there are limited data on their dietary intake and intake with fruits and vegetables. Some surprising data were reported on imidacloprid content in squash (427.2 ng/g) and spinach (569.2 ng/g), which were selected from the U.S. Congressional cafeteria (Chang et al., 2018). Nevertheless, both of these estimated imidacloprid contents were below the current chronic reference dose. However, due to wide use of neonicotinoids, their contamination of fruits and vegetables should not be ignored as a risk factor. Furthermore, the residues of neonicotinoids represented in fruits and vegetables should be considered as a risk factor for human health due to permanent exposure to both neonicotinoids and their metabolites. Therefore, the intestine system of non-insect organisms could be a target of neonicotinoids toxicity.

Neonicotinoid-caused defects of intestine barrier function. The Caco-2 monolayer is a well-developed model to study the transport of compounds across the intestinal epithelial barrier. Moreover, Caco-2 monolayer is a convenient model to detect the toxic agents causing intestinal injuries and disruption of intestine epithelial cell barrier function. The Caco-2 model is

widely accepted as the standard to forecast intestinal absorption of drugs, natural products and environmental contaminants. In the present study, we used the human colon cancer cell culture model to clarify the imidacloprid cytotoxicity in the intestine epithelial cells. There are limited data concerning toxic effects of imidacloprid on intestine epithelial cells. Recent findings of imidacloprid absorbance in enterocytes indicated that this neonicotinoid is strongly absorbed by inward and outward active transporters *in vivo* (Brunet et al., 2008). Moreover, *in vitro* data was reported on oxidative stress and histological injury in the gut of zebrafish (Luo et al., 2021). Besides, the dual role of gut microbiota was demonstrated in relation to oral bioaccessibility and intestinal transport of pesticides in a cell culture model (Shi et al., 2021). Thus, a large number of factors can affect enterocyte integrity and intestine barrier function. In spite of recent progress in pesticide toxicity, there are no data on the effect of neonicotinoids on tight junction proteins and homophilic tissue specific adhesion of intestine enterocytes. Since neonicotinoids and their metabolites could be absorbed by intestine epithelial cells, we hypothesized that imidacloprid can affect the adhesion proteins involved in the intercellular junctional complex and consequently disrupt the intestinal barrier function.

Different toxicants including mycotoxins and heavy metals were confirmed to be able to damage the intestinal barrier function through the disruption of both tight junctions and adherens junctions in the intestine epithelial cells (Luo et al., 2019). The food contaminants are the most critical factors that are considered as risk factors of the disturbance in gastrointestinal tract barrier function (Pinton et al., 2009). Furthermore, the first target of their harmful effect is the intestine epithelial cells. The analysis of the possible interactions between food and environmental contaminants has drawn a particular interest over the recent years (Le et al., 2018). The results we obtained in the study indicated the imidacloprid-caused decrease in Caco-2 cells viability as well as upregulation of the ROS production. These data are consistent with the recently published results on *in vitro* and *in vivo* study of neonicotinoids toxicity (Brunet et al., 2008; Luo et al., 2021; Shi et al., 2021). Therefore, low doses of imidacloprid may suppress the enterocyte viability and induce the redox imbalance. It should be mentioned that redox imbalance is not specific to neonicotinoid's toxicity and the oxidative stress generation was detected in a large number of cytotoxicity models for different kinds of pollutants (Koz et al., 2011; Nedzvetskii et al., 2012).

The role of tight and adherent junctions in imidacloprid intestine toxicity. The intercellular adhesion contacts play a crucial role in maintenance of all epithelial barriers, including the intestinal one. The specific targeting of neonicotinoids could affect the individual pathways, which are unique for its cytotoxicity, especially the regulation of enterocytes' intercellular adhesion. Decrease in E-cadherin content, which we observed in our study, suggests that imidacloprid exposure may affect E-cadherin-dependent enterocyte adhesion. According to the available literature data, our results on downregulation of E-cadherin under the imidacloprid exposure are the first published data on this particular aspect. E cadherin is considered as a supporter of the epithelial cells integrity and barrier function (Hartsock & Nelson, 2008). Therefore, the disruption of tight and adherens junctions could be an important part of the intestine epithelial cell toxicity. Cadherins form the family of calcium-dependent cell adhesion molecules that are responsible for adherens junctions of various cell types (Bartle et al., 2020). Extracellular cadherin domains form the cell-cell adhesion while intracellular domains interact with numerous structural and signalling proteins (Hülken et al., 1994; Huang et al., 2019). E-cadherin is expressed in epithelial cells and usually exhibits homophilic adhesion to bind the same type cells. Adherens junctions based on E-cadherin adhesion are observed in all kinds of epithelial sheets. This adhesion type is involved in apicobasal polarity machinery, which is a unique feature of all epithelium types. Therefore, the abnormalities in E-cadherin adhesion can initiate the intercellular communication disturbance and disrupt epithelial barrier. Furthermore, the role of E-cadherin expression in the maintaining of intestinal epithelium integrity was demonstrated in the model of necrotizing enterocolitis while the disturbance in barrier function was accompanied by disruption of tight junctions (Buonpane et al., 2020).

The role of tight junction protein occludin in intestinal epithelial cell barrier permeability was reported *in vitro* and *in vivo* studies. Occludin forms epithelial tight junctions and is the key membrane protein that sup-

ports the epithelial barrier functions. Decrease in occludin content is considered one of the most serious abnormalities in gastrointestinal diseases (Kou et al., 2019). Decrease in occludin content, which we observed in the study, indicates that imidacloprid can affect the occludin-based tight junctions and inhibit the enterocyte adhesion. The results we obtained on imidacloprid-caused downregulation of occludin content are presented for the first time. Intestinal epithelial tight junctions are an important part of the gut barrier structure and function. The decrease in barrier function may be caused by both endocytic removal of tight junctions proteins and degrading adhesive proteins with extracellular proteases. Another way of weakening the intestinal barrier is the inhibition of expression of tight junction proteins, including occludin, claudins and junction adhesion molecules. Among them, occludin is considered as the most critical intracellular adhesion component that plays an important role in supporting the barrier function (Rawat et al., 2020). The studies reported that occludin upregulation is able to support both intestinal and vascular barrier functions in the model of human colon epithelial cell and human endothelial cell barrier function (Grothaus et al., 2018). Moreover, the most extensive decrease in the E-cadherin content, which we seen in the study, compared with such of occludin, suggests that E-cadherin-dependent adhesion is most susceptible to the imidacloprid toxicity. Nevertheless, various factors can affect epithelial cells' barrier function through absorbance and direct interaction with signalling pathways. On the other hand, the proteases activity could be an alternative cause of adhesive protein decrease. For instance, bacterial proteases were recognized as environmental factors, which are permanently present in an intestine system. Pesticides affect the gut microbiota and provoke the reactive response in a form of excretion of defensive factors including proteases. Bacterial proteases are involved in virulence and can disrupt tight junctions of epithelial cells as it was reported for serine protease High temperature requirement protein A (HtrA) produced by *Campylobacter jejuni* (Harrer et al., 2019). Thus, one of the realistic scenarios of detrimental effects of the neonicotinoids could be related to stimulation of pathogenic intestine microbiota to secrete proteases and digest the tight junctions of epithelial cells. However, this hypothesis requires further *in vivo* studies. Nonetheless, the results we obtained confirm that low doses of imidacloprid may induce the decrease in intestine epithelial cell adhesion, affecting the epitheliocytes layer integrity. Therefore, imidacloprid exposure exhibits potential deleterious effects on intestine epithelial cell barrier function.

The role of inflammatory processes in neonicotinoid toxicity. The defects in epithelial tight junction barrier are closely related to progress of intestinal inflammation. The increase in gut-blood barrier permeability stimulates the innate immunity and release of inflammatory factors. Both of them are responsible for leukocytes infiltration and local inflammation in intestine epithelium, which increase the digestion of tight junction proteins and disrupt the gut-blood barrier. Recently, it was demonstrated that inflammation induces the occludin downregulation in mice and in Caco-2 cell line (Kuo et al., 2019). The disruption of interaction of intestinal epithelial cells is associated with diseases. Furthermore, similar disturbances are accompanied by intestinal inflammation and disruption of epithelial intercellular adhesion. Recently, a novel mechanism was reported that can mediate the intestinal epithelial barrier function by suppressing the occludin (Rawat et al., 2020). The exposure to IL-1 Caco-2 cells can induce several miRNAs that bind the occludin messenger RNA and consequently suppress the occludin expression. The inhibition of occludin synthesis was accompanied by the increase in intestinal tight junction permeability as well as disturbance of its barrier function.

The production of cytokines is considered as one of the important modulators of the intestinal epithelial barrier function (Brufau et al., 2017). Despite the fact that the strengthening of adhesiveness of tight junctions is regulated by different factors, proinflammatory cytokines were reported as the most critical factor that generates the defects in epithelial barrier function (Ma et al., 2005, 2004; Ye et al., 2006). The TNF- α -caused disturbance in the intestine barrier permeability was shown in the HT-29/B6 colonic cells (Gitter et al., 2000a). Moreover, it was demonstrated that TNF- α can directly affect tight junctions and epithelial barrier function independently by activating the inflammatory cascade (McKay & Singh 1997; Gitter et al., 2000a, 2000b). Upregulation of TNF- α release from Caco-2 cells exposed to imidacloprid, which we observed in the study, is

consistent with the earlier presented data that low doses of imidacloprid are potent to induce gut toxicity, accompanied by oxidative damages and increase in production of inflammatory factors (Luo et al., 2021). Recently, it was shown that TNF- α exerts a disruptive effect on intestine barrier function through the activation of transcription factor NF- κ B and p38MAPK signal pathways. This activation initiates the production of other proinflammatory cytokines such as interleukin-6 and interleukin-8, which are involved in a feedback loop and consequently can induce TNF- α release from both epithelial and immune cells (Nie et al., 2020). Furthermore, the combination treatment with interferon- γ and TNF- α resulted in a significant decrease in epithelial cells permeability and downregulation of occludin and claudins expression (Fischer et al., 2013). Furthermore, the modulation of NF- κ B activity in Caco-2 was reported as one of the principal mechanisms, which underlies the regulation of intestine barrier function (Ma et al., 2004). NF- κ B inhibitors including curcumin and triptolide were observed to abrogate TNF- α -induced increase in permeability in the intestine epithelial cells model (Ye et al., 2006). Since tight junctions are confirmed as the most important structure that supports the epithelial cell barrier function, NF- κ B could be one of the key factors in the regulation of intercellular adhesiveness of intestine epithelial cells. Another reported molecular mechanism regulating the intestine barrier function is the modulation of myosin light-chain kinase (MLCK) protein expression (Ma et al., 2005). In this scenario, to modulate intestine barrier function, the link between tight junctions and inflammatory factors was determined for protein kinase activity. Actually, it was shown that the increase in intestine epithelial cells Caco-2 permeability is accompanied by the upregulation in MLCK protein expression. Moreover, MLCK protein expression was controlled by transcriptional regulation, while permeability of tight junctions required the upregulation in MLCK activity (Ye et al., 2006). Therefore, the administration of intestine epitheliocytes with proinflammatory cytokines can induce both functional and structural abnormalities.

The detection of iNOS, the other marker of proinflammatory response, was carried out to clarify the NO-dependent pathways of proinflammatory response initiation in the epithelial cells of intestine. iNOS, which is a calcium-insensitive NOS isoform, plays a crucial role in neural tissue cells (Reddi et al., 2021). Besides, iNOS is involved in cellular response to different innate immune signals and to toxic factors in the intestine system. The capability of this enzyme to generate reactive species determines the role played by iNOS in the innate immunity. Different NOS isoforms could be involved in both anti-microbial and anti-tumour activities through the activation of macrophages to oxidative burst (Li et al., 2000). However, toxic agents are able to initiate the iNOS overactivity and could be associated with cell injury despite the absence of any infection (Zhao et al., 2006).

Since proinflammatory response is initiated by different factors, we have detected iNOS expression as one of universal pathways responsible for cell reactivity. iNOS is a key factor in the cellular response to toxicity and oxidative environment. In the present study, we detected imidacloprid-induced significant upregulation in iNOS expression in Caco-2 cells. Usually, in the normal physiological conditions the expression of iNOS is quite low. However, different stimuli including innate immune signals and toxic factors may induce extensive upregulation of the iNOS expression. Nitric oxide (NO), which is a product of almost all NOS activities can react with superoxide. This reaction leads to the peroxynitrite formation and consequent multiple macromolecular damages. Furthermore, upregulated iNOS produces large quantities of NO, which mediate the production and release of proinflammatory cytokines, including interferon- γ and TNF- α (Chen et al., 2006). According to the available literature data, increase in iNOS expression in Caco-2 cells exposed to imidacloprid is described for the first time. Therefore, the increase in iNOS and TNF- α expressions which we observed in our study could be at least partially involved in the suppression of tight junctions and adherent junction proteins. Thus, the imidacloprid toxicity looks like a multifaceted action that targets both proinflammatory response and the intestinal barrier function deficiency. Moreover, the presented results provide evidence that imidacloprid-caused disturbances in intestine epithelial cells may be mediated with the abnormal upregulation of NO-dependent signalling pathways.

On the other hand, the intestine microflora and nutritional factors can also affect intestine barrier function through the modification of the ex-

pression of tight junction proteins and their location (Ulluwishewa et al., 2011). Besides, similar to the aforementioned disturbances in the epithelial barrier integrity, proinflammatory cytokine releases were reported in epithelial cell cultures exposed to food contaminants, micro- and nanoparticles (Pinton et al., 2009; Busch et al., 2021). Thus, the mechanisms that are responsible for epithelial cell intercellular adhesion and intestine barrier function are susceptible targets for xenobiotics including pesticides. Overall, the present study provides new data on the toxicity of neonicotinoids to intestine epithelial cells. Taking into account the literature data and the data obtained in our study, the initiation of proinflammatory reactivity, suppression of tight and adherens junctions could be important parts of the neonicotinoid toxicity in the intestine epithelial cells. This mechanism may be responsible for the disruption of intestinal barrier function under exposure to neonicotinoids.

Conclusion

Low doses of imidacloprid induce TNF- α and iNOS upregulation in human intestine epithelial cells, which should be considered as a biomarker of proinflammatory changes. Furthermore, imidacloprid exposure downregulated tight and adherens junction proteins such as occludin and E-cadherin. Thus, imidacloprid can affect intestine barrier function through the increase of the proinflammatory cytokine production and decrease of the adhesiveness of enterocytes. Further assessment of the role of adhesion proteins and inflammatory cytokines in neonicotinoid pesticide cytotoxicity which affects the enterocytes barrier function is required to highlight the risk factor of neonicotinoids' application.

References

- Abdel salam, M. E., Elawady, E. H., Khater, A. S., Eweda, S. A., & Abd El Mo-neam, M. H. (2021). Neuropsychiatric sequelae of acute carbon monoxide poisoning: The predictive role of neuron specific enolase and glial fibrillary acidic protein. *NeuroToxicology*, 85, 115–120.
- Al-Sadi, R., Khatib, K., Guo, S., Ye, D., Youssef, M., & Ma, T. (2011). Occludin regulates macromolecule flux across the intestinal epithelial tight junction barrier. *American Journal of Physiology: Gastrointestinal and Liver Physiology*, 300(6), 1054–1064.
- Banks, W. A., Gray, A. M., Erickson, M. A., Salameh, T. S., Damodarasamy, M., Sheibani, N., Meabon, J. S., Wing, E. E., Morofuji, Y., Cook, D. G., & Reed, M. J. (2015). Lipopolysaccharide-induced blood-brain barrier disruption: Roles of cyclooxygenase, oxidative stress, neuroinflammation, and elements of the neurovascular unit. *Journal of Neuroinflammation*, 12, 223.
- Bartle, E. I., Rao, T. C., Beggs, R. R., Dean, W. F., Umer, T. M., Kowalczyk, A. P., & Mattheyses, A. L. (2020). Protein exchange is reduced in calcium-independent epithelial junctions. *Journal of Cell Biology*, 219(6), e201906153.
- Buonpane, C., Yuan, C., Wood, D., Ares, G., Klonoski, S. C., & Hunter, C. J. (2020). ROCK1 inhibitor stabilizes E-cadherin and improves barrier function in experimental necrotizing enterocolitis. *American Journal of Physiology – Gastrointestinal and Liver Physiology*, 318(4), 781–792.
- Busch, M., Bredeck, G., Kämpfer, A. A. M., & Schins, R. P. F. (2021). Investigations of acute effects of polystyrene and polyvinyl chloride micro- and nanoparticles in an advanced in vitro triple culture model of the healthy and inflamed intestine. *Environmental Research*, 193, 110536.
- Chang, C. H., MacIntosh, D., Lemos, B., Zhang, Q., & Lu, C. (2018). Characterization of daily dietary intake and the health risk of neonicotinoid insecticides for the U.S. population. *Journal of Agricultural and Food Chemistry*, 66(38), 10097–10105.
- Chao, L. S., & Casida, J. E. (1997). Interaction of imidacloprid metabolites and analogs with the nicotinic acetylcholine receptor of mouse brain in relation to toxicity. *Pesticide Biochemistry and Physiology*, 58(1), 77–88.
- Chen, C. J., Ou, Y. C., Lin, S. Y., Liao, S. L., Chen, S. Y., & Chen, J. H. (2006). Manganese modulates proinflammatory gene expression in activated glia. *Neurochemistry International*, 49(1), 62–71.
- Chen, Q., Chen, O., Martins, I. M., Hou, H., Zhao, X., Blumberg, J. B., & Li, B. (2017). Collagen peptides ameliorate intestinal epithelial barrier dysfunction in immunostimulatory Caco-2 cell monolayers via enhancing tight junctions. *Food and Function*, 8(3), 1144–1151.
- Coopman, P., & Alexandre, A. (2016). Adherens junction and E-cadherin complex regulation by epithelial polarity. *Cellular and Molecular Life Sciences*, 73(18), 3535–3553.
- Fernandes, E. F. A., & Özcelik, D. (2021). Imaging biomarkers for monitoring the inflammatory redox landscape in the brain. *Antioxidants*, 10, 528.
- Fischer, A., Gluth, M., Pape, U.-F., Wiedenmann, B., Theuring, F., & Baumgart, D. C. (2013). Adalimumab prevents barrier dysfunction and antagonizes distinct effects of TNF- α on tight junction proteins and signaling pathways in intestinal epithelial cells. *American Journal of Physiology: Gastrointestinal and Liver Physiology*, 304(11), 970–979.
- Gasso, V. Y., Hahut, A. N., Yermolenko, S. V., Hasso, I. A., Agca, C. A., Sukhareenko, E. V., & Nedzvetsky, V. S. (2020a). Local industrial pollution induces astrocyte cytoskeleton rearrangement in the dice snake brain: GFAP as a biomarker. *Biosystems Diversity*, 28(3), 250–256.
- Gasso, V., Yermolenko, S., Bobyliov, Y., Hahut, A., Huslysty, A., Hasso, I., & Petrushevsky, V. (2020b). Biomarkers of the influence of pyrethroids and neonicotinoids on amphibian larvae. *Ecology and Neospherology*, 31(1), 46–51 (in Ukrainian).
- Gitter, A. H., Bendfeldt, K., Schmitz, H., Schulzke, J. D., Bentzel, C. J., & Fromm, M. (2000a). Epithelial barrier defects in HT-29/B6 colonic cell monolayers induced by tumor necrosis factor- α . *Annals of the New York Academy of Sciences*, 915, 193–203.
- Gitter, A. H., Bendfeldt, K., Schulzke, J. D., & Fromm, M. (2000b). Leaks in the epithelial barrier caused by spontaneous and TNF- α -induced single-cell apoptosis. *The FASEB Journal*, 14(12), 1749–1753.
- Grothaus, J. S., Ares, G., Yuan, C., Wood, D. R., & Hunter, C. J. (2018). Rho kinase inhibition maintains intestinal and vascular barrier function by upregulation of occludin in experimental necrotizing enterocolitis. *American Journal of Physiology – Gastrointestinal and Liver Physiology*, 315(4), 514–528.
- Harrer, A., Bückner, R., Boehm, M., Zarzecka, U., Tegtmeyer, N., Sticht, H., Schulzke, J. D., & Backert, S. (2019). *Campylobacter jejuni* enters gut epithelial cells and impairs intestinal barrier function through cleavage of occludin by serine protease HtrA. *Gut Pathogens*, 11, 4.
- Hartsock, A., & Nelson, W. J. (2008). Adherens and tight junctions: Structure, function and connections to the actin cytoskeleton. *Biochimica et Biophysica Acta*, 1778(3), 660–669.
- Hernandez, J., Volland, A., Leyshon, B. J., Juda, M., Ridlon, J. M., Johnson, R. W., & Steelman, A. J. (2018). Effect of imidacloprid ingestion on immune responses to porcine reproductive and respiratory syndrome virus. *Scientific Reports*, 8(1), 11615.
- Hong, Y., Huang, Y., Wu, S., Yang, X., Dong, Y., Xu, D., & Huang, Z. (2020). Effects of imidacloprid on the oxidative stress, detoxification and gut microbiota of Chinese mitten crab, *Eriocheir sinensis*. *Science of the Total Environment*, 729, 138276.
- Huang, A., van den Brink, N. W., Buijse, L., Roessink, I., & van den Brink, P. J. (2021). The toxicity and toxicokinetics of imidacloprid and a bioactive metabolite to two aquatic arthropod species. *Aquatic Toxicology*, 235, 105837.
- Huang, H., Wright, S., Zhang, J., & Brekken, R. A. (2019). Getting a grip on adhesion: Cadherin switching and collagen signaling. *Biochimica et Biophysica Acta – Molecular Cell Research*, 1866(11), 118472.
- Hülksen, J., Birchmeier, W., & Behrens, J. (1994). E-cadherin and APC compete for the interaction with beta-catenin and the cytoskeleton. *Journal of Cell Biology*, 127(6), 2061–2069.
- Koz, S. T., Baydas, G., Koz, S., Demir, N., & Nedzvetsky, V. S. (2011). *Ginkgo biloba* extract inhibits oxidative stress and ameliorates impaired glial fibrillary acidic protein expression, but can not improve spatial learning in offspring from hyperhomocysteinemic rat dams. *Phytotherapy Research*, 26(7), 949–955.
- Kozak, V. M., Romanenko, E. R., & Brygadyrenko, V. V. (2020). Influence of herbicides, insecticides and fungicides on food consumption and body weight of *Rossius kessleri* (Diplopoda, Julidae). *Biosystems Diversity*, 28(3), 272–280.
- Kuo, W.-T., Shen, L., Zuo, L., Shashikanth, N., Ong, M. L. D. M., Wu, L., Zha, J., Edelblum, K. L., Wang, Y., Wang, Y., Nilsen, S. P., & Turner, J. R. (2019). Inflammation-induced occludin downregulation limits epithelial apoptosis by suppressing caspase-3 expression. *Gastroenterology*, 157(5), 1323–1337.
- Lansdell, S. J., & Millar, N. S. (2000). The influence of nicotinic receptor subunit composition upon agonist, alpha-bungarotoxin and insecticide (imidacloprid) binding affinity. *Neuropharmacology*, 39(4), 671–679.
- Latli, B., D'Amour, K., & Casida, J. E. (1999). Novel and potent 6-chloro-3-pyridinyl ligands for the $\alpha 4\beta 2$ neuronal nicotinic acetylcholine receptor. *Journal of Medicinal Chemistry*, 42(12), 2227–2234.
- Le, T.-H., Alassane-Kpembi, I., Oswald, I. P., & Pinton, P. (2018). Analysis of the interactions between environmental and food contaminants, cadmium and deoxynivalenol, in different target organs. *Science of the Total Environment*, 622–623, 841–848.
- Li, H., Hu, J., Xin, W., & Zhao, B. (2000). Production and interaction of oxygen and nitric oxide free radicals in PMA stimulated macrophages during the respiratory burst. *Redox Report*, 5(6), 353–358.
- Liu, M. Y., & Casida, J. E. (1993). High affinity binding of [3 H]-imidacloprid in the insect acetylcholine receptor. *Pesticide Biochemistry and Physiology*, 46(1), 40–46.
- Luo, S., Terciolo, C., Bracarense, A. P. F. L., Payros, D., Pinton, P., & Oswald, I. P. (2019). *In vitro* and *in vivo* effects of a mycotoxin, deoxynivalenol, and a trace metal, cadmium, alone or in a mixture on the intestinal barrier. *Environment International*, 132, 105082.

- Ma, T. Y., Boivin, M. A., Ye, D., Pedram, A., & Said, H. M. (2005). Mechanism of TNF- α modulation of Caco-2 intestinal epithelial tight junction barrier: Role of myosin light-chain kinase protein expression. *American Journal of Physiology – Gastrointestinal and Liver Physiology*, 288(3), 422–430.
- Ma, T. Y., Iwamoto, G. K., Hoa, N. T., Akotia, V., Pedram, A., Boivin, M. A., & Said, H. M. (2004). TNF- α -induced increase in intestinal epithelial tight junction permeability requires NF- κ B activation. *American Journal of Physiology – Gastrointestinal and Liver Physiology*, 286(3), 367–376.
- Markwell, M. A., Haas, S. M., Bieber, L. L., & Tolbert, N. E. (1978). A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Analytical Biochemistry*, 87, 206–210.
- McKay, D. M., & Singh, P. K. (1997). Superantigen activation of immune cells evokes epithelial (T84) transport and barrier abnormalities via IFN- γ and TNF α : Inhibition of increased permeability, but not diminished secretory responses by TGF- β 2. *The Journal of Immunology*, 159(5), 2382–2390.
- Nedzvetskii, V. S., Kirichenko, S. V., Baydas, G., & Nerush, O. P. (2012). Effects of melatonin on memory and learning deficits induced by exposure to thinner. *Neurophysiology/Neirofiziologiya*, 44(1), 42–48.
- Nie, N., Bai, C., Song, S., Zhang, Y., Wang, B., & Li, Z. (2020). *Bifidobacterium* plays a protective role in TNF- α -induced inflammatory response in Caco-2 cell through NF- κ B and p38MAPK pathways. *Molecular and Cellular Biochemistry*, 464, 83–91.
- Okazawa, A., Nakagawa, Y., Akamatsu, M., Ueno, T., & Nishimura, K. (2000). Comparison of the binding activities of chloronicotinyl insecticides toward the nicotinic acetylcholine receptors from rats and houseflies. *Journal of Pesticide Science*, 25(1), 40–43.
- Özdemir, S., Altun, S., & Arslan, H. (2017). Imidacloprid exposure cause the histopathological changes, activation of TNF- α , iNOS, 8-OHdG biomarkers, and alteration of caspase 3, iNOS, CYP1A, MT1 gene expression levels in common carp (*Cyprinus carpio* L.). *Toxicology Reports*, 5, 125–133.
- Pinton, P., Nougayrède, J. P., Del Rio, J. C., Moreno, C., Marin, D. E., Ferrier, L., Bracarense, A. P., Kolf-Clauw, M., & Oswald, I. P. (2009). The food contaminant deoxynivalenol, decreases intestinal barrier permeability and reduces claudin expression. *Toxicology and Applied Pharmacology*, 237(1), 41–48.
- Rawat, M., Nighot, M., Al-Sadi, R., Gupta, Y., Viszwapriya, D., Yochum, G., Koltun, W., & Ma, T. Y. (2020). IL1B increases intestinal tight junction permeability by up-regulation of MIR200C-3p, which degrades occludin mRNA. *Gastroenterology*, 159(4), 1375–1389.
- Reddy, Y. P., Tiwari, S., Tomar, L. K., Desai, N., & Shama, V. K. (2021). Fluoride-induced expression of neuroinflammatory markers and neurophysiological regulation in the brain of wistar rat model. *Biological Trace Element Research*, 199(7), 2621–2626.
- Schulz-Jander, D. A., Leimkuehler, W. M., & Casida, J. E. (2002). Neonicotinoid insecticides: Reduction and cleavage of imidacloprid nitroimine substituent by liver microsomal and cytosolic enzymes. *Chemical Research in Toxicology*, 15(9), 1158–1165.
- Schulz-Jander, D. A., & Casida, J. E. (2002). Imidacloprid insecticide metabolism: Human cytochrome P450 isozymes differ in selectivity for imidazolidine oxidation versus nitroimine reduction. *Toxicology Letters*, 132(1), 65–70.
- Shen, K., Jia, Y., Wang, X., Zhang, J., Liu, K., Wang, J., Cai, W., Li, J., Li, S., Zhao, M., Wang, Y., & Hu, D. (2021). Exosomes from adipose-derived stem cells alleviate the inflammation and oxidative stress via regulating Nrf2/HO-1 axis in macrophages. *Free Radical Biology and Medicine*, 165, 54–66.
- Shyntum, H. N., Dovban, O. O., Kovalchuk, Y. P., Yaroshenko, T. Y., & Ushakova, G. A. (2017). Corvatin restores metallothionein and glial fibrillary acidic protein levels in rat brain affected by pituitrin-izadrin. *The Ukrainian Biochemical Journal*, 89(3), 36–45.
- Spande, T. F., Garaffo, H. M., Yeh, H. J. C., Pu, Q. L., Pannell, L. K., & Daly, J. W. (1992). A new class of alkaloids from a dendrobatid poison frog – a structure for alkaloid-251f. *Journal of Natural Products*, 55(6), 707–722.
- Stanley, J., & Preetha, G. (2016). *Pesticide toxicity to non-target organisms*. Springer, New York.
- Suchail, S., Guez, D., & Belzunces, L. P. (2001). Discrepancy between acute and chronic toxicity induced by imidacloprid and its metabolites in *Apis mellifera*. *Environmental Toxicology and Chemistry*, 20(11), 2482–2486.
- Tomizawa, M., & Casida, J. E. (1999). Minor structural changes in nicotinoid insecticides confer differential subtype selectivity for mammalian nicotinic acetylcholine receptors. *British Journal of Clinical Pharmacology*, 127(1), 115–122.
- Tomizawa, M., & Casida, J. E. (2003). Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. *Annual Review of Entomology*, 48, 339–364.
- Tomizawa, M., & Yamamoto, I. (1993). Structure-activity relationships of nicotinoids and imidacloprid analogs. *Journal of Pesticide Science*, 18(1), 91–98.
- Tomizawa, M., Lee, D. L., & Casida, J. E. (2000). Neonicotinoid insecticides: Molecular features conferring selectivity for insect versus mammalian nicotinic receptors. *Journal of Agricultural and Food Chemistry*, 48(12), 6016–6024.
- Ulluwishewa, D., Anderson, R. C., McNabb, W. C., Moughan, P. J., & Wells, J. M. (2011). Regulation of tight junction permeability by intestinal bacteria and dietary components. *The Journal of Nutrition*, 141(5), 769–776.
- van Roy, F., & Berx, G. (2008). The cell-cell adhesion molecule E-cadherin. *Cellular and Molecular Life Sciences*, 65(23), 3756–3788.
- Wang, J., Wang, J., Wang, G., Zhu, L., & Wang, J. (2016). DNA damage and oxidative stress induced by imidacloprid exposure in the earthworm *Eisenia fetida*. *Chemosphere*, 144, 510–517.
- Wu, C., Dong, F., Mei, X., Ning, J., & She, D. (2020). Isotope-labeled internal standards and grouping scheme for determination of neonicotinoid insecticides and their metabolites in fruits, vegetables and cereals – A compensation of matrix effects. *Food Chemistry*, 311, 125871.
- Ye, D., Me, I., & Ma, T. Y. (2006). Molecular mechanism of tumor necrosis factor- α modulation of intestinal epithelial tight junction barrier. *American Journal of Physiology – Gastrointestinal and Liver Physiology*, 290(3), 496–504.
- Zhang, H., Aspinall, J. V., Lv, W., Zheng, X., Zhang, H., Li, S., Zhang, J., Bai, N., Zhang, Y., & Wang, X. (2021). Differences in kinetic metabolomics in *Eisenia fetida* under single and dual exposure of imidacloprid and dinotefuran at environmentally relevant concentrations. *Journal of Hazardous Materials*, 417, 126001.
- Zhao, H., Barger, M. W., Ma, J. K., Castranova, V., & Ma, J. Y. (2006). Cooperation of the inducible nitric oxide synthase and cytochrome P450 1A1 in mediating lung inflammation and mutagenicity induced by diesel exhaust particles. *Environmental Health Perspectives*, 114(8), 1253–1258.