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Disrupting effects of antibiotic sulfathiazole on developmental process during sensitive life-cycle stage of *Chironomus riparius*

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HIGHLIGHTS

- Antibiotic exposure alters biological and physiological responses in invertebrates.
- The STZ induces significant effects on sex balance of emergent *C. riparius* adults.
- STZ exposure cause the development of mouthpart deformity in aquatic midges.
- STZ causes a dose and time-dependent toxicity in most of the selected biomarkers.
- Endocrine disrupting effects observed in *C. riparius* exposed to STZ.

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ABSTRACT

Antibiotics in the environment are a concern due to their potential to harm humans and interrupt ecosystems. Sulfathiazole (STZ), a sulfonamide antibiotic, is commonly used in aquaculture and is typically found in aquatic ecosystems. We evaluated the ecological risk of STZ by examining biological, molecular and biochemical response in *Chironomus riparius*. Samples were exposed to STZ for 12, 24 and 96 h, and effects of STZ were evaluated at the molecular level by analyzing changes in gene expression related to the endocrine system, cellular stress response and enzyme activity of genes on antioxidant and detoxification pathways. STZ exposure induced significant effects on survival, growth and sex ratio of emergent adults and mouthpart deformity in *C. riparius*. STZ caused concentration and time-dependent toxicity in most of the selected biomarkers. STZ exposure leads to significant heat-shock response of protein genes (HSP70, HSP40, HSP90 and HSP27) and to disruption by up-regulating selected genes, including the ecdysone receptor gene, estrogen-related receptors, ultraspiracle and E74 early ecdysone-responsive gene. Furthermore, STZ induced alteration of enzyme activities on antioxidant and detoxification responses (catalase, superoxide dismutase, glutathione peroxidase and peroxidase) in *C. riparius*. By inducing oxidative stress, antibiotic STZ disturbs the endocrine system and produces adverse effects in growth processes of invertebrates.

1. Introduction

In both human and veterinary medicine, antibiotics are used to treat and prevent disease as well as to promote growth in some livestock (Kemper, 2008), and these can be introduced into the environment through various routes during or after manufacture or consumption. The widespread use of antibiotics eventually leads to surface water contamination (Kolpin et al., 2002). Many of these compounds are endocrine-disrupting chemicals that can interfere with natural hormone balance (Ji et al., 2010). For example, sulfonamides (SA) of antibiotics are widely administered in agriculture and aquaculture (Hruska and Franek, 2012), and it is the most frequently administered antibiotics in medical feeds for pigs in Switzerland (Arnold et al., 2004). Sulfathiazole (STZ) of SA is extensively used in aquaculture, livestock production and human medicine to treat bacterial, protozoal and fungal infections. STZ induces the up-regulation of ribosomal protein S3 transcript on the aquatic invertebrate *C. riparius* (Park and Kwak, 2012). In Korea, SA concentrations ranged from 0.45 to 10.57 μg L^{-1} in twelve sewage treatment plant influents and wastewater (Choi et al., 2007). SA antibiotics are a growing concern due to their long persistence in the environment, giving rise to extensive antibiotic resistant

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bacteria in sediments, and soils respectively (Kim et al., 2013). Along with other SA, studies also point to the presence of STZ in ecosystem with aquatic environment, which in most part is due to the inability to achieve full elimination during sewage treatment (Braschi et al., 2010; Garcia-Galan et al., 2008; Hruska and Franek, 2012). However, the potential effects of continuous, low-level exposure to these compounds is not well understood.

Chironomids are a taxonomical diverse group of insects that commonly inhabit freshwater environments. Due to their widespread distribution, short life-cycle, ability to be reared in the laboratory, and their easily identified life-history stages, chironomid species are widely used as test organisms in standard aquatic toxicity tests when conducting ecological risk assessments (OECD, 2011; Park and Kwak, 2014; Morales et al., 2011). Ecotoxicological tests using chironomid larvae have traditionally determined biological endpoints assays using survival, growth rate, sex ratio of emerged adults, behavior, and physiology (Williams et al., 1987; Park et al., 2009; Park and Kwak, 2014). Mouthpart deformities are also significant indicators of chemical stress (Martinez et al., 2003; Park and Kwak, 2008; Park et al., 2010). Furthermore, a toxicity assay using molecular endpoints has been performed in *C. riparius* to investigate the patterns of gene expression in the presence of various aquatic contaminants (Park et al., 2009; Nair et al., 2013; Morales et al., 2013, 2014; Park and Kwak, 2014; Planelló et al., 2015; Herrero et al., 2015, 2016). Several molecular biomarkers have been characterized as responsive endpoints in aquatic midge *C. riparius*, including those for heat shock proteins (HSPs), ribosomal proteins, antioxidant and detoxification pathways, endocrine-related genes (Planelló et al., 2007, 2011; Park and Kwak, 2008, 2010, 2012; Park et al., 2010; Martinez-Paz et al., 2012, 2014; Morales et al., 2011; Herrero et al., 2015). However, there is limited information as to the response markers for antibiotic toxicity on aquatic species.

Major developmental transitions in multicellular organisms are also driven by steroid hormones. Steroid hormones have control functions in the regulation of developmental growth, metamorphosis, molting process, and reproduction in Arthropoda (He et al., 2015). The 20-hydroxyecdysone (20E) activity, an insect steroid hormone, is triggered by transcriptional process regulated through the binding of 20E to a heterodimer complex comprising the ultraspiracle (USP) and ecdysone receptor (EcR) (Bozzolan et al., 2015). Estrogen-related receptors (ERRs) are nuclear receptors that modulate the estrogen receptor (ER)-mediated pathway and perform a critical function as transcription factors that control essential developmental and physiological pathways (Park and Kwak, 2010; Yamanaka et al., 2013). Early ecdysone-responsive gene (E74) also acts as ligands for nuclear receptors and altering gene transcription (Morales et al., 2013).

Heat shock proteins (HSPs) are a protein family that act as molecular chaperones, named according to their molecular weight. They prevent nonspecific protein aggregation and induce protein folding in the formation of their normal architecture (Lianos et al., 2015). Moreover, HSPs are likely to have anti-apoptotic properties, regulation of cell development and differentiation, and signal transduction processes. The up-regulation of HSPs can also be triggered by various environmental stresses as well as heat shock. HSP90 activity is related to the regulation and maintenance of various signaling proteins (Seo, 2015). HSP70 has been evaluated as a biomarker of various exposures to metals and chemicals in chironomids (Morales et al., 2014; Ozáez et al., 2014; Planelló et al., 2011; Park et al., 2009, 2010). Small HSP27 and HSP40 genes also have a potential response as suitable biomarkers for ecotoxicological studies in aquatic systems (Martinez-Paz et al., 2014; Park and Kwak, 2008).

Oxidative stress refers to the over-production of reactive oxygen species (ROS) related to defense mechanisms, and the imbalance between oxidants and antioxidants in favour of oxidative stress leads to cellular damage (Carnevali et al., 2003). This is a potential indicator as a response to the toxic effects to antioxidant enzymes that help maintain cellular homeostasis by removing ROS. Superoxide dismutase (SOD) and catalase (CAT) are important enzymes that protect the cell from oxidative stress by ROS, and these are a potential biomarker of the ecotoxicity (Rodrigues et al., 2015; Wiseman et al., 2013; Park et al., 2012; Nair et al., 2011; Datkhile et al., 2009). Glutathione peroxidase (GPx) and peroxidase (Px) are also detoxification enzymes that mediate the detoxification of peroxides to protect an organism from oxidative damage (Park and Kwak, 2014; Colle et al., 2013).

The aim of this study was to evaluate the potential effects of STZ in aquatic invertebrates of *C. riparius*. Our work investigated the response of biological and developmental parts (survival and growth rate, sex ratio and morphological deformity), transcriptional endocrine signaling genes (EcR, ERR, USP, E74), cellular stress response (HSP70, HSP90, HSP40, HSP27), and antioxidant and detoxification (CAT, SOD, GPx, Px). In addition, the enzyme activities of SOD and GPx were analyzed in *C. riparius* larvae exposed to STZ as a biochemical response. Our study suggests sensitive biomarkers in response to exposure to STZ antibiotics in *C. riparius*.

2. Materials and methods

2.1. Test animals

Chironomus riparius larvae were obtained from laboratory-reared adults, with the original strain provided by the Korea Institute of Toxicology (Daejeon, Korea). *C. riparius* were reared using the methodologies outlined by Streloke and Kopp (1995). The *C. riparius* colony was maintained with constant aeration under a light: dark cycle of 16:8 h at 20 ± 1 °C. The larvae hatched from eggs were maintained in Duran crystallizing dishes (Schott, Mainz, Germany) containing approximately 500 mL of M4 culture medium (Elendt, 1990) and a 1 cm sediment layer of fine sand (<63 μm particle size) with continuous aeration. The larvae were fed with fish food (Tetra-Werke, Melle, Germany) at 0.5 mg per larvae per day (OECD, 2004).

2.2. Exposure conditions

STZ solution was prepared from the corresponding solid compound (99% pure; Sigma-Aldrich, St. Louis, MO, USA) using dimethylsulfoxide (DMSO) as the solvent (Oh et al., 2006). The nominal concentrations were 1, 10, 30 and 100 μg L^{-1} based on results of the acute toxicity test (data not shown), and were prepared from stock solutions of 0.1 g L^{-1}. Preliminary tests demonstrated that DMSO induced no effects on the organisms in any of the experiments (Kim et al., 2009). All experimental larvae were acquired 11 days after hatching from their egg masses. Thirteen fourth-instar *C. riparius* larvae were transferred to 300 mL Duran crystallizing dishes (Schott) filled with 200 mL of M4 media and then treated with one of the four mentioned concentrations of STZ. All organisms were exposed to treatment for 12, 24 or 96 h, and all experiments were conducted in triplicate using independent samples. Freshly manufactured test concentrations were renewed after 12 h of exposure. The solvent treated larvae used as control were also evaluated in triplicate. For further analysis, RNA and protein extraction were performed from surviving larvae, which were frozen and stored at −80 °C.