UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Biológicas e Ingeniería

TÍTULO: Neonicotinoid Neurotoxicity Evaluation on Human Retinoic Acid Differentiated Cholinergic Neuroblastoma 5H-SY5Y Cells.

Trabajo de integración curricular presentado como requisito para la obtención
del título de Ingeniera Biomédica

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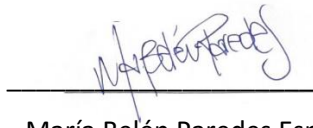

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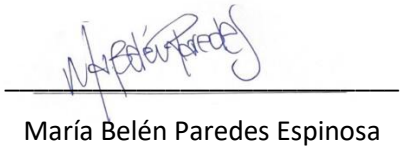

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María Belén Paredes Espinosa

ABSTRACT

Agrochemicals are considered the most common pollutants of the biosphere affecting not only humans and animals in agricultural areas, but everywhere through residues present in food. Neonicotinoids are the fastest-growing class of synthetic agrochemicals. Although these insecticides are used at low concentrations and their environmental levels are believed to be within safe limits, their synergic effects and health risks have not yet been thoroughly studied. Neonicotinoids are specific agonists of the post-synaptic nicotinic acetylcholine receptors (nAChRs) located in the central nervous system of insects. Even though neonicotinoids selectively act on insects, they are thought to have an unnoticeable activity on the nAChRs of vertebrates. Some recent evidence, however, suggests immunotoxic, neurotoxic, nephrotoxic, hepatotoxic, and reproductive cytotoxic effects of neonicotinoids in mammals. Ecuador is one of the leading exporters of tropical products, largely bananas, worldwide. Among the neonicotinoids that are intensively used to control insect plagues in banana plantations in Ecuador acetamiprid, imidacloprid, and thiamethoxam stand out. This study aimed to explore the putative neurotoxic effects of these neonicotinoids using an *in vitro* model of human neuroblastoma SH-SY5Y neurons. This cell line was selected because of its established high toxicity-testing validity. Neurons were treated with the neonicotinoids either alone or in combinations to detect possible interactions. Additionally, the cancer neurons were also exposed to neonicotinoids after being differentiated with retinoic acid (RA) to acquire a mature, cholinergic phenotype. Cell death was assessed by the MTT (mitochondrial respiration) and LDH (cellular membrane leakage) methods. All neonicotinoids caused statistically significant cell death at a millimolar range of concentrations, and acetamiprid was the most potent neurotoxin. When mixed pairwise, acetamiprid, imidacloprid and thiamethoxam showed either antagonist or synergic cytotoxic interactions depending on the cytotoxicity method used to assess viability.

KEYWORDS: Neonicotinoids; Neurotoxicity syndromes; Neuroblastoma; Receptors, Nicotinic

RESUMEN

Los agroquímicos se consideran los contaminantes más comunes de la biosfera que afectan no solo a humanos y animales en áreas agrícolas, sino en todas partes a través de los residuos presentes en los alimentos. Los neonicotinoides son la clase de agroquímicos sintéticos de uso de más rápido crecimiento. Aunque estos insecticidas se usan en bajas concentraciones y se cree que sus niveles ambientales están dentro de los límites seguros, sus efectos sinérgicos y riesgos para la salud aún no se han estudiado convenientemente. Los neonicotinoides son agonistas específicos de los receptores de acetilcolina nicotínicos postsinápticos (nAChR) ubicados en el sistema nervioso central de los insectos. Aunque los neonicotinoides actúan selectivamente sobre los insectos, también es probable que tengan una actividad imperceptible en los nAChR de los vertebrados. Alguna evidencia reciente sugiere inmunotoxicidad, neurotoxicidad, nefrotoxicidad, hepatotoxicidad y efectos citotóxicos reproductivos de los neonicotinoides en mamíferos. Ecuador es uno de los principales exportadores de productos agrícolas tropicales, principalmente banano, en todo el mundo. Entre los neonicotinoides que se usan intensamente para controlar las plagas de insectos en las plantaciones de banano en Ecuador se destacan el acetamiprid, el imidacloprid y el tiametoxam. Este estudio tuvo como objetivo explorar los efectos neurotóxicos de estos neonicotinoides utilizando un modelo in vitro de neuronas de neuroblastoma humano SH-SY5Y. Esta línea celular fue seleccionada debido a su validez establecida en pruebas de toxicidad. Las neuronas se trataron con los neonicotinoides administrados solos o en combinaciones para detectar posibles interacciones. Además, las neuronas cancerosas también fueron expuestas a neonicotinoides después de ser diferenciadas con ácido retinoico (AR) para adquirir un fenotipo colinérgico maduro. La muerte celular se evaluó mediante los métodos MTT (respiración mitocondrial) y LDH (filtración de la membrana celular). Todos los neonicotinoides causaron muerte celular estadísticamente significativa en un rango milimolar de concentraciones, y el acetamiprid fue la neurotoxina más potente. Cuando se mezclaron por pares, acetamiprid, imidacloprid y tiametoxam mostraron interacciones citotóxicas antagónicas o sinérgicas dependientes del método usado para medir citotoxicidad.

PALABRAS CLAVE: Neonicotinoides; Síndromes de neurotoxicidad; Neuroblastoma; Receptores Nicotínicos

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INTRODUCTION AND JUSTIFICATION

Agrochemicals include a wide variety of substances which are used in crops and food production, including pesticides -herbicides, insecticides, nematicides, fungicides- as well as fertilizers, feed additives, plant growth regulators, repellents, attractants, veterinary drugs, and other related compounds. These chemicals are not only widely used in agriculture, horticulture, and forestry, but are also used to improve conditions and maintain non-agricultural areas such households, public urban green areas, sports fields, gardens, and workplaces, thus protecting plants from diseases, pests or weeds and humans from vector-borne diseases. Yet to a lesser extent, they are also used in building materials, pet shampoos, and boat bottoms to prevent unwanted species. [1–4] According to the Food and Agriculture Organization (FAO), a total of 4,115,391.25 metric tons of pesticides are consumed around the world per year. Of a total of 34,252.80 metric tons of pesticides consumed per year in Ecuador, 4,700.84 metric tons are insecticides.[5]

Pesticides are considered global pollutants of the biosphere because they are widely sold in every continent thus making them readily available. They are believed to be one of the most common pollutants affecting humans and wildlife not only in agricultural areas, but everywhere due to their residues in food.[2] Human exposure to pesticides occurs by inhalation, ingestion, or direct contact with the skin, thus causing negative health effects, including carcinogenic, gastrointestinal, neurological, respiratory, dermatological, endocrine, and reproductive effects. There are several factors that determine the impact on health, such as the chemical nature of the pesticide, individual health condition, and time and route of exposure. Additionally, these chemicals can also be either metabolized and excreted or accumulated in the body. Although most insecticides act on the CNS of insects, they are also likely to be neurotoxic in humans, especially the developing brain (the most vulnerable), because of the similarity of neurochemical processes in animals. [3,4,6–8]

A large body of evidence suggests that pesticides constitute a serious health problem worldwide, even at minimal concentrations.[6,9–13] In recent years, several studies have demonstrated the harmful effects of environmental toxins like those of pesticides on mental health, including neurodevelopmental disorders in children. [9–11,13,14] Exposure to insecticides have been related to learning impairments, autism spectrum disorders, emotional and behavioral conditions. These chemicals can result in occupational or accidental exposure through the food

chain, cooked meals, animal feeds, human breast milk or groundwater, even when stored and handled properly, resulting in severe health problems or even death. Pesticides are typically used at low concentrations that are usually considered to be within the "safe limits." The current paradigm underestimates the possible synergic effects and health risks of the simultaneous exposure to two or more pesticides as common in real-life. [6,9,10,15]

Neonicotinoids are one of the fastest-growing class of synthetic insecticides. They are included in the group 4A (Nicotinic acetylcholine receptor, or nAChR, competitive modulators) of pesticides by the Insect Resistance Action Committee (IRAC).[16] Neonicotinoids are generally non-volatile, polar and hydrolytically stable compounds chemically and structurally related to nicotine containing nitromethylene and nitroimin as well as a cyanoimin groups. Nowadays, they are intensively used due to their instant action on insect pests at low doses and their versatile application. Their most important advantage is their high specificity and selectivity for insects, generally being thought to be safe for humans because of the poor penetrability to the blood-brain barrier; however, they might be toxic to the environment and vertebrate fauna. All neonicotinoid-based products are classified for general use and have been registered under EPA's Conventional Reduced Risk Program. Neonicotinoids are water-soluble and easily absorbed into the plant through the leaves or roots. Thus, they are used mainly as plant systemics, providing long-term protection to all parts of the pant from pierce-sucking insects. Thus, neonicotinoids control sap-feeding insects and insects that chew on plant tissue. Neonicotinoids tend to linger in the environment due to their high water solubility, persistence, and the sheer volume of use. Neonicotinoids account for 10-15% of the total insecticide market and are the most used insecticide worldwide, being licensed in 120 countries with a global market value of about 3.5 billion dollars. The main consumers are Asia, Latin America, North America (75%), and Europe (11%). The most popular neonicotinoids nowadays used are (year of patent): imidacloprid (1985), nithiazine (1977), thiamethoxam (1992), thiacloprid (1985), nitenpyram (1988), clothianidin (1989), acetamiprid (1989), and dinotefuran (1994). The most commonly used neonicotinoids acetamiprid, imidacloprid and thiamethoxam were evaluated in this study. [8,17–26]

The pharmacological mechanism of action of neonicotinoids include agonistic, antagonistic and allosteric modulation of nicotine acetylcholine receptors (nAChR). In insects, they act as agonists at the postsynaptic nAChRs, which are located entirely in the central nervous system (CNS).

These ligand-gated ion channels play a critical role in the fast-cholinergic transmission since they produce continuous excitation of the neurons of the CNS of insects. At high concentration, however, they block nerve propagation, leading to paralysis and finally death. Neonicotinoids also have an affinity for vertebrate nAChRs and could bind to the α4β2 nAChRs subtype in mammals. This receptor is present at high densities in the thalamus where it may play a role in the developing brain (causing neural proliferation, migration, apoptosis, synapse formation and/or neural circuit formation) as well as in CNS disorders. Despite the current paradigm that neonicotinoids represent a low risk for mammals, gathering evidence points to their immunotoxic, neurotoxic, nephrotoxic, hepatotoxic, and reproductive cytotoxic effects in both vertebrates and invertebrates. [3,8,13,16,17,22,25,27–29] Knowledge of the putative toxic effects of neonicotinoids in the human brain is still very limited.

Acetamiprid (ACE) N-[(6-chloro-3-pyridyl) methyl]-N'-cyano-N-methylacetamidine is considered the gold standard for neonicotinoids. It been detected in many agricultural products such as tomato, cotton, nuts, potato, and tobacco. ACE has a higher affinity for nAChR in rodents than other neonicotinoids, so it may have harmful effects on off-target organisms. Studies indicate that prenatal exposure to ACE in mice produces abnormal microglial activation, resulting in histological abnormalities and neurodevelopmental toxicity in newborn mice.[22] Also in mice, neonatal exposure to ACE significantly impairs neurogenesis and alters microglial profiles in the Dentate Gyrus. [30] Low doses of ACE cause neuropathic changes at the level of the axons and myelin in isolated frog sciatic nerve.[23] Neurodevelopmental toxicity of ACE has been investigated in zebrafish embryos, where ACE induces teratogenic effects at concentrations of 263 mg/L and higher. [31] In the male reproductive system of adult male Sprague Dawley rats, it was seen that ACE could lead to Leydig cell testosterone synthesis being inhibited, causing decreases in epididymal sperm motility and sperm count as well as reduced plasma testosterone levels.[24] A study showed that ACE has other harmful effects on male reproductive function, likely by inducing oxidative stress in the testes through its metabolites.[32] One study suggested that *in vitro* human peripheral blood lymphocytes, ACE, in combination with alpha-cypermethrin, causes a synergistic genotoxic/cytotoxic effect. [33] In mouse PC12 neurons, ACE significantly decreases cell viability, reduces the potential of the mitochondrial membrane, and provokes DNA damage. [34] In rat cerebellar granule primary cell cultures ACE, imidacloprid (IMI), and nicotine have similar effects on nAChRs at concentrations of 1 μM and higher. [35]

Despite these recent insights, our knowledge of the neurotoxic effects of ACE in the mammal nervous system still is wanting and in a nascent stage.

Imidacloprid (IMI), 1-[(6-Chloro-3-pyridyl) methyl)-4, 5-dihydroimidazol-2-yl) nitramide, is the fastest-growing neonicotinoid insecticide worldwide in terms of production and consumption. Even if IMI seems to be less toxic to mammals than traditional pesticides, more and more studies have demonstrated that IMI could put the health of mammals at risk. Several studies in rats concluded that IMI induced oxidative stress and genotoxicity in the female [36–38] and male reproductive systems. [39,40] Also, *in vitro* studies suggest that IMI at concentrations near 10 mM damage cell membranes in mice.[41] In rats, *in utero* exposure to a sublethal dose of IMI produced significant neurodevelopmental and behavioral abnormalities at an early adolescent age.[42] IMI has cytotoxic and potential genotoxic and cytotoxic effects in Chinese hamster ovary cells.[43] Genotoxicity of IMI has been demonstrated in human lymphocytes. [44] Finally, in a chick embryo model, IMI causes neural tube defects and neuronal differentiation dysplasia as well as increased cell apoptosis and reduced cell proliferation, leading to malformations during neurogenesis. [45]

Thiamethoxam (TMX), 3-[(2-Chloro-1,3-thiazol-5-yl) methyl]–5-methyl-N-nitro-1, 3, 5 -oxadiazinan-4-imine is a broad-spectrum neonicotinoid insecticide. Several studies have suggested the off-target toxicity of TMX in mammals and even in humans. A study with wood frogs showed that the exposure to high concentrations of TMX could decrease the number of circulating blood cells and levels of corticosterone. [46] An *in vitro* study with rat cells demonstrated cardiotoxicity induced by TMX. [47] TMX has hepatotoxic and hepatocarcinogenic properties in mice [49,50] and induces carcinogenesis in human cells. [51] Finally, a study reported neurotoxicity of TMX metabolites in Chinese lizards. It should be noted that TMX did not act on nAChRs, but directly increases the concentrations of acetylcholine in the brain and blood by causing up-regulation of the expression of the *ach* gene.[52] Our understanding of the neurotoxicity of neonicotinoids still falls short, and more research is required to evaluate their potential risk for humans.

PROBLEM STATEMENT

Ecuador is one of the leading banana exporters of the world (30% of the global banana supply in the world). According to Ministerio de Agricultura y Ganadería (MAGAP), the banana sector is responsible for about 2 to 2.5 million jobs, both direct and indirect. In 2015, Ecuador exported around 120 million boxes of banana largely coming from Los Rios (38.55%) and Guayas (24.26%) provinces.[53–55] Studies conducted in Costa Rica established an association between the incidence of mental health problems and exposure to neonicotinoids, most of which are also used in banana crops in El Oro and Los Rios.[10] In 2016, the incidence of several mental conditions, including schizophrenia and mental retardation was 831.21 (Pasaje), 636.64 (Machala), and 141.26 (El Guabo) per 100,000 inhabitants; that is, in areas with the highest production of banana in the province of El Oro. These rates were calculated based on RDACAA data provided by Health Districts 07D01 and 0D02.[56] It is imperative to conduct more studies on the putative neurotoxic effects cause by the most extensively used neonicotinoids in banana plantations of Ecuador, as well as to determine possible synergistic effects among pesticides. This study will use a human *in vitro* model as the experimental approach.

OBJECTIVES

The objective of this work is to evaluate *in vitro* the neurotoxicity of the neonicotinoid pesticides acetamiprid, imidacloprid and thiamethoxam, using the MTT and LDH methods to measure cell viability.

SPECIFIC OBJECTIVES

To expose human neurons to either the treatment of a single pesticide or a combinations of two pesticides to determine the existence of synergic or antagonistic interactions.

To differentiate SH-SY5Y neuroblastoma cells to display their cholinergic phenotype.

To assess retinoic acid differentiated cholinergic SH-SY5Y neuroblastoma cell viability and evaluate which neonicotinoid poses higher neurotoxicity.

To detect synergic effects between the neonicotinoids.

To assess whether a mature phenotypes posses a higher sensitivity to neonicotinoids.

METHODS

Reagents

Dulbecco's modified Eagle minimum essential/Ham's F-12 (DMEM/F12) plus Glutamax media, fetal bovine serum (FBS), penicillin G/streptomycin mix, Trypsin PBS-based cell dissociation buffer, and LDH cytotoxicity kits were purchased from Gibco (Carlsbad, CA). Thiamethoxam and imidacloprid, and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were acquired from Sigma-Aldrich (St Louis, MO), and acetamiprid from Chemservice (Gorizia, Italy).

Culture of SH-SY5Y Cells

Primary mammalian neurons cannot be used in this context because once they are differentiated, they can no longer be propagated. For this reason, SH-SY5Y cell lines were used to overcome this limitation. These cells have been shown to consititute an *in vitro* model with high "prediction" validity. The SH-SY5Y neuroblastoma cell line is a subline of SK-N-SH cells with the capacity of proliferating for long periods of time without contamination. The SK-N-SH cells were originally identified in the 1970s from a bone marrow biopsy of a sympathetic adrenergic ganglial neuroblastoma patient. SH-SY5Y cells were derived from immature neoplastic neural crest cells that display properties of stem cells. SH-SY5Y cells show neuronal marker enzyme activity, muscarinic, opioid and nerve growth factor receptors, express one or more neurofilament proteins and exhibit specific uptake of norepinephrine. These cells can be induced to differentiate to a more neuron-like phenotype using staurosporine, retinoic acid, brain-derived neurotrophic factor (BDNF), phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), purine or dibutyryl cyclic AMP (dBcAMP). [57,58]

The SH-SY5Y cells were a generous gift of Dr. Javier Sáez de Castresana (University of Navarra, Spain). The growth medium for the SH-SY5Y cells was prepared using Thermo Fischer DMEM medium (high glucose, GlutaMAX, phenol red), 10% FBS and 1% pen/strep. Before mixing, each component was previously filtered through a 0.22 µm pore filter apparatus. The SH-SY5Y cells were thawed and added to a T-75 Thermo Fischer tissue culture flask containing the growth media. Then the cells were cultured in an incubator at 37 °C with 5% CO_2. The growth media were renewed every 3-5 days, and the cells were checked for confluence every day. When an 80-90% confluence was reached, the cells were sub-cultured. For sub-culturing, the medium was aspirated under sterile conditions, and the adherent cells were rinsed with pre-warmed (37 °C) 1 x PBS (Gibco, pH 7.4). The PBS was removed by aspiration immediately after the cell monolayer

was rinsed. Subsequently, 0.05 % trypsin was added to the adherent cells which were incubated for 5 minutes to allow the cells to detach from the flask. Then, trypsin was neutralized using growth medium, and the SH-SY5Y cells were collected in tubes for centrifugation at 2500 rpm for 5 minutes at room temperature. The supernatant was removed, and the pellet resuspended in fresh medium, allowing cells to form a homogenously solution. Finally, the SH-SY5Y cells were plated at a density considered adequate for them to have a regular growth rate. This procedure was repeated as needed throughout the experimentation. For every experiment low passage cells (no more than 15 passages) were used.

Retinoic Acid-Induced Differentiation

A well-known method for the differentiation of SH-SY5Y cells into a more mature (non-dividing) phenotype is the treatment with retinoic acid (RA), which is a vitamin A derivative with growth-inhibiting and cellular differentiation-promoting effects. The differentiation with RA promotes the survival of the cells through the activation of the phosphatidylinositol 3-kinase/Akt signaling pathway and upregulation of the anti-apoptotic Bcl-2 protein. In response to RA treatment the SH-SY5Y cells differentiate mainly to a more cholinergic neuron phenotype with an increase in choline acetyl transferase (ChAT) activity and vesicular monoamine transporter (VMAT) expression.[58] SH-SY5Y cells were treated with 10μM retinoic acid, which was directly dissolved in DMEM medium supplemented with 10% FBS and 1% pen/strep. The differentiation medium was changed every 2 days prior to treatment with the neonicotinoids.

Treatments

Cells were seeded in 96-well plates at 5 x 10^3 cells/well and incubated in 100 μL of growth medium in the presence or absence of RA. Ready-to-use differentiated SH-SY5Y neurons were achieved either on day 2 or on day 7 after RA regimen. One μL of stock solutions of neonicotinoids disolved in pure DMSO were directly given to cells to achieve final concentrations of 1, 2, and 4 mM in the growth medium. To analyze putative synergies among neonicotinoids, cells were treated with the following mixtures: acetamiprid (1 mM) + imidacloprid (1 mM), acetamiprid (1 mM) + thiamethoxam (1 mM), imidacloprid (1 mM) +-thiamethoxam (1 mM), acetamiprid (2 mM) + imidacloprid (2 mM), acetamiprid (2 mM) + thiamethoxam (2 mM), imidacloprid (2 mM) + thiamethoxam (2 mM). A control with no neonicotinoid (1% DMSO), as well as a control of maximal cytotoxicity (10% DMSO), was included in every microplate to serve as a reference for the calculations in the cytotoxicity assays. Cells were then incubated for 24

and 48 h at 37 °C with 5% of CO_2. Before the cytotoxicity measurements each treatment condition was replicated from four to twelve times in each independent experiment.

Neurotoxicity Assessment by the MTT Method (Figure 1)

The MTT assay is a colorimetric assay that is based on the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide). This assay measures the viability of cells by evaluating the capacity of mitochondrial enzymes of cells to transform MTT tetrazolium salt into MTT formazan. [59,60] After either 24 or 48-hours of treatment with thiamethoxam, imidacloprid, and acetamiprid, a fresh MTT solution (5mg/mL) in PBS (Gibco, pH 7.4) was prepared, and 10 μL were added to each well, except the blanks, and incubated for 1 hour at 37 °C with 5% CO_2. Subsequently, the media were removed from the wells, and the MTT formazan was resuspended with 50 μL of DMSO in every well. Optical density was recorded at a wavelength of 570 nm, using an Omega spectrofluorometer (BMG Labtech, Ortenberg, Germany). Absorbance of wells with no treatment (control DMSO 1%) was set equal to 100 to standardize absorbance values of treated wells. Finally, obtained data (as the percentage of control survival) was graphed for evaluation and analysis.

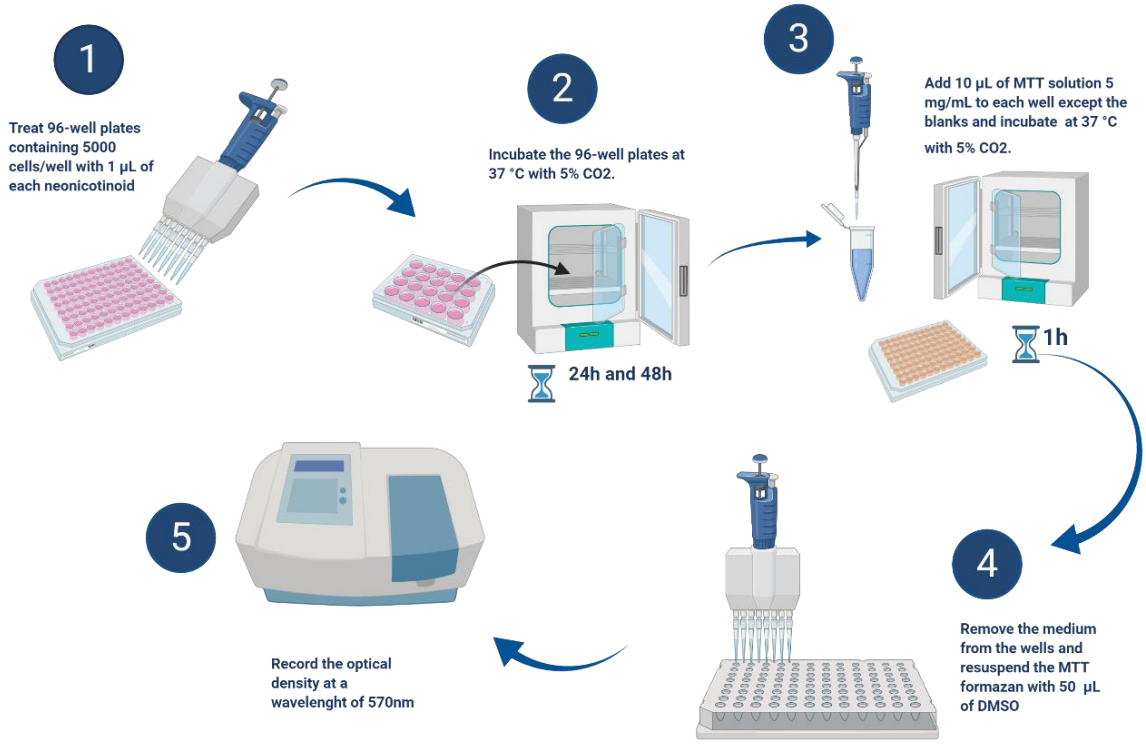

Figure 1. Procedure for the MTT assay (Created in Biorender.com)

Neurotoxicity Assessment by the LDH Method (Figure 2)

The LDH assay is an enzyme-release assay for cytotoxicity based on the amount of the enzyme lactate dehydrogenase (LDH) that leaks from lysed cells. [61] To evaluate the cytotoxicity of the neonicotinoids an LDH assay was carried out. After either 24 or 48-hours of treatment with thiamethoxam, imidacloprid, and acetamiprid, microplates were centrifuged at 250 x G for 4 minutes to pellet the cells. Then, aliquots of the supernatant were transferred to a clean flat bottom microplate (~50 μL). According to the LDH kit manufacturer instructions, the LDH mixture was prepared by mixing equal volumes of the LDH assay substrate, LDH dye solution, and the assay cofactor. Then, 100 μL of the mixture were added to each well in the dark to avoid photodegradation, and the plates were incubated for 30 minutes at 37 °C with 5% CO_2. The reaction was terminated with 10 μL of 1N HCl per well. Finally, the optical density was recorded at a wavelength of 490 nm and subtracted from a background optical density at 690 nm using an Omega spectrofluorometer to obtain the net absorbance**.**

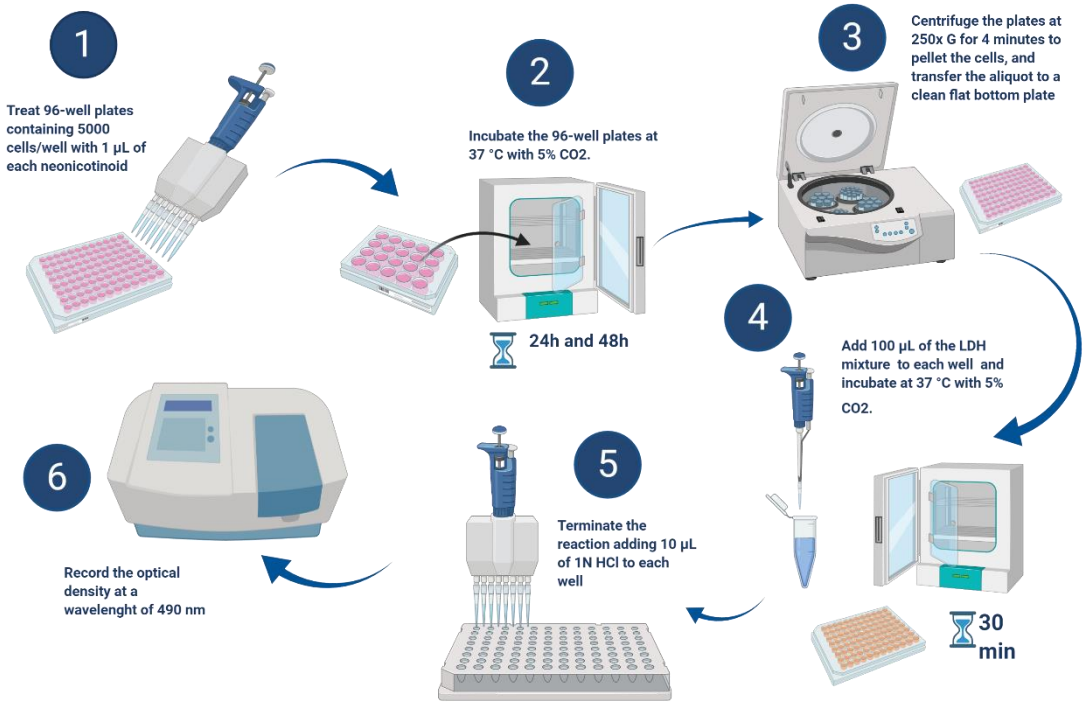

Figure 2. Procedure for the LDH assay (Created in Biorender.com)

Statistics

Statistical analyses were carried out using the SPSS 16 software for Windows. MTT and LDH data were analyzed by multi-factorial (three-way) ANOVA, with differentiation (RA+ and RA-), neonicotinoid-treatment time (24h and 48h), and concentration (1, 2, and 4 mM) or concentration mixtures (1:0, 2:0; 4:0, 1:1, and 2:2 mM) of neonicotinoids as the factors. Pairwise comparisons were made using the Tukey's HSD test for *post hoc* analyses. The alpha value was set at 0.05.

RESULTS

RA-induced differentiation of SH-SY5Y neurons

The differentiation treatment with RA was conducted for either 2 days or 7 days prior to the cytotoxic treatment with neonicotinoids. **Figure 3** shows the morphological change following differentiation with RA at several points in time under brightfield illumination. Compared to untreated (control) cells, RA-differentiated cells showed abundant neurite extensions resembling the mature neuronal phenotype. There was a peak of neuronal differentiation at 72 hours of RA treatment with no further changes thereafter.

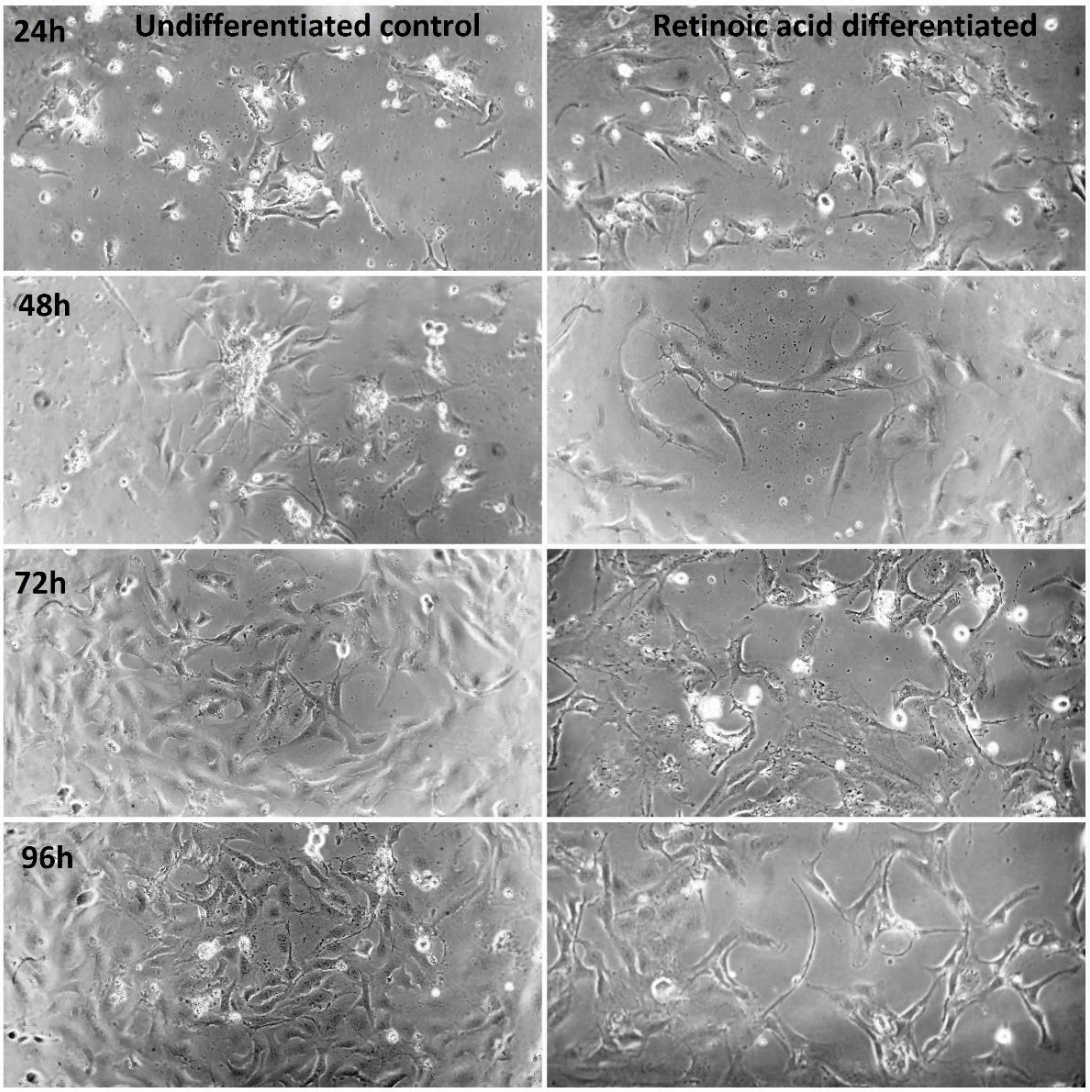

Figure 3. Morphology of SH-SY5Y cells differentiated with RA at X20 magnification. Notice the prolonged neurite extensions and decreased clusters of proliferating cells throughout the RA differentiation.

Viability of RA-differentiated (2 days) SH-SY5Y neurons after treatment with neonicotinoids according to the LDH method

The viability, as determined by the LDH method, of SH-SY5Y neurons differentiated with RA for 2 days after the 24-h (**Fig. 4**) and the 48-h (**Fig. 5**) of neonicotinoid treatment revealed various patterns. The three-way ANOVA revealed a significant interaction between differentiation and treatment time for acetamiprid ($F(11,1)= 11.36$, $P < 0.001$), imidacloprid ($F(11,1)= 9.11$; $P < 0.003$), and thiamethoxam ($F(11,1)= 24.84$, $P < 0.0001$). It also revealed a significant interaction between differentiation and concentration for acetamiprid ($F(11,2)= 3.81$, $P < 0.02$). There was also a significant interaction between concentration and treatment time for imidacloprid ($F(11,2)= 2.96$; $P < 0.05$), and thiamethoxam ($F(11,2)= 14.58$, $P < 0.0001$). All the three neonicotinoids, and particularly acetamiprid, induced cell death that increased in parallel with pesticide concentrations (($P < 0.0001$, 4 mM compared to 1 mM; $P < 0.0001$, 4 mM compared to 2 mM). Cytotoxic effects were more noticeable for acetamiprid, although they were less evident at 48 h. A reduction of cytotoxicity over time was also observed in imidacloprid and thiamethoxam treatments.

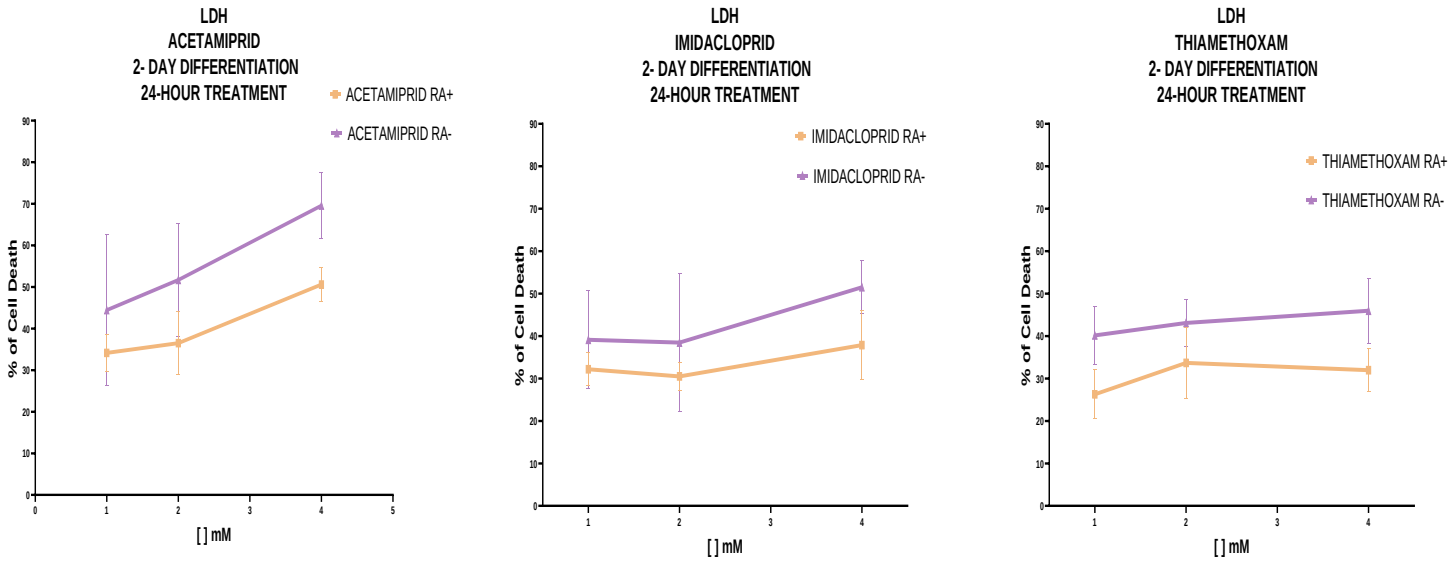

Figure 4. Cell viability based on the LDH method after 24-h neonicotinoid treatment in RA differentiated (2 days) cells *compared to undifferentiated cells.* Results are displayed as mean ± SD. *N*= 8

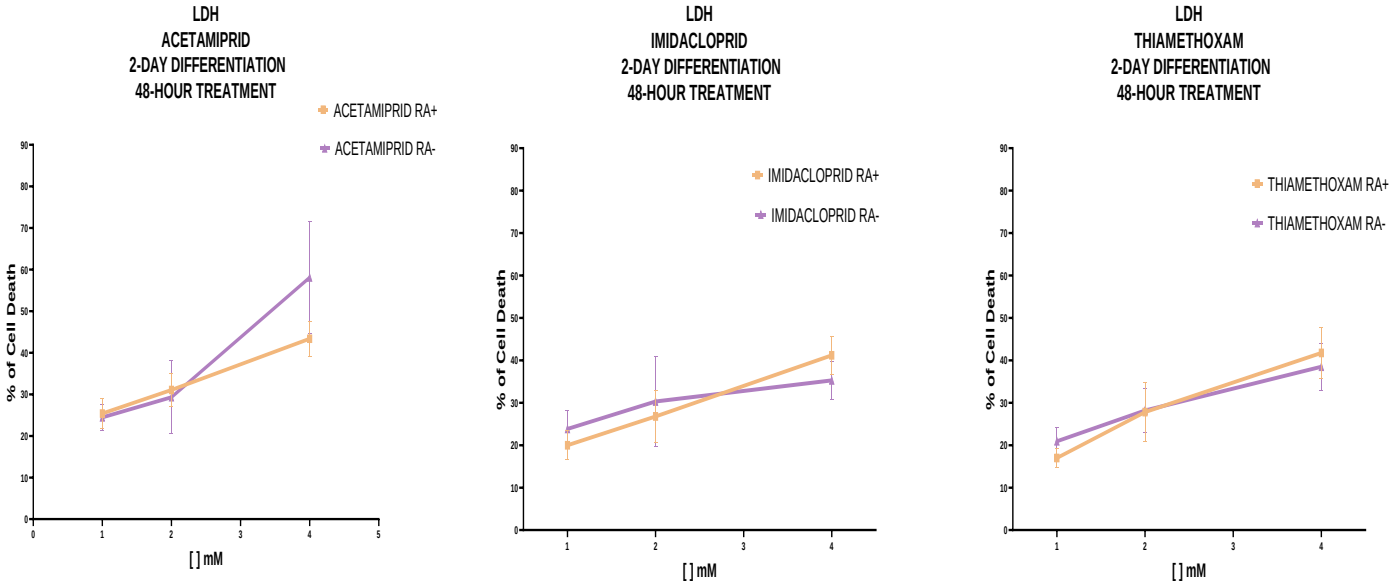

Figure 5. Cell viability based on the LDH method after 48-h neonicotinoid treatment in RA-differentiated (2 days) cells compared to undifferentiated cells. Results are displayed as mean ± SD. N= 8

Viability of RA-differentiated (2 days) SH-SY5Y neurons after treatment with neonicotinoids according to the MTT method

(**Fig. 6 & 7**) show the viability, as determined by the MTT method, of SH-SY5Y neurons differentiated with RA for 2 days after the 24-h (**Fig. 6**) and the 48-h (**Fig. 7**) of neonicotinoid treatment. The three-way ANOVA revealed a significant interaction between differentiation and concentration for acetamiprid ($F(11,2)= 6.35$, $P < 0.003$), imidacloprid ($F(11,2)= 11.9$; $P < 0.0001$), and thiamethoxam ($F(11,2)= 6.46$, $P < 0.002$). It also revealed a significant interaction between differentiation and treatment time for thiamethoxam ($F(11,1)= 5.61$, $P < 0.02$). Similar to the LDH assays, acetamiprid showed the clearest dose-toxic response curve ($P < 0.0001$, 4 mM compared to 1 mM; $P < 0.0001$, 4 mM compared to 2 mM; $P < 0.01$, 2 mM compared to 1 mM).

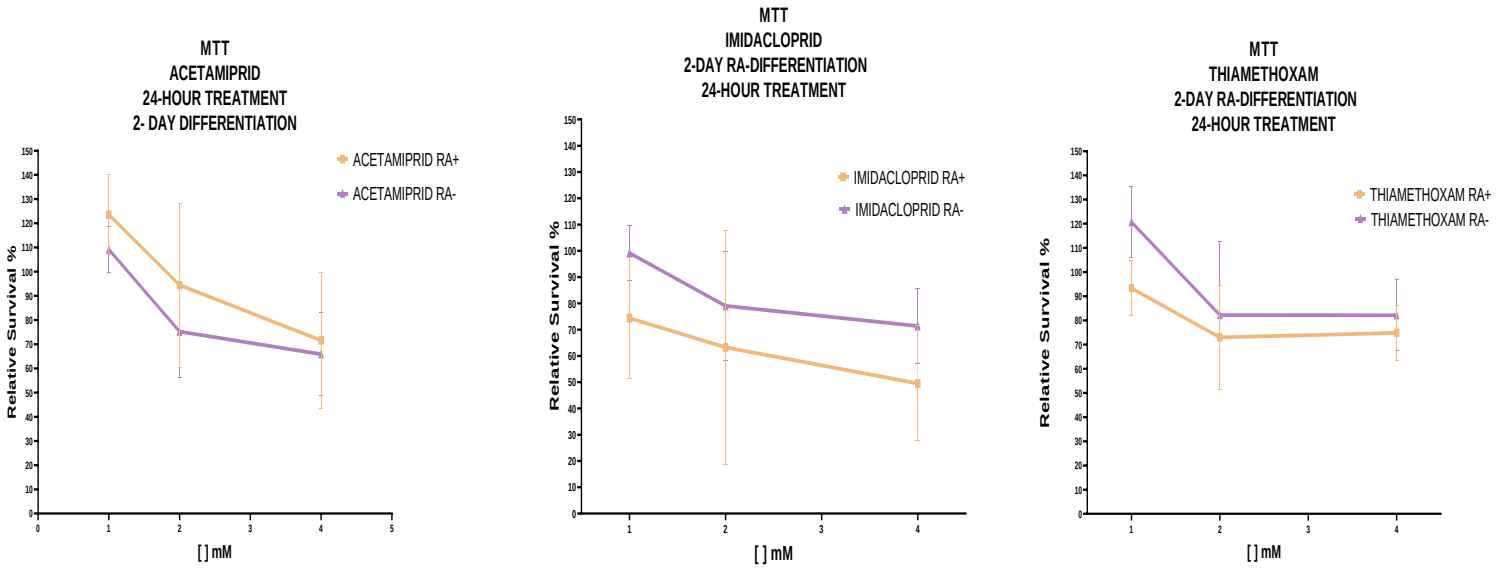

Figure 6. Cell viability based on the MTT method after 24-h neonicotinoid treatment in RA-differentiated (2 days) cells compared to undifferentiated cells. Results are displayed as mean ± SD. N= 8

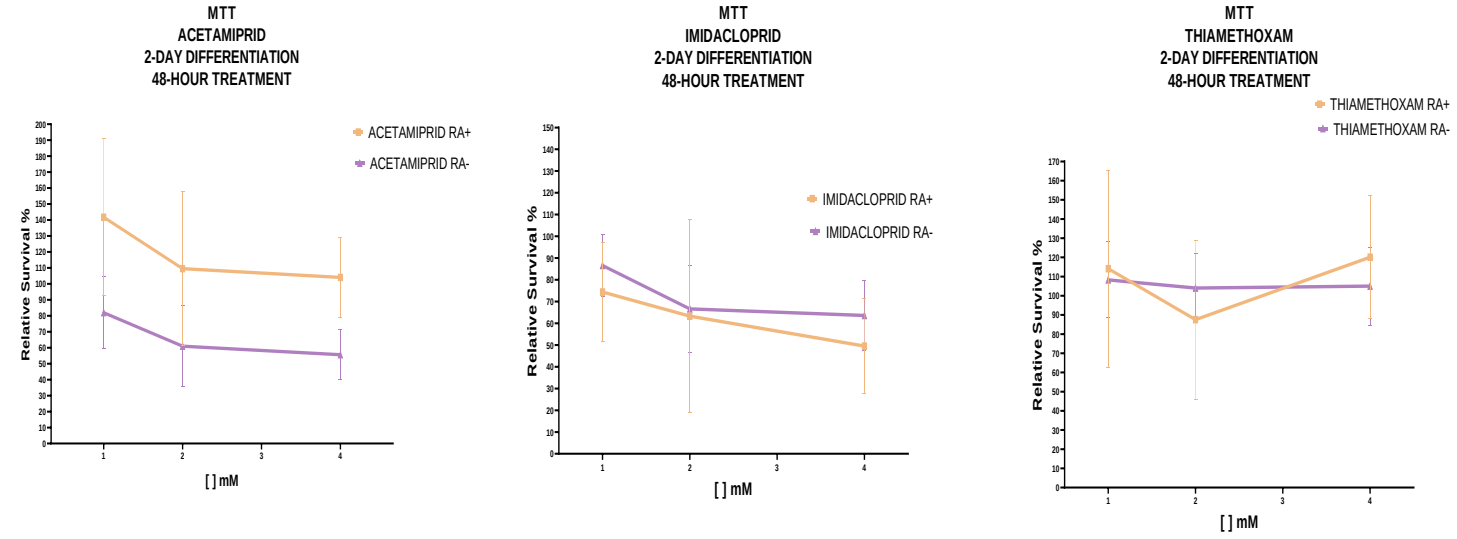

Figure 7. Cell viability based on the MTT method after 48-h neonicotinoid treatment in RA-differentiated (2 days) cells compared to undifferentiated cells. Results are displayed as mean ± SD. N= 8

Cytotoxicity of pairwise combinations of the neonicotinoids in RA-differentiated (2 days) SH-SY5Y neurons according to the LDH method.

Pairwise combinations of neonicotinoids were administered to cells in an attempt of searching for synergistic effects, as determined by the LDH method. SH-SY5Y neurons were differentiated with RA for 2 days and then treated with acetamiprid **(Fig.8**), imidacloprid (**Fig.9**), and thiamethoxam (**Fig.10**) for 24 h and 48 h. The three-way ANOVA revealed a significant interaction between treatment time and the state of differentiation ($F(23,1)= 11.27$, $P < 0.001$). The mixture of acetamiprid and thiamethoxam was significantly different from acetamiprid alone in every case [RA+,24h (acetamiprid 1 mM + thiamethoxam 1 mM vs. acetamiprid 2 mM, $P < 0.05$); RA+,48h (acetamiprid 1 mM + thiamethoxam 1 mM vs. acetamiprid 2 mM, $P < 0.001$); RA-,24h (acetamiprid 1 mM + thiamethoxam 1 mM vs. acetamiprid 2 mM, $P < 0.001$), (acetamiprid 2 mM + thiamethoxam 2 mM vs. acetamiprid 4 mM, $P < 0.0001$); RA-,48h (acetamiprid 1 mM + thiamethoxam 1 mM vs. acetamiprid 2 mM, $P < 0.01$)]. The mixture of acetamiprid and imidacloprid was significantly different from acetamiprid alone in [RA-, 24h (acetamiprid 1 mM + imidacloprid 1 mM vs. acetamiprid 2 mM, $P < 0.05$), (acetamiprid 2 mM + imidacloprid 2 mM vs. acetamiprid 4 mM, $P < 0.00001$); RA+,48h (acetamiprid 1 mM + imidacloprid 1 mM vs. acetamiprid 2 mM, $P < 0.005$)]. **(Fig.8**). The mixture of acetamiprid and imidacloprid was significantly different from imidacloprid alone in [RA+, 24h (acetamiprid 1 mM + imidacloprid 1 mM vs. imidacloprid 2 mM, $P < 0.005$); RA-,24h (acetamiprid 2 mM + imidacloprid 2 mM vs. imidacloprid 4 mM, $P < 0.001$); RA+,48h (acetamiprid 1 mM + imidacloprid 1 mM vs. imidacloprid 2 mM, $P < 0.002$)]. The mixture of thiamethoxam and imidacloprid was significantly different from imidacloprid alone in [RA-, 24h (thiamethoxam 2 mM + imidacloprid 2 mM vs. imidacloprid 4 mM, $P < 0.00001$); RA+,48h (thiamethoxam 1 mM + imidacloprid 1 mM vs. imidacloprid 2 mM, $P < 0.01$), (thiamethoxam 2 mM + imidacloprid 2 mM vs. imidacloprid 2 mM, $P < 0.0001$)] **(Fig.9**). The mixture of acetamiprid and thiamethoxam was significantly different from thiamethoxam alone in [RA+,24h (acetamiprid 1 mM + thiamethoxam 1 mM vs. thiamethoxam 2 mM, $P < 0.008$), (acetamiprid 2 mM + thiamethoxam 2 mM vs. thiamethoxam 4 mM, $P < 0.002$); RA+,48h (acetamiprid 1 mM + thiamethoxam 1 mM vs. thiamethoxam 2 mM, $P < 0.009$)]. The mixture of imidacloprid and thiamethoxam was significantly different from thiamethoxam alone in [RA-,24h (imidacloprid 2 mM + thiamethoxam 2 mM vs. thiamethoxam 4 mM, $P < 0.003$); RA+,48h (imidacloprid 1 mM + thiamethoxam 1 mM vs. thiamethoxam 2 mM,

P < 0.005), (imidacloprid 2 mM + thiamethoxam 2 mM vs. thiamethoxam 4 mM, *P* < 0.0001)] **(Fig.10**).

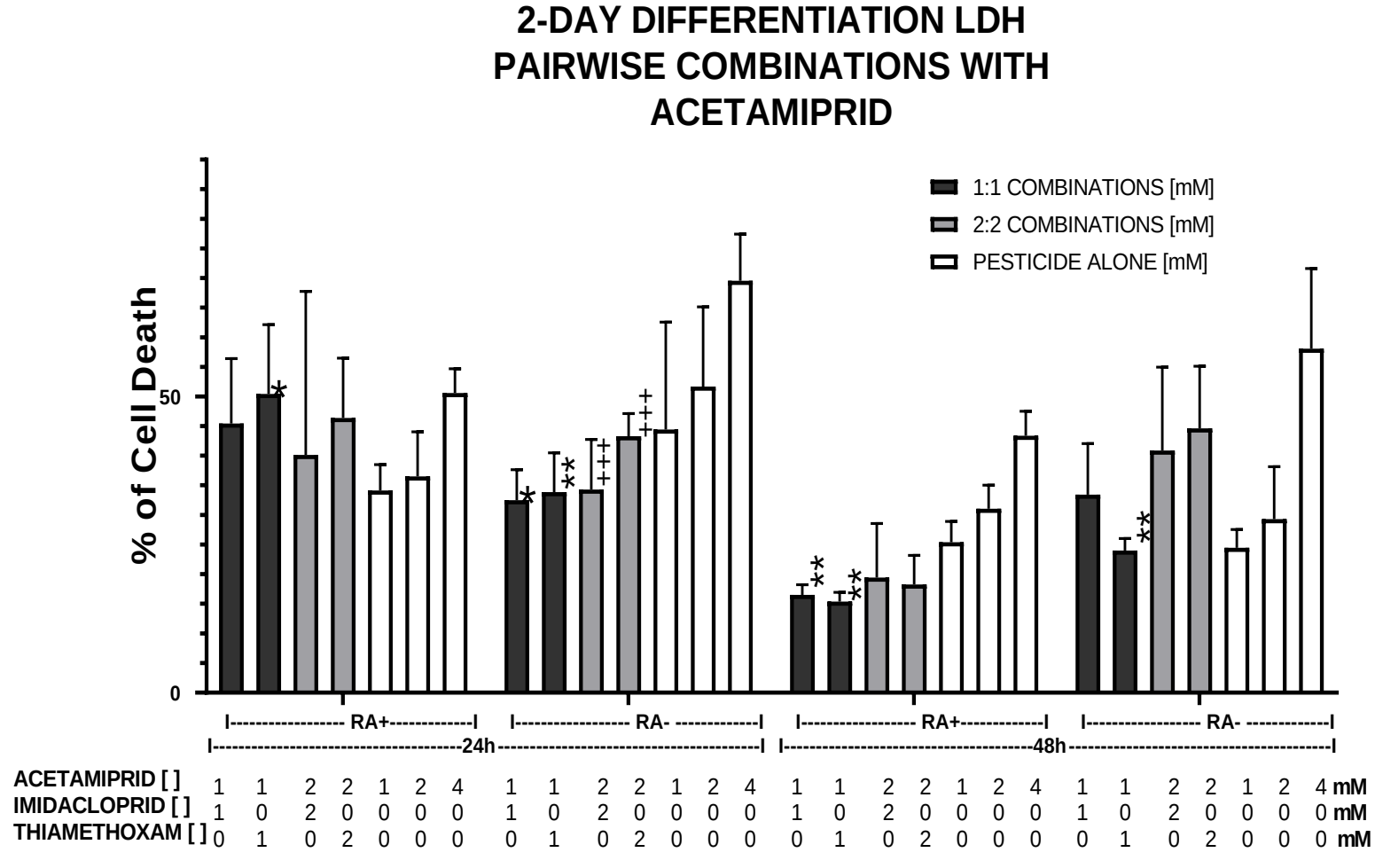

Figure 8. Interactions of acetamiprid with two other neonicotinoids in cell viability after 24-hour and 48-hour treatments according to the LDH method. The figure shows significant pairwise comparisons between one nicotinoid given alone and the concentration mixtures. *Results are displayed as mean ± SD.* **P* < 0.05; ***P* < 0.01, ****P* < 0.0001 compared to 2 mM; +*P* < 0.05; ++*P* < 0.01, +++*P* < 0.0001 compared to 4 mM, *N*= 6-8.

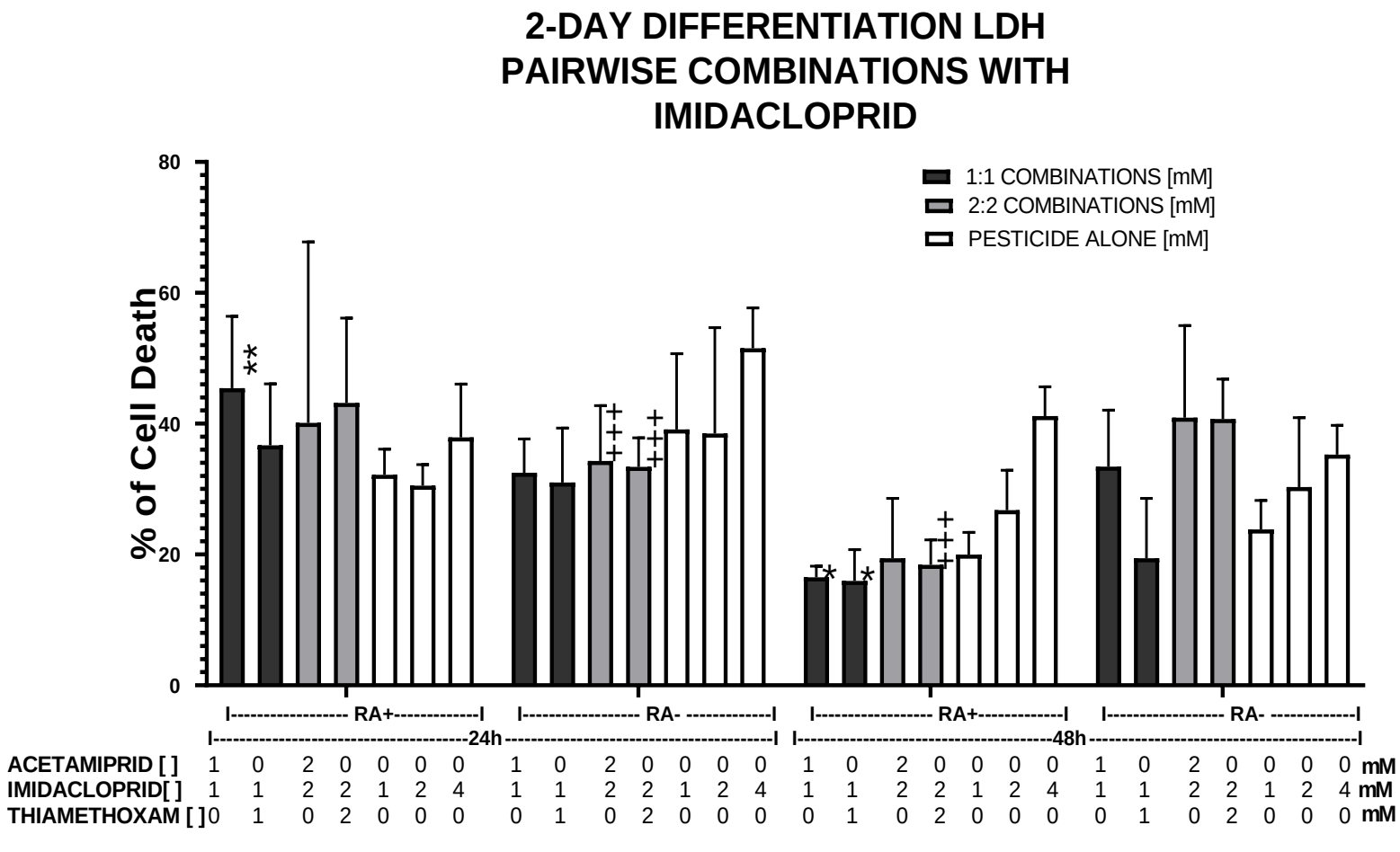

Figure 9. Interactions of imidacloprid with two other neonicotinoids in cell viability after 24-hour and 48-hour treatments according to the LDH method. The figure shows significant pairwise comparisons between one nicotinoid given alone and the concentration mixtures. *Results are displayed as mean ± SD.* **P* < 0.05; ***P* < 0.01, ****P* < 0.0001 compared to 2 mM; +*P* < 0.05; ++*P* < 0.01, +++*P* < 0.0001 compared to 4 mM, *N*= 6-8.

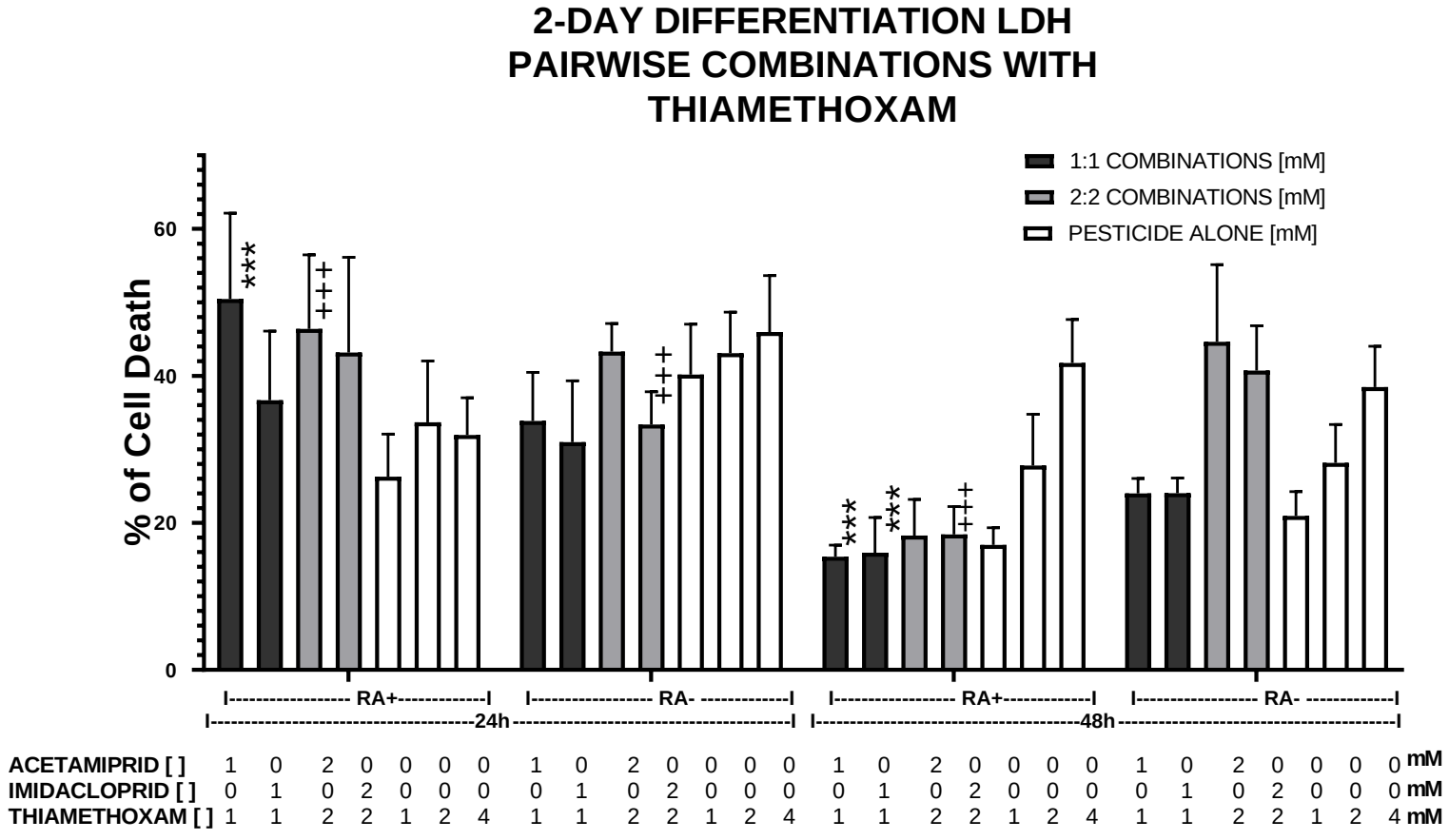

Figure 10. Interactions of thiamethoxam with two other neonicotinoids in cell viability after 24-hour and 48-hour treatments according to the LDH method. The figure shows significant pairwise comparisons between one nicotinoid given alone and the concentration mixtures.

Results are displayed as mean ± SD. **P* < 0.05; ***P* < 0.01, ****P* < 0.0001 compared to 2 mM; +*P* < 0.05; ++*P* < 0.01, +++*P* < 0.0001 compared to 4 mM. *N* = 6-8.

Cytotoxicity of pairwise combinations of the neonicotinoids in RA-differentiated (2 days) SH-SY5Y neurons according to the MTT method.

Pairwise combinations of neonicotinoids were administered to cells in an attempt of searching for synergistic effects, as determined by the MTT method. SH-SY5Y neurons were differentiated with RA for 2 days and then treated with acetamiprid **(Fig.11**), imidacloprid (**Fig.12**), and thiamethoxam (**Fig.13**) for 24 h and 48 h. The three-way ANOVA pointed to significant interactions between neonicotinoids concentration, differentiation and treatment time (*F*(23,5)= ; *P* < 0.0001). The mixture of acetamiprid and thiamethoxam was significantly different from acetamiprid alone in [RA+,48h (acetamiprid 1 mM + thiamethoxam 1 mM vs. acetamiprid 2 mM, *P* < 0.005); RA-,48h (acetamiprid 1 mM + thiamethoxam 1 mM vs. acetamiprid 2 mM, *P* < 0.002)] The mixture of acetamiprid and imidacloprid was significantly different from acetamiprid alone in [RA+, 24h (acetamiprid 2 mM + imidacloprid 2 mM vs. acetamiprid 4 mM, *P* < 0.01); RA-,48h (acetamiprid 2 mM + imidacloprid 2 mM vs. acetamiprid 4 mM, *P* < 0.001); RA-,48h (acetamiprid 1 mM + imidacloprid 1 mM vs. acetamiprid 2 mM, *P* < 0.002)]. **(Fig.11**). The mixture of acetamiprid and imidacloprid was significantly different from imidacloprid alone in [RA+, 24h (acetamiprid 2 mM + imidacloprid 2 mM vs. imidacloprid 4 mM, *P* < 0.004); RA-,24h (acetamiprid 2 mM + imidacloprid 2 mM vs. imidacloprid 4 mM, *P* < 0.005); RA-,48h (acetamiprid 1 mM + imidacloprid 1 mM vs. imidacloprid 2 mM, *P* < 0.002), (acetamiprid 2 mM + imidacloprid 2 mM vs. imidacloprid 4 mM, *P* < 0.00001)]. The mixture of thiamethoxam and imidacloprid was significantly different from imidacloprid alone in [RA+, 48h (thiamethoxam 1 mM + imidacloprid 1 mM vs. imidacloprid 2 mM, *P* < 0.00001); RA-,48h (thiamethoxam 1 mM + imidacloprid 1 mM vs. imidacloprid 2 mM, *P* < 0.03), (thiamethoxam 2 mM + imidacloprid 2 mM vs. imidacloprid 2 mM, *P* < 0.002)] **(Fig.12**). The mixture of acetamiprid and thiamethoxam was significantly different from thiamethoxam alone in [RA+,48h (acetamiprid 1 mM + thiamethoxam 1 mM vs. thiamethoxam 2 mM, *P* < 0.001); RA-,48h (acetamiprid 1 mM + thiamethoxam 1 mM vs. thiamethoxam 2 mM, *P* < 0.01), (acetamiprid 2 mM + thiamethoxam 2 mM vs. thiamethoxam 4 mM, *P* < 0.00001)]. The mixture of imidacloprid and thiamethoxam was significantly different from thiamethoxam alone in [RA-,48h (imidacloprid 2 mM + thiamethoxam 2 mM vs.

thiamethoxam 4 mM, $P < 0.001$)] **(Fig.13**). The reduction of the cytotoxic effect by the mixture observed in LDH was not replicated in the MTT assays.

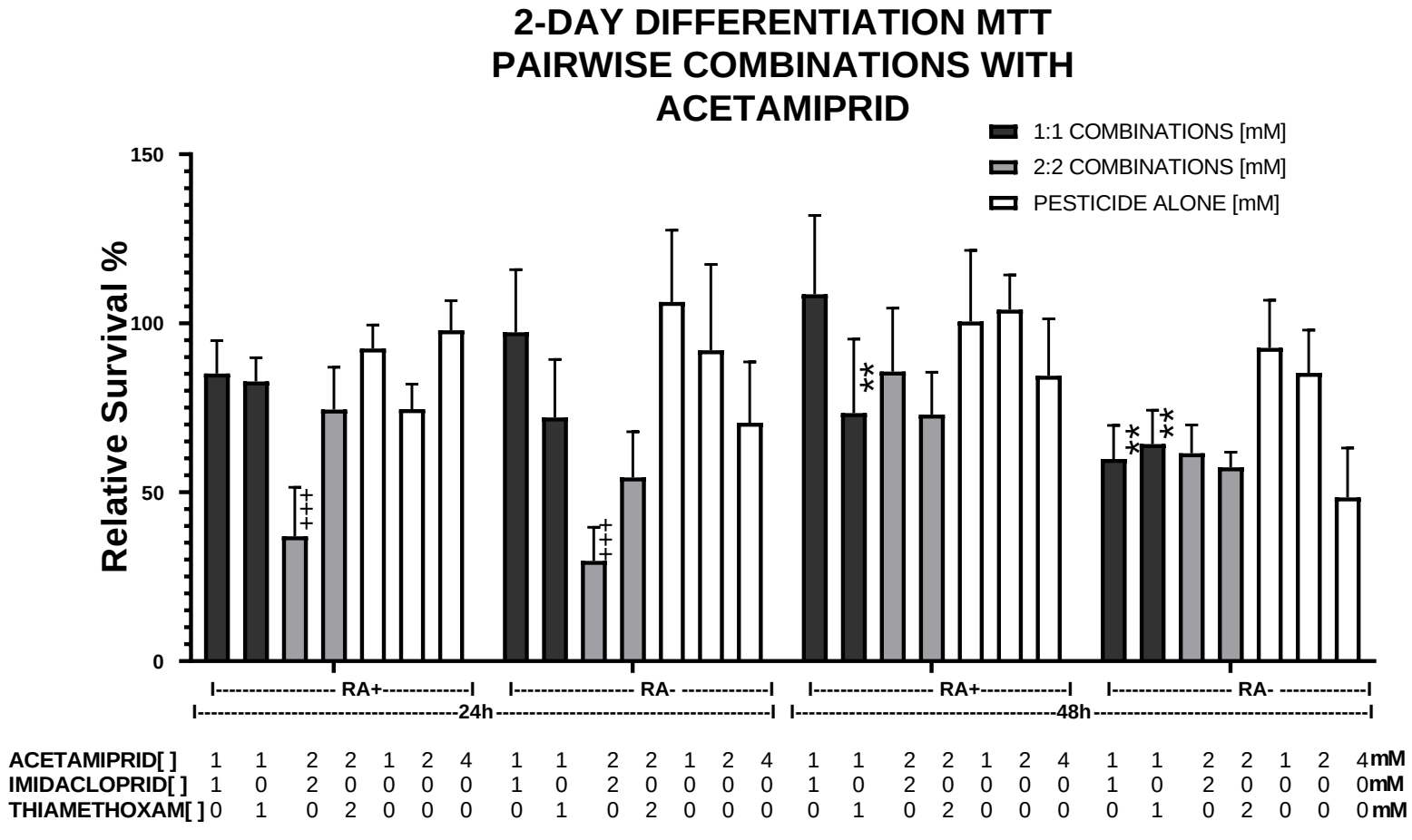

Figure 11. Synergic interactions of acetamiprid with two other neonicotinoids in cell viability after 24-hour and 48-hour treatments according to the MTT method. The figure shows significant pairwise comparisons between one nicotinoid given alone and the concentration mixtures. *Results are displayed as mean ± SD.* **P* < 0.05; ***P* < 0.01, ****P* < 0.0001 compared to 2 mM; +*P* < 0.05; ++*P* < 0.01, +++*P* < 0.0001 compared to 4 mM. *N* = 8-20.

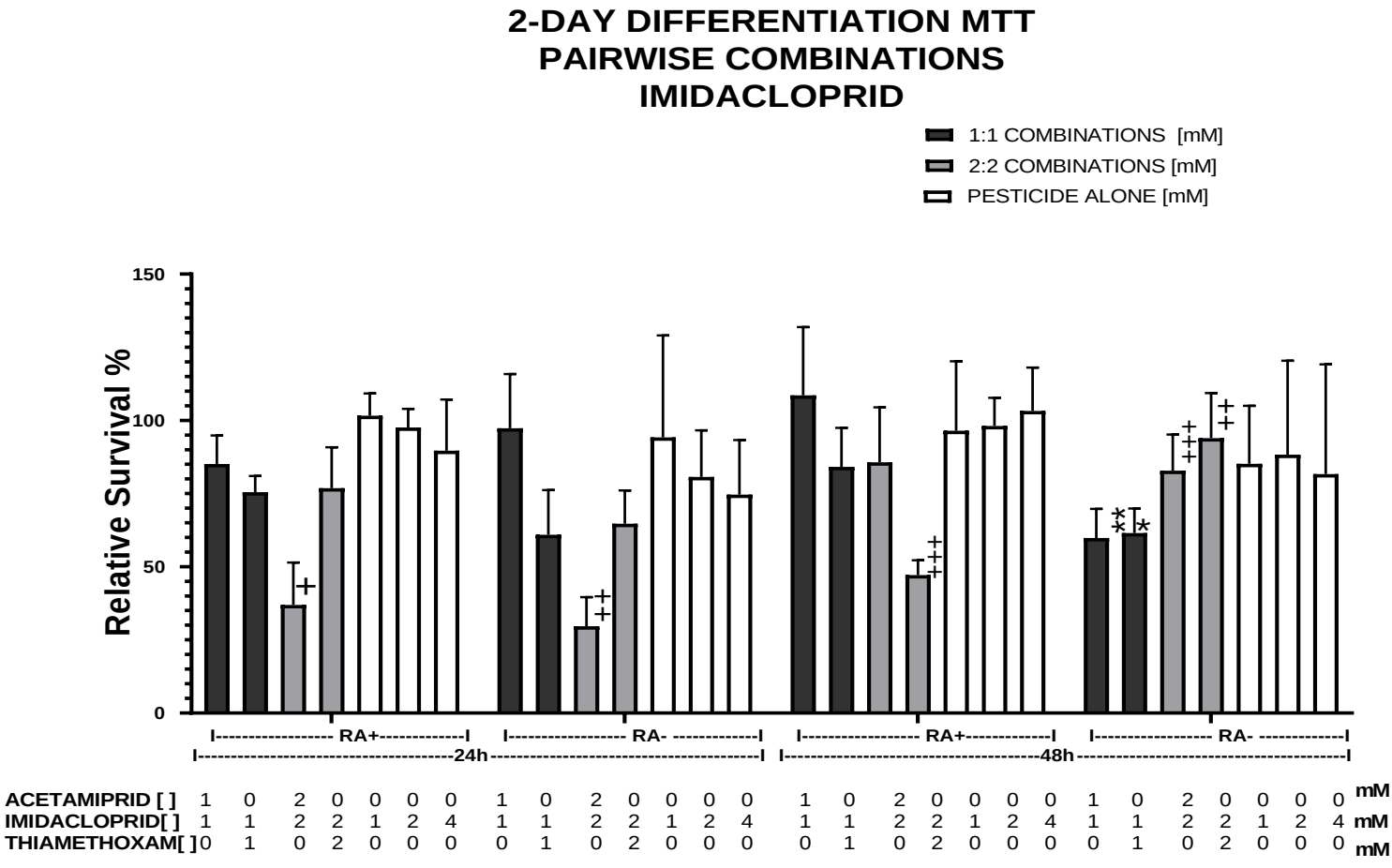

Figure 12. Synergic interactions of imidacloprid with two other neonicotinoids in cell viability after 24-hour and 48-hour treatments according to the MTT method. The figure shows significant pairwise comparisons between one nicotinoid given alone and the concentration

mixtures. Results are displayed as mean ± SD. *P < 0.05; **P < 0.01, ***P < 0.0001 compared to 2 mM; +P < 0.05; ++P < 0.01, +++P < 0.0001 compared to 4 mM, N= 8-20.

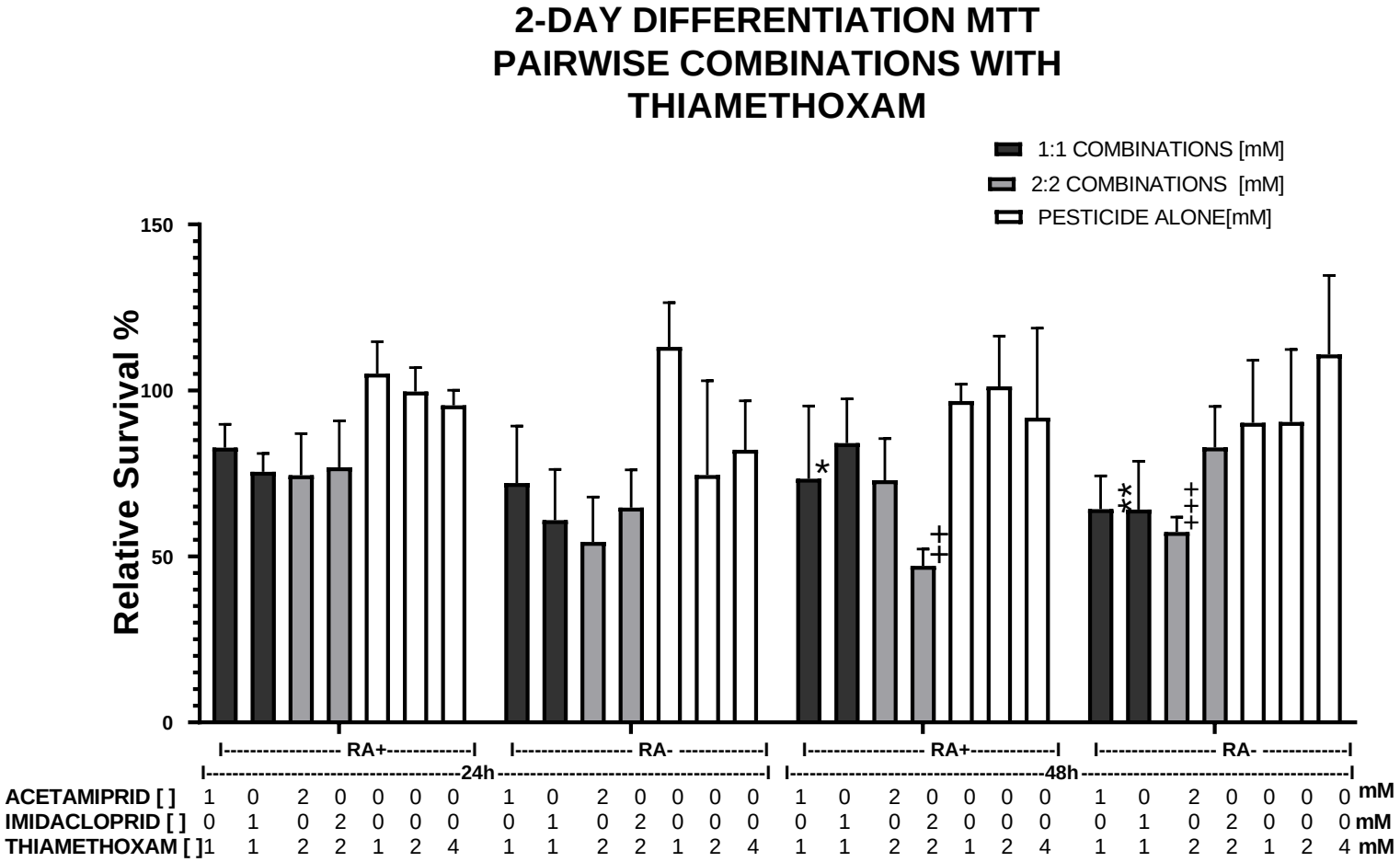

Figure 13. Synergic interactions of thiamethoxam with two other neonicotinoids in cell viability after 24-hour and 48-hour treatments according to the MTT method. The figure shows significant pairwise comparisons between one nicotinoid given alone and the concentration mixtures. *Results are displayed as mean ± SD.* **P* < 0.05; ***P* < 0.01, ****P* < 0.0001 compared to 2 mM; +*P* < 0.05; ++*P* < 0.01, +++*P* < 0.0001 compared to 4 mM. *N* = 8-20.

Viability of RA-differentiated (7 days) SH-SY5Y neurons after treatment with neonicotinoids according to the LDH method

Figures 14 and 15 represent the viability, as determined by the LDH method, of SH-SY5Y neurons differentiated with RA for as long as 7 days to reinforce their cholinergic phenotype. The multifactorial (three-way) ANOVA revealed significant interaction between concentration and differentiation effects only for acetamiprid ($F(11,2)= 5.26$; $P < 0.008$) and imidacloprid ($F(11,2)= 3.47$; $P < 0.035$). It also showed significant interaction between differentiation and treatment time for acetamiprid ($F(11,1)= 13.71$; $P < 0.0001$), imidacloprid ($F(11,1)= 70.24$; $P < 0.0001$) and thiamethoxam ($F(11,1)= 40.92$; $P < 0.0001$). It also showed significant interaction between concentration and treatment time for acetamiprid ($F(11,2)= 3.77$; $P < 0.029$) and thiamethoxam ($F(11,2)= 3.15$; $P < 0.048$). The neonicotinoids caused a rate of cellular death below 50% that reached a maximum at 4 mM ($P < 0.0001$ compared to 1 mM).

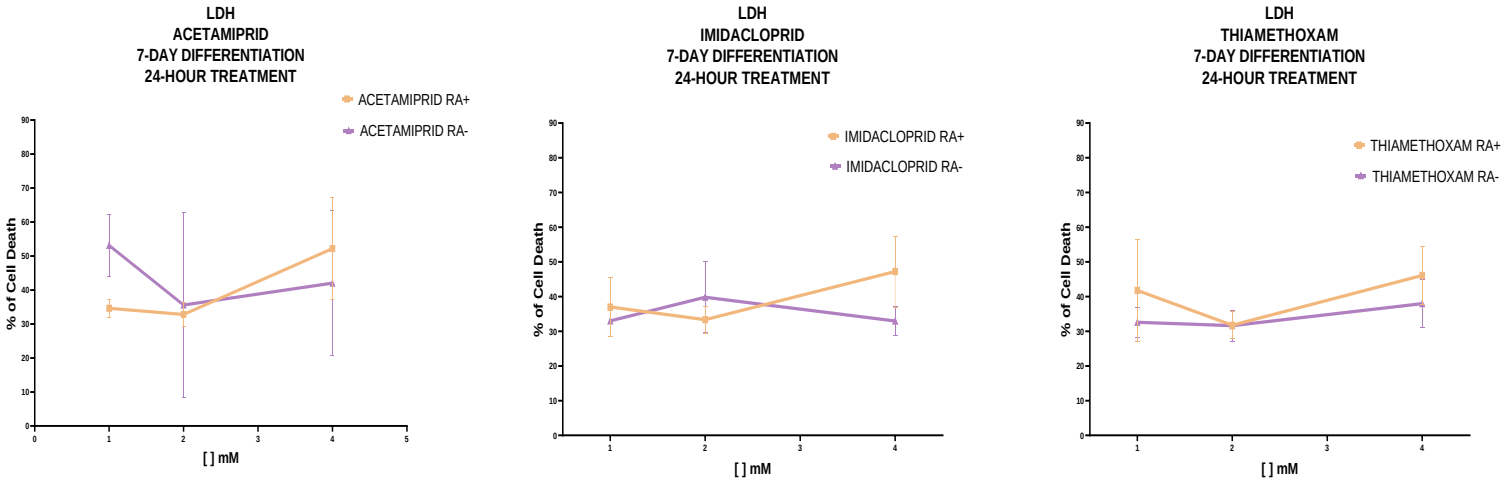

Figure 14. Cell Viability, according to the LDH method, of RA-differentiated (7 days) SH-SY5Y neurons after 24-h neonicotinoid treatment. The compounds show a moderate cytotoxicity regardless concentrations and differentiation. Results are displayed as mean ± SD. N = 8-20

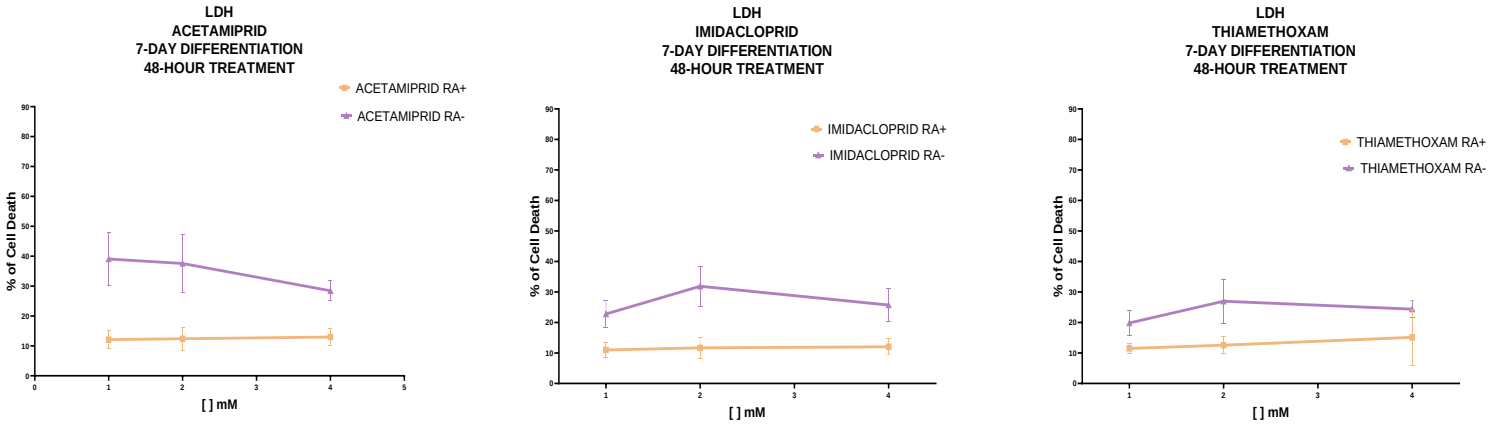

Figure 15. Cell Viability, according to the LDH method, of RA-differentiated (7 days) SH-SY5Y neurons after 48-h neonicotinoid treatment. Differentiated (RA-treated) showed less susceptibility to the cytotoxic effects of neonicotinoids. Cytotoxicity was not dose-dependent. Results are displayed as mean ± SD. N = 8-20

Viability of RA-differentiated (7 days) SH-SY5Y neurons after treatment with neonicotinoids according to the MTT method

The viability, as determined by the MTT method, of SH-SY5Y neurons differentiated with RA for 7 days and then treated with neonicotinoids for 24 h and 48 h are represented in **Figures 16 and 17**, respectively. Conversely to the LDH test, the multifactorial ANOVAs revealed a significant interaction between differentiation and treatment time for acetamiprid ($F(11,1)= 4.7$; $P < 0.03$) and thiamethoxam ($F(11,1)= 8.5$; $P < 0.005$). There was also a significant interaction between concentration and treatment time for imidacloprid ($F(11,2)= 4.54$; $P < 0.013$), and thiamethoxam ($F(11,2)= 4.9$; $P < 0.01$). These results were in agreement with the low cytotoxicity that the neonicotinoids yielded in the LDH assays under the same experimental conditions.

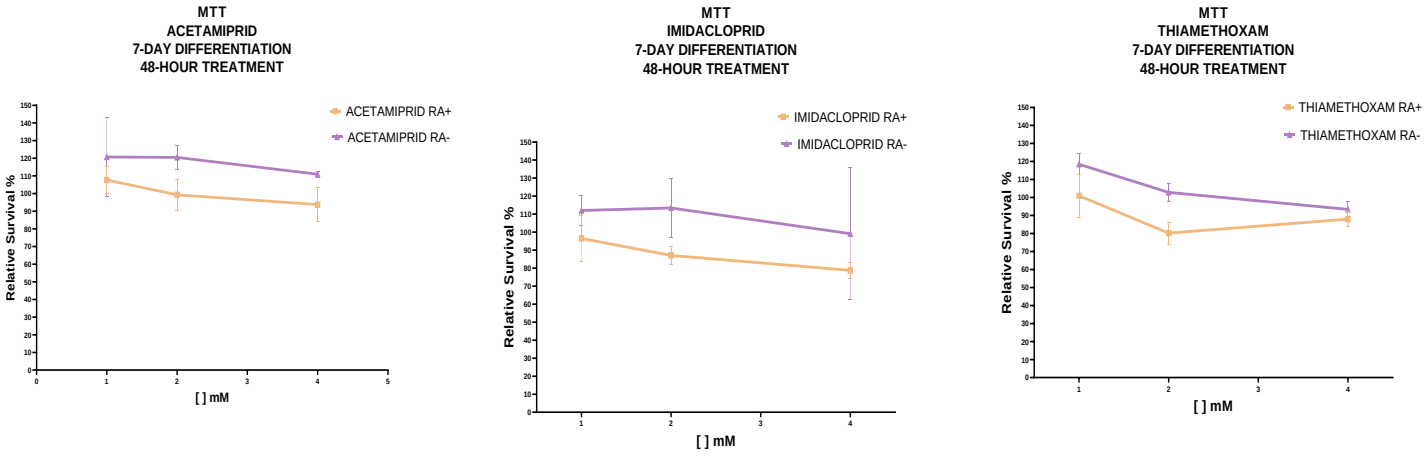

Figure 16. Cell Viability, according to the MTT method, of RA-differentiated (7 days) SH-SY5Y neurons after 24-h neonicotinoid treatment. Cytotoxicity was almost inexistent and not dose-dependent. Results are displayed as mean ± SD. *N* = 8-20

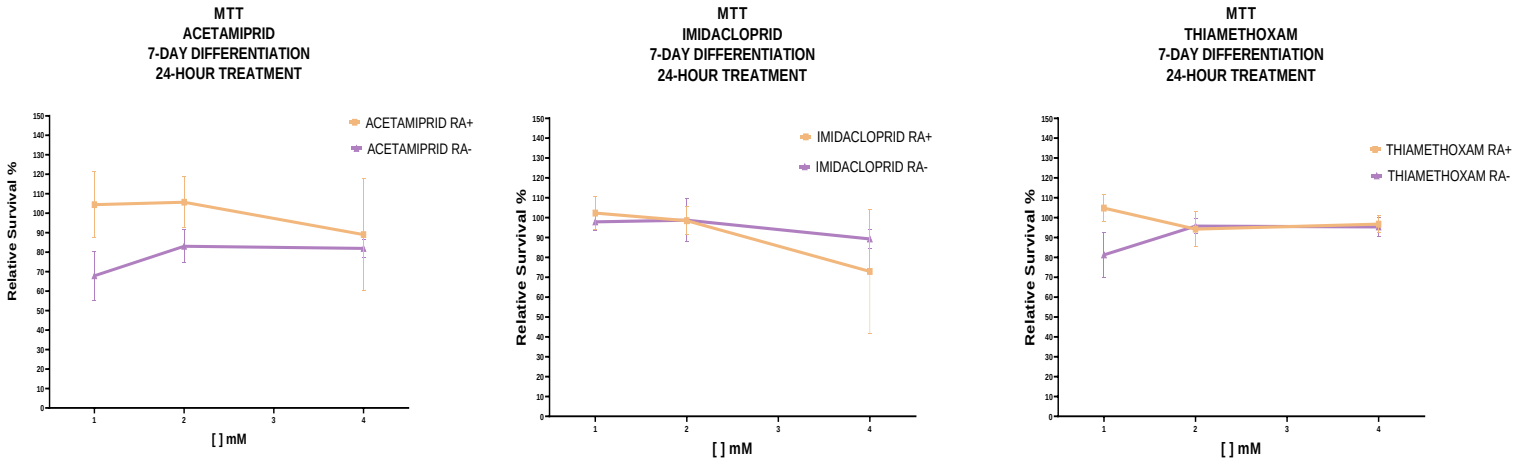

Figure 17. Cell Viability, according to the MTT method, of RA-differentiated (7 days) SH-SY5Y neurons after 24-h neonicotinoid treatment. Cytotoxicity was almost inexistent. Results are displayed as mean ± SD. *N = 8-20*

Cytotoxicity of pairwise combinations of the neonicotinoids in RA-differentiated (7 days) SH-SY5Y neurons according to the LDH method.

Pairwise combinations of neonicotinoids were administered to cells in an attempt of searching for synergistic effects, as determined by the LDH method. SH-SY5Y neurons were differentiated with RA for 7 days and then treated with acetamiprid **(Fig.18**), imidacloprid (**Fig.19**), and thiamethoxam (**Fig.20**) for 24 h and 48 h. The multifactorial ANOVAs revealed a significant interaction between differentiation, concentration and treatment time ($F(23,5)= 3.88$, $P < 0.002$). In most of the cases, the neonicotinoid combinations were more cytotoxic than the individual treatments. For instance, in RA-differentiated neurons observed after a 24-h treatment, the combinations of any pesticide with acetamiprid enhanced their cytotoxicity (acetamiprid 1 mM + imidacloprid 1 mM vs. acetamiprid 2 mM, $P < 0.0001$; acetamiprid 1 mM + imidacloprid 1 mM vs. imidacloprid 2 mM, $P < 0.0001$; acetamiprid 1 mM + thiamethoxam 1 mM vs. acetamiprid 2 mM, $P < 0.0001$; acetamiprid 1 mM + thiamethoxam 1 mM vs. thiamethoxam (2 mM), $P < 0.0001$). A similar pattern was observed in RA-differentiated neurons at 48-h treatment, although at a higher range of concentrations (acetamiprid 1 mM + imidacloprid 1 mM vs. acetamiprid 2 mM, $P < 0.0001$; acetamiprid 1 mM + imidacloprid 1 mM vs. imidacloprid 2 mM, $P < 0.0001$; acetamiprid 2 mM + imidacloprid 2 mM vs. acetamiprid 4 mM , $P < 0.0001$; acetamiprid 2 mM + imidacloprid 2 mM vs. imidacloprid 4 mM, $P < 0.0001$; acetamiprid 2 mM + thiamethoxam 2 mM vs. acetamiprid 4 mM, $P < 0.0001$; acetamiprid 2 mM + thiamethoxam 2 mM vs. thiamethoxam 4 mM, $P < 0.0001$). Like the 2-day differentiation experiments with LDH, a protective effect of some mixtures for RA-, 48h (acetamiprid 1 mM + imidacloprid 1 mM vs. acetamiprid 2 mM, $P < 0.0001$; acetamiprid 1 mM + thiamethoxam 1 mM vs. acetamiprid 2 mM $P < 0.0001$; imidacloprid 1 mM + thiamethoxam 1 mM vs. imidacloprid 2 mM, $P < 0.0001$) was observed. Thiamethoxam when combined with other neonicotinoids at 24h either RA+ or RA- enhanced its cytotoxicity [RA+ (acetamiprid 1 mM + thiamethoxam 1 mM vs. thiamethoxam 2 mM $P < 0.0001$); (imidacloprid 1 mM + thiamethoxam 1 mM vs. thiamethoxam 2 mM $P < 0.0001$); RA- (acetamiprid 1 mM + thiamethoxam 1 mM vs. thiamethoxam 2 mM $P < 0.02$); (imidacloprid 1 mM + thiamethoxam 1 mM vs. thiamethoxam 2 mM $P < 0.0001$).

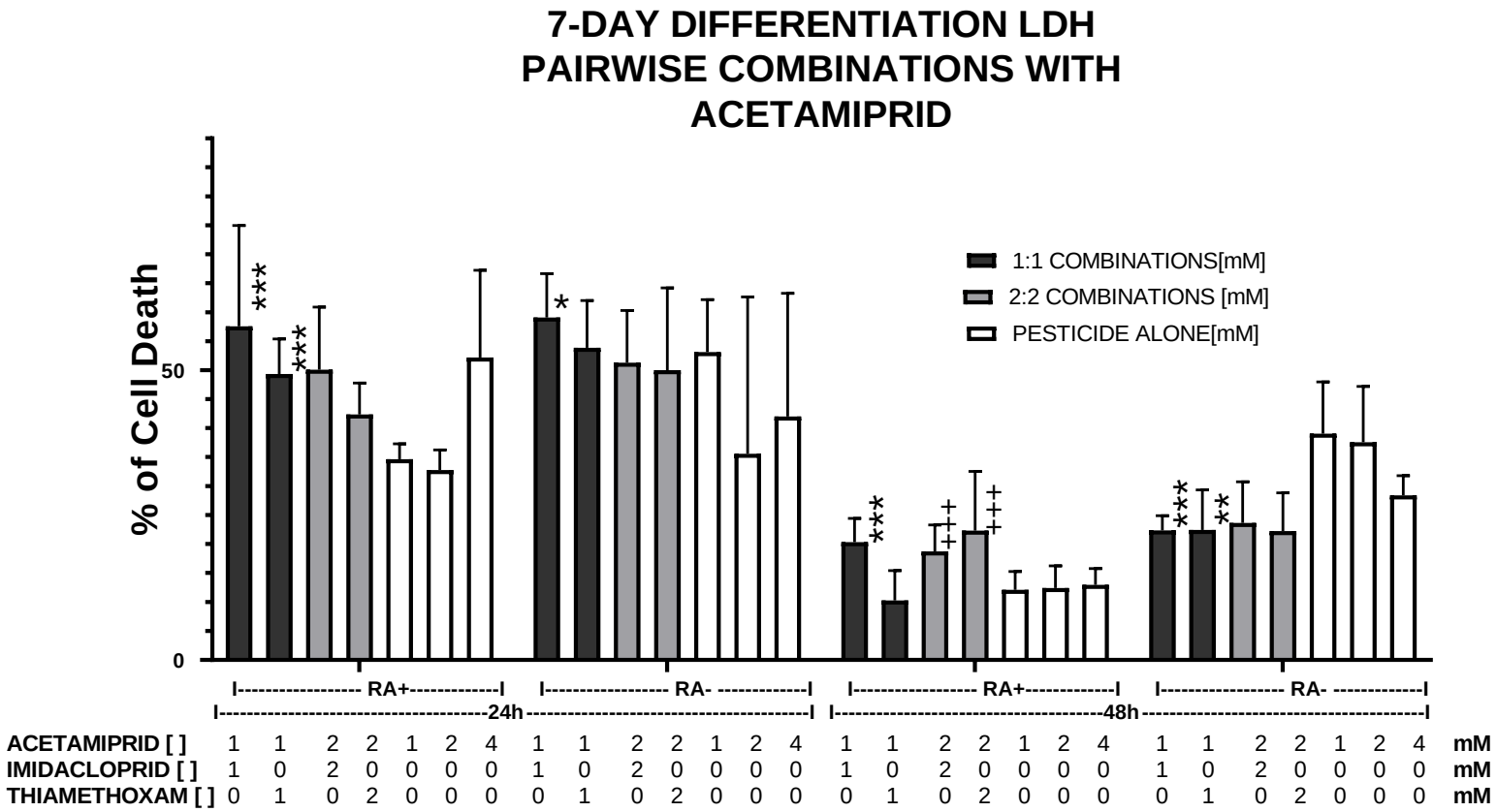

Figure 18. Synergic effects of the combinations of acetamiprid with two other neonicotinoids in cell viability after 24-hour and 48-hour treatments according to the LDH method. The figure shows significant pairwise comparisons between one nicotinoid given alone and the concentration mixtures. *Results are displayed as mean ± SD.* **P* < 0.05; ***P* < 0.01, ****P* < 0.0001 compared to 2 mM; +*P* < 0.05; ++*P* < 0.01, +++*P* < 0.0001 compared to 4 mM. *N* = 8-12.

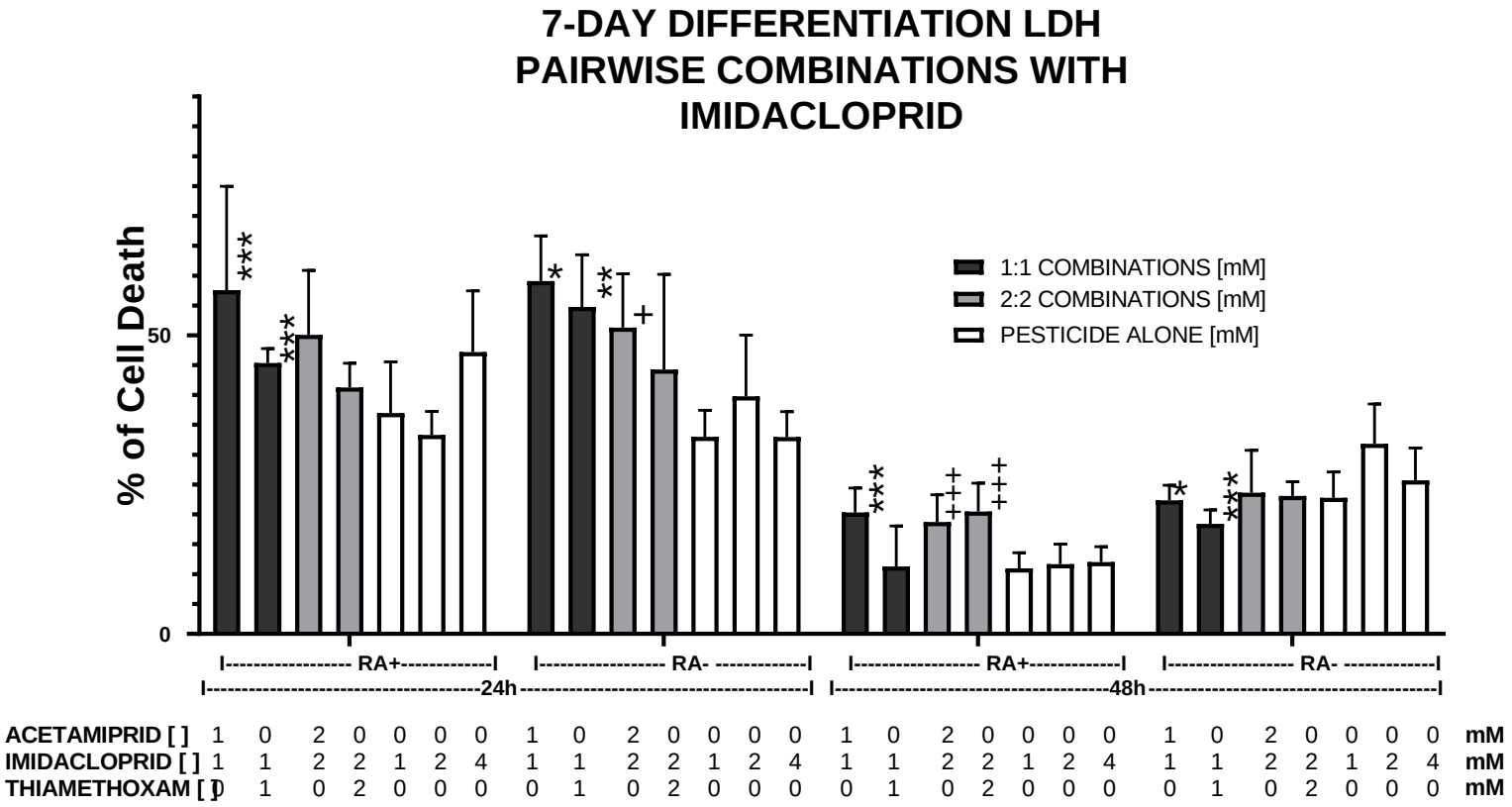

Figure 19. Synergic effects of the combinations of imidacloprid with two other neonicotinoids in cell viability after 24-hour and 48-hour treatments according to the LDH method. The figure shows significant pairwise comparisons between one nicotinoid given alone and the concentration mixtures. *Results are displayed as mean ± SD.* **P* < 0.05; ***P* < 0.01, ****P* < 0.0001 compared to 2 mM; +*P* < 0.05; ++*P* < 0.01, +++*P* < 0.0001 compared to 4 mM. *N* = 8-12.

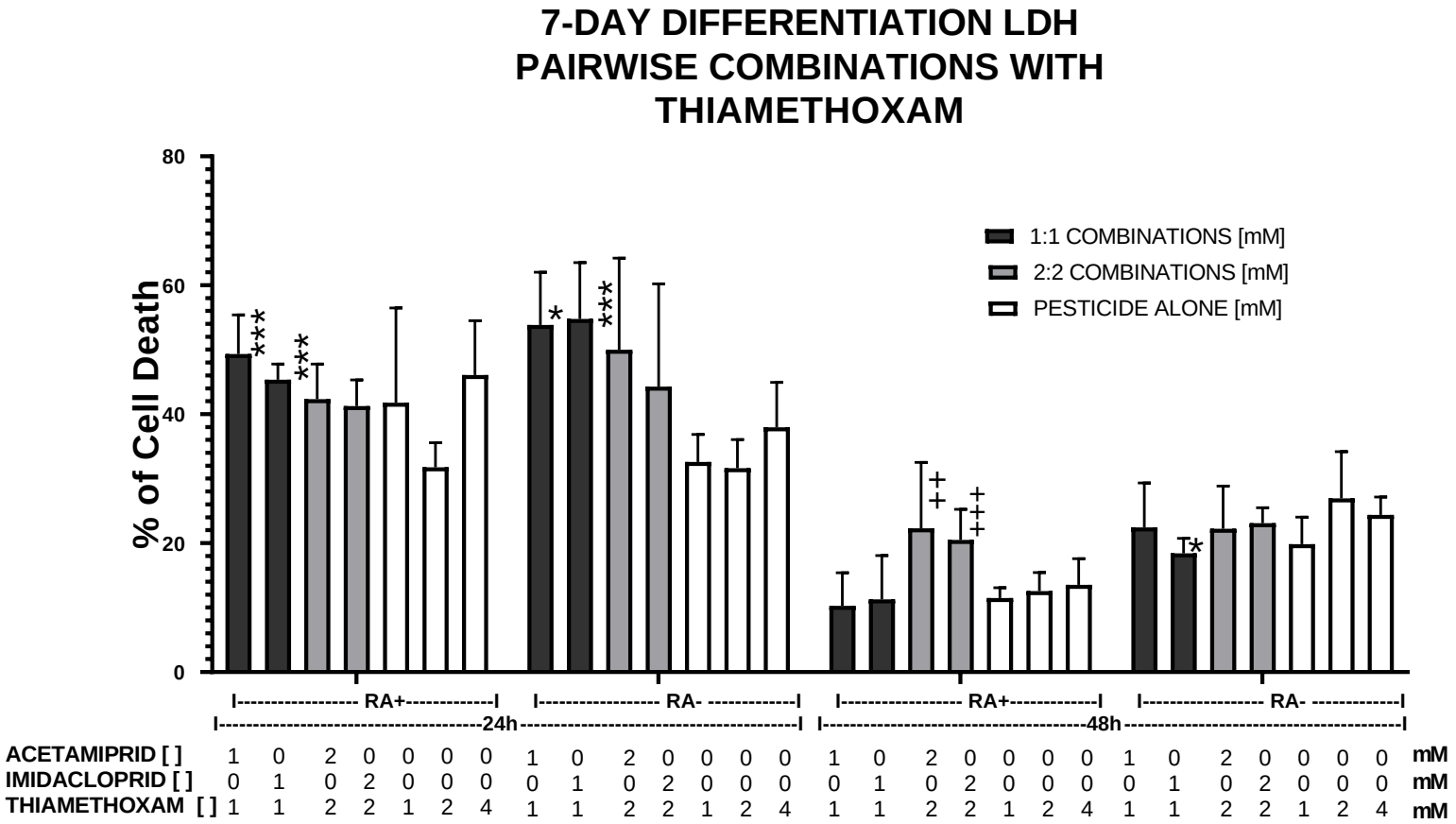

Figure 20. Synergic effects of the combinations of thiamethoxam with two other neonicotinoids in cell viability after 24-hour and 48-hour treatments according to the LDH method. The figure shows significant pairwise comparisons between one nicotinoid given alone and the concentration mixtures. Results are displayed as mean ± SD. *P < 0.05; **P < 0.01, ***P < 0.0001 compared to 2 mM; +P < 0.05; ++P < 0.01, +++P < 0.0001 compared to 4 mM. N = 8-12.

Cytotoxicity of pairwise combinations of the neonicotinoids in RA-differentiated (7 days) SH-SY5Y neurons according to the MTT method.

Pairwise combinations of neonicotinoids were administered to cells in an attempt of searching for synergistic effects, as determined by the MTT method. SH-SY5Y neurons were differentiated with RA for 7 days and then treated with acetamiprid **(Fig.21**), imidacloprid (**Fig.22**), and thiamethoxam (**Fig.23**) for 24 h and 48 h. The cytotoxic effects were not as robust as in the LDH method because of the loss of sensitivity in the MTT method (see Discussion section). The multifactorial ANOVAs revealed a significant interaction between differentiation and

concentration ($F(23,5)= 11.33$, $P < 0.00001$) and between differentiation and treatment time ($F(23,1)= 56.22$, $P < 0.00001$) and between concentration and treatment time ($F(23,1)= 6.57$, $P < 0.00001$). The differences between individual and combined neonicotinoid treatments were moderate (25% max.), remaining far above 50% viability.

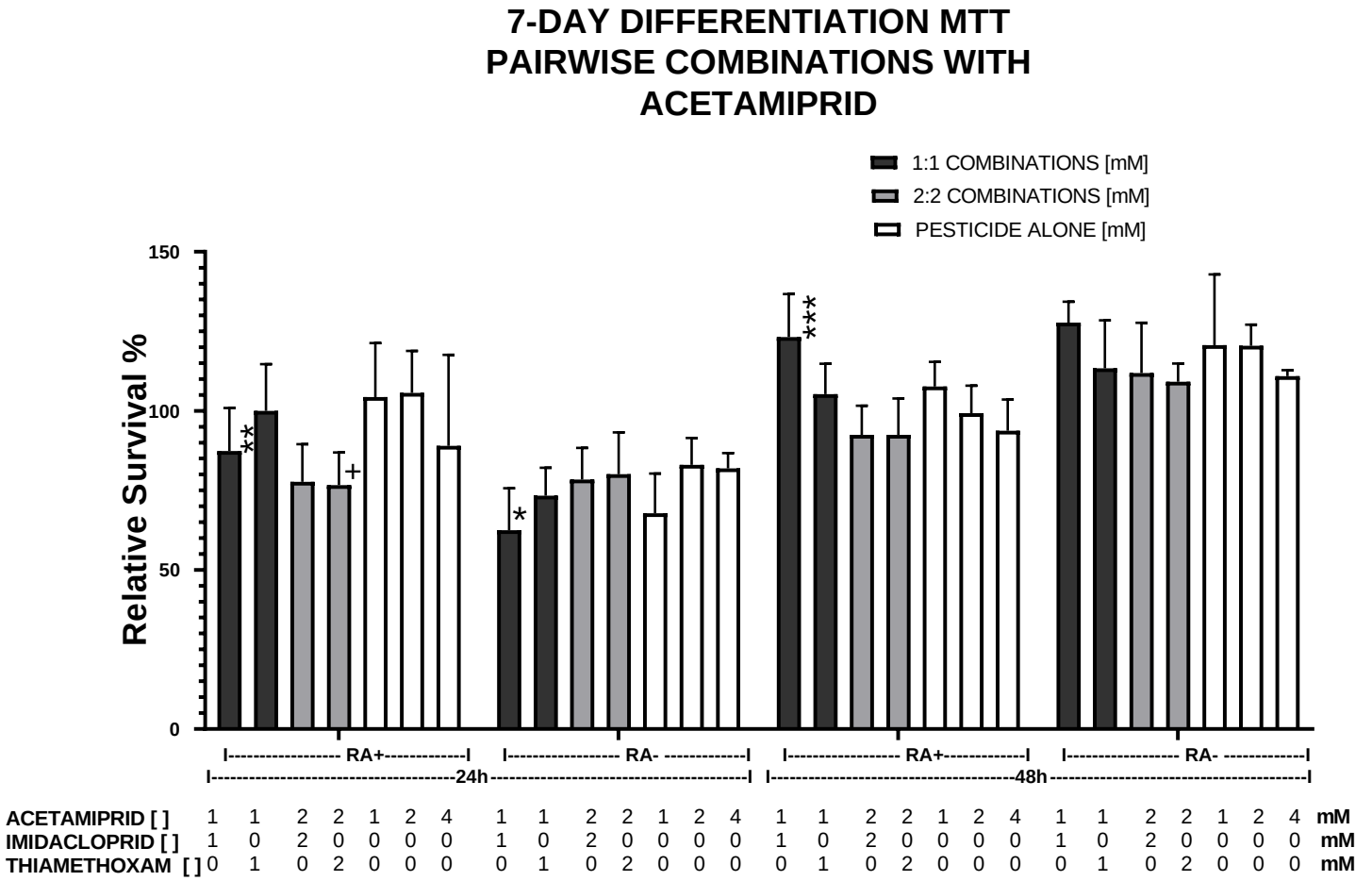

Figure 21. Synergic effects of the combinations of acetamiprid with two other neonicotinoids in cell viability after 24-hour and 48-hour treatments according to the MTT method. The figure shows significant pairwise comparisons between one nicotinoid given alone and the concentration mixtures. *Results are displayed as mean ± SD.* **P* < 0.05; ***P* < 0.01, ****P* < 0.0001 compared to 2 mM; +*P* < 0.05; ++*P* < 0.01, +++*P* < 0.0001 compared to 4 mM. *N* = 6-12.

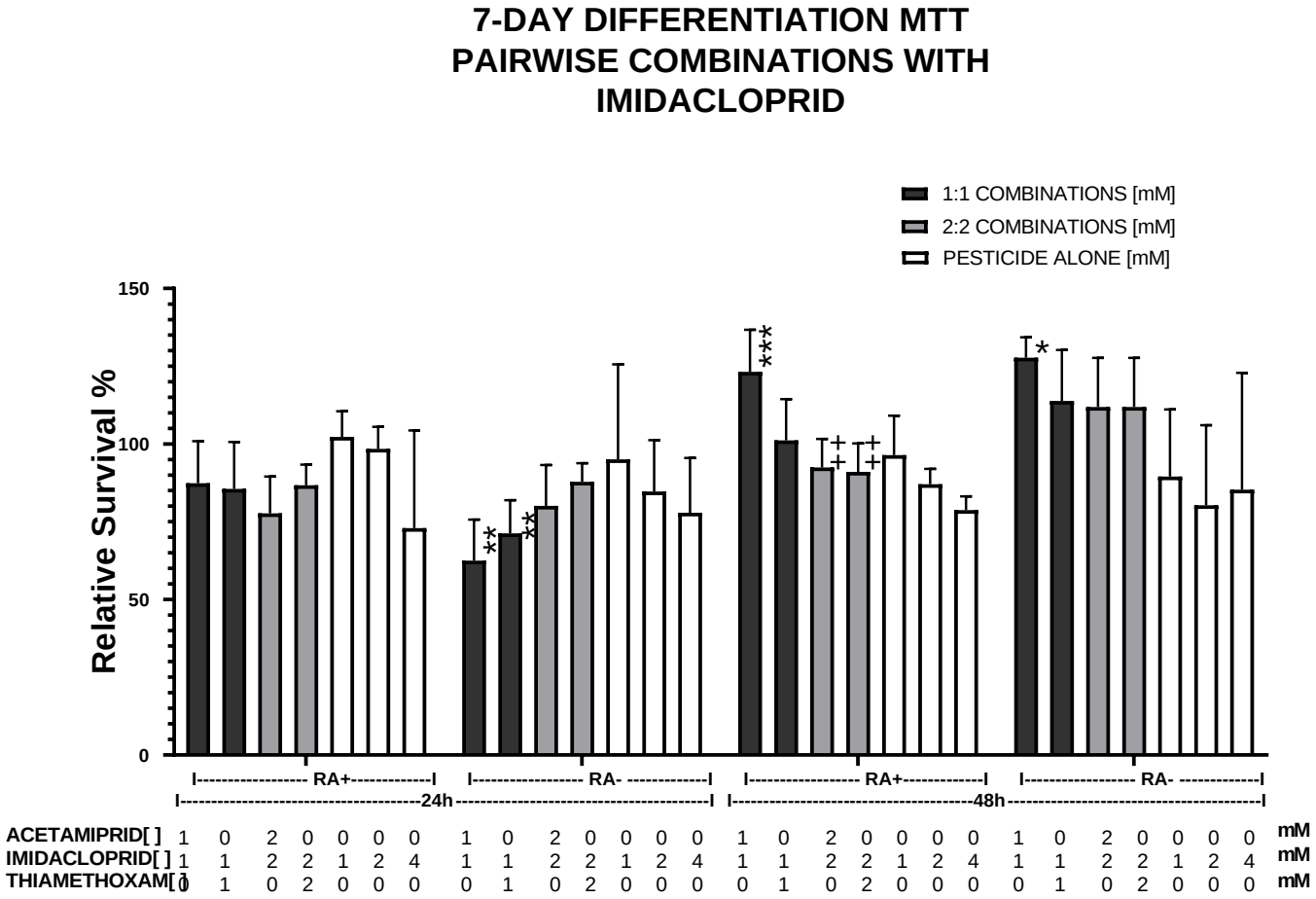

Figure 22. Synergic effects of the combinations of imidacloprid with two other neonicotinoids in cell viability after 24-hour and 48-hour treatments according to the MTT method. The figure shows significant pairwise comparisons between one nicotinoid given alone and the concentration mixtures. *Results are displayed as mean ± SD.* **P* < 0.05; ***P* < 0.01, ****P* < 0.0001 compared to 2 mM; +*P* < 0.05; ++*P* < 0.01, +++*P* < 0.0001 compared to 4 mM. *N* = 6-12.

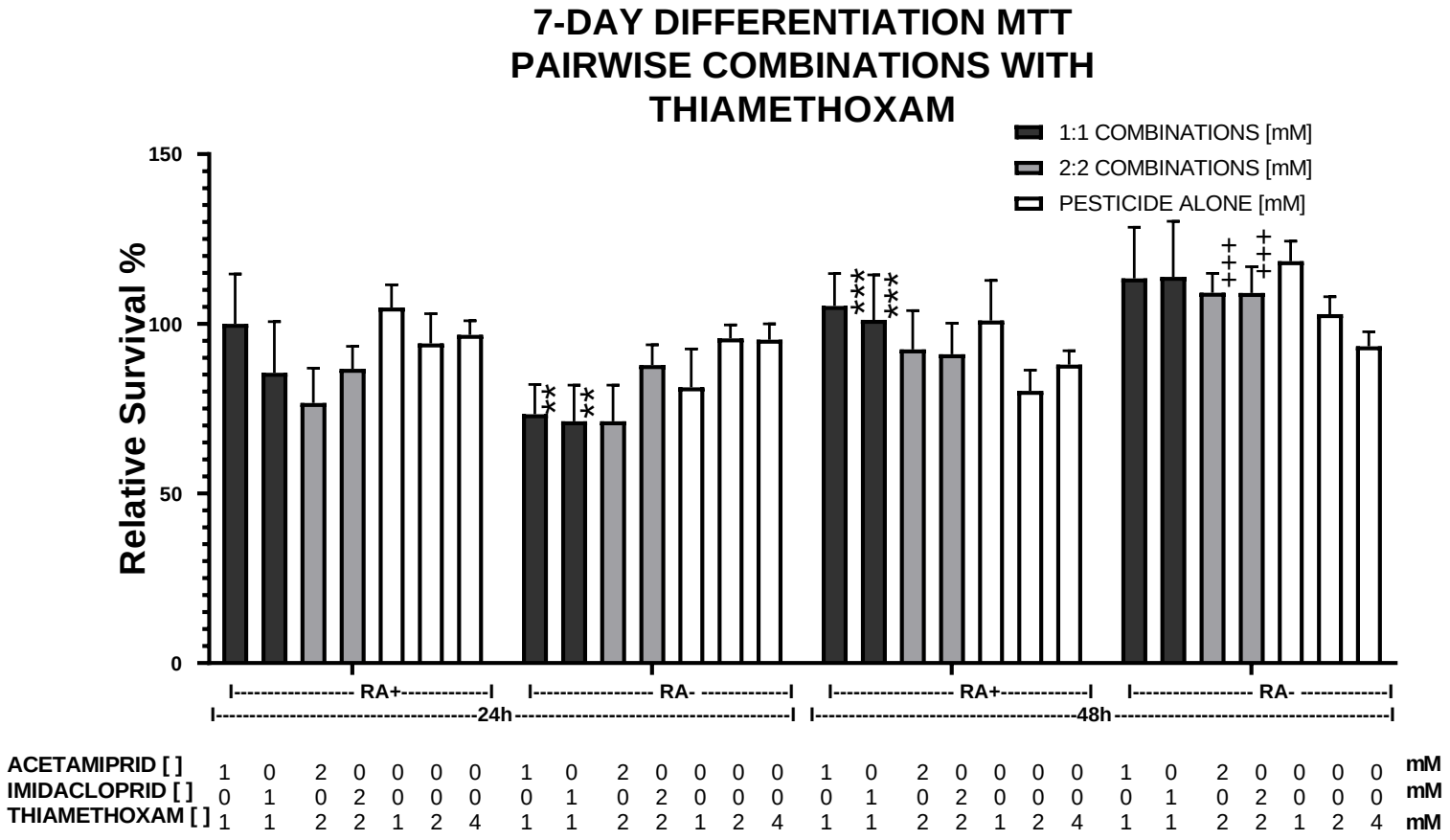

Figure 23. Effects of the combinations of thiamethoxam with two other neonicotinoids in cell viability after 24-hour and 48-hour treatments according to the MTT method. The figure shows significant pairwise comparisons between one nicotinoid given alone and the concentration mixtures. *Results are displayed as mean ± SD.* **P* < 0.05; ***P* < 0.01, ****P* < 0.0001 compared to 2 mM; +*P* < 0.05; ++*P* < 0.01, +++*P* < 0.0001 compared to 4 mM. *N* = 6-12.

DISCUSSION

The results herein presented showed that the neonicotinoid with the highest rate of cytotoxicity in almost every experimental condition was acetamiprid. The percentage of cell death was up to 70% for undifferentiated and 50% for RA-treated cells under a 2-day differentiation regime. A cell culture model was used for the evaluation of neurotoxicity of neonicotinoids under different maturation conditions, that is, RA-differentiated versus proliferating SH-SY5Y cells. Whereas other models could have been used, such as an animal model which could mimic more accurately the response of neonicotinoids in neurons, this human neuroblastoma cell line was chosen due to its *in vitro* cytotoxicity reliability, time, and low cost. SH-SY5Y neurons divide continuously (short doubling time) and provide the necessary number of cells without much variability due to its clonogenicity. Even if they do not have all the characteristics of adult neurons, they differentiate in the presence of RA (**Figure 3)** to achieve a more pronounced cholinergic, non-dividing phenotype that is closer to that of adult neurons. In fact, after a 48-h treatment with RA, neurite length increased, and slower proliferation was observed. Some studies suggest that performing the differentiation procedure for 48 and 72-hours for assessment of only neurite length and arrest on proliferation is viable. [62,63] Because non-morphological changes could not be detected without detailed molecular analyses, a longer differentiation time (up to 7 days in the current study) was required. No standardized protocol that specifies the time that is required for a complete differentiation has been published. The differentiation is a complex process regulated by many extracellular signaling molecules, cytokines, morphogens, trophic factors, and growth factors.[64] In this study, differentiation was carried out for either 2- or 7-days to evaluate differences in the toxicity of neonicotinoids since some research has shown that shorter RA-differentiation induces neuroblastic (N-type) cells whereas longer differentiations promote substrate adherent (S-type) cell proliferation. [65,66] The difference between these two types of cells is that following RA treatment N-type (immature nerve cells) cells extend their neurite processes whereas S-type cells (non-neuronal lineage of the neuronal crest) show no apparent morphological changes, lose their adherence and become apoptotic. [67,68] A medium supplemented with 10% FBS without heat inactivation (no inactivation of complement proteins present in the serum) was used. When non-heat-

inactivated FBS is used, the S-type phenotype in the SH-SY5Y culture progresses more quickly (**Figure 3)**. [69,70] It can also be seen that even after 96 hours, a total arrest of proliferation was not seen (no FBS deprivation). This is likely to account for the reduction of the cytotoxic effects of the neonicotinoids after differentiation for seven days.

RA treatment induced resistance of cells against neonicotinoids, an effect that was stronger after 7 days. This could be caused by a predominant population of S-type cells that developed a stronger attachment to the culturing surface. Accordingly, the 2-day differentiated cultures, where N-type cells were expected, displayed lower relative survival (higher cell death) than cells differentiated for 7days (S-type cells).

When looking at the combinations of neonicotinoids containing acetamiprid after a 2-day differentiation scheme, a higher cytotoxicity was observed after 24 h with RA (LDH method) and 48 h without RA (MTT method) compared to single neonicotinoid treatments. RA differentiated cells in general show higher relative survival (lower death) than undifferentiated cells, which could be due to the role of RA in the transcriptional activation of anti-apoptotic genes and survival of neural cells by resistance of apoptosis-inducing chemicals or drugs by the activation of the PI3K/Akt signaling pathway. Also, it is believed that RA contributes to cell survival by activating the transcription of genes encoding receptors for neurotrophic factors. [64] Despite the higher relative survival of differentiated cells, synergic cytotoxic effects of cocktails containing acetamiprid were also seen after 7-day RA-differentiation, both in 24-h and 48-h treatments. Interestingly, reductions in cytotoxicity were also observed in the combinations with acetamiprid regardless of RA differentiation treatment time, but only when using the LDH method, which was more sensitive than MTT.

One cytotoxicity assay used in this study was the MTT assay, which measures cell viability based on the enzymatic conversion of tetrazolium to water insoluble formazan crystals by cleavage of the tetrazolium ring by succinate dehydrogenases in the mitochondria of living cells. However, it is considered that reducing agents and enzymes located in other organelles such as the endoplasmic reticulum can also catalyze the chemical reaction which can be a source of variability in the results obtained. [71] To double check the MTT results and avoid potential variability, the LDH assays were performed, which in most of the cases validated the MTT-based cytotoxicity results. Contrary to our expectations, in some cases these cell viability methods

showed cell proliferation instead of cell death. Proliferation of cells was seen mostly in TMX (RA+) for undifferentiated cells and both 2 and 7-day differentiated cells. IMI also showed proliferation mostly on differentiated cells. These findings were in agreement with a study in which the effect of Clothianidin (CTD) was evaluated in SH-SY5Y cells and showed that CTD disrupted intracellular signaling leading to an increase in the influx of intracellular calcium, alteration of global gene expression, and phosphorylation of ERK, causing to growth and neurite stimulation. [72] That neonicotinoids may favor the progression of neuroblastoma in healthy individuals deserves further research. Finally, putative antagonistic interactions between neonicotinoids could only be detected by the LDH method because of its higher sensitivity compared to MTT.

It is important to consider that this study has been carried out exclusively on the active principle or pesticide alone. However, commercial formulations also include adjuvants like antifoaming agents, ionic and non-ionic surfactants, dyes, drift retardants among many others to promote pesticide action through the stabilization, absorption, and penetration of the active principle, while protecting it from degradation. Most of these adjuvants are known to be more toxic than the pesticide alone, a fact that is generally ignored. For example, adjuvants increase the penetration of the active ingredient not only on plants but also on the skin of those exposed. [73] Actually, a discrepancy in tests containing neonicotinoid-based formulations and the active principle (neonicotinoid alone) has been confirmed. For instance, the Apache 50 WG® formulation containing clothianidin (CTD) and CTD alone were evaluated in *Daphnia magna*, and it was found that the formulation was 46.5 times more toxic than CTD alone. [74]

CONCLUSION

This study evaluated the agrochemicals used in the banana plantations of Ecuador (acetamiprid, imidacloprid and thiamethoxam) and the synergistic effects between them. The model used for this was an *in vitro* neurotoxicology model consisting of exposure of the human neuroblastoma SH-SY5Y cell line differentiated or not with RA cholinergic and using the MTT and LDH method as a measure or cell viability and cell death, respectively. Results showed that in 2-day differentiated RA+ cells exhibited a lower rate of cell death than proliferating cells, an effect that was robust after a 7-days of culture. All three evaluated neonicotinoids show some degree of cytotoxic effects at concentrations in *mM* range, and among them, acetamiprid displayed the

highest neurotoxic activity. In addition, the highest synergic effects occur with those combinations with acetamiprid. The cytotoxic effects of neonicotinoids occurred at elevated concentrations and depended either on the maturation stage of the culture or on the method of cell death assessment. That cytotoxicity relied on the specific settlement of the culture (adherence time and RA treatment) thus, the putative toxic effects of neonicotinoids might take place during the neurodevelopment rather than by direct action on neurons. Further research is warranted to explore this possibility.

ANNEXES

Source	*Type III Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
Corrected Model	16590.760	11	1508.251	21.201	.000
Intercept	164147.644	1	164147.644	2307.320	.000
Differentiation	1950.757	1	1950.757	27.421	.000
Concentration	9092.740	2	4546.370	63.905	.000
Treatment time	4022.132	1	4022.132	56.537	.000
Differentiation * Concentration	542.218	2	271.109	3.811	.026
Differentiation * Treatment time	808.347	1	808.347	11.362	.001
Concentration * Treatment time	69.516	2	34.758	.489	.615
Differentiation * Concentration * Treatment time	105.050	2	52.525	.738	.481
Error	5975.940	84	71.142		
Total	186714.344	96			

Annex 1. Multi-factor ANOVA Between-Subjects Effects for 2-d RA differentiated cells treated for 24 and 48-hours with acetamiprid according to the LDH method. The dependent variable is % of cell death and factors are RA treatment (with and without), treatment time (24 and 48-hours) and concentration of acetamiprid (1, 2, 4 mM).

(I) Concentration	*(J) Concentration*	*Mean Difference (I-J)*	*Std. Error*	*Sig.*
2	1	5.0325*	210.864	.050
4	1	22.6961*	210.864	.000
	2	17.6637*	210.864	.000

Annex 2. Tukey HSD Analysis comparison between groups for 2-d RA differentiated cells treated for 24 and 48-hours with acetamiprid according to the LDH method. The dependent variable is % of cell death and the factor is concentration of acetamiprid (1, 2, 4 mM).

Source	*Type III Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
Corrected Model	6482.247	11	589.295	10.401	.000
Intercept	110898.801	1	110898.801	1957.335	.000
Concentration	2786.038	2	1393.019	24.586	.000
Differentiation	632.068	1	632.068	11.156	.001
Treatment time	1878.126	1	1878.126	33.148	.000
Differentiation * Concentration	19.240	2	9.620	.170	.844
Concentration* Treatment time	335.563	2	167.781	2.961	.057
Differentiation * Treatment time	516.192	1	516.192	9.111	.003
Differentiation * Concentration * Treatment time	315.021	2	157.511	2.780	.068
Error	4759.276	84	56.658		
Total	122140.325	96			

Annex 3. Multi-factor ANOVA Between-Subjects Effects for 2-d RA differentiated cells treated for 24 and 48-hours with imidacloprid according to the LDH method. The dependent variable is % of cell death and factors are RA treatment (with and without), treatment time (24 and 48-hours) and concentration of imidacloprid (1, 2, 4 mM).

(I) Concentration	*(J) Concentration*	*Mean Difference (I-J)*	*Std. Error*	*Sig.*
4	1	12.4862*	1.88179	.000
	2	9.9400*	1.88179	.000

Annex 4. Tukey HSD Analysis comparison between groups for 2-d RA differentiated cells treated for 24 and 48-hours with imidacloprid according to the LDH method. The dependent variable is % of cell death and the factor is concentration of imidacloprid (1, 2, 4 mM).

Source	*Type III Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
Corrected Model	7408.989[a]	11	673.544	19.090	.000
Intercept	104164.386	1	104164.386	2952.355	.000
Concentration	2891.424	2	1445.712	40.976	.000
Differentiation	981.422	1	981.422	27.817	.000
Treatment time	1472.143	1	1472.143	41.725	.000
Differentiation * Concentration	76.835	2	38.418	1.089	.341
Concentration * Treatment time	1029.344	2	514.672	14.587	.000
Differentiation * Treatment time	876.564	1	876.564	24.845	.000
Differentiation * Concentration * Treatment time	81.256	2	40.628	1.152	.321
Error	2963.671	84	35.282		
Total	114537.046	96			

Annex 5. Multi-factor ANOVA Between-Subjects Effects for 2-d RA differentiated cells treated for 24 and 48-hours with thiamethoxam according to the LDH method. The dependent variable is % of cell death and factors are RA treatment (with and without), treatment time (24 and 48-hours) and concentration of thiamethoxam (1, 2, 4 mM).

(I) Concentration	*(J) Concentration*	*Mean Difference (I-J)*	*Std. Error*	*Sig.*
2	1	7.0823*	148.496	.000
4	1	13.4364*	148.496	.000
	2	6.3541*	148.496	.000

Annex 6. Tukey HSD Analysis comparison between groups for 2-d RA differentiated cells treated for 24 and 48-hours with thiamethoxam according to the LDH method. The dependent variable is % of cell death and the factor is concentration of thiamethoxam (1, 2, 4 mM).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	33751.964	11	3068.360	8.513	.000
Intercept	780377.041	1	780377.041	2165.221	.000
Concentration	23525.132	2	11762.566	32.636	.000
Differentiation	3.286	1	3.286	.009	.924
Treatment time	3795.800	1	3795.800	10.532	.002
Differentiation * Concentration	4582.433	2	2291.217	6.357	.003
Concentration * Treatment time	251.822	2	125.911	.349	.706
Differentiation * Treatment time	7.558	1	7.558	.021	.885
Differentiation * Concentration * Treatment time	1585.932	2	792.966	2.200	.117
Error	30274.823	84	360.415		
Total	844403.828	96			

Annex 7. Multi-factor ANOVA Between-Subjects Effects for 2-d RA differentiated cells treated for 24 and 48-hours with acetamiprid according to the MTT method. The dependent variable is % of relative survival and factors are RA treatment (with and without), treatment time (24 and 48-hours) and concentration of acetamiprid (1, 2, 4 mM).

(I) Concentration	(J) Concentration	Mean Difference (I-J)	Std. Error	Sig.
1	2	14.7017*	4.74615	.007
	4	38.0206*	4.74615	.000
2	1			
	4	23.3190*	4.74615	.000

Annex 8. Tukey HSD Analysis comparison between groups for 2-d RA differentiated cells treated for 24 and 48-hours with acetamiprid according to the MTT method. The dependent variable is % of relative survival and the factor is and concentration of acetamiprid (1, 2, 4 mM).

Source	*Type III Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
Corrected Model	22846.952[a]	11	2076.996	7.415	.000
Intercept	797776.482	1	797776.482	2848.246	.000
Concentration	1427.171	2	713.586	2.548	.084
Differentiation	11536.073	1	11536.073	41.186	.000
Treatment time	817.181	1	817.181	2.918	.091
Differentiation * Concentration	6671.135	2	3335.568	11.909	.000
Concentration * Treatment time	386.429	2	193.215	.690	.504
Differentiation * Treatment time	.620	1	.620	.002	.963
Differentiation * Concentration * Treatment time	2008.342	2	1004.171	3.585	.032
Error	23527.891	84	280.094		
Total	844151.325	96			

Annex 9. Multi-factor ANOVA Between-Subjects Effects for 2-d RA differentiated cells treated for 24 and 48-hours with imidacloprid according to the MTT method. The dependent variable is % of relative survival and factors are RA treatment (with and without), treatment time (24 and 48-hours) and concentration of imidacloprid (1, 2, 4 mM).

Source	*Type III Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
Corrected Model	16335.706	11	1485.064	3.869	.000
Intercept	839739.445	1	839739.445	2188.011	.000
Concentration	2929.606	2	1464.803	3.817	.026
Differentiation	1094.138	1	1094.138	2.851	.095
Treatment time	2422.943	1	2422.943	6.313	.014
Differentiation * Concentration	4964.542	2	2482.271	6.468	.002
Concentration * Treatment time	1805.614	2	902.807	2.352	.101
Differentiation * Treatment time	2155.371	1	2155.371	5.616	.020
Differentiation * Concentration * Treatment time	963.491	2	481.746	1.255	.290
Error	32238.460	84	383.791		
Total	888313.610	96			

Annex 10. Multi-factor ANOVA Between-Subjects Effects for 2-d RA differentiated cells treated for 24 and 48-hours with thiamethoxam according to the MTT method. The dependent variable is % of relative survival and factors are RA treatment (with and without), treatment time (24 and 48-hours) and concentration of thiamethoxam (1, 2, 4 mM).

(I) Concentration	*(J) Concentration*	*Mean Difference (I-J)*	*Std. Error*	*Sig.*
1	2	13.5311*	4.89765	.019

Annex 11. Tukey HSD Analysis comparison between groups for 2-d RA differentiated cells treated for 24 and 48-hours with thiamethoxam according to the MTT method. The dependent variable is % of relative survival and the factor is concentration of thiamethoxam (1, 2, 4 mM).

Source	*Type III Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
Corrected Model	16864.910[a]	23	733.257	2.183	.003
Intercept	168337.663	1	168337.663	501.268	.000
Mixture Concentration	5177.227	5	1035.445	3.083	.012
Differentiation	64.124	1	64.124	.191	.663
Treatment time	2340.297	1	2340.297	6.969	.009
Differentiation * Mixture Concentration	691.125	5	138.225	.412	.840
Mixture Concentration * Treatment time	3319.114	5	663.823	1.977	.087
Differentiation * Treatment time	3786.223	1	3786.223	11.274	.001
Differentiation * Mixture Concentration * Treatment time	1486.799	5	297.360	.885	.493
Error	40298.802	120	335.823		
Total	225501.375	144			

Annex 12. Multi-factor ANOVA Between-Subjects Interactions for 2-d RA differentiated cells treated for 24 and 48-hours according to the LDH method. The dependent variable is % of cell death and factors are RA treatment (with and without), treatment time (24 and 48-hours) and mixture concentrations (1:1 and 2:2 mixtures of all neonicotinoids)

RA (+/-)	*Time (Hours)*	*(I) Concentration*	*(J) Concentration*	*Mean Difference (I-J)*	*Std. Error*	*Sig.*
+	24	1:1 AI	2I	14.92764*	406.302	.004
		1:1 AT	2A	13.94255*	490.603	.027
			2T	16.78290*	490.603	.008
		2:2 AT	4T	14.44693*	351.168	.002
-		1:1 AI	2A	18.58180*	712.849	.044
		1:1 AT	2A	20.97281*	497.440	.001
		2:2 AI	4A	39.03789*	465.473	.000
			4I	20.98414*	465.473	.001
		2:2 AT	4A	26.26127*	375.020	.000
		2:2 IT	4I	22.19918*	439.729	.000
			4T	16.65147*	439.729	.003
+	48	1:1 AI	2A	12.79373*	355.247	.005
			2I	10.25920*	355.247	.024
		1:1 AT	2A	13.93760*	371.469	.004
			2T	12.41707*	371.469	.009
		1:1 IT	2I	10.83881*	331.048	.011
			2T	11.85281*	331.048	.005
		2:2 IT	4I	24.81294*	296.122	.000
			4T	25.38163*	296.122	.000
-		1:1 AT	2A	7.02605*	221.920	.013

Annex 13. Tukey HSD Analysis comparison between groups for 2-d RA differentiated cells treated for 24 and 48-hours with Interactions according to the LDH method. The dependent

variable is % of cell death and the factor is mixture concentrations (1:1 and 2:2 mixtures of all neonicotinoids and pesticides alone).

Source	*Type III Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
Corrected Model	35307.847	23	1535.124	6.114	.000
Intercept	604916.779	1	604916.779	2409.152	.000
Mixture Concentration	12576.270	5	2515.254	10.017	.000
Differentiation	48.022	1	48.022	.191	.663
Treatment time	921.232	1	921.232	3.669	.058
Differentiation * Mixture Concentration	9264.171	5	1852.834	7.379	.000
Mixture Concentration * Treatment time	3470.549	5	694.110	2.764	.022
Differentiation * Treatment time	1850.386	1	1850.386	7.369	.008
Differentiation * Mixture Concentration * Treatment time	7018.810	5	1403.762	5.591	.000
Error	24104.755	96	251.091		
Total	687290.511	120			

Annex 14. Multi-factor ANOVA Between-Subjects Interactions for 2-d RA differentiated cells treated for 24 and 48-hours according to the MTT method. The dependent variable is % of relative survival and factors are RA treatment (with and without), treatment time (24 and 48-hours) and mixture concentrations (1:1 and 2:2 mixtures of all neonicotinoids)

RA (+/-)	*Time (Hours)*	*(I) Concentration*	*(J) Concentration*	*Mean Difference (I-J)*	*Std. Error*	*Sig.*
+	24	2:2 AI	4I	36.04728*	1.360.167	.042
-		2:2 AI	4A	29.45974*	961.438	.018
			4I	34.97602*	961.438	.005
+	48	1:1 AT	2A	30.62593*	852.779	.005
			2T	27.83213*	852.779	.011
		2:2 IT	2I	56.10646*	1.017.615	.000
			4T	44.67892*	1.017.615	.001
-		1:1 AI	2A	38.63716*	837.831	.001
			2I	34.01880*	837.831	.002
		1:1 AT	2A	34.19465*	836.109	.002
			2T	26.25770*	836.109	.014
		1:1 IT	2I	29.75050*	1.090.730	.034
		2:2 AI	4I	65.60213*	882.758	.000
		2:2 AT	4T	53.46556*	912.219	.000
		2:2 IT	4I	44.28970*	1.114.076	.002

Annex 15. Tukey HSD Analysis comparison between groups for 2-d RA differentiated cells treated for 24 and 48-hours with Interactions according to the MTT method. The dependent variable is % of relative survival and the factor is mixture concentrations (1:1 and 2:2 mixtures of all neonicotinoids and pesticides alone).

Source	*Type III Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
Corrected Model	15065.441	11	1369.586	13.043	.000
Intercept	68425.589	1	68425.589	651.639	.000
Concentration	314.788	2	157.394	1.499	.232
Differentiation	2786.039	1	2786.039	26.532	.000
Treatment time	5216.191	1	5216.191	49.675	.000
Differentiation * Concentration	1105.037	2	552.519	5.262	.008
Concentration * Treatment time	791.727	2	395.863	3.770	.029
Differentiation * Treatment time	1439.630	1	1439.630	13.710	.000
Differentiation * Concentration * Treatment time	209.526	2	104.763	.998	.375
Error	6300.325	60	105.005		
Total	88337.475	72			

Annex 16. Multi-factor ANOVA Between-Subjects Effects for 7-d RA differentiated cells treated for 24 and 48-hours with acetamiprid according to the LDH method. The dependent variable is % of cell death and factors are RA treatment (with and without), treatment time (24 and 48-hours) and concentration of acetamiprid (1, 2, 4 mM).

Source	*Type III Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
Corrected Model	11246.690[a]	11	1022.426	26.588	.000
Intercept	65735.550	1	65735.550	1709.414	.000
Concentration	304.163	2	152.081	3.955	.023
Differentiation	688.342	1	688.342	17.900	.000
Treatment time	5374.057	1	5374.057	139.749	.000
Differentiation * Concentration	267.600	2	133.800	3.479	.035
Concentration * Treatment time	86.189	2	43.095	1.121	.331
Differentiation * Treatment time	2701.214	1	2701.214	70.243	.000
Differentiation * Concentration * Treatment time	406.073	2	203.037	5.280	.007
Error	3230.222	84	38.455		
Total	88010.739	96			

Annex 17. Multi-factor ANOVA Between-Subjects Effects for 7-d RA differentiated cells treated for 24 and 48-hours with imidacloprid according to the LDH method. The dependent variable is % of cell death and factors are RA treatment (with and without), treatment time (24 and 48-hours) and concentration of imidacloprid (1, 2, 4 mM).

(I) Concentration	*(J) Concentration*	*Mean Difference (I-J)*	*Std. Error*	*Sig.*
4	1	5.8893*	1.55030	.001

Annex 18. Tukey HSD Analysis comparison between groups for 7-d RA differentiated cells treated for 24 and 48-hours with imidacloprid according to the LDH method. The dependent variable is % of cell death and the factor is concentration of imidacloprid (1, 2, 4 mM).

Source	*Type III Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
Corrected Model	11281.401	11	1025.582	20.065	.000
Intercept	67234.761	1	67234.761	1315.395	.000
Concentration	898.774	2	449.387	8.792	.000
Differentiation	374.365	1	374.365	7.324	.008
Treatment time	5783.028	1	5783.028	113.140	.000
Differentiation * Concentration	209.500	2	104.750	2.049	.135
Concentration * Treatment time	322.498	2	161.249	3.155	.048
Differentiation * Treatment time	2091.929	1	2091.929	40.927	.000
Differentiation * Concentration * Treatment time	120.339	2	60.169	1.177	.313
Error	4293.554	84	51.114		
Total	89498.121	96			

Annex 19. Multi-factor ANOVA Between-Subjects Effects for 7-d RA differentiated cells treated for 24 and 48-hours with thiamethoxam according to the LDH method. The dependent variable is % of cell death and factors are RA treatment (with and without), treatment time (24 and 48-hours) and concentration of thiamethoxam (1, 2, 4 mM).

(I) Concentration	*(J) Concentration*	*Mean Difference (I-J)*	*Std. Error*	*Sig.*
4	1	7.6172*	178.735	.000
	2	7.2920*	178.735	.000

Annex 20. Tukey HSD Analysis comparison between groups for 7-d RA differentiated cells treated for 24 and 48-hours with thiamethoxam according to the LDH method. The dependent variable is % of cell death and the factor is concentration of acetamiprid (1, 2, 4 mM).

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	15025.339[a]	11	1365.940	4.228	.000
Intercept	714925.422	1	714925.422	2213.119	.000
Concentration	2897.108	2	1448.554	4.484	.014
Differentiation	1722.419	1	1722.419	5.332	.023
Treatment time	3736.695	1	3736.695	11.567	.001
Differentiation * Concentration	1599.835	2	799.918	2.476	.090
Concentration * Treatment time	449.748	2	224.874	.696	.501
Differentiation * Treatment time	1518.767	1	1518.767	4.701	.033
Differentiation * Concentration * Treatment time	2168.083	2	1084.041	3.356	.040
Error	27135.335	84	323.040		
Total	916404.221	96			

Annex 21. Multi-factor ANOVA Between-Subjects Effects for 7-d RA differentiated cells treated for 24 and 48-hours with acetamiprid according to the MTT method. The dependent variable is % of relative survival and factors are RA treatment (with and without), treatment time (24 and 48-hours) and concentration of acetamiprid (1, 2, 4 mM).

(I) Concentration	(J) Concentration	Mean Difference (I-J)	Std. Error	Sig.
1	4	16.9488*	449.333	.001
2	4	17.2358*	449.333	.001

Annex 22. Tukey HSD Analysis comparison between groups for 7-d RA differentiated cells treated for 24 and 48-hours with acetamiprid according to the MTT method. The dependent variable is % of cell death and the factors is concentration of acetamiprid (1, 2, 4 mM).

Source	*Type III Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
Corrected Model	13960.987	11	1269.181	3.931	.000
Intercept	742405.186	1	742405.186	2299.196	.000
Concentration	1273.051	2	636.526	1.971	.146
Differentiation	248.887	1	248.887	.771	.382
Treatment time	2643.247	1	2643.247	8.186	.005
Differentiation * Concentration	1956.796	2	978.398	3.030	.054
Concentration * Treatment time	2933.146	2	1466.573	4.542	.013
Differentiation * Treatment time	1086.744	1	1086.744	3.366	.070
Differentiation * Concentration * Treatment time	192.565	2	96.283	.298	.743
Error	27123.414	84	322.898		
Total	931895.550	96			

Annex 23. Multi-factor ANOVA Between-Subjects Effects for 7-d RA differentiated cells treated for 24 and 48-hours with imidacloprid according to the MTT method. The dependent variable is % of relative survival and factors are RA treatment (with and without), treatment time (24 and 48-hours) and concentration of imidacloprid (1, 2, 4 mM).

Source	*Type III Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
Corrected Model	5379.220	11	489.020	2.535	.008
Intercept	738139.896	1	738139.896	3826.844	.000
Concentration	509.246	2	254.623	1.320	.273
Differentiation	.059	1	.059	.000	.986
Treatment time	25.284	1	25.284	.131	.718
Differentiation * Concentration	752.675	2	376.338	1.951	.149
Concentration * Treatment time	1888.851	2	944.426	4.896	.010
Differentiation * Treatment time	1639.930	1	1639.930	8.502	.005
Differentiation * Concentration * Treatment time	75.964	2	37.982	.197	.822
Error	16202.320	84	192.885		
Total	903283.103	96			

Annex 24. Multi-factor ANOVA Between-Subjects Effects for 7-d RA differentiated cells treated for 24 and 48-hours with thiamethoxam according to the MTT method. The dependent variable is % of relative survival and factors are RA treatment (with and without), treatment time (24 and 48-hours) and concentration of thiamethoxam (1, 2, 4 mM).

Source	*Type III Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
Corrected Model	63486.734	23	2760.293	39.875	.000
Intercept	373276.721	1	373276.721	5392.322	.000
Mixture Concentration	1768.491	5	353.698	5.109	.000
Differentiation	466.742	1	466.742	6.743	.010
Treatment time	52583.263	1	52583.263	759.613	.000
Differentiation * Mixture Concentration	1279.028	5	255.806	3.695	.003
Mixture Concentration * Treatment time	5181.113	5	1036.223	14.969	.000
Differentiation * Treatment time	862.423	1	862.423	12.458	.000
Differentiation * Mixture Concentration * Treatment time	1345.674	5	269.135	3.888	.002
Error	18275.068	264	69.224		
Total	455038.523	288			

Annex 25. Multi-factor ANOVA Between-Subjects Interactions for 7-d RA differentiated cells treated for 24 and 48-hours according to the LDH method. The dependent variable is % of cell death and factors are RA treatment (with and without), treatment time (24 and 48-hours) and mixture concentrations (1:1 and 2:2 mixtures of all neonicotinoids)

RA (+/-)	*Time (Hours)*	*(I) Concentration*	*(J) Concentration*	*Mean Difference (I-J)*	*Std. Error*	*Sig.*
+	24	1:1 AI	2A	24.76765*	541.850	.000
			2I	24.22176*	541.850	.000
		1:1 AT	2A	16.56003*	221.213	.000
			2T	17.59952*	221.213	.000
		1:1 IT	2I	12.02691*	150.660	.000
			2T	13.61230*	150.660	.000
-		1:1 AI	2A	23.50524*	748.613	.016
			2I	23.37986*	748.613	.016
		1:1 AT	2T	22.22428*	765.871	.026
		1:1 IT	2I	19.05434*	426.480	.001
			2T	23.14497*	426.480	.000
		2:2 AI	4I	19.46888*	667.168	.025
+	48	1:1 AI	2A	10.39144*	216.829	.000
			2I	11.12041*	216.829	.000
		2:2 AI	4A	15.30022*	149.836	.000
			4I	16.23520*	149.836	.000
		2:2 AT	4A	18.88553*	401.983	.000
			4T	16.71447*	401.983	.001
		2:2 IT	4I	20.88218*	345.056	.000
			4T	17.77614*	345.056	.000
-		1:1 AI	2A	15.20305*	307.833	.000
			2I	9.49788*	307.833	.017
		1:1 AT	2A	15.12009*	433.689	.008
		1:1 IT	2I	13.43870*	262.134	.000
			2T	8.54855*	262.134	.012

Annex 26. Tukey HSD Analysis comparison between groups for 7-d RA differentiated cells treated for 24 and 48-hours with Interactions according to the LDH method. The dependent variable is % of cell death and the factor is mixture concentrations (1:1 and 2:2 mixtures of all neonicotinoids and pesticides alone).

Source	*Type III Sum of Squares*	*df*	*Mean Square*	*F*	*Sig.*
Corrected Model	66384.240	23	2886.271	20.678	.000
Intercept	2039358.194	1	2039358.194	14610.774	.000
Mixture Concentration	3487.477	5	697.495	4.997	.000
Differentiation	41165.038	1	41165.038	294.923	.000
Treatment time	187.603	1	187.603	1.344	.248
Differentiation * Mixture Concentration	7908.618	5	1581.724	11.332	.000
Mixture Concentration * Treatment time	4588.435	5	917.687	6.575	.000
Mixture Concentration * Treatment time	7847.218	1	7847.218	56.221	.000
Differentiation * Mixture Concentration * Treatment time	893.125	5	178.625	1.280	.273
Error	31824.028	228	139.579		
Total	2454442.986	252			

Annex 27. Multi-factor ANOVA Between-Subjects Interactions for 7-d RA differentiated cells treated for 24 and 48-hours according to the MTT method. The dependent variable is % of relative survival and factors are RA treatment (with and without), treatment time (24 and 48-hours) and mixture concentrations (1:1 and 2:2 mixtures of all neonicotinoids)

RA (+/-)	*Time (Hours)*	*(I) Concentration*	*(J) Concentration*	*Mean Difference (I-J)*	*Std. Error*	*Sig.*
+	24	1:1 AI	2A	18.25068*	546.381	.007
		2:2 AI	2A	22.25468*	620.384	.023
-		1:1 AI	2A	20.50882*	738.965	.044
			2I	36.17679*	738.965	.001
		1:1 AT	2T	22.40613*	495.543	.002
		1:1 IT	2I	27.42975*	607.548	.002
			2T	24.47469*	607.548	.005
+	48	1:1 AI	2A	23.95322*	473.328	.000
			2I	36.16492*	473.328	.000
		1:1 AT	2T	25.03776*	385.447	.000
		1:1 IT	2T	22.03476*	453.345	.000
		2:2 AI	4I	13.71615*	380.395	.004
		2:2 IT	4I	12.26165*	313.090	.002
-		1:1 AI	2I	14.34680*	530.860	.038
		2:2 AT	4T	15.78696*	287.031	.000
		2:2 IT	4T	17.31780*	283.021	.000

Annex 28. Tukey HSD Analysis comparison between groups for 7-d RA differentiated cells treated for 24 and 48-hours with Interactions according to the MTT method. The dependent

variable is % of relative survival and the factor is mixture concentrations (1:1 and 2:2 mixtures of all neonicotinoids and pesticides alone).

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