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Copper Induced Lysosomal Membrane Destabilisation in Haemolymph Cells of Mediterranean Green Crab (*Carcinus aestuarii*, Nardo, 1847) from the Narta Lagoon (Albania)

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ABSTRACT

Destabilisation of blood cell lysosomes in Mediterranean green crab Carcinus aestuarii *was investigated using Neutral Red Retention Assay (NRRA). Crabs collected in Narta Lagoon, Vlora (Albania) during May 2014 were exposed in the laboratory to sub-lethal, environmentally realistic concentrations of copper. Neutral Red Retention Time (NRRT) and glucose concentration in haemolymph of animals were measured. The mean NRRT showed a significant reduction for the animals of the treatment group compared to the control one (from 118.6 ± 28.4 to 36.4 ± 10.48 min, $p<0.05$), indicating damage of lysosomal membrane. Haemolymph glucose concentration was significantly higher in the treatment group (from 37.8 ± 2.7 to 137.8.4 ± 16.2 mg/dL, $p<0.05$) than in control group, demonstrating the presence of stress on the animals. These results showed that* C. aestuarii *could be used as a successful and reliable bioindicator for evaluating the exposure to contaminants in laboratory conditions. NRRA provides a successful tool for rapid assessment of heavy metal pollution effects on marine biota.*

Key words: *Carcinus aestuarii*, neutral red retention assay, lysosomal membrane destabilization, bioindicator, copper

INTRODUCTION

In recent years, environmental pollution of water by heavy metals has increased worldwide due to extensive use of heavy metals in agriculture, and chemical and industrial processes, posing so a serious threat to living organisms (Gupta et al. 2013; Gohil et al. 2013; Pandey and Madhuri 2014; Fazio et al. 2014; Ramos et al. 2014). Heavy metals are among the major contaminants in the aquatic environment. The uptake of metals is mainly achieved via the digestive tract by endocytosis; thereafter they are transferred to lysosomes and then to residual bodies, especially in the digestive cells (Marigomez et al. 2002). Investigating the mechanisms by which living organisms cope with the chemical toxicity is very important to understand the adaptation as well as the recovery responses of the animals to stress.

Many studies have been performed using invertebrate to assess the cellular responses to these contaminants (Matozzo et al. 2012; Torre et al. 2013; Ramos et al. 2014). Aquatic invertebrates are known to accumulate high levels of heavy metals in their tissues and yet survive in the polluted environments (Dallinger and Rainbow 1992; Chiarelli and Roccheri 2014). Often metals penetrate the cells via transport mechanisms normally used for other purposes and are irreversibly accumulated in the cells where they interact with the cellular components and molecular targets. Invertebrates occupy a key

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position as intermediate consumers in the pelagic and benthonic food chains of aquatic ecosystems. Then, aquatic organisms may represent excellent bioindicators of the marine water quality (Viarengo et al. 2000; Rainbow (2002); Chiarelli and Roccheri 2014). Many studies have been carried out to assess the occurrence of chemical pollutants in marine ecosystems using different bioindicator organisms for evaluating possible risks on human health (Nunes et al. 2008; Corsi et al. 2011; Fazio et al. 2013; Giacomini et al. 2014; Ramos et al. 2014; Messina et al. 2014). Several studies have been carried out using the Green crab *Carcinus maenas* as an experimental test organism. In general, *C. maenas* is sensitive to a wide range of aquatic pollutants and a reliable estuarine/marine model for routine testing in ecotoxicology research and environmental quality assessment, especially in what concerns the application of the bioindicator approach. However, there are very few studies using *in vivo* toxicity testing assays in *Carcinus aestuarii*, especially relating with its response to copper pollution (Ricciardi et al. 2009; Corsi et al. 2011).

Lysosomes are recognized as the target sites for most environmental contaminants, which can cause destabilization of the lysosomal membrane. The main lysosomal stress responses are changes in size, lysosomal content, rate of fusion events, lysosomal permeability and alterations in uptake and detoxification ability (Hawkins 1980). The Neutral Red Retention Time (NRRT) assay evaluates the lysosomal membrane integrity, which can be used as an indicator of exposure to xenobiotics (Moore 1990; Lowe et al. 1995; Cheung et al. 1998; Zaroni et al. 2001; Abessa et al. 2005; Matozzo et al. 2012). In this assay, cells are incubated with the vital dye Neutral Red (NR), which is taken up by the lysosomes. Healthy cells retain the dye for more time than damaged cells, in which the dye rapidly leaks out into the cytoplasm. In Albania, the use of biomarkers in environmental pollution evaluations has been introduced recently (Sadikaj et al. 2010; Aliko et al. 2011; Morina et al. 2012; Aliko et al. 2013; Morina et al. 2013). Few studies have performed such assays using micronuclei frequency, oxidative stress enzymes and haematological alterations as biomarkers of stress response in fish and amphibians (Morina et al. 2013; Aliko et al. 2013).

In this study, the NRRT assay was applied for the first time in the crab *C. aestuarii*, a common native species in the estuarine waters in Mediterranean Sea, including transitional waters such as lagoons. The aim of this study was to determine the acute toxic effects of copper chloride on the lysosomal membrane stability of *C. aestuarii* haemocytes under laboratory conditions with the aim to apply the assay in the future in biomonitoring of marine and estuarine ecosystem's health.

MATERIAL AND METHODS

Collection site

The Narta Lagoon (Fig.1) is one of the most important lagoons of Albania and one of the sites included in the national monitoring system (Miho 2011). This is situated in the northern part of the Vlora Bay, about 3 km from Vlora City (40°31'52"N 19°25'26"E). Narta Lagoon has a surface of 41.8 km^2, with a maximum depth of 1.5 m and the average depth of 0.7 m. About 1/3 of its surface is used for salt extraction. Narta Lagoon is divided from the Adriatic Sea by the low hills of Zverneci-Treporti and by a littoral cordon of about 8 km long and width of 100-1400 m. The Narta Lagoon is connected to the Adriatic Sea by two artificial channels, the South (110 m long, 18 m wide and 1.67 m deep) and the North Channel (650 m long, 20 m wide and 0.54 m deep) that realize water exchange process between the lagoon and the Adriatic Sea.

Figure 1 - Narta Lagoon and the location of the sampling site.

Thirty adult male specimens of crab *C. aestuarii* (Decapoda, Portunidae) were collected during May 2014 in Narta Lagoon, Vlora, Albania. The specimens were collected along the South tidal inlet channel connecting the lagoon with the sea.

All the animals were transferred quickly to the laboratory in the buckets containing seawater from the collection site and allowed to acclimate for 48-96 h to laboratory conditions before exposure to copper.

Test solutions

To prepare the primary stock copper solution, chloride hydrate ($CuCl_2$ x $2H_2O$; Sigma-Aldrich) was used. Exposure concentration of copper was chosen with reference to the published sublethal data on biomarker responses for *Carcinus* species (Brown et al. 2004), which was 70 µg/L as described previously (Lazo et al. 2004; Arapi et al. 2008).

Experimental design

In the laboratory, crabs were kept in 30 L aquaria filled with continuously aerated seawater (SNSW, Nutri-SeaWater®Aquarium Saltwater, pH: 8 ± 0.1; salinity: 36±1 ppt; temperature: 17 ± 1°C) for acclimatization for 1/2 week before the onset of any experimental procedure. Water was changed every two days. Animals were fed once a day with algal slurry (Liquifry marine, Interpet, Dorking, England). After acclimatization, crabs were divided in two groups: a control group (n=10) kept in artificial sea water and the experimental group (n=20) exposed to $CuCl_2$ added directly to the tank water at 70 µg/L for 24 h. When water was changed, copper concentration was re-established. Only adult male crabs were used in copper exposure. No mortality was registered during the exposure. For both the groups, neutral red retention time (NRRT) and glucose concentration in hemolymph were measured.

Hemolymph collection and glucose measurement

Hemolymph was withdrawn through the athrodial membrane at the base of the fourth moving leg of the crab into a 2.5 mL hypodermic syringe fitted with a 25 gauge needle and containing 0.5 mL of physiological Ringer solution for crustaceans: [20 mM (4.77g) Hepes, 436 mM (25.48g) NaCl, 53 mM (13.06g) MgSO4, 10 mM (0.75g) KCl, 10 mM (1.47g) CaCl2), pH 7.4]. The obtained solution was discarded into a 2.0 mL siliconised (Sigmacote) Eppendorph tube held in ice water. Animals were manipulated very carefully in order to avoid excessive stress. Measurement of the hemolymph glucose levels in the crabs was done using glucometer (One Touch-Ultra).

Neutral Red Retention Assay

Neutral Red Retention Assay was realized according to Standard Operative Procedure (SOP) proposed by Lowe et al. (1995), prescribed from Martinez-Gomez et al. (2008) and adopted for the specimen taken under the study. The physiological saline and neutral red stock solutions were prepared prior to the beginning of the assay. Initially, 0.5 mL haemolymph from each crab was collected with the syringes containing 0.5 mL of physiological saline solution and transferred to an Eppendorph tube. Immediately, 50 µL haemocyte cells solution was pipetted and dropped onto a glass slide. The slides were placed into a dark and humid chamber and incubated for 15 min. Then, the neutral red stock solution was prepared by the dilution of 20 mg Neutral Red dye in 1.0 mL dimetil sulphoxid (DMSO). Working solution was prepared by the dilution of 5.0 µL of stock solution in 995 µL physiological solutions. After incubation, 50 µL NR working solution was dropped onto each slide. At the end of 15 min, the slides were quickly examined by microscopy. The cells were observed for the structural abnormalities and for the retention time of the neutral red dye. The slides were observed every 15 min until 50 % of the haemocytes lost the dye in the cytosol. A mean of neutral red time and glucose concentrations were calculated for each group.

Statistical Analysis

The results of each parameter obtained for each experimental group were compared among the treatment groups using either the parametric analysis of variance (ANOVA), or the non-parametric analysis (Kruskal Wallis test) based on the data distribution (normality and homogeneity of variance). When an indication of a significant difference ($p<0.05$) was observed, differences were analyzed by the post-hoc Dunn's test. Simple linear correlation (Pearson test) conducted with the mean values, was used to establish the significant relationships between the biological responses.

RESULTS

There was no significant mortality of *C. aestuarii* in the Cu exposure used. Instead, a significant reduction in the retention time of neutral red dye in the haemocytes was observed at 70 µg Cu L^{-1}, compared with the control (One-Way ANOVA, $F=8.902$, $p<0.05$) (Fig. 2).

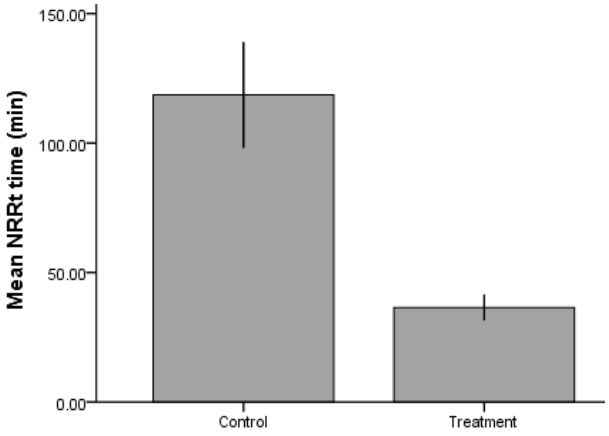

Figure 2 - NR retention times observed for each group of studied crabs, in control condition and in presence of 70µg/L of $CuCl_2$. The values are means ±SD. The difference between control and treatment is significant ($p< 0.05$).

The neutral retention assay indicated that whilst lysosomes of haemocytes from the control group had the capacity to retain the dye for 118.6 ± 28.4 min, those from the treated group lost the dye to the cytosol by 36.4 ± 10.48 min. Haemocytes tended to obtain a round spherical shape. A significant (One-Way ANOVA, $F=7.973$, $p< 0.05$) increase of plasmatic glucose level was observed in the crabs treated with $CuCl_2$ (from 37.8 ± 2.7 mg/dL to 137.8.4 ± 16.2 mg/dL) (Fig. 3).

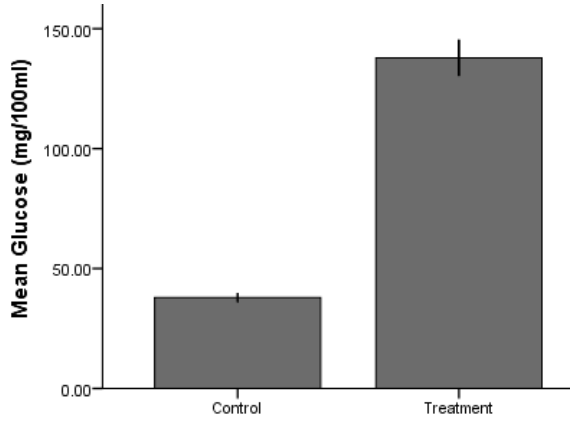

Figure 3 - Glucose levels in haemolymph observed for each group of studied crabs, in control condition and in presence of 70 µg/L of $CuCl_2$. The values are means ±SD. The difference between control and treatment is significant ($p< 0.05$).

The analysis for any possible correlation between the neutral retention time and plasmatic glucose levels indicated that no significant correlation between the NRRT and plasmatic glucose level ($r=0.452$, $p<0.05$) existed.

DISCUSSION

Evidently the membrane permeability was severely affected by copper ions in *C. aestuarii* as indicated by the reduction in NRR time in the crab haemocytes exposed to 70 µg Cu L^{-1}. It is well-known that one of the main modes of copper toxicity action is the reduction of the membrane permeability, and lysosomal membrane stability is particularly sensitive endpoint for demonstrating the effects of this metal in several invertebrate species (Svendsen and Weeks 1997; Ringwood et al. 1998; Viarengo et al. 2007; Aguirre-Martínez et al. 2013; Matozzo 2015). The NRR assay has been used previously in the field trials with *C. means* to demonstrate the differences between the polluted and non-polluted sites (Wedderburn et al. 1998; Astley et al. 1999). Lowe et al. (1992) postulated that the release of the neutral red dye into the cytosol following membrane damage could be due to impairment of lysosomal membrane proton pump. The internal acid environment of lysosomes is maintained by a membrane Mg^{2+}ATPase dependent H^+ ion proton pump (Ohkuma et al. 1982). Dysfunction of the pump would lead to a marked increase of the intralysosomal pH and, in the absence of any gradient, free passage of the lysosomal contents, including neutral red into the cytosol. Failure or dysfunction of the proton pump may be a direct consequence of the contaminant action, or alternatively the result of a reduction in ATP synthesis following contaminant damage to the mitochondria. Thus, damage of the lysosome membrane stability could be the beginning of apoptotic pathway (Nicholson 2003). The NRR assay has been used previously in the field trials with *C. maenas* to demonstrate the differences between the polluted and non-polluted sites (Wedderburn et al. 1998; Astley et al. 1999). In this study, baseline NRR times were similar to those reported by Wedderburn (1998) and the assay was sensitive enough to detect the cellular stress at 70 µg CuL^{-1}. In the individuals treated with $CuCl_2$, simultaneously with decreasing of NRR time, a significant increase of haemolymph glucose levels ($p<0.05$) was observed. Interestingly, there was not any significant correlation between the NRRT and blood glucose

level (r=0.452; P>0.05). This might be explained by the fact that hyperglycaemia was a common stress response of many aquatic animals including crabs, and it was not related specifically with copper exposure.

In all the species, stress appears to induce primarily a significant increase of glucose levels in blood, showing that glucose level is a general biomarker of stress response of the animals (Giacomin et al. 2014). Lorenzon et al. (2000) reported that heavy metals, such as Cd, Hg and Cu produced significant hyperglycaemic response in blood glucose levels of *P. elegans* exposed to sub lethal concentrations of these heavy metals. Cu contamination induced variation of serotonin (5-HT) of the eyestalk and haemolymph of *P. elegans* (Lorenzon et al. 2005). The release of 5-HT from the eyestalk into the haemolymph after Cu exposure precedes in its time course the release of crustacean hyperglycaemic hormone (cHH), confirming its role as neurotransmitter acting on cHH neuroendocrine cells. The rapid and massive release of 5-HT from the eyestalk of individual species following exposure to Cu might have induced release of the cHH resulting in hyperglycaemia in crabs.

The combination of NRR time alteration in case of copper toxicity, with increasing of blood glucose levels, can provide a "diagnosis of stress" for the organism by integrating overall physiological status with specific, molecular effects of copper ions on membrane permeability. Thus, haemolymph glucose level alteration cannot withstand alone as bioindicator of copper stress response, but it should be evaluated in combination with other more specific biomarkers, like lysosomal membrane stability in this study. Understanding how different biomarkers relate to each other on exposure to particular contaminants is a key to interpreting the effects of biomarkers in the field (Brown et al. 2004).

In conclusion, significant membrane destabilisation of *C. aestuarii* blood cells lysosomes in response to copper toxicity could be a very good biomarker of effects and it could be used successfully in field biomonitoring programmes for early detection of cellular damages. The lysosomal integrity is a cost-effective, simple, reproducible and biologically relevant biomarker suitable for use in routine environmental monitoring.

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