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In vitro effects of metals on isolated head-kidney and blood leucocytes of the teleost fish *Sparus aurata* L. and *Dicentrarchus labrax* L.

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ABSTRACT

The *in vitro* use of fish leucocytes to test the toxicity of aquatic pollutants, and particularly the immunotoxicological effects, could be a valuable alternative to fish bioassays but has received little attention. In this study, head-kidney and peripheral blood leucocytes (HKLs and PBLs, respectively) from gilthead seabream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax* L.) specimens were exposed to Cd, MeHg (methylmercury), Pb or As for 24 h being evaluated the resulting cytotoxicity. Exposure to metals produced a dose-dependent reduction in the viability, and MeHg showed the highest toxicity followed by Cd, As and Pb. Interestingly, leucocytes from European sea bass are more resistant to metal exposure than those from gilthead seabream. Similarly, HKLs are always more sensitive than those isolated from blood from the same fish species. Moreover, fish leucocytes incubated with metals exhibited alterations in gene expression profiles that were more pronounced in the HKLs in general, being Pb the metal provoking less effects. Concretely, genes related to cellular protection (metallothionein), stress (heat shock protein 70) and oxidative stress (superoxide dismutase, catalase and glutathione reductase) were, in general, down-regulated in seabream HKLs but up-regulated in seabream PBLs and sea bass HKLs and PBLs. In addition, this profile leads to the increase of expression in genes related to apoptosis (Bcl2 associated X protein and caspase 3). Finally, transcription of genes involved in immunity (interleukin-1β and immunoglobulin M) was down-regulated, mainly in seabream leucocytes. This study points to the benefits for evaluating the toxicological mechanisms of marine pollution using fish leucocytes *in vitro* and insight into the mechanisms at gene level.

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1. Introduction

Industrial development has caused an increase of metals such as mercury (Hg), lead (Pb), cadmium (Cd) or arsenic (As) in marine ecosystems, being detectable in water and organism including fish [1,2]. These pollutants in the marine environment produces severe problems, especially because they persist in the environment, and fish show the ability to absorb and accumulate higher levels of metals in their tissue than the level of toxic concentration in their environment [3] with a consequent negative influence on fish homeostatic mechanisms but also for human consumers. Concretely, some studies have showed the adverse effects of metals in fish including mortality, alterations in hematological parameters, metabolism, nutrition, reproduction, development and immunodeficiency [4–6]. In light of increasing social and political pressure to use non-mammalian systems for predicting human health risks and the recent impetus to develop biomarkers for assessing the biological effects of environmental stress, more and deeper studies are needed to better understand chemical-induced effects on aquatic species. Apart from classical biomarkers of toxicity such as oxidative stress or lipid peroxidation, other studies have revealed the impact of metals on the ecosystem and organism health suggesting the importance of including more and new markers. In this regard, though the fish immune system is considered a non-specific marker for environmental biomonitoring it has direct implications in individual health and population growth [7].

In vivo studies in fish, usually by waterborne exposure to pollutants, they have confirmed alterations in innate and acquired immune functions, such as respiratory burst, phagocytosis, lymphocyte proliferation or antibody levels as well as interfering with host resistance against infectious pathogens [4,8,9]. Despite the fact that fish may be negatively impacted by different kind of

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pollutants, little is known about the toxicological role of metals on the immune system and the oxidative stress in fish leucocytes *in vitro*, which could serve as good models for immunotoxicological studies. Previous studies carried out in our laboratory demonstrated alterations in the immune functions (phagocytosis, respiratory burst and transcription of immune-relevant genes) and overproduction of reactive oxygen species (ROS) of gilthead seabream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax* L.) head-kidney leucocytes (HKLs) after 30 min or 2 h of exposure to metals [10,11], which is in agreement with previous studies in fish [12–15]. Similarly, few papers have dealt with the toxicological effects of metals in peripheral blood leucocytes (PBLs). For instance, common carp (*Cyprinus carpio*) PBLs exposed to chromium (Cr) decreased the lymphocyte proliferation as it did Cd or Zn, but not Pb or Cu, with the respiratory burst activity [16]. In the case of red drum (*Sciaenops ocellatus*) PBLs, exposure to sub-toxic doses of Hg increased cell proliferation whilst toxic doses induced massive calcium flux and activation of tyrosine kinase activity [17]. However, only few works have focused in the toxicological mechanisms caused by metals at gene level. Thus, the regulation of gene expression of genes related to oxidative stress, cell death, metal protection or immunity has been evaluated *in vitro* in sea bass PBLs [18] and HKLs [11], seabream HKLs [10] or rainbow trout (*Oncorhynchus mykiss*) HK macrophages [19]. In general, they have showed an up-regulation of apoptosis marker genes, such as Bcl-2 associated X protein (*bax*) or caspase 3 (*casp3*), and oxidative stress-related genes, such as superoxide dismutase (*sod*) and catalase (*cat*) after HKLs exposure to metals [10,11]. Regarding immunity, seabream HKLs showed variable up- or down-regulation of genes related to lymphocyte markers, pro-inflammatory cytokines, antiviral, antimicrobial peptides or respiratory burst that differed with the metal used [10] whilst in the case of sea bass very few changes occurred after Hg exposure [11]. In rainbow trout macrophages, Cu exposure up-regulated the transcription of interleukin-1β (*il1b*), tumour necrosis factor-α (*tnfa*), interleukin-6 (*il6*), serum amyloid A (*saa*), NADPH oxidase, glutathione peroxidase and trout C-polysaccharide binding protein (*tcpbp*) [19]. Therefore, more studies are needed to clarify the toxicological effects and mechanisms of metals in fish leucocytes since it is known that *in vitro* cytotoxicity assays with fish cell lines are very well correlated to acute lethality tests *in vivo* [20].

Thereby, the present study aimed to compare the cytotoxicity of metals (Cd, Hg, Pb) and a metalloid (As) on HKLs and PBLs isolated from two teleost fish species: gilthead seabream (*Sparus aurata* L.) and European sea bass (*Dicentrarchus labrax*), the most important farmed fish species in the Mediterranean area. Thus, HKLs and PBLs were exposed to Cd, MeHg, Pb or As for 24 h and viability and transcription of genes related to cellular and oxidative stress, protection, death and immunity was determined. Comparisons between source of leucocytes and fish species will be discussed towards their implication at research and toxicological level.

2. Material and methods

2.1. Animals

Twenty specimens (80–100 g mean body weight) of the seawater teleost gilthead seabream (*S. aurata* L.) and European sea bass (*D. labrax* L.), sexually immature, obtained from a local fish farm were kept in seawater aquaria (250 l) in the Marine Fish Facilities at the University of Murcia (Spain). The water was maintained at 20 ± 2 °C, with a flow rate of 900 L h^{-1}, and 28‰ salinity. The photoperiod was 12 h light: 12 h dark and fish were fed with a commercial pellet diet (Skretting) at a rate of 2% body weight day^{-1}. Fish were allowed to acclimatise for 15 days before the start of the experimental trial. All experimental protocols were approved by the Ethical Committee of the University of Murcia.

2.2. Leucocyte isolation

Fish were anaesthetised with 0.21 mM benzocaine (stock dissolved in 4% acetone) (Sigma) and samples were taken under sterile conditions. For HKL isolation, fish were bled from the caudal vein to avoid tissue contamination with erythrocytes and HK tissue was excised, cut into small fragments and transferred to 7 ml of sRPMI [RPMI-1640 culture medium (Life Technologies) supplemented with 0.35% sodium chloride, 100 IU ml^{-1} penicillin (Life Technologies), 100 mg ml^{-1} streptomycin (Life Technologies) and 5% foetal bovine serum (Life Technologies)]. Cell suspensions were obtained by forcing fragments of the organ through a nylon mesh (mesh size 100 μm), washed twice (400 g, 10 min), counted and adjusted to 10^7 cells ml^{-1} in sRPMI. For PBL isolation, 200 μl of blood was immediately withdrawn from the caudal vein with a heparinized syringe and fish were returned to aquaria. Blood was mixed with 4 ml of sRPMI and layered over a 51% Percoll density gradient (Pharmacia), centrifuged (400 g for 30 min at 4 °C) and PBLs, located in the interface, were collected, washed twice, counted and adjusted to 10^6 cells ml^{-1} in sRPMI. In all cases, leucocyte viability was determined by the trypan blue exclusion test and resulted higher than 98%.

2.3. Metals exposure

Different salts of the tested metals (Sigma) were used: cadmium chloride ($CdCl_2$), methylmercury (II) chloride [CH_3HgCl (MeHg)], lead (II) nitrate ($Pb(NO_3)_2$) and trioxide arsenic (As_2O_3). Each salt was initially dissolved in sterile purified water (Milli-Q) and dilutions for each concentration were daily prepared. Prior to carrying out the assays, the osmolarity of these solutions was measured in an osmometer (Wescor) to avoid effects due to osmolarity.

For leucocyte treatments, 180 μl of freshly isolated HKLs and PBLs were dispensed into separate wells, always in triplicate, of flat-bottomed 96-well plates (Nunc). Then, 20 μl $well^{-1}$ of water (controls) or metal solutions, to make final concentrations of 50–5000 μM for Cd, 5–100 μM for MeHg, 500–5000 μM for Pb or 2000–5000 μM for As, were added. Cells were exposed for 24 h at 25 °C in an incubator. Leucocytes from 6 independent specimens (not pooled) were assayed separately (tested in different days) except in the gene expression studies that we used 4 independent fish specimens.

2.4. Cytotoxicity assays

2.4.1. PI (propidium iodide) uptake

In order to determinate the viability of the seabream and sea bass leucocytes, we assessed the abundance of dead HKLs and PBLs using a flow cytometry technique based on fluorochrome labelling (Ormerod, 1990). Following 24 h of metal exposure, samples were mixed by pipetting and 200 μl of each sample were transferred to 5 ml tubes (Becton Dickinson) containing 400 μl of phosphate buffered saline (PBS) and 100 μl of PI (400 μg ml^{-1}; Sigma-Aldrich). All samples were analysed in a flow cytometer (Becton Dickinson) with an argon-ion laser adjusted to 488 nm. Analyses were performed on 10,000 cells, which were acquired at a rate of 300 cells s^{-1}. Data were collected in the form of two-parameter side scatter (SSC, granularity) and forward scatter (FSC, size) and red fluorescence (FL2) dot plots or histograms were made on a

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