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Dimethoate-induced oxidative stress and DNA damage in *Oncorhynchus mykiss*

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ABSTRACT

The present study was conducted in order to investigate pro-oxidant activity of dimethoate in liver and brain tissues following sublethal pesticide exposure for 5, 15 and 30 d by using SOD, GPx, CAT enzyme activities and lipid peroxidation as biomarkers as well as DNA damaging potential via detecting % Tail DNA, Tail moment and Olive tail moment as endpoints in erythrocytes of *Oncorhynchus mykiss* in an *in vitro* experiment. Antioxidant enzyme activities were found to elicit two staged response which was an initial induction followed by a sharp inhibition in liver tissue while a sustained increase in GPx activity and slight stimulation in SOD activity were detected in brain tissue. Lipid peroxidation showed an ascending pattern throughout the exposure period in both tissues and a decreasing trend was determined in tissue protein levels which was proved to be positively correlated with duration. Similar findings were obtained from outcomes preferred to quantify DNA damage and TM was decided to reflect the extent of damage more sensitively because of determined positive correlation with concentrations applied. Considering these results, it can be concluded that oxidative stress condition evoked by dimethoate could not be responded effectively and genotoxic nature of pesticide was proven by determined clastogenic effect possibly via being an alkylation agent or stimulating the production of reactive species.

1. Introduction

Pesticides are deliberately released to the environment for the purpose of controlling agricultural pests because of their quick effectiveness and extensive use is resulted in environmental pollution and toxicity risk to non-target organisms leading to a number of pathological and disturbed biochemical processes. Dimethoate (*O*,*O*-dimethyl *S*-methylcarbamoylmethyl phosphorodithioate) is a broad use, systemic organophosphorus insecticide and acaricide which has been in use since 1956 against insects and mite on agricultural crops and ornamental plants. Dimethoate is primarily recognized for its neurotoxic effects caused by inhibition of acetylcholinesterase (AChE) by its oxon metabolite, dimethoxon. Despite of low environmental persistence, diminished carbohydrate metabolism (Begum and Vijayaraghavan, 1995), histopathological changes (Rodrigues and Fanta, 1998) and respiratory disturbance in fish (Shereena et al., 2009), endocrine disruption (Astiz et al., 2009) and carcinogenic effects (Reuber, 1984) in rat were reported.

The production of reactive oxygen species has long been regarded as a possible mechanism of pesticide-induced toxicity as evidenced by triggered oxidative stress in a number of studies (Monteiro et al., 2006; Modesto and Martinez, 2010). Macromolecules are sensitive to interactions with reactive species and their highly reactive nature results in severe threat to normal cellular functions. Under normal conditions, damaging effects of oxidative stress are counteracted by antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) providing first line defense (Livingstone, 2001). Damage to membrane lipids, lipid peroxidation, is considered as one of the molecular mechanisms involved in pesticide toxicity and its predictive importance as a biomarker for oxidative stress is indicated in different investigations (Kavitha and Rao, 2008; Ballesteros et al., 2009).

Since presence of genotoxins in the aquatic environment is a well-known fact, attempt to develop sensitive biomarkers to evaluate genotoxic effects in aquatic organisms has gained importance. The single cell gel electrophoresis (SCGE), known as comet assay, is recognized as one of the most sensitive and reliable methodologies available for DNA strand break detection with the advantages of being fast, simple and applicable to any eukaryotic cell type, *in vivo* as well as *in vitro* (Mitchelmore and Chipman, 1998). The effects of pesticides on DNA integrity have been reported in studies in which comet assay has been successfully applied to erythrocytes of different fish species (Ateeq et al., 2005; Simoniello et al., 2009).

Fish are favored as bioindicators of pollutant effects because of their usefulness in not only providing information related to

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ecotoxicological consequences of potentially harmfull xenobiotics but also extrapolating results to humans. Because of its fundamental anatomy, high metabolic potential and role in accumulation, biotransformation and excretion of contaminants, the liver represents a target organ for toxic substances. On the other hand, the nervous system is a common target for toxic agents and brain is particularly susceptible due to high amounts of polyunsaturated fatty acids and low antioxidant capacity (Timbrell, 2002).

Oncorhynchus mykiss is a freshwater fish preferring well oxygenated, clean and cold water. Because of its importance in fishing and food industry, it is one of the sensitive test organisms commonly used as an early warning of biological changes caused by pollutants before physiological problems arise. Also, it is recommended to use in acute and chronic toxicity studies as bioindicator (OECD, 1992). Therefore the present investigation was undertaken to assess and interpret oxidative stress inducing potential of dimethoate in liver and brain tissues of *O. mykiss* together with genotoxic effect by using alkaline comet assay in erythrocytes exposed to same concentrations *in vitro*.

2. Materials and methods

2.1. Study species

Adult rainbow trout, *O. mykiss* (65.61 ± 10.01 g and 18.61 ± 0.97 cm) were obtained from local breeder and acclimated to laboratory conditions for a month. During acclimatization, fish were held in aerated tap water (150 L aquariums) and fed daily with commercial trout food at a rate of 2% of their body weights. Fish were not fed for 24 h prior to experiment and dissection. Throughout the experimental period, the water quality was as follows: temperature 15.2 ± 1.0, pH 8.08 ± 0.25, alkalinity 216.43 ± 8.41 mg L^{-1} $CaCO_3$ and hardness 218.50 ± 16.9 mg L^{-1} $CaCO_3$ dissolved oxygen 7.3 ± 0.4 mg L^{-1}. Temperature, pH and dissolved oxygen were analyzed daily and 12:12 photoperiod was used.

2.2. Experimental design

The insecticide used in this investigation was obtained commercially (*O*,*O*-dimethyl *S*-[2-(methylamino)-2-oxoethyl] phosphorodithioate, 400 g L^{-1} and Poligor®). 96 h LC_{50} value of dimethoate for *O. mykiss*, 7.35 mg L^{-1}, (Johnson and Finley, 1980) was used as reference in selection of exposure concentrations. Then, three sublethal concentrations of 0.0735 mg L^{-1}, 0.3675 mg L^{-1} and 0.7350 mg L^{-1} were used that corresponding to 1%, 5% and 10% of LC_{50} value, respectively. After acclimatization, fish were randomly divided into four groups each with 12 individuals and placed in separate glass aquaria (150 L). Group 1 was reared in pesticide-free tap water and treated as control. Fish belonging to groups 2, 3 and 4 were exposed to indicated sublethal concentrations of dimethoate for 5, 15 and 30 d. Stock solutions were prepared by dissolving insecticide in test water and further diluted to obtain determined concentrations in aquariums. Throughout the experimental period, fish were fed daily with commercial trout food at a rate of 2% of their body weights. Food was not given for 24 h prior to experiment and dissection. Experiments were performed under semi-static conditions and 2/3 of aquaria water was changed every 24 h with the appropriate pesticide amount (APHA, AWWA, WPCF, 1980).

2.3. Tissue sampling

At the end of each exposure period, four randomly selected fish from each aquarium were dissected. Liver and brain tissues were carefully removed, washed with ice-cold physiological saline (0.59% NaCl) and immediately frozen in liquid nitrogen and stored at −80 °C until analysis. Frozen tissue samples were homogenized (1:5 w/v) in 0.5 M pH 7.4 sucrose buffer and centrifuged at 10.000 rpm for 30 min at 4 °C (Hettich Universal 320R) to obtain post-mitochondrial supernatant for biochemical analyses by using a spectrophotometer (Cintra 202) (de Duve, 1971; Monteiro et al., 2006).

2.4. Antioxidant enzyme assays

Superoxide dismutase (SOD) activity was measured using the method based on the ability of the enzyme to inhibit the INT (iodo-*p*-nitro tetrazolium violet) reduction by monitoring change in optical density at 505 nm for 3 min at 37 °C as described by McCord and Fridovich (1969). The assay mixture consisted of 0.01 M phosphate buffer (pH 7.0), xanthine oxidase (XOD, 80 U L^{-1}), substrate (0.05 mM xanthine and 0.025 mM INT) and tissue homogenate in a total volume of 1 mL. SOD activity was expressed in mU mg of $protein^{-1}$, with one U of SOD is equivalent to the quantity of enzyme that promoted the inhibition of 50% of the reduction rate of INT.

Glutathione peroxidase (GPx) activity was determined by monitoring dismutation of *t*-butylhydroperoxide at 37 °C and 340 nm for 5 min (Beutler, 1984). The assay mixture consisted of 0.5 M Tris buffer (pH 8.0), 0.1 GSH, glutathione reductase (GR, 10 U mL^{-1}), 2 mM NADPH, 7 mM *t*-butylhydroperoxide, distilled water and tissue homogenate in a total volume of 1 mL. One unit of GPx activity represents the amount of enzyme that oxidizes 1 μmol of NADPH per minute.

Catalase (CAT) activity was assayed according to Beutler (1984) by recording the rate of hydrogen peroxide (H_2O_2) degradation by CAT at 230 nm for 5 min at 37 °C in a reaction mixture consisting of 1 M Tris buffer (pH 8.0), 10 mM H_2O_2, distilled water and tissue homogenate in a final volume of 1 mL. One unit of CAT activity is defined as the amount of the enzyme that degrades 1 μmol of H_2O_2 per minute; activities were expressed as milliunits per milligram of protein (mU mg^{-1} protein).

2.5. Lipid peroxidation

Lipid peroxidation was estimated with the method of Ohkawa et al. (1979) by measuring concentration of thiobarbituric acid reactive substances (TBARS). After incubating of reaction mixture (8.1% sodium dodecyl sulphate, 20% acetic acid (pH 3.5) and 0.8% thiobarbituric acid (pH 3.5) and tissue homogenates at 95 °C for 30 min, samples were allowed to cool in ice, centrifuged at 4000 rpm for 10 min and optical density at 532 nm was recorded. Lipid peroxidation levels were expressed as nmols per gram wet weight.

2.6. Protein estimation

Tissue protein levels were determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.7. Comet assay

Blood samples of fish were taken from caudal vein into tubes with heparin and processed immediately. Cell viability for erythrocytes was determined using trypan blue exclusion method and viability was expressed as the percentage of viable cells in the total number of cells counted (100). 25 μL of blood sample added to 2.5 mL of sterile cell medium (RPMI 1440). Two groups as control and dimethoate exposed samples, (0.0735 mg L^{-1}, 0.3675 mg L^{-1} and 0.7350 mg L^{-1} concentrations), three replicates for each, were incubated at 37 °C for 1 and 2 h. After exposure periods, DNA

damage was analysed by alkaline comet assay according to Singh et al. (1988) with some modifications.

Incubated medium containing erythrocytes (6 μL) were mixed with 100 μL of 0.7% low-melting-point agarose (LMA, Sigma) in phosphate buffered saline (PBS) at 37 °C. Then, 80 μL of this cellular suspension was spread onto slides that had previously been coated with 1.0% normal melting point agarose (60 °C) (NMA) and covered with a cover slip at 4 °C for at least 5 min to allow the agarose to solidify. After removing cover slips, the slides were submersed in freshly prepared cold (4 °C) lysing solution (2.5 M NaCl, 100 mM EDTA-2Na, 10 mM Tris–HCl pH 10–10.5, 1% Triton X-100 and 10% DMSO added just before use) for at least 1 h. Slides were then placed side by side in a horizontal gel electrophoresis tank containing freshly prepared alkaline electrophoresis buffer (0.3 M NaOH and 1 mmol L^{-1} Na_2ETDA, pH > 13). The slides were left in this solution for 25 min to allow DNA unwinding and expression of alkaline-labile sites as DNA strand breaks. Electrophoresis was conducted at a current of 300 mA for 25 min at 4 °C.

All procedures were conducted under minimal illumination and the electrophoresis tank was covered with black paper in order to avoid additional DNA damage due to stray light. After electrophoresis, the slides were neutralized (0.4 M Tris–HCl, pH 7.5) for 5 min. The dried microscope slides were stained with ethidium bromide (2 μg mL^{-1} in distilled H_2O; 70 μL/slide), covered with a coverslip and 100 randomly selected and non-overlapping cells per slide were scored using a CCD camera attached fluorescence microscope (Olympus BX 51, Japan) with a magnification of 20×. Imagining was performed by using an image analysis system (CASP Analyzing programme). %Tail DNA (percent of DNA in the comet tail), tail moment (%Tail DNA × tail length, μm) and olive tail moment (%tail DNA × tail CoG (center of gravity) – head CoG, arbitrary units) were selected as parameters to quantify DNA damage.

2.8. Statistics

Statistics were performed using the SPSS 16.0 computer program (SPSS Inc. Chicago, Illinois, USA). The analysis of variance (One-way ANOVA) followed by Post Hoc LSD multiple comparison test was performed to determine statistical difference between mean values of control and experimental groups. Pearson correlation analysis was used to quantify the strength of association between two variables. Results were expressed as means ± standard error.

3. Results

3.1. Antioxidant enzyme activities

Change in SOD activity in liver and brain tissues of dimethoate exposed *O. mykiss* is presented in Table 1 and Fig. 1. In liver tissue, there were no noticeable changes in activity at lowest concentration on 5 and 15 d of application while time- and dose-dependant increase was observed at 0.3675 and 0.7350 mg L^{-1} concentrations as 179% and 340%, respectively ($r = 0.799$ and $r = 0.857$, $p < 0.01$). However the activity decreased dose dependently on day 30 with the magnitude of inhibition of 83% ($r = 0.842$, $p < 0.01$). In brain tissue, only change in SOD activity was an induction observed on day 5 at 0.3675 and 0.7350 mg L^{-1} concentrations reaching maximum of 47% ($p < 0.01$).

GPx specific activity did not show any change on day 5 in liver tissue (Table 1 and Fig. 1, $p > 0.05$). A remarkable and dose-dependant increase on day 15 followed by a significant decrease on day 30 was detected ($r = 0.970$, $p < 0.01$). The maximum induction and inhibition rates were recorded as 134% and 54%, respectively. GPx activity showed same pattern in brain tissue for 5 d of

Table 1
Effects of sublethal dimethoate exposure on SOD (mU mg^{-1} protein), GPx (mU mg^{-1} protein), CAT activities (U mg^{-1} protein), TBARS concentration (nmol g^{-1} ww) and protein levels (mg mL^{-1}) in liver and brain tissues of *Oncorhynchus mykiss*.

	Duration (d)					
	5	15	30	5	15	30
	Liver			Brain		
SOD						
Control	79.1 ± 10.0^{ax}	84.0 ± 10.0^{ax}	84.2 ± 10.0^{ax}	5.9 ± 1.00^{ax}	5.7 ± 1.00^{ax}	5.8 ± 1.00^{ax}
0.0735	76.9 ± 10.0^{ax}	147 ± 10.0^{by}	54.5 ± 10.0^{abx}	7.3 ± 1.00^{abx}	6.0 ± 1.00^{ax}	6.0 ± 1.00^{ax}
0.3675	136 ± 10.0^{bx}	$239 \pm 20.0^{cy*}$	23.9 ± 10.0^{bz}	7.9 ± 1.00^{bx}	6.4 ± 1.00^{ax}	6.2 ± 1.00^{ax}
0.7350	$221 \pm 20.0^{cx*}$	$370 \pm 50.0^{dy*}$	$13.7 \pm 10.0^{bz*}$	$8.7 \pm 1.00^{bx*}$	6.0 ± 1.00^{ax}	6.1 ± 1.00^{ax}
GPx						
Control	178 ± 10.0^{ax}	193 ± 10.0^{ax}	190 ± 10.0^{ax}	63.0 ± 2.00^{ax}	59.1 ± 5.00^{ax}	60.8 ± 4.00^{ax}
0.0735	188 ± 10.0^{ax}	$278 \pm 20.0^{by*}$	154 ± 20.0^{abx}	60.6 ± 4.00^{ax}	64.7 ± 4.00^{abxy}	71.5 ± 4.00^{by}
0.3675	182 ± 10.0^{ax}	$376 \pm 40.0^{cy*}$	126 ± 10.0^{bcz}	61.5 ± 4.00^{ax}	66.1 ± 1.00^{abxy}	72.9 ± 2.00^{by}
0.7350	197 ± 10.0^{ax}	$454 \pm 30.0^{dy*}$	$86.4 \pm 20.0^{cz*}$	68.3 ± 2.00^{ax}	71.7 ± 4.00^{bxy}	$77.9 \pm 6.00^{bx*}$
CAT						
Control	72.7 ± 2.54^{ax}	69.2 ± 2.21^{ax}	72.6 ± 2.26^{ax}	1.41 ± 0.09^{ax}	1.41 ± 0.05^{ax}	1.45 ± 0.07^{ax}
0.0735	72.5 ± 2.36^{axy}	$81.5 \pm 2.97^{bx*}$	70.6 ± 1.83^{ay}	1.41 ± 0.06^{ax}	1.42 ± 0.11^{ax}	1.50 ± 0.08^{ax}
0.3675	71.9 ± 2.58^{ax}	$91.8 \pm 3.15^{cy*}$	65.9 ± 1.99^{abx}	1.46 ± 0.04^{ax}	1.47 ± 0.08^{ax}	1.57 ± 0.10^{ax}
0.7350	72.4 ± 2.48^{ax}	$131 \pm 8.07^{dy*}$	$58.9 \pm 2.41^{bz*}$	1.46 ± 0.05^{ax}	1.49 ± 0.06^{ax}	1.58 ± 0.09^{ax}
TBARS concentration						
Control	8.28 ± 0.58^{ax}	7.34 ± 0.71^{ax}	5.75 ± 0.40^{ax}	22.2 ± 1.31^{ax}	28.7 ± 3.36^{ax}	25.1 ± 1.78^{ax}
0.0735	9.33 ± 0.79^{abx}	9.05 ± 0.55^{ax}	8.28 ± 1.25^{ax}	23.3 ± 2.55^{ax}	36.2 ± 6.87^{aby}	27.5 ± 0.40^{abxy}
0.3675	10.1 ± 0.83^{abx}	9.43 ± 1.21^{ax}	$12.3 \pm 1.99^{bx*}$	26.5 ± 1.71^{abx}	30.9 ± 0.51^{abx}	35.9 ± 7.26^{bx}
0.7350	12.8 ± 0.90^{bx}	$14.0 \pm 1.86^{bx*}$	$22.4 \pm 2.42^{cy*}$	34.6 ± 0.82^{bx}	38.6 ± 1.19^{bxy}	$47.5 \pm 3.36^{cy*}$
Protein level						
Control	21.9 ± 1.82^{ax}	20.4 ± 0.96^{ax}	21.2 ± 1.59^{ax}	7.71 ± 0.53^{ax}	7.95 ± 0.11^{ax}	8.12 ± 0.10^{ax}
0.0735	19.1 ± 1.77^{ax}	15.8 ± 0.43^{bx}	$15.9 \pm 1.32^{bx*}$	7.66 ± 0.83^{ax}	7.70 ± 0.76^{ax}	7.36 ± 0.44^{abx}
0.3675	18.8 ± 2.82^{ax}	$14.9 \pm 1.27^{bxy*}$	$13.2 \pm 1.45^{bcy*}$	7.76 ± 0.81^{ax}	7.26 ± 0.63^{ax}	6.47 ± 0.20^{bcx}
0.7350	18.9 ± 2.06^{ax}	$12.7 \pm 0.85^{by*}$	$10.9 \pm 0.49^{cy*}$	6.53 ± 0.78^{ax}	5.91 ± 0.27^{bx}	$5.31 \pm 0.53^{cx*}$

Values are given as mean ± standard error ($n = 4$). Superscript letters a, b, c and d indicate differences among exposure concentrations; superscripts x and y indicate differences among durations ($p < 0.05$).
* Significance at $p < 0.01$ level.

pesticide treatment while continuous and dose-dependant increase was determined on days 15 and 30 ($r = 0.654$, $p < 0.05$).

CAT specific activity showed a sharp dose-dependant increase on day 15 in liver tissue ($r = 0.930$, $p < 0.01$) while no remarkable change was observed on day 5 (Table 1 and Fig. 1). The induction rates were determined as 17%, 32% and 88% for 0.0735, 0.3675 and 0.7350 mg L^{-1} concentrations, respectively. It was observed that CAT activity decreased to control level for the lowest pesticide concentration and diminished by 18% at highest concentration on day 30 ($p < 0.01$). Dimethoate treatment did not cause any change in CAT activity in brain tissue except recorded increase on days 15 and 30 which was statistically insignificant.

3.2. Concentration of TBARS

An increasing trend in TBARS significantly correlated with duration was recorded in liver tissue ($r = 0.801$, $p < 0.01$). The highest induction detected on 5, 15 and 30 d of application were 55%, 91% and 290%, respectively (Fig. 2). In brain tissue, TBARS concentration increased only at highest concentration on days 5 and 15

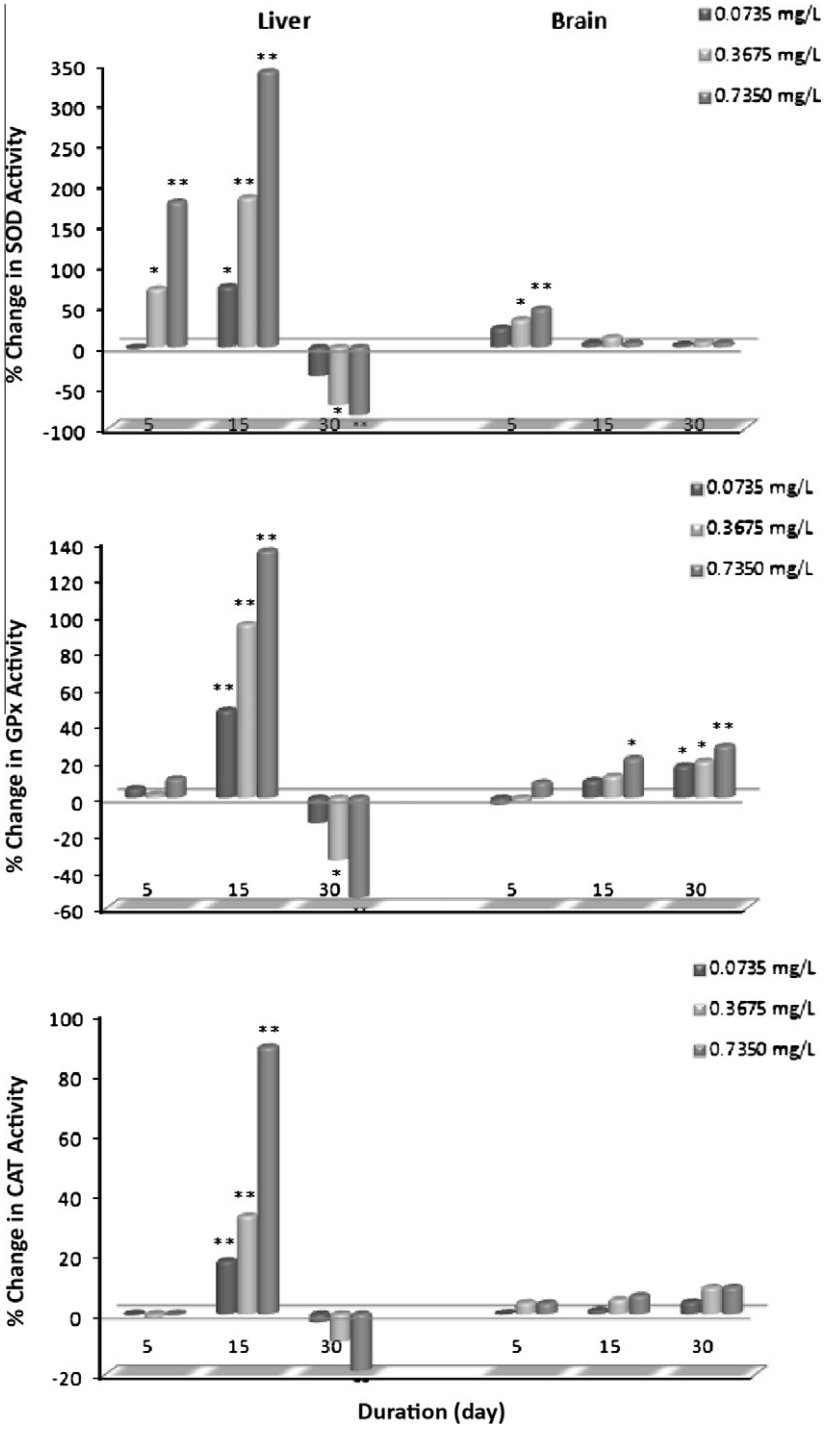

Fig. 1. Percentage change of mean values of SOD, GPx and CAT enzyme activities following dimethoate exposure in liver and brain tissues of *O. mykiss* with respect to control group. $^{*}p < 0.05$ and $^{**}p < 0.01$.

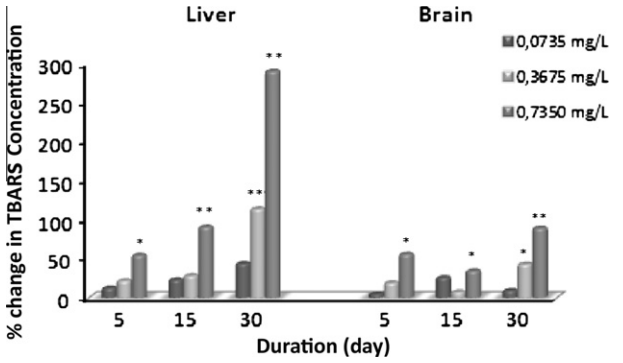

Fig. 2. Percentage change of mean values of TBARS concentration with respect to control group in liver and brain tissues of *O. mykiss* following dimethoate application. $^{*}p < 0.05$ and $^{**}p < 0.01$.

Table 2
Pearson correlation coefficients (r) between TBARS concentration and antioxidant enzyme activities/protein level in liver and brain tissues of *O. mykiss* following dimethoate exposure ($n = 16$).

		Duration (d)		
		5	15	30
Liver				
	SOD	0.567*	0.840**	−0.658**
	GPx	0.198	0.593*	−0.670**
	CAT	− 0.046	0.680**	−0.736**
	Protein	0.019	−0.685**	−0.710**
Brain				
	SOD	0.514*	0.213	0.056
	GPx	0.141	0.422	0.170
	CAT	0.328	0.289	0.211
	Protein	−0.223	−0.617**	−0.567*

Correlation is significant at $^{*}p < 0.05$ and $^{**}p < 0.01$ levels (2-tailed).

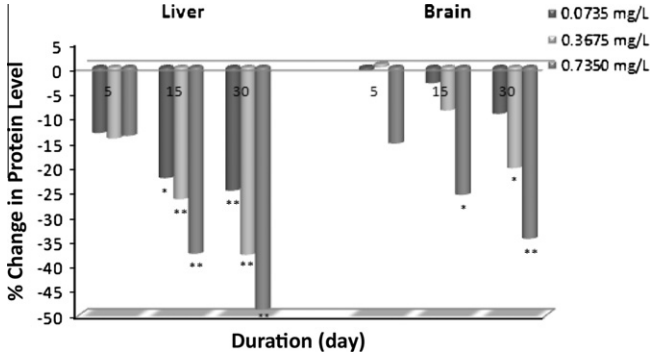

Fig. 3. Percentage change of mean values of protein level with respect to control group in liver and brain tissues of *O. mykiss* after dimethoate exposure. $^{*}p < 0.05$ and $^{**}p < 0.01$.

($p < 0.05$) and the most remarkable induction was determined at 0.7350 mg L^{-1} on day 30 as 89% which was highly correlated with duration ($r = 0.787$, $p < 0.01$).

Pearson correlation was applied to obtain information regarding relationship between changes recorded in TBARS concentration and antioxidant enzyme activities/protein levels in liver and brain tissues of dimethoate exposed *O. mykiss* (Table 2). In liver tissue, concentration of TBARS was found to be positively on day 15 and negatively on day 30 correlated with SOD, GPx and CAT activities while negative and significant relation was determined between TBARS concentration and protein level. In a similar way, analyse

Table 3
Effects of sublethal dimethoate exposure on %Tail DNA, TM and OTM values in erythrocytes of *Oncorhynchus mykiss*.

	Duration (h)	
	1	2
%Tail DNA		
Control	20.2 ± 1.79^{ax}	16.9 ± 2.70^{ax}
0.0735	19.1 ± 1.06^{ax}	20.9 ± 1.35^{abx}
0.3675	$32.4 \pm 4.40^{bx*}$	25.3 ± 2.97^{bx}
0.7350	20.3 ± 2.69^{ax}	$26.4 \pm 2.06^{cx*}$
TM (μm)		
Control	9.74 ± 1.67^{ax}	6.44 ± 2.26^{ax}
0.0735	10.6 ± 0.41^{ax}	10.8 ± 1.14^{abx}
0.3675	$23.9 \pm 5.54^{bx*}$	14.4 ± 2.54^{by}
0.7350	11.8 ± 1.32^{ax}	$16.5 \pm 2.22^{bx*}$
OTM (arbitrary units)		
Control	7.54 ± 0.97^{ax}	4.56 ± 1.05^{ax}
0.0735	7.22 ± 0.36^{ax}	7.45 ± 0.45^{abx}
0.3675	$14.3 \pm 2.88^{bx*}$	10.0 ± 1.33^{bcy}
0.7350	7.35 ± 0.42^{ax}	$11.2 \pm 1.43^{cx*}$

Values are given as mean ± standard error ($n = 3$). Superscript letters a, b and c indicate differences among exposure concentrations; superscripts x, y and z indicate differences among durations ($p < 0.05$).
* Significance at $p < 0.01$ level.

results revealed significantly negative r values for protein level in brain tissue although no significant relation between TBARS concentration and antioxidant enzyme activities was figured except for positive correlation detected for SOD on day 5.

3.3. Protein level

Protein level followed same pattern in liver and brain tissues on day 5 by not showing significant change over control values (Table 1 and Fig. 3, $p > 0.05$). However, time-dependant decrease at all applied concentrations was observed on days 15 and 30 in liver tissue ($r = 0.708$, $p < 0.01$). Similarly, significant reduction on days 15 (0.7350 mg L^{-1}) and 30 (0.3675 and 0.7350 mg L^{-1}) was determined in brain tissue which was correlated with durations ($r = 0.689$, $p < 0.01$). The maximum values of percent change recorded for liver and brain tissues were 48% and 34%, respectively.

3.4. DNA damage

Cell viability was found to be more than >90% in all treatments allowing the comet assay to be performed. The results of %Tail DNA, TM and OTM showing similar pattern are given in Table 3 and Fig. 4. There was no remarkable change after 1 h dimethoate treatment with the exception of drastic increase determined at 0.3675 mg L^{-1} whereas a consistent increase was observed following 2 h pesticide application which was more pronounced at the highest concentration ($p < 0.01$). Pearson correlation analyses revealed that this enhancement was positively correlated with applied concentrations for TM ($r = 0.894$, $p < 0.05$).

4. Discussion

The antioxidant enzyme activities having potential to provide oxidative stress markers are increasingly studied although connection between biomarker induction/inhibition and occurrence of physiological effects is not always fully understood (Lerner et al., 2007). Triggered oxidative stress has been reported as a consequence of pesticide exposure resulting from excess production and/or insufficient removal of highly reactive molecules such as reactive oxygen and nitrogen species (Sayeed et al., 2003; Ballesteros et al., 2009).

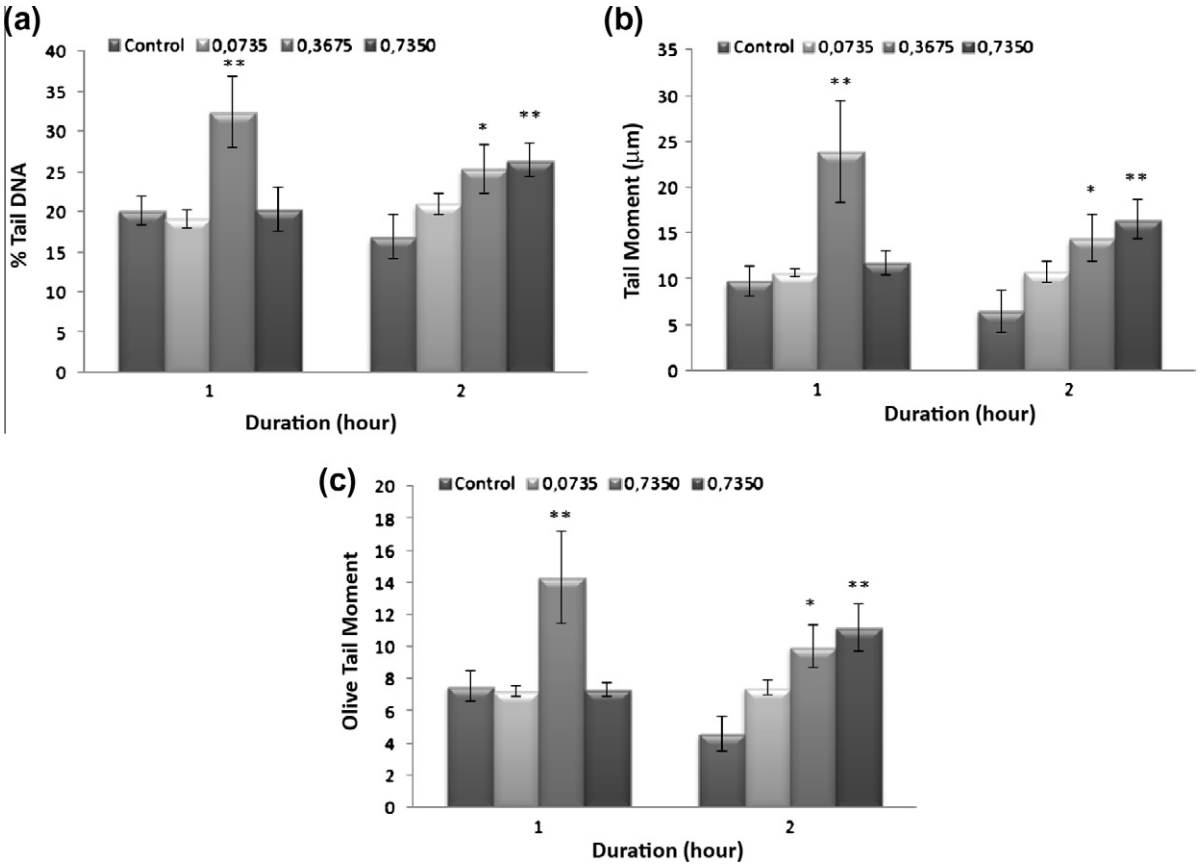

Fig. 4. Effects of *in vitro* dimethoate treatment for 1 h and 2 h on (a) % Tail DNA, (b) Tail moment (μm) and (c) Olive tail moment (arbitrary units) in erythrocytes of *O. mykiss*. Values are given as mean ± standard error, $^{*}p < 0.05$ and $^{**}p < 0.01$.

Response to chemical stress is not characterized with induced antioxidant enyzmes, both enhancement and diminishment can occur depending on time and dose of application as well as susceptibility of exposed species (Cheung et al., 2001). Li et al. (2010) classified results they obtained in brain tissue of *O. mykiss* under carbamazepine stress as adaptive and inhibitive stages and indicated that short-term application resulted in adaptation by induction of antioxidant enzymes while oxidative stress evoked after long-term exposure.

The detoxification pathway is the result of multiple enzymes consisting of SOD which functions to catalyse dismutation of superoxide anion to water and H_2O_2, CAT and GPx detoxifying resulted H_2O_2. Elevation in activities of these enzymes is a response to stress and reflects attempt to eliminate free radicals produced (Sies, 1986). Induction in SOD activity observed as an early signal was assumed as an adaptive response to dimethoate-triggered superoxide anion radical production while similar pattern in CAT and GPx enzyme activities was supposed to reflect altered cellular H_2O_2. Persistent and dose-dependant increase in GPx activity in brain tissue was caused by protective effect of enzyme against H_2O_2 as well as lipid hydroperoxide toxicity caused by membrane lipid peroxidation. It was stated that deceleration of termination reaction of hydroperoxide radical cycle causes hydroxyl radical accumulation and lipid peroxidation is triggered when H_2O_2 production is restricted by SOD (Michiels et al., 1994). Increase in enzyme activities arises either from altering enzyme amount or post-translational covalent modifications. The antioxidant enzymes analysed in this study are known not to undergo posttranslational regulation, therefore elevated enzyme activities under dimethoate stress are probably resulted from increased transcription and/or translation (Lushchak et al., 2005).

Differences in basal enzyme levels and responses to dimethoate toxicity were determined for liver and brain tissues. It is known that brain tissue is more sensitive to reactive oxygen species because of low antioxidant capacity and high amount of unsaturated fatty acid (Mates, 2000). However, liver has high metabolism and oxygen consumption due to being main organ in detoxification of xenobiotics and different toxic metabolites and therefore reflects the status of antioxidant defense better (Wilhelm Filho et al., 1993). Early elevation followed by reduction in SOD activity in liver tissue of *Cyprinus carpio* was interpreted as overwhelmed antioxidant capacity due to hexachlorobenzene triggered oxidative stress. Moreover, parallel increase in GPx activity and lipid peroxidation in brain tissue was reported and it was introduced that brain is more susceptible to toxicity than liver (Song et al., 2006).

Lipid peroxidation is one of the well recognized mechanisms of cell damage analysed as oxidative stress biomarker (Monteiro et al., 2006; Işık and Çelik, 2008). The reactive aldehydes produced during lipid peroxidation can diffuse from production site and cause damage to inter and intra cellular targets (Esterbauer et al., 1990). Organophosphorus pesticides can lead lipid peroxidation either by direct interaction with cellular plasma membrane (Hai et al., 1997) or reactive oxygen accumulation caused by excitotoxicity triggered by their anti-cholinesterase activity (Yang et al., 1996). Elevation determined in lipid peroxidation in tissues of *O. mykiss* shows that reactive species produced under dimethoate stress were not eliminated effectively by antioxidant enzymes. In *Prochilodus lineatus*, suppressed activities of antioxidant enzymes and simultaneous increase in lipid peroxidation in liver tissue after roundup exposure were attributed to oxidative stress arising from insufficient neutralization of reactive species (Modesto and Martinez, 2010).

Reduction in protein content is one of the commonly observed effects under pesticide stress in fish (Luskova et al., 2002; Ogueji and Auta, 2007) and analyzing of protein content is preferred as a diagnostic tool to evaluate physiological status of cells. Dimethoate treatment resulted in remarkable decrease in protein level in liver and brain tissues of *O. mykiss* in a time dependant manner. Similar findings were stated in *Clarias batrachus* after cypermethrin application (Begum, 1995) and in *Anguilla anguilla* under fenitrothion stress (Sancho et al., 1998). Decrease of tissue protein content was accepted as a generalised stress response and this reduction was assumed as reflection of physiological adaptability of fish to stress situation (Fernandez-Vega et al., 2002).

The single cell gel electrophoresis, also known as comet assay, provides advantage over other traditional cytogenetic methods by not needing mitotically active cell. Therefore, comet assay is used as a strong tool evaluating relationship between DNA damage and exposure to genotoxins in genetic toxicology and environmental monitoring areas. The percent DNA in the tail reflecting amount of DNA migrated out of nucleus is strongly recommended as the parameter of choice and directly linked to DNA break frequency. The other endpoints TM and OTM are derived from % Tail DNA and preferred in sensitive quantification of DNA damage (Olive et al., 1990; Kumaravel and Jha, 2006). Observed high DNA damage in *O. mykiss* erythrocytes after *in vitro* dimethoate treatment shows parallelism with related investigations (Ventura et al., 2008; Nwani et al., 2010) and confirms that comet assay using fish erythrocytes is effective in determination of genotoxic effects of chemicals. Morever, revealed positive correlation between increasing concentration of dimethoate and TM resulted in TM to be considered as an endpoint providing more accurate reflection of DNA damage in this study.

Alkylating property of organophosphates is proposed as a mechanism by reason of alkylation of DNA bases is involved in DNA damage (Wild, 1975). The electron-rich atoms in DNA are readily attacked by electrophiles and transfer of methyl, ethyl or alkyl groups causes phosphorylation or alkylation resulting in mutagenic or clastogenic effects (Jayashree et al., 1992). The direct action of genotoxic compounds and interaction with oxygen radicals or reactive intermediates were also emphasized as possible reasons of strand breaks (Eastman and Barry, 1992). Dimethoate, as an organophosphate, requires metabolic activation to exert its toxic effects, however induction of oxidative stress and increase in DNA damage were reported in *in vitro* studies conducted with organophosphorus pesticides (Gultekin et al., 2000; Parra et al., 2010). DNA damage detected in *P. lineatus* erythrocytes following roundup application was explained as occurence of reactive oxygen species during metabolism of pesticide and it was suggested that interaction between reactive species and DNA resulted in lesions determined with comet assay (Cavalcante et al., 2008). Cypermethrin triggered a dose-dependant increase in tail moment and olive tail moment in *Danio rerio* hepatocytes in an *in vitro* experiment and this finding was interpreted as oxidative DNA damage as supported by induced oxidative stress in liver tissue (Jin et al., 2010). DNA damaging effect of dimethoate detected in *O. mykiss* erythrocytes may be attributed to both oxidative stress producing potential, as confirmed with altering antioxidant enzyme activities and increased lipid peroxidation, and alkylating property of this pesticide.

5. Conclusion

It can be concluded that stress condition provided by dimethoate exposure at sublethal concentrations evoked tissue specific responses in *O. mykiss*. The biphasic response, induction followed by inhibition, in liver and continuous stimulation of GPx activity concomitant with increasing lipid peroxidation in brain clearly demonstrate that the lack of success to neutralize the reactive species produced during biotransformation process resulted in occurrence of oxidative stress. Significant reduction in tissue protein levels may be considered as a general adaptive response to cope with stressful situation. Comet assay performed *in vitro* in erythrocytes elicited potentiality of dimethoate to be considered as a genotoxic compound promoting damage either by provoking reactive oxygen species production or direct interaction with DNA because of its electrophilic nature.

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