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REPORT

Hepatopancreatic cells of a stone crab *Menippe frontalis* from Perú: separation, viability study, and evaluation of lipoperoxidation against cadmium contamination

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Abstract Crustaceans are frequently used as bioindicators, and changes in their metabolism at the hepatopancreas (HP) level are often followed in these studies. The HP is the site of digestion, absorption, nutrient storage, and toxic metal detoxification, enabling crabs to survive in metal contaminated regions. Cellular damage and high lipid peroxidation (LPO) levels have been found in crab populations under high cadmium (Cd) concentrations. The aim of this work was to separate and characterize the HP cells of the stone crab *Menippe frontalis* from the Pacific Ocean, Perú (5° 5′ 21″ S–81° 6′ 51″ W) and to measure the cellular viability and LPO after exposure to the non-essential metal Cd. The HP cells were dissociated by magnetic stirring, with posterior separation by sucrose gradient at concentrations of 10, 20, 30, and 40%. We found the same cell types that were described for other species (e.g., *Ucides cordatus*, Atlantic Ocean, Brazil). High cellular viability against 1 mmol L^{-1} of Cd was observed for resorptive (R) cells in 20% sucrose layer (88 ± 8%, $*P < 0.05$, ANOVA), and blister (B) cells in the 40% sucrose layers (92 ± 7%, $*P < 0.05$, ANOVA). Cd (1 mmol L^{-1}) caused an increase in LPO levels, suggesting that crabs from polluted areas can be affected by toxic metals, generating a physiological stress. The gradient sucrose methodology can be used for different species and results in a similar separation, viability, and cellular identification. The results are a starting point for toxic metal studies for species distributed across different geographic coordinates.

Keywords *Menippe Frontalis* · Hepatopancreas · Isolated cells · Lipid peroxidation

Introduction

Crustaceans are frequently used as bioindicators, providing data for variation in the availability of contaminants through cumulative concentrations in their body, tissues, organs, and cells (Rinderhagen et al. 2000; Ortega et al. 2016). Contact between the crustaceans and the environment occurs through the gills and hepatopancreas (HP). The HP is composed of four cell types: embryonic cells (E), resorptive cells (R), fibrillar cells (F), and blister cells (B) (Hopkin & Nott 1980; Al-Mohanna & Nott 1989; Ortega et al. 2011, Ortega et al. 2014a). The HP are involved on digestion, absorption, secretion, and detoxification of xenobiotics such as toxic metals.

The E cells are able to create the other cell types and have a nucleus, with few cellular organelles. The endoplasmic reticulum and Golgi are small, with some vesicles and cisternae and the lysosomal system includes primary lysosome and autophagosomes. It contains small lipids or glycogen particles (Vogt 1994; Chavez-Crooker et al. 2001; Ortega et al. 2011, Ortega et al. 2014a). The R cells present few mitochondria and three polarization zones with microvilli, endoplasmic reticulum, nutrient reserve, and some cellular organelles (Vogt 1994; Chavez-Crooker et al. 2001; Ortega et al. 2014a). The F cells are characterized by a large number of mitochondria, endoplasmic reticulum, cytoplasmic granules, Golgi system, and digestive enzymes, indicating hepatic functions, including absorption of small molecules, transport through cell apical membrane, reserve storage, and detoxification of toxic metals

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(Vogt 1994; Chavez-Crooker et al. 2001; Ortega et al. 2014a). The B cells have few mitochondria and intense presence of Golgi vesicles, microtubules, and a simple and large vacuole specific for detoxification and storage of toxic metal ions, such as cadmium, copper, zinc, and mercury (Vogt 1994; Chavez-Crooker et al. 2001; Ortega et al. 2014a).

Toxic metals ions such as cadmium are known to accumulate in crustacean cells and can cause cell damage and physiological stress. Lipid peroxidation (LPO) is a stress index of environmental pollution and metal excess (Hermes-Lima et al. 1995; Monserrat et al. 2003; Wang et al. 2013; Vitorino et al. 2015; Ortega et al. 2016). Several studies with vertebrates and invertebrates have shown that in heavily polluted areas with presence of cadmium, there is an increase in the oxidative biomarkers such as LPO (Xuan et al. 2011).

Thus, the aim of the present study is to assess the effect of cadmium in the viability and levels of LPO of the HP cells of the stone crab *Menippe frontalis*. Such work can be pivotal for future investigations on the transport of toxic metals and their impact on the physiology of this bioindicator.

Methods

Menippe frontalis specimens were collected in Lima, Perú coast (5° 5′ 21″ S–81° 6′ 51″ W), and brought to Universidad Nacional de Ingeniería (Lima campus) where they were acclimatized. They were kept in tanks filled with sea water at 30 ppt of salinity, gravel, water filter, and pieces of brick for their emersion. The individuals were divided in two groups: control (without cadmium, 5 animals) and experimental (1 mmol L^{-1} of cadmium, 1 h of exposition, 5 animals). In previous studies conducted by Ortega et al. (2014a, b; 2016), gill cells and hepatopancreas of crustaceans were viable up to 1 mmol L^{-1} Cd, with high levels of lipoperoxidation and metallothioneins. Thus, *Menippe frontalis* was also submitted to 1 mmol L^{-1} Cd in order to verify how the crab would react in contaminated environments in a short period of time.

HP cell dissociation was achieved by magnetic stirring according to a reported method (Ortega et al. 2011). The organ was separated from the animal and put in a beaker with 15 mL of the extracting solution (NaCl 395 mmol L^{-1}; KCl 10 mmol L^{-1}; $NaHCO_3$ 2.5 mmol L^{-1}; NaH_2PO_4 2.5 mmol L^{-1}; HEPES 3.75 mmol L^{-1}; Glucose 1.0 mmol L^{-1}; EDTA 0.9 mmol L^{-1}). After, it was stirred for 15 min. Then, the solution was filtered in 30 μm mashed and centrifuged for 5 min at 1000 rpm. The pellet was resuspended and stored, and later used for the separation by a discontinuous sucrose gradient. In this gradient, the HP cells were separated at different sucrose concentrations: 10, 20, 30, and 40%, which were diluted in the extracting solution to the desired concentration. The tube was centrifuged for 5 min at 1000 rpm. The HP cell layers were sampled for viability verification by Trypan Blue method.

Cellular viability was tested using the Trypan Blue method. In 200 μL of cells were added 20 μL of Trypan Blue (Tennant 1964), and put in the Neubauer chamber. After that, the viability was quantified by the visualization of the stained cells (unviable) and translucent cells (viable) in four quadrants, with the result multiplied by 10^4.

LPO quantification in tissue extracts (HP organ) was evaluated according to existing protocols (Jiang et al. 1991, 1992; Hermes-Lima et al. 1995). Frozen HP tissues samples were rapidly weighed and homogenized in cold 100% methanol (5°C) (1:9 w:v). Homogenates were centrifuged (12,000 rpm), and then supernatants were removed. For the standard assay, the following reagents were added sequentially in different cuvettes: 0.25 mmol L^{-1} iron (II) salt (ferrous ammonium sulfate), 25 mmol L^{-1} sulfuric acid, 0.1 mmol L^{-1} xylenol orange, and water for a final volume of 0.9 mL. A sample of tissue extract (45 μL) was then added, and the final volume was adjusted to 1 mL with deionized water. Blanks were prepared by replacing tissue extracts with deionized water. Samples were incubated at room temperature for 1 h, and absorbance was measured at 580 nm. Then, 30 μL of CHP (175 μmol L^{-1}) were added to the sample, and absorbance (580°nm) was read again after 15 min.

Kruskal-Wallis OneWay Analysis of Variance on Ranks followed by Tukey Test were performed with Stat 3.2 software. For cellular viability data, average values of the five populations ± SD were used.

Results and Discussion

The dissociation and sucrose gradient experiments were realized with the stone crab *Menippe frontalis*, and the results show the presence of four cell layers with four specific cells, as reported previously (Ortega et al. 2011). In addition, embrionary cells (E) were found to be present in the first layer (10% sucrose), resorptive cells (R) in the second layer (20%), fibrillar cells (F) in the third layer (30%), and blister cells (B) in the fourth layer (40%). Studies developed by Chavez-Crooker et al. (2001) showed that the lobster *Homarus americanus* HP was composed of all four major cell types (E, R, F, and B), and each of them were believed to contribute to the functions like digestion, absorption, secretion, osmoregulation, and detoxification. Moreover, it was reported that each cell type appears in different layers because each group of cells has different densities and amount of granules. Previously, our group (Ortega et al. 2014a) has used coloring methods (DASPEI and Triple Mallory) to confirm the presence of E cells in the 10% layer, R cells in the 20%, F cells in the 30%, and B cells in the 40% layer in *Ucides cordatus*. We also established (Ortega et al. 2014a) that E and R cells have a

small number of mitochondria, and hence have higher concentration in the 10 and 20% layer, respectively. Similarly, F and B cells have a large number of mitochondria and vacuoles, concentrating preferably in the 30 and 40%, respectively.

Animals from estuarine, sandy–muddy, and coastal regions, as *Menippe frontalis*, are directly affected by local pollution through the disposal of chemicals and toxic metals such as cadmium originating from industries and mining companies (Harris & Santos 2000; Rotter et al. 2010; Ortega et al. 2014a, b). Cadmium, a non-essential metal, when in contact with organs and tissues viz. HP, could cause physiological and morphological damage affecting the cellular viability (Ortega et al. 2016). In the absence of cadmium, control HP cells showed a viability of 77 ± 8%, $N = 5$. After 1 h, the viability for control HP decreased to 68 ± 7% (results not showed). High cellular viability against 1 mmol L^{-1} of Cd was observed for resorptive cells (R) in 20% sucrose layer (88 ± 8%, $^*P < 0.05$, ANOVA, $N = 5$), and blister cells (B) in the 40% sucrose layers (92 ± 7%, $^*P < 0.05$, ANOVA, $N = 5$) (Fig. 1). Furthermore, in the presence of 1 mmol L^{-1} Cd, a decrease in viability (55 ± 7%, $N = 5$) of the R, F, and B cells was observed (ANOVA, $P < 0.05$, $N = 5$) (Fig. 1). These results demonstrate that the presence of cadmium, in high concentration (1 mmol L^{-1}) and acute exposition (1 h), could decrease cell viability. According to Silvestre et al. (2004), in environments with high cadmium and low calcium and sodium concentrations, cadmium can be transported to the cells through calcium channels and sodium exchangers, as in *Ucides cordatus* mangrove crab (Ortega et al. 2014a, b), and can cause cellular damages. Lan et al. (2004) demonstrated that the crab *Sinopotamon yangtsekiense* showed damage to the ultrastructure of HP cells with partially disintegrated membrane along with damages in the vesicles and reticulum, when the animal had chronic exposition of Cd at low concentrations. Our results show that an acute and high exposition of Cd affected the physiology of the cells, resulting in a decrease in viability.

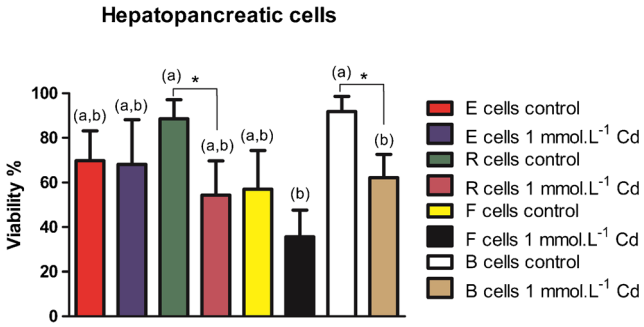

Figure 1. Cell viability of embryonic (E), resorptive (R), fibrillar (F), and blister (B) hepatopancreas cells of the crab *Menippe frontalis* in absence (control group) and presence of $CdCl_2$ (1 mmol L^{-1}). *Asterisks* represent significant difference inside the same group (control and Cd exposition of same cell types). *Different letters* represent significance difference in different groups (control and Cd exposition of different cell types). Average ± SD, $N = 5$; (ANOVA, $P < 0.05$).

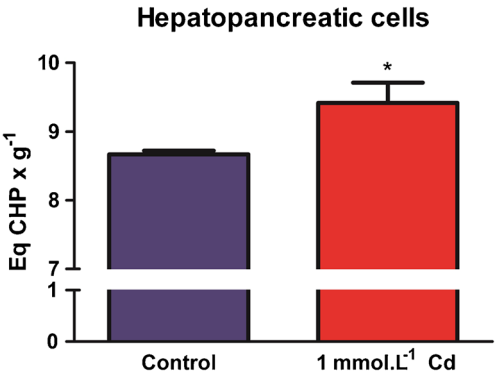

Figure 2. Level of lipoperoxidation (Eq CHP×g^{-1}) in the hepatopancreas of the crab *Menippe frontalis* in absence (control group) and presence of $CdCl_2$ (1 mmol L^{-1}). *Asterisk* represents the significance between control and experimental groups. Average ± SD, $N = 5$; (ANOVA, $P < 0.05$).

In addition, through bioindicators such as *Menippe frontalis*, some strategies can be used to detection of pollution degree in a location. One strategy is the LPO, where there is an increase in the oxidative phenomena, with the detection of pollution levels (particularly in respect to Cd) (Ortega et al. 2016). In our study, we could verify a significant increase of LPO levels in the presence of Cd (Fig. 2). The HP is known to act as an organ for detoxification (Chavez-Crooker et al. 2001). With the accumulation of metals, like Cd, there is a stimulus for the formation of hydroxyl radicals, which cause oxidative stress by their reaction with macromolecules, ultimately leading to cell damage, decreased viability, and LPO (Yang et al. 2013; Ortega et al. 2016). Our results show that the presence of Cd during 1 h could affect the HP, decreasing cell viability (Fig. 1) and increasing oxidative stress (Fig. 2).

Conclusions

The cell types found in *Menippe frontalis* are similar to other decapods, such as *Ucides cordatus*. In addition, the technique used for cell separation through different sucrose gradients is also valid for the crab *Menippe frontalis*. It was observed that, in the presence of Cd, there was a decrease in cell viability and an increase in oxidative stress, leading to LPO. This also demonstrates that *Menippe frontalis* can be used as a promising bioindicator of polluted environments, serving as a model for future studies in ecotoxicology.

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