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In vitro effect of five pharmaceuticals on the viability of the European abalone hemocytes, Haliotis tuberculata

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Introduction

Pharmaceutical compounds are a class of emerging contaminants with potential concern for the aquatic environment.1,2 Although they are mainly used for medical and veterinary purposes, their inadvertent release in the environment may pose a potential threat to non-target aquatic organisms. Pharmaceutical residues are continuously discharged into surface waters and many recent studies have revealed the presence of measurable concentrations from nanogram to low microgram-per-liter range.3 Thus, aquatic organisms are exposed during their entire life cycle and studies are necessary to evaluate their potential effects, particularly on marine organisms for which data are still missing.

The aim of the present study was to investigate the potential effects of four pharmacological compounds to Haliotis tuberculata hemocytes in vitro. Hemocytes represent an interesting model due to their key role in defenses of organisms against foreign particles and microorganisms.4,5 These cells are routinely used in in vitro ecotoxicological studies for specific endpoints with great precision and reproducibility6 allowing to chemically-induced immunological disorders to be well documented in an increasing number of species.5,11 Phagocytosis is one of the main immune response and is complemented by an array of bacterial killing mechanisms, where lysosomes are involved. In this study, the four pharmacological compounds of interest belong to β-blockers (acebutolol), antiepileptics (gabapentin), antihistamines (cetirizine) and serotonin-norepinephrine reuptake inhibitors (venlafaxine). Hemocytes were exposed to different concentrations of the selected compounds. Toxicities of these different compounds were assessed on the cell viability by the MTT assay and the neutral red uptake assay.

Materials and Methods

Animals

Abalones with shell length between 9 and 11 cm were collected by France Haliotis (S. Huchette, Plougerneau, France). Organisms were maintained in naturally and continuously aerated seawater at 17°C and fed with a mixed algal diet (Laminaria sp. and Palmaria sp.) at the Centre de Recherche en Environnement Côtier (C.R.E.C., Luc-sur-Mer, Basse-Normandie, France). Prior to their use in our study, abalones were acclimated for at least 2 weeks.

Primary cell cultures

Hemocytes were cultured as previously described.14 Briefly, the hemolymph was collected from the pedal sinus of each abalone with a 20 mL syringe with a 25G needle. Hemolymph was transferred into a sterile tube and diluted 1:4 in cooled sterile modified Alsever’s solution to avoid hemocyte aggregation.15 Cells were rapidly placed into 12-well plates at a density of 5x10⁵ cells per well (MTT assay) or into 24-well plates at a density of 3x10⁵ cells per well (neutral red uptake assay) with the addition of three volumes of artificial sterile seawater (ASSW), and maintained at 17°C during 90 min. Then, the ASSW were replaced by Hank’s sterile 199 medium modified.16,17 Cell cultures were incubated at 17°C overnight.

Exposure design

Pharmaceuticals, all supplied by Sigma-Aldrich at analytical grade were acebutolol (β-blocker), cetirizine (antihistamine), gabapentin (antiepileptic), venlafaxine (serotonin-norepinephrine reuptake inhibitor). All pharmaceuticals were dissolved in dimethyl sulfoxide (DMSO) and the final concentration of the solvent was 0.01%. The tested pharmaceuticals were assessed on the cell viability by the MTT assay and the neutral red uptake assay. Each concentration was tested in quadruplicate (i.e. four wells per concentration). Cells were exposed during 48 h and the medium was changed every day. The cell exposure was repeated three times, i.e. using three abalones (experiment replicates).

Cytotoxicity assays

MTT assay

The effect of pharmaceuticals on hemocytes survival was measured using the MTT reduction assay.18 Briefly, 10 % (v/v) of the MTT stock solution (5 mg MTT/mL PBS IX) was added to the culture dishes. After 24 h incubation at 17°C, an equal volume of isopropanol containing 0.04 N HCl was added to each culture to dissolve the converted formazan dye. The absorbance was measured at a wavelength of 570 nm with a 630 nm reference point.

Neutral red uptake assay

The neutral red uptake assay is a cell viability assay based on the ability of viable cells to incorporate and bind neutral red (NR) within lysosomes.17 After treatments, the neutral red uptake assay was used to determine lysosome membrane stability following the method adapted to microplate cultures.10,11 Briefly, 30 µL of NR stock solution (0.5 % NR in PBS IX) was added to each well containing 270 µL of culture medium. After 1 h incubation at 17°C the medium was removed, and wells were washed first with 3% formaldehyde in ASSW to fix cells and then twice with PBS IX. NR was extracted using 1% glacial acetic acid in 50% ethanol. After 30 min at room temperature, the plates were transferred to a TECAN Infinite M200 microplate reader, and the absorbance was measured at a wavelength of 540 nm with a 650 nm reference point.

Data analysis

Results are expressed as means±standard deviations (SD). Statistical analyses were undertaken with R software version 2.15.1,
and performed on values resulting from quad-
replicate wells containing cells repeated at
least three times. Data normality and
homoscedasticity were checked but were not
assumed even with data transformation.
Moreover, as we used pseudoreplication (i.e.
the same organism was used to test all the con-
centrations), the significance of the differ-
ences between mean values was estimated
using Wilcoxon tests.

Results and Discussion

The extension of pseudopods by hemocytes
and their adhesion capacity play key roles in
the cellular migration and the immune
defence of mollusks. The morphological results
obtained in this study were presented in
Figure 1. No morphological changes were
observed with increasing pharmaceutical con-
centrations. Cultured cells displayed an elon-
gated shape with large pseudopods and were
mostly interconnected whatever the pharma-
ceutical tested. Concomitantly, results of MTT
assays showed no effects of pharmaceuticals
on cell viability under our experimental condi-
tions (data not shown).

In contrast, a significant increase of NR
retention was measured for venlafaxine,
gabapentin and cetirizine. For venlafaxine and
gabapentin, this increased retention was only
observed from 250 µg/L (P<0.01), and can
reach 77% and 50% of increase at 500 µg/L of
venlafaxine (Figure 2A) and Gabapentin
(Figure 2B) respectively, compared to control.
For Cetirizine (Figure 2C), a significant
(P<0.05) increase of NR uptake by cells was
observed when hemocytes were exposed to a
concentration of 1 µg/L and reached 85% of
increase compared to the control at 50 µg/L.
On the contrary, acebutolol (Figure 2D) did not
affect NR uptake. Neutral red assays are con-
sidered reliable tools to quantify the number
of viable and uninjured cells after exposure to
pollutants where lysosomal integrity is a
generic common target for environmental
stressors.20 The assay used here is based on
the ability of viable cells to uptake the dye and
to concentrate it in the lysosomes. Non-viable
cells do not take up the dye. A change in the
number of cells or in their physiological state
related to lysosomes, results in a concomitant
change in the amount of dye incorporated by
cells.17 The abalone hemocytes are not prolifer-
ative cells. Thus, the increase of dye incorpora-
tion observed in hemocytes exposed to ven-
lafaxine, gabapentin and cetirizine, suggest an
increase in lysosome number and/or size. In
general, exposure to pollutants is known to
cause an increase of lysosomal size which is
often linked with a destabilization of lysosomal
membranes.10-23 These three pharmaceuticals
affected the uptake of NR, but displayed differ-
et effect profiles. For venlafaxine and
gabapentin, the NR uptake was still in the
ascending phase of dye accumulation whereas
for cetirizine, the maximum accumulation was
already observed at 50 µg/L followed by a
descending phase of dye accumulation from
250 µg/L. From our results, the four tested
pharmaceutical compounds would not display
any particular danger for abalone hemocytes
and immunity, when considered individually.
However, in the field, aquatic organisms are
exposed to contaminant mixtures. Further
investigations are needed to address the toxic-

Figure 1. Light microscopy images showing the morphology of Haliotis tuberculata
hemocytes. Cells were seeded at a density of 5×10^5 cells per well and grown at 17°C in
culture medium for 48 h in the absence (A) or presence of 500 µg/L of venlafaxine (B),
gabapentine (C) and cetirizine (D).

Figure 2. Results of neutral red uptake by control and exposed abalone hemocytes. NR
uptake by cells exposed to different concentrations (µg/L) of venlafaxine (A), gabapen-
tine (B), cetirizine (C) or acebutolol (D) were given compared to the 100% control. Each
data point represents the mean percentage±S.D. of triplicate cultures. Significant differ-
ences from control cells were indicated by stars: *P<0.05, **P<0.01.
ity of pharmaceutical mixture on aquatic organisms.

References