Faculty of Medicine

An in vivo study of the role of Cytochrome P450 and Aldehyde Oxidase on Imidacloprid and Cypermethrin metabolism and related oxidative stress and DNA damage

ΙΝ VIVO ΜΕΛΕΤΗ ΤΟΥ ΡΟΛΟΥ ΤΟΥ ΚΥΤΟΧΡΩΜΑΤΟΣ P450 ΚΑΙ ΤΗΣ ALDEHYDE OXIDASE ΣΤΟΝ ΜΕΤΑΒΟΛΙΣΜΟ ΤΟΥ IMIDACLOPRID ΚΑΙ ΤΟΥ CYPERMETHRIN ΚΑΙ ΕΚΤΙΜΗΣΗ ΤΟΥ ΟΞΕΙΔΩΤΙΚΟΥ ΣΤΡΕΣ ΚΑΙ ΒΛΑΒΩΝ ΤΟΥ DNA

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Dedication

This thesis is dedicated to the memory of my mother

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ABBREVIATIONS

CON, Control; CY, Cypermethrin; CY LD, Cypermethrin low dose; CY HD, Cypermethrin high dose; PBO, Piperonyl Butoxide; PBO LD, Piperonyl Butoxide low dose; PBO HD, Piperonyl Butoxide high dose; IMI, imidacloprid; IMI+NH, desnitro-imidacloprid; CYP450, Cytochrome P450; ST, sodium tungstate dihydrate; AOX, aldehyde oxidase; PBMC, peripheral blood mononuclear cell; CS-syndrome, choreoathetosis or clonic seizures and salivation; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances; Hb, hemoglobin; GSH, reduced glutathione; CAT, catalase activity; CARBs/Crbnls, protein carbonyls; MDP, methylenedioxyphenyl; BD, benzodioxole; EDTA, ethylenediamine tetraacetic acid; EDC's, endocrine-disrupting compounds ; ROS, reactive oxygen species; OS, oxidative stress; TNF, tumor necrosis factor; IL, interleukins; WBC, white blood cells; IUPAC, The International Union of Pure and Applied Chemistry; EPA, (Environmental Protection Agency, USA); MDP, methylenedioxyphenyl; BD, benzodioxole; CBMN, Cytokinesis Block Micronucleus Assay; BNMN, Binucleated cells with micronucleus; CBPI, Cytokinesis Block Proliferation Index; MN, Micronuclei; WHO, World Health Organization; OECD, Organization for Economic Co-operation and Development; H&E stain, Hematoxylin and Eosin; USEPA, United States Environmental Protection Agency; CLP regulation, classification, labelling, and packaging; IGHRC, The intergovernmental group on health risks from chemicals; SCGE, single cell gel electrophoresis; TA, telomerase activity; ECHA, European Food Safety Authority; PBMC, peripheral blood mononuclear cell; LMA, low melting agarose; EtBr, ethidium bromide; TCA, trichloroacetic acid; Tris–HCL, tris(hydroxymethyl)aminomethane hydrochloride; DNPH, 2,4 dinitrophenylhydrazine; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ETH, ethirimol; LOD, limit of detection; LOQ, limit of quantitation; SD, standard deviation; ANOVA, analysis of variance; LSD, least significant difference.

THESIS OUTLINE

This thesis is based on a number of studies each one designed to address a different aspect of the topic.

Chapter 1 — A general introduction is given on the toxicological features of two pesticides, cypermethrin (CY) a synthetic pyrethroid, and imidacloprid (IMI) a neonicotinoid, and their relevant functions, adverse effects and each pesticide's primary metabolic pathway. Many pesticides are used in combination with piperonyl butoxide (PBO), which is a powerful inhibitor of the oxidative function of Cytochrome P450 (CYP450) known for its synergistic actions. Descriptions of CY, IMI, PBO and CYP450 are given followed by the overall and specific objectives of the thesis.

Chapter 2 — To date, CY has been neither extensively studied regarding its effects in combination with PBO nor has PBO been studied for its inhibition capabilities and ability to induce toxicity on its own. These comparisons are described in Chapter 2 that focuses on the systemic condition of New Zealand male rabbits after long-term exposure to CY and PBO, based on oxidative stress and telomerase activity test results.

Chapter 3 — A continuation of Chapter 2, a further study was conducted to evaluate additionally the liver and kidney inflammation and genotoxicity in New Zealand white male rabbits after long-term exposure to CY and PBO.

Chapter 4 — The metabolism of IMI is known to be metabolized in vitro both by AOX (reduction) and by cytochrome P450s enzymes (CYPs) (oxidation). In Chapter 4, we evaluate the inhibition effectiveness of sodium tungstate dihydrate (ST), an AOX inhibitor, in order to elucidate the relative contribution of CYP 450 and AOX metabolic pathways in IMI metabolism so as to clarify which metabolic pathway is actually more detrimental to New Zealand male rabbits.

Chapter 5 — Finally, the results and conclusions of the previous chapters are integrated and discussed in a broader perspective and implications for future research are given.

ABSTRACT

In this PhD thesis we examined the toxicology aspect of two pesticides, cypermethrin (CY) a synthetic pyrethroid and imidacloprid (IMI) a neonicotinoid and their relevant functions, adverse effects and each pesticide's more dominant metabolic pathway. Regarding in vivo studies, many pesticides are used in conjunction with Piperonyl Butoxide (PBO) as it is a powerful inhibitor of the oxidative function of Cytochrome P450 (CYP450) and it is also known for its synergistic actions when combined. PBO has become a diagnostic tool for two important aspects of insecticide toxicology which is to determine if an in vivo metabolism of an insecticide is oxidative and to determine if cases of insecticide resistance involve oxidative metabolism by CYP450. The CYP450 mono-oxygenases are a large and functionally diverse family of enzymes that carry out the initial oxidation of a wide variety of lipophilic compounds. These enzymes play a major role in the metabolism of xenobiotics such as drugs, pesticides, carcinogens and other environmental chemicals. It is also known that the metabolites (produced when CYP450 is participated) of CYP450 are responsible for toxicity damage arising from the use of pesticides. Furthermore, neonicotinoids are simultaneously metabolized in vivo by aldehyde oxidase (AOX) from the reduction of the nitro-imino group as well as by CYP450 oxidation reactions. Reduced AOX activity is closely correlated with reduced IMI metabolism to IMI-NNO and IMI-NH, two main metabolic products from the AOX pathway. Finally, metabolites from PBO and AOX reactions are believed to be as potent as the parent compound. This thesis is based on a number of studies each one designed to address a different aspect of the topic. In older studies, CY has not been extensively studied regarding its combination with PBO and neither has PBO been studied based on its inhibition capabilities and its ability to induce toxicity on its own. These comparisons are described in the first publication that focus on the systemic condition of New Zealand male rabbits after long term exposure to CY and PBO based on oxidative stress and telomerase activity test results. In the second publication, a continuation of the first part of the study was conducted to evaluate additionally the liver and kidney inflammation and genotoxicity in New Zealand white male rabbits after long term exposure to CY and PBO. In the last publication, we evaluated the inhibition effectiveness of sodium tungstate dihydrate (ST), an AOX inhibitor, in order to elucidate the relative contribution of CYP 450 and AOX metabolic pathways in IMI metabolism so as to clarify which metabolic pathway is actually more detrimental in New Zealand male rabbits.

ΠΕΡΙΛΗΨΗ

Στην παρούσα διδακτορική διατριβή διερευνήθηκε (μελετήθηκε/εκτιμήθηκε) η δράση από τοξικολογικής πλευράς δύο φυτοφαρμάκων, του cypermethrin (CY), ενός συνθετικού πυρεθροειδούς και του imidacloprid (IMI), ενός νεονικοτινοειδούς, των σχετικών λειτουργιών τους, των ανεπιθύμητων παρενεργειών καθώς και το επικρατέστερο μεταβολικό μονοπάτι κάθε φυτοφαρμάκου. Στο πλαίσιο της πραγματοποίησης in vivo μελετών, πολλά φυτοφάρμακα χρησιμοποιούνται σε συνδυασμό με το Piperonyl Butoxide (PBO) το οποίο αποτελεί έναν ισχυρό αναστολέα της οξειδωτικής δράσης του Κυτοχρώματος P450 (CYP450) και είναι επίσης γνωστό για τις συνεργιστική του δράση, σε αυτές τις περιπτώσεις. Το PBO αποτελεί ένα διαγνωστικό εργαλείο ως προς δύο σημαντικές τοξικολογικές δράσεις των εντομοκτόνων και συμβάλλει στον προσδιορισμό του αν ο in vivo μεταβολισμός ενός εντομοκτόνου είναι οξειδωτικός καθώς και αν η αντοχή στα εντομοκτόνα περιλαμβάνει οξειδωτικό μεταβολισμό από το CYP450. Οι μονο-οξυγενάσες του κυτοχρώματος P450 ανήκουν σε μια μεγάλη οικογένεια ενζύμων με πολλαπλή δράση που διεξάγουν την αρχική οξείδωση σε μια πληθώρα λιποφιλικών ενώσεων. Τα ένζυμα αυτά διαδραματίζουν κυρίαρχο ρόλο στο μεταβολισμό ξενοβιοτικών ουσιών όπως τα φάρμακα, φυτοφάρμακα, καρκινογόνα και άλλα περιβαλλοντικά χημικά. Είναι επίσης γνωστό πως η τοξικολογική βλάβη που προκύπτει από τη χρήση των φυτοφαρμάκων οφείλεται στους μεταβολίτες που παράγονται από τη συμμετοχή του κυτοχρώματος CYP450. Επιπρόσθετα, τα νεονικοτινοειδή in vivo μεταβολίζονται συγχρόνως από την αλδεϋδική οξειδάση (AOX), λόγω αναγωγής της νιτρο-ιμινο ομάδας, όπως και από τις CYP450 οξειδωτικές αντιδράσεις. Η μειωμένη δραστικότητα της AOX συσχετίζεται στενά με μειωμένο μεταβολισμό του IMI στα δύο κύρια μεταβολικά προϊόντα (IMI-NNO και IMI-NH) τα οποία θεωρούνται εξίσου δραστικά με την μητρική ουσία. Η παρούσα διδακτορική διατριβή βασίστηκε σε μια πληθώρα μελετών καθεμία σχεδιασμένη για να διαλευκάνει διαφορετική πλευρά του θέματος. Σε προηγούμενες μελέτες, το CY δεν έχει εκτενώς μελετηθεί σε συνδυασμό με το PBO όπως επίσης δεν έχει μελετηθεί το PBO ως προς τις ανασταλτικές δυνατότητες και την ικανότητα του να μειώνει την τοξική δράση όταν χρησιμοποιείται μόνο του. Η σύγκριση της παρούσας διδακτορικής διατριβής με τις προηγούμενες έρευνες παρουσιάζονται στη πρώτη δημοσίευση, στην οποία το ενδιαφέρον μας επικεντρώνεται στη συστημική κατάσταση αρσενικών κουνελιών Νέας Ζηλανδίας μετά την μακροπρόθεσμη έκθεση τους σε CY και PBO και η εκτίμηση αυτής βασίζεται στα εργαστηριακά αποτελέσματα του οξειδωτικού στρες και της ενεργότητας της τελομεράσης. Στη δεύτερη δημοσίευση, διεξήχθη μια συνέχιση του πρώτου μέρους της μελέτης, ώστε επιπλέον να εξετασθεί η φλεγμονή στο ήπαρ και τους νεφρούς καθώς και η γενοτοξικότητα σε αρσενικά κουνέλια Νέας Ζηλανδίας μετά την μακροπρόθεσμη έκθεση τους

σε CY και PBO. Στην τελευταία δημοσίευση, μελετήσαμε την ανασταλτική αποτελεσματικότητα του sodium tungstate dihydrate (ST), ενός ανασταλτικού παράγοντα της AOX, ώστε να διασαφηνιστεί η σχετική συνεισφορά των CYP 450 και AOX μεταβολικών μονοπατιών στο μεταβολισμό του IMI για να μπορέσουμε τελικά να διευκρινίσουμε ποια μεταβολική οδός είναι περισσότερο επιζήμια για τα αρσενικά κουνέλια Νέας Ζηλανδίας.

CHAPTER 1

General Introduction

1. Introduction

In this PhD thesis we examine toxicological features of two pesticides, cypermethrin (CY) a synthetic pyrethroid, and imidacloprid (IMI) a neonicotinoid, and their relevant functions, adverse effects and each pesticide's primary metabolic pathway. Many pesticides are used in combination with piperonyl butoxide (PBO), which is a powerful inhibitor of the oxidative function of Cytochrome P450 (CYP450) known for its synergistic actions. PBO has become a diagnostic tool for two important aspects of insecticide toxicology: the determination of whether an in vivo metabolism of an insecticide is oxidative, and if resistance to the insecticide involves oxidative metabolism by CYP450. The CYP450 mono-oxygenases are a large and functionally diverse family of enzymes that carry out the initial oxidation of a wide variety of lipophilic compounds. These enzymes play a major role in the metabolism of xenobiotics such as drugs, pesticides, carcinogens and other environmental chemicals. It is also known that CYP450 metabolism is frequently responsible for toxicity arising from the use of pesticides. Neonicotinoids are simultaneously metabolized in vivo by Aldehyde Oxidase (AOX) from the reduction of the nitro-imino group as well as by CYP450 oxidation reactions. Reduced AOX activity is closely correlated with reduced IMI metabolism to IMI-NNO and IMI-NH, two main metabolic products from the AOX pathway. Finally, metabolites from PBO and AOX reactions are believed to be as potent pesticides as the parent compound.

1.1 Cypermethrin (CY)

Cypermethrin (CY) is classified as a moderately hazardous (Class II) pesticide by the World Health Organization (WHO) and a Type II pyrethroid that contains an alpha-cyano group (Fig. 1). CY causes a long-lasting protraction of the sodium permeability of the nerve membrane during excitation, which generally causes choreoathetosis or clonic seizures and salivation syndromes (CS-syndrome) that may cause hypersensitivity to external stimuli, choreoathetosis (sinuous writhing) and salivation, and in some cases even paralysis. Although toxicity studies conducted on single parent compounds are important for obtaining basic toxicological information, combinations of pesticides have gained popularity in plant protection and public health programs in order to overcome possible pesticide resistance and increase of efficiency.

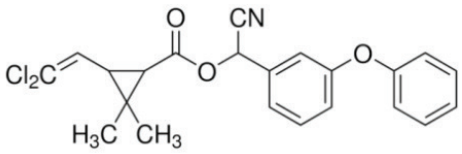

Fig. 1. The chemical structure of CY

Synthetic pyrethroids gain their efficiency from the addition of PBO, a potent inhibitor of Cytochrome P450 enzymes. Various combination-toxicity studies were earlier performed on synthetic pyrethroids, but data on the toxicological effects of PBO and CY, alone or combined, are limited. Toxicity levels of CY alone as a compound have been studied since the 1980s in insects, animals and humans to assess clinical outcomes. Cockroach brain cells exposed to very small doses (up to 0.02 micrograms per gram of brain weight or µg/g) of CY exhibited a nervous system response, which in cockroaches would result in restlessness, incoordination, prostration and paralysis (Gammon et al. 1981). Mice exposed to small doses (0.3 to 4.3 µg/g) of CY displayed symptoms that included writhing, convulsions and salivation (Lawrence and Casida 1982). Rats exposed to CY exhibited similar symptoms, including tremors, seizures, writhing and salivation, as well as burrowing behavior (Klaassen et al. 1996). People handling or working with pyrethrins and pyrethroids sometimes developed tingling, burning, dizziness and itching feelings (WHO 1989, Klaassen et al. 1996).

Synthetic pyrethroids are generally metabolized in mammals through ester hydrolysis, oxidation and conjugation processes (WHO 1989). The major urinary metabolites of CY are a variety of conjugates of cis and trans (DCVA) 3-phenoxybenzoic acid (3PBA) and 3-(4'-hydroxyphenoxy) benzoic acid (4OH3PBA). Marked differences in the urinary metabolite profile by oral and dermal routes in human volunteer studies suggest that CY could be significantly metabolized in the skin before systemic circulation occurs (Woollen et al. 1992). Rats excreted 30% of CY in their feces within 3 days (WHO 1989) compared to humans that excrete CY rapidly. Men who voluntarily ingested low doses of CY (0.25, 0.5, 1 or 1.5 milligrams per kilogram of body weight or mg/kg) in corn oil excreted between 49 and 78% of CY within 24 hours.

Such studies have indicated that CY is unlikely to bio-accumulate since the excretion rate is high. Effects of CY on human health and the environment depend on how much is present and on the length and frequency of exposure. Effects also depend on the health of a person and/or certain environmental factors.

1.2 Piperonyl Butoxide (PBO)

Piperonyl butoxide (Fig. 2) is used in a wide variety of pesticides for its potentiation (enhancement) action (Tozzi 1998). Synergists are chemicals that are believed to lack pesticidal effects on their own but potentiate the pesticidal properties of other chemicals (Olkowski et al. 1991). PBO is used in pesticides containing chemicals such as pyrethrins, synthetic pyrethroids, rotenone and carbamates (Knowles 1991). It is a colorless to pale yellow liquid, does not dissolve in water, is stable against breakdown from water and ultraviolet light, and is considered to be non-corrosive (RSC 1994). PBO's effectivness is based on inhibiting the breakdown procedures of pesticides by insects (Jones 1998). Without PBO, an insect may degrade a pesticide before an effect occurs, hence the addition of PBO to a pesticide reduces the amount of pesticide initially required to be effective.

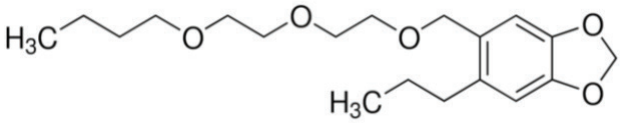

Fig. 2. The chemical structure of PBO

Initial toxicity studies of PBO have shown that when consumed by mammals, it has a low toxicological impact. More specifically, PBO toxicity was found to be low when inhaled by rats (Jones 1998, Moretto 1995, WHO 1995), very low in mammals when absorbed by the skin (Knowles 1991, Jones 1998, Moretto 1995, WHO 1995), while the eyes of rabbits exposed to PBO suffered temporary eye irritation. Dogs fed capsules containing PBO for one year died only when dosed at the highest level, with those dosed at the lowest level exhibiting some effects (Sarles and Vandegrift 1952). Human male volunteers aged 22–57 years were exposed to a single oral dose of PBO, and monitored for 31 hours but showed no changes in their metabolism (Knowles 1991, Jones 1998), as was also the case when researchers applied a commercial pesticide that contained PBO to their forearms (Wester et al. 1994).

1.3 The Cytochrome P450

The Cytochrome P450 mono-oxygenases are a large and functionally diverse family of enzymes that play a major role in the metabolism of xenobiotics (Jones 1998). Cytochrome P450-dependent mono-oxygenases are a very important enzymatic system involved in the metabolism of a phenomenal number of endogenous and exogenous compounds (Hodgson 1985). The overall reaction of P450 monooxygenase-mediated metabolism can be expressed as follows:

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+,$$

where RH is the selected substrate. P450 mono-oxygenases are capable of metabolizing numerous substrates and can carry out multiple oxidative reactions (Guengerich 1996). Insecticide resistance is the development, by some individual insects in a large population, of the ability to survive doses of a toxicant that would be lethal to the majority of individuals in a normal population of the same species (WHO 1957). P450-mediated detoxification is one of the most important mechanisms by which insects become resistant to insecticides (Hodgson 1985, Scott 1991, Oppenoorth 1985). Although P450s have long been associated with insecticide resistance, identification of specific P450 isoforms responsible for insecticide resistance has proved to be difficult (Scott 1999).

P450 enzymes are categorized into families and subfamilies by their sequence similarities. The human genomes comprise 57 P450 genes and about the same number of pseudogenes, which are grouped according to their sequence similarity into 18 families and 44 subfamilies. The P450 enzymes in families 1 to 3 are active in the metabolism of a wide variety of xenobiotics (Rendic and Di Carlo 1997, Pelkonen et al. 2005, Zanger et al. 2008). P450s are found in high concentrations in the liver, but are present in a variety of other tissues, including lung, kidney, the gastrointestinal tract, nasal mucosa, skin and brain (Lawton et al. 1990, Hjelle et al. 1986, Tremaine et al. 1985, Dutcher and Boyd 1979, Peters and Kremers 1989, Adams et al. 1991, Eriksson and Brittebo 1991, Khan et al. 1989, Dhawan et al. 1990, Bergh and Strobel 1992) and located primarily in the endoplasmic reticulum.

1.4 Imidacloprid (IMI)

Imidacloprid (IMI) is a systemic, chloro-nicotinyl (Fig. 3) insecticide for soil, seed and foliar uses to control sucking insects, including rice hoppers, aphids, thrips, whiteflies, termites, turf insects, soil insects and some beetles. It is most commonly used on rice, cereal, maize, potatoes, vegetables, sugar beets, fruit, cotton, hops and turf, and is especially systemic when used for seed or soil treatment. Imidacloprid is found in a variety of commercial insecticides. The products Admire, Confidor, Gaucho, Premier, Premise, Provado, and Marathon, all contain IMI as the active ingredient.

IMI is a General Use Pesticide and classified by the EPA (Environmental Protection Agency, USA) as both a toxicity Class II and Class III agent, and must be labeled with the signal words 'Warning' or 'Caution' (Meister 1994). There are tolerances for residues of IMI and its metabolites on food/feed additives, ranging from 0.02 ppm in eggs to 3.0 ppm in hops (EPA 1995). IMI is a neonicotinoid insecticide in the chloronicotinyl nitroguanidine chemical family (Wismer 2004, Tomlin 2006). The International Union of Pure and Applied Chemistry (IUPAC) name

is 1-(6-chloro-3-pyridylmethyl)-N-nitroimidazolidin-2-ylideneamine and the Chemical Abstracts Service (CAS) registry number is 138261-41-3 (Tomlin 2006). Neonicotinoid insecticides are synthetic derivatives of nicotine, an alkaloid compound found in the leaves of many plants in addition to tobacco (Costa 2008, Copping 2001, Ware and Whitacre 2004). The chemical operates by interfering with the transmission of stimuli in the insect's nervous system. Specifically, it causes a blockage in a type of neuronal pathway (nicotinergic) that is more abundant in insects than in warm-blooded animals (making the chemical selectively more toxic to insects). This blockage leads to the accumulation of acetylcholine, an important neurotransmitter, resulting in the insect's paralysis, and eventual death. It is effective on contact and via stomach action (Kidd and James 1991). IMI acts on several types of post-synaptic nicotinic acetylcholine receptors in the nervous system (Buckingham et al. 1997, Matsuda and Sattelle 2005). In insects, these receptors are located only within the central nervous system. Following binding to the nicotinic receptor, nerve impulses are spontaneously discharged at first, followed by failure of the neuron to propagate a signal (Schroeder and Flattum 1984, Sheets 2001). Sustained activation of the receptor results from the inability of acetylcholinesterases to break down the pesticide (Matsuda and Sattelle 2005). This binding process is irreversible (Ware and Whitacre 2004). IMI is moderately toxic if ingested (EPA 2009) with an Oral LD50 value in rats estimated at 450 mg/kg for both sexes in one study, and 500 and 380 mg/kg in males and females, respectively, in another study (Tomlin 2006). In mice, LD50 values were estimated at 130 mg/kg for males and 170 mg/kg for females (WHO 2008, Thyssen and Machemer 1999). Salivation and vomiting have been reported following oral exposure (Wismer 2004, Hovda and Hooser 2002). Very high oral exposures may lead to lethargy, vomiting, diarrhea, salivation, muscle weakness and ataxia, which are all indicative of IMI action on nicotinic receptors (Wismer 2004). Other signs of exposure at high doses are uncoordinated gait, tremors and reduced activity (Thyssen and Machemer 1999).

Reports of three cases of attempted suicide described signs of toxicity that included drowsiness, dizziness, vomiting, disorientation and fever. In two of these cases, the authors concluded that the other ingredients in the formulated product ingested by the victims were more likely to account for many of the observed signs (Wu et al. 2001, Shadnia 2007, Deepu et al. 2007). A 69-year-old woman ingested a formulated product containing 9.6% IMI in a N-methyl pyrrolide solution. The woman suffered severe cardiac toxicity and death 12 hours after the exposure. Signs of toxicity soon after the ingestion included disorientation, sweating, vomiting plus increased heart and respiratory rates (Huang et al. 2006). A 24-year-old man who accidentally inhaled a pesticide containing 17.8% IMI while working on his farm became disoriented, agitated, incoherent, sweaty and breathless following the exposure (Agarwal 2008).

Pet owners have reported contact dermatitis following the use of veterinary products containing imidacloprid on their pets (WHO 2008). No studies were found involving human subjects chronically exposed to IMI.
Mammals metabolize IMI via two major pathways, primarily though in the liver (Thyssen and Machemer 1999). In the first pathway (Fig. 3), IMI may be broken by oxidative cleavage to 6-chloronicotinic acid and imidazolidine. Imidazolidine is excreted in the urine, and 6-chloronicotinic acid undergoes further metabolism via glutathione conjugation to form mercaptonicotinic acid and a hippuric acid. IMI may also be metabolized by hydroxylation of the imidazolidine ring in the second major pathway (Thyssen and Machemer 1999, Klein and Karl 1990). Metabolic products from the second pathway include 5-hydroxy and olefin derivatives (Klein 1987).

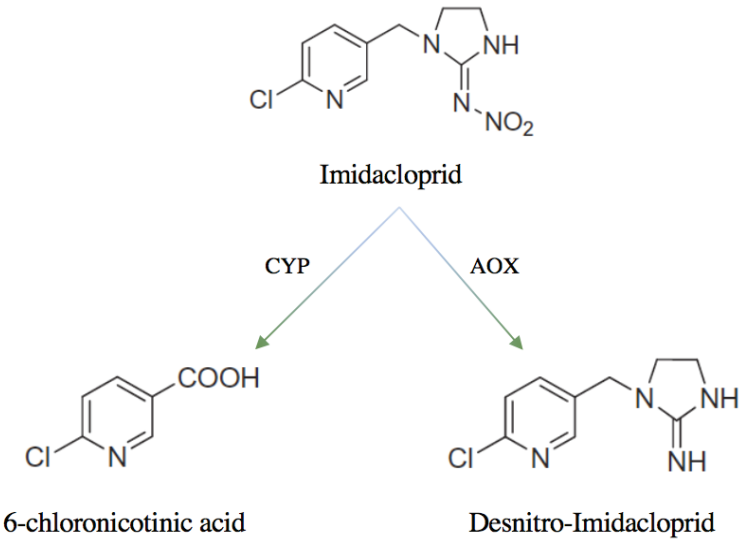

Fig. 3. The chemical structure of IMI and the two main metabolites from CYP and AOX metabolism

The metabolic products 5-hydroxy and olefin derivatives resulting from hydroxylation of the imidazolidine ring are excreted in both the feces and urine (Klein and Karl 1990, Schulz-Jander and Casida 2002). Metabolites found in urine include 6-chloronicotinic acid and its glycine conjugate and account for roughly 20% of the original radio-labeled dose (Roberts and Hutson 1999). Metabolites in the feces account for roughly 80% of the administered dose in

rats and include monohydroxylated derivatives in addition to unmetabolized IMI, which made up roughly 15% of the total. Olefin, guanidine and the glycine conjugate of methylthionicotinic acid were identified as minor metabolites (Roberts and Hutson 1999, Tomlin 2006). Rats excreted 96% of radio-labeled IMI within 48 hours following an unspecified oral dosing, with 90% excreted in the first 24 hours. Radio-labeled IMI was present in low amounts in organs and tissues 24 hours after male rats were orally dosed with 20 mg/kg (Klein 1987). No information was found on the specific metabolism of IMI in humans.

1.5 Overall and specific objectives of the thesis

CY was studied regarding its effects in combination with PBO, while PBO was studied for its own inhibition capabilities and ability to induce toxicity by itself. These comparisons are described based on oxidative stress, telomerase activity, organ inflammation and genotoxicity in New Zealand white male rabbits after a long-term exposure.

Furthermore, the evaluation of the inhibition effectiveness of sodium tungstate dihydrate (ST), an AOX inhibitor, to elucidate the relative contribution of the CYP 450 and AOX metabolic pathways in IMI metabolism, was studied to clarify which metabolic pathway is actually more detrimental to New Zealand male rabbits.

The initiation of this PhD study was approved by the University of Crete, School of Medicine, on 12 June 2013 (Ref. No. 296/5-6-2013). Animal experiments were approved by the Veterinary Administration Office on 26 September 2013 (Ref. No. 16089/26-09-13, 94763/2-5-2018). The facilities (Animal House Facilities of the School of Medicine, University of Crete) where the experiments were conducted, were approved by the Veterinary Administration Office of Crete (Ref. No. 2288/21-4-2010).

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CHAPTER 2

Downgrading the Systemic Condition of Rabbits after Long-Term Exposure to Cypermethrin and Piperonyl Butoxide

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Abstract

Aim: The aim of this study was to clarify the effect of cypermethrin (CY) on the oxidative stress (OS) and inflammation status of animals exposed to it and the synergistic role of piperonyl butoxide (PBO).

Main methods: Markers of oxidative stress, such as total antioxidant activity (TAC), protein carbonyls, hemoglobin (Hb), reduced glutathione (GSH), thiobarbituric-acid reactive substances (TBARS), along with the telomerase activity in PBMCs (peripheral blood mononuclear cells) were analyzed.

Key findings: Oxidative stress markers showed statistically significant differences between groups in TAC ($p=0.001$), GSH ($p=0.018$) and CAT activity ($p=0.029$), which depended on dose and combined effect of both compounds. Telomerase activity also showed a statistically significant difference between all groups ($F=43.48$, $df=6,14$, $p<0.001$) with cypermethrin, piperonyl butoxide and the co-exposed groups being significantly different from the control group ($p<0.05$).

Significance: The observed results for TBARS, GSH, Hb, TAC, Crbnls and CAT from our exposed groups showed altered levels compared to control groups that could be linked to doses and combined effects of each chemical substance (cypermethrin and piperonyl butoxide). Oxidative stress markers suggest that cypermethrin, piperonyl butoxide and the co-exposed groups, induce oxidative stress as well as induction of telomerase activity.

1. Introduction

The insecticide Cypermethrin (CY) is characterized by the World Health Organization (WHO) as a moderately hazardous Class II type pyrethroid and classified in Annex VI of the CLP (regulation, classification, labeling, packaging) Regulation 1272/2008/EC. The toxic effects vary, depending especially on the way the pesticide is prepared and applied [1,2]. Therefore,

there has been an interest in assessing the toxicity of pyrethroids induced in mammals, especially concerning its endocrine disrupting effects [3]. In fact, synthetic pyrethroids have hormonal activities and are classified as endocrine-disrupting compounds (EDCs) possibly posing a threat to humans and wildlife [4,5]. Metabolism of synthetic pyrethroids in mammals occurs through ester hydrolysis, oxidation and conjugation processes [6]. Of note, in vivo and in vitro studies have shown that some pyrethroids metabolites maintain endocrine disrupting properties [7] and could even affect the quality of semen [8]. Pesticides induce oxidative stress (OS) via the overproduction of free radicals or by altering the antioxidant defense mechanisms [9] causing damage to genetic material. Telomerase activity could be regarded as an indicator of already induced OS and such alterations in this activity could have clinical implications [10]. Telomerase is a target for regulatory mechanisms, while highly susceptible to OS [11]. Telomeres are regulated by groups of telomeric proteins [12]. Without these proteins, telomeres are not hidden from the DNA damage surveillance and chromosome ends are inappropriately processed by DNA repair pathways [13]. Telomeres shorten with cell division, which result in aging and cell death [14,15] and are primarily due to an end replication problem [16] and damage from chemicals and toxicants [17]. Enhancement of pyrethrins is accomplished by adding synergists to increase the toxicity of the initial pesticide. Synergists do not have pesticidal effects but enhance the pesticidal properties of other chemicals [18]. The most popular is piperonyl butoxide (PBO) [19]. PBO belongs to methylenedioxyphenyl (MDP) compounds or benzodioxole (BD) compounds. In mammals these compounds have been studied for their abilities to inhibit P450 metabolism and to induce P450 enzymes. A PBO metabolite binds to the Cytochrome P450 enzymes preventing pesticide detoxification [20]. PBO does not have a harmonized classification according to Annex VI of the CLP regulation, but has been recognized as an active biocide. Adding PBO to a pesticide reduces the amount of pesticide initially needed to reach the same toxic effect and this is achieved by inhibiting metabolic enzyme systems, P450s and esterases, inside insects [21,22,23]. Thus, PBO is used in mixture with pesticides such as pyrethroids, rotenone and carbamates [24]. Considering the above rationale, this study aims to clarify the effect of CY on the oxidative stress and inflammation status of New Zealand white male rabbits and the putative synergistic role of PBO. For this purpose, markers of OS, such as total antioxidant activity (TAC), protein carbonyls, hemoglobin (Hb), reduced glutathione (GSH), thiobarbituric-acid reactive substances (TBARS), along with the telomerase activity in PBMCs, which is a marker of systemic inflammation and activation of lymphocytes, were monitored in animals exposed to CY and PBO under isolated and combination paradigms.

2. Materials and Methods

2.1 Animals and administration protocol

Twenty-one New Zealand white male rabbits (weighing between 3200 and 3900 g each) were used in this study. The animals were housed in individual metal cages at the laboratory animal house facilities of the School of Medicine, University of Crete, Heraklion. Conditions of the room where the animals were kept consisted of a 12-hour dark/light cycle and a steady temperature set to be maintained between 20–23°C. Commercial rabbit pellets and drinking water were provided ad libitum. Before initiating the experiment, animals were acclimatized under these conditions for approximately 1 month. CY was a gift from Agriphar (Belgium) and PBO was purchased from Sigma Aldrich. Animals were divided into 7 groups, consisting of 3 animals per group in the following order: control, cypermethrin low dose (CY LD), cypermethrin high dose (CY HD), piperonyl butoxide low dose (PBO LD) and piperonyl butoxide high dose (PBO HD). Whilst the control group only received tap water and corn oil, CY LD was treated with an exposure of 25 mg/kg-bw (bw – body weight) per day, CY HD was treated with an exposure of pesticide 50 mg/kg-bw per day, PBO LD was treated with an exposure of 22.5 mg/kg-bw per day, PBO HD was treated with an exposure of 45 mg/kg-bw per day, and finally the combinations of each category together regarding low and high doses of each exposed group. The administered doses of CY corresponded to 1/120 and 1/60 of the respective LD_{50} (3000 mg/kg), respectively. For PBO the same rationale was followed. (LD50 = 2650 mg/kg). All doses were administered orally diluted in 0.5 mL corn oil three times per week. The animals were treated for 4 months and then sacrificed by veterinarians at the age of 6 months (weight 3.7–4.9 kg) by administering first a sedative injection of Xylapan/Narketan (2/1) then an injection of Dolethal (5 mL/5 kg-bw), which is a euthanasia agent. The present study was approved by the Veterinary Administration Office of Heraklion (Crete, Greece), the Animal Investigation Committee of the University of Crete (Heraklion, Crete, Greece) and conformed to the National and European Union directions for the care and treatment of laboratory animals. The required relative limitation number of animals per group may constitute a study limitation. All efforts were made to minimize any possible suffering. During the study period all rabbits were regularly observed and their condition was closely monitored. No clinical signs were observed throughout the experiment concerning food and water consumption, skin and eye conditions, excretion of urine and feces and abnormal body weight changes. After euthanasia, blood samples were collected (3 mL for telomerase and 3–5 mL for the oxidative stress assays) and stored at ideal temperatures (2–8°C for telomerase assays and −20°C for the oxidative stress assays) so as not to deteriorate before the various tests and assays of the experiment were to be conducted.

2.2 Blood sampling

Blood was collected in ethylenediamine tetraacetic acid (EDTA) tubes for measuring: total antioxidant activity (TAC), thiobarbituricacid reactive substances (TBARS), protein carbonyls (CARB), reduced glutathione (GSH) levels and catalase activity. Blood samples were centrifuged immediately at 1370g for 10 min at 4°C and the plasma was collected and used for the above measurements. The packed erythrocytes were lysed with distilled water (1:1 v/v), inverted vigorously, centrifuged at 4020g for 15 min at 4°C and the erythrocyte lysate was collected for measurement of catalase activity. A portion of erythrocyte lysate (500 µL) was treated with 5% trichloroacetic acid (TCA) (1:1 v/v), vortexed vigorously, and centrifuged at 28000g for 5 min at 4°C. The supernatants were removed, treated again with 5% TCA (1.3:1 v/v) and centrifuged again at 28000g for 5 min at 4°C. The clear supernatants were transferred to Eppendorf tubes and were used for the determination of GSH. Plasma and erythrocyte lysate were stored at −80°C prior to biochemical analyses.

2.2.1 Assessment of oxidative stress markers

For TBARS determination, a slightly modified assay of Keles et al. [25] was used. According to this method, 100 µL of plasma were mixed with 500 µL of 35% TCA and 500 µL of trishydroxymethylaminomethane hydrochloride (Tris–HCl) (200 mM, pH7.4) and incubated for 10 min at room temperature. One mL of 2 M Na2SO4 and 55 mM thiobarbituric acid solution was added and the samples were incubated at 95°C for 45 min. Samples were cooled on ice for 5 min and were vortexed after adding 1 mL of 70% TCA. Samples were then centrifuged at 15000g for 3 min and the absorbance of the supernatant was read at 530 nm. A baseline absorbance was taken into account by running a blank along with all samples during the measurement. Calculation of TBARS concentration was based on the molar extinction coefficient of MDA. Protein carbonyls were determined based on the method of Patsoukis et al. [26]. In this assay, 50 µL of 20% TCA were added to 50 µL of plasma and this mixture was incubated in an ice bath for 15 min and centrifuged at 15000g for 5 min at 4 °C. The supernatant was discarded and 500 µL of 10 mM 2,4 dinitrophenylhydrazine (DNPH) [in 2.5 N hydrochloride (HCl)] for the sample, or 500 µL of 2.5 N HCl for the blank, were added to the pellet. The samples were incubated in the dark at room temperature, for 1 h, with intermittent vortexing every 15 min and were centrifuged at 15000g for 5 min at 4°C. The supernatant was discarded and 1 mL of 10% TCA was added, vortexed and centrifuged at 15000g for 5 min at 4°C. The supernatant was discarded and 1 mL of ethanol-ethyl acetate (1:1 v/v) was added, vortexed and centrifuged at 15000g for 5 min at 4°C. This washing step was repeated twice. The supernatant was discarded and 1 mL of 5 M urea (pH2.3) was added,

vortexed and incubated at 37°C for 15 min. The samples were centrifuged at 15000g for 3 min at 4°C and the absorbance was read at 375 nm. Calculation of protein carbonyl concentration was based on the molar extinction coefficient of DNPH. Total plasma protein was assayed using a Bradford reagent from Sigma–Aldrich. GSH was measured according to Reddy [27]. Twenty μL of erythrocyte lysate treated with 5% TCA were mixed with 660 μL of 67 mM sodium potassium phosphate (pH=8) and 330 μL of 1 mM 5,5′ -dithiobis-2 nitrobenzoate (DTNB). The samples were incubated in the dark at room temperature for 10 min and the absorbance was read at 412 nm. GSH concentration was calculated relative to a calibration curve made using commercial standards. Catalase activity was determined using the method of Aebi [28]. Briefly, 4 μL of erythrocyte lysate (diluted 1:10) were added to 2991 μL of 67 mM sodium potassium phosphate (pH7.4) and the samples were incubated at 37°C for 10 min. Five μL of 30% hydrogen peroxide (H_2O_2) were added to the samples and the change in absorbance was immediately read at 240 nm for 130 s. Calculation of catalase activity was based on the molar extinction coefficient of H_2O_2. The determination of TAC was based on the method of Janaszewska and Bartosz [29]. In particular, 20 μL of plasma were added to 480 μL of 10 mM sodium potassium phosphate (pH7.4) and 500 μL of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical and the samples were incubated in the dark for 60 min at room temperature. The samples were centrifuged for 3 min at 20000g and the absorbance was read at 520 nm. TAC is presented as mmol of DPPH reduced to 2,2-diphenyl-1-picrylhydrazine (DPPH:H) by the antioxidants of plasma.

2.3 Telomerase activity

Telomerase activity in PBMCs was measured using a commercial telomerase PCR–ELISA (Roche Diagnostics Corp., Indianapolis, IN, USA), based on the telomeric repeat amplification protocol [30]. The method for the isolation of PBMCs is described elsewhere [31].

2.4 Statistical methods

The activity and the studied parameters of oxidative stress were expressed in the form of mean ± standard deviation (SD). The differences in mean telomerase activities and oxidative stress parameters were examined using independent samples t-test for two group comparisons. For more than two groups, one-way ANOVA (analysis of variance) was assessed, followed by pair-wise comparisons using a LSD (least significant difference) post-hoc tests. The IBM SPSS Statistics package was used for data analysis and for the graphic representation of data. Level of acceptance of null hypothesis was set at the 0.05 level.

3. Results

3.1. Telomerase activity

In Table 1 levels of telomerase activity in all the study groups are presented. Statistically significant differences between all groups (F=43.48, df=6,14, p<0.001) were noticed. Telomerase activity in PBMCs in all CY, PBO and co-exposed groups of CY with PBO increased significantly compared to the control group (p<0.05) in a dose dependent manner. Telomerase activity of CY groups compared to the control groups exhibits a significant increase (388% for CY LD and 539% for CY HD) both in low and high dose groups. PBO exposure caused a significantly higher increase in telomerase activity, for both low and high dose groups, compared to the control group. Compared to the CY groups, the increase was more than 300%. The presence of PBO further increased, but to a lesser extent, telomerase activity in the co-exposed groups, compared to the CY groups (134% for CY + PBO LD and 437% for CY + PBO HD) (Fig. 1). However, no addiction or synergistic effect was observed.

Table1. Telomerase activity (mIU/mL) shown in mean ± standard deviation, and statistical differences (p-values) from the comparison between study groups

		p-values					
Group	**Mean ± SD**	**CY** (LD)	**CY** (HD)	**PBO** (LD)	**PBO** (HD)	**CY+PBO** (LD)	**CY+PBO** (HD)
Control	0.34 ± 0.03	0.023	<0.001	<0.001	<0.001	<0.001	<0.001
CY (LD)	1.12 ± 0.23	-	0.004	<0.001	<0.001	0.005	<0.001
CY(HD)	2.18 ± 0.05		-	0.071	<0.001	0.854	<0.001
PBO (LD)	2.78 ± 0.04			-	<0.001	0.050	0.013
PBO (HD)	4.50 ± 0.65				-	<0.001	0.015
CY+ PBO (LD)	2.13 ± 0.67					-	<0.001
CY+ PBO (HD)	3.65 ± 0.22						-
F, df, p	F=43.482, df=6,14, p<0.001						

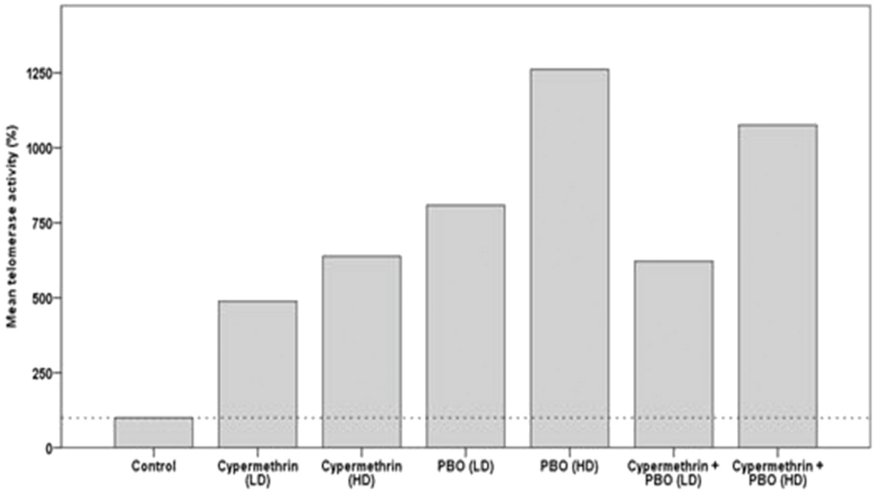

Fig. 1. Mean telomerase activity (%) of all studied groups (Control, CY, PBO and CY+ PBO).

3.2 Oxidative stress markers

The levels of oxidative stress markers for all studied groups are presented in Table 2. In particular, in the CY group at both low dose and high dose, TAC increased significantly (p=0.010 and p=0.002, respectively) by 20% compared to the control (Table 2). Similarly, in PBO low dose group, TAC increased significantly (p=0.004) by 20% compared to control, while at high dose there was just a tendency (p=0.067) for decrease by 20% (Table 2). The co-administration of CY and PBO at low dose did not affect TAC levels compared to control, while at high dose there was a tendency (p=0.079) for decrease in TAC by 20% (Table 2). In CY groups, GSH levels were not affected at any dose compared to control (Table 2). However, PBO administration at high dose decreased significantly (p=0.007) GSH levels by 73% compared to control (Table 2). Moreover, co-administration of CY and PBO at high dose decreased significantly (p=0.013) by 65% GSH levels compared to control, while at low dose there was just a tendency (p=0.08) for decrease by 43% in GSH (Table 2). Catalase activity was increased significantly (p=0.046) by 59% in CY low dose group compared to control (Table 2). There was also a tendency (p=0.086) for decrease in catalase activity by 50% in (CY + PBO) group at high dose compared to control (Table 2). In the other groups, catalase activity was not affected significantly compared to control (Table 2). CARB levels were not affected significantly by either CY or PBO exposure at any dose compared to control (Table 2). CY and PBO administration did not affect at any dose TBARS levels compared to control (Table 2). Thiobarbituric acid reactive substances (TBARS) showed no significant difference between the studied groups. The Glutathione antioxidant system (GSH) concerning CY

exposure is known to decrease GSH levels [32] but in this study CY does not significantly alter GSH levels compared to the control group. However, with the co-exposure to PBO, a dose-dependent significant decrease in GSH is observed. In this context, PBO results in increased Hb levels, while CY alone depletes Hb levels. In the co-exposed animals, PBO clearly inhibits CY-induced Hb damage. No significant differences were observed based on either the given dose, synergetic action or by independent action regarding protein carbonyl levels. However, there was a tendency for increased protein carbonyl levels when CY was present.

Table 2. Markers of oxidative stress shown in mean ± standard deviation and statistical differences (p-values) from the comparison between study groups

		Mean ± SD	p	CY (LD)	CY (HD)	PBO (LD)	PBO (HD)	CY +PBO (LD)	CY +PBO (HD)
TAC (mmol DPPH/L plasma)	**Control**	1.0 ± 0.1	**<0.001**	**0.010**	**0.002**	**0.004**	0.067	0.337	0.079
	CY (LD)	1.2 ± 0.1		-	0.427	0.609	**<0.001**	0.070	**<0.001**
	CY (HD)	1.2 ± 0.1			-	0.773	**<0.001**	**0.015**	**<0.001**
	PBO (LD)	1.2 ± 0.0				-	**<0.001**	**0.026**	**<0.001**
	PBO (HD)	0.8 ± 0.0					-	**0.010**	0.925
	CY+PBO (LD)	1.0 ± 0.1						-	**0.012**
	CY+PBO (HD)	0.8 ± 0.1							-
Hb (g/dL)	**Control**	7.3 ± 1.6	0.060	0.387	0.211	0.148	0.084	0.771	0.948
	CY (LD)	6.1 ± 1.0		-	0.682	**0.029**	**0.016**	0.254	0.354
	CY (HD)	5.6 ± 2.0			-	**0.013**	**0.007**	0.130	0.190
	PBO (LD)	9.2 ± 2.6				-	0.748	0.237	0.165
	PBO (HD)	9.6 ± 1.0					-	0.140	0.094
	CY+PBO (LD)	7.6 ± 0.8						-	0.821
	CY+PBO (HD)	7.3 ± 1.1							-
GSH (µmol/g Hb)	**Control**	3.7 ± 1.9	0.018	0.478	0.634	0.193	**0.007**	0.080	**0.013**
	CY (LD)	3.0 ± 0.3		-	0.245	0.533	**0.027**	0.265	0.053
	CY (HD)	4.1 ± 0.9			-	0.085	**0.002**	**0.032**	**0.005**
	PBO (LD)	2.5 ± 0.6				-	0.090	0.611	0.163
	PBO (HD)	1.0 ± 0.6					-	0.214	0.732
	CY+PBO (LD)	2.1 ± 1.2						-	0.358
	CY+PBO (HD)	1.3 ± 0.6							-

TBARS (µmol/L)	**Control**	7.8 ± 1.7	0.330	0.234	0.468	0.541	0.150	0.831	0.891
	CY (LD)	10.2 ± 2.9		-	0.066	0.547	0.785	0.322	0.188
	CY (HD)	6.4 ± 1.6			-	0.191	**0.040**	0.351	0.554
	PBO (LD)	9.1 ± 1.3				-	0.386	0.689	0.456
	PBO (HD)	10.8 ± 4.6					-	0.213	0.119
	CY+PBO (LD)	8.3 ± 0.4						-	0.726
	CY+PBO (HD)	7.6 ± 1.4							-
Catalase Activity (U/mg Hb)	**Control**	53.8 ± 8.9	**0.029**	**0.046**	0.581	0.817	0.249	0.722	0.086
	CY (LD)	85.6 ± 21.4		-	0.127	**0.030**	**0.004**	**0.023**	**0.001**
	CY (HD)	62.0 ± 11.8			-	0.437	0.099	0.369	**0.030**
	PBO (LD)	50.4 ± 11.0				-	0.350	0.900	0.130
	PBO (HD)	36.4 ± 10.7					-	0.416	0.531
	CY+PBO (LD)	48.6 ± 34.9						-	0.161
	CY+PBO (HD)	27.0 ± 9.30							-
Crbnls (nmol/mg protein)	**Control**	0.8 ± 0.2	0.337	0.079	0.158	0.817	0.894	0.125	0.269
	CY (LD)	1.0 ± 0.1		-	0.691	0.119	0.100	0.795	0.468
	CY (HD)	0.9 ± 0.1			-	0.230	0.197	0.890	0.739
	PBO (LD)	0.8 ± 0.1				-	0.922	0.184	0.375
	PBO (HD)	0.8 ± 0.0					-	0.157	0.327
	CY+PBO (LD)	0.9 ± 0.2						-	0.639
	CY+PBO (HD)	0.9 ± 0.1							-

3.3. Correlation between study groups

In CY treated animals there is no significant association between telomerase activity in PMBCs and catalase activity (rs=–0.429, p=0.397), or Crbnls (rs=–0.143, p=0.787), or even TAC (rs=0.657, p=0.156) and telomerase activity. Only TBARS levels showed a significant negative correlation with telomerase activity in PMBCs (rs=–0.829, p=0.042) (Fig. 2). Interestingly enough, in animals co-exposed to CY + PBO, the significant correlation between telomerase activity in PBMC cells and TBARS in plasma is not present (rs=–0.257, p=0.623), but the negative association is still valid. In co-exposed animals, catalase activity (U/mg Hb) vs telomerase activity showed a significant correlation (rs=–0.811, p<0.001) (Fig. 3). Additionally, when the effect of the PBO was investigated, telomerase activity does not correlate significantly with any of the aforementioned oxidative stress markers. Only GSH is correlated negatively (rs=–0.661, p=0.038) with telomerase activity with CY + PBO and PBO groups.

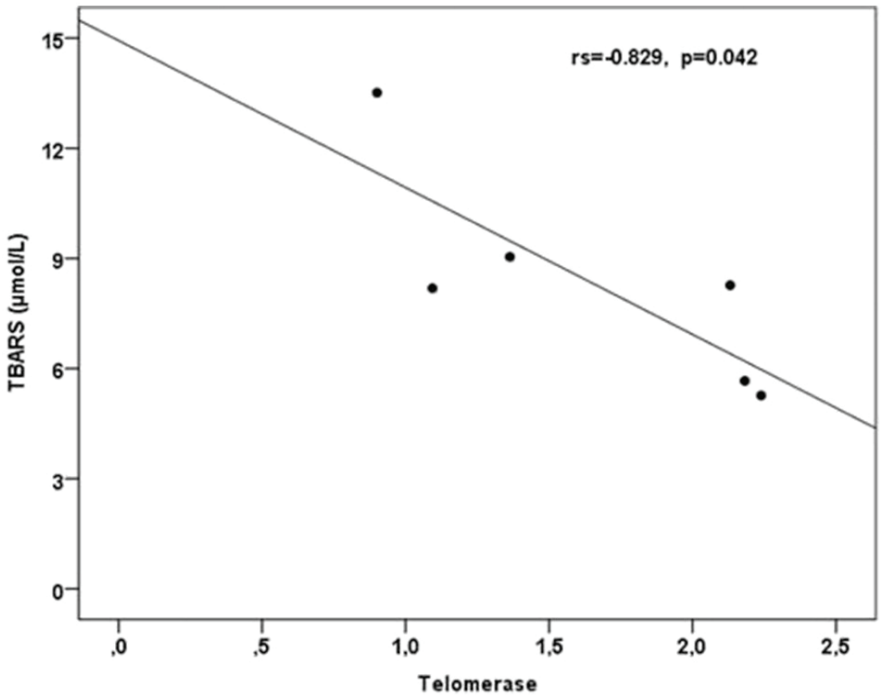

Fig. 2. Correlation graph of telomerase activity in PBMC cells and TBARS as measured in plasma.

4. Discussion

Monitoring inflammation can be achieved among others, either by measuring multifunctional pro-inflammatory cytokines, like TNF-α (tumor necrosis factor alpha), and interleukins (IL), a very sensitive but rather unspecific approach, or white blood cell count (WBC) and leukocyte subpopulations, such as PMBCs, proliferation and activity, aiming according to some researchers more to systemic inflammation [33]. In our study, telomerase activity of CY compared to the control groups exhibits a significant increase in low and high dose groups. CY exposure has previously been shown to increase cytokines in animal studies focusing on a-cypermethrin [34], a similar type synthetic pyrethroid of CY that we used, as well as on cypermethrin [35] on rats. Interestingly enough, in a recent study [36] on interleukins and TNF cytokines in workers exposed to a-cypermethrin little or no changes in a white blood cell count (WBC) and leukocyte subpopulations were reported. Effects of CY in human PBMCs were also evaluated in a study by Rajesh [37], where CY caused slight changes in cytokines, but no significant changes in proliferation. Other studies focusing on

carbamates pesticides, have also found an increase in systemic inflammation, as depicted by telomerase activity in PBMCs [38].

To our knowledge, this is the first study to show that PBO exposure can also cause an increase in telomerase activity in PMBCs, for both low and high dose groups, even higher compared to the CY groups. Such activation has a pivotal role to atheromatosis and coronary syndrome in humans, thus representing a very specific kind of inflammation [39]. Up to now, studies in mice have linked inflammation due to PBO with induction of allergic reactions and dermatitis [40,41]. Although PBO alone produces higher levels of telomerase activity compared to CY alone, their combination results in lower levels compared to the PBO alone. This could be explained by the fact that when both the pesticide and the biocide are present, there is an interference in the action of CY, possibly by the designed mode of action of PBO that creates conditions that lead to CY's reduced metabolism and hence to reduced inflammatory contribution. This would also explain why when PBO is administered alone there is more significant damage.

TBARS are byproducts of lipid peroxidation and when there are high levels of reactive oxygen species (ROS) an increase in lipid peroxidation is observed. In the present study, TBARS were not affected significantly after either CY or PBO administration. However, a study in Wistar rats showed that CY exposure can result in increased lipid peroxidation as measured in TBARS [42].

Glutathione antioxidant system (GSH) plays an important role in cellular defense against reactive pro-oxidant products that have escaped decomposition by the antioxidant enzymes [43]. Studies on rat erythrocytes showed that CY depletes GSH levels [32]. However, our results showed no effect at any dose. On the other hand, PBO caused a depletion compared to control groups in a dose dependent manner. A study on fish [44] found that PBO likely inhibited the cytochrome P450 in a short-term exposure but in a long-term exposure the opposite was noticed. Of note, glutathione S-transferase (GST) has been shown to be induced by PBO in Drosophila melanogaster. Such effect under our experimental conditions could also result in the observed effects of GSH levels but remains to be studied [45].

Hemoglobin (Hb) is a complex protein not often used as an indicator of OS alone but used additionally due to its connection with elevated OS levels. In a study on CY treated rabbits reduced levels of Hb occurred [46]. CY exposure did indeed cause a reduction in our study but PBO alone increased levels above those of the control groups. From the co-exposed groups of CY and PBO (low and high doses) there is a tendency of PBO to inhibit damage that CY causes meaning that the addition of PBO in this case works to counteract the depletion of Hb levels. Another study [47] examined CY and PBO treated rats and found elevated levels of Hb compared to the control group. In our study Hb levels were lower than control levels for CY alone and higher with PBO alone. Thus,

the addition of PBO did in fact result in elevated Hb levels for the co-exposed groups. Thus, it appears that the addition of PBO can actually inhibit the reduction in Hb levels by CY.

The use of CARBs as biomarkers of OS have some advantages in comparison with the measurement of other oxidation products. In determining whether to use lipids, DNA or proteins as a marker of OS, the nature of the ROS will play the significant role [48]. In our study no differences were noticed based on given doses, combined action or by independent action. Only in the CY low dose group there was a tendency to increase.

Catalase activity (CAT) is an important cellular antioxidant enzyme. It was previously suggested that lipid peroxidation might be a contributing factor for a decrease in the CAT during CY toxicity on Wistar rats [49]. Control groups compared to CY low-dose groups show a significant difference but compared to the co-exposed groups regarding high levels of exposure, there is a noticeable tendency to become significant as well. The increase in CAT may contribute as a rescue mechanism against tolerable oxidative stress conditions. However, CY and PBO combined at high levels of exposure decreased CAT activity significantly compared to the exposed groups of CY (low and high), suggesting a role of CY metabolism in this effect. This finding could suggest that when CY and PBO are combined, they induce more severe OS than each of them alone, and some ROS such as hydrogen peroxide at high concentrations can reduce catalase activity [28].

TAC gives information about the capability of averting negative effects of oxidative stress. In general, increase in TAC means enhancement of the antioxidant mechanisms. We observed that both CY doses increased TAC significantly compared to the control groups. Thus, the observed increase in TAC by CY, an oxidative agent, seems intriguing. However, this increase in TAC may be explained as a rescue mechanism in order to counteract the CY induced oxidative stress. Indeed, sometimes under tolerable oxidative stress conditions the organism increases the production of antioxidant molecules as a compensatory mechanism against increase in free radicals [50,51]. In previous studies, we have also reported that the pesticides diazinon and propoxur increased TAC, although, at the same time, they induced OS [52]. Moreover, this increase in TAC may account for the absence of any effect on TBARS and CARB levels after CY administration. Namely, exposure to CY at the doses used may cause a mild oxidative stress that in turn induced antioxidant mechanisms, which protected lipids or proteins from oxidative damage. The CY low dose-induced increase in CAT also conforms to the above hypothesis, that is, there was an increase in antioxidant molecules. Like CY, PBO low dose also increased TAC levels compared to the control groups. Low dose PBO induced increase in TAC and may also be explained as a compensatory mechanism for counteracting OS. As mentioned above for CY, this increase in TAC might

have prevented increases in TBARS and CARB levels. However, high dose PBO exhibited a tendency for decrease in TAC levels. This could be explained by the fact that PBO high dose induced more severe OS compared to low dose, leading to depletion of the antioxidant molecules. The significant decrease in GSH levels following exposure to high dose PBO supported the high OS occurring in that case. Co-exposure to CY and PBO at low doses did not affect TAC levels suggesting that the two compounds in combination may induce a pro-oxidant effect sufficient to eliminate the TAC compensatory outcome. Moreover, like exposure to PBO high dose, co-exposure to CY and PBO at high doses showed a tendency for decrease in TAC indicating that there was an even more severe pro-oxidant effect than that achieved by low doses, resulting in decreased levels of antioxidant molecules. The significant decrease in GSH levels after co-exposure to high doses CY and PBO supports the previous assumption. Similarly, the significant decrease in CAT after co-exposure to CY and PBO compared to CY exposure alone supports that more severe OS is occurring when the two compounds are combined due to possibly a combined effect between them.

5. Conclusions

The observed results for TBARs, GSH, Hb, TAC, CARBs and CAT showed that CY and PBO deteriorate the oxidative and inflammatory status via different mechanisms of action. PBO present in an animal co-exposed to CY and PBO has a noticeable counteraction of CY's pro-oxidant effects probably through its effect on CY metabolism and therefore PBO action prevails with regards to systemic inflammation. In all cases the general systemic condition of the animals appeared impaired. We conclude that PBO as an addition to CY could cause a potential danger even in cases of absence of CY. PBO is generally thought to be an innocent additive, however our results indicate that it poses a potential danger to human health. Further studies are needed to clarify this and to develop ways of ascertaining potential interactions between pesticides and biocides resulting in modifications to the system response to their mixture.

Conflicts of interest

The authors declare that there are no conflicts of interest or funding sources.

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CHAPTER 3

Long-Term Exposure to Cypermethrin and Piperonyl Butoxide Causes Liver and Kidney Inflammation and Induces Genotoxicity in New Zealand White Male Rabbits

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Abstract

Cypermethrin (CY) is frequently used as a Class II pyrethroid pesticide, while piperonyl butoxide (PBO) plays a major role in the pesticide formulation of synthetic pyrethroids. Synthetic pyrethroids are metabolized in mammals via oxidation and ester hydrolysis. PBO can prevent the metabolism of CY and enhances its pesticide effect. While this potentiation effect reduces the amount of pesticide required to eliminate insects, it is not clear how this mixture affects mammals. In our in vivo experiment, New Zealand white male rabbits were exposed to low and high doses of CY, PBO and their combinations, for 4 months. Genotoxicity and cytotoxicity were monitored by measuring binucleated cells with micronuclei (BNMN), micronuclei (MN) and the cytokinesis block proliferation index (CBPI) in lymphocytes. After two months of exposure, a statistically significant increase in the frequency of BNMN was observed for all exposed animals ($p<0.001$) in a dose-dependent way. MN were significantly elevated compared to controls ($p<0.001$), with high dose groups reaching a 442% increase when co-exposed. BNMN and MN continued to increase after 4 months. Histopathological examination of lesions showed damage involving inflammation, attaining lymphoplasmatocytic infiltration in the high dose groups. Both CY and PBO cause liver and kidney inflammation and induce genotoxicity.

1. Introduction

Cypermethrin (CY) is a Class II pyrethroid pesticide, classified in Annex VI of the Regulation 1272/2008/EC as toxic if swallowed (H301), suspected to cause respiratory irritation (H335), suspected to cause organ damage after repeated exposure (H373) and is very toxic to aquatic life with long-lasting effects (H400, H401). CY is widely used, though, in various insecticide applications, both in

domestic and agricultural settings. Pyrethroids are known to affect the nervous system, specifically the synaptic membranes and consequent potential toxic effects are anticipated in insects, mammals and certainly to humans (Kavvalakis et al. 2014, Vlastos et al. 2006, Saillenfait et al. 2015). The procedure of manufacturing formulations of synthetic pyrethrins comprises the addition of other agents to increase the desired pesticide effect at low concentrations. Popular additives are MGK-264 (n-Octyl bicycloheptene dicarboximide) and piperonyl butoxide (PBO), with PBO being the most used and preferred. Adding PBO to a pesticide reduces the total amount of pesticide initially needed to reach the same effects through inhibition of metabolic enzyme systems of insects, namely P450s and esterases (Wilkinson et al. 1984, Young et al. 2005, 2006). PBO does not yet have a harmonized classification in Europe but is classified by the US EPA as acute toxicant as Category III by oral and dermal, and Category IV by inhalation exposure routes, and minimally irritant to eyes and skin. On the other hand, WHO concluded that PBO is unlikely to present acute hazard to humans after normal use.

The genotoxic and mutagenic effects of various pesticides have been studied by a variety of in vitro and in vivo methods (Stivaktakis et al. 2010 and 2012, Titenko-Holland et al. 1997, Villarini et al. 1998, Papapaulou et al. 2001). Assessment of DNA damage is considered important because DNA is generally very sensitive to chemicals. A most commonly used technique is the micronucleus test, which measures both the clastogenic and aneugenic potential of a chemical. As a verification method, histopathology tests are usually conducted, focusing on biological cells and target tissues. Micronucleus (MN) is a biomarker widely used in various biomonitoring studies to determine possible genetic damage and in combination with histopathology it is often applied to assess pesticide-induced damage (Holland et al. 2008, Palanikumar and Panneerselvam, 2011, Speit et al. 2011, Norppa et al. 1993). MN contains acentric chromosome fragments or whole chromosomes, and are recognized as distinct formations in daughter cells separated from the main nucleus (Fenech 1993 and 1997). These are the results of chromosome breakage and/or chromosome loss caused by abnormal chromosome distribution during the mitosis procedure. The highlight of the method is the use of cytochalasin-B, which plays the role of an inhibitor of actin polymerization, hence preventing cytokinesis whilst nuclear division is performed (Fenech and Morley 1985, MacLean-Fletcher and Pollard 1980) resulting in the production of binucleated (BN) cells. The mammalian in vivo MN test is used for the detection of damage induced by the test substance to the chromosomes or the mitotic apparatus of erythroblasts by analysis of erythrocytes as sampled mostly from peripheral blood cells of animals, usually rodents and mammals and also in bone marrow. The method is an official regulatory tool in the European Legislation (B.12, Regulation 440/2008/EC) validated by OECD (OECD TG 474, 1997). The main target of the micronucleus test is to identify substances that cause cytogenetic damage resulting in the

formation of micronuclei containing lagging chromosome fragments or whole chromosomes. Furthermore, other techniques allow examination of cases of induced damage or diseases via processing, embedding, sectioning and then staining of the tissues. Hematoxylin and Eosin (H&E stain) is the most commonly used histology stain for light microscopy. Hematoxylin stains the nuclei within cells blue and Eosin stains the cytoplasm of cells pink. Results from histopathology tests help to clarify in combination with genotoxicity tests, the adverse effects from the exposure of chemical substances, such as pesticides, and their potential impact as shown in various studies (Abdel-Tawab et al. 2015, Marx-Stoelting et al. 2014, Tsitsimpikou et al. 2013, Zafiropoulos et al. 2014).

The possible toxic outcome when one or more chemical substances are applied together is the result of agent-to-agent interactions, toxicodynamic and toxicokinetic interactions. It is important to understand the chemical reactivity, the toxicokinetics (as well as their metabolic pathways) and finally the mechanisms of action for each chemical substance used. These interactions mainly alter the relationship between the external dose and the corresponding level of a pesticide at its target site, leading to a final alteration in the threshold of effects (IGHRC 2009). Exposure to various and multiple pesticides could cause changes in the toxicokinetics of each separate substance. These interactions occur when one pesticide alters the distribution, absorption, metabolism or elimination of others (Reffstrup et al. 2010). Toxicodynamic interactions require that a sufficient quantity of a pesticide reaches its target and causes its toxic effect and that a sufficient quantity of the additional substance also reaches the same initial target causing a second toxic effect that could either potentiate or antagonize the final outcome of the first chemical substance.

The aim of the present study is to clarify the combined toxicology of PBO and CY by studying its genotoxic and cytotoxic effects. Once there is an exposure to CY a metabolic pathway acts as a defense system trying to excrete its metabolites. PBO inhibits this defense action therefore we expect a possibly altered outcome. Furthermore, PBO might be able to affect the toxic levels enough by itself without the addition of CY. Exposure and co-exposure to CY and PBO at different concentrations is studied using the Cytokinesis Block Micronucleus Assay (CBMN) focusing on binucleated cells with micronuclei (BNMN), Micronuclei (MN) and the Cytokinesis Block Proliferation Index (CBPI). Furthermore, the impact on target organ tissues sampled from the liver, kidney and the lymph nodes is examined using histopathology.

2. Materials and Methods

2.1 Animals and administration protocol

Twenty-one New Zealand white male rabbits were used in this study. The animals were housed in individual metal cages at the laboratory animal house facilities of the School of Medicine, University of Crete, Heraklion. The animals

were kept under a 12-hour dark/light cycle and a steady ambient temperature between 20–23°C. Commercial rabbit pellets and drinking water were provided ad libitum. The animals were acclimatized under these conditions for approximately 1 month. CY was provided gratis by Agriphar (Belgium) while PBO was purchased from Sigma Aldrich.

Animals were divided into 7 groups, consisting of 3 animals per group, as shown in Table 1. The dosage scheme has been developed according to previously published data (El-Demerdash et al. 2011, Kavvalakis et al. 2014, Vardavas et al. 2016). All doses were administered orally diluted in 0.5 mL corn oil three times per week. All efforts were made to minimize any possible suffering. During the study period, all rabbits were regularly observed and their health condition was closely monitored. No adverse signs were observed throughout the experiment concerning food and water consumption, skin and eye conditions, excretion of urine and feces. The animals were exposed for 4 months and then sacrificed by veterinarians at the age of 6 months by administering first a sedative injection of Xylapan (20 mg/mL xylazine hydrochloride) and Narketan (100 mg/mL ketamine hydrochloride) with a ratio of 2/1 then an injection of Dolethal (200 mg/mL pentobarbitone sodium), which is a euthanasia agent. The present study was approved by the Veterinary Administration Office of Heraklion (Crete, Greece), the Animal Investigation Committee of the University of Crete (Heraklion, Crete, Greece) and conformed to the National and European Union directions for the care and treatment of laboratory animals. After euthanasia, whole blood samples (3 mL per animal) were collected in heparinized bottles (Collection Test Tube 13×75 mm with Lithium Heparin × 4 mL, Sterile, FL Medical-Vacumed) and stored at 2–8°C until further analysis. For histological examination, organs were removed and preserved in formaldehyde.

Table 1. Administration dosages of all experimental groups.

Experimental Group	Administration	Dose (mg/kg-bw per day)	
		Cypermethrin	Piperonyl Butoxide
Control Group (CON)	Corn oil	-	-
Cypermethrin Low Dose (CY LD)	Cypermethrin	25	-
Cypermethrin High Dose (CY HD)	Cypermethrin	50	-
Piperonyl Butoxide Low Dose (PBO LD)	Piperonyl Butoxide	-	22.5
Piperonyl Butoxide High Dose (PBO HD)	Piperonyl Butoxide	-	45
Cypermethrin + Piperonyl Butoxide Low Dose (CY +PBO LD)	Cypermethrin + Piperonyl Butoxide	25	22.5
Cypermethrin + Piperonyl Butoxide High Dose (CY +PBO HD)	Cypermethrin + Piperonyl Butoxide	50	45

2.2 Micronucleus assay in rabbit lymphocytes

Whole blood (0.5 mL) was added to 6.5 mL Ham's F-10 medium, 1.5 mL fetal calf serum, and 0.3 mL phytohemagglutinin (to stimulate cell division). Cultures were incubated at 37°C for a period of 72 h. Six µg/mL of cytochalasin-B was added 44 h after culture initiation. Cells were collected by centrifugation 72 h post incubation. A mild hypotonic solution of Ham's F-10 medium and milli-Q water (1:1, v/v) was added to the cell solution and left for 3 min at room temperature. Cells were fixed with a methanol: acetic acid solution (5:1, v/v) placed on microscope slides and stained with Giemsa (Stivaktakis et al. 2010, Fenech 1993). These slides were then placed under a Nikon Eclipse E200 microscope where the binucleated cells (BN) and micronuclei (MN) could be viewed (Fig. 1). One thousand BN with intact cytoplasm were scored per slide for each sample, in order to calculate the frequency of MN. Standard criteria were used for scoring MN (Fenech 1997). The Cytokinesis Block Proliferation Index (CBPI) is given by the equation:

CPBI = [M1+2M2+3(M3+M4)]/N ,

where M1, M2, M3, M4 correspond to the number of cells with one, two, three and four nuclei and N is the total number of cells. These parameters were calculated by counting 2000 cells, in order to determine possible cytotoxic effects (Suralles et al. 1995).

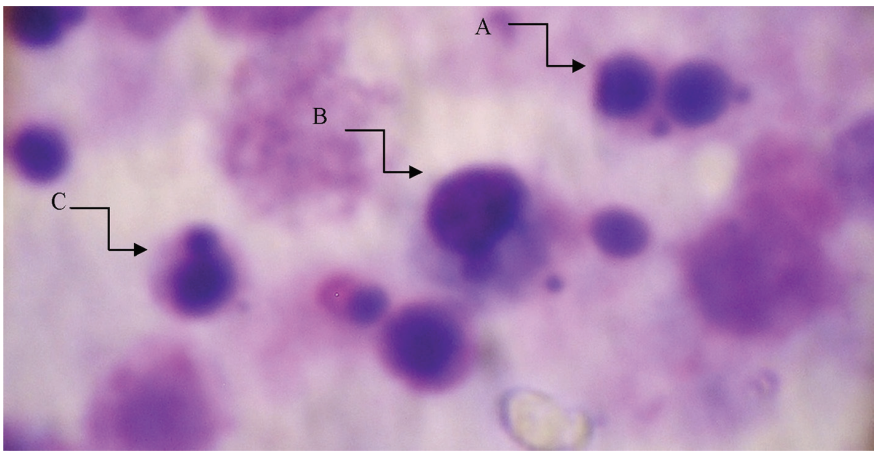


Fig. 1. A microscopic picture taken during the scoring process (100×/1.25, ∞/0.17 WD 0.23). (A). Binucleated cell with two micronuclei. (B and C) Mononucleated lymphocyte cells with present MN. According to the methods of this assay, such MN are not considered measurable.

2.3 Histopathological lesions

Tissue block samples, embedded in paraffin and sectioned at 4 mm, were stained with H&E (Hematoxylin and Eosin), a key staining procedure in histology giving a violet, blue and red outcome, and were subsequently examined under light

microscopy by a histopathologist without knowing the source of each tissue or the type of each exposure.

2.4 Statistical methods

Statistical analysis of the MN data was performed with the G-test for independence on 2×2 tables. The chi-squared test was used for the analysis of CBPI data. A level of significance was set at a 0.05 level. Differences in mean weight of organs of experimental animals upon autopsy were assessed using one-way analysis of variance (one-way ANOVA). Grouped bar charts were used for presenting the weight differences in experimental animal's organs. The IBM SPSS Statistics package 21.0 was used for data analysis and for the graphic representation of data. The level of acceptance of null hypotheses was set at the 0.05 level.

3. Results

3.1 Micronucleus assay

The number of binucleated cells examined for the presence of micronuclei was set at 1000, but as there were three experimental animals per group the total binucleated cells counted were 3000, as indicated in the second column of Table 2. For the estimation of CBPI, 2000 cells (mononucleated, binucleated, three nucleated and four nucleated cells) were scored for each experimental animal. No statistically significant differences between the control group and the animals to be exposed were observed, both for MN and BNMN frequencies ($p>0.05$), at baseline prior to the initiation of the exposure. After two months of exposure there was a statistically significant increase in the frequency of BNMN, for all exposed groups ($p<0.001$) (Table 2). The frequency of MN was also significantly increased compared to the control groups ($p<0.001$) (Table 2) by 268% for PBO LD, 373% for PBO HD, 231% for CY LD, 300% for CY HD, 310% for CY.PBO LD and 442% for CY.PBO HD. After 4 months of exposure, the increase both in BNMN and MN continued and remained statistically significant for the control animals in all exposed groups ($p<0.001$), possibly indicating a time-dependent genotoxic effect. The most prominent increase, by 19.2%, was observed from 2 to 4 months in the CY.PBO LD group and a 10% increase in the CY.PBO HD groups, respectively. Nevertheless, the increase of frequencies observed in four months was not statistically significant compared to the levels observed after two months in all exposed groups. The CBPI showed no statistically significant difference between control and exposed groups at any time of exposure ($p>0.05$), which indicates that there is no noticeable cytotoxic effect after all. Any genotoxic effect of CY, PBO and their combined administration, as presented in Table 2, appears to peak after about 2 months of exposure. Higher frequencies of BNMN (PBO 23%, CY 23%, CY.PBO 38%) and MN (PBO 28.5%, CY 20.6%, CY.PBO 32%) are observed in the HD groups,

compared to the LD groups, after a 2 month period of exposure indicating a possible dose-dependent effect for CY, PBO and their combination, respectively. The total damage in the co-exposed groups after 4 months is 4–12% higher, indicating, although not statistically significant, that the co-exposed groups underwent a greater amount of damage that can be justified from the addition of each chemical's toxicity, with the effect of PBO higher than that of CY when administered alone.

Table 2. Micronucleus Assay in cultures of rabbit lymphocytes showing BN, BNMN, MN and CBPI from the exposure of CY, PBO and CY+PBO.

Exposed Group	BN scored	BNMN ± s.e.	G	p	MN ± s.e.	G	p	CBPI ± s.e.
0 months								
CONTROL	3000	6.33 ± 0.47			6.33 ± 0.47			1.45 ± 0.04
PBO LD	3000	7.33 ± 0.47	0.151	0.697	7.33 ± 0.47	0.151	0.697	1.45 ± 0.02
PBO HD	3000	7.00 ± 0.82	0.069	0.793	7.33 ± 0.94	0.151	0.697	1.42 ± 0.01
CY LD	3000	7.00 ± 0.00	0.069	0.793	7.33 ± 0.47	0.151	0.697	1.43 ± 0.02
CY HD	3000	7.00 ± 0.82	0.069	0.793	6.33 ± 0.47	0.000	1.000	1.45 ± 0.03
CY+PBO LD	3000	6.00 ± 0.00	0.018	0.894	6.00 ± 0.00	0.018	0.894	1.44 ± 0.03
CY+PBO HD	3000	7.00 ± 0.82	0.069	0.793	6.33 ± 0.47	0.000	1.000	1.43 ± 0.02
2 months								
CONTROL	3000	6.33 ± 0.47			7.33 ± 0.47			1.32 ± 0.01
PBO LD	3000	20.33 ± 0.47	33.063	<0.001*	23.33 ±0.47	22.281	<0.001*	1.26 ± 0.02
PBO HD	3000	25.00 ± 0.82	31.691	<0.001*	30.00 ± 0.82	39.735	<0.001*	1.20 ± 0.02
CY LD	3000	17.33 ± 0.47	13.030	<0.001*	21.00 ± 0.94	17.056	<0.001*	1.22 ± 0.01
CY HD	3000	21.33 ± 1.25	22.052	<0.001*	25.33 ± 0.47	27.147	<0.001*	1.27 ± 0.01
CY+PBO LD	3000	22.33 ± 1.25	24.559	<0.001*	26.00 ± 0.82	28.852	<0.001*	1.21 ± 0.01
CY+PBO HD	3000	31.00 ± 0.82	49.776	<0.001*	34.33 ± 0.94	52.775	<0.001*	1.23 ± 0.02
4 months								
CONTROL	3000	7.00 ± 0.82			8.00 ± 0.82			1.34 ± 0.01
PBO LD	3000	25.33 ± 0.94	28.833	<0.001*	28.33 ± 0.47	31.405	<0.001*	1.23 ± 0.01
PBO HD	3000	30.00 ± 0.82	41.854	<0.001*	33.33 ± 0.47	45.117	<0.001*	1.22 ± 0.01
CY LD	3000	21.00 ± 0.82	18.340	<0.001*	24.33 ± 0.47	21.733	<0.001*	1.23 ± 0.03
CY HD	3000	22.33 ± 0.47	21.384	<0.001*	26.33 ± 0.47	26.413	<0.001*	1.27 ± 0.01
CY+PBO LD	3000	27.33 ± 0.47	34.210	<0.001*	31.00 ± 0.82	38.519	<0.001*	1.22 ± 0.01
CY+PBO HD	3000	34.00 ± 0.82	54.211	<0.001*	38.00 ± 0.82	59.335	<0.001*	1.22 ± 0.01

*BN: Binucleated cells, BNMN: Binucleated cells with micronuclei, MN: micronuclei, CBPI: Cytokinesis Block Proliferation Index, s.e.: standard error, p: statistical parameters, * p<0.05, comparison made with the control group, G = 2∑Oi ln(Oi/Ei), where Oi is the observed frequency in a cell, Ei is the expected frequency under the null hypothesis, ln denotes the natural logarithm and the sum is taken over all non-empty cells.*

3.2 Histopathological lesions

In liver tissues, cases of inflammation were observed throughout all exposed groups especially at the portal triads (Fig. 2A) and at the hepatic lobule. Worth noting that even in the control groups such inflammation signs were noticed (2/3 rabbits). CY LD, PBO HD and CY.PBO HD groups were found to have congested vessels. Only in the CY LD and PBO groups sinusoid dilatation was noticed (Fig. 2B). Kidney tissues regarding CY LD, PBO LD and PBO HD groups had interstitial inflammation with PBO LD having acute inflammation focally and topically as well. Interestingly enough, once again the control group had lymphocytic infiltration as well as renal tubules with calcium deposits. In the CY.PBO HD groups of kidney samples, focal inflammation, lymphoplasmatocytic infiltration and a small inflammation near the pelvicalyceal system was noticed (Fig. 3A). Finally, when tissues from lymph nodes were examined no alteration was noticed. Detailed findings are shown in Table 3.

3.3 Macroscopic overview of the anatomy

During organ sampling, a gross macroscopical examination was performed. Various symptoms were found, although not for all of the rabbits and not connected with any clinical signs of the rabbits throughout the experiment. In the control group only one of the three specimens had a larger right kidney (25.3 g compared to the others of the same group that were 12.6 g and 13.1 g). For the CY LD group one of the specimens had liver damage with white specks (possibly coccidiosis) and palpable nodules (Fig. 4A) and another had very easy detachment of the mucosa of the stomach and signs of bleeding in the submucosa (Fig. 4B) but the last rabbit did not show any abnormal clinical symptom. An increase in body fat was noticed for all the CY HD dose groups and one of them had a smaller left lung (10.2 g compared to the others of the same group that were 13.7 g and 12.5 g). Similar to the CY HD groups, PBO LD groups showed an increase in body fat for one, very easy detachment of the mucosa of the stomach and signs of bleeding in the submucosa for another. A rabbit of the PBO HD group had a noticeable distension in the renal pelvic cavitation with an abscess near the left lung (Fig. 4C) and another had an increase in body fat like the CY HD and PBO LD groups (Fig. 4D), as well as an increased collection of liquid in the peritoneal cavity. A rabbit of the CY.PBO HD group had a serious inflammation on the left testicle as well as parts of necrosis (possibly not due to the exposure of the substances but as a habit between male rabbits when kept originally together, prior the initiation of the study,

which tend to damage each other's genitals for supremacy) see Fig. 4E, and another one had red hepatization of the right lung. Based on the veterinarians and the animal husbandry personnel most of these symptoms are possibly due to slight infections or to the fodder consumed.

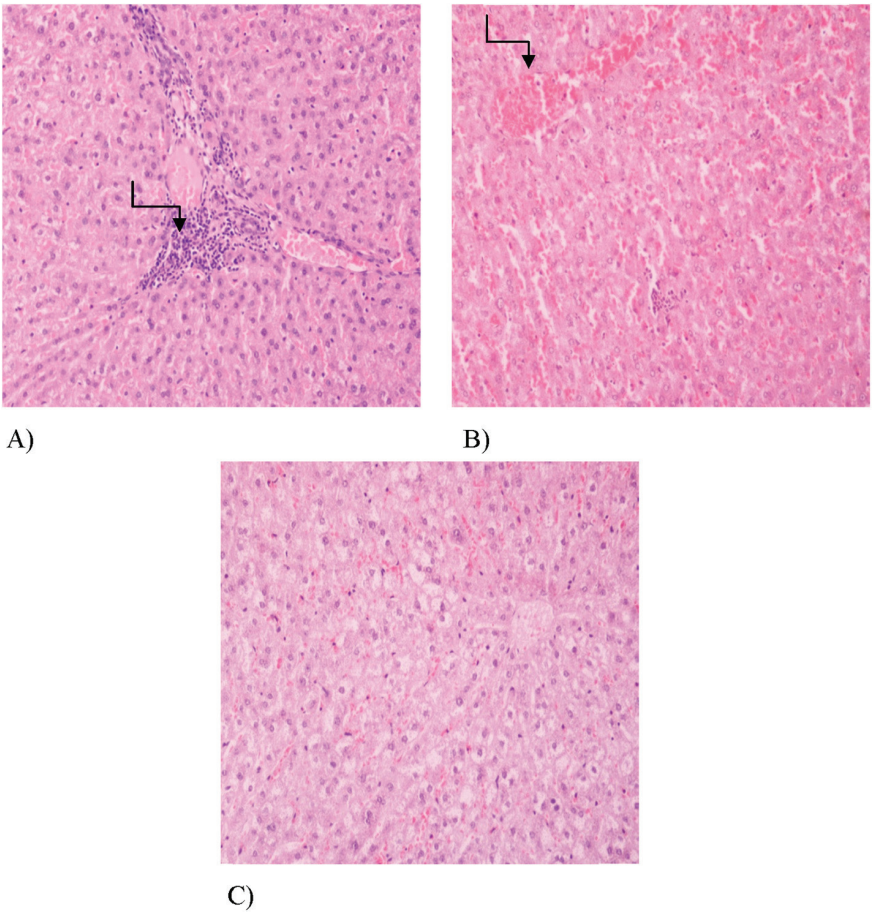
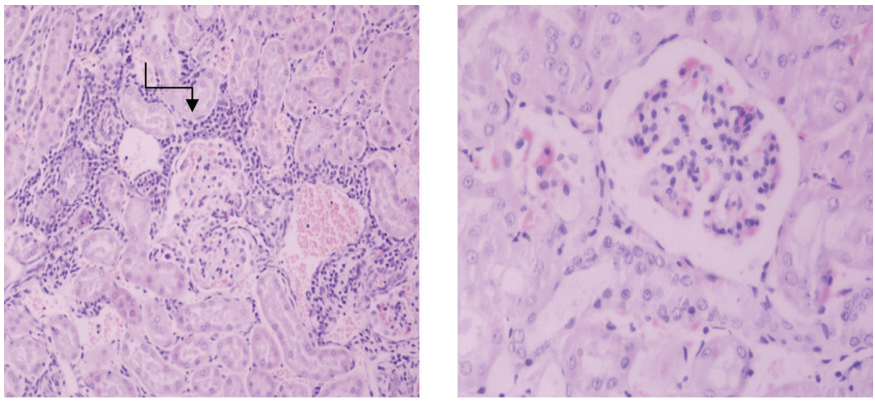


Fig. 2. A) Liver tissue from the CY HD group showing the portal triads with clear signs of inflammation. B) Liver tissue from the PBO HD group showing the dilatation of the sinusoids with erythrocytes. C) Liver tissue from the control group.



A) B)

Fig. 3. A) Kidney tissue from the CY.PBO HD group showing inflammatory infiltrations. B) Kidney tissue from the control group.

3.4. Body and organ weight changes

All exposed rabbits to CY, PBO and CY.PBO, as well as for the control groups did not differ in weight at the start (p=0.154) and during the experiment (p=0.165). Exposed rabbits present a significantly lower weight at the end of the experiment (p=0.002) when compared to control groups, but the rate their weight increased did not change significantly (p=0.211) (Fig. 5). Most of the organs during autopsy (Table 4) did not show any significant difference in weight (p>0.05). The only changes in mean weight were observed in: a) Left testicle (p=0.043) where the exposed rabbits in groups CY LD, CY.PBO LD, CY.PBO HD, were significantly heavier than the controls, b) Lymph nodes that showed significantly lower values (p=0.007) in all exposed animals than control animals, and c) Liver where all exposed groups except PBO HD group had significantly lower weight values than controls (p< 0.001).

Table 3. Anatomical pathological findings in liver, kidney and lymph nodes of exposed rabbits to CY, PBO and CY+PBO.

Group	Rabbit Number	Pathological findings
		Liver
CONTROL	1	Minor inflammation in the portal triad
CONTROL	2	No alterations
CONTROL	3	Minor inflammation in the portal triad
PBO LD	1	Medium sinusoid dilatation
PBO LD	2	Medium portal triad inflammation

PBO LD	3	Medium sinusoid dilatation, medium portal triad inflammation, hepatic lobule inflammation
PBO HD	1	Minor hepatic lobule inflammation
PBO HD	2	Minor hepatic lobule inflammation, sinusoid dilatation
PBO HD	3	Minor hepatic lobule inflammation, sinusoid dilatation, many congested vessels
CY LD	1	Fibrous tissue, minimum inflammation
CY LD	2	Minimum inflammation, lipid degeneration, congested vessels
CY LD	3	Fibrous tissue, minimum inflammation, lipid degeneration, congested vessels, sinusoid dilatation, hyperemic conditions
CY HD	1	Inflammation at the portal triads
CY HD	2	Inflammation at the portal triads, slightly more congested vessels
CY HD	3	Increased inflammation at the portal triads, hepatic lobule inflammation
CY+PBO LD	1	No alterations
CY+PBO LD	2	Inflammation at the portal triads, lipid degeneration
CY+PBO LD	3	Medium interlobular inflammation at the portal triads, lipid degeneration
CY+PBO HD	1	Minor portal triads inflammation
CY+PBO HD	2	Highly congested vessels
CY+PBO HD	3	Minor portal triads inflammation, less inflammation at the hepatic lobule, highly congested vessels
		Kidney
CONTROL	1	No alterations
CONTROL	2	Renal tubes with calcium deposits, focal inflammation
CONTROL	3	lymphocytic infiltration, renal tubes with calcium deposits, focal inflammation
PBO LD	1	Interstitial inflammation
PBO LD	2	Interstitial inflammation
PBO LD	3	Interstitial inflammation, acute inflammation focal-topically
PBO HD	1	Interstitial inflammation at the renal tubes
PBO HD	2	Hyperemic conditions
PBO HD	3	Interstitial inflammation at the renal tubes, hyperemic conditions
CY LD	1	Hyperemic conditions, minimum inflammation
CY LD	2	Minimum inflammation
CY LD	3	Hyperemic conditions, minimum inflammation
CY HD	1	Interstitial inflammation

CY HD	2	No alterations
CY HD	3	Interstitial inflammation
CY+PBO LD	1	Hyperemic conditions
CY+PBO LD	2	Interstitial inflammation, hyperemic conditions
CY+PBO LD	3	Foci of small inflammations, hyperemic conditions
CY+PBO HD	1	Focal inflammation
CY+PBO HD	2	Focal inflammation
CY+PBO HD	3	Focal inflammation, lymphoplasmacytic infiltration, small inflammation near the pelvicalyceal system
		Lymph nodes
	All	No alterations were noticed for any of the tested groups

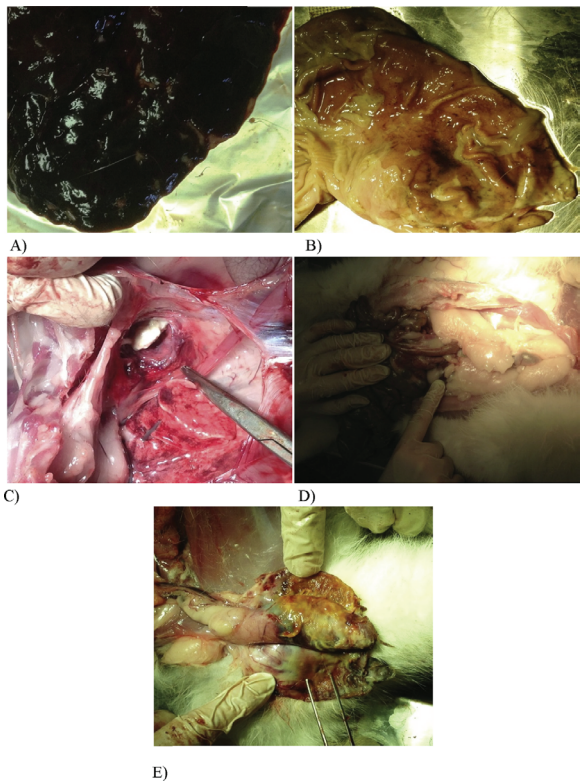


Fig. 4. A) White specks on the liver of CY LD group rabbit. B) Signs of bleeding in the submucosa and easy detachment of the mucosa of the stomach found in CY LD and PBO LD groups. C) The abscess noticed in the PBO HD group. D) Increase of body fat noticed in the CY HD, PBO LD and PBO HD groups. E) Inflammation on the left testicle as well as parts of necrosis.

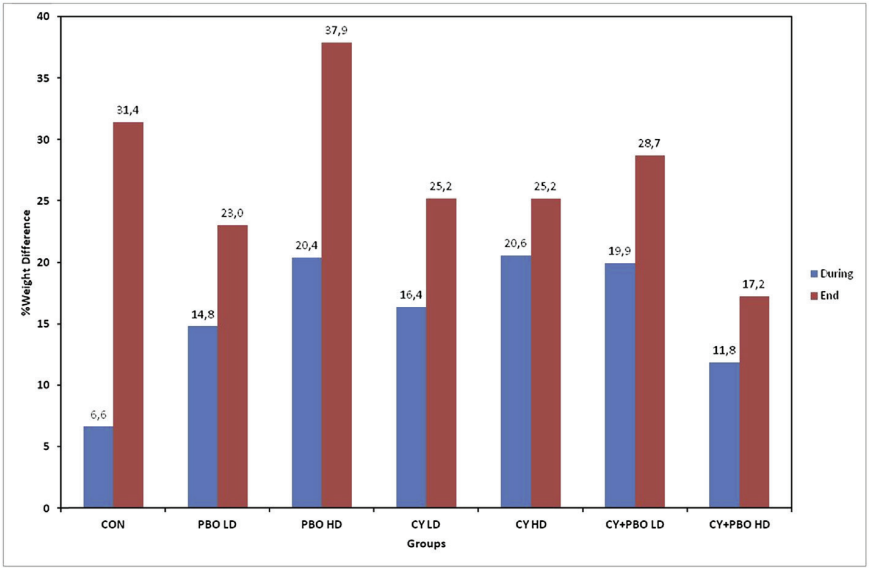


Fig. 5. %Weight differences of rabbits from baseline (start of experiment), during and end of experiment, time points.

Table 4. Vital organs absolute weight and %weight differences (in grams) from baseline comparisons upon autopsy.

	CON		PBO LD		PBO HD		CY LD		CY HD		CY+PBO LD		CY+PBO HD		p**
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	
Heart	15.5	8.7	12.1	2.7	13.9	2.8	12.9	0.6	17.4	1.5	11.2	0.5	12.8	1.7	0.467
%*	0.34	1.93	0.31	1.61	0.29	0.66	0.35	0.81	0.45	0.41	0.29	0.66	0.35	0.61	
Left lung	11.3	4	8.7	2.2	11.5	0.7	9.9	0.5	12.1	1.7	9.1	1.4	12.9	3.8	0.318
%	0.25	0.89	0.22	1.31	0.24	0.16	0.27	0.68	0.31	0.46	0.23	1.84	0.36	1.37	
Right lung	14.3	7.5	12.4	3.2	16.3	0.4	13.2	1.6	18.3	1.9	11.3	6.7	17.7	4.7	0.413
%	0.32	1.66	0.32	1.90	0.34	0.09	0.36	2.16	0.47	0.51	0.29	8.82	0.49	1.69	
Left kidney	15.2	8.2	12.6	1	16.9	5.2	11.3	0.7	12.4	0.4	12.1	0.3	11.8	1.3	0.519
%	0.34	1.82	0.32	0.60	0.35	1.22	0.31	0.95	0.32	0.11	0.31	0.39	0.33	0.47	
Right kidney	16.8	7.4	12.6	1.1	16.2	4.5	11.4	1.2	12.1	1.4	12	0.3	12.8	1.9	0.366
%	0.37	1.64	0.32	0.65	0.33	1.05	0.31	1.62	0.31	0.38	0.31	0.39	0.35	0.68	
Left testicle	6.6	1.0	8.9	1.3	8.2	0.4	*11*	*1.5*	8.3	2.5	*10.7*	*2.0*	*9.4*	*1.0*	0.043
%	0.15	0.22	0.23	0.77	0.17	0.09	*0.30*	*2.03*	0.21	0.68	*0.27*	*2.63*	*0.26*	*0.36*	
Right testicle	9.3	0.3	10	1.9	8.1	0.2	9.9	2.3	7.7	1.6	10.7	2.7	10.2	1.1	0.299
%	0.21	0.07	0.26	1.13	0.17	0.05	0.27	3.11	0.20	0.43	0.27	3.55	0.28	0.40	
Liver	187	29.2	*146*	*12.2*	175.4	10.1	*119.4*	*12.3*	*122.7*	*2.4*	*132.9*	*13.8*	*140.7*	*11.4*	<0.001
%	4.13	6.47	*3.75*	*7.26*	3.62	2.37	*3.22*	*16.62*	*3.14*	*0.65*	*3.39*	*18.16*	*3.89*	*4.10*	
Aorta	0.7	0.3	1.3	0.6	0.8	0.1	1.5	0.5	0.9	0.6	1.2	0.6	1.0	0.2	0.289
%	0.02	0.07	0.03	0.36	0.02	0.02	0.04	0.68	0.02	0.16	0.03	0.79	0.03	0.07	
Lymph nodes	3.5	0.7	*1.9*	*1.5*	*1.1*	*0.5*	2	*0.4*	*0.8*	*0.2*	*1.6*	*0.6*	*1.1*	*0.2*	0.007
%	0.08	0.16	*0.05*	*0.89*	*0.02*	*0.12*	*0.05*	*0.54*	*0.02*	*0.05*	*0.04*	*0.79*	*0.03*	*0.07*	

** % weight difference from baseline (before drug administration).*
*** p-values resulted from ANOVA*
Italics indicate significant differences of exposed groups compared to controls. Resulted from Dunnet's post-hoc

4. Discussion

In the current study the possible genotoxic and cytotoxic effects of the pesticide CY and the enzyme inhibitor PBO in rabbits after long-term administration, was investigated and additional information was gathered from histopathology. CY and PBO when administered alone were found to increase micronuclei and binucleated cells after their exposure period, but from the combinations of the two substances a noticeable cumulative effect was present, indicating in that way that the greater amount of damage could be justified from the addition of each substance's toxicity, with the effect of PBO being higher than that of CY when administered alone.

The CBMN assay constitutes a very reliable technique, used for the evaluation of the genotoxic effect of certain compounds to mammals and humans (Kirsch-Volders et al. 2014). This assay is a very effective tool for the prediction of the genetic risk posed by exposure to chemicals, including pesticides. Furthermore, it can help to understand the mode of action of a pesticide, categorizing them as clastogens or aneugens (Bolognesi et al. 2011). Genotoxic properties of CY have been confirmed in many studies based on in vitro assays such as the alkaline comet assay (Patel et al. 2006) and the MN assay in whole blood and isolated human lymphocyte cultures (Surralles et al. 1995b), which are in line with our in vivo results on rabbits. Notwithstanding such data, Demir et al. (2014) studied CY alone and in combination with PBO using the Drosophila SMART Assay and found negative results for genotoxicity, suggesting a lack of sensitivity of this model for this pesticide formulation. Regarding PBO genotoxicity only a few in vitro studies focusing on reverse mutation (Lawlor 1991, White et al. 1977) and unscheduled DNA synthesis (Lake 1995) are available, although reporting no genotoxic effect. Furthermore, an in vivo study by FAO/WHO (2011) on male and female mice using the micronucleus assay also found PBO to be non-mutagenic while our results show a statistically significant increase of BNMN and MN after 2 and 4 months exposure, suggesting a cumulative stressful effect that culminates in DNA damage. It is worth noting that the CBPI measured in our study indicated a lack of cytotoxic effect in general for all the exposed groups.

It is well known that the liver plays an important role in many essential functions of basic metabolism and that the kidney also plays a pivotal role in facilitating the elimination of pesticide residues from the mammal's body. Our results showed that the long-term exposure of rabbits to CY and PBO, at relatively low doses, caused histopathological lesions involving inflammation and dilatation of the sinusoids in the liver (Fig. 2B), as well as prominent kidney damage, with chronic inflammatory infiltrations and hyperemia (Fig. 3). Ahmad et al. (2011) concluded that rabbits exposed to CY at various doses developed hemorrhages in renal tubules, different stages of degeneration, cast deposition and increased urinary spaces, with a dose-dependent frequency and incidence. All these

are regarded as moderate histological lesions and are in agreement with our findings. Similarly, in another study (Dahamna et al. 2009), CY treated rabbits were found to have fat deposition and necrosis in the liver and tubular necrosis and pink homogeneous tubular casts in the kidneys. Diazinon treatment on male New Zealand Rabbits caused on the other hand degeneration of renal tubules, hypertrophy of glomeruli and leucocytic infiltrations in the liver and kidneys (Sarhan and Sarhhaf 2011). Mitsumori et al. (1996) studied atrophic changes in lymphemapoietic organs, the best known being the lymph nodes, in rats given a diet containing 3% PBO. Atrophy of lymphoid tissues and bone marrow and marked inhibition of body weight gain were reported. However, they found it hard to decide whether PBO had a direct toxicity to lymph hematopoietic tissues based on classical histopathological examination of sections stained with hematoxylin and eosin. They concluded that the atrophic changes of lymphhemapoietic organs seen in animals exposed to PBO are primarily due to under-nutrition resulting from reduced food intake. In our study, all animals were provided with a very good level of nutrition and did not have food or water intake fluctuations so we conclude that possibly this is why we did not notice any alterations in the lymph nodes when tested for histopathological lesions and did not show any type of damage possibly due to the low dose of exposure. As was mentioned earlier it is possible that the level of exposure might also be affecting the level of damage and that the liver and kidney are more susceptible organs.

A number of in vivo studies reporting histopathological lesions of the liver and kidneys have been conducted regarding exposure to PBO, primarily on rats and mice, but rarely on rabbits, as shown in Table 5. Most studies on the effects of CY and PBO have focused on rats or mice, while studies focusing on rabbits are few and to the best of our knowledge, histopathological lesions have not been extensively studied in rabbits. Our study shows that kidney tissues of rabbits exposed to PBO LD and PBO HD had interstitial inflammation with PBO LD exhibiting also acute inflammation focally and topically. In the kidney samples of the CY.PBO HD groups, focal inflammation, lymphoplasmatocytic infiltration and a small inflammation near the pelvicalyceal system were observed, obviously being the most damaged tissue sample compared to the other organ tissues sampled.

Table 5. In vivo studies reporting histopathological lesions of liver and kidneys conducted on rats/mice and rabbits regarding the exposure to PBO.

Author	Organ: Liver	Organ: Kidney
Graham (1987) (rats)	Hyperplasia and hypertrophy of the centrilobular hepatocytes and enlarged eosinophilic cells	Chronic interstitial glomerulonephritis

Chun and Wagner (1993) (mice)	Liver necrosis, centrilobular hypertrophy and polymorphonuclear cell infiltrates	-
Bond et al. (1973) (mice)	Liver damage	Kidney damage
Fujitani et al. (1993) (mice)	Hypertrophy of hepatocytes, single cell necrosis and inflammatory cell infiltration	No alterations
Sarles et al. (1949) * (rabbits)	No alterations	No alterations

** Rory Breathnach 1999. The Safety of Piperonyl Butoxide, in: Denys Glynne Jones, Piperonyl Butoxide, The Insecticide Synergist. Academic Press, London, 1999, Pages 7–39.*

5. Conclusions

Our study shows that CY, PBO, and combinations of the two, cause liver and kidney inflammation and induce genotoxicity, which is consistently confirmed in the co-exposed groups. Our results showed that the long-term exposure of rabbits to CY and PBO, at relatively low doses, causes histopathological lesions involving inflammation and dilatation of the sinusoids in the liver, as well as prominent kidney damage, with chronic inflammatory infiltrations and hyperemia. Our results also show a statistically significant increase of micronuclei and binucleated cells with micronucleus after a few-months exposure to CY, PBO and combinations of the two, suggesting a cumulative stressful effect that culminates in DNA damage. Further studies are required to evaluate the implications of such effects in public and environmental health resulting from the application of pesticides formulated with CY and PBO.

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Conflicts of interest

The authors declare that there are no conflicts of interest or any funding sources.

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CHAPTER 4

The Metabolism of Imidacloprid by Aldehyde Oxidase Contributes to its Clastogenic Effect in New Zealand Rabbits

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Abstract

Imidacloprid (IMI) is a systemic, chloro-nicotinyl insecticide classified in Regulation Nº 1272/2008 of the European Commission as 'harmful if swallowed and very toxic to aquatic life, with long-lasting effects'. IMI is metabolized in vitro both by aldehyde oxidase (AOX) (reduction) and by cytochrome P450s enzymes (CYPs). In the present study, the AOX inhibitor sodium tungstate dihydrate (ST) was used to elucidate the relative contribution of CYP 450 and AOX metabolic pathways on IMI metabolism, in male rabbits exposed to IMI for two months. To evaluate the inhibition effectiveness, various metabolite concentrations in the IMI and IMI+ST exposed groups were monitored. DNA damage was also evaluated in micronucleus (MN) and single cell electrophoresis (SCGC) assays in both groups, along with oxidative stress (OS) with the inflammatory status of the exposed animals, in order to clarify which metabolic pathway is more detrimental in this experimental setting. A significant increase in the frequency of binucleated cells with MN (BNMN, 105%) and micronuclei (MN, 142%) was observed after exposure to IMI ($p<0.001$). The increase in the ST co-exposed animals was less pronounced (BNMN 75%, MN 95%). The Cytokinesis Block Proliferation Index (CBPI) showed no significant difference between controls and exposed animals at any time of exposure ($p>0.05$), which indicates no cytotoxic effect. Similarly, comet results show that the IMI group exhibited the highest achieved tail intensity, which reached 70.7% over the control groups, whereas in the IMI+ST groups the increase remained at 48.5%. No differences were observed between all groups for oxidative-stress biomarkers. The results indicate that the AOX metabolic pathway plays a more important role in the systemic toxicity of IMI.

1. Introduction

Imidacloprid (IMI) (EC 428-040-8) is a systemic, chloro-nicotinyl insecticide in the chloro-nicotinyl nitro-guanidine chemical group [1, 2]. In the European level, it is recognized as biocidal-active substance that is approved under the Regulation Nº 528/2012/EC. It is classified in Annex VI of the Regulation No. 1272/2008/EC as harmful if swallowed (H302) and very toxic to aquatic life, with long-lasting effects (H400, H401).

IMI is quickly absorbed by the oral route and rapidly distributed in most organs and tissues. In rats, the oral absorption is estimated to be 92-99%. IMI degrades to many metabolites formed via multiple pathways. Common or structurally similar metabolites have been found in rats, goats and hens. Based on structural considerations, the following metabolites are of toxicological significance to humans: 6-chloronicotinic acid (6-CNA), imidazolidine 4- and 5-hydroxy compounds, olefinic imidacloprid, desnitro-imidacloprid (IMI-NH) and the nitrosoimine compound. IMI metabolites are primarily excreted in the urine as glutathione and glycine conjugates of mercaptonicotinic acid and hippuric acid [3]. IMI-NH is of particular interest, due to its nicotinic-type action that prefers mammalian versus insect nicotinic acetylcholine receptors (nAChRs) [4, 5], and therefore it binds very strongly to mammalian nerve receptors but not to insect nerve receptors. IMI-NH is not toxic to insects, but it is about four to five times more toxic than IMI to mice [6, 7].

In vitro studies have indicated the importance of cytochrome P450s (CYPs) in IMI oxidation and aldehyde oxidase (AOX) in IMI reduction. Currently, the most frequently used insecticides are neonicotinoids that are metabolized in vitro by AOX on reduction of the nitro-imino group and by CYPs via oxidation reactions. Similarly, in vitro reduction of the nitro-imino group of IMI by AOX leads to the desnitro-imidacloprid metabolite (IMI-NH), while oxidation reactions by CYP lead to other primary metabolites, such as 6-CNA. AOX metabolizes many xenobiotics in vitro, but its in vivo importance is usually not clear compared to that of cytochrome P450 (CYP) and other detoxification systems. Swenson and Casida [8] established the relative importance of AOX and CYP in vivo using the mouse model.

Sodium tungstate dihydrate (ST) is a substance that does not have yet a registration number according to Article 2 of the REACH Regulation (EC) No. 1907/2006, but is classified with warning statements such as 'Category 4', 'Acute toxicity', 'Oral', 'harmful if swallowed' by Regulation No. 1272/2008/EC. It has been shown to reduce AOX activity in vivo in mammals by replacing molybdenum at the active center, rendering it inactive [9, 10, 11].

The aim of the present study is to elucidate which IMI metabolic pathway, the AOX or the CYP, could be more detrimental in a systemic way. To accomplish this, concentrations of AOX and CYP metabolites, along with the major metabolite of IMI, 6-CNA, were monitored in various matrixes such as hair, urine and blood

of New Zealand male rabbits exposed either to IMI alone or co-exposed to IMI and ST, in order to inhibit AOX activity. Various parameters including DNA damage (micronuclei test, comet assay), oxidative stress (total antioxidant activity-TAC) and systemic inflammation, were measured. The study hypothesis is summarized in Table 1 and can be found in the supplementary information section.

2. Materials and methods

2.1 Animals and administration protocol

Nine 3-month-old New Zealand white male rabbits were used in this study. The animals were housed in individual metal cages at the laboratory animal house facilities of the School of Medicine, University of Crete, Heraklion, under a 12-hour dark/light cycle and a steady ambient temperature between 20–23°C. Commercial rabbit pellets and drinking water were provided ad libitum. The animals were acclimatized under these conditions for approximately 2 weeks. IMI was provided gratis by Vapco (Jordan) and ethirimol (ETH), which is the internal standard used, while 6-CNA, IMI-NH and ST were purchased from Sigma–Aldrich, Chemie GmbH. Animals were divided into 3 groups, consisting of 3 animals per group, as shown in Table 2 (see supplementary information section).

Since oral LD50 values of IMI for rabbits have not yet been estimated, based on the known oral values for rats [12], dermal values for rabbits, and similar dosage schemes developed according to Kavvalakis [13], an exposure dose was decided with an oral dose of 30 mg/kg-bw per day. The administered doses for ST were estimated based on the available literature, bearing in mind that a dose of 0.7mg/mL/water was found to inhibit AOX metabolism in mice [8]. All doses were administered orally, diluted in 500mL water, three times per week. All efforts were made to minimize any possible suffering. During the study period, all rabbits were regularly observed and their health condition was closely monitored. No adverse signs were observed throughout the experiment concerning food and water consumption, skin and eye conditions, excretion of urine and feces. The animals were exposed for 2.5 months and then sacrificed by veterinarians at approximately the age of 6 months by administering first a sedative injection of Xylapan (20mg/mL xylazine hydrochloride) and Narketan (100mg/mL ketamine hydrochloride) with a ratio of 2/1, and then an injection of Dolethal (200mg/mL pentobarbitone sodium), which is a euthanasia agent. The present study was approved by the Veterinary Administration Office of Heraklion (Crete, Greece), the Animal Investigation Committee of the University of Crete (Heraklion, Crete, Greece) and conformed to the National and European Union Directions for the care and treatment of laboratory animals. After euthanasia, blood samples were collected into heparinized vials (Collection Test Tube 13×75 mm with Lithium Heparin × 4 mL, Sterile, FL Medical-Vacumed) to

be used for the MN assay, comet assay and for the metabolite detection and in vials containing EDTA (Vacuette Blood Collection Tubes, spray dried K3EDTA × 3mL, Greiner bio-one) to be used for the OS and TA assay, and all sampled vials were then stored at 2–8°C, until further analysis.

2.2 Micronucleus assay (MN) in rabbit lymphocytes

The MN test is an official regulatory 'tool' in the European Legislation (B.12, Regulation 440/2008/EC) validated by OECD (OECD TG 474, 1997). Whole blood (0.5 mL) was initially added to 6.5 mL Ham's F-10 medium, 1.5 mL fetal calf serum, and 0.3 mL phytohemagglutinin (this is done to stimulate cell division). Cultures were then incubated at a temperature of 37′ C for a period of 72 h. Six µg/mL of cytochalasin-B was added 44 hours after culture initiation. Cells were collected by centrifugation 72 hours after the incubation process. A mild hypotonic solution of Ham's F-10 medium and milli-Q water (1:1, v/v) was then added to the cell solution and left for 3 min at room temperature. Cells were fixed with a methanol: acetic acid solution (5:1, v/v) placed on microscope slides and stained with Giemsa [14, 15]. These slides were then placed under a Nikon Eclipse E200 microscope where the binucleated cells (BN) and micronuclei (MN) could be clearly viewed. One thousand BN with intact cytoplasm were scored per slide for each sample, in order to calculate the frequency of MN. Standard criteria were used for scoring the MN [16]. The Cytokinesis Block Proliferation Index (CBPI) is calculated by counting 2000 cells and based on a specific equation, to determine additional possible cytotoxic effects [17].

2.3 Comet assay in rabbit lymphocytes

The comet assay is another fast and efficient method for obtaining details regarding DNA damage and possible repair procedures in individual cells [18]. It is widely accepted in the field of in vivo research experiments, besides human and environmental studies, up until today and is an official test validated by OECD (OECD TG 489, 2014). The slide preparation for the alkaline comet assay was performed conventionally, as described by Singh [19], with some slight modifications. All microscopic slides were covered with 0.65% normal melting agarose (NMA) prepared in PBS (Ca2+- and Mg2+- free). Isolated lymphocytes were mixed with 100µL of LMA at a temperature of 37′ C to form a cell suspension. Slides were maintained at 4′ C for 10 min to solidify the cell suspension layer. Coverslips were removed laterally and the slides were immersed in freshly prepared cold lysing solution (2.5M NaCl, 100mM Na2EDTA, 10mM Tris, pH10) including 1% Triton X and 10% DMSO. Slides were left in the lysing solution, at 4′ C, for at least 1 hour. Following the lysing step, the slides were placed in a horizontal electrophoresis tank filled with cold freshly prepared electrophoresis solution (1mM Na2EDTA and 300mM NaOH, pH13) at 4′ C for 20 min to prepare the DNA for electrophoresis by loosening the tight double-helix structure. After

the electrophoresis, the slides were removed from the tank and neutralized by immersing in Tris buffer (0.4M Tris, pH7.5) for 15 min, then refreshing the buffer every 5 min. Finally, the slide scoring was done using double slides prepared for each subject and a hundred cells were analyzed per subject at ×400 magnification, under an Olympus fluorescent microscope equipped with an excitation filter of 546 nm wavelength and a barrier filter of 590 nm. Comet Assay IV image analysis system (Perceptive Instruments) was used blindly by a one slide reader to score DNA damage, shown as tail intensity.

2.4 Assessment of oxidative stress markers

Sampled blood was collected in ethylenediaminetetraacetic acid (EDTA) tubes for measuring: total antioxidant activity (TAC), thiobarbituric acid reactive substances (TBARS), protein carbonyls (CARB), reduced glutathione (GSH) levels and catalase activity. Blood samples were centrifuged immediately at 1370g for 10 min at 4°C and the plasma was collected and used for measuring the oxidative stress indicators. The packed erythrocytes were lysed with distilled water (1:1 v/v), inverted vigorously, centrifuged at 4020g for 15 min at 4°C and then the erythrocyte lysate was collected for the measurement of catalase activity. A portion of erythrocyte lysate (500 µL) was treated with 5% trichloroacetic acid (TCA) (1:1 v/v), vortexed vigorously, and centrifuged at 28000g this time for 5 min at 4°C. The supernatants were then removed, treated again with 5% TCA (1.3:1 v/v) and centrifuged once again at 28000g for 5 min at 4°C. The clear supernatants were transferred to Eppendorf tubes and were used for the estimation of GSH. Plasma and erythrocyte lysate were stored at −80 °C prior to biochemical analyses. For TBARS determination, a slightly modified assay of Keles [20] was applied, protein carbonyls were determined based on the method of Patsoukis [21], GSH was measured according to Reddy [22], catalase activity was determined based on the method of Aebi [23], and determination of TAC was based on the method of Janaszewska and Bartosz [24].

2.5 Telomerase activity

Telomerase activity in PBMCs was measured using a commercial telomerase PCR–ELISA (Roche Diagnostics Corp., Indianapolis, IN, USA), based on the telomeric repeat amplification protocol [25]. The method for the isolation of PBMCs is described elsewhere [26].

2.6 Rabbit hair and urine sampling

Hair and urine were collected initially prior to exposure and then once every month. In order to assess the accumulation of IMI and IMI-NH metabolites, the hair specimens were collected from the back of each animal just below the head. This pattern remained the same throughout the experiment. Collected hair samples were sealed in aluminium foil, labeled and stored in A4 paper

envelopes in a dry dark place, at room temperature until analysis. Urine samples were collected in falcons (Tube 15mL, 120×17mm, PP tube, HD-PE cap, SARSTEDT) and stored at a temperature of -20°C until analysis.

2.7 Hair sample preparation procedures

For the removal of the external contaminants a procedure used in previous studies [27, 28], was employed with slight modifications. Briefly, hair specimens were washed once in 5 mL of water (for 10 min) and twice in 5 mL of methanol (for 1 min) at room temperature. Washed hair specimens were dried in the oven. The methanol wash was tested for IMI and 6-CNA to confirm that external contamination was eliminated. Subsequently, an amount of 50 mg of hair was cut in 2–3 mm pieces, weighed and finally transferred in a glass test-tube with a screw top. Ten μL of ethirimol (ETH) were added (10 μg/mL) as internal standard. In order to isolate the analytes from the hair samples, the hair was incubated with 2 mL of methanol at room temperature in an ultrasonic bath for 3 hours. The temperature of the bath during the ultrasonic extraction did not exceed 50°C. The methanol extract was filtered through 0.2 μm membrane filter (Econofilter, Agilent Technology) to a glass test-tube. The same procedure was repeated once more and finally the total 4 mL of methanol were evaporated to dryness under a gentle nitrogen stream at room temperature. The residue was dissolved in 100 μL of methanol and 10 μL were injected to the LC–MS system.

2.8 Urine and serum sample preparation procedures

To 0.5 mL of urine, 10 μL of ETH solution (10 μg/mL in methanol) were added. The specimen was further diluted with 1.5 mL of water (HPLC grade) and 2 mL of dichloromethane were added. The mixture was shaken mechanically for 10 min, and centrifuged at 4000 rpm for 5 min, after which the organic layer was collected. The aquatic phase was acidified with 10 μL of HCl 6 M and then 2 mL of dichloromethane were added again. The extraction procedure was repeated once more. Both organic layers (total 4 mL) were mixed and evaporated to dryness under a gentle nitrogen stream at 25°C. The residue was reconstituted in 100 μL of methanol and 10 μL were injected into LC–MS.

2.9 Liquid chromatography and mass spectrometry conditions

Liquid chromatography was carried out using the Shimadzu Prominence LC system consisting of a binary LC pump, a vacuum degasser, an auto sampler and a column oven. A gradient of 0.1% formic acid in water (solvent A) and methanol (solvent B) were selected for routine use: starting at 20% of solvent B, 100% B (15.0 min linear ramp) and finally 20% B (5 min). Total mobile phase pumped at 0.6 ML/min through a Discovery C18 HPLC column (25 cm × 4.6 mm, 5μm) thermostated at 30°C. A 10 μL volume of each sample was injected in the mobile phase flow. A mass spectrometer (LCMS-2010 EV Shimadzu),

comprising an atmospheric pressure chemical ionization (APCI) interface and a single quadrupole mass filter, was used to detect and quantify IMI and IMI-NH metabolites in column effluent (see Fig.1, supplementary information section). Interface, curved desolvation system (CDL) and heat block temperatures were 400°C, 200°C and 200°C, respectively. The detector voltage was 1.5 kV and the nebulizing gas flow 2.5 L/min. Drying gas was set at 0.02 MPa. Ion signals were acquired in time-selected ion monitoring (SIM) mode: with ions m/z = 256.10, 211.85, 174.95 for IMI; 157.85, 189.90, 96.00 for 6-CNA; 211.05, 213.00 for IMI-NH; and 210.10 for ETH; used as internal standard (see Table 3). The mass-spectrometry operating conditions were tuned according to the manufacturer procedure. Data acquisition and processing were performed using LC–MS Solution software (Shimadzu, version 3.40.307). Stock and working solutions of the parent compound of IMI and their metabolites, IMI-NH and 6-CNA at a concentration of 1 mg/mL were prepared in methanol. Working solutions of mixed analytes were prepared weekly by dilutions in methanol in the concentrations of 0, 0.05, 0.1, 0.25, 0.5, 1 and 10 µg/mL. All working solutions were stored at -20°C. The analytical parameters of the developed LC-APCI(+)-MS base method for the determination and quantification of IMI, 6-CNA and IMI-NH in hair, urine and blood samples are shown in Table 3.

Table 3. Analytical parameters of the developed LC-APCI (+)-MS base method for the determination and quantification of IMI, IMI-NH, ETH and 6-CNA. Parameters from the LC consist of: analytical parameters, such as retention time (Rt) and m/z Fragments; and performance parameters, such as linearity (r2), LOD and LOQ values.

	IMI-NH	**IMI**	**Ethirimol (ETH)**	**6-CNA**
Rt *(min)*	7.26	10.86	11.20	12.06
target m/z	211.05	256.10	210.10	157.85
Q1 m/z	213.00	211.85	182.05	189.90
Q2 m/z	-	174.95	206.05	96.00
r^2 *(spiked) urine*	0.9909	0.9990	-	0.9805
r^2 *(spiked) blood*	0.9895	0.9941	-	0.9960
r^2 *(spiked) hair*	0.9884	0.9854	-	0.9976
r^2 *(standards)*	0.9995	0.9999	-	0.9891
LOD (pg/mg) urine	0.528	0.012	-	0.036
LOD (pg/mg) blood	0.068	0.003	-	0.010
LOD (pg/mg) hair	0.009	0.007	-	0.015
LOQ (pg/mg) urine	1.762	0.039	-	0.120
LOQ (pg/mg) blood	0.226	0.011	-	0.032
LOQ (pg/mg) hair	0.028	0.024	-	0.049

Rt: retention time, r2: linearity, Q1: qualification ion 1, Q2: qualification ion 2, LOD: limit of detection, LOQ: limit of quantitation

3. Statistical methods

Statistical analysis of the MN data was performed with the G-test for independence on 2×2 tables. The chi-squared test was used for the analysis of CBPI data. The level of significance was set at 0.05. Independent samples t-test or Mann-Whitney U test was used for comparing continuous variables between two groups. One-way ANOVA or Kruskal–Wallis was applied to estimate differences between 3 or more groups. Repeated measures ANOVA was used for examining the effects of inhibitors at two different time points, 1st and 2nd month of experiment. Mean plots with error bars and bar-charts were used for graphical presentation of data. The IBM SPSS Statistics Package 21.0 was used for data analysis and for the graphic representation of data. The level of acceptance of null hypotheses was set at the 0.05 level.

4. Results

4.1 Metabolite concentrations in urine, hair and blood serum

Levels of IMI and its metabolites, IMI-NH and 6-CNA, were measured after the first and second month of administration in all groups (CON, IMI, IMI-NH) in urine and hair samples, and in blood samples at the end the administration scheme (2 months). The results are summarized in Table 4. Overall mean IMI levels in urine significantly increased ($F=12.77$, $df=1,4$, $p=0.023$) through time. The above described pattern was not observed for IMI levels measured in hair samples. IMI-NH levels in urine were shown to be affected by ST ($F=109.99$, $df=1,4$, $p<0.001$) and decreased through time ($F=140.28$, $df=1,4$, $p<0.001$; 1st month $p=0.024$; 2nd month $p=0.019$). IMI-NH blood levels were not affected by the addition of ST. All measured levels in hair were below the limit of detection (LOD). The 6-CNA metabolite of IMI was not measured in hair samples due to LOD limitations. In blood samples there was no significant effect from the addition of ST after 2 months of exposure ($p=0.086$). Urine levels of 6-CNA were not affected.

Based on urine excretion, it is quite clear that from the addition of ST, the concentration of the IMI-NH metabolites in the IMI-ST group is reduced by 91% compared to the IMI group one month after exposure and the same pattern was observed at the end of the administration scheme. Although excretion of IMI-NH decreases with time, the efficiency of ST as an inhibitor of IMI-NH metabolites remained the same, 91% and 86%.

Table 4. Concentrations of IMI, IMI-NH and 6-CNA in urine (μg/mL), blood (μg/mL) and hair (μg/pg) of rabbits.

		IMI (μg/mL)				IMI-NH (μg/mL)				6-CNA (μg/mL)			
		1st Month		2nd Month		1st Month		2nd Month		1st Month		2nd Month	
	ST	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Urine	No	9.7	8.2	23.9	3.5	33.1	15.1	11.7	12.7	1.8	1.2	1.7	0.2
	Yes	18.9	9.1	31.3	4.6	2.9	0.3	1.6	1.0	1.3	0.5	4.6	2.1
	Overall	14.3	9.2	27.6	5.5	18.0	19.1	6.7	9.8	1.6	0.8	3.1	2.1
Effects	Months	F=12.77	df=1, 4	p=0.023*		F=140.28	df=1, 4	p<0.001*		F=6.58	df=1, 4	p=0.062	
	Months x ST	F=0.054	df=1, 4	p<0.827		F=109.99	df=1, 4	p<0.001*		F=7.48	df=1, 4	p=0.050	
Blood	No	-	-	4.9	3.7	-	-	0.3	0.6	-	-	0.2	0.0
	Yes	-	-	23.9	10.9	-	-	0.0	0.0	-	-	0.7	0.4
	Overall	-	-			-	-			-	-		
Effect	Inhibitor	-	-	p=0.045		-	-	p=0.374		-	-	p=0.086	
Hair	No	30.8	6.1	38.3	6.8	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Yes	27.7	7.7	29.2	1.3	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
	Overall	29.3	6.4	33.7	6.6	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD
Effects	Months	F=1.03	df=1, 4	p=0.367		F=-	df=-	p=-		F=-	df=-	p=-	
	Months x ST	F=0.47	df=1, 4	p=0.532		F=-	df=-	p=-		F=-	df=-	p=-	

LOD: limit of detection, SD: standard deviation, F: value of F distribution used in ANOVA, p is the probability of the statistical test, - = no sample

4.2 Metabolite excretions in urine, hair and blood serum

Calculating the excretion rate of urine can be accomplished by taking into account parameters such as daily urine excretion, detected concentration of the requested metabolite and the total administered amount of the substance, in this case IMI. For blood, the same pattern was followed, but the daily amount was considered stable due to controlled sampling procedures giving approximately 6 mL of blood. The excretion rate of IMI, IMI-NH and 6-CNA in hair is not used as a calculation method of excretion and can only be applied for accumulation rates.

The excretion rate of IMI as a parent compound in urine without the addition of ST during the first month was 148% and the second month 182%. With the concomitant administration of ST, urine excretions were 288% and 239%. Regarding IMI-NH as a metabolite of AOX metabolism, without the addition of ST during the first month it was 505% and for the second month 89.3%. With the concomitant administration of ST, urine excretions were 44.3% and 12.2%. Finally, the excretion rate of 6-CNA, the major metabolite of IMI, was 27.5% the first month and 12.9% the second month. Under the influence of ST, urine excretions were 19.8% and 35.1%, respectively (Fig.2, see supplementary information section). In blood samples, however, due to sampling only of the final month, excretion rate of IMI without the influence of ST was 0.68% and under the influence of ST was 3.3%. Regarding IMI-NH, without the influence of ST, the excretion rate was 0.04% and under the influence of ST was 0%. Finally, 6-CNA, was 0.02% and under the influence of ST was 0.09%.

4.3 Micronucleus assay

Table 5. Results of MN assay in cultures of rabbit lymphocytes showing BN, BNMN, MN and CBPI, from the exposure of IMI and IMI+ST.

Exposed group	BN cells scored	BNMN ± s.e.	G	p	MN ± s.e.	G	p	CBPI ± s.e.
Baseline values								
CON	3000	6.33 ± 0.82			6.33 ± 1.25			1.54 ± 0.24
IMI	3000	7.00 ± 0.82	0.069	0.793	7.33 ± 0.47	0.151	0.697	1.53 ± 0.02
IMI+ST	3000	6.33 ± 0.47	0.000	1.000	7.33 ± 0.94	0.151	0.697	1.49 ± 0.02
Two months of exposure								
CON	3000	7.00 ± 0.82			7.33 ± 0.47			1.46 ± 0.01
IMI	3000	14.33 ± 0.47	5.927	**0.015**	15.33 ± 0.47	6.687	**0.010**	1.47 ± 0.02
IMI+ST	3000	11.00 ± 0.82	1.960	0.162	12.33 ± 0.82	2.85	0.091	1.45 ± 0.01

*BN: Binucleated cells (for each rabbit 1000 BN cells were scored, three rabbits/group result in a total of 3000 BN cells scored), BNMN: Binucleated cells with micronuclei, MN: micronuclei, CBPI: Cytokinesis Block Proliferation Index, s.e.: standard error, p: statistical parameter, *p < 0.05, comparison made with the control group, G. 2POi ln(Oi/Ei), where Oi is the observed frequency in a cell, Ei is the expected frequency under the null hypothesis, ln denotes the natural logarithm and the sum is taken over all non-empty cells.*

The frequency of BNMN was increased compared to the controls after the 2-month exposure (Table 5) by 126% for IMI and 73% for IMI + ST, whereas regarding the frequency of MN, the increase reached 142% and 95%, respectively.

The CBPI showed no statistically significant difference between controls and exposed animals at any time of exposure ($p>0.05$), which indicates that there is no overall cytotoxic effect.

4.2 Comet assay

An increase in tail intensity (Fig. 3, see supplementary information section) was observed in both exposure groups after 2 months compared to controls (70.7% IMI group, 48.5% IMI+ST groups), although not statistically significant (Figure 4).

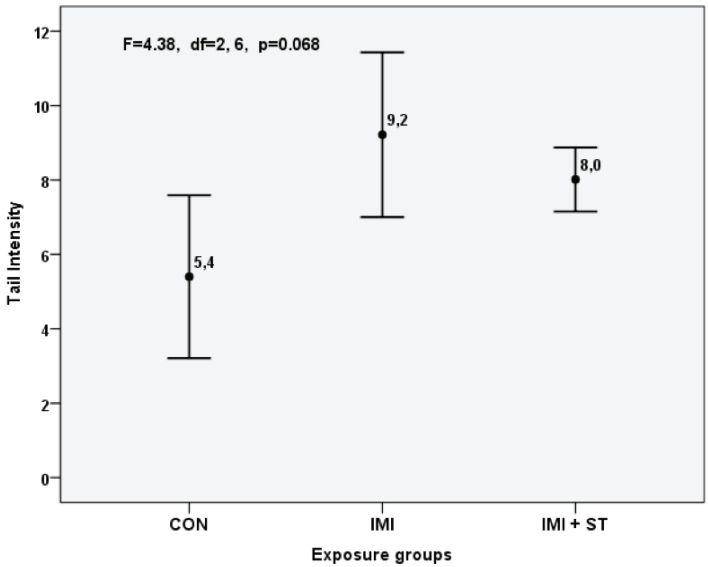

Fig.4 Comet assay results based on the tail intensity from the groups CON, IMI+ST and IMI.

4.4 Oxidative stress markers

The levels of oxidative stress markers for all studied groups are presented in Table 6. TAC and glutathione remained practically unchanged and unaffected by IMI exposure. A nearly significant increase (23% Crbnls) in protein oxidation accompanied by a decrease in lipid peroxidation (9% TBARS) was observed in the IMI group. Catalase activity was also non-significantly decreased (15%). This redox disturbance was not observed in the presence of ST (p=0.125).

Table 6. Markers of oxidative stress shown in mean ± standard deviation and statistical differences (p-values), between study groups (CON, IMI, IMI-ST).

OS MARKERS	Groups	N	Mean	SD	SEM	P
TAC (mmol DPPH/mL).	CON	3	0.62	0.09	0.05	0.573
	IMI	3	0.59	0.03	0.02	
	IMI+ST	3	0.64	0.02	0.01	
Catalase activity U/mg Hb)	CON	3	231.03	6.44	3.72	0.125
	IMI	3	196.73	15.45	8.92	
	IMI+ST	3	233.40	31.36	18.11	
TBARS (µmol/L)	CON	3	22.57	0.31	0.18	0.210
	IMI	3	20.70	1.25	0.72	
	IMI+ST	3	21.07	1.63	0.94	
Crbnls (nmol/mg protein)	CON	3	0.42	0.04	0.02	0.066
	IMI	3	0.52	0.05	0.03	
	IMI+ST	3	0.49	0.04	0.02	
GSH	CON	3	13.20	2.69	1.55	0.909
	IMI	3	14.10	2.66	1.54	
	IMI+ST	3	13.70	2.13	1.23	
Hb (g/dL)	CON	3	11.19	0.46	0.26	0.681
	IMI	3	10.24	2.37	1.37	
	IMI+ST	3	10.20	1.01	0.58	

4.5 Telomerase Activity

In Figure 5, telomere activity (%) does not differ significantly among exposed groups (ANOVA p=0.068, Kruskal-Wallis p=0.051). Nevertheless, the IMI+ST group showed the higher activity (37% increase in comparison to IMI group), probably indicating higher systemic inflammation levels.

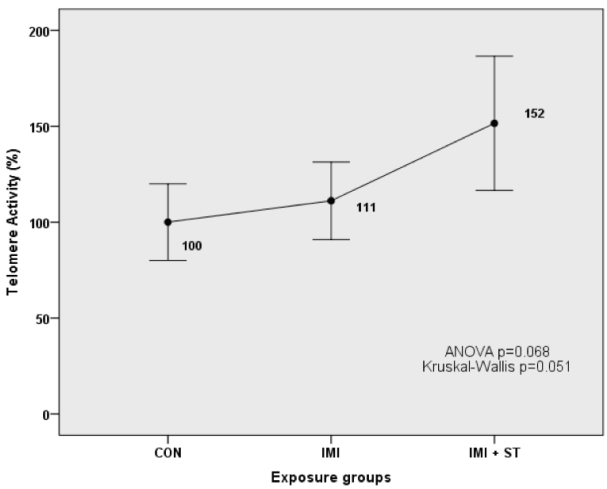

Fig.5. Telomerase activity from the groups CON, IMI and IMI+ST.

5. Discussion

In the present study, we investigated whether the AOX metabolic reduction of IMI is systemically more detrimental than the CYP oxidative metabolism thereof. Systemic damage was assessed by measuring genotoxic and cytotoxic effects, by assessing oxidative status of the animals, as well as the systemic inflammation state.

From the results, we can additionally note that the best matrix to observe IMI concentrations is via hair samples and for IMI-NH via urine samples, where we monitored the 24-hour urine production of the animals from the first week during the first month of administration and the same pattern was followed also for the second month. In order to calculate the excretion rate of IMI, IMI-NH and 6-CNA, the amount of urine excreted by an animal depends on many factors, including food and water consumption, activity and environmental temperature. A 24-hour urine volume of an adult rabbit, with free access to food and water, varies within a wide range (20–350 mL/kg-bw), with an average excretion rate of about 130 mL/kg-bw [29]. In our current study, the excretion rate fell between these levels with a urine excretion rate of 330 mL/day. The reduced metabolites of IMI (IMI-NH) are excreted first, while IMI as the parent compound, was excreted in urine and blood with increased concentrations by the end of the experiment. The oxidized IMI metabolites (6-CNA) are excreted steadily over the monitored 2-month period. The rate of excretion of IMI in urine samples increased slightly at the end of the 2-month period; while IMI-NH after the second month was reduced dramatically in the urine, obviously from the AOX inhibitory effect of ST, and in the hair samples because hair as a matrix cannot show the excretion rate of a substance but only the rate of accumulation.

The AOX-generated IMI metabolites are not all regarded detoxification products. IMI-NH is assumed to be a possible contributor to the nicotinic effects of IMI. IMI-NH can become over 300 times more potent than IMI at the mammalian nAChR and when referring to mouse toxicity, it can also be increased many times as studies have shown [6, 30, 31]. AOX is a potentially important factor in drug metabolism and ST treatments have provided a way to reduce AOX activity in vivo in mammals.

Frequent in vitro studies have revealed the contribution of cytochrome P450s and AOX in IMI oxidation and IMI reduction, respectively. IMI is oxidized to the 5-hydroxy and olefin metabolites and reduced to the nitrosoguanidine, aminoguanidine and IMI-urea metabolites by human CYP isozymes [32, 33]. Nitroreduction reactions are possible also with rabbit liver cytosol independently of NADPH [34, 32]. AOX activity can be different between members of a single taxonomical species, or within a species, and can occur among species. This intra-inter species variations could, therefore, affect the metabolism and detoxification processes of such neonicotinoids.

A study on rabbits by Stivaktakis [35] found that after a longer period of exposure to IMI than in our study, the genotoxic effect reached a plateau even in low dose groups, whereas we did not notice a plateau effect, but instead a noticeable MN increase, indicating a possible cumulative stressful effect that could lead to DNA damage with the CBPI index measured, however, to be lacking an overall cytotoxic effect for IMI and IMI+ST groups. IMI, besides this study in rabbits, has also been studied in loaches for which IMI had a genetic toxic effect on erythrocytes [36], and on the Argentinian frog for which signs of genotoxicity were also present [37]. Furthermore, the genotoxicity of IMI was found to have a potential adverse effect in human peripheral blood samples [38], but studying the relation of the metabolic activation and composition of a commercial product containing IMI on human peripheral blood samples did not show genotoxicity at a specific given dose [39].

The oxidative as well as the inflammatory status of mammals exposed to neonicotinoid insecticides, such as IMI, have been studied often. It is believed that insecticides affect the vital immune mechanisms and can induce various inflammatory conditions [40]. Oxidative stress has been also studied in rabbits following long-term exposure to diazinon and propoxur, showing a discrete and concentration-dependent effect in the liver and kidney [41].

Evaluation of the possible genotoxic and cytotoxic effects in human peripheral blood lymphocytes exposed in vitro to thiacloprid and clothianidin showed that at high concentrations they significantly reduced human lymphocyte viability and eventually caused cell death [42]. Possible cytotoxic and DNA damage effects of common neonicotinoid insecticides acetamiprid, clothianidin, IMI, thiacloprid and thiamethoxam in hepatocellular carcinoma (HepG2) and neuroblastoma (SH-SY5Y) cells showed changes in DNA damage at the high concentrations of these neonicotinoids after a 24-hour exposure by using the alkaline comet assay in HepG2 and SH-SY5Y cells [43]. Neonicotinoids not only control insect pests but also, independently, alter plant growth and responses to stress. IMI, thiacloprid, acetamiprid, thiamethoxam and clothianidin, but not nitenpyram and dinotefuran, induced foliar lesions and peroxidative damage in soybean (Glycine max) seedlings assayed with the 3,30-diaminobenzidine stain [44].

6. Conclusions

The results of the present study clearly demonstrate that IMI induces a clastogenic effect in exposed rabbits and that this effect is attenuated by co-administration of the AOX inhibitor ST. This highlights the AOX-mediated metabolism of IMI to IMI-NH as a prominent pathway to the mutagenic effects of this pesticide, in detriment to the CYPs-mediated metabolism.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Supplementary Information

Table 1

Study hypothesis flowchart: to elucidate the severity of the added toxicity from the AOX and the CYP metabolic pathways, in IMI exposed rabbits.

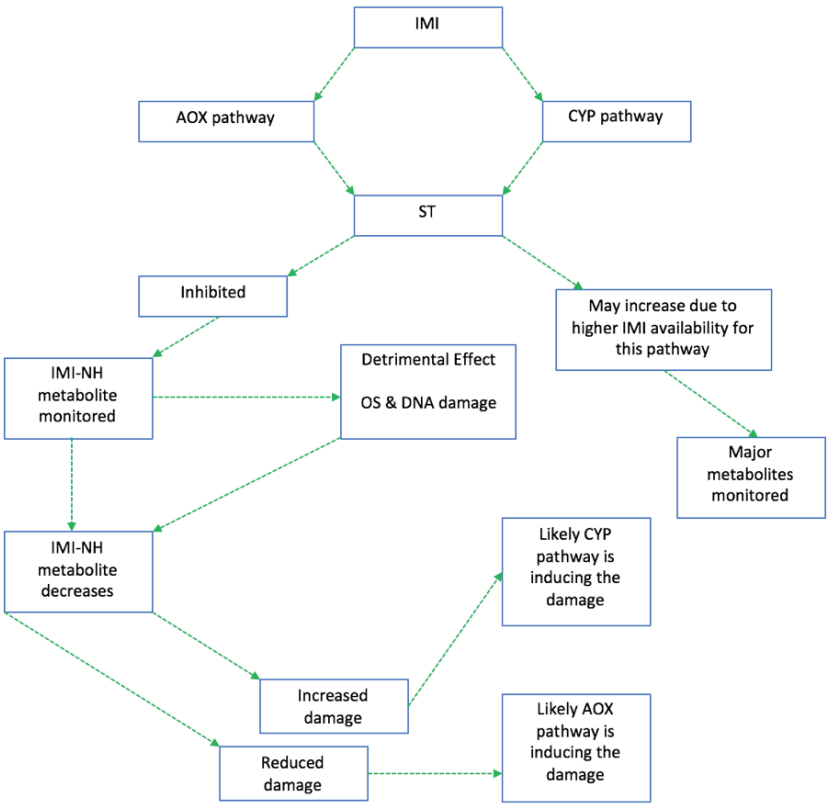


Table 2

Administration dosages of all experimental groups.

Experimental Group	Administration	Dose	
		IMI (mg/kg bw/per day)	ST (mg/mL/per exposure day)
Control Group (CON)	Tap Water	-	-
Imidacloprid (IMI)	Imidacloprid	30	-
Imidacloprid + Sodium Tungstate Dihydrate (IMI +ST)	Imidacloprid + Sodium Tungstate	30	2

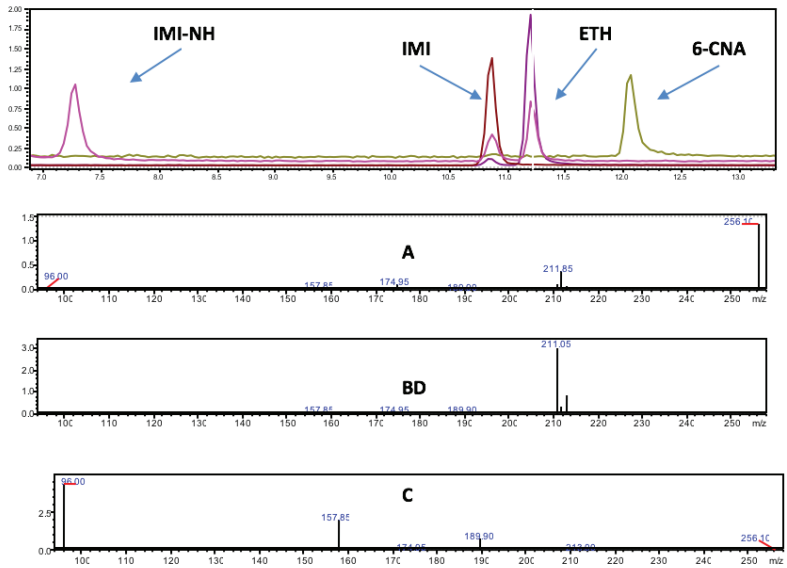


Fig.1 Chromatograms of standard solutions IMI, 6-CNA and IMI-NH at 500 ng/mL, and the spectra of IMI (A), IMI-NH (B) and 6-CNA (C).

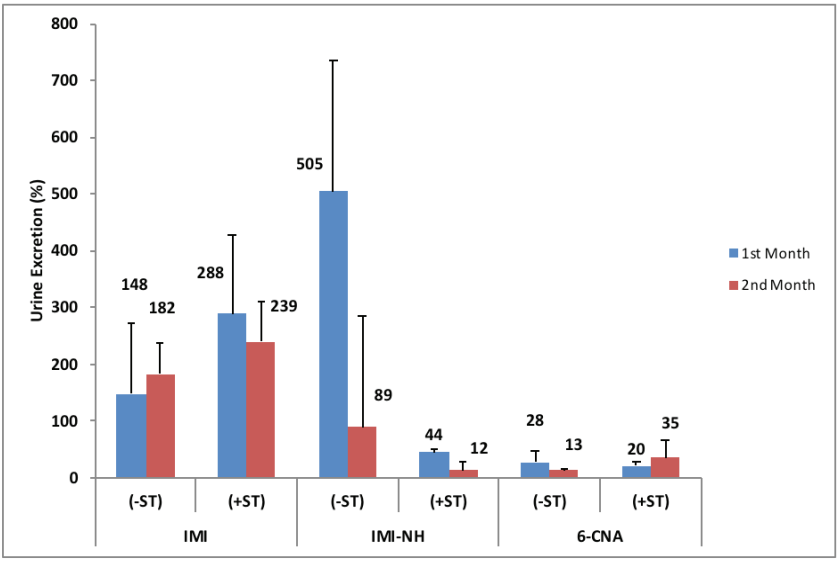
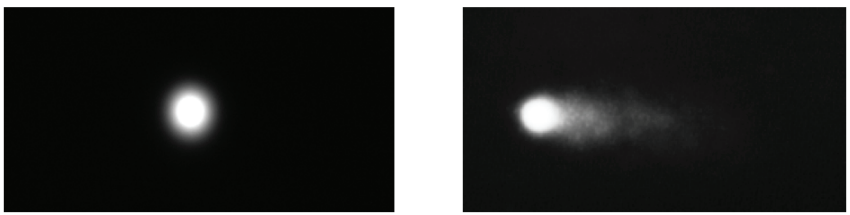


Fig.2 Excretion rate of IMI, IMI-NH and 6-CNA in the urine matrix.



A. B.

Fig.3. A picture of a cell from the CON group (A), and a picture of the 'comet' from the IMI group (B), both taken during the scoring process of the comet assay technique. Clearly, the increased damage to the cell is shown in the IMI group, hence the increased tail intensity and length, giving the known resemblance of a 'comet' (B).

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CHAPTER 5

General Discussion and Conclusions

1. Downgrading the systemic condition of rabbits after long-term exposure to cypermethrin and piperonyl butoxide

Through this study we were able to investigate the systemic condition of New Zealand rabbits, focusing on the systemic redox balance, namely TAC, CARB's, Hb, GSH, TBARS and the telomerase activity in PBMCs (peripheral blood mononuclear cells), after long-term exposure to CY and PBO. Telomerase is a target for regulatory mechanisms, while highly susceptible to oxidative stress [11], and alterations on telomerase activity could have clinical implications [10]. Monitoring inflammation can be achieved among others, either by measuring multifunctional pro-inflammatory cytokines, like TNF-α (tumor necrosis factor alpha), and interleukins (IL), a very sensitive but rather unspecific approach, or white blood cell count (WBC) and leukocyte subpopulations, such as PMBCs, proliferation and activity, directed according to some researchers more to systemic inflammation [33].

In our study, telomerase activity of CY groups exhibited a significant increase in low and high dose groups compared to the control groups. To our knowledge, this is the first study that has shown that PBO exposure can also cause an increase in telomerase activity in PMBCs, for both low and high dose groups, even higher compared to the CY groups. PBO alone produces higher levels of telomerase activity compared to CY alone, although their combination results in lower levels compared to PBO alone. This could be explained by the fact that when both the pesticide and the biocide are present, there is an interference in the action of CY, possibly by the designed mode of action of PBO that creates conditions that lead to CY's reduced metabolism and hence to reduced inflammatory contribution. This would also explain why when PBO is administered alone there is more significant damage. Oxidative stress markers suggest that CY, PBO and the co-exposed groups, induce oxidative stress as well as induction of telomerase activity. The observed results for TBARS, GSH, Hb, TAC, Crbnls and CAT from our exposed groups showed altered levels compared to control groups that could be linked to doses and combined effects of each chemical substance.

TBARS are byproducts of lipid peroxidation and when there are high levels of reactive oxygen species (ROS) an increase in lipid peroxidation is observed. In the present study, TBARS were not affected significantly after either CY or PBO administration.

The glutathione (GSH) antioxidant system plays an important role in cellular defense against reactive pro-oxidant products [43]. However, the results showed no effect at any dose, except for PBO that caused a depletion compared

to the control groups in a dose dependent manner.
Hemoglobin (Hb) is a complex protein not often used as an indicator of OS alone but used additionally due to its connection with elevated OS levels. CY exposure did indeed cause a reduction in our study but PBO alone increased levels above those of the control groups. From the co-exposed groups of CY and PBO (low and high doses) there is a tendency of PBO to inhibit damage that CY causes, meaning that the addition of PBO in this case works to counteract the depletion of Hb levels. In our study, Hb levels were lower than control levels for CY alone and higher with PBO alone. Thus, the addition of PBO did in fact result in elevated Hb levels for the co-exposed groups.
The use of CARBs as biomarkers of OS have some advantages in comparison with the measurement of other oxidation products but no differences were noticed based on given doses, combined action or by independent action. Only in the CY low dose group there was a tendency to increase.
Catalase activity (CAT) is an important cellular antioxidant enzyme. Control groups compared to CY low-dose groups showed a significant difference but compared to the co-exposed groups regarding high levels of exposure, there is a noticeable tendency to become significant as well. The increase in CAT may contribute as a rescue mechanism against tolerable oxidative stress conditions. However, CY and PBO combined at high levels of exposure decreased CAT activity significantly compared to the exposed groups of CY (low and high), suggesting a role of CY metabolism in this effect. This finding could suggest that when CY and PBO are combined, they induce more severe OS than each of them alone.
TAC gives information about the capability of averting negative effects of oxidative stress. In general, increase in TAC means enhancement of the antioxidant mechanisms. We observed that both CY doses increased TAC significantly compared to the control groups, although this increase in TAC, may be explained as a rescue mechanism in order to counteract the CY induced oxidative stress. Namely, exposure to CY at the doses used may cause a mild oxidative stress that in turn induced antioxidant mechanisms, which protected lipids or proteins from oxidative damage. CY and PBO low dose increased TAC levels compared to the control groups. As mentioned above for CY, this increase in TAC might have prevented increases in TBARS and CARB levels. However, high dose PBO exhibited a tendency for decrease in TAC levels. This could be explained by the fact that PBO high dose induced more severe OS compared to low dose, leading to depletion of the antioxidant molecules. The significant decrease in GSH levels following exposure to high dose PBO supported the high OS occurring in that case. Co-exposure to CY and PBO at low doses did not affect TAC levels, suggesting that the two compounds in combination may induce a pro-oxidant effect sufficient to eliminate the TAC compensatory outcome. Moreover, like exposure to PBO high dose, co-exposure to CY and

PBO at high doses showed a tendency for decrease in TAC, indicating that there was an even more severe pro-oxidant effect than that achieved by low doses, resulting in decreased levels of antioxidant molecules.
The significant decrease in GSH levels after co-exposure to high doses CY and PBO supports the previous assumption. Similarly, the significant decrease in CAT after co-exposure to CY and PBO, compared to CY exposure alone, supports that more severe OS is occurring when the two compounds are combined due to possibly a combined effect between them. The observed results for TBARs, GSH, Hb, TAC, CARBs and CAT showed that CY and PBO deteriorate the oxidative and inflammatory status via different mechanisms of action. PBO present in animals co-exposed to CY and PBO have a noticeable counteraction of CY's pro-oxidant effects probably through its effect on CY metabolism and therefore PBO action prevails with regards to systemic inflammation.

2. Long-term exposure to cypermethrin and piperonyl butoxide cause liver and kidney inflammation and induce genotoxicity in New Zealand white male rabbits

As noted in Chapter 3, the ability of CY and PBO exposure to downgrade the systemic condition of New Zealand rabbits was additionally evaluated based on genotoxicity and cytotoxicity studies from monitoring and measuring binucleated cells with micronuclei (BNMN), micronuclei (MN), the cytokinesis block proliferation index (CBPI) in lymphocytes and by histopathological examination of lesions. Both CY and PBO caused liver and kidney inflammation, besides many other organs examined, and induced genotoxicity.
CY and PBO when administered alone were found to increase micronuclei and binucleated cells after their exposure period but from the combinations of the two substances a noticeable cumulative effect was present, indicating, in that way, that the greater amount of damage could be justified from the addition of each substance's toxicity, with the effect of PBO being higher than that of CY when administered alone. This assay applied is a very effective tool for the prediction of the genetic risk posed by exposure to chemicals, including pesticides.
It is worth noting that the CBPI measured in our study indicated a lack of cytotoxic effect in general for all the exposed groups. It is well known that the liver plays an important role in many essential functions of basic metabolism and that the kidney also plays a pivotal role in facilitating the elimination of pesticide residues from the mammal's body. Our results showed that the long-term exposure of rabbits to CY and PBO, at relatively low doses, caused histopathological lesions, such as inflammation and dilatation of the sinusoids in the liver as well as prominent kidney damage, with chronic inflammatory infiltrations and hyperemia.
As was mentioned earlier it is possible that the level of exposure might also be

affecting the level of damage and that the liver and kidney are more susceptible organs. A number of in vivo studies reporting histopathological lesions of the liver and kidneys have been conducted regarding exposure to PBO, primarily on rats and mice, but rarely on rabbits. Most studies on the effects of CY and PBO have focused on rats or mice, while studies focusing on rabbits are few, and to the best of our knowledge, histopathological lesions have not been extensively studied in rabbits. Our study shows that kidney tissues of rabbits exposed to PBO LD and PBO HD had interstitial inflammation, with PBO LD exhibiting also acute inflammation focally and topically. In the kidney samples of the CY. PBO HD groups, focal inflammation, lymphoplasmatocytic infiltration and a small inflammation near the pelvicalyceal system was observed, obviously being the most damaged tissue sample compared to the other organ tissues sampled.
In all cases the general systemic condition of the animals appeared impaired. We conclude that PBO as an addition to CY could cause a potential danger even in cases of absence of CY. PBO is generally known to be an innocent additive, however our results indicate that it poses a significant potential danger to human health.

3. The metabolism of imidacloprid by aldehyde oxidase contributes to its clastogenic effect in New Zealand rabbits

Chapter 4 investigated whether the AOX metabolic reduction of IMI is systemically more detrimental than the CYP oxidative metabolism thereof. Systemic damage was assessed by measuring genotoxic and cytotoxic effects, by assessing oxidative status of the animals, as well as the systemic inflammation state.
From the results, we can additionally note that the best matrix to observe IMI concentrations is via hair samples and for IMI-NH via urine samples, where we monitored the 24-hour urine production of the animals from the first week during the first month of administration and the same pattern was followed also for the second month. In order to calculate the excretion rate of IMI, IMI-NH and 6-CNA, the amount of urine excreted by an animal depends on many factors, including food and water consumption, activity and environmental temperature. A 24-hour urine volume of an adult rabbit, with free access to food and water, varies within a wide range (20–350 mL/kg-bw). In our current study, the excretion rate fell between these levels, with a urine excretion rate of 330 mL/day. The reduced metabolites of IMI (IMI-NH) are excreted first, while IMI as the parent compound, was excreted in urine and blood with increased concentrations by the end of the experiment. The oxidized IMI metabolites (6-CNA) are excreted steadily over the monitored 2-month period. The rate of excretion of IMI in urine samples increased slightly at the end of the 2-month period; while IMI-NH after the second month was reduced dramatically in the urine, obviously from the AOX inhibitory effect of ST, and in the hair samples

because hair as a matrix cannot show the excretion rate of a substance but only the rate of accumulation.

However, the AOX-generated IMI metabolites are not all regarded detoxification products. AOX alone is a potentially important factor in drug metabolism and ST treatments have provided a way to reduce AOX activity in vivo in mammals. Frequent in vitro studies have revealed the contribution of cytochrome P450s and AOX in IMI oxidation and IMI reduction, respectively. IMI is oxidized to the 5-hydroxy and olefin metabolites and reduced to the nitrosoguanidine, aminoguanidine and IMI-urea metabolites by human CYP isozymes. Nitroreduction reactions are possible also with rabbit liver cytosol independently of NADPH. AOX activity can be different between members of a single taxonomical species or within a species, and can occur among species. This intra-inter species variations could, therefore, affect the metabolism and detoxification processes of such neonicotinoids.

A genotoxic effect was noticed regarding an MN increase, indicating a possible cumulative stressful effect that could lead to DNA damage with the CBPI index measured, however, to be lacking an overall cytotoxic effect for IMI and IMI+ST groups. The oxidative as well as the inflammatory status of mammals exposed to neonicotinoid insecticides, such as IMI, have been studied often. Neonicotinoids not only control insect pests but also, independently, alter plant growth and responses to stress.

The results of the present study clearly demonstrate that IMI induces a clastogenic effect in exposed rabbits and that this effect is attenuated by co-administration of the AOX inhibitor ST. This highlights the AOX-mediated metabolism of IMI to IMI-NH as a prominent pathway to the mutagenic effects of this pesticide, in detriment to the CYPs-mediated metabolism.

4. Final conclusions

- It was found that CY and PBO deteriorate the oxidative and inflammatory status of animals via different mechanisms of action. PBO present in animals co-exposed to CY and PBO have a noticeable counteraction of CY's pro-oxidant effects probably through its effect on CY metabolism and therefore PBO action prevails with regards to systemic inflammation.
- PBO as an addition to CY could cause a potential danger even in cases of absence of CY. PBO is generally known to be an innocent additive, however our results indicate that it poses a significant potential danger to human health.
- The results of the present study also clearly demonstrate that exposure to the neonicotinoid insecticide IMI induces a clastogenic effect in exposed rabbits and that this effect is attenuated by co-administration of the AOX inhibitor ST. This highlights the AOX-mediated metabolism of IMI to IMI-

NH as a prominent pathway to the mutagenic effects of this pesticide, in detriment to the CYPs-mediated metabolism.

5. Future perspectives

- PBO is generally thought to be an innocent additive, however our results indicate that it poses a potential danger to human health. Further studies are needed to clarify this and to develop ways of ascertaining potential interactions between pesticides and biocides resulting in modifications to system response to their mixture.
- Our results show a statistically significant increase of micronuclei and binucleated cells with micronucleus after a few-months exposure to CY, PBO and combinations of the two, suggesting a cumulative stressful effect that culminates in DNA damage. Further studies are required to evaluate the implications of such effects on public and environmental health resulting from the application of pesticides formulated with CY and PBO.
- The results of the present study also clearly demonstrate that IMI induces a clastogenic effect in exposed rabbits and that this effect is attenuated by co-administration of the AOX inhibitor ST. This highlights the AOX-mediated metabolism of IMI to IMI-NH as a prominent pathway to the mutagenic effects of this pesticide that need to be ascertained by further studies.

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List of Publications

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Tsiaoussis, Athanasios K. Alegakis, Christina Tsitsimpikou, Valerii N. Rakitskii, Félix Carvalho, Aristidis M. Tsatsakis, Long-term exposure to cypermethrin and piperonyl butoxide cause liver and kidney inflammation and induce genotoxicity in New Zealand white male rabbits, Food and Chemical Toxicology, Volume 94, 2016, Pages 250-259.
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8. Nikolaos Georgiadis, Konstantinos Tsarouhas, Christina Tsitsimpikou, Alexander I. Vardavas, Ramin Rezaee, Ioannis Germanakis, Aristides Tsatsakis, Dimitrios Stagos, Demetrios Kouretas, Pesticides and cardiotoxicity. Where do we stand?, Toxicology and Applied Pharmacology, Volume 353, 2018, Pages 1-14.

List of Abstracts/Posters

- Matthaios P. Kavvalakis, Manolis N. Tzatzarakis, Konstantina K. Vorou, Alexander I. Vardavas, Polychronis Stivaktakis, Andreas K. Tsakalof and Aristidis M. Tsatsakis. Determination of metabolites of cypermethrin in urine of exposed rabbits using gas chromatography-mass spectrometry, IMA, Thessaloniki 2013 (poster).
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