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In vitro effects of the nonsteroidal anti-inflammatory drug, ibuprofen, on the immune parameters of the colonial ascidian *Botryllus schlosseri*

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ABSTRACT

In this study, *in vitro* effects of ibuprofen (IBU) on the immune parameters of the colonial ascidian *Botryllus schlosseri* were evaluated. Haemocytes were exposed for 1 h to 0 (control), 100 and 1000 μg IBU/L and the effects on haemocyte viability and morphology (shape factor), lysosomal membrane stability (Neutral Red Retention Assay), phagocytic activity, apoptosis (TUNEL reaction), hydrolytic (acid phosphatase) and oxidative (phenoloxidase and peroxidase) enzyme activities were evaluated. The exposure of haemocytes to IBU did not affect significantly their viability, but increased the percentage of cells with round shape. IBU caused a significant reduction in both phagocytic activity and lysosomal membrane stability. The percentage of haemocytes positive to TUNEL reaction (indicative of DNA fragmentation) increased significantly after IBU exposure. Significant decreases in the percentage of haemocytes positive to acid phosphatase were recorded at 1000 μg/L of IBU. Conversely, no significant variations were recorded in the percentage of haemocytes positive to phenoloxidase and peroxidase. Results obtained indicate that exposure of ascidian haemocytes to IBU induces marked alterations in cell functionality. Immunomarkers measured in this study are sensitive, rapid and reproducible. However, their responsiveness and biological relevance will need to be verified for *in vivo* exposure.

1. Introduction

Ibuprofen (IBU) is a propanoic acid derivative used worldwide as an analgesic, antirheumatic and antipyretic in non-steroidal anti-inflammatory drugs (NSAIDs) (Fent et al., 2006; Praveen Rao and Knaus, 2008). IBU decreases the formation of prostaglandins and thromboxane by inhibiting cyclo-oxygenase COX-1 and COX-2 enzymes (Gierse et al., 1999; Fent et al., 2006). Its consumption is on the order of tons per year (see Fent et al., 2006 for a review), as reported for Germany (approximately 345 tons in 2001) (Huschek et al., 2004) and Italy (approximately 1.9 tons) (Calamari et al., 2003). IBU is not fully metabolised by humans, and can be excreted in its native form (70–80% of the therapeutic dose) or as metabolites (hydroxy- and carboxy-ibuprofen and carboxy-hydratropic acid) (Heberer, 2002; Pounds et al., 2008). In aquatic environments, IBU has been detected at concentrations ranging between ng/L and μg/L in wastewater and surface water. For example, IBU has been found in effluents (up to 22 μg/L) and influents (up to 84 μg/L) from sewage treatment plants (STPs) (Brun et al., 2006; Gómez et al., 2007), as well as in 84% of the STP effluent samples (median 3.086 μg/L) that have been analysed in the U.K. (Ashton et al., 2004). In Italy, IBU levels of approximately 120 ng/L have been measured in the effluents from nine STPs (Zuccato et al., 2005), and of approximately 17 ng/L in the Po River (Calamari et al., 2003).

In aquatic ecosystems, IBU can affect non-target species. In *Daphnia magna*, the 48 h immobilisation EC_{50} was 51.4 mg/L, and the 21 day reproduction NOEC (No Observed Effect Concentration) was <1.23 mg/L; in *Moina macrocopa*, the 48 h immobilisation EC_{50} was 72.6 mg/L, and the 7 day reproduction NOEC was 25 mg/L; for *Oryzias latipes*, the 120 day survival NOEC was 0.1 μg/L (Han et al., 2010). At cellular levels, *in vitro* exposure of haemocytes from the mussel *Dreissena polymorpha* to different IBU concentrations (45, 450, 909 μg/L) caused lysosomal membrane destabilisation and increased the percentage of apoptotic cells (Parolini et al., 2009). In the same bivalve species, *in vivo* exposure (0.2, 2 and 8 μg/L) affected antioxidant and detoxifying enzyme activities (Parolini et al., 2011), and increased oxidative stress in the digestive gland of IBU-exposed mussels (0.206, 2.06, 20.6 and 206.3 μg/L) (Contardo-Jara et al., 2011). In the clam *Ruditapes philippinarum* (now *Venerupis philippinarum*), exposure to various IBU concentrations (100, 500 and 1000 μg/L) for 7 days caused significant alterations in immunomarker responses (Matozzo et al., 2012). In the same clam species, exposure to IBU (0.1, 5, 10, 50 μg/L) affected

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significantly lysosomal membrane stability (Aguirre-Martínez et al., 2013).

In this study we evaluated the effects of IBU on immune parameters of the colonial ascidian *Botryllus schlosseri*, in order to provide further information about the immunotoxic effects of IBU to aquatic species. *B. schlosseri* is a filter-feeding organism common in shallow coastal waters all around the world. Ascidians rely on innate immune defences that are mediated by haemocytes. In *B. schlosseri*, at least three different categories of haemocytes can be detected: (1) undifferentiated cells (haemoblasts and lymphocyte-like cells); (2) immunocytes, represented by phagocytes (both hyaline amoebocytes and macrophage-like cells) and cytotoxic cells (morula cells and their precursors, granular amoebocytes); (3) storage cells (pigment cells and nephrocytes) (Ballarin et al., 1994, 2008; Ballarin and Cima, 2005). *B. schlosseri* has already been used as a reference organism to study the effects of various pollutants on the functionality of immunocytes, with particular reference to phagocytes (Cima et al., 1995, 1998, 2002, 2008; Cima and Ballarin, 1999, 2004, 2012). Considering that information about the toxic effects of IBU on ascidian haemocytes is lacking in the literature, in this study we investigated the effects of IBU on the viability of haemocytes, induction of apoptosis, phagocyte morphology, lysosomal membrane stability, hydrolytic and oxidative enzyme activities and phagocytic activity. An *in vitro* approach was adopted since *in vitro* assays are increasingly used for assessing or predicting the toxic effects of chemicals and for elucidating their mechanisms of action. Moreover, *in vitro* assays reduce the use of experimental animals, have low costs, rapid performance and high reproducibility (Olabarrieta et al., 2001).

2. Material and methods

2.1. Animals

Colonies of *B. schlosseri* (Pallas, 1766) were collected in the Lagoon of Venice. They were kept in aerated aquaria, attached to glass slides and fed with Liquifry Marine (Liquifry Co., Dorking, England). Ascidians were acclimatised in the laboratory for 1 week before their use in experiments.

2.2. Haemocyte cultures and exposure to IBU

Haemolymph was collected with a glass micropipette after puncturing, with a fine tungsten needle, the tunic marginal vessels of colonies previously immersed in anti-clotting solution (0.38% Na-citrate in 0.45 μm filtered seawater (FSW, pH 7.5). It was then centrifuged at 780*g* for 10 min and pellets were finally resuspended in FSW to give a final concentration of 10^6 cells/mL. Sixty μL of haemocyte suspension were placed in the centre of culture chambers, prepared as described elsewhere (Ballarin et al., 1994) and left to adhere to coverslips for 30 min at room temperature.

After adhesion of haemocytes to the coverslips, cells were exposed for 60 min to IBU (60 μL per culture chamber) at room temperature. The duration of exposure to IBU was chosen on the basis of our previous surveys concerning the evaluation of *in vitro* effects of contaminants in *B. schlosseri* (Cima and Ballarin, 2000; Cima et al., 2008). A stock solution of IBU (1 g/L) was prepared in ethanol and stored at room temperature in the dark. Working solutions (100 and 1000 μg/L) were obtained by dissolving the stock solution in FSW. Solvent (0.1% in FSW) was added in controls. The nominal concentrations were chosen based on the reported data on *in vitro* IBU toxicity in aquatic organisms (Parolini et al., 2009). The reason of this choice was to provide further information concerning the negative effects of IBU to differing species of aquatic organisms.

2.3. Haemocyte viability assay

Cell monolayers were incubated with 0.25% Trypan Blue in FSW for 5 min at room temperature and observed *in vivo* under a Leitz Dialux 22 light microscope (LM) at 1250×. The percentage of stained haemocytes was then estimated.

2.4. Effects of IBU on cell morphology: shape factor

Haemocyte monolayers, exposed for 60 min to IBU, were fixed for 30 min at 4 °C in a solution of 1% glutaraldehyde and 1% sucrose in FSW, washed in phosphate buffered saline (PBS: 1.37 M NaCl, 0.03 M KCl, 0.015 M KH_2PO_4, 0.065 M Na_2HPO_4, pH 7.2), for 10 min, and stained for 5 min in 10% Giemsa solution. A computer-assisted image analysis (Casting Image NT) was performed on fixed haemocyte monolayers to evaluate the phagocyte shape factor, as defined by Ottaviani et al. (1997). Briefly, the shape factor is equal to AC/AT, where AT is the area of a circle with the same perimeter of that of the given cell, and AC is the actual area of the cell. Lower shape factors indicate larger perimeters with respect to the areas, and increased amoeboid shape.

2.5. Assay for actin cytoskeleton

After exposure to IBU, haemocyte monolayers were fixed in 4% paraformaldehyde in a 0.2 M Na-cacodylate buffer containing 1% NaCl and 1% sucrose. They were then washed in PBS, permeabilised with 0.1 M Triton X-100 in PBS for 5 min, washed again in PBS and incubated in a solution of 0.1 μM FITC-conjugated phalloidin (Sigma), in PBS for 30 min. Phalloidin binds specifically to F-actin and allows the visualisation of microfilaments. Coverslips were then washed in PBS and mounted on glass slides with Aquovitrex (Carlo Erba). They were then observed under a Leitz Dialux 22 light microscope equipped with I2/3 filter block for FITC excitation, at the magnification of 1250×.

2.6. Phagocytosis assay

After adhesion of haemocytes to coverslips, cells were incubated for 30 min at room temperature with three different solutions containing 60 μL of FSW plus ethanol (control), 100 and 1000 μg/L of IBU. After treatment, haemocyte monolayers were washed three times in FSW to eliminate IBU. Cells were then incubated for 60 min at room temperature with 60 μL of a suspension of yeast (*Saccharomyces cerevisiae*) cells (yeast:haemocyte ratio = 10:1) in FSW. Haemocyte monolayers were gently washed several times in FSW to eliminate uningested yeast cells, fixed, washed in PBS, stained with Giemsa and mounted on glass slides as previously described. Slides were then observed under the LM at 1250× and the percentage of phagocytosing cells was evaluated.

2.7. Neutral Red Retention Assay

Lysosomal membrane stability was assessed using a modification of the Neutral Red Retention (NRR) assay (Lowe et al., 1995), as previously described (Matozzo et al., 2001). A stock solution of 0.4% NR in FSW was prepared. The working solution was obtained by diluting 10 μL of stock solution in 5 mL of FSW. After exposure of haemocytes, IBU solution was discarded from culture chambers and 60 μL of NR working solution were added. After 5 min, the slides were observed under the LM at 1250×. The lysosomal stability index was expressed as the percentage of haemocytes showing dye loss from lysosomes into the cytosol which, consequently, appears reddish-pink.