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Inorganic mercury accumulation in brain following waterborne exposure elicits a deficit on the number of brain cells and impairs swimming behavior in fish (white seabream—*Diplodus sargus*)

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ABSTRACT

The current study contributes to fill the knowledge gap on the neurotoxicity of inorganic mercury (iHg) in fish through the implementation of a combined evaluation of brain morphometric alterations (volume and total number of neurons plus glial cells in specific regions of the brain) and swimming behavior (endpoints related with the motor activity and mood/anxiety-like status). White seabream (*Diplodus sargus*) was exposed to realistic levels of iHg in water (2 μg L^{-1}) during 7 (E7) and 14 days (E14). After that, fish were allowed to recover for 28 days (PE28) in order to evaluate brain regeneration and reversibility of behavioral syndromes. A significant reduction in the number of cells in hypothalamus, optic tectum and cerebellum was found at E7, accompanied by relevant changes on swimming behavior. Moreover, the decrease in the number of neurons and glia in the molecular layer of the cerebellum was followed by a contraction of its volume. This is the first time that a deficit on the number of cells is reported in fish brain after iHg exposure. Interestingly, a recovery of hypothalamus and cerebellum occurred at E14, as evidenced by the identical number of cells found in exposed and control fish, and volume of cerebellum, which might be associated with an adaptive phenomenon. After 28 days post-exposure, the optic tectum continued to show a decrease in the number of cells, pointing out a higher vulnerability of this region. These morphometric alterations coincided with numerous changes on swimming behavior, related both with fish motor function and mood/anxiety-like status. Overall, current data pointed out the iHg potential to induce brain morphometric alterations, emphasizing a long-lasting neurobehavioral hazard.

1. Introduction

Fish brain was demonstrated to be a target organ for mercury compounds, mainly methylmercury (MeHg). This Hg counterpart can easily cross the blood-brain-barrier (BBB), reaching the brain where it exerts toxicity (Farina et al., 2013). Conversely, so far, little is known about the neurotoxicity of inorganic mercury (iHg) in fish. Rouleau et al. (1999) postulated that the BBB is relatively impervious to iHg, working as a protective barrier against this Hg form. However, iHg compounds (e.g., $HgCl_2$) can act as a direct BBB toxicant, increasing thus its permeability in rodents (Zheng et al., 2003). In fact, iHg was able to reach fish brain after three days of exposure to environmentally realistic levels in water (Pereira et al., 2015). This is in line with other studies that documented the occurrence of iHg in the brain of fish (Berntssen et al., 2003; Mieiro et al., 2010; Korbas et al., 2011; Wang et al., 2015).

The prevalence of information on MeHg is likely based in the perception of its higher toxicity associated with a rapid uptake and distribution. Nevertheless, it has been also stated that the different forms of Hg share the same toxic chemical entity (De Flora et al., 1994) and, thus, neurotoxicity may depend mainly on the exter-

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nal bioavailability. For instance, iHg (as $HgCl_2$) appeared somewhat more toxic than MeHg to glial cells and neurons in immature aggregate cultures of rat telencephalon (Monnet-Tschudi et al., 1996). Furthermore, the majority of Hg in natural waters occurs in inorganic forms, while MeHg often contributes to less than 5% of the total waterborne Hg (Watras et al., 1998). In the light of such evidences, the neurotoxicity of iHg in fish is worthy of investigation. Such relevance is consubstantiated by the fact that MeHg can be demethylated in the brain, leading to iHg retention over time (Vahter et al., 1995; Allen et al., 2002). Autopsy samples taken years after exposure to MeHg revealed that inorganic species account for most of the remaining Hg in monkeys' brain (Charleston et al., 1995). It has been suggested that the long residence time of iHg in the brain is due to the formation of an insoluble complex with selenium (WHO, 1990).

Only a few neurotoxicological endpoints have been employed to evaluate the biological effects of iHg in fish, both in laboratory experiments (Berntssen et al., 2003; Wang et al., 2011) and under field exposures (Mieiro et al., 2011). Both Berntssen et al. (2003) and Mieiro et al. (2011) searched for changes in oxidative stress profiles, while Wang et al. (2011) assessed alterations in the protein expression. Moreover, a histopathological examination of fish brain was performed by Berntssen et al. (2003), revealing a widespread neuronal degradation as an effect of iHg deposition. Such evidences are in line with observations in the brain of mammals exposed to iHg, either humans (Eto, 1997) or rodents (Fujimura and Usuki, 2012). Apoptosis is a key mechanism of neuronal destruction in degenerative brain damage and it has been widely related with Hg exposure, mainly MeHg (Aschner and Ceccatelli, 2010; Aschner et al., 2013). For instance, the accumulation of Hg in glial cells leads to apoptosis, thus, contributing to neuronal degeneration (Ohgoh et al., 2000). Moreover, the exposure of a developing rat brain to iHg elicited a rapid inhibition of cell proliferation, particularly in the hippocampus and cerebellum, two regions of postnatal neurogenesis (Burke et al., 2006). In contrast, iHg led to a significant increase in the number of reactive glial cells in monkeys (Charleston et al., 1994). This study also revealed a significant increase in the volume of brain regions of iHg treated monkeys, possibly related with edema (Charleston et al., 1994). Despite iHg triggered apoptosis of rodents' neuronal cells and interfered with regeneration of brain cells, fish were never examined for such processes. In this context, the assessment of brain morphometric alterations in specific brain regions upon iHg exposure could provide, for the first time, an indication of potential brain degradation and regeneration.

Behavioral changes are widely described as an integrated manifestation of biochemical and structural disturbances (Scott and Sloman, 2004). As previously stated, certain anatomical regions of the rodents' brain seemed to be more vulnerable to iHg exposure, namely cerebellum and brain stem (Møller-Madsen and Danscher, 1986). Structural alterations in those regions regulating the motor system could lead to neurobehavioral impairments (e.g., ataxia, paresthesia, insomnia, tremors) in humans and wildlife (reviewed by ATSDR, 1999). In fact, the motor function of rats was compromised after exposure to iHg, as demonstrated by the negative geotaxis and beaker test (Moraes-Silva et al., 2014). Moreover, the chronic exposure to iHg can impair memory formation in rats, leading to a deficit on object recognition and aversive memories (Mello-Carpes et al., 2013). Fish exposure to iHg provided contrasting results, namely on the Atlantic salmon where no alterations were found on overall activity (Berntssen et al., 2003), while *Pomatoschistus microps* exhibited a reduced ability to swim (Vieira et al., 2009). In addition, Vieira et al. (2009) found concentration-dependent effects on swimming resistance and covered distance at concentrations equal or higher than 3 $\mu g\,L^{-1}$.

The evaluation of swimming performance is considered a paradigmatic endpoint of the fish motor status that is being widely used to evaluate neurobehavioral effects of aquatic contaminants (e.g., Little and Finger, 1990; Vieira et al., 2009; Almeida et al., 2010). In addition, the evaluation of the anxiety-like status of fish has been recently proposed to assess behavioral effects of toxic substances (Maximino et al., 2010, 2012). Though Hg was never associated with such symptoms in fish, human epidemiological studies established a relationship between Hg accumulation and depression (Wojcik et al., 2006), a major mood alteration.

According to Korbas et al. (2010), the accumulation of Hg in brain of fish does not imply *per se* toxicity, which can be partially related to the chemical form of Hg in the cells and protection mechanisms. Particularly, iHg can be stored as Hg selenide (HgSe) that is considered an inert nontoxic form (Korbas et al., 2010). Nevertheless, iHg can induce effects in the brain by causing a deficiency of essential Se-dependent enzymes, which is an indirect mechanism of iHg toxicity called the "selenium depletion hypothesis" (Korbas et al., 2010). Hg deposits were located exclusively in the lysosomes of brain cells of rats exposed to $HgCl_2$ and presented as a sequestration strategy (Møller-Madsen and Danscher, 1991).

The neurotoxicity of iHg in fish was never assessed by a comprehensive approach, comprising brain morphometric and behavioral evaluations. Hence, this study tackles the impact of iHg in the brain of fish (white seabream—*Diplodus sargus*) by the implementation of such approach. The study aims to clarify in what extent iHg accumulation induces morphometric alterations and impairment of swimming behavior, as well as the processes of brain recovery and reversibility of behavior alterations along with iHg depuration. Thus, a combined approach was designed to answer these questions, comprising: (i) stereological evaluation of the total number of cells (neurons plus glia) and volume of specific regions of the encephalon (medial and lateral pallia, optic tectum, hypothalamus and cerebellum); (ii) assessment of fish swimming behavior through diverse exploratory endpoints that include the evaluation of motor performance and potential mood/anxiety-like status of fish. Fish were surveyed after 7 and 14 days of iHg exposure, as well as after a 28 days post-exposure period. A realistic waterborne Hg concentration was tested (2 $\mu g\,L^{-1}$) in order to produce reliable data for environmental health assessment. The exposure level is comparable to those found in contaminated water of rice fields in China (1.5 $\mu g\,L^{-1}$) (Horvat et al., 2003) or during a flooding event in Kazakhstan (ranging between 1.6 and 4.3 $\mu g\,L^{-1}$) (Li et al., 2009).

2. Material and methods

2.1. Experimental set-up

The white seabream *D. sargus* was selected as a test organism since it is an abundant fish in estuarine systems, where Hg contamination is a frequent scenario (Pereira et al., 2009). In this context, *D. sargus* was previously employed to investigate the toxicokinetics of iHg (Pereira et al., 2015). Moreover, it is effortlessly maintained in the laboratory and is easy to handle, which is an important trait to perform behavior studies.

Juvenile specimens (sexually immature) were used in the experiment, provided by an Aquaculture Research Station (IPMA—Olhão, Portugal), from the same cohort (weight: 146 ± 14 g; total length: 19 ± 1 cm). At this stage of development, *D. sargus* have an undifferentiated gender. Fish were kept in 300 L fiberglass tapered-cylindrical tanks with an average initial density of 0.0068 $kg\,L^{-1}$, under a 10:14 light:dark photoperiod. A total of 12 tanks with the same characteristics were used in the experiment (6 for control condition and 6 for exposure to iHg). Each tank contained at the beginning of the experiment a total of 14 individuals. All tanks were placed in the same aquaria room. Seawater was renewed daily (around 80%) and fish were fed once a day with a commer-