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

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ABSTRACT

Cypermethrin (CY) is a frequently used 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 four 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 at 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

Abbreviations: CY, Cypermethrin; CON, Control; 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; 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, labeling, and packaging; IGHRC, The intergovernmental group on health risks from chemicals.

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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, 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 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, 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 which 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-h dark/light cycle and a steady ambient temperature between 20 and 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 microgram per milliliter 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) can 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:where M_1, M_2, M_3, M_4 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 (Surallès et al., 1995).

2.3. Histopathological lesions

Tissue block samples, embedded in paraffin and sectioned at 4 μm, were stained with H&E (eosin and hematoxylin), 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-square 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 barcharts 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,

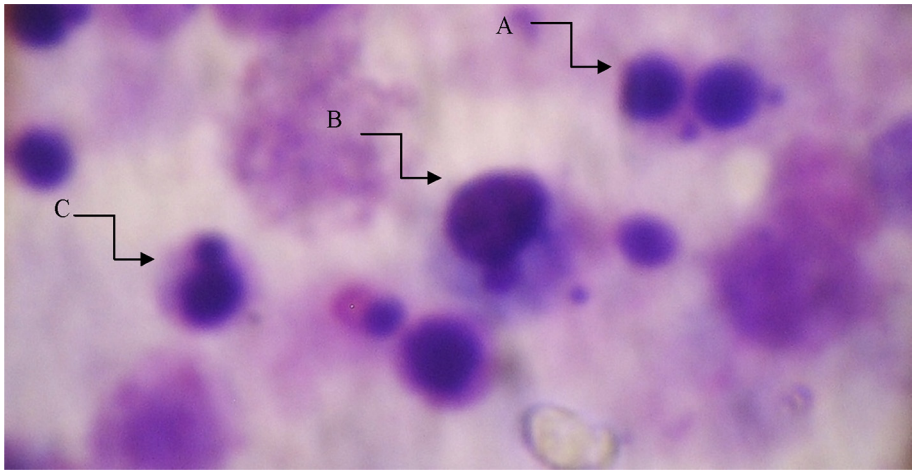

Fig. 1. A microscopic picture taken during the scoring process (100×/1.25, ∞/0.17 WD 0.23). (A). Binucleated cell with two micronuclei. (B and C) Mononucleated lymphocyte cells with present MN. According to the methods of this assay, such MN are not considered measurable.

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								
CON	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								
CON	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								
CON	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, $G = 2\sum O_i \, ln(O_i/E_i)$, where O_i is the observed frequency in a cell, E_i is the expected frequency under the null hypothesis, *ln* denotes the natural logarithm and the sum is taken over all non-empty cells.

300% for CY HD, 310% for CY+PBO LD and 442% for CY+PBO HD. After four 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 two to four 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 two 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 two 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 from 4% to 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.

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 CON 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 CON 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. They found various symptoms, although not for all of the rabbits and not connected with any clinical signs of the rabbits throughout the experiment. In the CON group only one of the three specimens had a larger right kidney (25.3gr compared to the others of the same group that were 12.6gr and 13.1gr). 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 sub mucosa (Fig. 4B) but the last rabbit didn't 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 (10.2gr compared to the others of the same group that were 13.7gr and 12.5gr). Similarly 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 sub mucosa 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 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

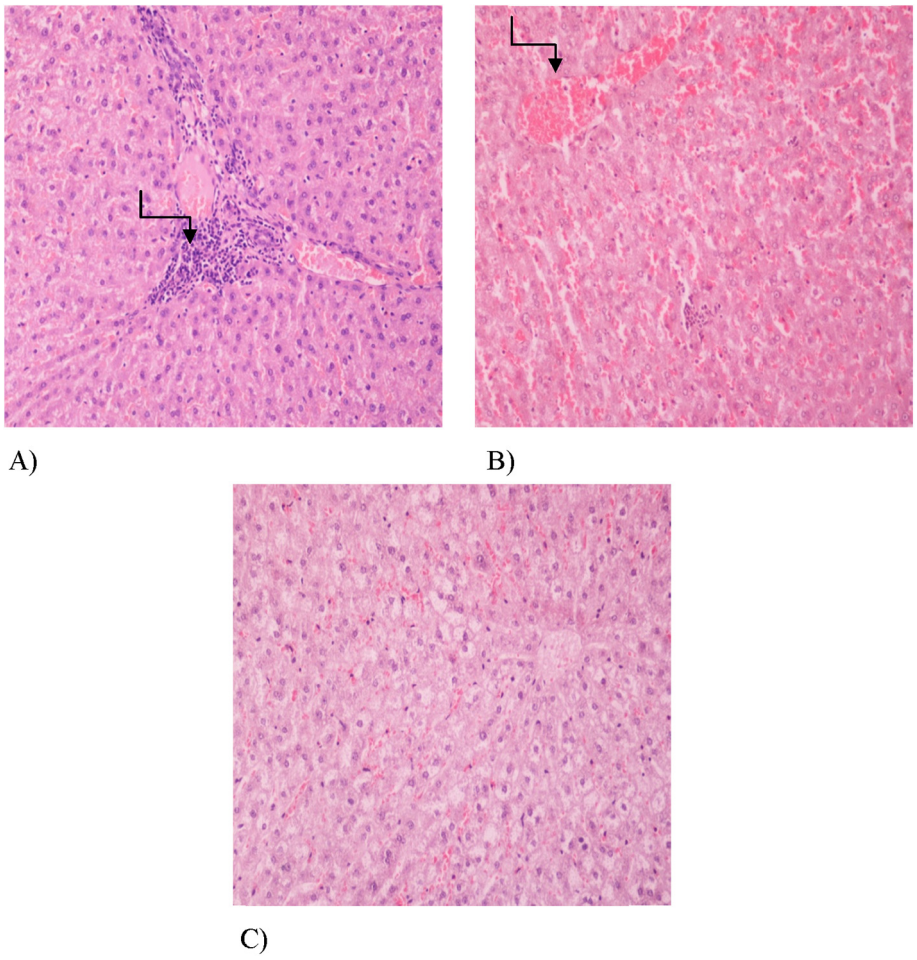

Fig. 2. A) Liver tissue from the CY HD group showing the portal triads with clear signs of inflammation. B) Liver tissue from the PBO HD group showing the dilatation of the sinusoids with erythrocytes. C) Liver tissue from the CON group.

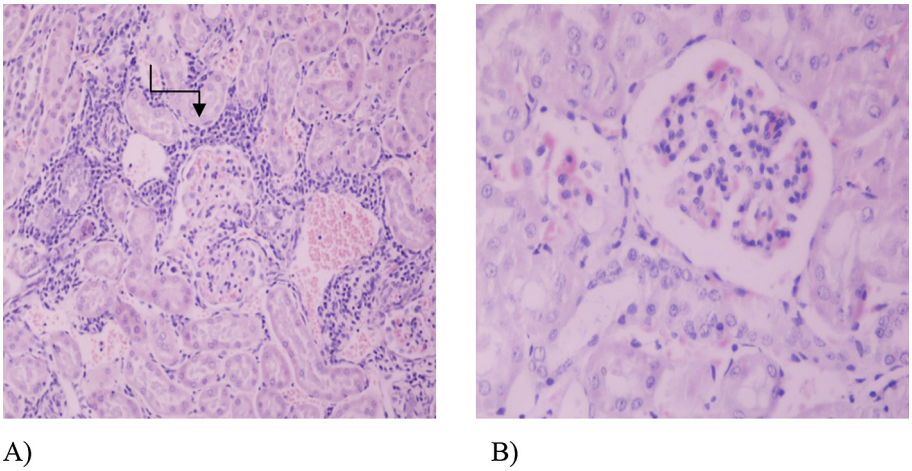

Fig. 3. A) Kidney tissue from the CY + PBO HD group showing inflammatory infiltrations. B) Kidney tissue from the CON group.

husbandry personnel most of these symptoms are possibly due to slight infections or to the fodder consumed.

3.4. Body and organ weight changes

All exposed rabbits to CY, PBO and CY+PBO as well as for the CON groups do 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 of which the weight increased did not change significantly ($p = 0.211$) (Fig. 5.). Most of the organs during autopsy (Table 4) did not show any significant

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

Group	Rabbit no	Pathological findings
		Liver
CON	1	Minor inflammation in the portal triad
CON	2	No alterations
CON	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 interlobule 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
CON	1	No alterations
CON	2	Renal tubes with calcium deposits, focal inflammation
CON	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, lymphoplasmatocytic infiltration, small inflammation near the pelvicalyceal system
		Lymph nodes
	All	**No alterations were noticed for any of the tested groups.**

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 finally c) liver where all exposed groups −except PBO HD group had significantly lower weight values than controls ($p < 0.001$).

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

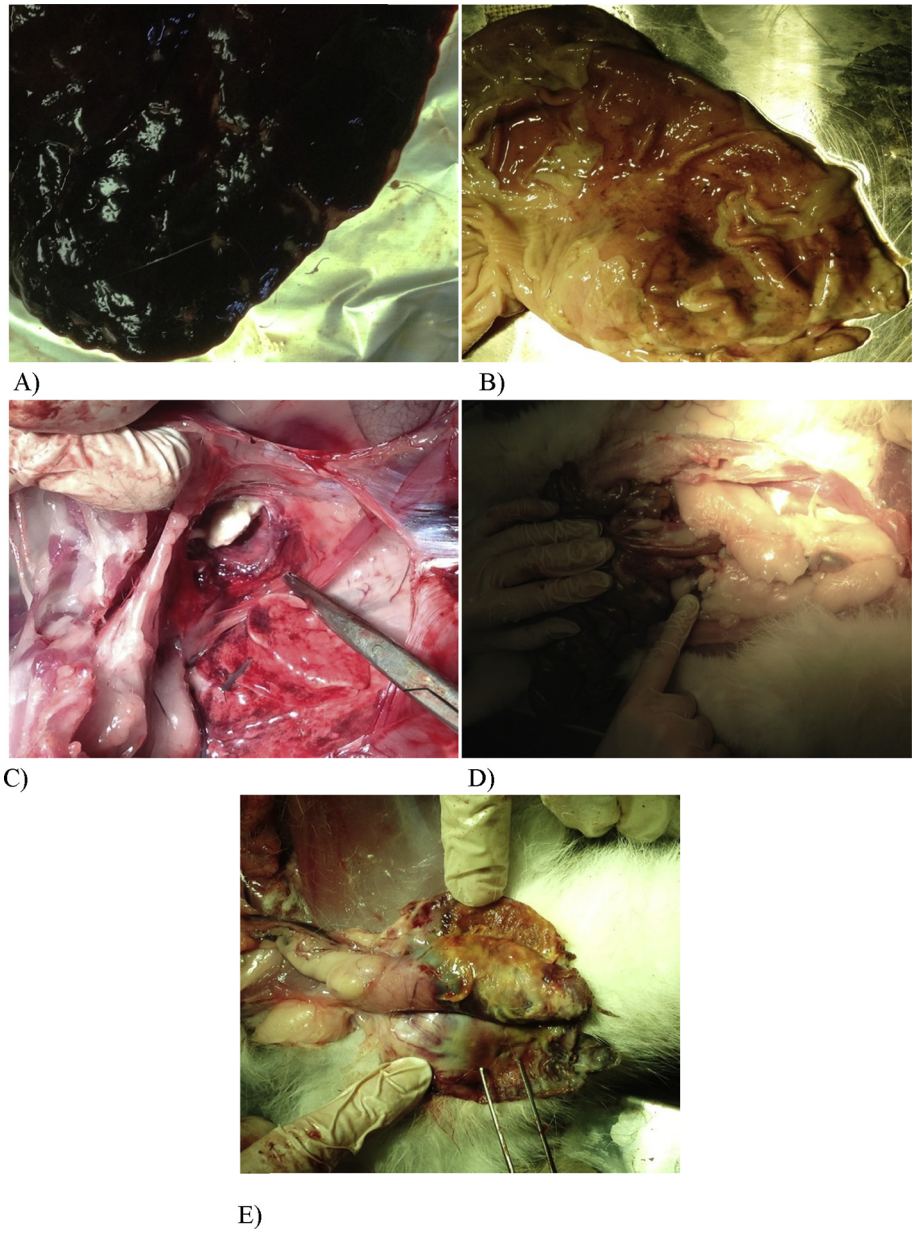

Fig. 4. A) White specks on the liver of CY LD group rabbit. B) Signs of bleeding in the sub mucosa and easy detachment of the mucosa of the stomach found in CY LD and PBO LD groups. C) The abscess noticed in the PBO HD group. D) Increase of body fat noticed in the CY HD, PBO LD and PBO HD groups. E)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

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

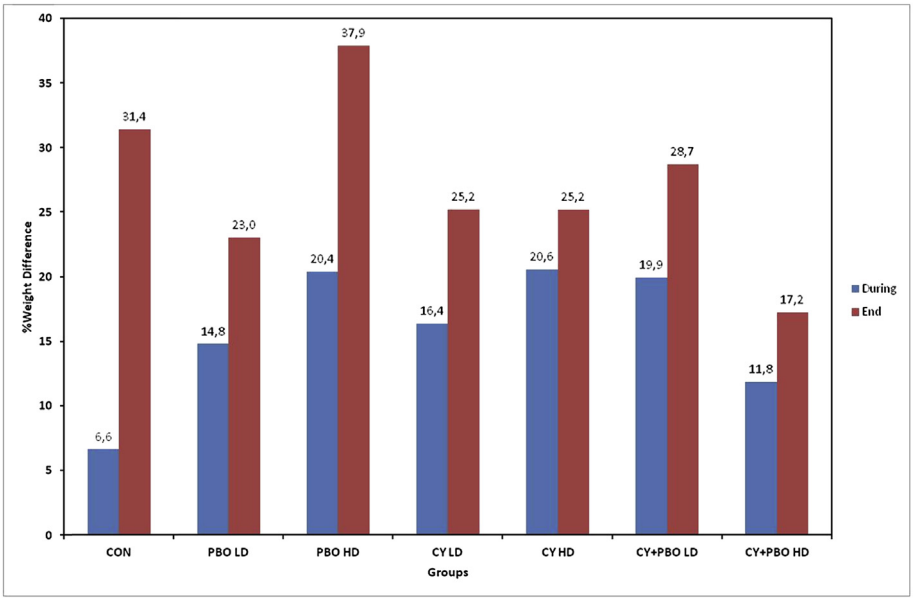

Fig. 5. %Weight differences of rabbits from baseline (start of experiment) at during and end of experiment time points.

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.

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) [a] (rabbits)	No alterations	No alterations

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

tubules, hypertrophy of glomeruli and leucocytic infiltrations in the liver and kidneys (Sarhan and Sarhhaf, 2011).

Mitsumori et al. (1996) studied atrophic changes in lymphohemapoietic 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 lymphohemapoietic 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 was observed, obviously being the most damaged tissue sample compared to the other organ tissues sampled.

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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Conflict of interest

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

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