Ecotoxicology and Environmental Safety 147 (2018) 809–816

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Alteration in DNA structure, molecular responses and Na^+ -K^+ -ATPase activities in the gill of Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) in response to sub-lethal verapamil

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ARTICLE INFO

Keywords:
Fish
DNA damage
Gene expression
Oxidative stress
Drug

ABSTRACT

The ecotoxicological consequences of residues from pharmaceutical drugs on aquatic biota have necessitated the development of sensitive and reliable techniques to assess the impact of these xenobiotics on aquatic organisms. This study investigated the alteration in DNA structure, molecular responses and the activities of Na^+ -K^+ -ATPase and antioxidant enzymes in the gill of Nile tilapia, *Oreochromis niloticus,* exposed to long-term effects at the concentrations (0.14, 0.28 and 0.57 mg L^{-1}) of verapamil in static renewal system for 15, 30, 45 and 60 days. Evaluation of DNA structure, using single cell gel electrophoresis, revealed certain degree of DNA damages in the gill in a time and concentration-dependent relationship. Transcription of mRNA of superoxide dismutase (*sod*), catalase (*cat*) and heat shock protein (*hsp70*) genes in the gill of the fish showed the genes were up-regulated. Na^+-K^+-ATPase activity was inhibited in a concentration and time dependent manner. The indices of oxidative stress biomarkers (lipid peroxidation and carbonyl protein) as well as superoxide dismutase, glutathione peroxidase, glutathione-S-transferase were elevated in the treated fish in comparison to the control. Further, the level of reduced glutathione and catalase activity were inhibited at 0.28 mg L^{-1} after day 30. Long-term exposure to sub-lethal concentration of verapamil can cause DNA damages, molecular effects and oxidative stress in *O. niloticus*. The biomarkers analysed can be used as early warning signals in environmental biomonitoring and assessment of drug contamination in aquatic ecosystem.

1. Introduction

Verapamil is L-type voltage gated calcium channels inhibitor drug, used in the treatment of hypertension, angina pectoris, cardiac arrhythmia and cluster headaches (Leone et al., 2011). Verapamil inhibits Ca^{2+} ions influx to the cells, especially in muscle and endocrine cells initiating many activities such as muscle contraction, hormone secretion, neurotransmitter release and neurons migration (Kania et al., 2015). Verapamil is also used in cell biology as an inhibitor of drug efflux pump proteins such as p-glycoprotein (Bellamy, 1996). Nallani et al. (2016) noted that higher residence times of verapamil in the plasma and fish tissues could impose greater pharmacodynamic activity and potential adverse effects, if similar pharmacological receptor responses between mammals and fish are assumed. Le et al. (2011) showed that verapamil, at a concentration of 4.2 mg L^{-1}, reduced the expression level of Vtg gene in *Daphnia magna*, which might decrease their reproduction ability. L-type calcium blocker was recognized as p-glycoprotein-mediated multixenobiotic resistance mechanism inhibitor which may influence protein-related transport and reactivity in the intestine and liver (Doi et al., 2001). Verapamil is a potent inhibitor of the drug efflux pump proteins such as P-glycoprotein and hence may enhance an organism's susceptibility to toxic effects by other xenobiotics (Kurelec, 1995). In aquatic environment, verapamil has been found at the concentration of 0.058–0.9 μg L^{-1} in the groundwater (Al-Rifai et al., 2007; Khan and Ongerth, 2004). As fish are capable of retaining the xenobiotics, generated through food chains and from aquatic environment, they are considered as one of the ideal organism for studying aquatic ecotoxicology and are usually used as bioindicator of environmental pollution (Audu et al., 2015; Lakra and Nagpure, 2009).

Na^+ -K^+-ATPase plays a significant role in both, whole body ion regulation, and cellular water balance in aquatic animals and in active transport of the ions (Lavanya et al., 2011; Towle, 1981). Pollutants are known to inhibit Na^+ -K^+ -ATPase activity in a variety of marine organisms by disrupting energy-producing metabolic pathways (Haya and

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http://dx.doi.org/10.1016/j.ecoenv.2017.09.050
Received 17 July 2017; Received in revised form 16 September 2017; Accepted 17 September 2017
Available online 25 September 2017

Waiwood, 1983; Watson and Beamish, 1981) and can be used as a useful toxicological tools for evaluation of cellular dysfunction organisms (Agrahari and Gopal, 2008).

Bioaccumulation of toxic compounds including, pharmaceutical drugs in fish together with environmental stress, can also invoke production of excess ROS commonly known as free radicals- such as superoxide anion (O_2), hydrogen peroxide (H_2O_2), the hydroxyl radical (.OH) which can elicit physiological alterations, oxidative dysfunction including lipid peroxidation and DNA damage in the tissues (Ajima et al., 2016a; Islas-Flores et al., 2013; Woo et al., 2006).

However, Steinbach et al. (2013) reported malformations and edemas at various developmental stages of *Cyprinus carpio* on exposure to 46.3– 463 mg L^{-1} of verapamil. Further, Kania et al. (2015) documented that at relatively low concentration (8 mg L^{-1}), the behavior of male Siamese fighting fish was negatively impacted by verapamil. Overturf et al. (2012) noted that exposure of fathead minnow (*Pimephales promelas*) larvae at 600 μg L^{-1} of verapamil caused a significant decrease in the growth rate.

Information on alteration in DNA structure, molecular responses and Na^+ -K^+ -ATPase activities in the gill of Nile tilapia, *O. niloticus,* in response to long-term effects of verapamil are limited. Nile tilapia is an economically important aquaculture species, and has wide range of global distribution, it is therefore necessary to understand the issues concerning this species. Gills are the organ that play an important role in ion osmoregulation, gas exchange, acid-base balance and are directly exposed to many toxic compounds that may usually militate against their function. The present study was designed to ascertain if long-term effects of verapamil can alter the DNA structure, molecular responses and activities of Na^+ -K^+ -ATPase as well as oxidative stress in *O. niloticus*.

2. Materials and methods

2.1. Experimental fish and drug

Juveniles (n = 120) of Nile tilapia (42.55 ± 1.14 g; mean length 13.48 ± 1.01 cm) were obtained from Nallasopora, India through the help of local fish farmers. They were transported to the Wet Laboratory of Central Institute of Fisheries Education, Mumbai, India and were held in four fiber reinforced plastic (FRP) tanks, containing 500 L of dechlorinated tap water that were continuously aerated to maintain dissolved oxygen contents. Fish were acclimatized for 14 days before the commencement of the experiment and fed with a commercial fish pellet, containing 30% protein. Verapamil (CAS number: 52–53-9, purity- 99%), was obtained from Themis Chemicals, India. It was dissolved in distilled water (80 mg L^{-1}) to make a stock solution that was used in the study. From the stock solution, different sub-lethal concentrations of the drug were prepared. Stock solution was prepared fresh each time the concentrations were renewed.

2.2. Exposure to sub-lethal concentration of verapamil

The experiment was conducted in a semi-static renewal bioassay, using 150 L glass aquaria. The concentrations (0.14, 0.28 and 0.57 mg L^{-1}) of verapamil were used following our earlier reports Ajima et al. (2017). One group was exposed to only de-chlorinated tap water which served as control. Each of the concentration was prepared in triplicate. Ten fish from the acclimatized group were distributed randomly to each of the twelve 150 L capacity plastic tanks. The fish were fed daily, with commercial fish pellets (30% protein) at the rate of 2% total body weight at 9.00 and 16.00 h. The exposure solution was renewed each day and was also analysed using LC-MS/MS to ensure the agreement between nominal and actual concentrations of the drug in the aquaria (Li et al., 2011). The experiment was conducted under the natural photoperiod of 12:12 light-dark cycle. Unconsumed feed and faeces from the fish were siphoned out daily shortly after feeding. The physico-chemical parameters of the test water were analysed daily, using standard methods APHA (2005) and were recorded to be within an optimal range (dissolved oxygen 5.5–7.50 mg L^{-1}, temperature 27.50–28.00 °C, pH 7.7–7.8 and free carbon dioxide 4.25–4.32 mg L^{-1}). The test fish were sampled on days 15, 30, 45 and 60 to determine the toxic effects of verapamil on the fish. The gills of two fish from each replicate were dissected. One was used to estimate DNA damage and the antioxidant as well as other enzyme profile. The remaining fish was used for gene expression study.

2.3. Single-cell gel electrophoresis (comet assay)

Single-cell gel electrophoresis (comet assay) was carried out according to Singh et al. (1988) as modified by (Klaude et al., 1996). The gill tissues were placed in 1 mL of cold Hanks balanced salt solution (HBSS), containing 20 mM EDTA in 10% dimethyl sulfoxide (DMSO) and centrifuged at 3000 rpm at 4 °C for 5 min. Ten percent DMSO was added to HBSS to prevent lipid peroxidation and was suspended in chilled phosphate buffered saline (PBS). Viability cell exceeding 84% were processed for the comet assay after evaluation by trypan blue exclusion test methods (Anderson et al., 1994). Normal melting agarose (NMA) of 1% (in distilled water) was smeared on frosted glass slides and kept for air drying (base layer). A second layer of 200 μL of 1% agarose (in PBS) solution was coated over the base layer to provide attachment and binding of the cells and the agarose to the slide. More so, 20 μL of cell suspension was mixed with 80 μL low melting point agarose (LMPA, 0.5% in PBS) and layered on the agarose coated slides. Further, the slides were coated with third layer of 100 μL LMPA (0.5% in PBS). The slides were immersed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, with 10% DMSO and 1% Triton × 100 added fresh) and kept overnight in dark at 4 °C after solidification of the gel. The slides were placed in horizontal gel electrophoresis unit, immersed in electrophoresis buffer (300 nM NaoH, 1 mM Na_2EDTA, 0.2% DMSO, pH 13.5) and were left for 20 min for DNA unwinding and conversion of alkali–labile sites to single strand breaks followed by electrophoresis in buffer at 15 V (0.8 V/cm) and 300 mA at 4 °C. The slides were neutralized with (0.4 M Tris buffer at pH 7.5), dehydrated using methanol and were stained with 75 μL ethidium bromide (20 μg mL^{-1}). Gill cells were also treated with 100 μM of H_2O_2 for 10 min at 4 °C which served as positive control. Finally, 25 cells per slide (250 cells per concentration) were screened and analysed using software (Comet Imager Meta Systems, Germany) for scoring DNA damage as per cent tail DNA (i.e. % tail DNA = 100 −% head DNA).

2.4. Gene expression analysis by Real-time PCR

The expression levels of *sod, cat* and *hsp70* were determined by quantitative real-time-polymerase chain reaction (qRT-PCR). Gill tissues of the fish (n = 1) from each replicate were dissected and homogenized separately in TRIzol reagent (Invitrogen, Carlsbad, CA), and subsequently the total RNA was extracted. Concentration of RNA samples were quantified using NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). One microgram of total RNA was reverse transcribed using reverse transcriptase enzyme and random primers (Promega, Madison, WI) for making cDNAs. qRT- PCR was performed with cDNAs and gene specific primer pairs of *sod, cat* and *hsp70* (Table 1) mixed with ABI SYBR Green PCR master mix (Applied Biosystems, Foster City, California, USA) in an ABI 7500 Sequence Detection System (Applied Biosystems). Beta actin gene was used as an endogenous control because of the non-significant changes in the Ct value between the treatments. The relative mRNA expression data were analysed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).