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INFLUENCE OF THE POLYPHENOLIC TANNIC ACID ON THE TOXICITY OF THE INSECTICIDE DELTAMETHRIN TO FISH. A COMPARATIVE STUDY EXAMINING BOTH BIOCHEMICAL AND CYTOPATHOLOGICAL PARAMETERS

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Humics and pesticides are present in aquatic environment and the toxicological consequences of their chemical interaction is well studied. However, data concerning the mechanism of the biochemical action of humic-pesticide combinations are scarce, especially in vertebrates. Thus we have chosen to study the *in vivo* effects of the plant polyphenolic tannic acid and the pyrethroid insecticide deltamethrin [Decis] alone or in combination on hepatic xenobiotic-metabolizing enzyme activities and the associated redox-parameters in carp, as the complex assessment of these systems are regarded to serve as a relevant biomarker of environmental pollution. Stress effects and tissue damage were followed by determination of the plasma glucose level, the activities of plasma transaminases, and by electron microscopy. Tannic acid alone exerted weak prooxidant effect due to its marked antioxidant enzyme inhibitory activity. Deltamethrin, applied in a very low dose, induced oxyradical production in fish via activation of cytochrome P450 isozymes. This effect was promoted by the antioxidant enzyme inhibitory action of tannic acid, when the two chemicals were combined; however, the ultrastructural damage of the hepatocytes was reduced by the common cytoprotective capacity of the phenolic. Numerous humics are known to alter the toxicity of pesticides and their influence depends on their type and concentration. Therefore, our work taken together with other comparative studies may contribute to the assessment of the impact of humics in nature, especially in case of environmental pollution.

Keywords: Cytochrome P450-dependent monooxigenases – antioxidant enzymes – humics – pyrethroid – carp

INTRODUCTION

The aquatic environment is loaded by numerous natural and artificial chemicals. Deltamethrin (DM) a type II pyrethroid insecticide, used in agricultural protection and as a mosquitocide. In spite of the fact that pyrethroid II is considered to be the safest class of insecticides (because of its rapid degradation), under laboratory conditions it has high potential toxicity to fish [2, 44], and was found to be responsible for numerous fish devastation in aquatic ecosystems [3]. Xenobiotics may undergo

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redox cycling via the cytochrome P450 system [10, 23], and the involvement of the CYP 1A isoenzymes in the biotransformation of certain planar organic contaminants [24], pyrethroid pesticides [5, 23], e.g. deltamethrin [12, 36] has also been demonstrated. Elevated oxyradical production as a consequence of induction of cytochrome(s) P450 activities [10, 23, 32, 35] may result in toxicological consequences as oxidative damage in the tissues [10, 17, 19], mutagenesis and carcinogenesis [32]. Therefore, measuring cytochrome P450 1A isoenzymes together with redox parameters has a potential use as a biomonitoring system for certain environmental contaminants [19, 23, 24].

Plant-derived phenolics have since long been in the focus of interest, because of their potential scavenging activity towards reactive oxygen intermediates (ROI) [15]. Owing to this antioxidant property [11] many of them may exert beneficial physiological effects in living organisms [15, 31]. However, some plant polyphenols (e.g. quercetin) may behave as both antioxidant and prooxidant depending on their concentration and the source of free radicals [27], as well as the pH [11]. Typical aqueous concentration of dissolved humic materials (DHM) in freshwater is ranged from 5 to 25 mg l^{-1} [16] and its major portion are polyphenolic substances containing carboxyl groups. DHM may form complexes with inorganic and organic compounds [41], thereby affecting the bioavailability, the toxicity, the degradation and transport of these chemicals in the environment [29]. Tannins, as plant cell wall materials, are common components of humics; they are defined as water-soluble polymeric phenolics that precipitate proteins [18]. Tannins may exert various biological effects: many of them are reported to be enzyme inhibitors [e.g. 1, 39], antimutagenic and antitumorigenic agents [15], but some of them show carcinogenic activity [21]. High concentration of tannins may induce tissue necrosis [13].

The observation, that the toxicity of pesticides in aquatic ecosystems is different than under laboratory conditions [43], has evoked the examination of humic acid mixtures and pesticides concerning their molecular interactions, to find possible explanation to the discrepancies and to set up better modeling systems [29]. Examination of the modifying effects of humics on toxicity of pollutants on organisms belonging to different evolutional level (e.g. fish, reptiles, etc.) may contribute to the understanding of the nature of molecular interactions and the mechanism of detoxifying reactions occurring in the mammalian organism, and make appropriate prediction possible relating the toxicity of agricultural and industrial chemicals on the aquatic ecosystem. Consequently, examination of single humic-pesticide combinations in a simple system is principal. This paper underlines different mechanism of action of the model plant polyphenolic tannic acid (TA) and the insecticide DM [Decis] on liver xenobiotic-metabolizing enzymes (the CYP 1A isoenzymes) and on the linked antioxidant defense system of common carp, and examines whether TA actually alters DM toxicity in fish and if so what is the mechanism of this modifying effect. Plasma glucose (GLU) level was determined as general index of stress [3] and tissue necrosis was followed by measurement of the activities of plasma transaminases (aspartate aminotransferase (AspAT) and alanine aminotransferase (AlaAT)) [3]. The study was supplemented by an electron microscopic analysis of the hepatic tissue.

MATERIALS AND METHODS

Materials

Tannic acid (catalog number: 1401-55-4, M_r 1700.2) was purchased from Sigma (St. Louis, MO), the common and biochemical reagents were obtained from Sigma (St. Louis, MO) or from Reanal (Budapest, Hungary) and all were of analytical grade. Durcupan ACM was obtained from Fluka (Buchs, Switzerland) and Decis 2.5 EC (with an active ingredient content of 2.5% deltamethrin) was purchased from Chemark Ltd. (Budapest, Hungary).

In vivo *experiments*

Animals, treatment and sample preparation

All experimental procedures were carried out on common carp (*Cyprinus carpio* L.) weighing 600–800 g obtained from Szeged Fish Ltd., Hungary. Groups of 6–8 individual fish were used for each treatment. Two fish were kept in air-saturated water (pH ~7.0) at 13-14 °C in each 100-litre aquarium and acclimatized for 7 days before the treatment. Controls were injected intraperitoneally (i.p.) with distilled water. In the experimental group, fish were injected i.p. with 5.9 μmol kg^{-1} (= 10 mg kg^{-1}) body weight (b.w.) TA and 0.4 nmol kg^{-1} (= 0.2 μg kg^{-1}) b.w. DM singly or in combination. TA and DM were dissolved in distilled water immediately before the treatment and the pH of these solutions was adjusted to 7.0, excluding the disturbing effect of varying acidity. After an exposure time of 24 or 48 h, venous blood was taken with the addition of heparin as anticoagulant, and centrifuged to remove the blood cells. GLU-concentration, AspAT and AlaAT activities were determined from the plasma. Animals were decapitated and the liver was removed and carefully washed free of blood with cold fish physiological saline solution (0.62% NaCl), the wet weight was measured after drying the surface between filter papers and the organ was then frozen in liquid nitrogen and stored at –80 °C until use. Measurements were prepared with homogenization of liver in a Braun 853302/4 homogenizer (1/5, w/v) at 4 °C in 0.1 M K-PO_4 containing 0.15 M KCl and 0.1 M EDTA-Na_2 (pH 7.4). The homogenate was centrifuged at 10,000 × *g* for 20 min. The supernatants were used for the redox-parameter determinations (excluding thiobarbituric acid (TBA) test, which was assessed from the whole homogenate). Microsomes, for cytochrome P450-dependent enzyme assays, were prepared by differential centrifugation of liver homogenate as described by Förlin [14].

Measurement of biochemical parameters in the liver and in the plasma

The CYP 1A isoenzyme (EC 1.14.14.1) activities were followed by determination of 7-ethoxyresorufin-O-deethylase (EROD) by the method of Burke et al. [8] and 7-ethoxycoumarin-O-deethylase (ECOD) by Kamataki et al. [20]. Superoxide dismutase (SOD, EC 1.15.1.1) and manganese-superoxide dismutase (Mn-SOD) activities were determined by the method of Misra and Fridovich [28]; catalase (CAT, EC 1.11.1.6) activity by the method of Beers and Sizer [6]; the amount of thiobarbituric acid-reactive substances (TBARS), expressed as malonyl dialdehyde (MDA) equivalents, as products of lipid peroxidation (LP), was assessed by the method of Placer et al. [30]; reduced tissue glutathione (GSH) by the method of Sedlak and Lindsay [34]; cytosolic glutathione-peroxidase (GPx, EC 1.11.1.9) activity by the method of Chiu et al. [9]; protein by the method of Lowry et al. [26]; the GLU by the method of Trinder [37]; and the AspAT (EC 2.6.1.1) and AlaAT (EC 2.6.1.2) activities by the method of Reitman and Frankel [33]. CYP 1A isoenzyme activities are presented as pmol substrate min^{-1} mg^{-1} protein, antioxidant enzyme activities are given in nmol substrate min^{-1} mg^{-1} protein, except SOD, which is expressed in U mg^{-1} protein. One unit (U) of SOD activity is defined as the amount of SOD decreasing the oxidation rate of the organic substrate by 50% [28]. MDA and GSH concentrations are presented in nmol mg^{-1} protein. Plasma GLU is expressed in mmol l^{-1}.

In vitro *experiments*

Preliminary investigations revealed the occurrence of possible artifacts under measurement of certain redox-parameters, therefore *in vitro* experiments with TA and/or DM were performed and used as controls for the *in vivo* study, facilitating the interpretation of the *in vivo* data.

The influence of TA and DM alone or in combination on the antioxidant enzyme activities was studied in the following reaction media: 50 mM pH 7.0 phosphate buffer for CAT; 50 mM pH 10.2 carbonate buffer for SOD and Mn-SOD (plus 5 mM KCN for Mn-SOD) and 50 mM pH 7.5 TRIS buffer for GPx. The preincubation and reaction mixtures contained 1.2–75 μM TA and/or 4–100 nM DM and supernatant of control fish liver homogenate. The final dilutions of the supernatant in the reaction mixtures were the same both under *in vivo* and *in vitro* experiments: 600-fold, 1500-fold, 150-fold and 50-fold for CAT, total-SOD, Mn-SOD and GPx activity measurements, respectively. After preincubation for 10 min at 20 °C, the enzymatic reaction was started by adding substrate and the enzyme activities were estimated as referenced above. As reference for *in vitro* CAT and SOD measurements the possible H_2O_2 or O_2^- generating nature and apparent CAT and SOD activity of TA and/or DM were also tested under enzyme-free conditions (the supernatant was omitted).

The possible interaction between the effectors and GSH, a major component of the antioxidant defense system, was studied *in vitro*, too. TA and/or DM was dissolved in distilled water (the pH was adjusted to 7.0) and the reactions were set up either in the presence of supernatant (from control fish, diluted two-fold in the preincubation mixture) or lacking supernatant, with the equivalent 190 μM (initial) GSH concentration. The latter reactions, performed in 'pure' chemical environment, were used as reference for reactions with the supernatant. After preincubation for 30 min at 20 °C, the GSH concentration was measured according to Sedlak and Lindsay [34]. Each reaction had its own control, with the same ingredients, but with 0-min preincubation. The result of each 30-min reaction is expressed as percentage of the value of its own control. GSH-assays examined the apparent GPx activity of the effectors therefore regarded as reference for the above *in vitro* GPx assays. Three to five parallel determinations were carried out. Data are mean ± SD. Statistical significance was assessed with Student's *t*-test. The level of statistical significance was taken as $*P < 0.05$.

Electron microscopy

Small cuboidal blocks were cut from the marginal area of the carp liver, the blocks were prefixed for 4 h in Karnovsky-solution [22], rinsed in (pH 7.2, 50 mM) phosphate buffer, postfixed for 1 h in 1% OsO_4 solution buffered at pH 7.4 with Millonig's phosphate, dehydrated in graded ethanol and embedded in Durcupan ACM (Fluka). Ultrathin sections (100 nm) were stained with 4% uranyl acetate, contrasted with lead citrate by Reynold's procedure, and coated with carbon, then examined in a Tesla BS 500 electron microscope.

RESULTS

Response of carp to TA and/or DM treatments in vivo

Parameters of treated fish were compared to untreated controls (Table 1). TA alone did not change the activities of ECOD and EROD one day after the treatment, but decreased both considerably after two days. Either DM alone or combined with TA doubled ECOD activity both after 24 and 48 h (except after 48 h the combined treatment), while EROD activity remained unchanged. Either DM alone or the TA-DM mixture resulted in higher CYP 1A isoenzyme activities than TA treatment after 48 h. All three treatments elevated the level of LP and decreased GSH concentration to the same extent after an exposure for 48 h. Exposure of fish to DM alone for 24 and 48 h and to the TA-DM combination for 24 h resulted in the same high antioxidant enzyme activities (excluding Mn-SOD) compared to the control values (or to the low activity values induced by TA). CAT and GPx activities were similarly low both one and two days after the TA treatment, total- and Mn-SOD activities diminished only

Table 1

Parameters of carps after i.p. injection with 5.9 μmol kg^{-1} b.w. tannic acid and/or 0.4 nmol kg^{-1} b.w. deltamethrin [Decis][a]

Biomarkers	Exp.	Control	TA	DM	TA+DM	
CYP 1A isozymesECOD	24 h	52.81±9.97	38.02±11.12	90.63±13.30*	84.53±18.23*	pmol mg^{-1} min^{-1}
	48 h	40.16±12.13	16.80±8.52*	80.36±9.29*	63.93±26.10	
EROD	24 h	4.25±0.61	3.75±1.52	3.55±0.87	8.02±4.52	
	48 h	5.09±1.49	1.93±0.62*	4.23±1.64	6.50±2.80	
Redox parametersLP (MDA)	24 h	0.35±0.02	0.39±0.07	0.42±0.02*	0.41±0.05	nM mg^{-1}
	48 h	0.38±0.02	0.47±0.02*	0.50±0.03*	0.55±0.08*	
CAT	24 h	5.91±0.58	5.02±0.29*	7.10±0.61*	8.31±1.48*	nmol mg^{-1} min^{-1} ×10^4
	48 h	6.33±0.34	5.13±0.54*	7.09±0.33*	4.49±1.33*	
SOD	24 h	9.50±2.00	7.24±2.29	14.75±3.01*	13.36±2.56*	U mg^{-1}
	48 h	10.44±1.78	8.25±0.63*	14.00±1.20*	7.87±1.49	
Mn-SOD	24 h	1.71±0.23	1.48±0.46	1.97±0.12	1.92±0.32	
	48 h	1.98±0.29	1.32±0.21*	2.55±0.14*	1.07±0.30*	
GSH	24 h	15.43±0.80	14.59±2.13	13.67±1.70	12.78±1.05*	nM mg^{-1}
	48 h	15.36±1.99	12.62±0.67*	10.91±1.95*	11.31±1.66*	
GPx	24 h	4.15±0.95	2.82±0.25*	7.93±1.58*	8.72±1.20*	nmol mg^{-1} min^{-1}
	48 h	4.35± 0.80	2.70±0.52*	7.62±0.65*	5.97±0.97*	
Blood parametersGLU	24 h	3.27±0.29	3.72±0.26*	4.22±0.69*	3.59±0.93	mM
	48 h	3.03±0.39	1.84±0.51*	4.45±0.60*	2.83±1.14	
AspAT	24 h	5.64±1.20	8.12±1.40*	8.44±1.42*	5.31±1.73	nmol mg^{-1} min^{-1}
	48 h	6.08±0.53	4.36±0.38*	7.25±1.76	5.18±1.28	
AlaAT	24 h	6.81±0.79	8.08±2.04	9.17±1.45*	5.97±0.85	
	48 h	7.53±0.84	6.01±1.39	7.90±0.48	4.29±0.58*	

[a] Determination method of CYP 1A activities and redox-parameters in liver, and glucose and aminotransferases in plasma are indicated in the Materials and Methods section. Values are mean ± SD of 3-5 determinations. * statistically different at $P < 0.05$ (*t*-test).

Abbreviations: AlaAT, alanine aminotransferase; AspAT, aspartate aminotransferase; b.w.; body weight; CAT, catalase; ECOD, 7-ethoxycoumarin-O-deethylase; EROD, 7-ethoxyresorufin-O-deethylase; GLU, glucose; GPx, glutathione-peroxidase; GSH, glutathione; i.p., intraperitoneally; LP, lipid peroxidation; MDA, malonyl dialdehyde; (Mn-)SOD, (manganese-)superoxide dismutase; TA, tannic acid.

after two days. Following 48 h exposure to TA alone or to the TA-DM combination the antioxidant enzyme activities were similarly low relative to controls with one exception: GPx activity level was elevated above the control. The difference between the GPx activities of control fish and carps exposed to TA after an 48-h exposure is equal to that developed two days after treatment with DM singly and the TA-DM mixture.

Stress and injury to the liver by chemical action

Changes in redox-parameters revealed the induction of oxidative stress triggered by TA and DM alone or in combination in the tissues and the consequent deleterious effects to liver have been well demonstrated by the changes in plasma aminotransferase activities (Table 1) and by electron microscopy (Fig. 1). TA treatment resulted in slightly elevated GLU level and enhanced AspAT activity after one day, but both parameters declined relative to controls after two days; however, AlaAT activity was not changed at all. All three blood parameters were enhanced 24 h after the DM injection, but remained unchanged following 24 and 48 h the TA-DM treatment (Table 1); except AlaAT, where a considerable loss was found in the activity two days after the combined treatment relative to controls (and also relative to TA or DM measurements). Lower transaminase activities were found one day after the combined treatment than after DM injection alone. Exposure for 48 h to DM led to higher GLU level and AlaAT activites than either TA alone or its combination with DM; and DM alone initiated higher AspAT activity than TA (Table 1).

Ultrastructural alterations in the hepatocytes were studied two days after the treatments. Control hepatocytes were very rich in compact, dense glycogen granules and lamellar-structures of rough endoplasmic reticulum (rEr) (Fig. 1A). TA treatment induced non-deleterious ultrastructural alterations: the electron-density but not the number of the glycogen granules decreased in the cytoplasm; both the rEr elements and the perinuclear cisterns were enlarged; the electron-density of the nuclei and mitochondria was enhanced relative to controls (Fig. 1B). The most obvious change was the formation of light and dark cells as a consequence of the toxic effects of DM alone [2]. Both the number and the electron-density of the glycogen granules in the cytoplasm were reduced and both the rEr elements and the perinuclear cisterns were strongly swollen. The nuclei were swollen and the matrix of the mitochondria was extremely electrondense (Fig. 1C). Following the TA-DM combined injection the glycogen disappeared in the cytoplasm. The oval-shaped swelling of rEr cisterns was moderate compared to that after treatment with DM, and the rEr structure was more confuse. Formation of light and dark cells was not observed, and the structure and electron-density of nuclei and mitochondria was similar to those affected by TA alone (Fig. 1D).

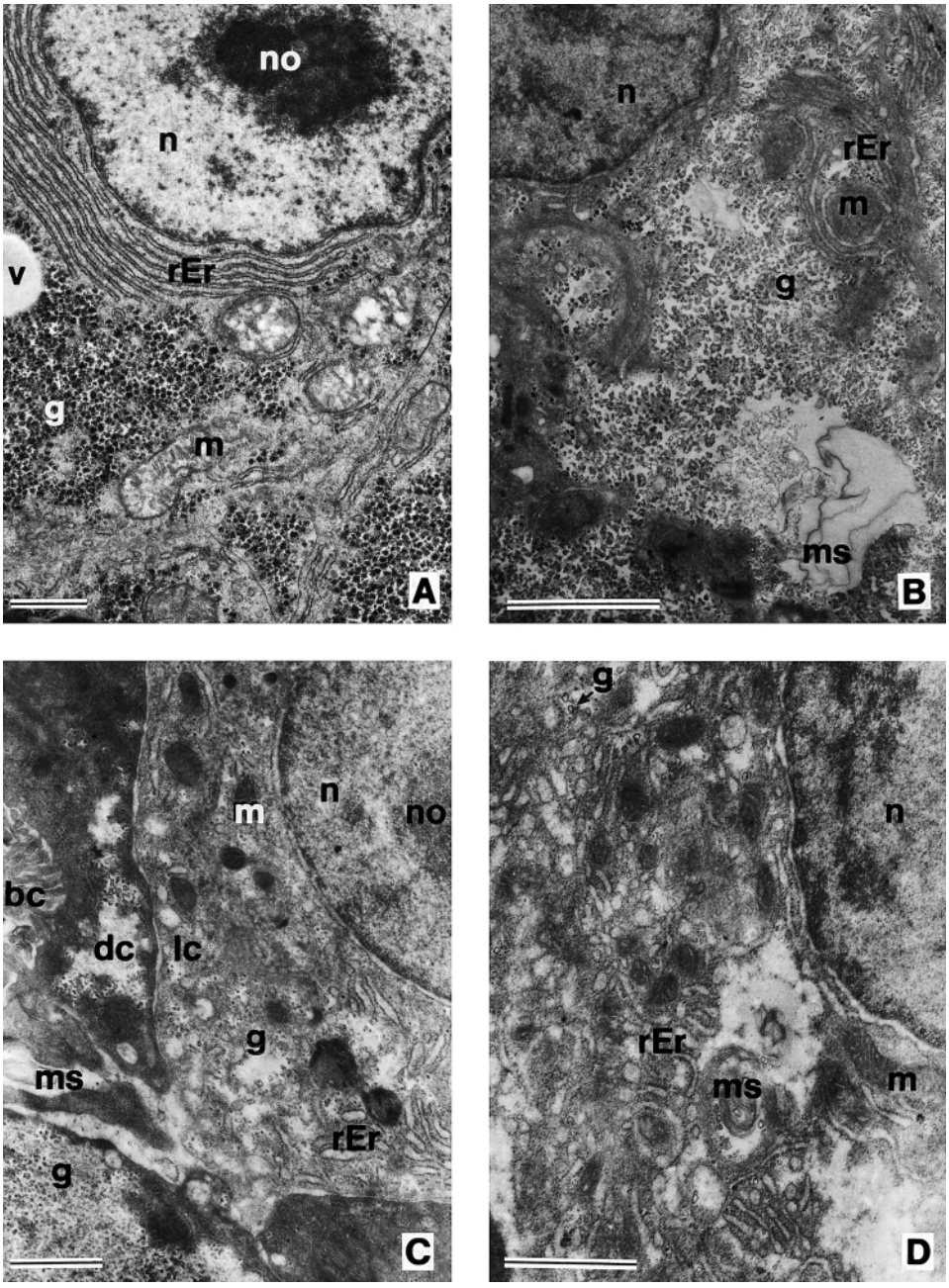

Fig. 1. Detail of hepatocytes from carp control sample (A), from carp treated with tannic acid (B), with deltamethrin (C) or with tannic acid and deltamethrin combined (D). Abbreviations: n, nucleus; no, nucleolus; m, mitochondrion; rEr, rough endoplasmic reticulum; g, glycogen or remains of glycogen; ms, myeline-membrane specialization; v, vacuole; bc, bile canaliculi; dc, dark cell; lc, light cell. Bar represents 1 μm

Effect of TA and/or DM in vitro

Our non-enzymatic assays proved that TA and/or DM did not exert apparent CAT activity (data not shown) in the examined concentration range, but they either alone and in combination inhibited CAT in a dose-dependent fashion. The drugs were applied in different concentrations, where DM alone was the weakest, and its combination with TA the strongest CAT inhibitor, except at the highest applied concentrations, where TA alone or its combination with DM exhibited the same CAT inhibitory capacity (Fig. 2). Only the highest applied TA or DM concentration enhanced (slightly) the apparent concentration of GSH in supernatant-free reaction mixture, while their combination was effective even at their lowest concentration. Apparent GSH level was the highest in reaction mixtures lacking supernatant but containing 75 μM TA alone or its combination with 100 nM DM (Fig. 3a). In contrast, a notable GSH degrading capacity of the effectors was found in the presence of supernatant, which effect was the mildest at the highest applied TA and/or DM concentration (excluding the no effect of 3 μM TA) (Fig. 3b). This moderate GSH loss may be related to the relatively high apparent GSH concentrations measured in the equivalent *in vitro* system lacking supernatant (Fig. 3a). TA and/or DM affected GSH even at lower concentrations in mixtures containing supernatant than in supernatant-free systems (Fig. 3a, b). TA alone or combined with DM inhibited GPx in dose-dependent manner *in vitro*. Preincubation with the insecticide alone resulted in inhibition of GPx activity at the lowest, but elevated GPx activity at the highest DM concentration applied relative to controls (Fig. 3c). The rate of epinephrine autooxidation was strongly enhanced by the addition of TA and/or DM, and the *in vitro* SOD activities therefore could not be evaluated properly (data not shown).

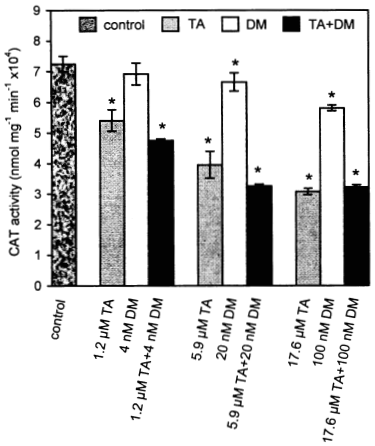

Fig. 2. The *in vitro* concentration-dependent effects of tannic acid (TA) and/or deltamethrin (DM) on catalase (CAT) activity. Carp liver homogenate supernatant was added to the reaction mixtures containing of TA and/or DM and enzyme activity was measured after 10-min preincubation at 20 °C and expressed as nmol substrate min^{-1} mg^{-1} protein. Values are mean ± SD of 3–5 determinations. * statistically different at $P < 0.05$ (*t*-test)

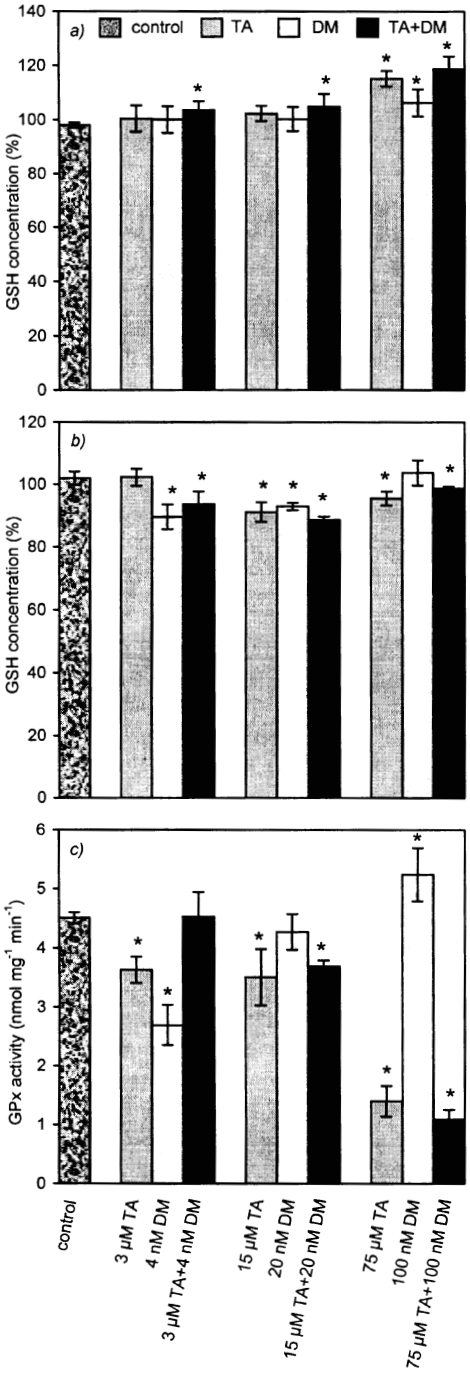

Fig. 3. The *in vitro* concentration-dependent effects of tannic acid (TA) and/or deltamethrin (DM) on glutathione (GSH) concentration in reaction mixtures (a) lacking carp liver homogenate supernatant and (b) containing supernatant; and on (c) glutathione-peroxidase (GPx) activity. GSH (initial concentration: 190 nM) or supernatant was preincubated with various concentrations of TA and/or DM for 30 min at 20 °C and the GSH concentration was then measured. The result of each 30-min reaction is expressed as percentage of the value of its own control. 10-min preincubation was applied at 20 °C for GPx assays and activity was given in nmol substrate min^{-1} mg^{-1} protein. Values are mean ± SD of 3–5 determinations. * statistically different at $P < 0.05$ (*t*-test)

DISCUSSION

Significant enzyme inhibitory capacity of nanomolar concentration of DM towards antioxidant enzymes (Fig. 2 and 3c) and low micromolar concentration of DM towards CYP 1A isoenzymes [4] were proven *in vitro*. The crucial role of cytochrome P450-dependent monooxygenases in the metabolism of pyrethroids has been demonstrated by numerous studies [4, 10, 12, 36]. Although, DM was found to be a strong enzyme inhibitor [e.g. 4], its very low bioconcentration in the liver (undetectable by gas chromatography-mass spectrometry) rose ECOD activity (Table 1). Higher doses of the xenobiotic at similar temperature initiated identical effects on CYP 1A isoenzymes *in vivo* [12] with stronger induction of ECOD, however, further elevation in DM dose caused serious inhibition of various cytochrome(s) P450 [4]. Enhanced cytochrome P450-dependent metabolism have long been known to lead to elevated oxyradical production [10, 25, 32, 35], as demonstrated clearly by the marked changes in redox parameters (Table 1). *In vitro* study revealed the following dual effects of the pesticide on GSH, which may result in artifact formation and consequently in erroneous interpretation of the results: (a) certain concentration of the insecticide seems to influence positively the absorbance of nitromercaptobenzoic acid (the yellow pigment, product of the reaction of GSH and Ellmann's reagent) at 412 nm leading to detection of elevated GSH concentration (Fig. 3a); moreover, (b) the pesticide initiated increased GPx activity both *in vitro* (Fig. 3b, c) and *in vivo* (Table 1). Enhanced *in vitro* GPx activity was measured in the presence of the supernatant only (Fig. 3), which may be the consequence of conformational changes induced by the xenobiotic. Whereas, elevated GPx activity measured *in vivo* is obviously related to the remarked GSH-degrading potency of the elevated oxyradical bioconcentration [42] derived from increased cytochrome(s) P450 activity (Table 1) [e.g. 10, 17, 19, 25].

Interestingly, according to non-enzymatic redox-parameters (Table 1) TA itself induced oxidative stress, although, phenolics are generally reported to behave as antioxidants [11, 15]; however, their potential prooxidant action has also been described [11, 27]. The prooxidant effect exerted by TA may be due to the strong antioxidant enzyme inhibitory nature of TA (Fig. 2, 3c and Table 1) as a consequence of formation of hydrogen-bindings between the numerous hydroxy groups of TA and the enzyme molecules [39, 41]. ROI-elimination processes were decreased in fish relative to the control rate. Furthermore, the observation that TA together with liver homogenate supernatant has remarkable GSH degrading ability *in vitro* (Fig. 3a vs. Fig. 3b), may refer to enzyme-phenolic interactions. The possible metabolic activation of TA (as described by Metodiewa et al. [27] for quercetin) accompanied with increased ROI-production in fish organism should be taken into account when estimating the potential toxicity of TA, as well as, the varying pro/antioxidant behavior of a phenolic depending on pH [11]. TA exerted moderate deleterious alterations in hepatocytes than DM alone (Fig. 1) which may be in connection with the considerably reduced oxyradical production of the strongly inhibited CYP 1A isoenzymes [1] following treatment with TA. It is possible that not the enzyme-inhibitory and weak

tissue-damaging activity, but the free radical scavenging capacity of TA [15] might dominate when very low TA concentration is used at acidic pH *in vivo* [11].

Other studies have reported that phenolics may influence the toxicity of agricultural chemicals via their molecular interaction [e.g. 29]. Notwithstanding, time-course analysis of antioxidant enzyme activities *in vivo* (Table 1) led to a conclusion that the time-course action of DM and TA are simply additive in fish during the combined treatment (no signs of their interaction or their joint effect have been revealed by the results), and the stress response of fish to DM was modified by TA hereby. Accordingly, rise in the antioxidant enzyme activities after 24 h is the consequence of the enhanced H_2O_2 and O_2^- generation by the fast metabolism of the xenobiotic [10, 12, 19], and the falloff after 48 h reflects the characteristic enzyme inhibitory action of TA (Table 1). The difference between the time-course action of DM and TA is due to the low absorption rate of the large TA molecules [M_r 1700.2] [31]. Notwithstanding, moderate alterations/damage in nuclear structure were noticed in consequence of treatment with the TA-DM combination (or with TA alone) than after single injection of DM (Fig. 1). Therefore, TA is suggested to exert some nucleo-protective capacity against DM toxicity, probably via complexation of DNA-molecules [38]. The formation of "dark cells" after the combined treatment has been prevented by TA, therefore some common cytoprotective capacity is due to TA, as well. Elevated LP after the combined treatment (similarly, like after the single injections, Table 1) refers to membrane damage and therefore to the impairment of membrane-linked transport leading to swollen rEr cisterns (Fig. 1) due to protein-accumulation [7]. The partial inhibition of biosynthetic enzymes by TA action two days after the combined treatment manifested by moderate swelling of cisterns relative to that following DM treatment alone (Fig. 1). When the two chemicals were applied together their metabolism required high energy, which resulted in total glycogen loss in hepatocytes (Fig. 1), demonstrating high metabolic stress and serious impairments in glucose-metabolism. Collation of electronmicroscopic data with transaminase activities led to a conclusion: one should be cautious in using transferases as indicator of tissue damage when strong enzyme inhibitors (such as TA) are applied.

In summary, DM, even in a dose as low as 0.4 nmol kg^{-1} b.w., behaved as prooxidant in fish via activation of cytochrome P450 isozymes. The oxidative stress, manifested in marked changes in biochemical parameters (Table 1) and in the ultrastructure of hepatocytes (Fig. 1), demonstrated an interference with in nucleic acid-, protein-, lipid- and glucose-metabolism [2, 7]. TA-DM combined treatment yielded more complex stress response in fish, including increased ROI-production (by the biotransformation of the two agents) combined with reduced antioxidant capacity in fish and the common cytoprotective effect (against DM toxicity) exerted by TA, as well. The accurate prediction of chemical toxicity in aquatic ecosystems requires information both on the potential for chemical interaction with (single or mixed) components of the aqueous matrix and also on their complex biochemical effects in living organisms. Consequently, further comparative studies have to be carried out with different concentrations of the characteristic/dominant species of DHM, which may contribute to the assessment of the impact of humics in nature.

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