Dietary exposure to continuous small doses of α-cypermethrin in the presence or absence of dietary curcumin does not induce oxidative stress in male Wistar rats

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ABSTRACT

α-Cypermethrin is a widely used insecticide and, at high doses, induces oxidative stress in mammals. Curcumin is an antioxidant phytochemical commonly used for food coloring and flavoring. We aimed to investigate the effects of continuous dietary exposure to low doses of α-cypermethrin, as is the case in exposed humans, on oxidative stress and its potential prevention by dietary curcumin. Four groups of ten male Wistar rats were ad libitum-fed a control diet or identical diets fortified with α-cypermethrin (350 mg/kg diet), curcumin (1000 mg/kg diet), or α-cypermethrin and curcumin (350 and 1000 mg/kg diet, respectively) for 7 weeks. α-Cypermethrin accumulated in adipose tissues and was detectable in kidney, liver, and brains. Dietary α-cypermethrin did not alter concentrations of malondialdehyde, ascorbic and uric acid, retinol, liver damage markers, or the activities of CAT and SOD, but reduced vitamin E in blood. α-Cypermethrin did not affect malondialdehyde or reduced glutathione concentrations in any of the tissues, but significantly increased glutathione disulfide in kidney and subcutaneous adipose tissue. In conclusion, dietary exposure to small doses of α-cypermethrin did not induce oxidative stress in rats and may be less toxic than exposure to comparable quantities administered as single high doses by gastric intubation.

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1. Introduction

Cypermethrin is a type II synthetic pyrethroid that is widely used as pest control in agriculture, forestry, horticulture, health programs, and private homes. Cypermethrin is a racemic mixture of eight stereoisomers of which four exist in cis- and four in trans-configuration. Two of the cis-isomers, namely the 1R,cis- and 1S,cis-stereoisomers make up α-cypermethrin, the most active pair of the cypermethrin isomers [4,7]. The urinary concentrations of 3-phenoxybenzoic acid, a cypermethrin metabolite, were positively correlated with the frequency

of spraying in Japanese pest control operators [20]. Because of its widespread use, not only persons working with insecticides, but large parts of the population are regularly exposed to cypermethrin. Cypermethrin metabolites were found in 60% of the collected urine samples in a German population [15] and in >70% of urine samples in a population in the USA [1]. Ingestion of cypermethrin with the diet was identified as the most likely route of exposure in Germany [33].

In rats, oral gavage of single or repeated doses of cypermethrin induces oxidative stress and subsequent oxidative damage in erythrocytes, brain, and liver [9,13,17,32] and is accompanied by reductions in the activities of the antioxidant enzymes catalase [9,32] and superoxide dismutase [9]. In these studies, simultaneous treatment with antioxidants, such as vitamin E, phytochemicals from propolis, or curcumin, reduced cypermethrin-induced oxidative stress and increased the activities of antioxidant enzymes [9,13,17,32].

Curcumin is a yellow pigment and lipophilic phenolic compound derived from the rhizome of the plant turmeric (*Curcuma longa* of the ginger family (Zingiberaceae)) and is used for food coloring and flavoring in private homes and by the food industry. Among the reported health beneficial effects of dietary curcumin are its potent antioxidant and anti-inflammatory activities [2].

Previous animal studies have applied single or repeated doses of cypermethrin, equivalent to 10% LD_{50} or higher, dissolved in oil via gastric intubation [9,12,32], which would represent the rather unrealistic scenario of a high-dose oral exposure once a day in humans. The present experiment was therefore designed to mimic a constant low-level dietary exposure spread out over the day, which is a more realistic simulation of the exposure pattern observed in humans [33]. The aim of this experiment was to test whether or not this continuous dietary exposure to small doses of α-cypermethrin would induce oxidative stress in rats and, if so, whether or not simultaneous ingestion of curcumin would reduce pesticide-induced oxidative

damage. As previous studies focused on acute effects shortly after high-dose exposure, we furthermore aimed to investigate the potential accumulation of pyrethroid-induced oxidative damage over a longer period of seven weeks.

2. Methods

2.1. Chemicals

α-Cypermethrin (CAS # 52315-07-8; purity ≥97%) was a kind gift from Nanjing Essence Fine-Chemical Co., Ltd. (Nanjing, China). Curcumin (LKT-C8069; CAS # 458-37-7; purity >90%) was from LKT Laboratories Inc. (St. Paul, MN, USA). Malondialdehyde bis (diethyl acetal) was from Merck KGaA (Darmstadt, Germany); 2,4-dinitrophenylhydrazine (DNPH), catalase (CAT; product no. C30), Red cell lysing Buffer Hybri-Max™ (product no. R7757), potassium periodate, iodonitrotetrazolium chloride, superoxide dismutase from bovine erythrocytes, xanthine, xanthine oxidase, and Purpald® were from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). Hydrogen peroxide solution (35%) was purchased from Carl Roth GmbH + Co. KG (Karlsruhe, Germany).

2.2. Experimental animals and diets

The animal experiment was performed in accordance with the guidelines for the care and use of animals for experimental procedures and approved by the Regional Council of Stuttgart, Germany. Forty male Wistar rats (200–250 g; Janvier, Le Genest Saint-Isle, France) were used because male rats, contrary to female rats, can be housed in groups and randomized into groups of ten animals with similar mean body weights (Table 1) and kept in groups of 3–4 animals per cage under standard conditions ($22 \pm 2\,^\circ$C, $55 \pm 5\%$ relative humidity, 12 h light/dark cycle). Cages (type IV) were equipped with softwood bedding, a

Table 1
Feed intake,[a] body and organ weights of rats fed control diets or identical diets supplemented with α-cypermethrin (350 mg/kg), curcumin (1000 mg/kg), or a mixture of α-cypermethrin and curcumin (350 and 1000 mg/kg, respectively) for seven weeks.

	Control	α-Cypermethrin	Curcumin	α-Cypermethrin + Curcumin	P
Feed intake (g/rat/day)[a]					
Day 0–14	20.9 ± 4.5	20.0 ± 1.1	24.0 ± 1.7	20.1 ± 1.1	0.255
Day 15–28	26.6 ± 0.9	25.5 ± 1.4	25.1 ± 4.7	24.7 ± 0.1	0.812
Day 29–49	34.9 ± 1.1	34.4 ± 1.7	39.3 ± 6.2	33.4 ± 1.1	0.209
Day 1–49	28.7 ± 1.7	27.9 ± 0.8	31.2 ± 2.6	27.3 ± 0.8	0.087
Body weight (g)					
Day 0	240.1 ± 21.0	245.5 ± 17.9	247.7 ± 20.5	240.5 ± 13.0	0.743
Day 14	348.5 ± 21.1	340.4 ± 25.6	355.4 ± 15.4	337.2 ± 21.5	0.230
Day 28	418.4 ± 33.2	405.8 ± 32.2	433.7 ± 19.3	403.4 ± 31.5	0.104
Day 49	462.7 ± 37.8	459.7 ± 36.2	483.9 ± 26.4	448.5 ± 42.7	0.193
Organ weight (g)					
Liver	12.2 ± 1.3	13.2 ± 1.7	13.3 ± 0.8	12.7 ± 1.6	0.271
Kidney	3.3 ± 0.3	3.5 ± 0.4	3.4 ± 0.3	3.2 ± 0.4	0.199
Brain	2.1 ± 0.1	2.1 ± 0.2	2.1 ± 0.1	2.2 ± 0.1	0.697
Heart	1.7 ± 0.2	1.7 ± 0.2	1.8 ± 0.2	1.6 ± 0.2	0.244
Lung	2.1 ± 0.3	2.1 ± 0.2	2.3 ± 0.2	2.1 ± 0.1	0.258

[a] Feed intake per rat was calculated by dividing the daily feed consumption of all animals in a cage by the number of animals in the respective cage; *n* = 3 (cages per group).

water bottle, and a plastic tube. Animals were fed a modified standard rodent diet (C1000; modifications: vitamin A, 2500 IU; vitamin E, 30 mg; selenium, 150 μg; all values per kg diet; Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) that was free from synthetic antioxidants, plant polyphenols, and ascorbic acid for an acclimation period of one week and then assigned to one of four treatments: (1) the control group received the standard diet only, (2) the cypermethrin group received the standard diet fortified with 350 mg/kg α-cypermethrin, (3) the curcumin group the standard diet fortified with 1000 mg/kg curcumin, and (4) the cypermethrin + curcumin group the standard diet fortified with a combination of 350 mg/kg α-cypermethrin and 1000 mg/kg curcumin. Animals had free access to water and feed during the entire experiment, which lasted 7 weeks. Blood was collected from the jugular vein into separate K-heparinized tubes after CO_2 anesthesia and decapitation. Blood samples were centrifuged ($3000 \times g$, 10 min) to obtain plasma and both whole blood and plasma samples were stored at $-80\,°C$ until analyzed.

2.3. Quantification of malondialdehyde in whole blood and tissues

Malondialdehyde (MDA) in whole blood and tissues was analyzed according a method described by [25]. Briefly, whole blood or homogenates of liver, kidney, brain and fat (25 μL) mixed with 1% sulphuric acid (75 μL) and 6 M NaOH solution (20 μL) were incubated at 60 °C for 30 min (waterbath). After de-proteinisation with 25% perchloric acid (50 μL) supernatant (100 μL) was mixed with 5 mM 2,4-dinitrophenyl-hydrazine (10 μL) and incubated for 30 min before analysis on a Shimadzu Prominence HPLC. The MDA-2,4-dinitrophenyl-hydrazine adduct was separated on a Reprosil-Pur 120 C18 AQ (250 mm × 4.6 mm, 5 μm; Trentec) with 50% methanol in formic acid buffer (0.05 M, pH 3.75) at 1 mL/min and detected by UV–vis at 310 nm. Pooled whole blood ($n = 7$) analyzed along with the samples gave intra-batch and inter-batch coefficients of variation of 2.4% and 3.1%, respectively.

2.4. Liver enzymes and lipids in plasma

Aspartate aminotransferase (AST), alanine transaminase (ALT), and alkaline phosphatase (ALP) were measured in plasma using diagnostic kits (OSR6009, 6007, and 6004, respectively; Beckman Coulter) adapted for the Olympus AT200 auto analyser. Plasma cholesterol and triacylglycerols were determined using diagnostic kits OSR6116, 61118, and OE66300 (Beckman Coulter).

2.5. Quantification of lipid-soluble vitamins in plasma and tissues

Retinol and tocopherols in plasma (40 μL) were analyzed by reversed phase HPLC as recently described [40], with minor modifications. Retinol was quantified by UV–vis (325 nm) and tocopherols by fluorescence detection (excitation at 298 nm/emission at 328 nm). α-Tocopherol in liver, kidney, brain, and adipose tissues was

determined by HPLC with electrochemical detection as previously described [14].

2.6. Quantification of ascorbic and uric acid in plasma

Plasma ascorbic and uric acid were analyzed by RP-HPLC and UV–vis detection (245 nm) after reduction with tris-(2-carboxyethyl)-phosphine (abcr GmbH & Co. KG, Karlsruhe, Germany). Briefly, 100 μL of plasma was mixed with 25 μL of 20% (w/w) tris-(2-carboxyethyl)-phosphine and de-proteinised with 75 μL of 10% (w/w) meta-phosphoric acid. After centrifugation (13,500 rpm, 4 °C), whole supernatant was transferred to an HPLC vial and 20 μL was analyzed on a Shimadzu Prominence HPLC. Separation of ascorbic and uric acid was achieved using a 5 μm analytical column (Reprosil-Pur 120 C18 AQ 250 mm × 4.6 mm; Trentec, Gerlingen, Germany) set at 40 °C and a mobile phase consisting of 0.05 M sodium phosphate buffer (pH 2.5) at a flow rate of 1 mL/min.

2.7. Quantification of total glutathione in whole blood, and glutathione and glutathione disulfide in tissues

Total glutathione in whole blood was analyzed after reduction with 1,4-dithiothreit using 5,5′-dithiobis-2-nitrobenzoic acid (Ellman). Briefly, 100 μL of whole blood or glutathione standard was first reduced with 100 μL 1,4-dithiothreit (12.5 mol/L) and de-proteinised with 200 μL of 10% (w/v) trichloroacetic acid. Two hundred μL of the supernatant was buffered with 100 μL 2 M di-potassium hydrogen phosphate and finally mixed with 50 μL of Ellman reagent (30 mmol/L dithiobis-2-nitrobenzoic acid in 0.5 M K_2HPO_4-buffer, pH 7.5); 20 μL was injected for analysis on a Shimadzu Prominence HPLC using a Reprosil-Pur 120 C18 AQ column (5 μm, 250 mm × 4.6 mm, Trentec) at 40 °C, a mobile phase consisting of 15% methanol and 0.05 M acetate buffer (pH 5, v/v) at 1 mL/min and UV–vis detection at 326 nm.

Tissue samples were thawed on ice and ca. 200 mg weighed into a 2 mL test tube. One milliliter ice-cold 10% PCA solution (0.4 N perchloric acid and 100 nM EDTA, both from Sigma) was added and samples sonicated thrice for 15 s each. Homogenates were centrifuged (13,250 × g, 15 min, 4 °C) and 100 μL supernatant transferred to an HPLC vial, diluted with 100 μL mobile phase, and 10 μL sample injected. Reduced glutathione (GSH) and glutathione disulfide (GSSG) were separated on a Reprosil C18 column (5 μm, 250 mm × 3 mm; Trentec-Analysentechnik, Rutesheim, Germany) with 25 mM sodium dihydrogen-phosphate; 1.4 mM 1-octanesulfonic acid, and 6% acetonitrile (adjusted to pH 2.65) as mobile phase at a flow rate of 0.6 mL/min. The HPLC system consisted of an AS-2057 Plus autosampler, PU-2080 Plus pump, LG-2080-02 gradient unit, and a 3-line degasser (Jasco, Groß-Umstadt, Germany) and an ESA 5600A electrochemical detector equipped with a Boron Doped Diamond electrode model 5040 (Dionex, Idstein, Germany) that was set to +1500 mV (vs. PD reference). Between injections, the electrode was cleaned by applying +1900 mV for 30 s, and allowing a re-equilibration time of 5 min. Peaks were recorded and integrated with the chromatographic software CoulArray 3.10 (ESA) and

GSH and GSSG were quantified against authentic external standards (Sigma).

2.8. Catalase activity

Catalase (CAT) activities were determined according to the method of [6]. Briefly, 60 μL of diluted whole blood (see above) or CAT standard was added to 70 μL working reagent (phosphate buffer, pH 7.0, methanol, and hydrogen peroxide; 3:3:1 by vol) and incubated for 10 min at room temperature. Ninety μL Purpald® (22.8 mmol) was added, the sample incubated at room temperature for 20 min, and the absorbance read at 540 nm after addition of 30 μL of potassium periodate (65.2 mmol/L). A standard curve was constructed from dilutions of CAT standard and used to calculate the CAT activities of the samples. Results are reported as U/mg protein.

2.9. Superoxide dismutase activity

Whole blood superoxide dismutase (SOD) activity was determined using a procedure modified from the original method published by [16] and the modifications published by [28]. Samples were prepared by diluting whole blood with H_2O (1:20, v/v). Twenty microliter diluted sample was mixed with 20 μL chloroform/ethanol (1:2, v/v) solution. Thirty microliter of the resulting sample or of SOD standards of known concentrations, respectively, were pipetted onto a 96-well plate and 250 μL buffer (prepared from 48 mL of 24 mmol/L $NaHCO_3$ and 15 mmol/L NaOH, with 1 mL of 50 mmol/L xanthine in 100 mmol/L NaOH, and 1 mL of 5 mmol/L iodonitrotetrazolium chloride diluted in ethanol and water (0.11: 0.89)), and 20 μL of 0.15 U/mL xanthine oxidase in water were added to each well. Absorbance was read at 505 nm at 37 °C in 1 min intervals for 30 min on microplate reader (BioTek™ Synergy HT, BioTek™ Instruments GmbH, Bad Friedrichshall, Deutschland). The standard curve was generated from the linear rate of reaction SOD standards of known concentration and SOD activity is reported as U/mg protein.

2.10. Quantification of α-cypermethrin in tissues

α-Cypermethrin from liver, kidney, brain and adipose tissues were extracted and purified as previously described [10]. Briefly, tissue samples (500 mg) were homogenized with 5 g of sodium sulphate in a mortar, transferred to screw cap tubes, and well mixed with 5 mL of hexane. Samples were extracted with 5 mL acetonitrile and the mixture vigorously shaken for 5 min. After centrifugation (15 min at 2000 rpm), the whole lower acetonitrile phase was transferred and extracted using C18 solid phase extraction cartridges (VertiPak, Thailand). Residues were eluted with acetonitrile, evaporated (rotary evaporator at 35 °C, 100 mbar) to dryness, re-dissolved in 500 μL ethyl acetate, of which 1 μL was injected into and analyzed by GC-ECD on a HP 6890 with ChemStation B.04.02 (Agilent Technology, USA) and the following operating conditions: HP-5 column (30 m × 0.32 mm × 0.25 μm film thickness, cross-linked 5% PH ME siloxane); injector in split-less mode operated at 250 °C; oven temperature (column) at 100 °C for 1 min, then changed to 250 °C with 25 °C/min ramp rate, then changed to 280 °C with 5 °C/min ramp rate, held at 280 °C for 5 min, and post run at 290 °C for 5 min. Oxygen free nitrogen as make-up gas and helium as carrier gas were from TIG, Bangkok. The limit of detection for α-cypermethrin was 0.1 μg/kg tissue. Pooled liver samples spiked with α-cypermethrin (80 μg/kg) and analyzed along the study samples gave intra-batch ($n = 8$) and inter-batch ($n = 10$) coefficients of variation of 8.5% and 13.7%, respectively.

2.11. Statistical analyses

All statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL; Version 11.5) and GraphPad Prism 5 for Mac OS X (version 5.0c; GraphPad Software, Inc., La Jolla, CA, USA). All data are given as mean with standard deviation. Differences between groups were assessed by means of one-way ANOVA with Bonferroni's multiple comparisons test and considered significant at $P < 0.05$.

3. Results and discussion

The toxic and pro-oxidative effects of the pesticide α-cypermethrin have been investigated in rats [3,9,11–13,23,27,30,32,38,41]. A major problem that limits the power of these studies to simulate the situation in humans is the fact that they did not study continuous low-level dietary exposure but a less realistic oral intake of individual high doses of the pesticide once per day. We thus designed the current experiment to investigate if the more realistic scenario of a continuous intake of small α-cypermethrin doses spread-out over the day [33], amounting to a total daily intake comparable to the doses applied in previous studies [9,12,32], would lead to impaired antioxidant defence mechanisms and increased lipid peroxidation and if so, whether or not dietary curcumin might counteract these harmful effects. Curcumin was chosen as test compound because of its antioxidant activity in various model systems in addition to its reported safety for human consumption, even at high doses (curcumin is generally recognized as safe by the US Food and Drug Administration), its widespread use as a colorant by the food industry and the high acceptance of this natural plant compound by the consumers [21].

The daily α-cypermethrin intake in the current study was in the range of 20–35 mg/kg bodyweight (BW) and thus 8–14% of the acute oral LD_{50} for adult rats, which is 250 mg/kg bodyweight [3]. Since rats consume their feed in approximately 14–18 meals over the course of one day [31,42], the individual doses were much lower and below 1% LD_{50}.

3.1. Animal performance and hepatotoxicity

In our rats, feed intake and body weight were not affected by α-cypermethrin and were not significantly different between groups (Table 1), which concurs with previous studies using individual high-dosage protocols [11,12,27]. On the other hand, gastric intubation with 25 mg cypermethrin per kg bodyweight (ca. 20–40% LD_{50};

Table 2

Antioxidants, oxidative stress markers and biomarkers of liver damage in plasma and whole blood of rats fed control diets or identical diets supplemented with α-cypermethrin (350 mg/kg), curcumin (1000 mg/kg), or a mixture of α-cypermethrin and curcumin (350 and 1000 mg/kg, respectively) for seven weeks.[a]

	Control	α-Cypermethrin	Curcumin	α-Cypermethrin + Curcumin	P
Plasma					
α-Tocopherol (μmol/L)	10.4 ± 1.3[ac]	7.2 ± 2.0[b]	12.2 ± 2.2[c]	8.6 ± 1.4[ab]	<0.001
γ-Tocopherol (μmol/L)	0.40 ± 0.06[a]	0.24 ± 0.12[b]	0.46 ± 0.11[a]	0.35 ± 0.09[ab]	<0.001
Cholesterol (mmol/L)	3.1 ± 0.4	2.7 ± 0.9	3.3 ± 0.8	2.8 ± 0.5	0.233
Triacylglycerols (mmol/L)	1.1 ± 0.2	1.2 ± 0.6	1.4 ± 0.4	1.1 ± 0.4	0.264
Retinol (μmol/L)	1.7 ± 0.2	1.8 ± 0.2	1.9 ± 0.4	2.0 ± 0.4	0.286
Ascorbic acid (μmol/L)	72.1 ± 21.2	77.8 ± 37.5	77.5 ± 23.4	79.7 ± 34.4	0.948
Uric acid (μmol/L)	84.2 ± 44.4	79.3 ± 33.7	89.9 ± 75.0	64.1 ± 17.4	0.655
Alanine transaminase (U/L)	45.6 ± 10.3[a]	58.1 ± 16.7[ab]	44.2 ± 9.6[a]	63.4 ± 7.3[b]	<0.001
Alkaline phosphatase (U/L)	118.3 ± 22.7	113.1 ± 26.2	110.6 ± 26.9	126.4 ± 34.2	0.609
Aspartate transaminase (U/L)	122.8 ± 28.0	145.6 ± 29.9	133.6 ± 34.6	138.1 ± 26.7	0.396
Whole blood					
Malondialdehyde (μmol/L)	9.9 ± 1.7[ab]	11.3 ± 2.5[a]	9.4 ± 1.2[ab]	8.4 ± 1.1[b]	0.006
Total glutathione (μmol/L)	1347 ± 81[a]	1246 ± 100[ac]	1093 ± 89[b]	1166 ± 121[bc]	<0.001
Catalase activity (U/mg protein)	1.21 ± 0.46	1.56 ± 0.91	1.56 ± 0.70	1.31 ± 0.57	0.564
Superoxide dismutase activity (U/mg protein)	3.40 ± 1.90	2.38 ± 1.32	2.68 ± 1.31	4.02 ± 1.85	0.150

[a] Values within a row not sharing a common superscript letter are significantly different at $P < 0.05$.

see below for discussion) for 28 days resulted in reduced bodyweight in male Wistar rats [32].

Consumption of α-cypermethrin or curcumin alone did not affect the activities of the liver damage markers ALT, ALP, and AST in plasma in the present experiment (Table 2). The combined intake of α-cypermethrin with curcumin significantly increased plasma ALT, but not ALP or AST activities. However, because the activities of liver enzymes remained within the reference ranges for healthy rats [26] in all groups, this statistically significant increase is likely without biological importance. In support of our data, even high-dose feeding of 420 mg cypermethrin/kg BW for 6 months did not result in increases in serum liver enzymes in rats [38]. Even the increases in the activities of liver enzymes in cypermethrin-exposed rats observed in some studies [23,32] remained within the reference ranges for healthy rats and are thus not indicative of hepatic injury. Hence, it appears that statistically significant effects on liver enzymes that remained within the boundaries of normal biological variation have in the past been incorrectly interpreted as pesticide-induced liver damage in some studies.

3.2. Accumulation of α-cypermethrin in tissues

α-Cypermethrin was only present in organs of animals fed the pesticide, but not of control and curcumin only-fed animals (Table 3). The fat-soluble α-cypermethrin accumulated in adipose tissues at concentrations of up to 9.8 μg/g tissue, whereas its contents (in descending order) were much lower in kidney, liver, and brain tissues. The simultaneous ingestion of curcumin did not alter α-cypermethrin concentrations in any of these tissues (Table 3).

The higher concentrations of α-cypermethrin residues in adipose compared to brain and other tissues is in agreement with observations in male Sprague-Dawley rats given a single oral dose of a mixture of four pyrethroids (each administered at 3 mg/kg bodyweight; including cypermethrin) dissolved in glycerol formal. These authors proposed

that the higher concentrations and longer persistence of the pesticides in adipose tissue may be due to its slower metabolism and lack of enzymes required for pyrethroid hydrolysis [24]. Similarly, cypermethrin concentrations in rats orally administered a single dose of a mixture of six pyrethroids (of which 29% were cypermethrin) in corn oil (total pyrethroids, 27.4 mg/kg bodyweight; cypermethrin, 8 mg/kg bodyweight) were higher in adipose tissue (1.07 μg/g), than in the brain (0.14 μg/g) and liver (0.40 μg/g) 2.5 h after dosing [39]. The higher α-cypermethrin concentrations in the adipose tissues of our animals are likely explained by the longer intervention period (7 weeks vs. single oral dose in the above studies) and the daily administration, which allowed tissue accumulation and the establishing of steady-state concentrations. The concentrations in the single-dosing experiments were likely determined at a point in time when concentrations were increasing or decreasing and may thus not represent maximum tissue concentrations and certainly not steady-state concentrations.

3.3. Redox status

Whole blood MDA concentrations were not significantly higher than in control rats after 7 weeks of low-level exposure to α-cypermethrin, curcumin or α-cypermethrin plus curcumin. Simultaneous ingestion of α-cypermethrin and curcumin, however, significantly reduced MDA concentrations in comparison with exposure to α-cypermethrin alone (Table 2). α-Cypermethrin-feeding, alone or in combination with curcumin, did not alter MDA concentrations in the liver, kidney, brain or visceral and subcutaneous adipose tissues (Table 3). Application of a single dose of 125 mg/kg bodyweight cypermethrin by oral gavage previously resulted in increased concentrations of MDA in plasma (by 54%), liver, and kidney in female Wistar rats [9]. Other authors also reported increased markers of oxidative damage (MDA, thiobarbituric acid reactive substances, or "oxidation index") in erythrocytes, brains,

Table 3
α-Cypermethrin, malondialdehyde, α-tocopherol, glutathione and glutathione disulfide in liver, kidney and adipose tissues of rats fed control diets or identical diets supplemented with α-cypermethrin, curcumin, or a mixture of α-cypermethrin and curcumin for seven weeks.[a]

	Control	α-Cypermethrin	Curcumin	α-Cypermethrin + Curcumin	P
α-Cypermethrin (ng/g)					
Liver	n.d.	53.2 ± 92.9	n.d.	84.2 ± 161.4	0.605
Kidney	n.d.	523.9 ± 522.3	n.d.	701.5 ± 548.3	0.468
Brain	n.d.	17.5 ± 11.5	n.d.	10.3 ± 4.9	0.085
Visceral adipose tissue	n.d.	9813 ± 2093	n.d.	8955 ± 2051	0.367
Subcutaneous adipose tissue	n.d.	9288 ± 2325	n.d.	9252 ± 5329	0.985
Malondialdehyde (μmol/g)					
Liver	0.099 ± 0.066	0.105 ± 0.084	0.124 ± 0.105	0.217 ± 0.147	0.058
Kidney	0.047 ± 0.007	0.051 ± 0.008	0.045 ± 0.009	0.051 ± 0.008	0.235
Brain	0.005 ± 0.001	0.004 ± 0.005	0.005 ± 0.001	0.004 ± 0.001	0.125
Visceral adipose tissue	142.0 ± 48.9[ab]	202.0 ± 53.6[a]	131.1 ± 40.2[b]	175.6 ± 76.0[ab]	0.031
Subcutaneous adipose tissue	116.4 ± 39.2[ab]	125.4 ± 53.0[ab]	84.7 ± 43.4[a]	144.8 ± 37.7[b]	0.032
α-Tocopherol (nmol/g)					
Liver	5.19 ± 3.51	5.36 ± 3.48	8.35 ± 3.80	6.32 ± 3.30	0.186
Kidney	2.92 ± 0.35[ab]	2.40 ± 0.44[a]	3.27 ± 0.46[b]	2.68 ± 0.58[a]	0.001
Brain	1.84 ± 0.32[a]	2.89 ± 0.74[b]	2.23 ± 0.45[ab]	2.21 ± 0.77[ab]	0.005
Visceral adipose tissue	6.04 ± 0.96[ab]	5.52 ± 0.85[a]	7.30 ± 1.69[b]	6.05 ± 1.09[ab]	0.015
Subcutaneous adipose tissue	3.83 ± 1.10[ab]	2.92 ± 0.62[a]	4.23 ± 1.04[b]	3.41 ± 0.42[ab]	0.009
Glutathione					
Liver (μmol/g)	3.0 ± 0.5	2.9 ± 0.5	3.2 ± 0.4	3.4 ± 0.5	0.240
Kidney (nmol/g)	14.3 ± 4.4[a]	20.8 ± 11.5[ab]	19.4 ± 8.4[ab]	25.6 ± 6.5[b]	0.031
Visceral adipose tissue (nmol/g)	3.7 ± 1.5	4.8 ± 1.7	5.2 ± 2.4	5.1 ± 2.0	0.343
Subcutaneous adipose tissue (nmol/g)	6.4 ± 3.8	7.1 ± 2.9	4.8 ± 1.2	6.9 ± 4.0	0.419
Glutathione disulfide					
Liver (μmol/g)	0.14 ± 0.05[ab]	0.23 ± 0.09[a]	0.12 ± 0.05[b]	0.20 ± 0.10[ab]	0.008
Kidney (nmol/g)	2.9 ± 0.9[a]	8.3 ± 2.4[b]	2.9 ± 1.5[a]	9.1 ± 2.4[b]	<0.001
Visceral adipose tissue (nmol/g)	32.1 ± 6.5[ab]	37.1 ± 6.8[a]	29.0 ± 3.9[b]	36.7 ± 5.8[a]	0.014
Subcutaneous adipose tissue (nmol/g)	23.6 ± 2.8[a]	37.8 ± 8.4[b]	26.4 ± 6.3[ac]	35.2 ± 9.4[bc]	<0.001

[a] Values within a row not sharing a common superscript letter are significantly different at $P < 0.05$.

livers, or kidneys after single (≥ 100 mg/kg bodyweight) or repeated (≥ 12.5 mg/kg bodyweight for ≥ 28 days) gastric intubation of rats [11,13,23,27,41]. At low doses of 2.5 mg cypermethrin per kg BW for 60 days, however, no lipid peroxidation was observed in erythrocytes [27].

Whole blood concentrations of total glutathione did not differ from control in α-cypermethrin- and α-cypermethrin plus curcumin-fed rats, but were significantly lower in only curcumin-fed rats (Table 2), although the extent of the effect was small. In liver and adipose tissues, reduced glutathione concentrations were similar in all groups. Only in the kidney, the combination of α-cypermethrin plus curcumin significantly increased reduced glutathione concentrations, whereas the pesticide and the phytochemical alone, did only numerically increase it (Table 3). The oxidized form of glutathione, glutathione disulfide, was significantly higher in the kidney and subcutaneous adipose tissue and numerically increased in the liver and visceral adipose tissue of α-cypermethrin-fed rats compared to control animals (Table 3). Ingestion of curcumin did not affect glutathione disulfide concentrations in these tissues and, consequently, its co-ingestion with α-cypermethrin did not mitigate the α-cypermethrin-induced increase in glutathione disulfide concentrations (Table 3). In comparison to control, α-cypermethrin, curcumin, or the combination of both did not alter SOD activity. Compared to α-cypermethrin-fed rats, the combination of α-cypermethrin and curcumin increased SOD activity (Table 2). CAT activity in whole blood was not different between groups (Table 2). In the literature, the impact of high doses of cypermethrin on the antioxidant enzymes SOD and CAT in rats has been somewhat contradictory. A single gastric dose of 125 mg/kg BW reduced the activity of both enzymes in plasma [9], whereas intubation with 25 mg/kg BW for 60 days increased their activities in erythrocytes [27]. Gastric application of lower doses of 12.5 or 2.5 mg/kg BW for 60 days did not alter SOD or CAT activities in erythrocytes [11,27].

Of the lipid- and water-soluble antioxidants measured in plasma, only α- and γ-tocopherol (vitamin E) were significantly reduced by exposure to α-cypermethrin ($P < 0.001$), while retinol, ascorbic acid and uric acid concentrations were similar in all groups (Table 2). Curcumin consumption alone did not significantly alter antioxidant status compared to control, but numerically increased vitamin E concentrations and attenuated the decreasing effect of α-cypermethrin in the combined α-cypermethrin plus curcumin group (Table 2). In a previous study, 4 week feeding of 4 g curcumin/kg diet to Sprague-Dawley rats only numerically increased plasma, but significantly increased lung vitamin E concentrations [18].

Since low-dose dietary exposure to α-cypermethrin did not induce overt oxidative stress in our animals, it is not surprising that curcumin did not reduce oxidative stress markers in blood in the present study. A previous study reporting protective effects of curcumin used cypermethrin (dissolved in oil) at a dose of 25 mg/kg BW/day and thus produced significant oxidant effects in liver, kidney, and brain [32]. The difference between their findings and ours can be partly explained by the use of younger

animals, which weighed 199–227 g at the end of the experiment [32], which is even less than the weight of our animals at the beginning (240–248 g) and half that at the end of our experiment (Table 1). Young rats are known to be more susceptible to the toxic effects of cypermethrin. While the oral LD_{50} of adult rats is 250 mg/kg BW, it is significantly lower for younger rats (21 days, 49; 16 days, 27; 8 days, 15 mg/kg BW) [3]. Thus, the dose used by Sankar and colleagues (2010) exceeded the intended 10% LD_{50} and is more likely to have been in the range of 20–40% LD_{50} for rats of that particular age. Better absorption and higher maximum plasma concentrations of the lipid-soluble insecticide when administered dissolved in oil may have further contributed to the observed differences (see also Section 3.4 below). Furthermore, it cannot be ruled out, that the positive effects observed in their animals, which were given curcumin 1 h prior to cypermethrin intubation, may have been confounded, as the used curcumin was diluted in gum arabic [32]. The oral toxicity of deltamethrin, another pyrethroid, was 100 times lower when dissolved in 10% gum arabic compared to oil or other solvents [29]. In humans, the absorption of amoxicillin, an antibiotic, was significantly reduced when gum arabic was ingested simultaneously with or 2 h prior to the drug (AUC: control, 21; simultaneous, 5; 2 h-prior, 11 Mg h/L) [8]. Thus, interactions of the gum arabic with cypermethrin absorption cannot be ruled out and may, at least in part, explain the lower oxidative stress observed in rats given curcumin prior to cypermethrin compared to those receiving only cypermethrin.

3.4. Matrix effects and bioavailability considerations

Overall, the above-mentioned studies used single or repeated high-doses of cypermethrin dissolved in oil, which probably enhances the oral bioavailability of the lipophilic insecticide and, thus, its toxicity, as previously described for deltamethrin [29]. The increased uptake of cypermethrin from oil suspensions, particularly when given as a single dose, may have resulted in much higher maximum plasma and tissue concentrations of cypermethrin than in our experiment and might explain the higher level of oxidative stress observed in blood and tissues of these rats. The animals in the current study, on the other hand, were exposed to low doses of α-cypermethrin in the diet. Matrix effects may have limited the absorption of the insecticide and resulted in lower concentrations of the pesticide compared to rats exposed by gastric intubation with oil suspensions and consequently resulted in cypermethrin concentrations that did not suffice to induce oxidative stress. In agreement, dose-dependent increases in malondialdehyde have been reported in organs of fish (*Clarias batrachus*) exposed for 96 h to increasing doses of cypermethrin in the water [22] and in plasma of mice given 5 or 10 mg cypermethrin for 15, 45 or 60 days [19]. However, further experiments are warranted to test if the food or vehicle matrix may significantly alter cypermethrin plasma and organ concentrations.

Overall, it appears likely that the potential pro-oxidative effects of α-cypermethrin are dose-dependent and that the small individual doses ingested in the present study and the thus likely lower maximum plasma and tissue concentrations of α-cypermethrin may explain the absence of oxidative stress in our animals.

The lack of biological activity of curcumin in the present study may be explained by its limited absorption, extensive metabolism and rapid elimination [21], which result in very low concentrations of free curcumin, if any at all, and the predominance of conjugated metabolites (mainly glucuronic acid and sulphate conjugates) in the organism [34]. Consequently, the parent compound, which is used in in vitro experiments and for which potent antioxidant activities have been reported, is not the form present in the organism and, importantly, the conjugates are attached at the functional groups associated with its antioxidant activity, rendering the metabolites much less potent antioxidants, if antioxidants at all (reviewed in [21]). The lack of any direct or indirect in vivo antioxidant activity of native curcumin in the present study is in agreement with previous findings from different animal models (e.g. [5,35,36]). The low oral bioavailability of native curcumin has led to the development of novel strategies, for example the administration of curcumin micelles, that can enhance its absorption and plasma concentrations and thus its bioavailability in healthy humans by up to 185-fold [37]. Such novel curcumin formulations with enhanced biological availability will be useful in future experiments aimed at studying the biological activities of this otherwise poorly absorbed phytochemical.

4. Conclusions

Even though high-dosage gastric intubation protocols in rats in the above cited studies resulted in statistically significant increases in oxidative stress and an inconsistent modulation of antioxidant enzymes, the extent of the observed changes was often small and their biological relevance may have been overrated. Using a more realistic scenario of continuous low-dose dietary exposure to α-cypermethrin, we did not observe liver damage or an overt induction of oxidative stress and impaired antioxidant defence in our rats. Our data suggest that previously performed studies using single high-dosing protocols may have overestimated the induction of oxidative stress by and the hepatotoxic effects of cypermethrin, possibly due to better bioavailability of the insecticide from oil. Additional studies are required to understand the impact of the food matrix on cypermethrin absorption kinetics, tissue distribution, and toxicity.

Conflict of interest statement

The authors declare they have no actual or potential competing financial interests.

Transparency document

The Transparency document associated with this article can be found in the online version.

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