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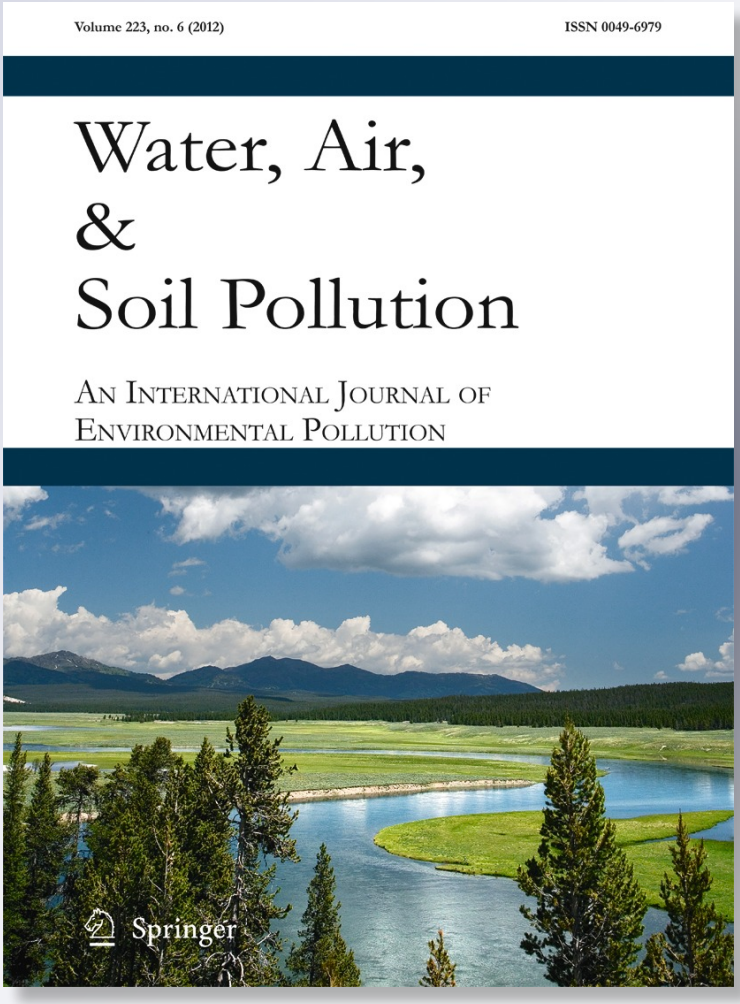

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Impact of Oxidative Stress Indicated by Thiobarbituric Acid Reactive Substances (TBARS) and Protein Carbonyl Levels (PC) on Ethoxyresorufin-*O*-deethylase (EROD) Induction in Common Carp (*Cyprinus carpio*)

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Abstract This study examined the impact of oxidative stress indicated by thiobarbituric acid reactive substances (TBARS) and protein carbonyl (PC), induced by intensive exercise and cadmium chloride ($CdCl_2$) on ethoxyresorufin-*O*-deethylase (EROD) activity in juvenile carp (*Cyprinus carpio*). In the first experiment, fish were divided into three groups: (1) control, (2) carp exposed to intensive exercise, and (3) carp that was not exercised but previously, as well as carp in group 2, received single dose of 3-methylcholantrene (3-MC). The third and sixth day fish were sacrificed and the measurements were conducted. In the second experiment, fish were divided into (1) control, (2) carp in water containing $CdCl_2$, and (3) carp in dechlorinated tap water (2 and 3 received single dose of 3-MC on the seventh day after exposure to $CdCl_2$). The carp were killed 6 days later and livers were excised for biochemical analyses. In the first experiment, on the sixth day after treatment with 3-MC, results show statistically significant increase in EROD activity in non-exercised carp, while that increase in carp exposed to intensive exercise was significantly lower. Three days after exposure to 3-MC, statistically significant increase in TBARS was observed in both exercised and non-exercised carp. Six days after exposure to 3-MC, PC levels were significantly higher in exercised carp. Pretreatment with $CdCl_2$, in the second experiment, caused oxidative stress and reduction of EROD activity. Results show linkage between expression of EROD activity and oxidative stress biomarkers and possible influence of oxidative stress on the cell membrane structures and consequently on EROD activity.

Keywords EROD · Oxidative stress · Lipid peroxidation · Protein carbonylation

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1 Introduction

An important group of biomarkers formed enzymatic components of antioxidative defense system remove reactive oxygen species (ROS), thus protecting organisms from oxidative stress (Almeida et al. 2002). ROS such as superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and highly reactive hydroxyl radical (·OH) can generate in oxidative stress (Livingstone

2001). Oxidative stress can be defined as an imbalance between the free radicals and the antioxidants levels. Beside fairly widespread pollutants, there have been many reports showing that exercise can also cause increased production of ROS and free radicals, thus exceeding the protective capacity of the antioxidant defense, which consequently leads to oxidative stress (Kelle et al. 1998; Ji 2000; Liu et al. 2000; Manna et al. 2003; Sen et al. 2005; Belviranli and Gökbel 2006; Chang et al. 2007).

Biomarkers of oxidative stress are directly connected with changes of ROS concentration in organism (Barker et al. 1994; Ahmad et al. 2004). When antioxidant defenses are impaired or overcome, oxidative stress may produce lipid peroxidation, protein carbonyl formation, and enzymatic inactivation (Halliwell and Chirico 1993; Shacter 2000).

Lipid peroxidation in fish, measured as thiobarbituric acid reactive substances (TBARS), has been frequently used as a marker of oxidative stress in response to different environmental pollutants in a number of studies (Almroth et al. 2005; Ando and Yanagida 1999; Choi and Oris 2000; Gabrzelak and Klekot 1985; Lafontaine et al. 2000; Oakes and Van Der Kraak 2003; Roméo et al. 2000). Increased levels of lipid peroxidation products are observed in liver of common carp (*Cyprinus carpio*) exposed to contaminants such as paraquat (Gabrzelak and Klekot 1985), then to heavy metals in liver of sea bass (*Dicentrachus labrax*), bream (*Lepomis macrochirus*) (Roméo et al. 2000; Choi and Oris 2000), and other. Furthermore, Liu et al. (2000) showed that acute exercise induced significant increases in malondialadehyde (MDA) content in rat muscle and liver (Liu et al. 2000). Heavy metals such as cadmium is a redox-inactive metal, but it can elevate the generation ROS indirectly by depletion of glutathione and antioxidant enzymes, such as superoxide dismutase and catalase (Banjerdkij et al. 2005; Fortuniak et al. 1996; Stohs and Bagchi 1995). Furthermore, cadmium is known to displace redox-active metals (Zn and Fe ions) from metalloproteins resulting in their inactivation as well as the release of free Fe that can then catalyze the generation of reactive oxygen species via the Fenton reaction (Banjerdkij et al. 2005). It is also known that cadmium produces ROS by inhibiting the electron transfer chain in the mitochondria (Wang et al. 2004).

Second, the most common perturbation resulting from oxidative stress is protein carbonyl formation (Shacter 2000). In some cases, the formation of carbonyl derivatives could be nonreversible, causing conformational changes and decreased catalytic activity in enzymes (Parvez and Raisuddin 2005). Numerous research refers on measurements of protein carbonylation in human tissues (Floor and Wetzel 1998; Romero et al. 1998); however, during the past several years, protein carbonyl levels have also been measured in fish tissues (Bagnyukova et al. 2005; Lushchak et al. 2005; Parvez and Raisuddin 2005; Shi et al. 2005).

A group of biomarkers which has been investigated most extensively and is one of the earliest biological responses of fish to a presence of certain types of xenobiotic in the water in both laboratory and field conditions is cytochrome P450 that belongs to the large family of hepatic mixed function oxidase enzymes of phase I xenobiotic biotransformation (Stegeman 1989; Lemaire-Gony and Lemaire 1992; Stein et al. 1992; Peters et al. 1994; Whyte et al. 2000; Široka and Drastichova 2004). Induction of cytochrome P450 1A (CYP1A) measured by 7-ethoxyresorufin-*O*-deethylase activity (EROD) has been widely used as an indicator of exposure to planar halogenated hydrocarbons, polycyclic aromatic hydrocarbons, and other structurally similar compounds found in pulp and paper mill effluent (Fenet et al. 1998). Many advantages of using EROD activity as a biomarker include high sensitivity, feasibility, and simplicity of its measurement (Förlin et al. 1986; Arniç et al. 2000). The aim of the present study was to investigate how oxidative stress, induced by intensive exercise and by cadmium chloride ($CdCl_2$), affects EROD induction, TBARS, and PC levels in common carp, a fish species commonly used in monitoring of freshwater systems.

2 Materials and Methods

2.1 Reagents and Animals

Phenylmethylsulfonyl fluoride (PMSF), trichloroacetic acid (TCA), thiobarbituric acid (TBA), butylated hydroxytoluene (BHT), 2,4-dinitrophenylhydrazine (DNPH), guanidine HCl, ethylenediaminetetraacetic acid (EDTA), nicotinamide adenine dinucleotide phosphate (NADPH), 7-ethoxyresorufin, and Coomassie Brilliant Blue G-250 were purchased from Sigma Chemical Co. (USA). All reagents used were of analytical grade.

Juvenile common carp (*C. carpio* L.) (mean body length 12.6±2.9 cm, mean weight 25.6±5.3 g) were

obtained from the Grudnjak fishpond. The fish were acclimatized for 1 week before experiments. They were kept unfed in tanks with aerated, filtered, dechlorinated tap water (hardness 380.3 mg L^{-1} as $CaCO_3$, pH 7.1±0.2). The temperature of the water was maintained at 15±1 °C and light in the room followed a 12:12-h photoperiod.

2.2 Exposure of Carp to Intensive Exercise

The acclimatized fish were divided into three groups: two experimental and one control, with 24 carp each (n=24). Fish were maintained in polyethylene tanks (250 L) containing aerated dechlorinated tap water at 15±1 °C and were not fed during the experiment.

At the beginning of the experiment, carp from experimental groups received a single intraperitoneal injection of 3-MC previously dissolved in sterile corn oil (model inducer of the CYP1A) at 50 mg/kg body weight. Group I was exposed to intensive exercise sustained by water mixing system constructed in the tank. Fish swim speed approximately classified in sustained category (Beamish 1978). Water flow rate was set at approximately 15 cm/s. The duration of exposure to exercise was 22 h daily (6 days), with two pauses (every 12 h for 1 h without mixing of water). Group II did not perform exercise. After 3 days, one half of the fish (n=12) from each group was killed, and at the end of the experiment, the other half was killed. Fish were decapitated, and their livers were carefully isolated, weighted, and immediately stored in liquid nitrogen for further processing.

2.3 Exposure of Carp to 3-MC Alone and After Pretreatment with Cadmium Chloride

In the second experiment, carp were divided into three groups. The first group (n=30) was the control and maintained in dechlorinated tap water during the experimental period (13 days). To induce oxidative stress, fish (n=30) in the second group were exposed to freshly prepared aqueous solution of $CdCl_2$ (10 μg/L) that was added every 24 h in aerated glass aquarium (100 L). The water in the aquarium was not recycled but renewed every 24th hour. On the seventh day, fish were transferred to the aquarium with clean, dechlorinated water and received a 3-MC. The third group of carp (n=30) were kept in dechlorinated tap water for 7 days, and after that period they received a single intraperitoneal injection of 3-MC at 50 mg/kg body weight in sterile corn oil. The control group was injected with sterile corn oil alone. The carp were killed 6 days later and the livers were removed for biochemical analyses. The exposure concentration of $CdCl_2$ was verified by performing analytical measurements of all solutions from the aquarium at the beginning of exposure and after every 48 h for 7 days. Analyses were performed by a combined system of gas chromatography and mass spectrometry (GC-MS-SIM-Perkin-Elmer Q-mass 910 benchtop mass spectrometer, Norwalk, USA). Reagent blanks were used as a part of quality assurance–quality control. Blank levels were found to be below detection limits in all cases.

2.4 Preparation of Homogenates

Livers were washed in chilled 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM EDTA and a few crystals of PMSF (protease inhibitor) and then minced (5 mL/g of tissue) for 10 s using a Potter–Elvehjem homogenizer, before being centrifuged at 9,000 × g for 15 min at 4 °C (Burgeot et al. 1994). The supernatant (S9) was used for EROD measurements.

A 500-μL aliquot of S9 extracts was mixed with 1 mL of 30 % (w/v) TCA and then centrifuged for 10 min at 5,000 × g (Rice-Evans et al. 1991). The pellet was used for protein carbonyl assay and the supernatants for measuring TBARS contents.

2.5 Biochemical Analysis

The catalytic activity of CYP1A was detected fluorometrically by measuring the conversion of 7-ethoxyresorufin into the fluorescent resorufin (Burke and Mayer 1974; Flammarion and Garric 1997; Grzebyk and Galgani 1991). The reaction was performed in 96-well plates and a fluorescent plate reader (Fluorolite 1000 (Dynatech)) at 4 °C using 20 μL of S9 in 200 μL of buffer (Tris 0.1 M, NaCl 0.1 M, pH 8.0) containing 2 μM of 7-ethoxyresorufin (10 μL) and 0.25 mM of NADPH (10 μL). The progressive increase in fluorescence resulting from the resorufin formation was measured for 5 min (excitation wavelength 544 nm, emission wavelength 590 nm). Results were expressed as picomoles per minute per milligram of microsomal protein.

Lipid peroxidation was measured by the TBARS assay (Rice-Evans et al. 1991). Liver supernatants

were combined with the same volume of TBA reagent, containing saturated solution of TBA in 0.1 M HCl and 10 mM BHT previously dissolved in ethanol; pH was adjusted to 2.5. BHT was added to avoid tissue peroxidation during heating of the samples. Control samples contained water instead of supernatant. The mixtures were immersed in a boiling water bath for 60 min. After quick cooling, a volume of butanol equal to the mixture total volume was added and mixed vigorously. Samples were centrifuged for 10 min at 5,000 × *g*; the butanol phase was removed and used to evaluate the level of TBARS. Absorption was measured at 535 nm and a molar extinction coefficient of 156×10^3 $M^{-1}cm^{-1}$ was used to calculate TBARS concentration (Rice-Evans et al. 1991). The values were expressed as nanomoles of TBARS per gram wet weight of tissue.

Carbonyl derivatives of proteins were detected by reaction with DNPH. This compound reacts with protein carbonyl groups that have been formed by ROS attack on side chains of amino acids (Lushchak et al. 2005). Resulting 2,4-dinitrophenylhydrazones were quantified spectrophotometrically (Lenz et al. 1989). The pellet from TCA-treated extract (above) was mixed with 1 mL of 10 mM DNPH in 2 M HCl. Control samples contained only 1 mL of 2 M HCl. Samples were incubated for 1 h at room temperature, then centrifuged for 10 min at 5,000 × *g*. Supernatants were discarded and pellets were washed three times with 1 mL of ethanol butyl acetate (1:1 *v*/*v*) mixture. Pellets were then dissolved in 1.5 mL of 6 M guanidine HCl. The amount of PC was evaluated spectrophotometrically at 370 nm using a molar extinction coefficient of 22×10^3 $M^{-1}cm^{-1}$ (Lenz et al. 1989). The values were expressed as nanomoles of PC per milligram protein in the guanidine chloride solution.

Protein concentration was measured by the Bradford method with Coomassie Brilliant Blue G-250 (Bradford 1976) and using bovine serum albumin as a standard. The protein assay was performed using a UV-Vis spectrophotometer (Shimadzu UV-1601) at 595 nm and expressed as milligrams per milliliter.

2.6 Statistical Analysis

Data values are given as mean ± standard deviation. Prior to the analysis, data were log-transformed and then tested for normality using Shapiro–Wilk test. All data were analyzed for significant differences ($P<0.05$) using *t* test and ANOVA followed by Bonferroni post hoc test when a significant difference was found.

3 Results

3.1 Impact of Intensive Exercise on EROD Activity and the Levels of TBARS and PC

Figure 1 presents the results of EROD activity after 3 and 6 days in control group (CG), group I that has been exposed to forced exercise, and finally the EROD activity results in the group II that did not perform the exercise but was otherwise as was previously as the carp from group I, pretreated with 3-MC. There are no significant differences in results of liver EROD activity at CG during the experiment. EROD activity in group I, after 3 days, was 17.59 pmol min^{-1} mg^{-1}, and after 6 days, activity was 28.1 pmol min^{-1} mg^{-1}, representing 7.2- and 11.3-time increase when compared to the control group, respectively. EROD activity in group II, after 3 days, was 19.1 pmol min^{-1} mg^{-1}, whereas after 6 days, activity was 48.7 pmol min^{-1} mg^{-1}, representing a 7.8- and 19.6-time increase when compared to control.

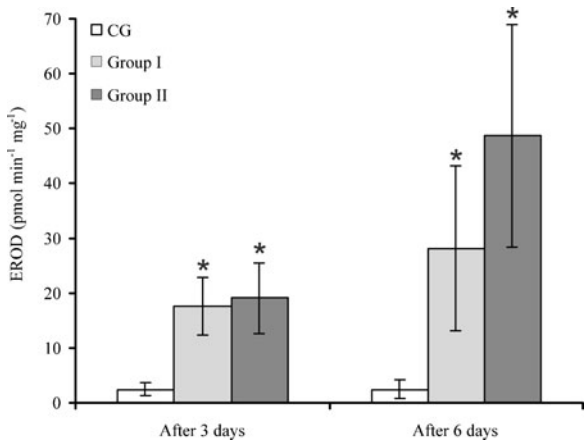

Fig. 1 7-Ethoxyresorufin-*O*-deethylase activity (EROD) in carp liver extracts (90,000 × *g* supernatants) 3 and 6 days after exposure to intensive exercise. *CG* control group, *group I* exercised carp, and *group II* non-exercised carp. Groups I and II received a single intraperitoneal injection of 3-MC (50 mg/kg body weight) at the beginning of the experiment. The values are expressed as means ± S.E. of 24 animals in each group. Liver EROD activity was expressed as picomoles of resorufin produced per minute per milligram of PMS protein. *$P<0.01$ significant differences to control group

In samples taken after 3 days, post hoc analysis showed statistical differences ($P<0.01$) between group I and CG and on the same level of significance between group II and CG. There are no significant differences ($P<0.05$) between samples from group I and group II. In fish taken after 6 days, there is significant difference ($P<0.01$) between group I and CG, and also between group II and CG. However, unlike from the samples taken after 3 days, post hoc analysis showed statistically significant differences ($P<0.05$) between both samples treated with 3-MC (group I and group II). The increase of EROD that is observable from the results was significantly higher, approximately 40 %, after 6 days in the group II, than in the group I.

Figure 2 presents TBARS results. The levels of TBARS 3 days after injection 3-MC were 13.6 nmol g^{-1} in CG, 17.6 nmol g^{-1} in group I, and 14.8 nmol g^{-1} in group II. Six days after injection of 3-MC, the levels of TBARS were 15.3 nmol g^{-1} in CG, 19.4 nmol g^{-1} in group I, and 12.3 nmol g^{-1} in group II. There are statistically significant ($P<0.01$) differences in the levels of TBARS between the control group and exercised group (group I) after 3 and 6 days of the experiment. It is obvious that increases in the level of TBARS after 6 days were detected only in group I, whereas, on the other hand, the levels of TBARS in group II were reduced for 14.8 % in relation with the level measured on the third day of the experiment.

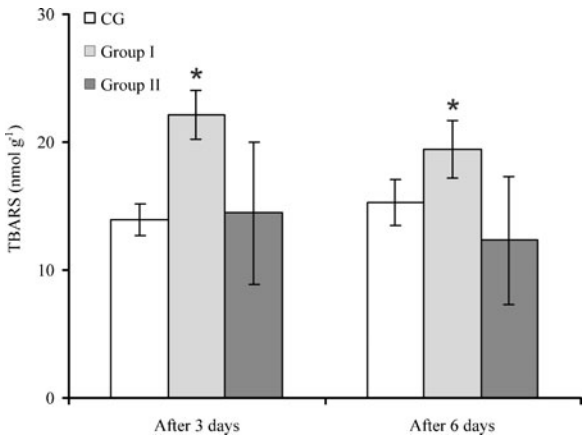

Fig. 2 Thiobarbituric acid reactive substance (TBARS) levels in carp liver extracts (9,000 × *g* supernatants) 3 and 6 days after exposure to intensive exercise. *CG* control group, *group I* exercised carp, and *group II* non-exercised carp. Groups I and II received a single intraperitoneal injection of 3-MC (50 mg/kg body weight) at the beginning of the experiment. Values are expressed as means ± S.E. of 24 animals in each group. The levels of TBARS were expressed as nanomoles of MDA per gram of PMS protein. *$P<0.01$ significant differences to control group

Figure 3 presents the results of PC levels. There were no statistically significant differences ($P<0.05$) 3 days after treatment with 3-MC between the groups. The level of PC was 164.1 mmol g^{-1} in the control group, 187.4 mmol g^{-1} in group I, and 172.7 mmol g^{-1} in group II. In samples taken 6 days after injection with 3-MC, statistically significant difference ($P<0.05$) in PC levels between the CG and the group I was proven. The level of PC was 171.5 mmol g^{-1} in the control group, 259.9 mmol g^{-1} in group I, and 174.3 mmol g^{-1} in group II. Significant negative correlation between concentration of PC and EROD activity in group I was proven (Fig. 4). The same negative correlation between these two parameters in the samples from group II is indicated but not statistically significant.

3.2 Impact of Preexposure to $CdCl_2$ on EROD Activity and Oxidative Stress Parameters

Figure 5 presents the results of PC, TBARS levels, and EROD activity after 3-MC injection to carp preexposed to $CdCl_2$. Carp that were preexposed to $CdCl_2$ have showed lower levels of EROD activity (14.4 times compared to control) than carp without preexposure (23.5 times compared to control). PC levels in preexposed carp were higher (1.7 times compared to

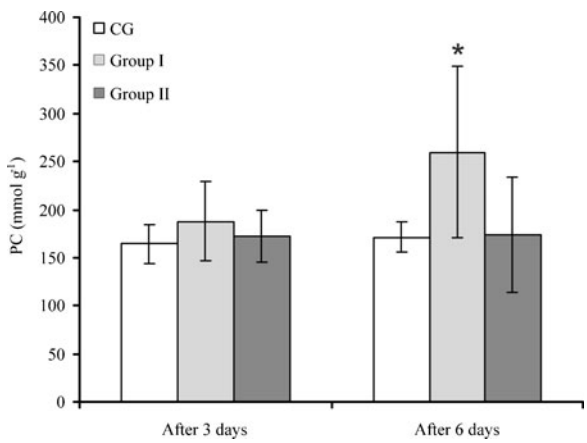

Fig. 3 Protein carbonyl level (PC) in carp liver extracts (9,000 × *g* supernatants) 3 and 6 days after exposure to intensive exercise. *CG* control group, *group I* exercised carp, and *group II* non-exercised carp. Groups I and II received a single intraperitoneal injection of 3-MC (50 mg/kg body weight) at the beginning of the experiment. The values are expressed as means ± S.E. of 24 animals in each group. Liver protein carbonyl levels were expressed as nanomoles of carbonyl groups per milligram of PMS protein. *$P<0.05$ significant differences to control group

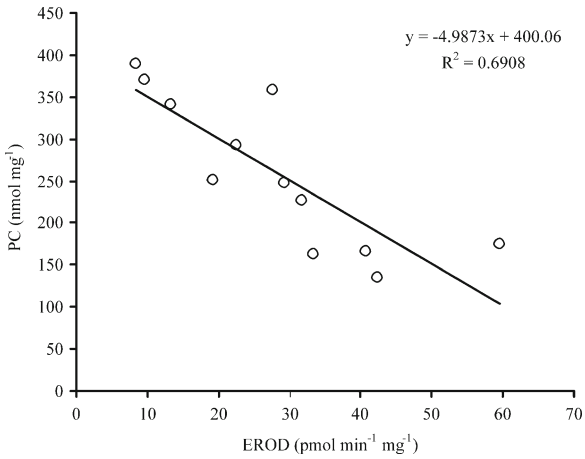

Fig. 4 Dependence between protein carbonyl level (PC) and 7-ethoxyresorufin-*O*-deethylase activity (EROD) in carp liver extracts (9,000 × *g* supernatants) after exposure to intensive exercise and treatment with 3-MC (group II). Significant negative correlation (r=−0.80) between concentration of protein carbonyl and EROD activity in this group of carp (n=24) was proven

the control) than in carp without preexposure (1.1 times compared to control). The levels of TBARS in preexposed carp sixth day after injection with 3-MC was 0.7 % values of TBARS in the control group, while in preexposed carp, levels of TBARS increased 1.7 times.

The analysis of the chemical concentrations at the beginning of exposure and after every 48 h for 7 days revealed that there was good agreement between the nominal and actual exposure levels in the experiment.

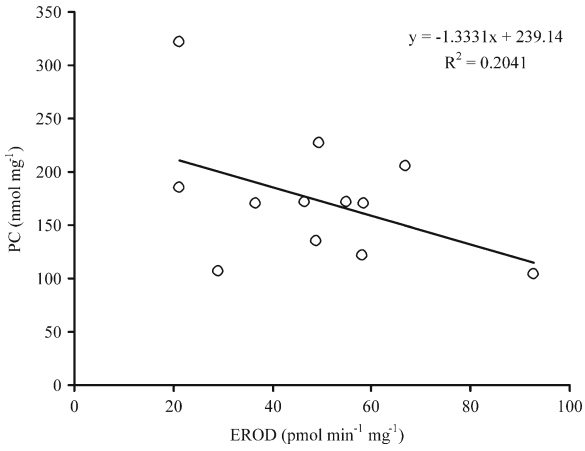

Fig. 5 Relative change in measured parameters (EROD, TBARS, and PC) compared to the control in carp liver extracts 6 days after exposure to 3-MC with and without $CdCl_2$ preexposure. Data are means ± S.E. of 30 animals in each group. *P< 0.05 significant differences to control group

The nominal concentration of $CdCl_2$ was 10 μg/L and the exposure concentrations after every 24 h were 9.52, 9.87, 9.53, 9.82, 9.75, and 9.70 μg/L, respectively. Since no significant differences were detected between the actual exposure levels and nominal concentrations, the nominal concentrations correspond to exposure concentrations.

4 Discussion

The present study investigated the effects of oxidative stress induced by intensive exercise and $CdCl_2$ on EROD inducibility, TBARS, and PC levels in common carp. In this study, we found that EROD activity in carp killed 3 days after administration of 3-MC increased by 17.4 times in exercised and 18.9 times in non-exercised carp relative to the control. However, after the sixth day, EROD activity was significantly lower in the carp exposed to intensive exercise compared with non-exercised carp (Fig. 1). These result undoubtedly lead to a conclusion that an increased physical activity during the experiment had a negative effect on induction of EROD activity by 3-MC. Similar results were obtained by Konstandi et al. (2000) who estimated the effect of restraint stress on EROD and pentoxyresorufin 7-dealkylase activity in the Wistar rat. They found that restraint stress significantly suppress basal EROD activity in the rat liver and also can alter CYPs and other drug-metabolizing enzymes (Konstandi et al. 2000).

EROD is a highly sensitive indicator of contaminant uptake in fish, providing evidence of receptor-mediated induction of cytochrome P450-dependent monooxygenases by xenobiotic chemicals (Whyte et al. 2000). However, EROD activity can be influenced by a large number of abiotic and biotic factors such as water temperature, health, condition, sex, age, nutritional status, metabolic activity, migratory behavior, reproductive and development status, population density, seasonal variations, etc. (Whyte et al. 2000; Van der Oost et al. 2003; Stanic et al. 2006). Furthermore, in environmental conditions, fish are often found in river flows where water flow rates can vary significantly. Our results however showed that this abiotic factor is very important and can influence on oxidative stress levels as well as on EROD inducibility. Neglecting abiotic and biotic factors that are proven to have influence on EROD activity can lead to misinterpretation of

biomonitoring results, in sense of overestimates or underestimates of pollutant concentrations.

Significantly higher levels of TBARS were measured in exercised carp, already 3 days after exposure to 3-MC. After the sixth day, TBARS levels in carp that did not perform exercise decreased compared to the control, while the TBARS level in exercised carp remained significantly increased. We concluded that the increase in TBARS levels in this experiment is a direct consequence of exposure to increased physical activity. In some other organisms such as rat, short intense exercise has been found to induce significant increase in MDA in liver mitochondria (Alessio et al. 1988; Liu et al. 2000). Increased lipid peroxidation may have influence on the membrane structures. Namely, peroxidized membranes become rigid and lose permeability and integrity (Kelle et al. 1998). Many enzymes are bound to membrane structures or are components of a membrane (such as EROD), therefore they are either directly involved in the reactions of lipids with ROS either exposed to effects of lipid peroxidation products (Shacter et al. 1994).

Data in the present study showed that PC level increased on the sixth day after exposure to 3-MC in the exercised group, suggesting that 3-MC exposure and increased physical activity, which carp were exposed to during the experiment, synergistically affect PC formation. Namely, it is known that polycyclic aromatic hydrocarbons (such as 3-MC) and halogenated aromatic hydrocarbons may itself enhance the generation of reactive oxygen species (Liu et al. 2001), which may with the increased physical activity contribute to a stronger oxidative stress and thus increased levels of PC.

It is also obvious from the previous experiment that an increase in PC level is directly related to the reduction of EROD activity, and on the one hand increased physical activity is directly related to the increased PC formation. Significant negative correlation ($r=-0.80$) between concentration of protein carbonyls and EROD activity in exercised carp was proven (Fig. 4). The same negative correlation between these two parameters was found in carp that did not perform exercise. This correlation was only suggested but not statistically significant ($r=-0.45$).

Interestingly, the levels of TBARS after oxidative stress caused by increased free radical production after intensive exercise increased much earlier than the PC concentrations levels, although the PC levels maintained increased much longer than the TBARS levels. That should also be taken into account when using these two biomarkers for the purposes of environmental biomonitoring.

To confirm that EROD activity, i.e., inducibility of CYP1A by 3-MC is closely associated with oxidative stress, we conducted an experiment in which we compared the inducibility of intact carp and carp preexposed to $CdCl_2$, a well-known inducer of ROS formation and oxidative stress (Shi et al. 2005). It is known that ROS such as H_2O_2 specifically inhibit the cytochrome P450 enzyme system, mainly at the transcriptional level by inhibiting the mRNA synthesis (Barker et al. 1994). Sometimes, CYP inhibition also occurs at the post-transcriptional level by the increasing degradation of mRNA (Delaporte and Renton 1997). On the seventh day after preexposure with cadmium(II) chloride, carp were treated with 3-MC, and sixth day after this treatment, the measurement of EROD activity was carried out. It should be noted that heavy metals and hydrocarbons are widely dispersed pollutants in the aquatic environments and are generally jointly present in polluted areas. As shown in Fig. 5, EROD activity was lower in cadmium/3-MC-treated carp than in fish treated with 3-MC alone. Significant increase in PC and TBARS levels, compared to the control after exposure to $CdCl_2$, indicate the presence of oxidative stress. Such results also indicated an impact which ROS, formed after preexposure to $CdCl_2$, had on EROD inducibility. These data are in agreement with the previous observation of Hassanain et al. (2007) whose study revealed that preexposure to 1/10 LC_{50} of $CdCl_2$ for 15 days decreased the effect of benzo(a)pyrene (BaP) on EROD activities in the liver of freshwater fish *Oreochromis niloticus* and *Clarias gariepinus*. This response of EROD activity may be explained by the suggestion that the cadmium exposure has global effects on the cells that can alter membrane structure of fish hepatocytes (Hassanain et al. 2007). However these results are contrary to those of Lemaire-Gony and Lemaire (1992) who reported that in cadmium-exposed eels (*Anguilla anguilla*), BaP treatment elicited a greater increase in EROD and BaP hydroxylase (BaPMO) activities (induction factor of 19- and 71-fold, respectively) and also caused a 1.8-fold increase in cytochrome P-450 microsomal content. That could be interpreted by long-term exposure and increased synthesis of toxic BaP metabolites and their slow elimination. In

conclusion, our main finding demonstrated a linkage between EROD activity and oxidative stress biomarkers (PC and TBARS), as well as possible influence of oxidative stress and its products (ROS, free radicals) on the cell membrane structures (enzymes), and consequently on the EROD activity.

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References

Ahmad, I., Pacheco, M., & Santos, M. A. (2004). Enzymatic and nonenzymatic antioxidants as an adaptation to phagocyte-induced damage in *Anguilla anguilla* L. following in situ harbor water exposure. *Ecotoxicology and Environmental Safety, 57*, 290–302.

Alessio, H. M., Goldfarb, A. H., & Culter, R. G. (1988). MDA content increases in fast- and slow-twitch skeletal muscle with intensity of exercise in a rat. *The American Journal of Physiology, 6*(1), 874–877.

Almeida, J. A., Diniz, Y. S., Marques, S. F. G., Faine, L. A., Ribas, B. O., Burneiko, R. C., & Novelli, E. L. B. (2002). The use of the oxidative stress responses as biomarkers in Nile tilapia (*Oreochromis niloticus*) exposed to in vivo cadmium contamination. *Environmental International, 27*, 673–679.

Almroth, B. C., Sturve, J., Berglund, A., & Förlin, L. (2005). Oxidative damage in eelpout (*Zoarces viviparus*), measured as protein carbonyls and TBARS, as biomarkers. *Aquatic Toxicology, 73*, 171–180.

Ando, S., & Yanagida, K. (1999). Susceptibility to oxidation of copper-induced plasma lipoproteins from Japanese eel: Protective effect of vitellogenin on the oxidation of very low density lipoprotein. *Comparative Biochemistry and Physiology. C, 123*, 1–7.

Arniç, E., Sen, A., & Bozcaarmutlu, A. (2000). Cytochrome P4501A and associated mixed-function oxidase induction in fish as a biomarker for toxic carcinogenic pollutants in the aquatic environment. *Pure and Applied Chemistry, 72* (6), 985–994.

Bagnyukova, T. V., Vasylkiv, O. Y., Storey, K. B., & Lushchak, V. I. (2005). Catalase inhibition by amino triazole induces oxidative stress in goldfish brain. *Brain Research, 1052*, 180–186.

Banjerdkij, P., Vattanaviboon, P., & Mongkolsuk, S. (2005). Exposure to cadmium elevates expression of genes in the OxyR and OhrR regulons and induces cross-resistance to peroxide killing treatment in *Xanthomonas campestris*. *Applied and Environmental Microbiology, 71*(4), 1843–1849.

Barker, C. W., Fagan, J. B., & Pasco, D. S. (1994). Down-regulation of P4501A1 and P4501A2 mRNA expression in isolated hepatocytes by oxidative stress. *Journal of Biological Chemistry, 269*, 3985–3990.

Beamish, F. W. H. (1978). Swimming capacity. In W. S. Hoar & D. J. Randall (Eds.), *Fish physiology. Vol. 7* (pp. 101–187). New York: Academic.

Belviranli, M., & Gökbel, H. (2006). Acute exercise induced oxidative stress and antioxidant changes. *European Journal of General Medicine, 3*(3), 126–131.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry, 72*, 248–254.

Burgeot, T., Bocquéné, G., Truquet, P., Le Dean, L., & Galgani, F. (1994). Induction of EROD activity in red mullet (*Mullus barbatus*) along the French Mediterranean coasts. *Science of the Total Environment, 142*, 213–220.

Burke, M. D., & Mayer, R. T. (1974). Ethoxyresorufin: Direct fluorometric assay of a microsomal O-dealkylation which is preferentially inducible by 3-methylcholanthrene. *Drug Metabolism and Disposition, 2*(6), 583–588.

Chang, C.-K., Huang, H.-Y., Tseng, H.-F., Hsuuw, Y.-D., & Tso, T. K. (2007). Interaction of vitamin E and exercise training on oxidative stress and antioxidant enzyme activities in rat skeletal muscles. *Journal of Nutritional Biochemistry, 18*, 39–45.

Choi, J., & Oris, J. T. (2000). Evidence of oxidative stress in bluegill sunfish (*Lepomis macrochirus*) liver microsomes simultaneously exposed to solar ultraviolet radiation and anthracene. *Environmental Toxicology & Chemistry, 19*, 1795–1799.

Delaporte, E., & Renton, K. W. (1997). Cytochrome P4501A1 and cytochrome P4501A2 are downregulated at both transcriptional and post-transcriptional levels by conditions resulting in interferon-alpha/beta induction. *Life Sciences, 60*, 787–796.

Fenet, H., Casellas, C., & Bontoux, J. (1998). Laboratory and field-caging studies on hepatic enzymatic activities in European eel and rainbow trout. *Toxicology and Environmental Safety, 40*, 137–143.

Flammarion, P., & Garric, J. (1997). Cyprinids EROD activities in low contaminated rivers: A relevant statistical approach to estimate reference levels for EROD biomarker? *Chemosphere, 35*(10), 2375–2388.

Floor, E., & Wetzel, M. G. (1998). Increased protein oxidation in human substantia nigra pars compacta in comparison with basal ganglia and prefrontal cortex measured with an improved dinitrophenylhydrazine assay. *Journal of Neurochemistry, 70*, 268–275.

Förlin, L., Haux, C., Karlsson-Norrgren, L., Runn, P., & Larsson, A. (1986). Biotransformation enzyme activities and histopathology in rainbow trout, *Salmo gairdneri*, treated with cadmium. *Aquatic Toxicology, 8*, 51–64.

Fortuniak, A., Zadrinski, R., Bilinski, T., & Bartosz, G. (1996). Glutathione depletion in the yeast *Saccharomyces cervisiae*. *Biochemistry and Molecular Biology International, 38*, 901–910.

Gabrzelak, T., & Klekot, J. (1985). The effect of paraquat on the peroxide metabolism enzymes in erythrocytes of freshwater fish species. *Comparative Biochemistry and Physiology, 81C*, 415–418.

Grzebyk, D., & Galgani, F. (1991). Measurement of the effect of organic pollution on marine organisms: Rapid determination of EROD induction using plate readers. *Aquatic Living Resources, 4*, 53–59.

Halliwell, B., & Chirico, S. (1993). Lipid peroxidation: Its mechanism, measurement and significance. *American Journal of Clinical Nutrition, 57*, 715S–725S.

Hassanain, M. A., Abdel-Rahman, E. H., Abo-Hegab, S., Tawfik, M. A. A., & Abbas, W. T. (2007). Induction of cytochrome

P450 1A1 as a biomarker of benzo-a-pyrene pollution in Egyptian fresh water fish. *Pakistan Journal of Biological Sciences, 10*(8), 1161–1169.

Ji, L. L. (2000). Antioxidants and oxidative stress in exercise. *Proceedings of the Society for Experimental Biology and Medicine, 222*, 283–292.

Kelle, M., Diken, H., Şermet, A., Atmaca, M., & Koçyiğit, Y. (1998). Changes in blood antioxidant status and lipid peroxidation following distance running. *Turkish Journal of Medical Sciences, 28*, 643–647.

Konstandi, M., Johnson, E., Lang, M. A., Camus-Radon, A. M., & Marselos, M. (2000). Stress modulates the enzymatic inducibility by benzo[α]pyrene in the rat liver. *Pharmacological Research, 42*, 3.

Lafontaine, Y., Gagne, F., Blaise, C., Costan, G., Gagnogn, P., & Chan, H. M. (2000). Biomarkers in zebra mussels (*Dreissena polymorpha*) for the assessment and monitoring of water quality of the St Lawrence river (Canada). *Aquatic Toxicology, 50*, 51–71.

Lemaire-Gony, S., & Lemaire, P. (1992). Interactive effects of cadmium and benzo-a-pyrene on cellular structure and biotransformation enzymes of the liver of the European eel *Anguilla anguilla*. *Aquatic Toxicology, 22*, 145–159.

Lenz, A.-G., Costabel, U., Shaltiel, S., & Levine, R. L. (1989). Determination of carbonyl groups in oxidatively modified proteins by reduction with tritiated sodium borohydride. *Analytical Biochemistry, 177*, 419–425.

Liu, J., Yeo, H. C., Övervik-Douki, E., Hagen, T., Doniger, S. J., Chu, D. W., Brooks, G. A., & Ames, B. N. (2000). Chronically and acutely exercised rats: Biomarkers of oxidative stress and endogenous antioxidants. *Journal of Applied Physiology, 89*, 21–28.

Liu, L., Bridges, R. J., & Eyer, C. L. (2001). Effect of cytochrome P450 1A induction on oxidative damage in rat brain. *Molecular and Cellular Biochemistry, 223*, 89–94.

Livingstone, D. R. (2001). Contaminant-stimulated reactive oxygen species production and oxidative damage in aquatic organisms. *Marine Pollution Bulletin, 42*, 656–666.

Lushchak, V. I., Bagnyukova, T. V., Lushchak, O. V., Storey, J. M., & Storey, K. B. (2005). Hypoxia and recovery perturb free radical processes and antioxidant potential in common carp (*Cyprinus carpio*) tissues. *International Journal of Biochemistry & Cell Biology, 37*, 1319–1330.

Manna, I., Jana, K., & Samanta, P. K. (2003). Effect of intensive exercise-induced testicular gametogenic and steroidogenic disorders in mature male Wistar strain rats: A correlative approach to oxidative stress. *Acta Physiologica Scandinavica, 178*, 33–40.

Oakes, K. D., & Van Der Kraak, G. J. (2003). Utility of the TBARS assay in detecting oxidative stress in white sucker (*Catostomus commersoni*) populations exposed to pulp mill effluent. *Aquatic Toxicology, 63*, 447–463.

Parvez, S., & Raisuddin, S. (2005). Protein carbonyls: Novel biomarkers of exposure to oxidative stress-inducing pesticides in freshwater fish *Channa punctata* (Bloch). *Environmental Toxicology and Pharmacology, 20*, 112–117.

Peters, L. D., Porte, C., Albaiges, J., & Livingstone, D. R. (1994). 7-Ethoxyresorufin *O*-deethylase (EROD) and antioxidant enzyme activities in larvae of sardine (*Sardina pilchardus*) from the North coast of Spain. *Marine Pollution Bulletin, 28*(5), 299–304.

Rice-Evans, C. A., Diplock, A. T., & Symons, M. C. R. (1991). Techniques in free radical research. In R. H. Brton & P. H. Knippenberg (Eds.), *Laboratory techniques in biochemistry and molecular biology* (pp. 147–149). Amsterdam: Elsevier.

Roméo, M., Bennani, N., Gnassia-Barelli, M., Lafaurie, M., & Girard, J. P. (2000). Cadmium and copper display different responses towards oxidative stress in kidney of the sea bass *Dicentrachus labrax*. *Aquatic Toxicology, 48*, 185–194.

Romero, F., Bosch-Morell, F., Romero, M. J., Jareno, J., Romero, B., Marin, N., & Roma, J. (1998). Lipid peroxidation products and antioxidants in human disease. *Environmental Health Perspectives Supplements, 106*, 1229.

Sen, C. K., Atalay, M., Agren, J., Laaksonen, D. E., Roy, S., & Hänninen, O. (2005). Fish oil and vitamin E supplementation in oxidative stress at rest and after physical exercise. *Journal of Applied Physiology, 83*, 189–195.

Shacter, E. (2000). Quantification and significance of protein oxidation in biological samples. *Drug Metabolism Reviews, 32*(3–4), 307–326.

Shacter, E., Williams, J. A., Lim, M., & Levine, R. L. (1994). Differential susceptibility of plasma proteins to oxidative modification: Examination by western blot immunoassay. *Free Radical Biology and Medicine, 17*, 429–467.

Shi, H., Sui, Y., Wang, X., Luo, Y., & Ji, L. (2005). Hydroxyl radical production and oxidative damage induced by cadmium and naphthalene in liver of *Carassius auratus*. *Comparative Biochemistry and Physiology. C, 140*, 115–121.

Široka, Z., & Drastichova, J. (2004). Biochemical markers of aquatic environment contamination—cytochrome P450 in fish. A review. *Acta Veterinaria Brno, 73*, 123–132.

Stanic, B., Andric, N., Zoric, S., Grubor-Lajsic, G., & Kovacevic, R. (2006). Assessing pollution in the Danube River near Novi Sad (Serbia) using several biomarkers in starlet (*Acipenser ruthenus* L.). *Ecotoxicology and Environmental Safety, 65*, 395–402.

Stegeman, J. J. (1989). Cytochrome P450 forms in fish: Catalytic, immunological and sequence similarities. *Xenobiotica, 19*(10), 1093–1110.

Stein, J. E., Collier, T. K., Reichart, K., Caillas, L., Hom, E., & Varanasi, U. (1992). Bioindicators of contaminant exposure and sublethal effects: Studies with benthic fish in Pudget Sound, WA. *Environmental Toxicology & Chemistry, 11*, 701–714.

Stohs, S. J., & Bagchi, D. (1995). Oxidative mechanisms in the toxicity of chromium and cadmium ions. *Journal of Environmental Pathology, Toxicology, and Oncology, 20*, 77–88.

Van der Oost, R., Beyer, J., & Vermeulen, N. P. E. (2003). Fish bioaccumulation and biomarkers in environmental risk assessment: A review. *Environmental Toxicology and Pharmacology, 13*, 57–149.

Wang, Y., Fang, J., Leonard, S. S., & Rao, K. M. (2004). Cadmium inhibits the electron transfer chain and induces reactive oxygen species. *Free Radical Biology & Medicine, 36*, 1434–1443.

Whyte, J. J., Jung, R. E., Schmitt, C. J., & Tillitt, D. E. (2000). Ethoxyresorufin-*O*-deethylase (EROD) activity in fish as a biomarker of chemical exposure. *Critical Reviews in Toxicology, 30*(4), 347–570.