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Antioxidant responses in different body regions of the polychaeta *Laeonereis acuta* (Nereididae) exposed to copper

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ABSTRACT

Antioxidant enzymes, total antioxidant capacity (TOSC) and concentration of reactive oxygen species (ROS) were measured in anterior (A), middle (M) and posterior (P) body regions of *Laeonereis acuta* after copper (Cu; 62.5 μg/l) exposure. A catalase (CAT) activity gradient observed in control group (lowest in A, highest in P) was not observed in Cu exposed group. Glutathione-*S*-transferase (GST) activity in A region of Cu group was higher than in A region of the control group. DNA damage (comet assay) was augmented in the A region of Cu group. Since copper accumulation was similar in the different body regions, sensitivity to copper in A regions seems to be related to lowest CAT activity. In sum, copper exposure lowered TOSC, a result that at least in part can be related to lowering of antioxidant enzymes like CAT. DNA damage was induced in the anterior region, where a lower CAT activity was observed.

1. Introduction

The nereidid polychaeta *Laeonereis acuta* is an epifaunal animal broadly distributed in shallow waters of estuaries, and previous studies have characterized this organism as a selective deposit feeder (Bemvenuti, 1998). The Nereididae family is described as anoxic and hypoxic tolerant, and several responses of the antioxidant defense system (ADS) have been described in this group (Abele-Oeschger et al., 1994; Abele-Oeschger and Oeschger, 1995; Abele et al., 1998; Rosa et al., 2005).

Copper pollution in the aquatic environment results from natural and anthropogenic sources such as mine washing or agricultural leaching. Although copper is a trace element essential to life it is also one of the most toxic heavy metals (Tóth et al., 1996). One of the main toxic mechanisms of this metal is due to oxidative stress generation through Fenton and Haber–Weiss reaction (Furuno et al., 1996). Pourahmad and O'Brien (2000) showed that ROS formation, glutathione oxidation and lipid peroxidation were induced in hepatocytes exposed to copper. Lloyd and Phillips (1999) observed the formation of double-strand breaks, 8-hydroxydeoxyguanosine and putative intra-strand cross-links mediated by copper, iron and nickel in salmon sperm DNA. In the freshwater olygochaeta *Tubifex tubifex*, higher concentrations of copper and lead were revealed in its posterior region than in its anterior region when the organisms were collected in a polluted site (Lucan-Bouché et al., 1999). In another study, the same species was exposed to different copper concentrations, and higher catalase (CAT) activity and reduced glutathione-*S*-transferase (GST) activity were observed (Mosleh et al., 2006). Morgan et al. (1989) verified that *Dendrodrilus rubidus* and *Lumbricus rubellus* sampled at a soil contaminated with cadmium showed major accumulation of this metal within the posterior alimentary canal.

The estuarine worm *L. acuta* (Nereididae) was formerly used as a biological model in copper toxicity assays and environment monitoring studies (Geracitano et al., 2002, 2004a, b), mainly taking into account its antioxidants responses. Furthermore, a gradient of antioxidant enzymes activity along the body of this worm was verified (Rosa et al., 2005), exhibiting higher CAT and superoxide dismutase (SOD) activities in the posterior region and higher GST activity in the anterior region. This type of gradient also has been demonstrated in the capitellid worm *Heteromastus filiformis*, with a correlation between an external gradient of PO_2 and pH and an internal gradient of ADS (Abele et al., 1998). Taking into consideration the existence of an antioxidant gradient along the body of *L. acuta* and also the previous reports showing differential metal accumulation (copper and lead) in different

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body regions of annelids (Lucan-Bouché et al., 1999), the aim of this study was to analyze copper accumulation and antioxidant and oxidative damage responses in anterior, middle and posterior body regions of *L. acuta* after experimental exposure to copper.

2. Material and methods

2.1. Collected animals and maintenance

Specimens of the polychaeta *L. acuta* weighting between 60 and 120 mg were collected in a salt marsh in Saco do Justino of Patos Lagoon (32°05′S–052°12′W; RS, Brazil) during the winters of the years 2003, 2004 and in the 3 years 2005–7. The worms were transferred to the laboratory in an ice-cold container in order to reduce their metabolism. Once in laboratory, worms were maintained under 12:12 h light:dark cycle, saline water 10‰, pH 8.0 and 20 °C for all assays. For the chronic assays the protocol of acclimation (10 days) depicted by Geracitano et al. (2002) was followed. For the *in vitro* assays the acclimation was of 2 days without feeding, in order to clean the intestines of the employed organisms.

2.2. Chronic assays

In the chronic assays (14 days) the worms were divided into two experimental groups: one was the control group (Ctr) and the other group was exposed to nominal copper concentration of 62.5 μg/l ($CuSO_4 \cdot H_2O$ from Merck, Rio de Janeiro, Brazil). This concentration was selected taking into account that one of the more sensitive parameters to copper exposure in oligochaetas is cocoon formation, which is inhibited at concentrations of copper about 50–60 μg/l (International Programme on Chemical Safety, 1993). Worms were placed in glass dishes (6.0 cm diameter), filled with 100 ml of saline water (with or without copper). Animals in the chronic assays were fed *ad libitum* with frozen *Artemia salina* every 2 days and the water was then 100% renewed.

After the end of the exposure, the organisms were sacrificed, dissected and subdivided in three regions: anterior region (A, first 20 settiger segments), middle region (M, next 20 settiger segments) and posterior region (P, the rest of the body) (Rosa et al., 2005). Pools of A, M or P sections were formed to make one sample. Because of low tissue quantity in A region, few samples were analyzed in this region ($n = 3$–6). In the other body regions, number of samples analyzed varied between 5 and 10. The number of worms employed for each treatment varied between 30 (control) and 40 (copper).

For copper analysis samples of each body region were dried at 60 °C until constant weight. Total content of copper in each region was evaluated using an atomic absorption spectrometer with a graphite furnace (ZEEnit 60). One milliliter of nitric acid 65% was added to samples weighing more than 0.03 g (0.5 ml of nitric acid was added to samples weighing less than 0.03 g). After 24 h in this solution, samples were heated (60 °C) for 2 h. NIST 2976 was the certified material used for method validation.

For the enzymatic assays, pools of each region were homogenized (1:3 w/v) in ice-cold buffer with pH adjusted to 7.60 (20 mM Tris-base, 1 mM EDTA, 1 mM DL-dithiothreitol, 500 mM sucrose and 150 mM KCl). Homogenates were centrifuged at 9000*g* for 45 min (4 °C) and the supernatants were collected and stored at −80 °C and employed later to determine total protein content, and CAT and GST activities (Geracitano et al., 2002). All the determination assays were performed at least in duplicate. The total protein content in the supernatant of homogenate extracts was determined at 550 nm using a commercial diagnostic kit (Doles reagents, Brazil) based on the Biuret method. Number of samples varied between 3 and 6 for each treatment and body region. As previously stated, few samples from anterior body region were analyzed in virtue of tissue availability.

The activity of the enzyme CAT was quantified by the consumption of 10 mM of H_2O_2 at 240 nm according to Beutler (1975). The activity of the enzyme was expressed in CAT units/mg of proteins, where one unit (U) is the amount of enzyme that hydrolyzes 1 μmol of H_2O_2 per minute and per mg of proteins at 30 °C and pH 8.0. The activity of the enzyme GST was measured by following the conjugation of 1 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene at 340 nm as described by Habig et al. (1974) and Habig and Jakoby (1981). The activity of the enzyme was expressed in GST units/mg of proteins, where one unit (U) is the amount of enzyme that conjugates 1 μmol of CDNB per minute and per milligram of protein at 25 °C and pH 7.0.

For total oxyradical scavenging capacity (total antioxidant capacity (TOSC) assay) pools of each body region from sampled worms were homogenized (1:4, w/v) in ice-cold phosphate buffer 50 mM, pH 7.50, plus NaCl (2.5%, w/v). Homogenates were centrifuged at 13,500*g* for 25 min (4 °C) and aliquots from the supernatant were collected for a second centrifugation at 33,000*g* during 60 min (4 °C) (Geracitano et al., 2004a). The supernatant of this last centrifugation was stored at −80 °C for TOSC measurements. Additional assays were conducted with the diet given to worms, *Artemia salina*, in order to analyze the antioxidant influence of the diet offered to the worms. Frozen *A. salina* was homogenized exactly as worm samples, and their antioxidant capacity determined. The total protein content in the supernatant was measured in triplicate as described above. TOSC was determined according to Winston et al. (1998) and Regoli and Winston (1999), using alpha-keto-γ-methiolbutyric acid (KMBA; 0.2 mM; Sigma, USA) as substrate. KMBA reacts with reactive oxygen species (ROS) producing ethylene. TOSC values reflect the sample ability to inhibit ethylene formation, through ROS scavenging. Two different kinds of ROS were generated *in vitro*. Peroxyl radicals were produced by thermal (35 °C) decomposition of 2,2′-azobis 2 methylpropionamidine dihydrochloride (ABAP; 20 mM; Aldrich) dissolved in potassium phosphate buffer 100 mM, pH 7.4. Hydroxyl radicals were produced using a Fenton system containing Fe^{3+} (2.7 μM)/EDTA (5.4 μM), and ascorbic acid (270 μM). Ethylene gas produced was measured using a Shimadzu GC-17A gas chromatograph equipped with an Agilent Technologies (USA) DB-5 capillary column (30 m × 0.25 mm × 0.25 μm) and a flame ionization detector (FID). TOSC values were calculated according to Winston et al. (1998) and referred to the total protein content in the homogenates. Number of samples analyzed varied between 6 and 10, for each body region and treatment.

The xylenol orange assay for lipid hydroperoxides (LPO) was performed as described by Monserrat et al. (2003). Frozen tissues of each region were homogenized in methanol (1:9 w/v) and centrifuged at 1000*g* for 5 min. LPO were determined using $FeSO_4$ (1 mM), H_2SO_4 (0.25 M) and xylenol orange (1 mM) added in this order. The assay mixture was measured at 580 nm in a microplate reader after 1 h of incubation at room tempeture. LPO values were expressed in terms of cumene hydroperoxide (CHP) equivalents, used as standard (1.75 nmol/ml).

The comet assay was executed according to Singh et al. (1988) and Steinert et al. (1998), with some modifications. Microscope slides were fully frosted and covered with 1% normal melting point agarose diluted in a solution with 0.04 Tris–acetate and 1 mM EDTA, cleaning the rear side with tissue and then drying. Each region (A, M and P, $n = 5$) of the two experimental groups was immediately homogenized in Petri dishes at the end of the 14-day experiment. The homogenates were filtered (pore: 145 μM) for cell separation. The cellular preparation was diluted (1:25 v/v) in Kenny's salt solution (400 mM NaCl, 9 mM KCl, 0.7 mM K_2HPO_4, 2 mM $NAHCO_3$, pH 7.50). After a second dilution (1:7.5 v/v) in 0.65% low-melting point agarose (diluted in Kenny's salt solution) the cellular preparations were added to the prepared frosted slide and roofed with a cover slip. Then, the slides were submitted to lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, 10% dimethyl sulfoxide, 1% Triton X-100, and 1% sodium sarcosyl) and kept at 4 °C overnight. For unwinding DNA strands, slides were transferred into chambers filled with electrophoresis and unwinding buffer (10 M NaOH, and 200 mM EDTA, pH 13.00) for 15 min. Eelectrophoresis was carried out for 20 min at 25 V and 280 mA. Slides were washed with 400 mM Tris (pH 7.50) and stained with 80 μl of ethidium bromide. DNA migration was visually determined in 100 cells per slide randomly selected in an epifluorescence Zeiss-Axioplan microscope (400 magnification). Comets were classified into five different groups: 0 for intact nucleoids; 1, 2 and 3 for intermediary damage, and 4 for maximum damage. Results were expressed as scores, 0 represents absence of damage and 400 indicates the highest damage registered in the 100 nucleoids analyzed.. Four or five slides of each body region and treatment was analyzed.

DNA–protein cross-link (DNAPC) levels were determined using K^+/SDS precipitation assay, following the method of Costa et al. (1996). Worm tissues were lysed in 500 μl of 0.5% SDS, 1 mM PMSF, 20 mM Tris–HCl, pH 7.5. The DNAPC fraction was digested with 200 μg of proteinase K (15 units/mg protein) for 3 h at 50 °C. The samples were dyed using SyberGold (Fluka) fluorescent dye. The amount of DNA in the samples was determined using the fluorometer (Victor 2, Perkin-Elmer), with excitation and emission wavelengths 485 and 535 nm, respectively.

2.3. In vitro assays

In the first *in vitro* assay the worms were divided into three experimental groups: control group (Ctra); 250 μg of copper/l (C1a), and 1.6 mg of copper/l (C2a). Copper concentrations were selected taking into account copper accumulation observed in the chronic assay (C2a) and a concentration previously assayed (C1a) in an acute experiment by Geracitano et al. (2002). In the second *in vitro* assay the worms were divided into three experimental groups: control group (Ctrb), 10 μM of H_2O_2 (C1b), and 50 μM of H_2O_2 (C2b), taking into account the concentrations assayed by Rosa et al. (2005) in *in vivo* experiments aimed to evaluate hydrogen peroxide effects on different body regions of *L. acuta*. In both assays the organisms were also dissected and subdivided in different regions A, M and P as in the chronic assays.

Measurements of ROS were conducted to according to Ferreira-Cravo et al. (2007). Homogenates were obtained from the regions A, M and P of each experimental group of worms (1:4 w/v) in ice-cold buffer (320 mM sucrose, 20 mM HEPES, 1 mM $MgCl_2$, pH 7.40). Homogenates were centrifuged at 20,000*g* for 20 min (4 °C). The supernatants were obtained (166 μg of total proteins) and incubated at 25 °C during 30 min with 30 mM HEPES (pH 7.2), 200 mM KCl, 1 mM $MgCl_2$ and 16 μM of 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA; Molecular Probes). The acetates groups of H_2DCF-DA are cleaved by intracellular esterases. After that, the non-fluorescent compound H_2DCF is oxidized by ROS to the fluorescent compound, DCF. The fluorescence intensity was determined, using a

fluorometer (Victor 2, Perkin-Elmer), with excitation and emission wavelengths 485 and 520 nm, respectively. Total fluorescence production was calculated by integrating the fluorescence units over the measurement time and expressed as area of fluorescence. Data were corrected to the background fluorescence and was expressed as a relative value: total fluorescence area in H_2O_2 or copper exposed/total fluorescence area in control group. These values were calculated for each body region (A, M and P).

2.4. Statistical analysis

Values in all determinations were computed as means ± SEM. Statistical analysis was executed through analysis of variance followed by Newman-Keuls *post-hoc* comparisons ($\alpha = 0.05$) for all determinations (Zar, 1984).

3. Results

3.1. Chronic assays

All the regions of Cu group (Fig. 1) had the same ($p > 0.05$) quantity of accumulated copper (mean: 62.15 µg of Cu/g of dry tissue) at the end of the 14th day of experiment, and this quantity was significantly different ($p < 0.05$) from the same regions of Ctr group (mean: 4.7 µg of Cu/g of dry tissue).

CAT activity (Fig. 2a) was different ($p < 0.05$) in different body regions of worms from control group, lowest in the anterior region A, intermediate in the middle region M, and highest in the posterior region P. No differences were observed ($p > 0.05$) in CAT activity in the distinct body regions of the exposed group Cu. GST activity (Fig. 2b) was higher ($p < 0.05$) in the A region, both in control and Cu group in respect of the M regions and P regions. Also GST activity in the A region of Cu group was higher ($p < 0.05$) than GST activity in the same body region of control group.

TOSC showed no differences ($p > 0.05$) against hydroxyl radicals (Table 1) between control and Cu groups and between the body regions. TOSC values against peroxyl radicals were higher ($p < 0.05$) in control group with respect to Cu group (Table 1); but no differences ($p > 0.05$) among the distinct body regions were observed. TOSC values for peroxyl radicals were higher ($p < 0.05$) than TOSC values for hydroxyl radicals comparing each region for both experimental groups (Table 1).

Considering damage variables, it was observed that LPO content (Fig. 3) was lower ($p < 0.05$) in the P region in both control and Cu groups. No influence ($p > 0.05$) of copper treatment was verified for this variable. In terms of DNA damage, the comet

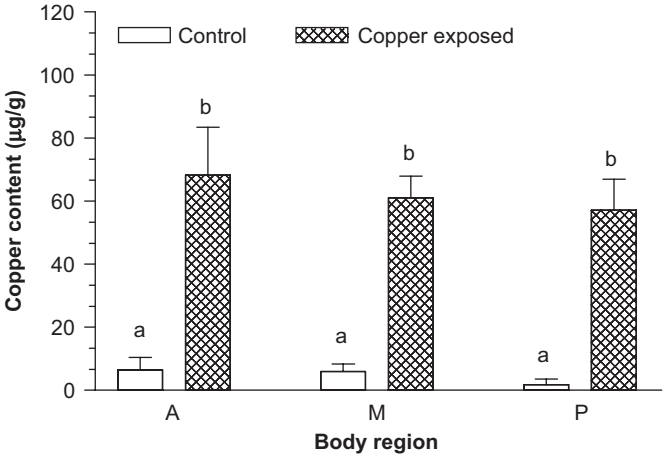

Fig. 1. Total content of accumulated copper in the different body regions of the worm *Laeonereis acuta* (Nereididae) at the end of the 14th day of the experiment. Data are expressed as means ± 1 standard error ($n = 5$). Identical letters indicate absence of statistical differences ($p > 0.05$). A: anterior, M: middle, and P: posterior.

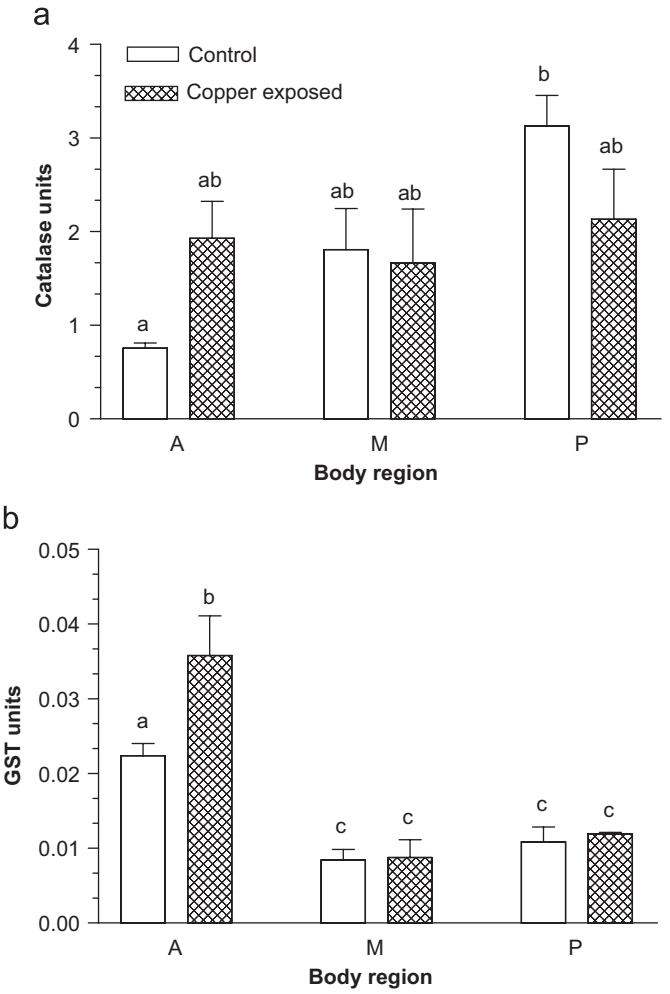

Fig. 2. (a) Catalase (CAT) and (b) glutathione-*S*-transferase (GST) activities in different body regions of the worm *Laeonereis acuta* (Nereididae). Data are expressed as means ± 1 standard error ($n = 3$–6). Identical letters indicate absence of statistical differences ($p > 0.05$). A: anterior, M: middle, and P: posterior. Enzymatic activities are expressed in units (U) as defined in Material and methods section.

Table 1
Total antioxidant capacity determined by TOSC assay in different body regions of *Laeonereis acuta* employing peroxyl (ABAP) and hydroxyl (Fenton) radical generators

Body region	Control	Copper exposed
Radical generator: ABAP		
Anterior	$517 \pm 88^{a,\dagger}$	$406 \pm 34^{a,\dagger}$
Middle	$427 \pm 44^{a,\dagger}$	$372 \pm 32^{a,\dagger}$
Posterior	$495 \pm 69^{a,\dagger}$	$386 \pm 28^{a,\dagger}$
Whole body	$478 \pm 39^{*,\dagger}$	$387 \pm 18^{\dagger}$
Radical generator: Fenton		
Anterior	204 ± 24^{a}	200 ± 18^{a}
Middle	198 ± 31^{a}	179 ± 14^{a}
Posterior	193 ± 25^{a}	210 ± 28^{a}
Whole body	199 ± 14^{a}	195 ± 10^{a}

Data (TOSC units/mg of proteins) are expressed as means ± 1 standard error ($n = 6$–10). Identical letters indicate absence of statistical differences ($p > 0.05$) between body regions. * Indicates statistical difference ($p < 0.05$) between control and copper exposed groups. † Indicates statistical difference ($p < 0.05$) between radical generator (ABAP and Fenton) inside each treatment (control and copper exposed) and body region.

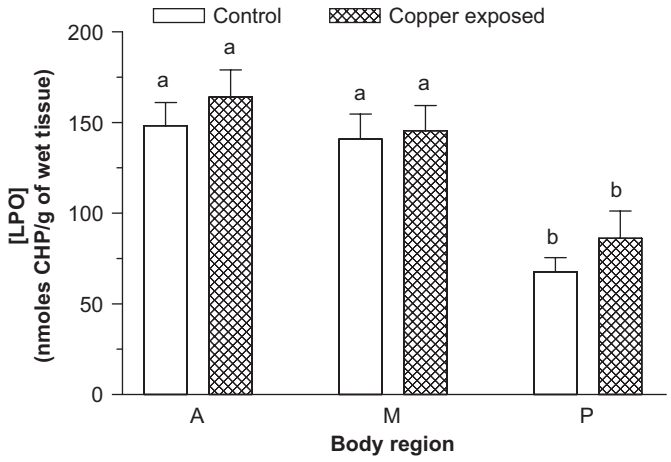

Fig. 3. Lipid peroxides (LPO) content in the different body regions of the worm *Laeonereis acuta* (Nereididae). Data are expressed as means ± 1 standard error ($n = 5$). Identical letters indicate absence of statistical differences ($p > 0.05$). A: anterior, M: middle, and P: posterior. CHP stands for cumene hydroperoxide, the standard employed for lipid hydroperoxides measurements.

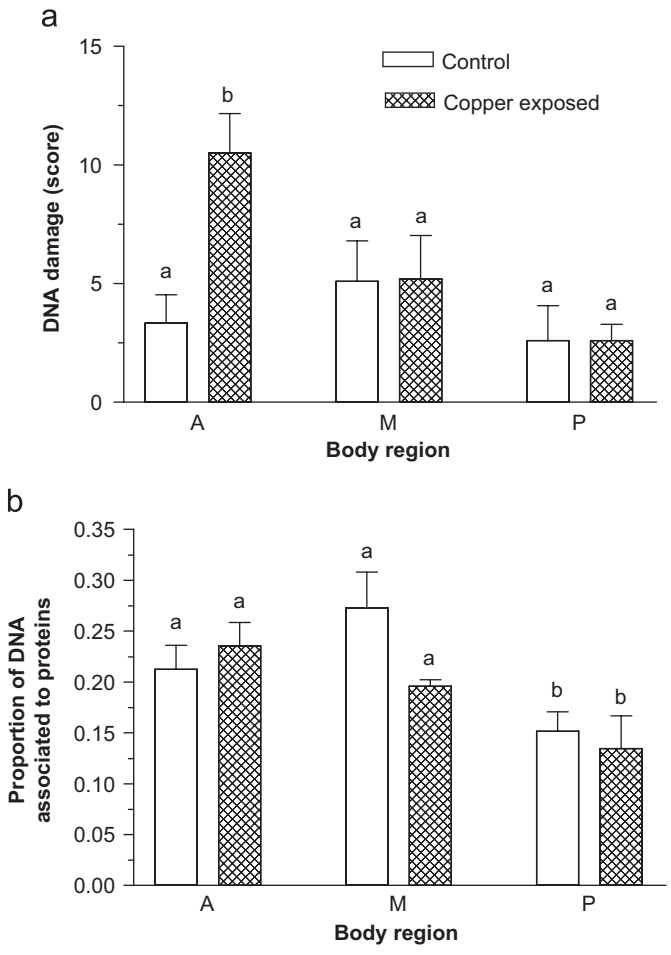

Fig. 4. DNA damage in different body regions of the worm *Laeonereis acuta* (Nereididae) measured through (a) comet assay, and (b) DNA–protein crosslink. Data are expressed as means ± 1 standard error ($n = 4$–5). Identical letters indicate absence of statistical differences ($p > 0.05$). A: anterior, M: middle and P: posterior.

assay (Fig. 4a) pointed higher score ($p < 0.05$) in the A region of Cu group in respect of the others regions and treatments. Although no effect of copper treatment was registered in terms of DNAPC

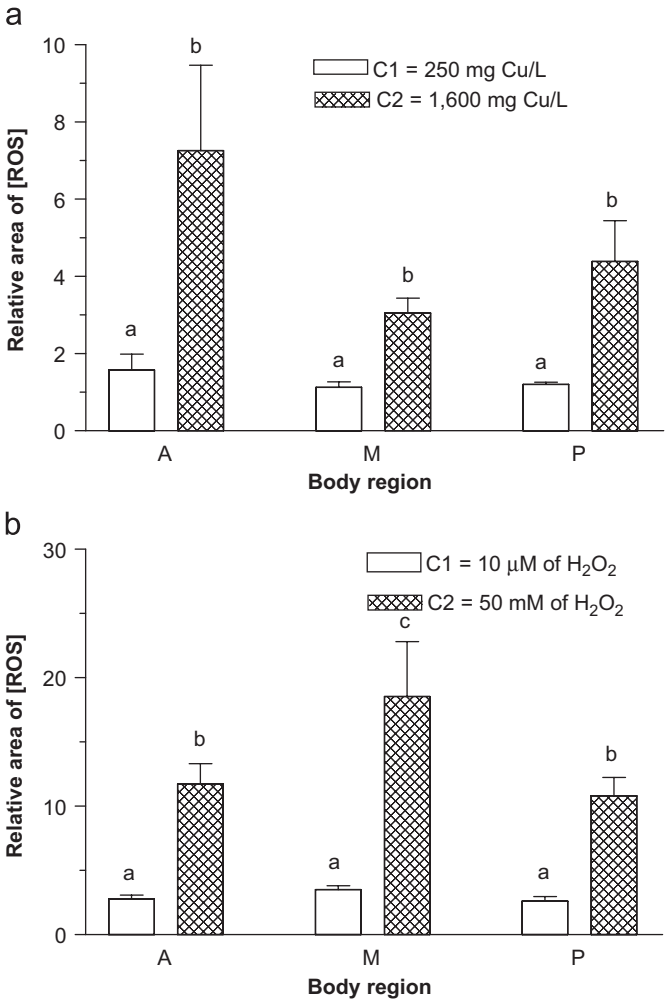

Fig. 5. Relative areas of fluorescence production in different body regions of the worm *Laeonereis acuta* (Nereididae). Data are expressed as means ± 1 standard error ($n = 6$). Identical letters indicate absence of statistical differences ($p > 0.05$). A: anterior, M: middle and P: posterior. In (a), C1 = 250 µg of Cu/l and C2 = 1.6 mg of Cu/l. In (b) C1 = 10 µM of H_2O_2 and C2 = 50 µM of H_2O_2.

($p > 0.05$), lower proportion of DNA was linked to proteins in the posterior region of the worms ($p < 0.05$; Fig. 4b).

3.2. *In vitro assays*

In the first experiment (Fig. 5a), the relative fluorescence area was significantly higher ($p < 0.05$) in extracts of anterior, middle and posterior region of the group C2 (1.6 mg/l of Cu) compared with the same regions of the group C1 (250 µg/l of Cu). In the second experiment (Fig. 5b), the relative fluorescence area was significantly higher ($p < 0.05$) in extracts of anterior, middle and posterior region of the group C2 (50 µM of H_2O_2) in respect of the samples of the group C1 (10 µM of H_2O_2). Even more, the middle region of C2 had the highest ($p < 0.05$) relative fluorescence area.

4. Discussion

Copper accumulation in *L. acuta* varied from 57 to almost 68 µg/g after 14 d exposure to a nominal concentration of 62.5 µg Cu/l, values that are little higher than that reported for the polychaete *Hediste japonica* after 6 d exposure to 100 µg Cu/l (20.19 µg Cu/g) and 150 µg Cu/l (22.41 µg Cu/g) (Sun and Zhou, 2007).

The lack of differences of copper content in the different body regions (Fig. 1) indicates that *L. acuta* possess a different strategy compared to that of *T. tubifex* (high capacity of copper accumulation in the posterior region; Lucan-Bouché et al., 1999) in managing copper pollution. Alternatively, an eventual difference in Cu compartmentalization along worm body should appear after a preliminary adaptation of the antioxidant system or after a more prolonged exposure period (or higher Cu concentrations). Also note the lack of difference in ROS levels in different body regions after *in vitro* copper exposure (Fig. 5a). It seems that the strategy is to maintain low levels of precursor of hydroxyl radicals like hydrogen peroxide (Fig. 2a) or anion superoxide radical (Rosa et al., 2005). In this context, it is interesting to note that the regions of Cu group lost the CAT gradient activity exhibited in the regions of control group, showing evidence of DNA damage. Higher CAT and SOD activities and thinner cuticle in the posterior region of *L. acuta* are considered to favor detoxification and diffusion of environmental pro-oxidants like H_2O_2 and O_2 (Rosa et al., 2005). Besides these strategies, another mechanism of detoxification observed in marine invertebrates is the formation of insoluble precipitates such as granules (Viarengo and Nott, 1993), and this is suggested as one of the mechanisms involved in cadmium accumulation in *L. acuta* (Sandrini et al., 2006).

The activity of GST in the A region of Cu group was the highest and it is known that GSTs constitute a large family of multifunctional enzymes involved in GSH conjugation to xenobiotics, fatty acids hydroperoxides and aldehydic products of lipid peroxidation (Hermes-Lima, 2004). This higher GST activity in A region of Cu group possibly explains the lack of differences in LPO content between control and Cu groups for this region. Also, as previously indicated by Rosa et al. (2005), higher GST in the A region indicates a different strategy to cope with oxidative stress, because of its repairing instead of preventive nature.

The lower TOSC values were obtained for hydroxyl radicals indicating that these reactive species is the most difficult to be neutralized by cellular antioxidants (Table 1). According to Regoli and Winston (1999) and Regoli (2000) low-molecular-weight scavengers (GSH, ascorbic acid, uric acid, vitamin E) generally accounted for 70% of the total scavenging capacity towards peroxyl radicals but their contribution was only 50% towards hydroxyl radicals. In this respect, it has been hypothesized that organisms can better prevent toxicity of hydroxyl radicals by reducing their cellular formation, and a fundamental role has been postulated for CAT (30%) in inhibiting the Fenton reaction (Regoli et al., 2000). The loss of CAT gradient activity in the Cu group seems to be problematic in this context, but the fact that no differences were observed between TOSC values against hydroxyl radicals in control and Cu groups, indicates that other antioxidants are important to cope with the oxidative stress generated by copper. However, the lower TOSC values for peroxyl radicals in the Cu group compared with control group showed that some antioxidants have been depleted after 14 days of exposure to copper. In this view, Regoli and Winston (1998) demonstrated that GSH, ascorbic acid, uric acid accounted for 35% of the measured TOSC values against peroxyl radicals.

Non-enzymatic compounds are more important to antioxidant defenses than enzymatic compounds in *L. acuta* exposed to copper chronically, as no difference in antioxidant competence against hydroxyl radicals was observed in spite of the loss of CAT activity gradient registered in worms exposed to copper. However, the reduction of TOSC values for peroxyl radicals in Cu group may reflect a high consumption of antioxidants in order to cope with oxidative stress. The lower LPO values for posterior regions may be due to higher CAT and SOD activity as observed in previous (Rosa et al., 2005) and present studies. Note that the posterior region presents interesting and complex responses in terms of oxidative stress: field animals possess higher antioxidant competence and ROS concentration and this pro-oxidant condition is also reflected in the lower values of sulfhydril groups associated to proteins, as observed by Ferreira-Cravo et al. (2007). The fact that this region also posses lower proportion of DNAPC indicates differential effects of oxidative stress in the posterior region of *L. acuta*.

When the obtained values of TOSC are compared with those of a previous study of Ferreira-Cravo et al. (2007), some differences are observed (Table 2) in the sense that these authors observed an antioxidant gradient of antioxidant competence against peroxyl and hydroxyl radicals, being lowest in A region and highest in P region. This gradient was not observed in the present study. Several factors can be considered, including:

(1) In the study of Ferreira-Cravo et al. (2007) the organisms were sampled and immediately sacrificed whereas in the present work, the worms spent more than 3 weeks on laboratory conditions.
(2) During maintenance in laboratory worms were fed with *Artemia salina*, and it was considered that this diet could be influencing the antioxidant capacity. A previous study of Regoli et al. (2002) mentioned altered TOSC values in the scallop *Adamussium colbecki* and the authors related that to a higher algal food supply that could alter levels of carotenoids and α-tocopherol.

Because of point (2), antioxidant competence against peroxyl radicals was determined in *Artemia salina*, and a value of 495.86 ± 34.89 TOSC units/mg of proteins ($n = 6$) was registered. Looking at results depicted in Table 2, it can be concluded that the diet is not the influencing factor that abolishes the antioxidant gradient in terms of TOSC in *L. acuta*, since TOSC values of *A. salina* samples are in the range (not much lower, not much higher) of antioxidant competence observed in the different body regions of *L. acuta*.

The DNA damage in the A region of Cu group had statistical significance ($p < 0.05$), although with a low score. However, it is important to note that this higher damage occurs in the anterior region, where low CAT activity was verified and previous studies have also shown low activity of SOD (Rosa et al., 2005). These two enzymes are important to degrade the precursors (hydrogen peroxide and superoxide anion) that generate hydroxyl radicals in presence of metals like copper. Note that this effect, as mentioned above, cannot be ascribed to different copper-binding capacity between the different body regions, as no differences in the accumulated copper was observed along the body of *L. acuta* (Fig. 1). In this way, the metal accumulation strategy of *L. acuta* is different from that of *T. tubifex*, where an increasing capacity for metal accumulation was observed from the anterior to posterior region (Lucan-Bouché et al., 1999).

Table 2
Total antioxidant capacity determined by TOSC assay in different body regions of *Laeonereis acuta* employing against peroxyl radicals in organisms sampled in field, at a reference non-polluted site (Ferreira-Cravo et al., 2007) and in worms maintained in laboratory (this study)

Body region	Worms sampled in field	Worms under laboratory conditions
Anterior	$300 \pm 35^*$	517 ± 84
Middle	$633 \pm 47^*$	427 ± 44
Posterior	648 ± 111	495 ± 69

Antioxidant competence against peroxyl radicals was also analyzed in the diet offered to *L. acuta* in laboratory, *Artemia salina*. Data (TOSC units/mg of proteins) are expressed as means ± 1 standard error ($n = 6$–10). * Indicates statistical difference ($p < 0.05$) between field and laboratory worms.

The *in vitro* assays showed that copper at the highest concentration induced higher ROS formation in all regions compared with the lowest concentration, but no differences between body regions were observed, indicating that no mechanism to intercept hydroxyl radicals generated by copper is triggered by *L. acuta*. Note that this result fits well with the fact that the copper-binding capacity was similar for all body regions (Fig. 1) and again suggests that the differential DNA damage observed in the anterior region (Fig. 4a) is more related to antioxidant strategy of each body region instead of its metal accumulation. The last point was observed by Lucan-Bouché et al. (1999) in *T. tubifex*. The *in vitro* results with hydrogen peroxide showed higher ROS content in the middle region, a result that fits with a previous report of Rosa et al. (2005), where higher LPO content was verified in this region after exposure to 50 μM of H_2O_2. Differences in CAT activity (higher in P and lower in M and A) can account for differences in ROS content observed in the *in vitro* assay, and should be important in the preventive strategy elicited by this worm against this pro-oxidant, whereas other non-enzymatic antioxidants could be important in the response against copper exposure.

5. Conclusions

The present study showed that toxicological responses after copper exposure vary according to the body region of the estuarine worm *L. acuta.* A higher DNA damage observed in the anterior region can be related to the lowest CAT activity, leading to a higher H_2O_2 concentration, a known precursor of hydroxyl radical in presence of copper. TOSC was lowered after copper exposure, indicating employment of antioxidant to cope with copper-induced ROS.

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