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Effect of pharmaceuticals exposure on acetylcholinesterase (AchE) activity and on the expression of *AchE* gene in the monogonont rotifer, *Brachionus koreanus*

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ABSTRACT

Pharmaceuticals are widely used in human and veterinary medicine. However, they are emerging as a significant contaminant in aquatic environments through wastewater. Due to the persistent and accumulated properties of pharmaceuticals via the food web, their potential harmful effects on aquatic animals are a great concern. In this study, we investigated the effects of six pharmaceuticals: acetaminophen, ATP; atenolol, ATN; carbamazepine, CBZ; oxytetracycline, OTC; sulfamethoxazole, SMX; and trimethoprim, TMP on acetylcholinesterase (AChE; EC 3.1.1.7) activity and its transcript expression with chlorpyrifos (as a positive control) in the monogonont rotifer, *Brachionus koreanus*. ATP, CBZ, and TMP exposure also remarkably inhibited Bk-AChE activity at 100 μg/L (24 h) and 1000 μg/L (12 h and 24 h). ATP, CBZ, and TMP exposure showed a significant decrease in the *Bk-AChE* mRNA level in a concentration-dependent manner. However, in the case of OTC and SMX, a slight decrease in *Bk-AChE* mRNA expression was found but only at the highest concentration. The time-course experiments showed that ATP positively induced *Bk-AChE* mRNA 12 h after exposure at both 100 and 1000 μg/L, while the *Bk-AChE* mRNA expression was significantly downregulated over 6 to 24 h after exposure to 1000 μg/L of CBZ, OTC, SMX, and TMP. Our findings suggest that Bk-AChE would be a useful biomarker for risk assessment of pharmaceutical compounds as an early signal of their toxicity in aquatic environments. Particularly, ATP, CBZ, and TMP may have a toxic cholinergic effect on rotifer *B. koreanus* by inhibiting AChE activity.

1. Introduction

Pharmaceuticals are widely used as therapeutic agents in human and veterinary medicines, and are increasingly found in aquatic ecosystems (Ternes et al., 2001). Therefore, these compounds are a growing concern as an emerging contaminant in aquatic environments. Pharmaceuticals have often been detected in sewage treatment plant (STP) effluents, drinking water, groundwater, and seawater (Fent et al., 2006). Among these aquatic contaminants, the most common pharmaceutical groups include analgesics, antibiotics, anti-epileptics, β-blockers, and lipid regulators (Fent et al., 2006; Kümmerer, 2009). In Korea, several pharmaceuticals including acetaminophen, carbamazepine, diclofenac, ibuprofen, and salicylic acid have been detected from treated wastewater (Park, 2005; Han et al., 2006). Of these, pharmaceuticals such as acetaminophen and carbamazepine have been the most frequently reported worldwide (Ternes et al., 2001; Kolpin et al., 2002; Fent et al., 2006). Sulfonamides including sulfamethoxazole are known as the second largest group in Korea and other countries (Thiele-Bruhn, 2003; National Veterinary Research and Quarantine Service, 2005; Park, 2005). Antibiotics such as oxytetracycline, sulfamethoxazole, and trimethoprim have been used extensively as therapeutic drugs in aquaculture (Kümmerer, 2009).

To date, the modes of actions of pharmaceuticals in humans and mammals are well-known (Fent et al., 2006), whereas knowledge of aquatic organisms, particularly invertebrates, is limited. This is problematic as these contaminants/pollutants have potentially harmful effects on wildlife organisms that have identical and/or similar target molecules as pharmaceuticals are developed to target-specific molecular pathways. Some receptors in lower invertebrates are structurally similar to humans and higher vertebrates, suggesting that the molecular mechanisms of pharmaceuticals in mammals may be functionally similar in lower invertebrates (Fent et al., 2006). Recently, acute and chronic toxicity experiments of these compounds have been conducted in aquatic organisms with the marine bacterium *Vibrio fischeri*, the water flea *Daphnia magna*, and the Japanese medaka *Oryzias latipes*

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(Kim et al., 2007) as well as the cnidarian *Hydra attenuata* (Quinn et al., 2009), the microalgae *Tetraselmis chuii*, and the cladoceran *Artemia parthenogenetica* (Ferreira et al., 2007). However, the traditional endpoints using mortality and growth inhibition cannot explain the in-depth mode of the actions of these pharmaceuticals. To better understand the molecular mechanisms of the toxicological effects of these compounds as an early signal of their toxicity, combined in vivo and in vitro studies are important (Fent, 2001). However, little information is available on the potential ecotoxicological effects at the molecular level of pharmaceuticals in aquatic organisms. Therefore, uncovering a reliable molecular endpoint with a small aquatic organism would be helpful in obtaining knowledge of the effect of pharmaceuticals.

The monogonont rotifer, *Brachionus koreanus* (*B. koreanus*) is widely distributed along costal lines, and is an important species in its role as an energy transmitter that acts as a primary producer and secondary consumer in aquatic food webs. Due to several advantages (e.g. small size, short generation cycle at ≈24 h, simple structure, genetic homogeneity, high fecundity, and easy maintenance in the laboratory), they are considered to be a model species in aquaculture, ecophysiology, ecotoxicology, and environmental genomics (Snell and Janssen, 1995; Dahms et al., 2011). To date, exploration of invertebrate nervous systems were focusing on model animals with diverse experimental approaches but little attention was given as yet on the Rotifera. This happened even though it was shown that rotifers have a primitive brain, located above the mastax, and a neural system throughout the whole body (Kotikova et al., 2005; Hochberg, 2009). Swimming speed alteration by neurotoxic chemicals (e.g. pentachlorophenol, γ-hexachlorocyclohexane, and eserine) revealed a disruption of the neural function in rotifers (Charoy and Janssen, 1999; Garaventa et al., 2010). Previously, Pineda-Rosas et al. (2005) showed acetylcholinesterase (AChE) receptors in six freshwater rotifers, suggesting that rotifer nervous system would be capable to transmit acetylcholine for further signal transduction. However, gene information or protein-relevant data on neurotoxicity were not identified in the Rotifera as yet.

As one of molecular endpoints of pharmaceuticals, AChE is a good candidate that is responsible for the hydrolysis of the neurotransmitter acetylcholine (Fukuto, 1990). This enzyme plays a key role in the nervous system and is found mainly in the brain. Inhibition of AChE disrupts the nervous system as accumulating the neurotransmitter, resulting in deleterious effects including death (Koelle, 1994). Thus, AChE activity has been used as a biomarker for environmental pollution, particularly pesticide in aquatic environments. To date, inhibition of AChE activity has been reported in aquatic organisms exposed to methanol (Rico et al., 2006), heavy metals (Banni et al., 2005; Richetti et al., 2011), and pesticides (Anquiano et al., 2009; Ezemonye and Ikpesu, 2011). More recent studies have also showed that some pharmaceuticals increased the inhibition of AChE activity in aquatic organisms (Solé et al., 2010; Li et al., 2012).

The purpose of the present study is (1) to characterize AChE from the monogonont *B. koreanus* and to analyze the modulation of the AChE activity and its transcription level after exposure to six pharmaceuticals, (2) to evaluate the usefulness of AChE as a molecular biomarker upon pharmaceutical exposure in this species, and (3) to unveil the mode of action of the pharmaceutical effect in *B. koreanus*.

2. Materials and methods

2.1. Rotifer culture and maintenance

The monogonont rotifer, *B. koreanus* was collected at Uljin (36°58′ 43.01″N, 129°24′28.40″E) in South Korea. To establish the rotifer strain as an experimental animal in the laboratory, a single and parthenogenetic individual was isolated, reared, and maintained in 0.2 μm-filtered artificial seawater (TetraMarine Salt Pro, Tetra™, OH, USA) adjusted to 25 °C under a LD 12:12 h photoperiod with 15 psu of salinity. The green algae *Chlorella* was used as a live diet (approximately 6×10^4 cells/mL). Species identification was confirmed by Lee et al. (2011) with the morphological characteristics and the mitochondrial genome sequence (Hwang et al., in press). The external morphology of *B. koreanus* was given as shown in Supplementary Fig. 1.

2.2. Retrievement and annotation of the acetylcholinesterase (AChE) gene from the monogonont rotifer, B. koreanus

To obtain the rotifer *AChE* gene, we searched the rotifer, *B. koreanus* genomic DNA database that was constructed in our previous study (Lee et al., 2011) using an internal local BLAST with the software BioEdit (ver. 7.0). The obtained contig was subjected to BLASTx analysis in the NR database at GenBank for annotation. Subsequently, the *AChE* gene was subjected to 5′- and 3′-Rapid Amplification of cDNA Ends (5′- and 3′-RACE) to confirm the full-length cDNA sequence according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA) and to check the exon/intron boundary. A series of RACE was performed with specific primers (Table 1) under the following conditions: 94 °C/4 min; 40 cycles of 98 °C/25 s, 55 °C/30 s, 72 °C/60 s; and 72 °C/10 min. The final RT-PCR products were isolated from 1% agarose/TBE gel, cloned into pCR2.1 TA vectors (Invitrogen), and sequenced with an ABI PRISM 3700 DNA analyzer (Bionics Co., Seoul, South Korea).

2.3. Preliminary characterization of Bk-AChE

Chlorpyrifos is an organophosphate insecticide being a strong inhibitor of AChE activity. Previously, Ferrando and Andreu-Moliner (1991) reported that lethal concentration of 50 (LC50) values of chlorpyrifos for 24 h were similar in two rotifer species, 11.85 mg/L for *Brachionus calyciflorus* and 10.67 mg/L for *Brachionus plicatilis*, respectively. To confirm whether Bk-AChE has an inhibitory mechanism compared to those of well-characterized AChEs, we checked the protein activity and mRNA expression after exposure to chlorpyrifos. Exposure concentrations (1, 10, 100, 500 μg/L) were calculated based on the no observed effect concentration (NOEC) (528.2 μg/L) and LC50 (3.9 mg/L) values for chlorpyrifos in *B. koreanus*. Subsequent mRNA expression patterns and activity changes were measured over 24 h using the following methods.

2.4. Dose–response and time course

To analyze the effects of pharmaceutical exposure on mRNA and the activity of the rotifer AChE, we exposed *B. koreanus* to six pharmaceuticals with three antibiotics (acetaminophen, ATP; atenolol, ATN; carbamazepine, CBZ; oxytetracycline, OTC; sulfamethoxazole, SMX; and trimethoprim, TMP). All the chemicals were purchased from Sigma (Sigma-Aldrich, Inc., St. Louis, MO, USA; purity > 99%). The stock

Table 1
Primer list used in this study.

Gene	Oligo name	Sequence (5′–3′)	Remarks	Amplicon (bp) (efficiency)
Bk-AChE	5GSP1	CAGATGTTGCTTCTAAAA TTCTCTC	5′-RACE	
	5GSP2	CTAAAAGTGTAATGCGA TTTGGATCTA		
	3GSP1	CTTATTAAACTATGACAAGAA TAGTG	3′-RACE	
	3GSP2	GAAGCTTGTCAATTTTGGAA TTCAT		
	RT-F	GAATGTAGAGATGATTAAA TACT	Real-time PCR	118
	RT-R	GTCATATTTCCACTATTCTT GTCATAG	Amplification	(97.81%)
Bk-18S rRNA	RT-F	GACTCAACACGGGAAATC TCACC	Real-time PCR	106
	RT-R	ACCAACTAAGAACGGCC ATGCAC	Amplification	(98.83%)