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Myofibrilar functional dysregulation in fish: A new biomarker of damage to pesticides

Andrea C. Crupkin[a], Fernando G. Iturburu[b], Marcos Crupkin[c], Mirta L. Menone[b,*]

[a] Laboratorio Ecotoxicología, Comisión de Investigaciones Científicas (CIC), Facultad Cs. Ex. y Nat.-UNMDP, Funes 3350, 7600 Mar del Plata, Argentina
[b] Instituto de Investigaciones Marinas y Costeras (IIMyC)- UNMDP, CONICET, Facultad Cs. Ex. y Nat. Lab. Ecotoxicología, Funes 3350, 7600 Mar del Plata, Argentina
[c] Facultad de Ciencias Agrarias, UNMDP, Ruta 226, km 73.5, Balcarce, Argentina

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ABSTRACT

Endosulfan (ES) modifies the ultrastructure of skeletal muscle fibers and causes changes to the swimming behavior of fish. The objectives of the present work were to evaluate, in fishes of *Australoheros facetus*, 1) the integrity of myofibrils (Mf) by the analysis of SDS-PAGE profiles, and 2) the functionality of Mf through the microscopically monitoring of the contraction and changes in Mg^{2+} Ca^{2+}- ATPase and Mg^{2+}(EGTA) -ATPase activities. As expected, after the addition of the contraction buffer, control fish Mf contracted. On the contrary, Mf from fish exposed at 0.5 µg/L ES showed a partial contraction and none of the fish exposed at 10 µg/L ES contracted. As judged by its high Mg^{2+} Ca^{2+} ATPase activity and low Mg^{2+} (EGTA) ATPase activity, control Mf showed good functionality. In Mf from fish exposed to 0.5 and 10 µg/L ES the activities of these enzymes were similar, suggesting denaturation or degradation of some component of tropomyosin–troponin complex. SDS-PAGE patterns of Mf from fish exposed to ES showed degradation of the myosin heavy chain and of tropomyosin. Similar values of lipid peroxidation (TBARS) were found in both control and exposed Mf, suggesting that lipid oxidation was not be related to the above-mentioned changes. The observed effects expand the knowledge of ES action in muscles and could be used as biomarkers of damage in fishes exposed to organochlorine compounds like the insecticide endosulfan.

1. Introduction

Some pesticides generate neurotoxic effects in aquatic organisms. To evidence these effects, the enzyme acetylcholinesterase (AChE) and perturbations in the swimming behavior of fish are the biomarkers commonly used in ecotoxicology. The AChE activity removes the neurotransmitter acetylcholine (ACh) from the synaptic cleft through hydrolysis (Kumar et al., 2016), mainly in organisms exposed to organophosphorus and carbamate pesticides. Inhibition of AChE activity correlates with a decrease in swimming behavior -a second type of biomarker- in fishes exposed to high concentrations of organophosphorus pesticides (Bonansea et al., 2016) and to sublethal concentrations of the organochlorine endosulfan (ES) (Ballesteros et al., 2009). However, there is not always a direct relationship between both AChE activity and behavior (Ballesteros et al., 2017). In this sense, Da Cuña et al. (2011) showed no changes in this enzyme but hypo-activity and erratic swimming in *Cichlasoma dimerus* exposed to ES, suggesting that alterations in the swimming activity can be caused through other pathways. As an alternative, we propose new biomarkers (contraction of isolated myofibrils and ATPase activity) to explore if ES directly alters the structure and functionality of the muscle tissues.

Myofibrils are the functional units of skeletal muscle. Muscle contraction occurs by cyclic movements of myosin heads on thin filaments produced by the mechanical force generated by conformational changes in tropomyosin- troponins complex after Ca^{2+} capture. The energy necessary to produce these movements is obtained by ATP hydrolysis in the presence of Mg^{2+} and Ca^{2+}, reaction catalyzed by the Ca^{2+}ATPase present in myosin heads (Roura, 1992). In this way, normally contraction of muscle occurs when neurotransmitter ACh produced by nervous stimulation induces membrane depolarization and releases Ca^{2+} from the reticulum sarcoplasmic. Relaxed muscle takes place in the absence of nervous stimulus. In this condition, Ca^{2+} ions return to reticulum sarcoplasmic membrane spending energy to overcome the concentration gradient. On the other hand, a significant higher activity of the Mg^{2+} Ca^{2+}- ATPase than Mg^{2+} (EGTA) –ATPase enzymes evidence the normal functioning of the tropomyosin- troponins complex while if it is deregulated there is no difference between these activities. Depending on fish species, its nutritional and gonadal status, Mg^{2+} Ca^{2+}- and Mg^{2+} (EGTA)- ATPase activities in healthy fish muscle range between 0.20 and 1.0 µmol P/ mg protein. min and 0.05–0.4 µmol P/

* Corresponding author.
E-mail address: lujanm@mdp.edu.ar (M.L. Menone).

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mg protein. min, respectively (Roura et al., 1995; Pagano et al., 2004; present work).

Mishra and Shukla (1995) demonstrated that ES exerts marked ultrastructural alterations in the skeletal muscle fibers of the fish, that correlate with an inhibitory effect on electron transport and affects ATP synthetase complex leading to impairment in mitochondrial bioenergetics. More recently, ultrastructural changes (breakage, collapse of Mf, disorganized myosin and actin filaments) in muscle tissues have been described in fish exposed to other synthetic compounds (Lee et al., 2014).

Considering the effects of ES on – the ultrastructure of the skeletal muscle fibers (Mishra and Shukla, 1995), – the swimming behavior (Ballesteros et al., 2009; Maynart Pereira et al., 2012), and that ES generates oxidative stress increasing the levels of thiobarbituric acid reactive substances (TBARs) able to react with proteins, lipids and nucleic acid producing denaturation and aggregation of biopolymers (Tironi and Tomas, 2000), the objectives of the present work were to evaluate, in fishes of *Australoheros facetus*, 1) the integrity of myofibrils (Mf) by the analysis of SDS-PAGE profiles, and 2) the functionality of Mf through the microscopically monitoring of the contraction and changes in Mg^{2+} Ca^{2+}- ATPase and Mg^{2+}(EGTA) -ATPase activities.

2. Material and methods

2.1. Fish exposure

Adult fish were collected in non- anthropized freshwater bodies around Mar del Plata (Buenos Aires Province, Argentina, 37° 53´ South, 57° 59´ West) and acclimatized for 2 months to laboratory conditions in 140 L tanks. Healthy specimens with approximately the same size (mean total length (± SD): 10.1 ± 1.4 cm; mean weight (± SD): 24.1 ± 11.4 g) were selected.

Two concentrations of ES were tested: 0.5 and 10 µg/L. These concentrations were selected according to previous studies of ES in *A. facetus* in which sublethal effects were detected (Crupkin et al., 2013). Both concentrations are environmentally relevant since values from 0.004 to over 2 µg/L ES have been reported in Argentina (Silva et al., 2005; Gonzalez et al., 2012), and higher than 100 µg/L in other countries like USA (Mersie et al., 2003).

A negative control containing tap water with 50 µl/L of DMSO (representing a concentration of 0.004%, the same concentration of DMSO used in ES treatments) was included. A solution of endosulfan (6, 7,8, 9, 10, 10 – hexachlor – 1, 5, 5, 6, 9, 9- exahydro – 6,9 – metane-2,4,3 –benzo (e) dioxatiepin -3-oxide) (70:30 alpha-: beta- isomers) was used. We prepared two stock solutions by diluting alpha- endosulfan (Riedel-de Haën, CAS 959-98-8) or beta-endosulfan (Riedel-de Haën, CAS 33213-65-9) in dimethyl sulfoxide-DMSO (Mallinckrodt). The concentrations of the stock solutions were 1600 mg/L for alpha-ES and 800 mg/L for beta-ES. Afterwards, we prepared the exposure medium using the following amounts of both isomers in tap water in order to obtain a relation according to commercial formulas (alpha-isomer 70% and beta-isomer 30%). For the treatment of 5 µg/L: 22 µl alpha-ES from a 1/10 dilution of the stock, plus 18.7 µl beta-ES from a 1/10 dilution of the stock were used. For the treatment of 10 µg/L: 44 µl alpha-ES from a 1/10 dilution of the stock, plus 7.5 µl beta-ES from a 1/2 dilution of the stock were used. The acute static experiment was conducted using one glass tank of 30 L per treatment, containing six fish per treatment (negative control, 0.5 and 10 µg/L). The concentrations of endosulfan in the exposure medium were quantified using a GC- ECD, data previously reported in Crupkin et al. (2013).

The experiments were conducted in an aquarium illuminated with fluorescent lamps setting 12:12 light: dark periods. Mar del Plata city tap water was used for the experiments. The water temperature was of 18 °C, pH of 8.2 ± 0.2, mean total hardness of 270.2 mg/L CaCO3 and mean alkalinity of 160 mg/L CaCO3. All fishes were fed during acclimation with a commercial food (pellet Shulet composed by 45% protein, 2% lipids, 3% fiber, 1.95–2.99% Ca, 1–1.4% P), but were kept starved during the exposure to ES.

All fish were sacrificed after an exposure period of 24 h by transecting the spinal cord using a fresh razor blade. This procedure was evaluated and approved by the Animal Ethical Committee at the National University of Mar del Plata (CICUAL/UNMDP) (OCA 146/15). Muscle was dissected, weighed and immediately stored for few hours at 4 °C before its analysis.

2.2. Extracts and measurement of biomarkers in muscle tissue

2.2.1. Isolation of myofibrils

The isolation of the myofibrils were prepared according to Yasui et al. (1975), with a little modification of the extraction and purification buffer: 20 mM Tris-maleate, 100 mM KCl, pH 7, without Triton X-100 because it inactivates contractile proteins (Roura et al., 1992). Muscle subsamples of 4 g were mechanically shredded and homogenized using 30 mL standard buffer standard (0,1 M KCL-20 mM Tris-maleate, pH = 7.0) at 4 °C during 3 min in a "Virtis 45" homogenizer. The homogenate was centrifuged at 850*g* during 15 min in a refrigerated centrifuge (HERMLE Z36HK). The pellet was resuspended in the same buffer and the centrifugation and resuspension was repeated two times more.

Protein concentration was determined in aliquots of myofibril suspensions by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard.

Contraction of isolated myofibrils was monitored microscopically, at ambient temperature, by Phase Contrast Microscopy OLYMPUS CX31 RTSF, according to the procedure previously used by Huxley (1983). Two determinations per sample (n = 6 per treatment) were done. A volume of 20 µl of the extract was poured on a slide and it was covered with a coverslip. A volume of 10 µl of contraction buffer containing: 50 mM KCl, 5 mM MgSO4, 1 mM ATP, 0,1 mM Ca^{2+} and 20 mM imidazol, pH = 7 was incorporated by capillarity. Myofibril contraction was registered by photography with Digital Panasonic DMC LZ8 camera with a maximum zoom (5 ×), before and 1–3 min after addition of contraction buffer. Two pictures per sample were taken. The time was registered using a chronometer. The time started (time = 0) with the addition of the buffer and finished after 3 min. In order to confirm the no contraction in samples from fishes exposed to ES, 2 min more were waited.

2.2.2. Myofibrillar ATPase activity

The activities of Mg^{2+} Ca^{2+}- ATPase and Mg^{2+}(EGTA) -ATPase are related to the actin/myosin interaction in the actomyosin complex in presence and absence of calcium ions respectively. The Mg^{2+} ATPase and Mg^{2+} ethylene glycol tetra acetic acid (EGTA) ATPase activities of myofibrils were measured at 37 °C in a 30 mM Tris-maleate buffer (pH 7) according to Roura (1992). For the inhibition of myofibrillar Mg^{2+}-ATPase in the absence of Ca^{2+} ions, 0.5 mM EGTA was used. Specific conditions for each enzyme were 0.12 mg/mL of protein, 0.75 mM ATP, 60 mM KCl, 2 mM $MgCl_2$ and 0.1 mM $CaCl_2$ for the Mg^{2+} Ca^{2+} ATPase. For the Mg^{2+} (EGTA) ATPase activity a mix of 0.25 mg/mL of protein, 0,75 mM ATP, 60 mM KCl, 2 mM $MgCl_2$ and 0.5 mM EGTA were used. A blank to evaluate the ATP hydrolysis was performed, consisting of the same mix than for enzymes but replacing the sample by distilled water. A final incubation volume of 3 mL was used in all cases. Incubation times were 4 min for both enzymes and for the blank. Reactions were stopped by addition of 1 mL of cold 40% trichloroacetic acid (TCA) solution (at 10% final concentration). Liberated phosphorous coming from the ATPsic activity was determined according to the method of Chen et al. (1956). The reactive was composed by 2 volumes of bidistilled water plus 1 vol of 10% ascorbic acid, 1 vol of 6 N sulfuric acid and 1 vol of 2.5% ammonium molybdate. An aliquot of 2 mL of reactive plus 2 mL of the sample were mix and incubated 1.5 h at 37 °C. Released inorganic phosphorus from the ATP hydrolysis was quantified by