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Impairment of mitochondrial energy metabolism of two marine fish by in vitro mercuric chloride exposure

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ABSTRACT

The goal of this work was to understand the extent of mercury toxic effects in liver metabolism under an episode of acute contamination. Hence, the effects of in vitro mercuric chloride in liver mitochondria were assessed in two commercial marine fish: Senegalese sole (*Solea senegalensis*) and gilthead seabream (*Sparus aurata*). Liver mitochondria were exposed to 0.2 mg L^{-1} of mercury, the average concentration found in fish inhabiting contaminated areas. Mercuric chloride depressed mitochondrial respiration state 3 and the maximal oxygen consumption in the presence of FCCP indicating inhibitory effects on the oxidative phosphorylation and on the electron transport chain, respectively. The inhibition of F1Fo-ATPase and succinate-dehydrogenase activities also corroborated the ability of mercury to inhibit ADP phosphorylation and the electron transport chain. This study brings new understanding on the mercury levels able to impair fish mitochondrial function, reinforcing the need for further assessing bioenergetics as a proxy for fish health status.

Mitochondria are the principal source of intracellular energy and thus, normal eukaryotic cell function relies entirely on its supply (McFarland and Turnbull, 2009). Besides ATP synthesis, mitochondria also regulate the mechanisms of calcium handling, as well as the metabolism of iron and certain amino acids, β-oxidation of free fatty acids, heme synthesis, production of free radical species, formation and export of Fe/S clusters and apoptosis (Duchen, 2004; Michel et al., 2012). Moreover, these organelles are being recognized as biosensors for drug-induced toxicity (Palmeira et al., 1995; Cannino et al., 2009; Moreira et al., 2011). The interaction with xenobiotics is recognized as inducing alteration of the bioenergetic reactivities and several stressors, such as cadmium (Cd), copper (Cu), nickel (Ni), mercury (Hg), iron (Fe) and dioxins have been identified as mitochondrial damaging agents (Belyaeva et al., 2004; Cannino et al., 2009; Cambier et al., 2009; Garceau et al., 2010; Nohl et al., 1989; Pardo et al., 2009). These environmental pollutants are often described as inducing mitochondrial permeability transition pore (MPTP), leading to mitochondrial swelling, activation of basal respiration and membrane depolarization, as well as decreasing the respiratory control ratio (RCR) and inducing oxidative stress (Belyaeva et al., 2004; Cannino et al., 2009; Cambier et al., 2009; Garceau et al., 2010; Nohl et al., 1989; Pardo et al., 2009).

Among environmental stressors, Hg is considered a priority hazardous substance with all its chemical forms exhibiting toxicological characteristics for both wildlife and humans (SEC, 2005). Mitochondria have been referred as the major target for Hg toxicity (Belyaeva et al., 2008). Specifically, Hg alters mitochondrial

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permeability by modifying transport membrane proteins (Nigsberg et al., 2001), and inducing efflux of intra-mitochondrial Ca^{2+} (Chávez and Holguín, 1988). Moreover, Hg acts as an uncoupling agent, stimulating state 4 respiration and decreasing the mitochondrial membrane potential (Palmeira and Madeira, 1997; Santos et al., 1997), affecting both the mitochondrial respiratory chain and the membrane permeability (Belyaeva et al., 2008). Messer et al. (2005) also found that inorganic Hg impairs mitochondria via oxidative stress mechanisms. Despite that mitochondria surveys are mostly associated with human health, they are being increasingly acknowledged as useful tools for environmental risk assessment, particularly in fish (Cambier et al., 2009; Lerebours et al., 2010).

In Hg contaminated areas, studies with fish have demonstrated high Hg burdens in the liver (Navarro et al., 2009; Mieiro et al., 2012), 70% of which was accumulated in the inorganic form (I-Hg) (Mieiro et al., 2009). Liver has been acknowledged to be the primary target organ for Hg (Wang et al., 2013) as well as the main detoxifying organ, being able to demethylate organic mercury (O-Hg) into I-Hg forms (Gonzalez et al., 2005). Yasutake and Hirayama (2001) found that Hg demethylation occurs mainly in mitochondria, which is in agreement with findings by Wang et al. (2013) that $HgCl_2$ affects several proteins related with mitochondrial function. Branco et al. (2012) also found more extensive and severe lesions in zebrafish livers when exposed to I-Hg than to O-Hg. For the above-mentioned reasons, the present work focused on the effects of $HgCl_2$ in fish liver mitochondria. In agreement, the main goal was to simulate an episode of acute contamination in order to understand the extent of Hg toxic effects in liver metabolism in two commercially important marine fish: Senegalese sole (*Solea senegalensis*) and gilthead seabream (*Sparus aurata*). To the best of our knowledge, this is the first study to evaluate in vitro I-Hg effects in fish mitochondria.

Juvenile hatchery-brood Senegalese sole (total length = 13.0 ± 2.4 cm; total wet weight = 40.0 ± 4.3 g) and gilthead seabream (total length = 14.3 ± 0.99 cm; total wet weight = 41.7 ± 11.2 g), all from the same cohort, were acquired from two local aquacultures and used as test subjects. At the laboratory fish were kept in a closed-system recirculation arrangement of polyvinyl tanks containing 180 L of seawater. Each species was kept in separated and replicated tanks. A weekly 25% water change was performed to maintain constancy of the parameters. Temperature was held constant at 17.9 ± 3.7 °C and the photoperiod was set at 12:12 h light:dark. Water parameters were monitored weekly and were similar to all rearing conditions: salinity = 26.2 ± 0.1, pH = 7.8 ± 0.1, dissolved oxygen = 91.1 ± 4.1% and unionized ammonia (NH_3) was never higher than 0.04 ± 0.02 mg L^{-1}. Fish were fed daily with species-specific commercial pellets (from Sorgal, Portugal). Total Hg was determined in commercial diets and both diets were in compliance with the European legislation for contaminants in fish feed (DI 2010/6/EU) showing less than 0.2 mg kg^{-1} of Hg.

After two weeks of acclimatization, randomly selected fish were dissected on ice for liver harvesting and mitochondria were isolated by conventional tissue disruption methods (Gazotti et al., 1979). Each independent replica (n = 3) consisted of a pool of 3 livers. Each pool was ground at 4 °C in a glass potter with a Teflon piston, applying 10 strokes. The homogenization buffer was adapted from Cambier et al. (2009) containing: 140 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES, 10 mM EDTA, 0.5% of bovine fatty acid free serum albumin (BSA) (pH 7.4). The final washing medium was the same as the homogenization buffer without BSA and adjusted at pH 7.4. Protein content was determined by the biuret method (Gornall et al., 1949) calibrated with bovine serum albumin.

Previous work showed that Hg concentrations found in the livers of fish chronically exposed to Hg ranged from 0.5 to 1 mg kg^{-1} wet weight (Mieiro et al., 2009). In the present study the same range of concentrations was tested directly in mitochondria to evaluate a condition of acute exposure to those concentrations. The different concentrations of exposure were prepared from a 100 mg L^{-1} stock solution of $HgCl_2$ that was prepared in ultra-pure water every week and kept refrigerated. Liver mitochondria were initially exposed to 0.5 mg L^{-1} of $HgCl_2$, since this concentration has been described as suitable to reflect an eventual Hg spill (Rocha et al., 2013) and was the lowest Hg concentration found in fish livers from contaminated areas (Mieiro et al., 2009). However, and since no mitochondrial respiration was observed at this concentration (0.5 mg L^{-1} of $HgCl_2$), lower concentrations of Hg were tested until it was possible to detect mitochondrial respiration at 0.2 mg L^{-1} of $HgCl_2$. Accordingly, mitochondria were incubated with 0.5, 0.4, 0.3 and 0.2 mg L^{-1} of $HgCl_2$ at 20 °C for 3 min, as previous work with liver mitochondria determined that time-dependent $HgCl_2$ effects are maximal at 3 min (Palmeira and Madeira, 1997).

The oxygen consumption of isolated mitochondria was monitored polarographically using a Clark oxygen electrode (Estabrook, 1967) in a 1 ml thermostatically controlled chamber (Hansatech, OXY1 System). Mitochondria (1 mg) were suspended under constant stirring, at 20 °C, in 1 ml of standard respiratory medium (MRB) (130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 5 mM KH_2PO_4, 50 μM EDTA, and 5 mM HEPES, pH 7.4). The oxygen consumption of isolated mitochondria was measured at least 4 times for each replica both with and without $HgCl_2$. $HgCl_2$ was added to the respiratory medium (MRB) with mitochondria and allowed to incubate for 3 min before the addition of succinate (5 mM). State 3 of respiration was induced by adding 100 nmol of ADP. The oxygen consumption was also measured in the presence of oligomycin (0.5 μg/mg protein) and 1 μM carbonylcyani de-p-trifluoromethoxyphenylhydrazon (FCCP). Any decline on the respiratory rate in the presence of FCCP reflects an inhibitor effect at the level of the respiratory chain. Oligomycin is a specific inhibitor of the ATP synthase. In this regard, it is important to note that oligomycin reduces respiration to a residual rate due to H^+ leak (phospholipid membrane H^+ permeability) since it promotes the full inhibition of ATP synthase by inhibiting the H^+ translocation through Fo-ATP synthase.

Evaluated according to Chance and Williams (1955) RCR: it is the ratio between the oxygen consumption rate in the presence of the respiratory substrate and ADP (state 3) and the rate after ADP consumption (state 4). State 3 corresponds to the maximum flow of electrons during ATP synthesis and translated in a rapid respiration rate, whereas state 4 is the respiratory rate necessary to counteract the passive leak of protons (increase in state 4 is often linked to the increase of proton leak through inner mitochondrial membrane) whereas the decrease of state 4 is often linked to inhibitory effects on the respiratory chain.

Succinate-dehydrogenase (SDH) activity was polarographically determined in agreement with Singer (1994). The reaction was carried out at 25 °C in 1.3 mL of standard respiratory medium (as in mitochondrial respiration) supplemented with 5 mM succinate, 2 μM rotenone, 0.1 μg antimycin A, 1 mM KCN and 0.3 mg Triton X-100. After the addition of freeze–thawed mitochondria (0.25 mg), the reaction was initiated by adding 1 mM phenazine-metasulfate (PMS).

F1Fo-ATPase activity was determined spectrophotometrically at 660 nm, in association with ATP hydrolysis. The reaction occurred at 37 °C, in 2 mL reaction medium (125 mM sucrose, 65 mM KCl, 2.5 mM $MgCl_2$ and 5 mM Hepes, pH 7.4). After the addition of freeze-thawed mitochondria (0.25 mg), the reaction was initiated by adding 2 mM Mg2+-ATP, in the presence or absence of oligomycin (1 μg/mg protein). After 10 min, 1 mL of 40% trichloroacetic acid was added to stop the reaction. Then, an aliquot of 1 ml was