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Short communication

Stability of oxidative stress biomarkers in flathead mullet, *Mugil cephalus*, serum during short-term storage

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ABSTRACT

The production of reactive oxygen species (ROS) by aquatic organisms in response to stressful factor is a common feature in aquatic environment. Thus, the evaluation of parameters related to oxidative stress in fish, in first instance those showing the redox potential, is notable to characterize the environmental conditions.

Short-term storage of samples is essential when blood must be transported from collection sites to laboratories. Therefore, excessive delays in processing might compromise the reliability of results. The aim of the present study was to assess the effect of short-term storage time at +4 °C on total oxidant status (TOS), total antioxidant capacity (TAC) and oxidative stress index (OSI) in flathead mullet (*Mugil cephalus*) serum. After blood collection, all sample were analyzed and the assays were repeated after 24, 48 and 72 h from sampling. results showed a significant effect of short-term storage on TOS and TAC ($P \leq 0.0001$), while the statistical significance of the linear regression study for these parameters was reduced as consequence of storage. These results highlight that the activities of oxidants and antioxidants in flathead mullet serum change during short-term storage at 4 °C and should be assesses as soon as possible from collection.

1. Introduction

During catabolic processes, free radicals and other free radical intermediates, also known as reactive oxygen species (ROS), are generated. They represent natural by-products of the normal metabolism of oxygen and have important roles in maintenance of cellular activity, including inter- and intracellular signaling (Cadenas, 1989; Halliwell and Gutteridge, 2007).

Several internal or external factors can lead to an increase of ROS, responsible of serious oxidative damage to cellular components. In order to counteract the potential oxidant activity of ROS and maintain the redox balance in cells, animal beings have an effective antioxidant defense system being, by definition, a combination of substances, partly depending on dietary supplement, capable to inhibit the oxidation of self-molecules (Davies, 2000). In spite of these antioxidant defenses, when the oxidant activity overcomes the neutralizing activity of antioxidants, the organism slips into the condition called oxidative stress (OS). This can damage several cellular components such as lipids, proteins, free amino acids, deoxyribonucleic acid (DNA) and carbohydrates (Lykkesfeldt and Svendsen, 2007; Toyokuni, 1999). Thus, ROS reaction with lipids is considered one of the most prevalent mechanism of cell damage (Halliwell and Gutteridge, 2007).

As regards aquatic animals, changes in environmental conditions, such as thermal stress, ultraviolet radiation and anthropogenic contamination, can lead to OS (Martínez-Álvarez et al., 2005; Grim et al., 2010; Madeira et al., 2013). According to Valavanidis et al. (2006), several xenobiotics are responsible for ROS production by aquatic organisms, causing oxidative damages and adverse effects on health status of living beings. Therefore, the production of ROS by organism is very common in aquatic environmental stress and OS is an important component of the stress response in aquatic organisms, being the evaluation of fish OS parameters a good biomarker in the assessment of the quality of the aquatic environment (Pathiratne and Hemachandra, 2010). So,

Abbreviations: ROS, reactive oxygen species; TOS, total oxidant status; TAC, total antioxidant capacity; OSI, oxidative stress index; ROMs, reactive oxygen metabolites; FRAP, ferric reducing antioxidant power; DEPPD, *N,N′*-diethyl-para-phenylendiamine; *t*-BHP, *tert*-butyl hydroperoxide; TPTZ, 2,4,6-tris(2-pyridyl)-*s*-triazine; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; CAT, catalase; GST, glutathione *S*-transferase.

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Lesser (2006) showed that in marine environments, among other alterations, oxidative damage to DNA induces lesions such as deletions and mutations. At the same time, the induction of antioxidant defense system as consequence of increased ROS levels is a common response of marine fish and invertebrates (Abele and Puntarulo, 2004). Thus, according to literature fish provide a good model for monitoring the toxicity in aquatic systems because they are extremely sensitive to pollutants, have the ability to metabolize xenobiotics and exhibit a very high bioaccumulation rate of dissolved chemicals relative to their concentration (da Rocha et al., 2011; Fazio et al., 2013a,b).

Accurate measurement of OS biomarkers is essential for correct interpretation of OS disorders in both clinical and research setting. On the other hand, analytical measurements of OS markers are difficult because of the short half-life and high reactivity of the majority of ROS and the applicability of measurement methods (Pitocco et al., 2010). Blood samples are the appropriate biological materials for assessing the status of oxidants and antioxidants. A unique system for the evaluation of oxidative stress levels and antioxidant capacity in blood has been developed (Alberti et al., 2000). This evaluation approach is based on the free radical analytical system that mainly analyses lipid hydroperoxides, which are relatively stable in blood. This system has been used for both animal and human samples, confirming its applicability (Vassalle et al., 2006; Pasquini et al., 2008). Anyhow, analytical methods describing the redox potential of samples are usually applied as first evaluation and, as regards fish, they are applied both in specimens kept in captivity (Hoogenboom et al., 2012; Di Marco et al., 2008) and in fish caught from natural environments for biomonitoring studies (Livingstone, 2001; Amado et al., 2009; Lushchak, 2011).

OS results are often influenced by a number of pre-analytical variables. Among these, the short-term storage represents an important variable to the stability of OS biomarkers (Cesarone et al., 1999). According to literature, not many reports on the storage stability of total oxidants/antioxidants in biological samples can be found (Cavalleri et al., 2004; Celi et al., 2010; Jansen et al., 2013a,b), but not research was found in fish. Some authors (Faggio et al., 2013) showed the influence of storage on hematological parameters, underlining that long-term storage modifies the results of analyses in flathead mullet (*Mugil cephalus* Linnaeus, 1758). Flathead mullet, known as a sentinel organism for its high sensibility to anthropogenic compounds (Ferreira et al., 2005; Gorbi et al., 2005; Tsangaris et al., 2011), is considered a suitable fish species for biomonitoring studies.

The aim of this study was to establish the effect of short-term storage (24, 48, 72 h at 4 °C) on the stability in flathead mullet serum of two OS parameters employed in the assessment of total oxidant and antioxidant conditions. Total oxidant status (TOS) and total antioxidant capacity (TAC) were analysed as reactive oxygen metabolites (ROMs), mainly represented by lipid hydroperoxides, and ferric reducing antioxidant power (FRAP), respectively.

2. Materials and methods

2.1. Experimental procedure

Thirty adult male flathead mullets, caught from Ganzirri Lake (Sicily, Italy), were used. The animals, weighing 300.00 ± 30.00 g and being 30.00 ± 2.00 cm fork length (mean ± SD), were considered as healthy on the basis of an external examination for any signs of abnormalities or infestation. Based on their weight and length, all fish were considered sexually mature and with an age between two and four years (McDonough et al., 2005). Fish were acclimated before sampling for 3 weeks in 800-L tanks with flowing seawater (temperature: 18 °C, salinity: 39 ppm and pH 7.5) to restore the effects of capture, handling and transport. Blood withdrawal was always performed between 8.00 a.m. and 12.00 a.m. and feeding was stopped 24 h before blood collection. Flathead mullets were quickly dip-netted from the tanks and immediately anesthetised with 2-phenoxyethanol (1:300 v/v) in a 60-L bucket, before submitting them to blood collection from caudal vein using a 2.5 mL syringe. For each fish, 0.6 mL of blood was collected and the sample, stored in eppendorf tubes with no additive, was left to clot. Serum samples, collected within two hours after centrifugation (3000 rpm for 10 min, 4 °C), were split into four aliquots. The first one (T0) was immediately analysed for TOS and TAC parameters. The other aliquots, stored at 4 °C, were analysed after 24, 48 and 72 h from sampling (T1, T2 and T3, respectively) in order to evaluate the effect of storage time on the assessed parameters.

2.2. Total oxidant status (TAS) and total antioxidant capacity (TAC) evaluation

TOS, measured as ROMs, was evaluated using the radical cation *N*,*N*′-diethyl-*para*-phenylendiamine (DEPPD), as described by Alberti et al. (2000) with some modifications. 10 μL of samples in duplicate were added to wells of a microtitre plate. Subsequently, 200 μL of a solution containing 0.37 mM DEPPD and 2.8 mM iron (II) sulfate heptahydrate in 100 mM acetate buffer, pH 4.8, was added to each well. After incubation (30 min at 25 °C) absorbance was recorded at 530 nm using a microplate reader (Model 550, BioRad). The standard curve was constructed using *tert*-butyl hydroperoxide (t-BHP) at concentrations ranging from 125 to 1000 μM (Pearson's correlation coefficient: $r = 0.99$).

TAC was evaluated performing the FRAP assay as indicated by Benzie and Strain (1996). Firstly, 300 mM sodium acetate buffer, pH 3.6, 10 mM 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ) in 40 mM HCl and 20 mM iron(III) chloride hexahydrate were mixed in a volume ratio of 10:1:1 to generate FRAP fresh daily prepared solution. Subsequently, 10 μL of samples in duplicate were added to 300 μL of FRAP solution in wells of a microtitre plate and the absorbance of the reaction mixture was recorded at 593 nm after 5 min of reaction using the microplate reader. The standard curve was constructed using iron(II) sulfate · $7H_2O$ at concentrations ranging from 62.5 to 1000 μM (Pearson's correlation coefficient: $r = 1$).

Moreover, TOS/TAC ratio was accepted as OSI, an indicator of redox balance.

2.3. Statistical analysis

Analytical data, represented as mean (M) ± standard error of mean (SEM), are the averages of three analyses carried out by the same operator. Samples exhibited parallel displacement to the standard curve. The overall intra-assay coefficient of variation was <9%.

The influence of sampling time on TOS, TAC and OSI was assessed by one-way analysis of variance (ANOVA) for repeated measures. Bonferroni's multiple comparison test was applied for post hoc comparison. Linear regression analysis was used to study the trend of values measured at T0, T1, T2 and T3 for TOS and TAC, respectively. Statistical differences were considered for P values ≤0.05. Data were analyzed using statistical software Prism v.4.00 (Graphpad Software Ltd., USA, 2003)

3. Results

Table 1 shows the values of TOS and TAC, measured as ROMs and FRAP, respectively, and OSI together with statistical differences during the different times of storage (2, 24, 48 and 72 h after collection) at 4 °C. One-way repeated measures ANOVA showed a