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Oxidative stress profiles in brain point out a higher susceptibility of fish to waterborne divalent mercury compared to dietary organic mercury

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ABSTRACT

This study examines, for the first time, the neurotoxicity of Hg(II) and MeHg in fish (*Diplodus sargus*) in a time-course comparative perspective and considering realistic exposure levels and routes. Both forms followed an identical time-variation pattern of accumulation in the brain, but dietary MeHg was more efficiently transported to the brain. MeHg was substantially eliminated from the brain in 28 days of depuration, which did not occur for Hg(II). Moreover, Hg(II) displayed a high neurotoxicity potential, as unveiled by the poor activation of brain antioxidant defenses and recurrent oxidative damage (as protein oxidation), while the opposite was recorded upon MeHg exposure. These results highlight the need to include Hg(II) in future environmental health assessment plans, preventing an underestimation of the risk for wild fish populations, which has probably been occurring due to the long-standing idea of the higher toxicity of MeHg in comparison with inorganic Hg forms.

1. Introduction

Mercury (Hg) has triggered major environmental and human health concerns. This element is present in aquatic environments in organic (primarily methylmercury - MeHg) and inorganic forms [e.g. Hg(II) and Hg(0)], and both can be bioaccumulated by fish, exerting toxicity at different biological levels. Some of the reported Hg effects in fish are inhibition of hepatic biotransformation enzymes (Guilherme et al., 2008a), oxidative stress in the brain (Berntssen et al., 2003), genotoxicity in blood (Guilherme et al., 2008b) and reproductive alterations (Crump and Trudeau, 2009). However, there are fundamental knowledge gaps concerning the effects of mercury species on the fish brain. This is an important issue since fish fitness and survival are significantly affected by the neurotoxic effects of Hg exposure (Farina et al., 2013; Pereira et al., 2016; Puga et al., 2016).

While there are a few studies on the neurotoxicity of MeHg in fish (Berntssen et al., 2003; Puga et al., 2016), there is very little information on the ability of divalent mercury [Hg(II)] to accumulate in fish brain and the resulting effects. The primary focus on MeHg in the literature is likely due to the perception of its higher toxicity associated with rapid uptake and partitioning to sensitive tissues such as the brain. However, some research has indicated that Hg(II) can also easily cross the blood-brain barrier (BBB) and result in neurotoxicity (Aschner and Aschner, 2007; Farina et al., 2013). In contrast to those works, Rouleau et al. (1999) postulated that the BBB is relatively impervious to Hg(II). However, $HgCl_2$ can act as a direct BBB toxicant in rodents, thus increasing its permeability (Zheng et al., 2003). Our previous work has shown that Hg(II) can reach fish brain after only three days of exposure to environmentally realistic levels in water (Pereira et al., 2015), resulting in a reduction in the number of cells in specific brain areas, as well as impairing swimming behavior (Pereira et al., 2016). This is in line with other studies that have documented the occurrence of inorganic forms of Hg in fish brain (Berntssen et al., 2003; Korbas et al., 2012; Wang et al., 2015). It has been also hypothesized that the different forms of Hg share the same toxic chemical entity and, thus, neurotoxicity depends mainly on the external bioavailability (De Flora et al., 1994). In fact, $HgCl_2$ displayed higher toxicity than MeHg in glial cells and neurons of immature aggregate cultures of rat telencephalon

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(Monnet-Tschudi et al., 1996). Within a different framework, Clarkson and Magos (2006) postulated that the conversion of inorganic Hg into MeHg by microorganisms in aquatic sediments could be a protective mechanism since Hg(II) is more toxic. In light of this controversy, more research is needed to evaluate and compare the neurotoxic mechanisms of Hg(II) and MeHg exposure in fish.

It is well established that oxidative stress, emerging from the imbalance between the production and removal of reactive oxidative species (ROS) (Ercal et al., 2001), is a key pathway to trigger Hg neurotoxicity in mammals (Aschner and Aschner, 2007; Farina et al., 2013). Mercury is highly reactive with sulfhydryl groups, forming covalent bonds with GSH and cysteine residues of proteins. In particular, GSH directly binds to MeHg acting as an endogenous ligand, and the complex formed contributes to MeHg efflux from the cells (Clarkson and Magos, 2006). In the same direction, it has been foreseen that MeHg promotes a decrease in intracellular GSH levels, which is considered one of its cytotoxic effects (Choi et al., 1996). Additionally, the inhibition of antioxidant enzymes has been referred as a relevant mechanism involved in oxidative stress due to Hg (Roos et al., 2009). Only a few studies had searched for the modulation of antioxidant enzymes and alterations in GSH content in fish brain after Hg exposure (Berntssen et al., 2003; Mieiro et al., 2011). Moreover, most of those studies were performed under field conditions where a simultaneous exposure of fish to Hg(II) and MeHg occurs, hindering conclusions about the neurotoxicity potential of each Hg counterpart. Additionally, laboratory exposures generally considered a single Hg species, not comparing organic and inorganic forms. So, there is still a lack of studies elucidating the modulation of the antioxidant system and subsequent emergence of oxidative damage in fish brain after exposure to Hg(II) and MeHg. Nevertheless, Berntssen et al. (2003) found a significant increase of lipid peroxidative products after dietary exposure to MeHg together with a decrease of antioxidant enzymes activity (superoxide dismutase – SOD; glutathione peroxidase - GPx), while no significant changes of those endpoints were observed upon exposure to inorganic Hg in food. Despite these contributions, Berntssen et al. (2003) did not assessed the time-evolution of Hg accumulation/depuration and oxidative stress responses, as well as the potential reversibility of toxicity events.

In order to examine these gaps in research, the present study compares the neurotoxic effects of Hg(II) and MeHg exposure on brain of fish (white seabream - *Diplodus sargus*) by the combination of bioaccumulation levels and oxidative stress profiles in a time-course experiment, incorporating both exposure and post-exposure periods. In order to do this, two separate experiments were performed with comparable daily exposure levels of both Hg forms. Realistic exposure levels and routes were tested, viz. waterborne exposure to Hg(II) (2 $\mu g\ L^{-1}$) and dietary exposure to MeHg (8.7 $\mu g\ g^{-1}$ feed dry weight). Ultimately, it was intended to clarify the toxicokinetics and toxicodynamics of both Hg forms, providing reliable data to environmental health assessment.

2. Material and methods

2.1. Experimental set-up

Two experiments, with the same design (Fig. 1), were performed with juvenile white seabreams (*Diplodus sargus*) provided by an Aquaculture Research Station (IPMA - Olhão, Portugal), under a 14:10 light:dark photoperiod. Fish were held in 300 L fiberglass tanks in an average density of 0.062 $kg\ L^{-1}$ in the Hg(II) experiment (fish weight: 146 ± 14 g; total length: 19 ± 1 cm) and 0.056 $kg\ L^{-1}$ in the MeHg experiment (fish weight: 124 ± 11 g; total length: 18 ± 0.6 cm). Fish were exposed to Hg(II) via water ($HgCl_2$), while MeHg (CH_3HgCl) was provided to fish through contaminated pellets. In both experiments, seawater was renewed daily (~80% renewal) and fish were fed once a day, namely 1–2 h before water renewal. In all sampling days, fish were not fed in the 12 h preceding fish handling. Water temperature, salinity and pH were monitored daily throughout the Hg(II) and MeHg experiments, varying as follows, respectively: 14 ± 0.3 °C and 17 ± 2.0 °C (mean ± standard deviation); 35 ± 2 and 35 ± 1 psu (mean ± standard deviation); 7.4–7.9 and 7.6–7.9 (range).

In the Hg(II) experiment, $HgCl_2$ (Sigma-Aldrich) was added to the water of exposure tanks in an aqueous solution in order to reach an initial nominal concentration of 2 $\mu g\ L^{-1}$. Divalent mercury was added on a daily basis after water renewal (i.e. daily water recontamination) during the exposure period. Exposure level of Hg(II) was established considering previous studies in contaminated areas (Horvat et al., 2003; Li et al., 2009), in order to mimic environmentally realistic conditions. Fish were exposed to Hg(II) in the current work since it is believed to be the proximate toxic agent for several inorganic forms of mercury (Clarkson and Magos, 2006). Control fish were kept throughout the experiment in tanks filled with clean seawater. In this experiment, fish were fed with a commercial dry food [standard 3 mm from Sorgal (Portugal)] with vestigial Hg levels (lower than 0.01 $\mu g\ g^{-1}$).

In the MeHg experiment, MeHg-contaminated pellets (8.7 $\mu g\ g^{-1}$ dry weight) were used to feed exposed fish. This MeHg exposure level is also environmentally realistic since natural food of *D. sargus* (e.g. *Nereis diversicolor*) from contaminated areas can have such high levels of MeHg (Pereira et al., unpublished data), which is also in agreement with levels found in benthic species from Hg contaminated areas (Locarnini and Presley, 1996). Contaminated feed (3 mm pellets) was produced by SPAROS company (Portugal) using a solution of MeHg chloride (CH_3HgCl; Sigma-Aldrich; prepared in ethanol) that was added during the process of pellet production, with a homogenous distribution of toxicant throughout the batch. Fish were feed at a daily feeding rate of 3% (as percentage of fish biomass, corresponding to 30 g food/day/kg of fish). Control fish were fed with food prepared in the same occasion but without adding MeHg (intrinsic MeHg levels lower than 0.01 $\mu g\ g^{-1}$).

As a premise to allow the comparison of the neurotoxic potential of both Hg forms, comparable daily exposure levels were sought in both experiments, which were translated in the values of 265 and 261 µg/day/kg of body weight, respectively for Hg(II) and MeHg. Daily exposure values were estimated considering the amount of food ingested and the corresponding mass of metal vehiculated for MeHg experiment, while for Hg(II) experiment it was considered that the difference between the initial nominal concentration and the concentration measured before recontamination (maximum value corresponding to 18% of the initial concentration) represents a rough measure of the Hg that was taken up, assuming negligible losses by volatilization and adsorption to tank surface (plausible due to daily recontamination) in line with previous findings (Oliveira Ribeiro et al., 2000).

In both experiments, fish were allowed to acclimatize to experimental conditions and routines for two weeks prior to Hg exposure. Fish were exposed to Hg(II) or MeHg for 1 (E1), 3 (E3), 7 (E7) and 14 (E14) days (Fig. 1). Thereafter, fish were transferred to clean water (post-exposure in Hg(II) experiment) or feed shifted to uncontaminated pellets (post-exposure in MeHg experiment) and allowed to recover for 14 (PE14) and 28 days (PE28). At each sampling time, 8 fish were sampled per condition ($n = 8$) and the brain was divided longitudinally in two sets, one for Hg quantification and the other for determination of oxidative stress related endpoints. Experiments had a total duration of 42 days and fish wellbeing deserved a permanent attention along that time, in accordance with national and international guidelines for the protection of animal welfare.

During the exposure period (at days 1, 3, 7 and 14) of Hg(II) experiment, water samples were collected in triplicate, 24 h after recontamination, to quantify total Hg (tHg) levels, in order to assess the toxicant bioavailability. Values of tHg in the exposure tanks immediately before recontamination varied between 0.05 and 0.36 $\mu g\ L^{-1}$, which would correspond to the minimum exposure concentration. Levels of tHg in the control tanks were below the detection limit (0.1 $ng\ L^{-1}$) throughout the experiment, as well as at days 28 and