Immunotoxicity of benzothiazole on *Mytilus edulis* following *in vitro* exposure of hemocytes

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Introduction

In recent years, a wide array of industrially-made substances have been classified as emerging organic pollutants due to their omnipresence in the aquatic environment.[1-6] Many environmental pollutants are the result of improper use and disposal and they are not properly eliminated by sewage treatments.[1,2] Benzothiazole (C_7H_5NS) is an aromatic heterocyclic molecule formed with a 1,3-thiazole ring linked to a benzene group.[1-3] Benzothiazole (BTs) possess a vast array of derivate molecules used in many consumer products ranging from rubber (tires), anticorrosive agents, as fungicide in the paper and leather industry and it is also commonly found in a wide range of household products.[1,2,4,5] BTs are not very soluble in water and are thus found in large quantities in urban particulate matter.[3] The remarkably stable chemical properties and the slow degradation rate of BTs contribute to their popularity and ubiquity in the aquatic environment.[1,2] Human exposure to benzothiazole is widespread, as it is also been documented in bottled water, food flavors, tea leaves and tobacco smoke.[4] Some benzothiazole derivates have been identified as carcinogenic agents in humans or have been associated with mutagenicity in different microorganisms.[4,5] Some studies have even shown that BTs derivates can accumulate in the human adipose tissues.[4] There is a clear lack of knowledge on the toxicity levels of benzothiazole and its derivates on aquatic environments. The goal of this study is to monitor the immunotoxicity of benzothiazole on the hemocytes of the blue mussel (*Mytilus edulis*) following an *in vitro* exposure.

Materials and Methods

Hemocytes collection

A total of 14 blue mussels (*M. edulis*) were collected in Mitis Bay located on the South shore of the St. Lawrence estuary (48° 40' N, 68° 00' W). Each animal was randomly selected and possessed similar shell lengths measuring between 45 and 60 mm.

Hemocytes were collected from the adductor muscle using a 23G needle and a 3.0 mL syringe.[7]

Exposure protocol

Due to the poor solubility of benzothiazole in water, BT was prepared in ethanol (99.8% Ethanol, ACS reagent, Sigma-Aldrich Company, St. Louis, MO, USA). Prior to the exposure, a response-dose curve was performed to determine the effect of ethanol alone on mussel hemocytes. The hemocytes from 5 animals were exposed to 6 final concentrations of ethanol (0.0, 1.5, 2.5, 5.0, 7.5, 10% V/V) during a 21-h period. Exposures were performed in microplates; to 180 µL of hemolymph was added a volume of 20 µL of each prepared solutions at the appropriate concentration. Then, viability and phagocytosis were evaluated.

The dose-response curve with benzothiazole was performed with 9 different mussels exposed to 5 concentrations (0, 10^{-3}, 10^{-5}, 10^{-7}, 10^{-9} M). The mother solution of benzothiazole was prepared in ethanol (99.8%) initially to a 10^{-2} M concentration. Then, serial dilutions were prepared in distilled water to prepare the solutions at the required concentrations. The maximal ethanol concentration was 10% found in the 10^{-3} M dilution. Exposures for a period of 21 h were performed in microplates; to 180 µL of hemolymph was added a volume of 20 µL of each prepared solution at the appropriate concentration. Then, viability and phagocytosis were evaluated.

Viability

The viability was determined for each animal before and after 21-h period using flow cytometry. To each cell suspension of 200 µL, a volume of 10 µL of propidium iodide was added (stock solution 10 µg/mL^{-1}) (Sigma Chemical Company).[7] A minimum of 5000 events was gated, acquired and analyzed according to their scattered properties of forward and right angle and fluorescence frequency distribution on FL3. Data collection and analysis were performed with BD CellQuest™ Pro software (Becton Dickinson, San Jose, CA, USA).

Phagocytosis

After 3 h of exposure with either ethanol or BT, yellow-green latex Fluospheres (Molecular Probes Inc., Eugene, OR, USA) with a diameter of 1.2 um, were added at a ratio of 1:100 (hemocytes:beads) and left in the dark at room temperature for another incubation period of 18 h.[7] Then, the microplates were drained and cells were resuspended and fixed with 0.5% formaldehyde in marine water. The samples were read via flow cytometry (FACScan system, Becton Dickinson). Phagocytosis was expressed in percentage of phagocytic activity (1 bead or more) as well as in percentage of phagocytic efficiency (3 beads or more).[7] A minimum of 5000 events was gated and acquired and analyzed according to their scattered properties of forward and right angle and fluorescence frequency distribution on FL1. Data collection and analysis were performed with BD CellQuest™ Pro software (Becton Dickinson).

Statistical analysis

The effects of ethanol and benzothiazole were expressed as mean ± standard deviation and were assessed using an analysis of variance (ANOVA) ($P \leq 0.05$) followed by a Shapiro-Wilk normality test using SigmaPlot® (version 12.5; Systat Software, Inc., San Jose, CA, USA). When the normality tests failed, a Kruskal-Wallis one-way ANOVA was performed.

Results

For the ethanol dose-response curve, results are presented in Figure 1. There are no significant differences in viability except for hemocytes exposed to 7.5 and 10% (V/V) of ethanol when compared to hemocytes from control group. Phagocytic activity and efficiency did not appear to be significantly affected by ethanol. For the benzothiazole response-curve, the results are presented in Figure 2. Due to the poor solubility of BT, the first working dilution of BT (10^{-3} M) contained 10% final concentration of ethanol. Due to poor hemocyte viability at this con-

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Key words: Immunotoxicity; benzothiazole; *Mytilus edulis.*

Conference presentation: part of this paper was presented at *ECOBIM meetin*g, 2015 May, Québec City, Québec, Canada.

Journal of Xenobiotics 2015; 5:5771
doi:10.4081/xeno.2015.5771

centration, the phagocytosis was not measured. For the others concentrations tested, the ethanol content was 0.1% and lower. No significant differences could be observed in phagocytic activity and efficiency as well as in viability of the hemocytes exposed from 10^{-9} up to 10^{-5} M of benzothiazole for a period of 21 h.

Discussion and Conclusions

Benzothiazole has been quantified in aquatic environments by many laboratories. Herrero *et al.*,[1] analyzed the occurrence of benzothiazole and its derivates in a few locations such as Greece, Germany, Spain and China. The highest level of contamination was found in effluent sewage water at a concentration of 12 μg/L in Germany.[1] The exposure concentrations in this study ranged from concentrations below the values found by Herrero *et al.*[1] to 100 times higher than the concentration of benzothiazole found in the environment and no effect could be found on the phagocytic capacity and viability of hemocytes in blue mussels. The only effects observed were a very high mortality rate of hemocytes exposed to 10^{-3} M of benzothiazole. However, because the solution contained 10% V/V of ethanol and the dose-response curve showed similar cytotoxicity at the same concentration, it is impossible to discriminate between the effects related to the ethanol or the possible effects of benzothiazole. Benzothiazole has low solubility and most studies only analyze the dissolved fraction of benzothiazole in waste water.[6] Some studies have shown that BTs compounds are found at higher concentration in the sewage sludge (up to 265 ng/g d.w.) than in the water column.[1] Those information suggest that although the molecules are very stable, once released in the environment, BTs can undergo processes; such as adsorption to sediment.[3] More studies need to be done to confirm this theory because data on the behavior of benzothiazoles in the water column are scarce.[1,3,6] Some studies suggest that BTs are not prone to bioaccumulate[2] but recent studies have shown that derivate from benzothiazoles have been found in human adipose tissues suggesting otherwise.[4] Due to the high concentrations of those pollutants found in the sewage sludge, studies should be done to determine whether or not benthic marine organisms could be affected by benzothiazole including its metabolites.

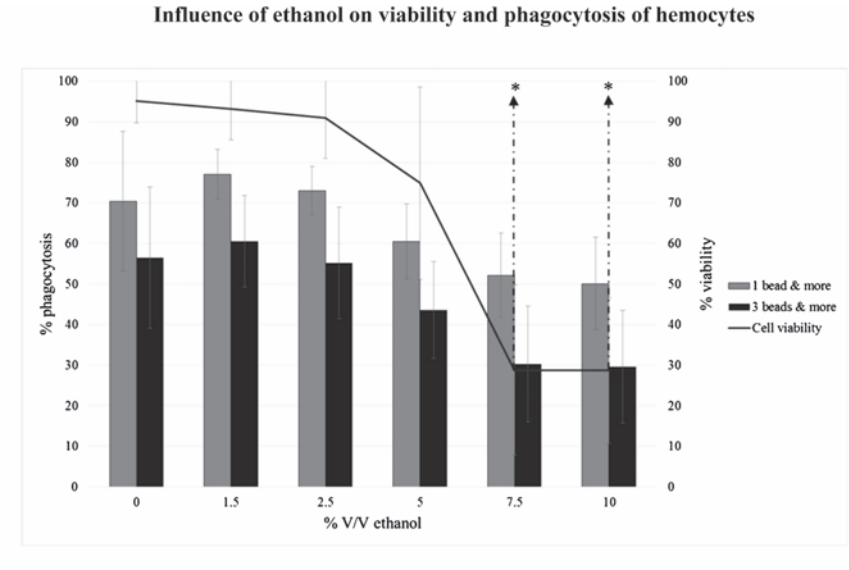

Figure 1. Percentage of phagocytosis (activity and efficiency) and viability of hemocytes exposed to different concentrations of ethanol for a period of 21 h (P≤0.05).

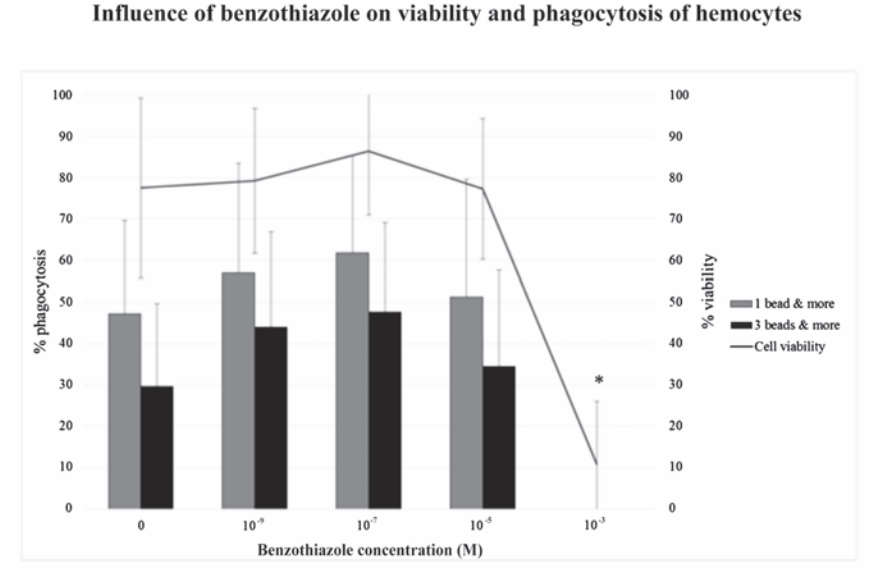

Figure 2. Percentage of phagocytosis (activity and efficiency) and viability of hemocytes exposed to different concentrations of benzothiazole (P≤0.05).

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