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A method to measure total antioxidant capacity against peroxyl radicals in aquatic organisms: Application to evaluate microcystins toxicity

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ABSTRACT

Determination of total antioxidant capacity, instead of the measurements of limited number of antioxidants, is very important for the understanding of how antioxidants interact with reactive oxygen species (ROS). Several techniques already exist with this propose, although some of them are extremely time-consuming. A new methodology is proposed, based on the detection of ROS by fluorometry (ex/em: 485/520 nm) employing 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA) as substrate. Supernatant of homogenized samples from different organs (gill, muscle, liver, and brain) of the teleost fish *Jenynsia multidentata* (Anaplebidae) were exposed to peroxyl radicals generated by thermal (35 °C) decomposition of 2,2′-azobis (2 methylpropionamidine) dihydrochloride (ABAP, 4 mM). Different protein concentrations (0.5, 1, 2 and 8 mg/ml) were assayed to get the best signal and curve fitting of fluorescence data over time (30 min). Total antioxidant capacity against peroxyl radicals was estimated as the difference in ROS area with and without ABAP, relative to the fluorescence registered without ABAP. For application of this methodology, *J. multidentata* specimens were exposed for 24 h to microcystins, cyanotoxins known to induce oxidative stress. Almost all organs showed a lower antioxidant capacity ($p<0.05$) in samples with 8 mg proteins/ml, when compared to protein content of 1–2 mg/ml. In liver samples, higher ($p<0.05$) free iron content was determined in samples with 8 mg proteins/ml. Sensitivity test employing GSH spiked in homogenized samples showed the protocol efficiency in detecting total antioxidant capacity. In the test with microcystins a dose-dependent decrease ($p<0.05$) of antioxidant competence in gills and brain and an inverse result with liver samples were observed. The use of antioxidant defenses was efficient in avoiding oxidative damage, as the content of oxidized proteins was not altered. Data obtained show the potential of this new methodology to be used in ecotoxicological studies.

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1. Introduction

The oxygen paradox arises from the fact that this gas is essential for energy production but at the same time cellular metabolism is continuously generating reactive oxygen species (ROS) that in high concentrations can be extremely harmful to cell constituents. Several exogenous molecules (such as organic or inorganic environmental pollutants) or endogenous processes (inflammation, respiratory burst) are known to enhance ROS formation leading to the depletion of one or more antioxidants and even to oxidative damage (Shackelford et al., 2000; Valavanidis et al., 2006). Organisms protect themselves from such harmful effects with a number of enzymatic and non-enzymatic defenses that together constitute a complex antioxidant defense system (Halliwell and Gutteridge, 2007). In this respect, the intracellular production of ROS does not necessarily imply cellular toxicity, but oxidative stress will occur when ROS formation exceeds antioxidant defense capability or disrupt redox signaling, affecting cell functionality (Jones, 2006). In fact, oxidative stress has been linked to a number of cellular toxic processes, including damages to proteins, membrane lipid peroxidation, enzyme inactivation and DNA breakage, which can lead to various pathologies including chemical carcinogenesis, heart disease, reperfusion injuries, rheumatoid arthritis and ageing (Sohal, 2002; Klaunig and Kamendulis, 2004; Halliwell and Gutteridge, 2007). Antioxidant responses and oxidative stress parameters have been also used as biomarkers of pollutants exposure, due to the fact that many pollutants are known to enhance intracellular formation of ROS through different mechanisms including the redox cycle, the cytochrome P450-dependent oxidative metabolism of aromatic hydrocarbons and the Fenton reaction in the presence of some transition metals (Regoli, 2000; Halliwell and Gutteridge, 2007; Monserrat et al., 2007).

From the stand point of pathologies comprehension as well as for the evaluation of pollution effects, it's useful to understand how antioxidants react to the enhancement of oxyradicals production. In this way, classical oxidative stress studies measure antioxidants efficiency individually, and variations in its levels or activities are used to indicate ROS mediated toxicity. However, since the antioxidant systems can act in a cooperative way (Winston et al., 1998), a more holistic determination of antioxidant capacity can provide a better understanding of organisms resistance to toxicity caused by ROS, than the measurements of limited number of antioxidants. Also, several studies with aquatic organisms like fish have been demonstrating that it is necessary to consider the different antioxidant capacity of each tissue when analyzing the responses of an organism to oxidative stress. Prieto et al. (2007) observed higher lipid peroxidation (LPO) in kidney compared with liver and gills of tilapia fish. In a study with golden grey mullet, however, gills had the greater susceptibility to LPO followed by liver. In this fish species kidney was the assessed organ that had the lower vulnerability to oxidative damage, what probably was related to its higher antioxidant basal levels (Oliveira et al., 2008). In another study, higher thiobarbituric reactive substances (TBARS, a measure of malondialdehyde content) were found in liver of rainbow trout when compared with gills, intestine and brain (Smith et al., 2007). For the fish *Corydoras paleatus*, Monserrat et al. (2008) observed the highest concentration of reduced glutathione (GSH) in muscle, followed by liver, gills and last by brain. Concerning activity of phase II enzymes, Ferrari et al. (2007) observed lower glutathione-S-transferase (GST) activity in kidney of juvenile rainbow trout when compared with liver. Oliveira et al. (2008) found greater GST activity in kidney compared to gills in golden grey mullet. Finally, Monserrat et al. (2008) registered maximum GST activity in liver of *C. paleatus*, followed by brain and, at last, by gills and muscle.

Therefore, in virtue of these sometime conflicting results, attempts have been made to determine the total antioxidant capacity of body fluids and tissues homogenates rather than go to the trouble of identifying what has happened to each component of the complex antioxidant defense network (Winston et al., 1998; Halliwell and Gutteridge, 2007). In fact, a number of methodologies have been developed and used to determine the total antioxidant capacity of various biologic samples (for a review see Pryor and Cao, 1999; Llesuy et al., 2001), although some of them are extremely time-consuming, a problem when large quantities of samples are needed to measure.

Herein, we describe a simple, rapid and reliable method, based on the detection of ROS by fluorometry (ex/em: 485/520 nm) employing 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA) as substrate. Peroxyl radicals are generated by thermal decomposition at 35 °C of 2,2′-azobis (2 methylpropionamidine) dihydrochloride (ABAP) in different tissues of the fish *Jenynsia multidentata* and the tissue total absorbance capacity of peroxyradical is monitored by the fluorescence signal emitted by the reaction between ROS and H_2DCF-DA.

2. Materials and methods

The fish used as biological model in this study was *J. multidentata* (Anaplebidae), collected in a reference site in Patos Lagoon estuary, considered non-polluted after an biomonitoring study that analyzed biomarker responses, including total antioxidant capacity (Geracitano et al., 2004). After being collected, fish were immediately brought to the laboratory and allowed to acclimate during one week in a 300 l tank equipped with a filtering system (2‰ salinity; pH 7.0; 7.20 mg O_2/l, 20 °C). After this period, 50 fish were used for the new procedure tests and other 77 fish were employed in the experiment with microcystin, which was done to validate the new method.

2.1. *Tissue samples preparation*

After the acclimation period or the exposure to microcystins, fish were anesthetized in ice-cold water for the dissection of gills, liver, brain and muscle. Tissue samples to be used in all measurements were prepared through the homogenization (1:5 — w/v) in a Tris–HCl (100 mM, pH 7.75) buffer plus EDTA (2 mM) and Mg^{2+} (5 mM) (Gallagher et al., 1992). The homogenates were centrifuged at 10,000 ×*g* during 20 min at 4 °C and the supernatants resulting from this centrifugation were employed for all measurements described here. Previously