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Evaluation of chlorpyrifos toxicity through a 28-day study: Cholinesterase activity, oxidative stress responses, parent compound/metabolite levels, and primary DNA damage in blood and brain tissue of adult male Wistar rats

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ABSTRACT

In this 28 day-study, we evaluated the effects of the insecticide chlorpyrifos orally administered to Wistar rats at doses 0.160, 0.015, and 0.010 mg/kg b. w./day. Following treatment, total cholinesterase activity and activities of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) were measured. Oxidative stress responses were evaluated using a battery of endpoints to establish lipid peroxidation, changes in total antioxidant capacity, level of reactive oxygen species (ROS), glutathione (GSH) level and activities of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD) and catalase. Using HPLC-UV DAD analysis, levels of the parent compound and its main metabolite 3,5,6-trichloro-2-pyridinol in plasma and brain tissue were measured. The genotoxic effect was estimated using alkaline comet assay in leukocytes and brain tissue. The exposure did not result in significant effects on total cholinesterase, AChE and BChE activity in plasma and brain tissue. Lipid peroxidation slightly increased both in plasma and brain tissue. Total antioxidant capacity, ROS and GSH levels were marginally influenced by the exposure. Treatment led to significant increases of GSH-Px activity in blood, SOD activity in erythrocytes and a slight increase of catalase activity in plasma. HPLC-UV DAD analysis revealed the presence of both the parent compound and its main metabolite in the plasma of all of the experimental animals and brain tissue of the animals treated at the two higher doses. All of the tested doses of chlorpyrifos were slightly genotoxic, both to leukocytes and brain tissue. Our results call for further research using other sensitive biomarkers of effect, along with different exposure scenarios.

1. Introduction

Chlorpyrifos (O,O-diethyl-O-3,5,6-trichloro-2-pyridyl phosphorothioate) is an organophosphate insecticide that plays a key role in pest management around the world. Considering the frequent use of chlorpyrifos-based pesticides to control insects in a wide range of crops, fruits and vegetables, its toxicity profile has gained the attention of numerous toxicological studies.

It has been well-established that the compound, once ingested, is rapidly absorbed and widely distributed in a mammalian organism, with low potential for accumulation. It is almost completely excreted after 48 h, mainly via urine [1]. The acute oral LD_{50} for rats is 66–195 mg/kg b. w [1]. Following oral exposure, chlorpyrifos is metabolized mainly in the liver by CYP450 enzymes via chlorpyrifos-oxon to 3,5,6-trichloro-2-pyridinol [2–5]. Although long considered less toxic than its parent compound, 3,5,6-trichloro-2-pyridinol metabolite

Abbreviations: AChE, acethylcholinesterase; BChE, butyrylcholinesterase; ROS, reactive oxygen species; GSH, glutathione; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase; HPLC-UV DAD, high performance liquid chromatography with UV diode array detector; ADI, acceptable daily intake; AOEL, acceptable operator exposure; ARfD, acute reference dose; MRL, maximum residue level; TBARS, thiobarbituric reactive substance; TAC, total antioxidant capacity; TPTZ, 2,4,6-tri[2-pyridyl]-s-triatine; DCF-DA, 2′,7′-dichlorofluorescin diacetate; MBCI, monochlorobimane

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was recently found hepatotoxic and nephrotoxic in mice [6]. Thus, from a toxicological point of view, both the parent compound and its metabolites pose risk for animals, plants and the environment [1].

Although it predominantly produces neurotoxic effects, and acetylcholinesterase inhibition is considered the main mechanism of chlorpyrifos toxicity [2,7–10], several other mechanisms also contribute to its toxic action. Chlorpyrifos elicits oxidative stress in various cell/tissue types [11–16], which results in the formation of free radicals, lipid peroxidation and impairments in the antioxidant system [17–21]. It alters mRNA expression profiles in brain cells [22], inhibits protein synthesis [23] and DNA synthesis, both *in vitro* [24] and *in vivo* [25]. Several studies suggested a genotoxic potential of chlorpyrifos [26–32]. A few animal studies reported histopathological changes in liver [26,33–35] and kidney [33] after exposure to chlorpyrifos. Chlorpyrifos also acts as the endocrine disruptor which alters the effects of estrogenic, androgenic, thyroid and parathyroid hormones [36,37]. Even a single high dose of chlorpyrifos (250 mg/kg) may cause behavioural effects in rats [38]. Finally, maternal exposure to chlorpyrifos in rats leads to neurobehavioral impairments in the offspring [39].

Residential human exposure to chlorpyrifos occurs mostly through ingestion of its residues from food commodities, but may also include inhalation and dermal routes, which are both more important for occupational exposure [5]. Owing to its lipophilic character, chlorpyrifos is efficiently absorbed through the skin [40,41], which makes it hazardous in occupational exposure. All of these facts indicate a need for gaining a better understanding of chlorpyrifos toxicity and point toward the need for its safe and controlled use.

The latest conclusion on human health risk assessment of the active substance chlorpyrifos, drawn by the European Food Safety Authority, was published in 2014. It proposed following reference values for chlorpyrifos: acceptable daily intake (ADI) of 0.001 mg/kg b. w./day, acceptable operator exposure level (AOEL) of 0.001 mg/kg b. w./day, and acute reference dose (ARfD) of 0.005 mg/kg b. w./day [1].

The European Union (EU), USA and other state regulatory bodies established maximum residue levels (MRLs) for active compound chlorpyrifos in various commodities. In 2016, the EU concluded that the current MRLs of chlorpyrifos for some commodities may raise concern among consumers and established new ones. They proposed lowering the MRLs especially for mandarins, apples, pears, peaches, table grapes, blackberries, raspberries, currants, gooseberries, kiwi, pineapples, potatoes, tomatoes, peppers, aubergines, melons, watermelons, head cabbage, chinese cabbage, globe artichokes, leek and sugar beet [42].

Although many adverse effects of chlorpyrifos exposure are already known or have been documented in evaluation reports and/or official papers issued by international regulatory or government bodies such as the European Food Safety Authority [1], United States Environmental Protection Agency [43], World Health Organisation [44], or the Australian National Registration Authority for Agricultural and Veterinary Chemicals [45], most recent animal studies relied on experiments in which the compound was applied at rather high doses. Furthermore, much more is known about the acute exposure, while the outcomes of sub-chronic low-level exposure have thus far not been documented well.

To more reliably assess possible adverse effects of chlorpyrifos, we considered a wide battery of biomarkers which enables insight in pesticide mode of action from different points of view. Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are both, enzymes responsible for signal transmition in nervous system. They both perform the breakdown of choline esters that function as neurotransmitters. Inhibition of their activity is a measure of organophosphorous pesticides poisoning [46]. However, it has been proved that other cells than neurons express acethycholine or butyrilcholine receptors on their membrane either [47]. Although the role of these receptors in signal transmission in non-neuronal cells is not clear, it has been deduced that their prolonged excitation due to inhibition of ChEs may act as growth factor stimulation and may be responsible for neoplastic growth [48].

Oxidative stress may be evidenced by increased production of free radicals and altered antioxidant defence capability. ROS reacts readily with lipids, proteins, carbohydrates, and nucleic acids, causing impairment of the structure and function of cellular components. When free radicals attack unsaturated fatty acids in cellular membranes that contain lipids, lipid peroxidation occurs. Malondialdehyde (MDA) formation often assayed with thiobarbituric acid (TBA) assay is the most widely used index of lipid peroxidation in animal studies [49]. Besides being a reliable biomarker of oxidative stress, MDA is known mutagenic agent [50]. Antioxidant defense system primarily includes antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px). Together with glutathione (GSH) they contribute to scavenging free radicals and protection against oxidative stress damage induced by various xenobiotics [51,52]. The total antioxidative capacity is a measure of susceptibility of the cell to oxidative stress. Due to its integrated approach encountering different cellular mechanisms of defense against ROS and ability to provide a quantitative measure of the capacity of plasma, tissues and organs for overall oxidative buffering, the assay has found its applicability in testing of chemicals [53].

The alkaline comet assay is a reliable cytogenetic technique for detection and quantification of primary damage DNA. Its advantage lies in ability to detect the damage which occurs in direct interaction of a substance with genotoxic activity and cell genome. It detects wide range of DNA damage types such as single and double DNA strand breaks, alkylation, oxidative damage of nitrogenous bases, DNA-adduct formation, DNA-protein covalent bonding, pyrimidine dimers, DNA cross-linking and sites of extensive DNA damage repair. Thus it is an efficient tool in assessing genotoxic potential of substances of the concern [54].

In this regard, to assess the potential effects of exposure to doses comparable to reference values for chlorpyrifos stipulated in current EU legislation, our study focused on an *in vivo* rat model. By employing a comprehensive experimental design, we tried to establish whether and how a 28-day exposure of adult male Wistar rats influenced cholinesterase activity, oxidative stress responses, parent compound/metabolite levels, and primary DNA damage in blood and brain tissue. Evaluations were performed using (1) biochemical assays of total cholinesterase activity, acetylcholinesterase and butyrylcholinesterase activity, (2) markers of oxidative stress (lipid peroxidation, changes in total antioxidant capacity, the reactive oxygen species (ROS) level, glutathione level and activities of enzymes glutathione peroxidase, superoxide dismutase and catalase), (3) HPLC-UV DAD analysis to measure the levels of parent compound, and its main metabolite 3,5,6-trichloro-2-pyridinol in the plasma and brain tissue, and (4) the alkaline comet assay to determine the levels of primary DNA damage in leukocytes and brain tissue.

2. Materials and methods

2.1. Chemicals and reagents

Chlorpyrifos 99.9% (CAS-No. 2921-88-2) was purchased as analytical standard PESTANAL® (Sigma-Aldrich Laborchemikalien GmbH; Germany). 3,5,6-Trichloro-2-pyridinol, 99.3% (CAS-No. 6515-38-4) was the product of Dr. Ehrenstorfer GmbH (Augsburg, Germany). Acetonitrile and methanol were Ultra gradient HPLC grade products (J.T.Baker, Deventer, Netherlands). LC grade water was prepared by purifying deionised water with a Milli-Q water purification system (Millipore, Bedford, MA, USA). β-Glucuronidase Type H-2, from *Helix pomatia* was purchased from Sigma-Aldrich, USA. Bakerbond SDB/C18polar+, 6 mL (100 mg SDB + 250 mg C18polar+) (J.T. Baker, Deventer, the Netherlands) was used for solid phase extraction of pesticide compounds from plasma and tissue samples. Before HPLC analysis extracts were filtrated through a 0.2 μm PTFE filter (Waters,

Miliford, USA).

Other chemicals and reagents used for biochemical analyses and in the alkaline comet assay were purchased form Sigma Chemical Co., St. Louis, MO, USA, unless specified otherwise.

2.2. Animals

Adult 3 months old male Wistar rats were obtained from the breeding colony at the Institute for Medical Research and Occupational Health, Zagreb (Croatia). A total of 30 rats were randomly assigned to control and treatment groups. Each group comprised 5 animals with minimal weight variation (300 g ± 10%). Animals were maintained under pathogen-free conditions in steady-state micro environmental conditions, 12 h light/dark cycle, 22 °C with *ad libitum* access to standard GLP certified food (Mucedola, 4RF21, Italy) and tap water. Appropriate enrichment was provided in animal cages. The study was approved by the Institutional Animal Care and Use Committee and the Croatian Ministry of Agriculture. It was carried out in compliance with international standards and national legislation to protect animal welfare.

2.3. Experimental design

To obtain a stock solution, chlorpyrifos was dissolved in ethanol. Further dilutions to obtain working pesticide solutions to be used in the experiment were done in saline. The final concentration of ethanol in the pesticide solutions given to rats did not exceed 0.03%.

Chlorpyrifos was orally administered to the rats for 28 consecutive days according to the treatment schedule listed in Table 1. We tested the effects of chlorpyrifos at doses of 0.010 mg/kg b. w./day (10-fold higher than current ADI and AOEL), 0.015 mg/kg b. w./day (3-fold higher than current ARfD) and 0.160 mg/kg b. w./day [(equal to 1/2000 of acute oral LD_{50} for rats, or within 1/400 and 1/1200 acute oral LD_{50} for rats), selected based on WHO [44], and current EU legislation [41], and data reported from previous studies. Appropriate negative, solvent and positive control groups were studied in parallel. Positive control was ethyl methanesulfonate, a well-established genotoxicant recommended for *in vivo* comet assay in rodents [55].

During the experiment, body weights were regularly monitored and the doses of chlorpyrifos were adjusted accordingly. Survival and clinical signs of intoxication were also evaluated on a daily basis.

All of the animals were sacrificed 24 h after the last treatment using an anaesthetic cocktail (Narketan, Vetoquinol UK Ltd., 80 mg/kg b. w.; Xylapan, Vetoquinol UK Ltd., 12 mg/kg b. w., *i. p.*), and tissues were harvested.

The blood samples were collected in heparinised vacutainers by dissection of carotid artery under general anaesthesia and further processed. Heparinised blood was divided into two portions. The first portion, which was further used for biochemical and HPLC analyses, was centrifuged (3000 rpm, *i.e.* 976 *g*, 10 min, at 4 °C) and the plasma removed. After removing the plasma, the remaining erythrocytes were resuspended with cold saline (0.9% NaCl) and centrifuged for 10 min at 3000 rpm (*i.e.* 976 *g*). The washing procedure and centrifugation were repeated three more times. Prior to measurement, the isolated erythrocytes were suspended in the saline up to 1 mL; solution was well-mixed and finally diluted (10×) in 0.1 M sodium phosphate buffer, pH 7.4. The second portion of whole blood was immediately used to prepare agarose microgels for the alkaline comet assay.

Brain tissue was dissected, rinsed with cold PBS, and also divided into two portions. The tissue samples used for biochemical and HPLC analyses were immediately frozen at −20 °C until further processing. Before the measurements of oxidative stress parameters, the brain samples were homogenized (100 mg tissue/mL 50 mM phosphate buffer, pH 7.8), and centrifuged at 13600 rpm and 4 °C for 30 min to obtain a supernatant. Before the measurements of cholinesterase activities, the brain homogenates were diluted to 40 mg/mL. The tissue samples used in the alkaline comet assay were isolated, cleaned from the adhering matters and washed in cold PBS, weighed, put into cold buffer (75 mM NaCl and 24 mM Na_2EDTA, pH 7.5), carefully minced with scissors and glass rod into fine pieces, and the obtained cellular suspension was immediately used for preparation of agarose microgels.

Table 1
Experimental schedule.

Experimental group	Treatment	Duration of the treatment
Negative control	Water	28 days
Solvent control	EtOH in saline (0.03% solution)	28 days
Exposed I	Chlorpyrifos at 0.010 mg/kg b.w./day dissolved in 0.03% EtOH sol.	28 days
Exposed II	Chlorpyrifos at 0.015 mg/kg b.w./day dissolved in 0.03% EtOH sol.	28 days
Exposed III	Chlorpyrifos at 0.160 mg/kg b.w./day dissolved in 0.03% EtOH sol.	28 days
Positive control	EMS at 300 mg/kg b.w. *p.o.*	last three days of the experiment

EtOH – ethanol; EMS – Ethyl methanesulfonate.
All rats received 1 mL of tested solutions once a day *per os.*

2.4. Cholinesterase activity assay

Blood and brain tissue samples were analysed for AChE and BChE activity using the spectrophotometric Ellman method [56]. Enzyme activity was measured in a 0.1 M sodium phosphate buffer, pH 7.4, at 25 °C using ATCh (1.0 mM) and DTNB (0.3 mM). AChE and BChE activities were distinguished using the BChE-selective inhibitor ethopropazine (20 μM). For the erythrocyte samples, increase in absorbance was monitored at 436 nm and for plasma and tissue samples at 412 nm over 4 min. All of the measurements were performed on a Cecil 9000 (Cecil Instruments Limited, Cambridge, UK) Spectrophotometer. Enzyme activity was expressed as $IU/g_{protein}$.

2.5. Lipid peroxidation

The concentration of thiobarbituric reactive substances (TBARS), as a measure of lipid peroxidation, was determined using a modification of the method by Drury et al. [57]. Butylated hydroxytoluene (BHT; 5 μL; 0.2%; w/v) and phosphoric acid (750 μL; 1%; v/v) were added to 50 μL of plasma/brain tissue homogenate sample. After mixing, 250 μL 0.6% (w/w) thiobarbituric acid (TBA) and 445 μL H_2O were added and the reaction mixture was incubated in a water bath at 90 °C for 30 min. The mixture was cooled and absorbance was measured at 532 nm on a Shimadzu UV Probe Spectrophotometer. The concentration of TBARS was calculated using standard curves of increasing 1,1,3,3-tetramethoxypropane concentrations, and expressed as μM.

2.6. Total antioxidant capacity

Total antioxidant capacity (TAC) was investigated using a FRAP assay, based upon the reduction of the Fe^{3+}-TPTZ complex under acidic conditions [58]. The reagents included 300 mM acetate buffer (pH 3.6) with 16 mL acetic acid per mL of buffer solution, 10 mM 2,4,6-tri [2-pyridyl]-s-triatine (TPTZ) in 40 mM HCl and 20 mM $FeCl_3$. Working FRAP reagent was prepared as required by mixing 20 mL acetate buffer, 2.0 mL TPTZ solution, 2.0 mL $FeCl_3$ solution and 2.4 mL distilled water. Thirty microliters of plasma/brain tissue homogenate sample was added to 1 mL of freshly prepared reagent warmed at 37 °C. The complex between Fe^{2+} and TPTZ gives a blue colour with absorbance at 593 nm. Water solutions of known $FeSO_4x7H_2O$ concentration, in the range of 0.1–1.0 mM, were used for obtaining the calibration curve.

2.7. ROS detection

The amount of ROS in blood plasma and brain tissue homogenates was measured using 2′,7′-dichlorofluorescin diacetate (DCF-DA). DCFH-DA is hydrolysed by cellular esterase to form 2′7′-dichlorofluorescin, which is oxidized to the fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of peroxides, including hydrogen peroxide [59]. Briefly, 1% tissue homogenate and 10% blood plasma samples were prepared by dilution with ice-cold PBS (pH 7.4). Portions of 0.07 mL PBS and 0.03 mL of 1% tissue homogenate as well as 0.1 mL of 10% blood plasma were pipetted into wells of black 96-well culture plate. Afterwards, 20 μL of 0.12 mM DCFH-DA dye in PBS was added to each well and incubated for 30 min at 37 °C. Control for dye autofluorescence was prepared without addition of dye. Negative (non-treated) samples were included in each experiment. All of the samples were analysed using a Victor3™ multilabel plate reader at an excitation wavelength of 485 nm and emission wavelength of 535 nm. Data are expressed as fluorescence arbitrary units (AU).

2.8. Quantification of glutathione (GSH)

Analysis of GSH level was performed using a fluorogenic bimane probe, monochlorobimane (MBCl), which reacts specifically with GSH to form a fluorescent adduct [60]. Brain tissue homogenates and plasma samples were prepared as previously described for ROS measurement following addition of 20 μL of 0.24 mM MBCl dye in PBS for 20 min at 37 °C. The amount of GSH in brain tissue homogenate and blood plasma samples was analysed using a Victor3™ multilabel plate reader at an excitation wavelength of 355 nm and emission wavelength of 460 nm. Negative (non-treated) control samples were included in each experiment. Data are expressed as fluorescence arbitrary units (AU).

2.9. Glutathione peroxidase (GSH-Px) activity

The GSH-Px activity in whole blood and the supernatant of the brain homogenate was measured by the European standardized method [61]. The amount of GSH oxidized by t-butyl hydroperoxide was determined by following the decrease in the β-NADPH concentration, and the decrease in absorbance at 340 nm was measured by spectrophotometry (Cary 50 UV–Vis, Varian Inc. CA, USA). One unit of GSH-Px is the number of micromoles of β-NADPH oxidized per minute. Activity of GSH-Px in whole blood was expressed per gram of haemoglobin (U/g Hb), while the results obtained in brain homogenate were expressed per gram of total protein (U/$g_{protein}$).

2.10. Superoxide dismutase (SOD) activity

SOD activity in the erythrocytes was measured by the European standardized method [62] using the Ransod kit (Randox, Crumlin, UK) according to the manufacturer's instructions. This method employs xanthine and xanthine oxidase to generate superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5 phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. SOD activity is then measured by the degree of inhibition of this reaction at 505 nm by spectrophotometry (Cary 50 UV–Vis, Varian Inc. CA, USA). One unit of SOD is that which causes a 50% inhibition of the rate of reduction of I.N.T. under the conditions of the assay. SOD activity was expressed per gram of haemoglobin (U/g Hb). Haemoglobin in erythrocytes and whole blood was measured at 540 nm (Cary 50 UV–Vis, Varian Inc. CA, USA) by standard cyanmethaemoglobin method using Haemiglobincyanide standard. To verify the analytical procedure, three levels of Hb were used.

SOD activity in plasma and brain tissue was measured spectrophotometrically according to Flohé and Ötting [63]. The reduction rate of cytochrome *c* by superoxide radicals was monitored at 550 nm utilizing the xanthine-xanthine oxidase system as the source for O_2.-. SOD competed for superoxide and decreased the reduction rate of cytochrome *c*. One unit of SOD was defined as the amount of enzyme that inhibits the rate of cytochrome *c* reduction by 50%. Enzyme activity was expressed as IU/$g_{protein}$.

2.11. Catalase activity

Catalase activity was measured in plasma and supernatants of the brain 25 °C, pH 7.0, at 240 nm [64]. Enzyme activity was calculated using the molar extinction coefficient (40.0 mM^{-1} cm^{-1}) and was expressed as IU/$g_{protein}$.

2.12. Protein quantification

Determination of protein concentration was carried out according to the method of Bradford [65] using bovine serum albumin as standard.

2.13. Plasma and brain tissue analysis for chlorpyrifos and its metabolite

2.13.1. Equipment

HPLC analysis was performed on a Varian ProStar system equipped with a 230SDM pump, 410 autosampler and 330 UV diode-array detector (Varian, Palo Alto, CA, USA). The chromatographic column was a 250 mm × 4 mm I.D. of 5 μm particle size Gemmini C18 (Phenomenex, Torrence, USA).

The mobile phase consisted of acetonitrile and water, pH 2–3. The elution was performed in a linear gradient mode from 45% to 50% of acetonitrile in 5 min, continued from 50% to 95% of acetonitrile over 10 min, hold down 95% of acetonitrile for 2 min and ended with 45% of acetonitrile in 8 min. The flow-rate was 1 mL/min. The amount injected was 100 μL. The UV spectra of all of the peaks were recorded from 200 to 400 nm. The working wavelength for quantitative analysis was 202 nm.

Pesticide compounds in extracts were identified by their retention time and UV-spectra, which were compared to the known standard.

2.13.2. Standard solution and sample preparation

Stock solutions of 1 mg/mL of chlorpyrifos and 3,5,6-trichloro-2-pyridinol were prepared in acetonitrile and further diluted with deionised water. The standards for HPLC analysis of spiked samples for recovery experiments were prepared in deionised water.

2.13.3. Extraction of chlorpyrifos and its metabolite from plasma samples

The plasma samples were prepared by acidifying the 200 μL of plasma with 10 μL of hydrochloric acid to pH 4.10 μL of β-glucuronidase was added and then allowed to stand for 1 h at 40 °C. The solid-phase extraction cartridge was conditioned by passing through 5 mL of methanol, 5 mL of acetonitrile and 5 mL of deionised water. A 200 μL plasma sample was forced through the cartridge at a flow rate 2–3 mL using the positive air pressure. The cartridge was washed with 2 mL of water. Elution was performed with 10 mL of acetonitrile. The acetonitrile eluate was evaporated under a gentle stream of nitrogen to dryness. The dry residue was dissolved in appropriate volume of mixture methanol/water, 1:1 and filtrated through a 0.2 μm PTFE syringe filter before HPLC analysis.

2.13.4. Extraction of chlorpyrifos and its metabolites from brain samples

The 500 mg of brain tissue sample with 3 mL of acidified (pH 4) deionised water was homogenized with a Powergen-125 homogenizer (Fisher Scientific, New Jersey, USA). Then, 50 μL of β-glucuronidase was added and after vortexing for 2 min allowed to stand for 1 h at 40 °C. The samples were centrifuged for 10 min at 425 *g*. The 3 mL of water layer was forced through the extraction cartridge previously conditioned with 10 mL of methanol, 10 mL of acetonitrile and 10 mL of deionised water. The sorbent was washed with 20 mL of water. The pesticide compounds were eluted with 10 mL of acetonitrile. The eluate

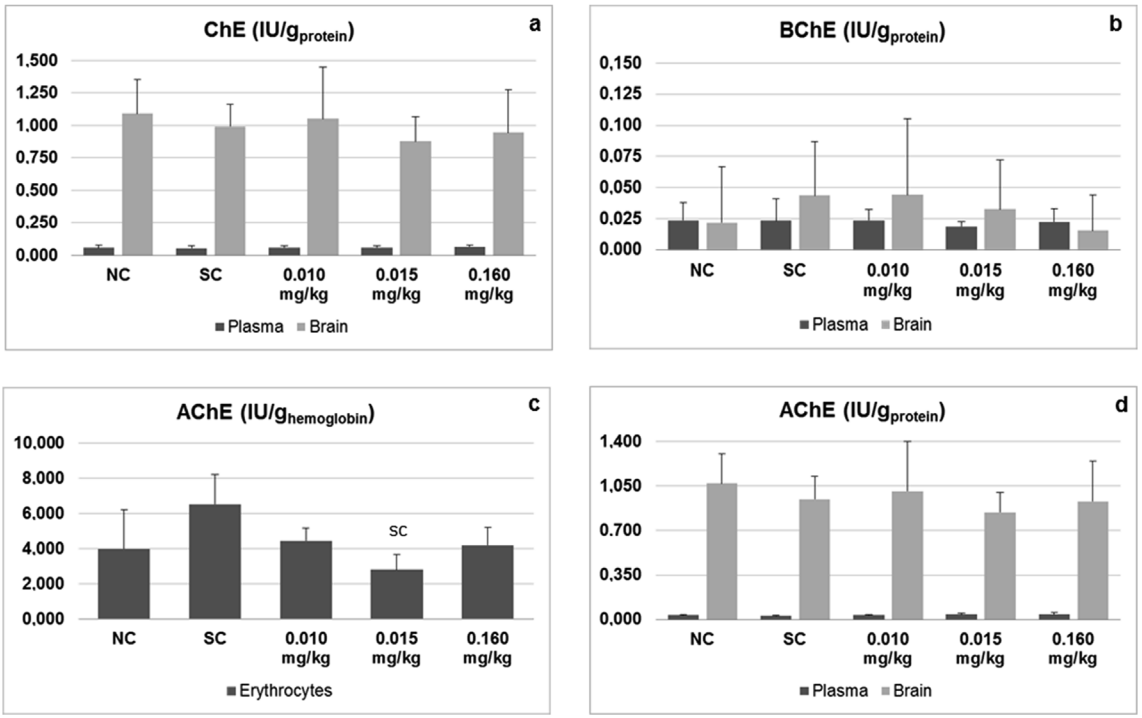

Fig. 1. (a) Total cholinesterase (ChE) activity in plasma and brain of Wistar rats treated for 28 days with chlorpyrifos and in the corresponding controls. (b) Butyrylcholinesterase (BChE) activity in plasma and brain of rats treated for 28 days with chlorpyrifos applied at three doses, and in the corresponding controls. (c) Acetylcholinesterase (AChE) activity in erythrocytes of rats treated for 28 days with chlorpyrifos and in the corresponding controls. (d) Acetylcholinesterase activity (AChE) in plasma and brain of rats treated for 28 days with chlorpyrifos and in the corresponding controls.
Significantly decreased value ($P < 0.05$) was: ^{sc} *vs.* solvent control.

was evaporated under a gentle stream of nitrogen to dryness. The dry residue was dissolved in an appropriate volume of mixture methanol/water, 1:1 and filtrated through a 0.2 μm PTFE syringe filter before HPLC analysis.

2.13.5. HPLC-UV DAD analysis and method validation

HPLC-UV DAD analysis of chlorpyrifos and its metabolite from plasma and tissue samples of treated rats, standard prepared in water, spiked tissue and plasma samples prepared by adding an amount of analytes to the tissue of the non-exposed group and blank samples were performed. Calibration lines for the determination of pesticide compounds were obtained in triplicate at five different concentration levels and were linear within the range from 0.03 μg/mL to 1 μg/mL ($r \geq 0.999$, five points in triplicate). For recovery experiments, tissue and plasma samples collected from non-exposed animals were spiked with a pesticide aqueous solution at a level of 75–80 ng/mL ($n = 5$). The recoveries were 83% and 84% for trichloro-pyridinol and chlorpyrifos, respectively (RSD < 10%). The detection limits were 30 ng/mL or 30 ng/g.

2.14. Alkaline comet assay

The assay was performed following the protocol by Singh et al. [66], with minor modifications. Agarose microgels were made on microscopic slides coated with 1% and 0.6% normal melting point (NMP) agarose. Samples of heparinised whole blood (V = 10 μL per slide) and suspensions of brain cells (V = 10 μL per slide) were embedded in a 0.5% low melting point (LMP) agarose. Two microgels per each rat per sample were prepared. All slides were coded. The gels were immersed overnight at 4 °C in a freshly prepared lysis solution (pH 10.0; 100 mM Na_2EDTA, 2.5 M NaCl, 1% Na lauroylsarcosinate, 10 mM Tris–HCl, 10% dimethyl sulfoxide, and 1% Triton X-100). After lysis, microgels were gently rinsed with distilled water and placed into a horizontal electrophoresis unit filled with cold freshly prepared alkaline buffer (300 mM NaOH and 1 mM Na-EDTA, pH > 13). After 20 min of denaturation, microgels were subjected to electrophoresis at 4 °C. Electrophoresis lasted 20 min both for leukocytes and brain cells at 4 °C. For leukocytes, it ran at 0.86 V/cm [67] and for brain cells at 1 V/cm [68]. Then, the microgels were neutralized in three changes of 0.4 M Tris-HCl buffer (pH 7.5) at 5-min intervals. They were stained with ethidium bromide (20 μg/mL). One well-trained scorer performed all of the comet measurements on coded/blinded slides. The level of DNA damage in individual cells was assessed with an image analysis system (Comet Assay IV, Perceptive Instruments Ltd., Suffolk, Halstead, UK) using an epifluorescence microscope (Olympus BX50, Japan) equipped with appropriate filters, under 200× magnification. A total of 300 randomly selected comets per each rat (i.e. 1500 comets per each tested dose) were measured on replicate slides in two independent evaluations. Tail intensity (i. e. DNA % in tail) and tail length were chosen as indicators of DNA damage.

2.15. Statistical analysis

Statistical calculations were performed using the Dell™ Statistica™ 13.2 software (USA). The data were first evaluated using descriptive statistics. Further evaluations were performed by ANOVA. Before data analysis, to normalize data distribution $\log_{10}$-transformation was used. Finally, *post-hoc* Tukey HSD test was used for calculations regarding pair-wise comparisons. P values < 0.05 were considered statistically significant.

3. Results

All of the rats survived the 28-day treatments with chlorpyrifos. In the course of study, the experimental rats did not show signs typical for cholinergic overstimulation, such as hypothermia, salivation, lacrimation, diarrhoea, incoordination, tremors and fasciculations.

3.1. Cholinesterase activity

Fig. 1a shows the effects of chlorpyrifos on the catalytic activity of total ChE in plasma and brain tissue. Application of chlorpyrifos did not significantly affect total ChE activity, either in the plasma, or in the brain tissue. The effects of chlorpyrifos on the activity of BChE in plasma and brain tissue are shown in Fig. 1b. The 28-day exposure to chlorpyrifos slightly, but non-significantly, inhibited BChE activity in plasma. Brain BChE activity was more inhibited by the chlorpyrifos, which was dose-dependent, but still not statistically significant compared to the solvent and negative control groups. Fig. 1c shows the effects of 28-day exposure to chlorpyrifos on the activity of AChE in erythrocytes. AChE activity in erythrocytes decreased in all of the treated rats, and the lowest was in rats treated with chlorpyrifos at 0.015 mg/kg b. w./day. The effects of chlorpyrifos on the activity of AChE in plasma and brain tissue are shown in Fig. 1d. The 28-day exposure to chlorpyrifos slightly changed the activity of AChE in plasma. AChE activities observed in brain tissue pointed to a mild inhibitory effects of treatment, most prominent in rats treated with chlorpyrifos at 0.015 mg/kg b. w./day. However, none of the values were statistically significant compared to the solvent and negative control groups.

3.2. Lipid peroxidation

The measured levels of lipid peroxidation expressed as concentration of thiobarbituric acid reactive species (TBARS) in the plasma and brain tissue of chlorpyrifos-treated rats are shown in Fig. 2a. The 28-day exposure to chlorpyrifos slightly changed the TBARS concentration, both in plasma and brain tissue. Most of the measured values were within the limits of the control values (Fig. 2a).

3.3. Total antioxidant capacity

The 28-day exposure to chlorpyrifos slightly changed the total antioxidant capacity of blood plasma and brain tissue. As reported in Fig. 2b, the ferric ion-reducing ability of plasma in rats following the 28-day exposure to chlorpyrifos at 0.010 and 0.015 mg/kg b. w./day was increased compared to the respective negative/solvent control samples. The FRAP levels measured in the brain tissue of the treated rats were lower than in the respective controls, but without statistical significance.

3.4. ROS level

Treatment slightly influenced ROS levels in plasma (Fig. 2c), without any statistical significance. ROS levels in brain tissue were comparable or lower than the respective negative/solvent control samples. The highest value was measured in the positive control sample (Fig. 2c).

3.5. Quantification of glutathione (GSH)

The reduced GSH content measured in the plasma following the 28-day exposure to chlorpyrifos indicated a marginal effect of the treatment on this parameter (Fig. 2d). GSH concentrations in brain tissue were similar or comparable to the control values.

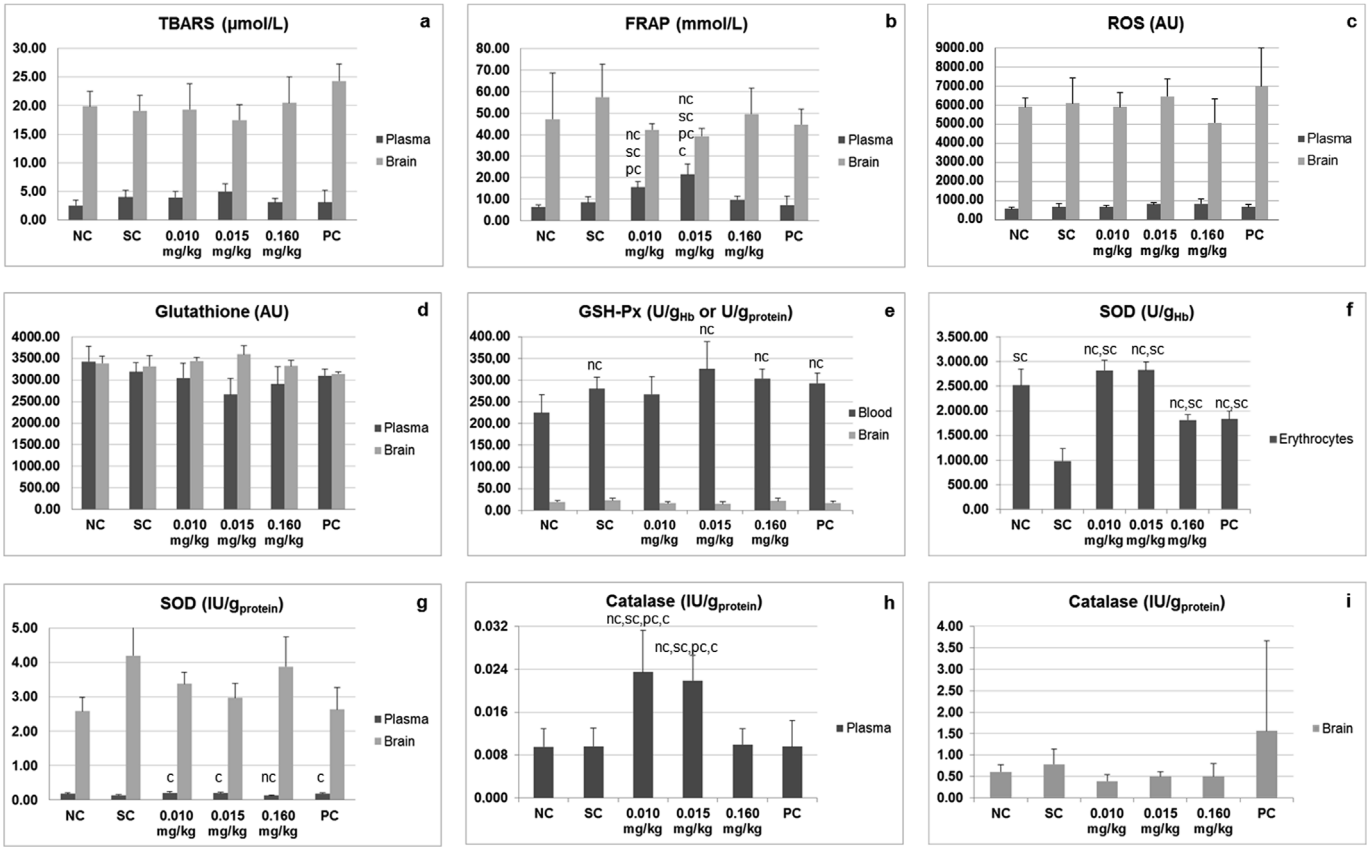

Fig. 2. Results regarding the biochemical markers of oxidative stress measured in the plasma and brain tissue of Wistar rats treated for 28 days with chlorpyrifos applied at three doses, and in the corresponding controls. (a) The levels of thiobarbituric reactive substances (TBARS) in the plasma and brain tissue; (b) the levels of FRAP in the plasma and brain tissue; (c) concentrations of ROS in the plasma and brain tissue; (d) concentrations of glutathione (GSH) in the plasma and brain tissue; (e) glutathione peroxidase (GSH-Px) activity in the whole blood and brain tissue; (f) superoxide dismutase (SOD) activity in erythrocytes; (g) superoxide dismutase (SOD) activity in the plasma and brain tissue; (h) catalase activity in the plasma; (i) catalase activity in the brain tissue.
Significantly increased values ($P < 0.05$) were: ^{nc} *vs.* negative control; ^{sc} *vs.* solvent control; ^{pc} *vs.* positive control; ^{c} vs. 0.160 mg/kg b. w./day exposed group.

3.6. Glutathione peroxidase (GSH-Px) activity

GSH-Px activity significantly increased in the blood of animals treated with two higher doses of chlorpyrifos (Fig. 2e) compared to negative control. A significant increase in GSH-Px activity, compared to negative controls, was also observed in the positive control and solvent control group. Treatment with chlorpyrifos had no significant effect on the activity of GSH-Px in the supernatant of the brain homogenate compared to negative controls (Fig. 2e).

3.7. Superoxide dismutase (SOD) activity

SOD activity in erythrocytes significantly increased in animals treated with chlorpyrifos at 0.010 mg/kg b. w./day and 0.015 mg/kg b. w./day, while in the group treated with chlorpyrifos at 0.160 mg/kg b. w./day, the positive control and solvent control group, SOD activity significantly decreased compared to the negative control group (Fig. 2f).

SOD activity in plasma slightly affected by the 28-day exposure to chlorpyrifos (Fig. 2g). In the brain tissue of all of the treated rats, we found SOD levels higher than in the negative control group, but without statistical significance (Fig. 2g).

3.8. Catalase activity

The 28-day exposure to chlorpyrifos resulted in increased catalase activity in plasma, especially in animals treated at 0.010 mg/kg b. w./day and 0.015 mg/kg b. w./day (Fig. 2h). Brain catalase activity was not significantly changed compared to the respective negative/solvent control animals.

3.9. Plasma and brain tissue analysis for chlorpyrifos and its metabolite

HPLC-UV DAD analysis of plasma samples of chlorpyrifos-treated rats revealed the presence of both the parent compound and its main metabolite 3,5,6-trichloro-2-pyridinol in all of the experimental animals. As shown in Fig. 3, in all of the plasma samples analysed, the average mass concentration of chlorpyrifos was greater than the average mass concentration of its metabolite. The levels of both compounds were dose-dependent. The highest average mass concentration of chlorpyrifos (554 ng/mL) was found in the plasma of rats treated at 0.160 mg/kg b. w./day. In the plasma of these rats, we found an average of 468 ng/mL of 3,5,6-trichloro-2-pyridinol, and both values were significantly increased (P_C = 0.0295; P_T = 0.0002) compared to the mass concentrations of both chlorpyrifos and 3,5,6-trichloro-2-pyridinol measured in the plasma of rats treated at 0.010 mg/kg mg/kg b. w./day (184 ng/mL of chlorpyrifos; 27 ng/mL of 3,5,6-trichloro-2-pyridinol). Statistically significant difference in plasma 3,5,6-trichloro-2-pyridinol mass concentration was also found between rats treated at 0.160 and 0.015 mg/kg b. w./day (P_T = 0.0002).

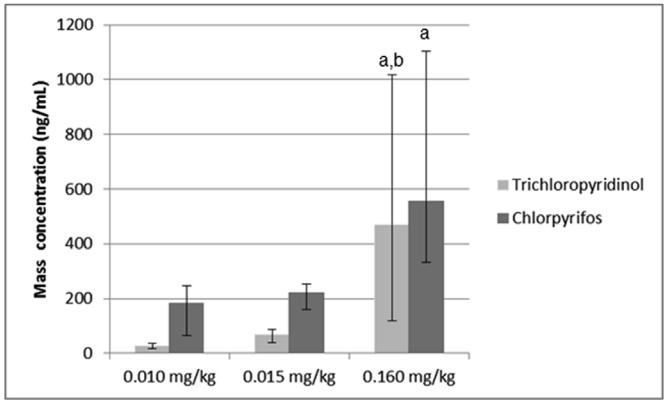

Fig. 3. Average mass concentration and ranges of chlorpyrifos and 3,5,6-trichloro-2-pyridinol in the plasma samples of Wistar rats treated for 28 days with chlorpyrifos at doses 0.010 mg/kg b. w./day, 0.015 mg/kg b. w./day and 0.160 mg/kg b. w./day.
Significantly increased values (P < 0.05) were: [a] *vs.* 0.010 mg/kg b. w./day exposed group; [b] *vs.* 0.015 mg/kg b. w./day exposed group.

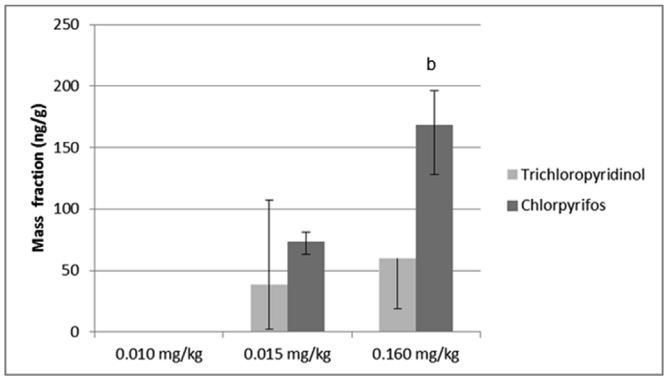

Fig. 4. Average mass fraction of chlorpyrifos and 3,5,6-trichloro-2-pyridinol in the brain tissue of Wistar rats treated 28 days with chlorpyrifos at doses 0.010 mg/kg b. w./day, 0.015 mg/kg b. w./day and 0.160 mg/kg b. w./day.
Significantly increased value (P < 0.05) was: [b] *vs.* 0.015 mg/kg b. w./day exposed group.

HPLC-UV DAD analysis of brain tissue dissected from chlorpyrifos-treated rats confirmed the presence of both the parent compound and its main metabolite 3,5,6-trichloro-2-pyridinol in experimental animals treated at two higher doses. The levels of both compounds in rats treated at 0.010 mg/kg b. w./day were below the level of detection (Fig. 4). Brain levels of both compounds were dose-dependent. However, they were lower than the levels of both compounds detected in the plasma samples of the same rats. The highest average mass fraction of chlorpyrifos (168 ng/g) was found in the brain tissue of rats treated at 0.160 mg/kg b. w./day. The corresponding average mass fraction of 3,5,6-trichloro-2-pyridinol was 60 ng/g. As shown in Fig. 4, the only statistically significant difference (P_C = 0.0079) was found between the mass fractions of chlorpyrifos measured in the brain tissue of rats treated at 0.160 mg/kg b. w./day and those treated at 0.015 mg/kg b. w./day.

3.10. Alkaline comet assay

The results of the alkaline comet assay are reported in Tables 2 and 3.

Control rats had a low level of spontaneous DNA damage in their leukocytes (Table 2). Results regarding both tail intensity (DNA %) and tail length indicate that 28-day exposure to chlorpyrifos resulted in statistically significant increases in primary DNA damage in leukocytes compared to the negative control. Detailed inter-group comparisons and their significances are shown in Table 2.

Since the comet assay allows for DNA damage to be estimated in single cells, the obtained results indicate that leukocytes responded to treatment with different levels of primary DNA damage. From the measured values of both comet parameters, it can be concluded that comets mostly had relatively long but rather thin tails, considering that the mean tail DNA % was not too high (mean tail intensities varied between 1.67 and 2.16%, while the maximum values for tail lengths in leukocyte nucleoids of chlorpyrifos-exposed rats increased up to 50.42 μm).

Spontaneous DNA damage in brain tissue of control rats was also low (Table 3). Brain cells of chlorpyrifos-exposed rats generally had lower levels of primary DNA damage than leukocytes. The highest mean tail intensity and tail length values were recorded in brain cells of rats exposed to 0.160 mg/kg b. w./day of CPF (Table 3). Detailed inter-group comparisons and their significances are shown in Table 3.

Table 2
Results of the alkaline comet assay in rat leukocytes.

Parameter	Tail intensity							
Experimental group	Mean	SD	SE	Min	Max	Lower quartile	Median	Upper quartile
Chlorpyrifos								
0.010 mg/kg b.w./day	1.99[nc,sc]	3.78	0.10	0.00	32.70	0.00	0.07	2.11
0.015 mg/kg b.w./day	2.16[c,nc,sc]	3.88	0.10	0.00	22.53	0.00	0.07	2.72
0.160 mg/kg b.w./day	1.67[nc]	3.31	0.09	0.00	26.37	0.00	0.08	1.57
Negative control	1.08	2.22	0.06	0.00	20.26	0.00	0.04	0.83
Solvent control	1.36	2.86	0.07	0.00	19.70	0.00	0.02	1.07
Positive control	23.18*	12.43	0.41	0.08	66.51	13.32	22.51	32.92
Parameter	Tail length							
Experimental group	Mean	SD	SE	Min	Max	Lower quartile	Median	Upper quartile
Chlorpyrifos								
0.010 mg/kg b.w./day	19.86[b,nc,sc]	4.59	0.12	9.58	49.17	16.25	18.75	22.92
0.015 mg/kg b.w./day	18.72[nc,sc]	3.82	0.10	9.17	41.25	15.83	17.92	21.25
0.160 mg/kg b.w./day	21.19[a,b,nc,sc]	4.41	0.11	8.75	50.42	17.92	21.25	23.75
Negative control	17.46	3.16	0.08	12.08	45.00	15.42	16.67	18.75
Solvent control	17.06	3.32	0.09	6.67	87.08	15.00	16.25	18.33
Positive control	48.09*	14.82	0.48	9.17	107.08	35.83	47.50	58.75

Significantly increased values ($P < 0.05$; ANOVA with *post-hoc* Tukey HSD test) are: [a] *vs.* 0.010 mg/kg b.w./day exposed group; [b] *vs.* 0.015 mg/kg b.w./day exposed group; [c] *vs.* 0.160 mg/kg b.w./day exposed group; [nc] *vs.* negative control; [sc] *vs.* solvent control; * *vs.* all groups.

4. Discussion

Chlorpyrifos toxicity has been the subject of many toxicological studies, and much knowledge has been gathered thus far, pointing to its main mechanisms of action and various adverse effects, both in animals and humans. However, what has not been answered yet, and our study tried to elucidate, is whether a repeated 28-day exposure to low and "realistic" concentrations of chlorpyrifos produces any signs of intoxication. Our results could shed new light on chlorpyrifos' toxicity profile and add new findings relevant to human risk assessment.

This study analysed changes in several blood and brain tissue biomarkers, considering that the nervous system is the main target of action of organophosphate compounds, and that the endpoints measured in blood and plasma may accurately reflect the various imbalances that occur in a body following ingestion of the tested compound.

What we have clearly shown using high performance liquid chromatography method with UV diode-array detector is that a 28-day exposure of rats to chlorpyrifos, applied at all three of the tested doses, results in detectable levels of both the parent compound and its primary metabolite in plasma (the average mass concentrations of chlorpyrifos were 184–554 ng/mL; average mass concentrations of 3,5,6-trichloro-2-pyridinol 27–468 ng/mL). In the brain tissue, we detected both compounds following the exposure to two higher doses (average mass fractions of chlorpyrifos were 73 and 168 ng/g; average mass fractions of 3,5,6-trichloro-2-pyridinol 38 and 60 ng/g). These results confirm that applied analytical method enables accurate detection of the tested pesticide and its metabolite levels, following exposure at two higher doses in both matrices studied. Using the alkaline comet assay, we further showed that such low doses of the parent compound and its metabolite had the potency to inflict primary DNA damage, in both biological matrices studied, despite slight changes in the oxidative biomarker levels. These findings suggest that direct interactions

Table 3
Results of the alkaline comet assay in rat brain cells.

Parameter	Tail intensity							
Experimental group	Mean	SD	SE	Min	Max	Lower quartile	Median	Upper quartile
Chlorpyrifos								
0.010 mg/kg b.w./day	0.24[b,nc,sc]	0.44	0.01	0.00	4.25	0.00	0.04	0.27
0.015 mg/kg b.w./day	0.15	0.34	0.01	0.00	6.65	0.00	0.02	0.14
0.160 mg/kg b.w./day	2.98[a,b,nc,sc]	2.05	0.05	0.00	19.93	1.53	2.64	4.11
Negative control	0.13	0.25	0.01	0.00	2.75	0.00	0.01	0.14
Solvent control	0.16	0.30	0.01	0.00	3.53	0.00	0.03	0.17
Positive control	30.18*	7.90	0.21	0.51	44.37	24.52	32.27	36.78
Parameter	Tail length							
Experimental group	Mean	SD	SE	Min	Max	Lower quartile	Median	Upper quartile
Chlorpyrifos								
0.010 mg/kg b.w./day	13.92	1.95	0.05	10.00	29.17	12.92	13.75	14.58
0.015 mg/kg b.w./day	15.52[a,nc,sc]	2.00	0.05	7.50	26.67	14.17	15.42	16.67
0.160 mg/kg b.w./day	21.19[a,b,nc,sc]	5.34	0.14	11.67	49.58	17.50	20.00	23.75
Negative control	14.13	1.62	0.04	10.42	27.92	12.92	13.75	15.00
Solvent control	14.48[a,nc]	1.74	0.04	10.42	22.50	13.33	14.17	15.42
Positive control	56.12*	14.12	0.38	12.50	99.58	46.67	56.25	65.83

Significantly increased values ($P < 0.05$; ANOVA with *post-hoc* Tukey HSD test) are: [a] *vs.* 0.010 mg/kg b.w./day exposed group; [b] *vs.* 0.015 mg/kg b. w./day exposed group; [c] *vs.* 0.160 mg/kg b. w./day exposed group; [nc] *vs.* negative control; [sc] *vs.* solvent control;* *vs.* all groups.

between the compounds and DNA played a more significant role than indirect ones, such as the effects of free radicals.

Taken together, what the present study adds to the existing knowledge is the evidence that, at very low doses of chlorpyrifos as the ones tested here, the most subtle biosensor was DNA, while the other biochemical and enzyme activity-based endpoints had lesser sensitivity to detect the adverse effects of the exposure. Nevertheless, they were useful in the assessment of harmful effects caused by much higher doses or, as in the case of cholinesterase enzymes, in acute exposure, which has already been well-documented in the existing literature. The practical significance of each endpoint is briefly discussed below.

The first issue that has to be mentioned is the selection of doses. To the best of our knowledge, no previous study evaluated such low doses of chlorpyrifos on a rat model, using a similar study design and similar array of biomarkers. The lowest level of chlorpyrifos tested so far on a rat model was 0.1 mg/kg in the study by Muller et al. [27]. Among other endpoints, they studied DNA damage using a comet assay on blood cells. However, in rats exposed at 0.1 mg/kg they did not found statistically significant differences in DNA damage levels compared to control. We, on the other hand, observed genotoxicity even at a dose ten-fold lower than Muller et al. [27]; however, it has to be stressed that in their study, the rats received chlorpyrifos during one week, which was a much shorter period than in our study. Furthermore, the routes of exposure also differed: they treated rats via subcutaneous injection, while the rats in our study were treated *per os*. We stress this because routes of exposure always have to be taken into account while discussing the outcomes of treatment in rodent studies [5,69]. As known, the subcutaneous administration of a tested substance (or dermal exposure in general) typically leads to slower release and absorption than oral administration [5,41,70]. It has to be also taken into account that in rats, the acute dermal toxicity of chlorpyrifos is far lower than the acute oral toxicity, which significantly affects values of the biochemical parameters studied [5]. Having in mind what has been stated so far, in discussing our results, we focus only on studies with the same route of exposure, irrespective of the chlorpyrifos doses tested.

The repeated 28-day exposure of rats to chlorpyrifos in our study changed only the AChE activity in erythrocytes, but did not significantly affect total ChE activity or AChE and BChE activities in the plasma and brain tissue. The AChE inhibitory effects observed in the erythrocytes could be explained by the repeated exposure to low doses of chlorpyrifos. The treatment schedule we applied caused the continuous exposure of erythrocytes to the parent compound in the systemic circulation. Chlorpyrifos itself is a weak inhibitor of AChE [62] and does not inhibit other serine-dependent esterases or proteases. Similar applies for its principal metabolite 3,5,6-trichloro-2-pyridinol [3]. However, the short-lived chlorpyrifos-oxon, formed upon metabolic activation of chlorpyrifos, is a potent inhibitor of AChE, BChE and carboxylesterase [2,9,72]. The differences observed between erythrocyte, plasma and brain ChE activities after chlorpyrifos treatment could be associated with their different susceptibility towards the tested compound. In rats, a tissue specific sensitivity of ChE toward the CPF-oxon has been observed [73], and is known that the different affinity and number of available binding sites contribute to the effectiveness of anticholinesterase detoxification. Furthermore, plasma AChE activity is also influenced by BChE, which has a protective function, preventing the inactivation of AChE. Considering this, a possible reason why we did not detect a salient inhibition of cholinesterases in plasma and brain tissue was that, during the 28 days of study, the rats repeatedly received chlorpyrifos in doses that were too low to exert a clear inhibitory effect on plasma and brain ChE, since oxon is short-lived and the overall turnover and clearance of chlorpyrifos from the organism is rapid, as known from previous pharmacokinetics and pharmacodynamics studies [2,3,9,70,71]. For comparison, in previous 28-day studies on a rat model where chlorpyrifos was administered orally, depressed cholinesterase activity was reported; e.g. by Wielgomas and Krechniak [16] in the plasma and brain of rats treated with 10 mg/kg b. w. of chlorpyrifos. Ahmed and Zaki [11] reported a significant inhibition of brain ChE activity following exposure to 16.4 mg/kg b. w. of chlorpyrifos. Mansour and Mossa [33] reported significant inhibition of serum AChE after exposure to 6.75 mg/kg b. w. of chlorpyrifos. Newairy and Abdou [34] reported significant inhibition of AChE activity both in serum and brain after exposure to 6.8 mg/kg b. w. of chlorpyrifos. In a study by Noaishi et al. [35], rats exposed to 2.84 mg/kg b. w. and 4.26 mg/kg b. w. of chlorpyrifos had significantly inhibited BChE in plasma. The inhibitory effects of chlorpyrifos on cholinesterases were also reported in studies with shorter exposures. For instance, Singh and Panwar [74] reported decreased AChE activity in rat brain after 15-day exposure to 89.4 mg/kg b. w. of chlorpyrifos, while Rosalovski et al. [20] found a significant decrease of BChE activity in serum of rats after a single exposure to 50 mg/kg b. w. of chlorpyrifos. What has to be taken into account here is that in the latter two studies near LD_{50} doses were tested, so inhibitory effects were to be expected.

The results obtained for lipid peroxidation in the plasma and brain tissue of the treated rats suggest that repeated daily exposure to low doses of chlorpyrifos did not produce significant amounts of lipid peroxidation products, which could inflict massive direct damage to lipids. This was further sustained by results regarding ROS levels and total antioxidant capacity. Almost all of these parameters, both in plasma and brain tissue were within the limits of the control values. Thus, the oxidative/antioxidative status was not significantly disturbed following repeated exposure to low doses. However, in previous 28-day studies with a similar study design but much higher doses, the above mentioned endpoints were found more valuable. For instance, Newairy and Abdou [34] reported significantly increased TBARS levels in serum after exposure to 6.8 mg/kg b. w. of chlorpyrifos. Baba et al. [17] found increased levels of malondialdehyde in blood of rats exposed to 1 and 10 mg/kg b. w. of chlorpyrifos. Łukaszewicz-Hussain [66] studied the effects of chlorpyrifos treatment at 0.2, 2 and 5 mg/kg b. w. for 14 and 28 days, and reported significantly increased brain malondialdehyde levels in comparison to the control group at 5 mg/kg b. w./day. Evidence for lipid peroxidation in rat brain tissue was also reported by Ahmed and Zaki [11] following exposure to 16.4 mg/kg b. w. of chlorpyrifos. Verma et al. [76] found increased levels of malondialdehyde in brains of rats exposed to 3.1 mg/kg b. w. of chlorpyrifos.

In the present study, we found a marginal effect on the reduced GSH content measured in plasma following the exposure, while GSH concentrations in the brain tissue were similar or comparable to the control values. Again, we assume that such findings could be associated with the very low doses applied. GSH has a vital part in protecting cells/tissues against chemically induced oxidative stress as well as in the detoxification of various substrates [77,78]. Its role as a valuable biomarker was confirmed in previous 28-day studies on a rat model where chlorpyrifos was administered orally at much higher doses. For example, the decreased GSH level in blood of rats was reported in a study by Baba et al. [17] after exposure to 1 and 10 mg/kg b. w. of chlorpyrifos. Newairy and Abdou [34] found a significantly decreased GSH level in the serum of rats after exposure to 6.8 mg/kg b. w. of chlorpyrifos. Decreased GSH level in brain of rats was reported by Ahmed and Zaki [11] following exposure to 16.4 mg/kg b. w. of chlorpyrifos, and by Verma et al. [76] after exposure to 3.1 mg/kg b. w. of chlorpyrifos.

Existing reports on the main antioxidant enzyme activities following exposure to chlorpyrifos are inconclusive. In the present study we evaluated the activities of glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT). GSH-Px and SOD scavenge free radicals to prevent oxidative damage and overcome increased ROS production [79]. We found that GSH-Px activity significantly increased in the blood of animals treated with two higher doses of chlorpyrifos, possibly as a response to a disturbed oxidative/antioxidative equilibrium due to treatment. However, treatment with chlorpyrifos had no significant effect on the activity of GSH-Px in the brain. The differences

in the enzyme activities observed between blood and brain cells apparently originated from the metabolic differences between these cell types, which results in varying antioxidant capacities. Furthermore, it has to also be taken into account that using HPLC-UV DAD method we detected significantly lower levels of both parent compound and its metabolite in the brain tissue than in the plasma. Several previous studies on rats exposed to chlorpyrifos also measured GSH-Px activity. Baba et al. [17], who also studied the effects of 28-day oral exposure, found decreased GSH-Px activity in blood after treatment with 10 mg/kg b. w. of chlorpyrifos. In contrast, Barski et al. [80] in rats exposed to 3 mg/kg b. w. chlorpyrifos for 28 days observed increased activity of GSH-Px during the experiment. Similarly, Łukaszewicz-Hussain [75], reported increased GSH-Px activity in the brains of rats treated for 14 days at 0.2, 2 and 5 mg/kg b. w., however, it returned to lower values with prolonged treatment (28 days). Other studies where the same parameter was evaluated did not last as long. For instance, Gultekin et al. [18] did not find significant changes in erythrocyte GSH-Px activity following oral exposure to two doses of chlorpyrifos-ethyl (41 mg/kg b. w.) given within 21 h. Rosalovski et al. [20] found a significant increase of GSH-Px activity in blood of rats after single exposure to 50 mg/kg b. w. of chlorpyrifos. Singh and Panwar [74] reported decreased GSH-Px activity in rat brain after a 15-day exposure to 89.4 mg/kg b. w. What has to be mentioned is that in these studies much higher doses of chlorpyrifos were administered to the animals than in ours.

Our results show that SOD activity in erythrocytes and plasma increased in animals treated with chlorpyrifos at 0.010 mg/kg b. w./day and 0.015 mg/kg b. w./day. However, SOD activity in brain tissue was not significantly affected. Increased rat brain SOD activity after 14 and 28 days of treatment with chlorpyrifos at 2 and 5 mg/kg b. w. reported Łukaszewicz-Hussain [75]. However, results regarding SOD activity in 28-day studies on rats comparable to our, where the same route of exposure was studied, but at much higher doses, are opposite. Mansour and Mossa [33] reported a significant decrease of SOD activity in rat plasma after exposure to 6.75 mg/kg b. w. of chlorpyrifos. Similar results were reported by Baba et al. [17] after exposure to 1 and 10 mg/kg b. w. of chlorpyrifos, and Newairy and Abdou [34] after exposure to 6.8 mg/kg b. w. of chlorpyrifos. However, Barski et al. [80] after 28 days of exposure to chlorpyrifos at a dose of 3 mg/kg b. w. observed significantly lowered SOD activity in rat erythrocytes at the beginning of the experiment (up to the 24th hour), while later on it started to increase. Decreased SOD activity was observed in some studies with shorter exposures. For instance, Gultekin et al. [18] found significantly decreased erythrocyte SOD activity following oral exposure to two equal doses of chlorpyrifos-ethyl (41 mg/kg b. w.) given within 21 h. Oyewole and Olabiyi [81] found significantly decreased brain SOD activity following a 21-day oral exposure to chlorpyrifos at 20 mg/kg b. w. However, in a study by Singh and Panwar [74], 15-day exposure to 89.4 mg/kg b. w. resulted in significantly increased brain SOD activity.

In the present study we found increased CAT activity in plasma of rats treated with chlorpyrifos at 0.010 mg/kg b. w./day and 0.015 mg/kg b. w./day, but brain CAT activity was not significantly disturbed by the treatment. Such a result may indicate that low levels of both the parent compound and its metabolite in brain possibly did not produce high amounts of hydrogen peroxide to be converted by CAT into water and oxygen, which is the main mechanism of the enzyme to prevent cell oxidative damage [82]. Results regarding CAT activity in comparable rat studies are rather ambiguous. Slightly increased CAT activity also observed Noaishi et al. [35] in rats orally exposed to 2.84 mg/kg b. w. of chlorpyrifos. Łukaszewicz-Hussain [75] found increased activity of brain catalase after 14 and 28 days of chlorpyrifos administration at doses 0.2, 2 and 5 mg/kg b. w. Increased CAT activity in erythrocytes was reported by Wielgomas and Krechniak [16] in rats treated *per os* with 10 mg/kg b. w. of chlorpyrifos. In some studies, 28-day oral exposure to chlorpyrifos resulted in decreased CAT activity. Such results were reported by Ahmed and Zaki [11], who following exposure to 16.4 mg/kg b. w. of chlorpyrifos found decreased CAT activity in brain. Barski et al. [80] observed significantly lowered erythrocyte CAT activity in rats exposed to 3 mg/kg b. w. chlorpyrifos at the beginning of the experiment (up to the 24th hour), while later on it started to increase. After exposure to 1 and 10 mg/kg b. w. of chlorpyrifos, Baba et al. [17] found significantly decreased CAT activity in blood. Newairy and Abdou [34] found a similar effect in rats exposed to 6.8 mg/kg b. w., and Noaishi et al. [35] in rats exposed to 4.26 mg/kg b. w. of chlorpyrifos.

Taken together, the ambiguous results obtained for the majority of biochemical markers studied here may also indicate that during 28 days of treatment some sort of adaptive response was achieved. Although repeated exposure was characterized by constant delivery of new amounts of the tested compound, chlorpyrifos-oxon, which is most toxic species formed following ingestion of the pesticide has a very short half-life in the body, tested pesticide has low potential for accumulation and is rapidly eliminated/cleared. Thus, the overall toxic effects of chlorpyrifos on cholinesterase enzymes and other endpoints of oxidative stress are obviously more important in acute exposures where the correlations between measured levels of tested compound and a particular biochemical marker are much accurate than in cases of repeated exposure – as studied here. However, to further elucidate changes in enzyme biomarkers following low-dose exposure, future research in that regard should focus on more subtle molecular methods, including the level of mRNA and gene expression as well.

Only a limited number of studies on rats treated with chlorpyrifos via the oral route applied a comet assay. Sandhu et al. [30] studied shorter exposures (7 and 14 days) and doses tested in their study were 3 and 12 mg/kg b. w. Ezzi et al. [26] used a 28-day regimen, but the doses tested were significantly higher than in our study (3.1 and 6.2 mg/kg b. w.). Sandhu et al. [30] reported a significant increase of the Olive moment in lymphocytes of male rats at a higher dose and longer duration of treatment. Ezzi et al. [26] reported a statistically significant increase of DNA damage in rat blood cells at 6.2 mg/kg b. w. Our results show that 28-day exposure to chlorpyrifos resulted in statistically significant increases in primary DNA damage, especially in leukocytes. Brain cells of chlorpyrifos-exposed rats generally had a lower level of primary DNA damage than leukocytes.

The genotoxic effects of chlorpyrifos using a comet assay were also reported in a study by Mehta et al. [83], where the rats were administered 50 and 100 mg/kg b. w. chlorpyrifos daily for 1, 2, and 3 days as well as 1.12 and 2.24 mg/kg b. w. for 90 days. Their results clearly indicate that both acute and chronic exposure caused a dose-dependent increase in DNA damage in rat brain cells.

The utility of the alkaline comet assay in detecting DNA damage following exposure to chlorpyrifos was also confirmed on other models *in vitro* and *in vivo* models. Sandal and Yilmaz [84] evaluated the genotoxic effects of chlorpyrifos on human peripheral blood lymphocytes and confirmed increased DNA damage at 10 μM Želježić et al. [32] also recorded DNA damage in a comet assay study on human peripheral blood lymphocytes and HepG2 cells exposed to chlorpyrifos at 0.000623 μg/mL, 0.016 μg/mL and 0.0262 μg/mL. A few comet assay studies were performed on animal models. For instance, Ali et al. [85] studied the toxicity of chlorpyrifos on the freshwater fish *Channa punctatus* Bloch and found significantly increased levels of DNA damage in blood and gill cells following exposure to 203, 102 and 68 μg/L of chlorpyrifos. Ismail et al. [86] applied the comet assay in erythrocytes and gill cells of the fish *Labeo rohita* exposed to chlorpyrifos at sublethal concentrations (221.4 μg/L, 110.7 μg/L and 73.8 μg/L) for 96 h. They detected concentration-dependent DNA damage in both cell types, but the gill cells were more sensitive to chlorpyrifos than erythrocytes. In another study, Ismail et al. [87] exposed tadpoles of the Common Indus Valley Toad, *Bufo stomaticus* to three sublethal concentrations (155, 233 and 465 μg/L) of chlorpyrifos for 96 h. Using the alkaline comet assay on blood cells, they observed concentration-dependent genotoxic effects of the tested compound.

In ours as well as in all of the previously mentioned studies, the alkaline version of the comet assay was used. It detects the broadest spectrum of DNA damage [54,80,88,89]. What we also observed is that low mean values of tail intensity were accompanied by relatively high mean values of tail length, especially in leukocytes. Such results suggest that, following treatment with chlorpyrifos, the amount of DNA breaks was low, but the fragments travelled to longer distances, giving the appearance of comets with long thin tails, which contained a low percentage of DNA. This is in line with previous reports that some genotoxins induce long, thin tails, while others induce short, dense tails [90], and also with the theory behind comet formation, which suggests that, when small amounts of breaks are present, lower amounts of DNA loops are relaxed, and consequently the intensity of fluorescence of the comet tail is lower [91].

Although endogenous levels of DNA damage in both cell types were low, leukocytes suffered more primary damage than brain cells. This could be attributed to the inherent differences between these cell types. The most important difference to mention is the cells' ability to repair DNA damage, and also their proliferation status needed to replace highly damaged cells. Considering that neurons cannot divide, our results which point to lower DNA damage in brain tissue are encouraging. As known, brain tissue due to intense oxidative metabolic activity, but relatively lower antioxidant levels, is particularly prone to the detrimental effects of organophosphate pesticides [92]. Our results unambiguously confirm that changes in DNA damage levels between leukocytes and brain cells were primarily influenced by different levels of the parent compound and its metabolite (as previously mentioned, HPLC-UV DAD confirmed they were higher in blood plasma than in the brain). It is evident from the genotoxicity studies conducted so far that both the parent molecule of chlorpyrifos and 3,5,6-trichloro-2-pyridinol exhibit DNA damaging properties. Mechanisms of DNA strand breaks due to chlorpyrifos have been insufficiently explained. In their study on chlorpyrifos-treated fish, Ali et al. [85] suggested that phosphorylation of DNA damage possibly contributed to DNA breaks and formation of comets, since previous reports [93] suggested that phosphorous moiety from organophosphate insecticides seems to be a good substrate for nucleophilic attacks. Kashanian et al. [94] reported that 3,5,6-trichloro-2-pyridinol could interact with DNA via a groove-binding mode. Therefore, if one takes into account the possibility that a chlorpyrifos metabolite is capable of intercalating into DNA, the effects at DNA level extend to lesions that have to be repaired by complex mechanisms involving the excision of damaged parts of DNA. As known, such repair processes induce new breaks in DNA, which contributes to increased DNA migration. What we could conclude from the comet assay results obtained is that the overall levels of DNA damage measured could be attributed to direct effects of both compounds, along with indirect effects mediated through free radicals (which based on the results of oxidative stress markers did not prevail). Both direct and indirect effects obviously contributed to the production of single-strand breaks, oxidized bases, and other DNA lesions, which were readily identified by the employed comet version. However, it also has to be stressed that we studied the effects of repeated 28-day exposure, and that in such cases an equilibrium between DNA damage infliction and repair might be achieved, which also contributed to the values measured in the comet assay.

5. Conclusions

Considering that most of the existing knowledge on chlorpyrifos' adverse effects originates from studies that investigated much higher doses, our study added new useful information regarding its toxicity profile following exposure to doses likely to occur in real-life situations.

In our study, the DNA instability in rat leukocytes was evident even at the lowest tested dose of chlorpyrifos (0.010 mg/kg b. w./day). Fortunately, this dose is 10-fold higher than the current ADI and AOEL values proposed by the EFSA [1]. However, former international legislative [44] proposed much higher reference values: the old estimated ADI for humans was 0.01 mg/kg b. w., while the previous ARfD was 0.1 mg/kg b. w. We also tested two higher chlorpyrifos doses; 0.015 mg/kg b. w./day (3-fold higher than current ARfD) and 0.160 mg/kg b. w./day (equal to 1/2000 of acute oral LD_{50} for rats based on WHO [44], or within 1/400 and 1/1200 acute oral LD_{50} for rats based on EFSA [1]). Although low, the doses we tested were able to produce measurable biological and biochemical responses in the treated rats. Therefore, our results confirm that it was reasonable to set lower values for all of the above mentioned reference values.

Although we did not reveal all aspects of chlorpyrifos toxicity, our findings suggest that 28-day exposure of rats to very low doses of chlorpyrifos, *i.e.* within the toxicology reference values, resulted in detectable plasma/tissue levels of both the parent compound and its metabolite, which through direct interaction with DNA, and to a lesser extent via induction of oxidative stress responses, contributed to DNA instability. Such results call for further research using other sensitive biomarkers of effect, along with different exposure scenarios. In that regard, sub-chronic as well as chronic studies, along with more proficient research focused on metabolic differences which contribute to differential susceptibility of various rat tissues towards both the parent chlorpyrifos compound and its metabolites are especially welcome.

Ethical statement

This study was conducted in accordance with the EU Directive 2010/63/EU on protection of animal used for scientific purpose authorised by the competent authorities.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cbi.2017.10.029.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.cbi.2017.10.029.

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