OXIDATIVE EFFECTS OF INORGANIC AND ORGANIC CONTAMINANTS IN HAEMOLYMPH OF MUSSELS: UTILITY OF RECENTLY- DEVELOPED METHODS FOR MEASURING TOTAL ANTIOXIDANT CAPACITY AND PROTEIN CARBONYLATION

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Abstract

In the present study, a firstly-established method in haemolymph of mussels exposed to different concentrations of heavy metals, such as zinc and cadmium and organic pollutants, such as PAHs and lindane, for the detection of total antioxidant capacity (TAC) was applied. The susceptibility of exposed mussels was increased in relation to oxidative stress induced by contaminants tested. Oxidative modifications of proteins were estimated by measuring protein carbonyl content (PCC) and malondialdehyde levels (MDA). For PCC measurement, a highly sensitive and accurate ELISA method was used. The results of the present study reinforce the role of MDA and PCC as biomarkers of oxidative stress. Significant correlation of TAC assay and oxidative status parameters reinforces the application of PCC method as useful tool for the determination of PCC alterations in haemolymph of mussels exposed to different levels of contaminants. TAC method promotes encouraged results, concerning its ability to predict antioxidant efficiency in haemolymph of mussels exposed to inorganic and organic contaminants.

Keywords: antioxidant capacity, malondialdehyde, protein carbonylation, heavy metals, organic contaminants.

1. Introduction

Haemocytes play a key role in the immune defense of mussels (Cheng, 1981). Metallic or organic xenobiotics are taken up by the haemocytes, are concentrated in their well developed endolysosomal system that results either in their detoxification (Cajaraville and Pal, 1995), or ROS generation (Winston *et al.*, 1996).

Protein carbonyl content (PCC; an irreversible modification of protein amino acid side-chains, mostly lysine, arginine, proline and hystidine, into ketone groups) and malondialdehyde (MDA; a product of different biochemical pathways such as lipid peroxidation, unsaturated fatty acids or arachidonic acid catabolism) has been recently reported as a biomarker of oxidative stress in several sentiment species (Dowling *et al.*, 2006; Box *et al.*, 2007).

Haemolymph of mussels contain various antioxidant enzymes to protect animal from oxyradicals (Pipe *et al.*, 1993). The method of total oxyradical scavenging capacity (TOSC), concerning the assessment of redox status in marine organisms has been widely used in the last decade (Regoli, 2000). Alamdari *et al.* (2007) developed a parallel method for determining the total antioxidant response of a sample, named as total antioxidant capacity (TAC). In the present laboratory study, we investigated the utility of a home-made Elisa assay, which requires only 5 μg of protein, as a useful method for determining protein carbonylation in the haemolymph of mussels, *Mytilus galloprovincialis* exposed to oxidative stress. Furthermore, the induction of lipid peroxidation in haemocytes of exposed mussels was determined, through the determination of MDA content, a well-known biomarker of oxidative stress. Moreover, the use of a firstly-established, in mussels, assay for the evaluation of total antioxidant susceptibility to oxidative stress induced by pollutants was carried out.

2. Materials and Methods

2.1. Mussel collection and experimental procedure

10 groups of mussels (20 mussels/group) were placed in static tanks, containing 40 l of natural aerated seawater (1 mussel/2 l of seawater). Each group of mussels was exposed either to 0.3 mg/l of a mixture of PAHs (stock solution, 1:1:1, of anthracene, phenanthrene and napthalin dissolved in acetone 100 μg/l), or to 30 μg/l of an organochlorine insecticide, gamma-hexachlorocyclohexane (γ-HCH), also known as lindane (stock solution of lindane dissolved in acetone 100 μg/l), or to different concentrations of zinc and/or cadmium chloride (1, 10 and 100 μg/l respectively) for 12 days. Control groups of mussels were consisted of non-exposed mussels and mussels exposed to acetone (acetone-exposed mussels) respectively. Both at 6 and 12 days of exposure, 9 mussels from each group collected, divided to 3 subgroups (3 mussels/subgroup) and prepared for haemolymph collection. Haemolymph of each mussel from each subgroup was collected from the posterior adductor muscle with a sterilized syringe and placed in plastic tubes. 1 ml of haemolymph (10^5 cells) was centrifuged for 10 min at 900x*g* and two fractions, the supernatant (S1 fraction) and the cell pellet, were collected. The supernatant (S1 fraction) was used for MDA and antioxidant capacity determination. The cell pellet was dissolved in 1 ml PS, lysed by sonication and centrifuged at 10.000x*g* for 10 min. Finally, the supernatant of the cell lysate (S2 fraction) was stored at -80^{o}C and used for the measurement of protein carbonyl groups.

2.2 ELISA (enzyme linked immunosorbent assay) for measuring carbonyl-group in haemocytes proteins:

For the quantitative estimation of PCC, a modified experimental procedure as reported by Alamdari *et al.* (2005) was used. This method is a modification of the ELISA assay described by Buss *et al.* (1997) that used 2.4-dinitrophenylhydrazine (DNPH) derivatisation, after attachment of the protein to the polystyrene plate.

2.3 Determination of MDA

MDA was detected in S1 fraction of mussels, based on method described by Niehaus and Samuelsson (1998).

2.4 Total antioxidant capacity (TAC) determination

Total antioxidant capacity (TAC) was measured according to Alamdari *et al.* (2007). This method utilizes the ability of antioxidants to reduce 3.3΄.5.5΄ tetramethylbenzidine (TMB) cation which causes decolourization of TMB cation and hence results in a decrease in absorbance at 450 nm (reference wavelength 570 or 620 nm). This absorbance is then compared with the absorbance given by a known concentration of a standard solution at the same wavelength. The amount of the oxidant reduced by the antioxidant is a reflection of the total antioxidant capacity of the sample.

2.5 Statistical analysis

Two-way analysis of variance (ANOVA) was used in order to identify significant differences between values obtained for each parameter, after 6 and 12 days of exposure to each compound (Bonferroni multiple comparison test, $p<0.05$). Statistical relationships among antioxidant capacity and oxidative status parameters were compared using non-parametric Spearman's rank correlation, $p<0.05$).

3. Results

3.1 Protein carbonyl and MDA content in haemolymph of mussels, after exposure to heavy metals and organic contaminants

Mussels exposed for 12 days to different concentrations of Zn showed significantly higher levels of PCC in their haemocytes, in relation to the control group at the respective time point (Fig. 1A). Exposure of mussels to 1 and 10 μg/l of Cd revealed significant increase of PCC in their haemocytes, only after 12 days of exposure, (Fig. 1B). Exposure of mussels to organic contaminants for 6 and 12 days revealed a time- dependent increase of protein carbonylation (Fig. 1C).

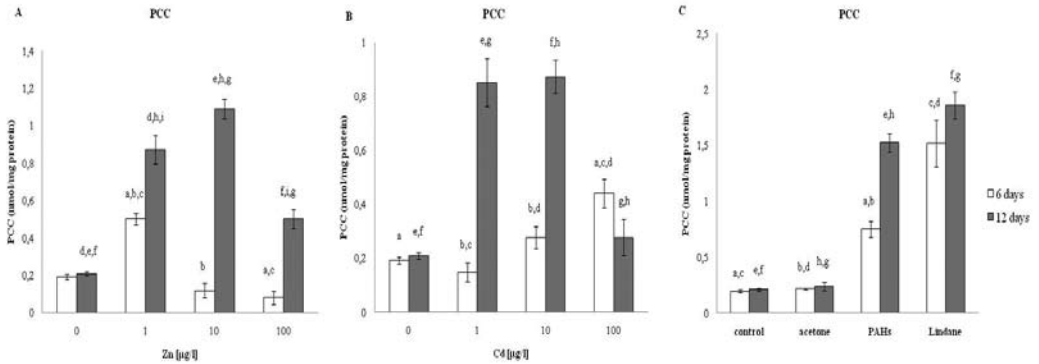

Fig. 1: *Protein carbonyl content (PCC) in haemocytes of mussels exposed to either zinc, or cadmium, or organic contaminants. Values are means ± standard deviations of 3 different measurements. In each measurement, haemolymph from 3 animals was used. Values that share the same letter are statistically significant different from each other.*

MDA content in S1 fraction of mussels exposed to different concentrations of Zn or Cd showed no statistical differences in relation to control mussels, during the exposure period (6 and 12 days respectively), with the exception of MDA content measured in the S1 fraction of mussels exposed to 10 μg/l of Zn for 6 days (Fig. 2A) and 1, 10 μg/l of Cd for 12 days (Fig. 2B). Exposure of mussels to both organic contaminants resulted in a significantly increase of MDA content in S1 fraction, in relation to those measured in control mussels (Fig. 2C).

3.2 Antioxidant capacity and relationship with oxidative stress parameters

Determination of TAC in haemolymph of metal-exposed mussels revealed no significant differences, at 6 days, while a rapid decrease was obtained in tissues of mussels exposed for 12 days, in all cases (Fig. 3A, B). Organic-exposed mussels showed significant decrease of TAC with the time passing (Fig. 3C). Correlation coefficient analysis between antioxidant and oxidative stress parameters showed a significant negative relationship between TAC and PCC in tissues of mussels exposed for 12 days either in Zn or to Cd (r = -0.65 and -0.90 respectively). Regarding organic-exposed mussels, significant negative correlations between TAC and PCC were obtained both at 6 days (r = -0.88 either in PAHs- or in Lindane- exposed mussels) and 12 days of exposure (r = -0.88 for PAHs- and r = -0.71 for Lindane-exposed mussels respectively).

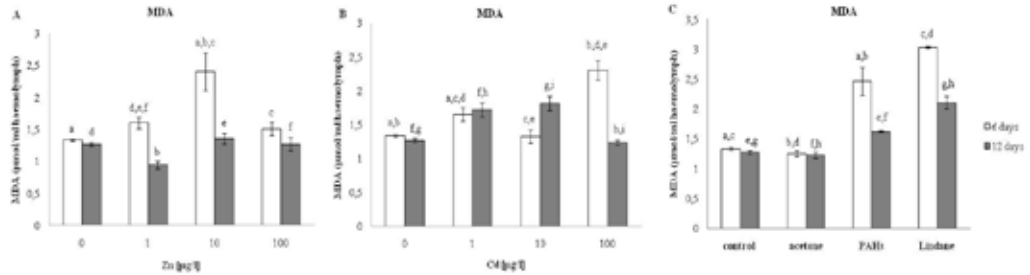

Fig. 2: *Malondialdehyde (MDA) content in haemocytes of mussels exposed to either zinc, or cadmium, or organic contaminants. Values are means ± standard deviations of 3 different measurements. In each measurement, haemolymph from 3 animals was used. Values that share the same letter are statistically significant different from each other.*

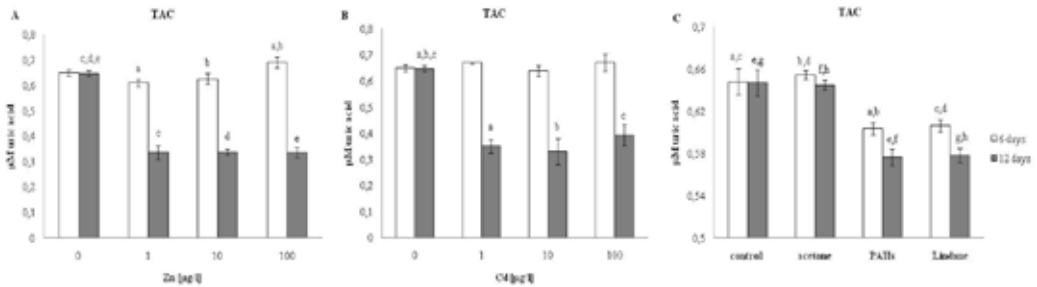

Fig. 3: *Total antioxidant capacity (TAC) in haemocytes of mussels exposed to either zinc, or cadmium, or organic contaminants. Values are means ± standard deviations of 3 different measurements. In each measurement, haemolymph from 3 animals was used. Values that share the same letter are statistically significant different from each other.*

4. Discussion

Elevated levels of PCC in tissue of mussels exposed to heavy metals, in relation to oxidative damage, could be due to their capability to induce directly the formation of protein carbonyls via metal catalyzed oxidation reactions (Stadtman & Oliver, 1991; Dailianis *et al.*, 2005; Kaloyianni *et al.*, 2005) and oxidative damage to proteins and lipids (Davies and Delsignore, 1987). The fact that PAHs metabolism in mussels occurs by radical oxidation, possibly involved oxygen radicals (Winston *et al.*, 1988) could give rise to the suggestion that organic contaminants could probably enhance ROS production in tissues of mussels, leading to protein carbonylation.

Different MDA alterations obtained, in relation both in exposure time and to concentrations of each contaminant tested, probably reflect the short half life of MDA in tissues (Siems *et al.*, 1995). PAHs may be liable to oxidative attack by radicals being present in the lysosomes of the haemocytes, producing derivatives such as anthraquinones, which then undergo redox cycling. Lipid peroxidation in haemocytes of mussels may be a consequence of elevated ROS production, which could be attributed to disturbance of membrane integrity. This is confirmed by the increase levels of MDA content, as well as the decrease levels of antioxidant resistance measured in the present study and demonstrated by others (Winston *et al.*, 1996; Nesto *et al.*, 2007).

The significant decrease of TAC value, either in heavy metal- or to organic- exposed mussels indicates that antioxidant defenses were depleted probably to oxidative stress occurred after exposure of mussels to high doses of contaminants. Antioxidant capacity seemed to be time-dependent, supporting the argument that antioxidants can transiently respond to various pollutants (Livingstone *et al.*, 1993; Doyotte *et al.*, 1997). Statistical analysis showed that oxidative damage, expressed at the level of PCC induction, was negatively correlated with the depletion of antioxidant defense in haemolymph of exposed mussels. According to these findings, we could hypothesize that mussels exposed to both inorganic and organic contaminants, at least under the present experimental conditions, possesses increased susceptibility to oxidative stress. The extent to which oxidative stress produces PCC might dependent on the effectiveness of antioxidant defenses, an argument which is in accordance with other studies (Diguiseppi & Fridovich, 1984; Michiels & Remacle, 1988).

The results of the present study reinforce the use of TAC assay for the detection of total antioxidant capacity in haemocytes of mussels as well as the use of an accurate ELISA method as a sensitive tool for the determination of PCC alterations in haemolymph of mussels exposed to different levels of contaminants. The significant increase of PCC in haemolymph of mussels exposed to pollutants, give rise to the suggestion that protein carbonylation could play an important role as a pro-survival signal, during oxidative stress. Significant relations between antioxidant assay and oxidative stress parameters encourage further investigation of the current methods, both in field and laboratory studies, in order to evaluate their use as tools in environmental biomonitoring.

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