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Acute toxicity and gene responses induced by endosulfan in zebrafish (*Danio rerio*) embryos

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ABSTRACT

Endosulfan has been listed as a persistent organic pollutant, and is frequently found in agricultural environments during monitoring processes owing to its heavy use and persistent characteristics. This study was conducted to understand the effects of endosulfan on the development of zebrafish (*Danio rerio*) embryos by exposing them to a specific range of endosulfan concentrations. Exposing zebrafish embryos to endosulfan for 96 h yielded no acute toxicity until the concentration reached 1500 μg L^{-1}, whereas malformed zebrafish larvae developed severely curved spines and shortened tails. About 50% of zebrafish larvae were malformed when exposed to 600 μg L^{-1} of endosulfan. Comparative gene expression using real-time quantitative polymerase chain reaction was assessed using endosulfan-exposed zebrafish embryos. *CYP1A* and *CYP3A* were significantly enhanced in response to endosulfan treatment. Two genes, *acacb* and *fasn*, encoding acetyl-CoA carboxylase b and fatty acid synthase proteins, respectively, were also up-regulated after treating zebrafish embryos with endosulfan. These genes are also involved in fatty acid biosynthesis. The genes encoding vitellogenin and Hsp70 increased in a concentration-dependent manner in embryos. Finally, biochemical studies showed that acetylcholinesterase activity was reduced, whereas glutathione *S*-transferase and carboxylesterase activities were enhanced in zebrafish embryos after endosulfan treatment. These biochemical and molecular biological differences might be used for tools to determine contamination of endosulfan in the aquatic environment.

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Introduction

Endosulfan has been widely used as a broad-spectrum insecticide since the 1950s, especially in areas of cotton cultivation to control the cotton bollworm *Helicoverpa zea* or *H. armigera*, which are some of the most problematic insect pests in the world owing to the development of insecticide resistance.[1,2] For example, the heavy use of endosulfan for the control of the cotton bollworm has caused endosulfan resistance in this species.[3,4]

Additionally, endosulfan use has been reduced because of its long persistence in agricultural areas, strong toxicity to fish in aquatic ecosystems, and harmful effects to agricultural workers.[5–9] Its long persistence has been reported in many countries, including Korea, Japan, and China.[5,10–12] After being included on the list of persistent organic pollutants, it has been banned and phased out of the market in several countries, including Korea.[5] Except for some countries that use endosulfan for certain applications, endosulfan may be permanently banned from use.[13]

In addition to its long persistence, ecological toxicity is its most considerable effect, such that many environment agencies and institutions should monitor its residual state in residential, industrial, and agricultural areas, including bodies of water.[5] Its toxicity to fish has been reported at LC_{50} values lower than those of terrestrial animals.[14,15] In tiger earthworms, *Eisenia fetida*, endosulfan showed acute toxicity with an LC_{50} value of 7.7 g L^{-1} following direct contact.[15] In Japanese medaka (*Oryzias latipes*), endosulfan exhibited toxicity with an LC_{50} value of 22.18 μg L^{-1}.[14] One of its metabolites, endosulfan sulfate, is more toxic than the parent compound, with LC_{50} values ranging from 2.1 to 3.5 μg L^{-1} for mosquitofish (*Gambusia affinis*), least killifish (*Heterandria formosa*), sailfin mollies (*Poecilia latipinna*), and fathead minnows (*Pimephales promelas*).

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[16] Therefore, the biotransformation of endosulfan to endosulfan sulfate has been shown to be a representative suicidal mechanism in environmental studies, with oxons forming from organophosphorus insecticides that affect acetylcholinesterases in animals.[17,18]

Accurate and precise techniques have elucidated the unknown toxic effects of compounds, including endosulfan.[19,20] We recently analyzed lipids and small proteins using MALDI-TOF MS/MS and SELDI-TOF MS in chlorpyrifos- and endosulfan-exposed fish samples.[14,15,21] In this study, MALDI-TOF MS/MS identified phospholipids that had been differentially biosynthesized after exposure to chlorpyrifos in Japanese rice fish.[21] In addition, a phospholipid with a mass ion of 556.32 significantly decreased in concentration after exposure to chlorpyrifos in Japanese medaka. Therefore, such differentially expressed biomarkers can be used to understand the environmental state when contaminated by pollutants.

Herein, zebrafish (*Danio rerio*) embryos were exposed to α-endosulfan at various concentrations to determine its acute toxicity and measure the extent of malformation during development to larvae. Biochemical studies were conducted to determine its adverse effects during exposure to endosulfan because of inhibition on acetylcholinesterase expression and activity in *Tigriopus japonicas*, and possible induction of two detoxifying enzymes carboxylesterases and glutathione *S*-transferases (GST).[22–24] Additionally, endosulfan enhances genotoxicity and mutagenicity through oxidative stresses in *Carassius carassius*.[8] Therefore, the expression of 12 genes were measured using quantitative polymerase chain reaction (qPCR) techniques and the genes were involved in antioxidant activities and defense systems, and lipid and carbohydrate metabolism. With these respects, the goal of the present study was to understand biochemical and molecular changes in zebrafish embryos after endosulfan treatment, which could be used to understand the response of fish to α-endosulfan, quickly monitor the aquatic environment, and assess ecological risk.

Materials and methods

Chemicals

α-Endosulfan was kindly supplied by Prof. Jang-Eok Kim at Kyungpook National University (Daegu, South Korea). 5,5-Dithio-bis(2-nitrobenzoic acid) (DTNB), Fast Blue B salt, tris(hydroxymethyl)-aminomethane (Tris), and acetylthiocholine iodide were used to determine acetylcholinesterase (AChE) activity; α-naphthyl acetate was used to determine carboxylesterase (CE) activity; and 1-chloro-2,4-dinitrobenzene was used to determine GST activity. All reagents were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). All chemicals, including acetone, were of the highest grade available.

Exposure of animals and embryos

Zebrafish embryos were obtained from wild-type zebrafish adults after mating. Adult zebrafish were purchased from a local shop in Korea and acclimatized to charcoal-filtered tap water. They were maintained under a 16:8 h light–dark cycle at 26 °C ± 1 °C. Ten zebrafish embryos, 6 h post fertilization (hpf), were randomly chosen and placed on an individual glass plate containing 25 mL egg water. They were then exposed to 20 µL of seven different concentrations of endosulfan in acetone, at concentrations ranging from 200 to 1500 µg L^{-1}.[25] Second exposure to endosulfan in acetone against zebrafish embryos followed by the first exposure at 6 hpf was on 30 hpf when we tore chorion before endosulfan treatment. The endosulfan-exposed embryos were left for 3 days without further treatment at 26 °C ± 1 °C. The experiments were replicated in triplicate, and the endosulfan-exposed embryos were observed every 24 h for 4 days to determine mortality and morphological alterations. Observations of spinal deformities were based on the level of curvature of the larval vertebral column.

Sample preparation and determination of enzyme activity

All larvae exposed to endosulfan were homogenized in 100 µL phosphate buffer (pH 7.4), followed by filtering with a cell strainer (SPL Life Science, Pocheon, South Korea). After the filtered homogenates were prepared, they were centrifuged at 12,000 × *g* at 4 °C for 20 min. The supernatant was retained and used as a crude enzyme preparation to measure enzyme activity, and the pellet was discarded. Proteins were measured using Coomassie Blue staining solution.[26] A method modified from Ellman et al. [27] was performed and used to determine AChE activity of zebrafish embryos after exposure to endosulfan. Carboxylesterase (CE) activity on the hydrolysis of α-natheyl acetate and GST activity as the conjugation reaction of 1-chloro-2,4-dinitrobenzene were determined following previously reported methods.[28,29] All enzyme activity was measured using a Thermo Multiskan GO 96 well spectrophotometer (Thermo Fisher Scientific Inc., Vantaa, Finland).

RNA extraction and cDNA synthesis

Total RNA from endosulfan-exposed zebrafish embryos was extracted following the manufacturer's guidelines using Tri-Reagent (QIAzol lysis reagent, Qiagen, Venlo, Netherlands). Zebrafish embryos (at least 50) exposed to endosulfan for 96 h were carefully washed five times with diethylpyrocarbonate (DEPC)-treated water. As soon as Tri-Reagent (1 mL) was added, the zebrafish embryos were homogenized using a DEPC-washed pencil-typed homogenizer. Total RNA was isolated following the

manufacturer's protocol, and the purity was determined by measuring the ratio of absorbance at 260/280 nm using a spectrophotometer. DNA/RNA quantification ratios were also determined using a μDrop™ plate (Thermo Fisher Scientific Inc.).

Five micrograms of isolated RNA were used to synthesize cDNA using a Maxima First Strand cDNA Synthesis Kit for qPCR (Thermo Fisher Scientific Inc.). Reverse transcription was conducted at 50 °C for 30 min. The total volume of the reaction mixture was 20 μL.

Quantitative PCR

Real-time quantitative PCR (qPCR) was conducted using a CYBR® Green Kit (Rotor-Gene SYBR® PCR Kit, Qiagen) with a qPCR instrument (Rotor-gene® Q, Qiagen) for 40 cycles. Primers were designed based on information gathered from an NCBI BLAST search of GenBank for acetylcholinesterase (AChE), β-actin, superoxide dismutase 1 (SOD), cytochrome P450 1A (CYP1A), cytochrome P450 3A (CYP3A), heat shock cognate 70-kd protein (HSP70), fatty acid synthase (FASN), acetyl-CoA carboxylase 2 (ACACB), vitellogenin 1 (VTG), catalase (CAT), GST, glutathione peroxidase 1a (GPX), and glyceraldyde-3-phosphate dehydrogenase (GAPDH). Those primers were exactly same as previously reported in Jeon and his coworkers.[30] ΔΔCt was analyzed using the method reported by Kenneth and Livark [31] to compare the degree of gene expression in zebrafish embryos after exposure to endosulfan. The *β-actin* gene were used as references to compare the expression level.

Statistical analysis

All experiments were conducted in triplicate and the data was expressed as mean ± standard deviation. Statistical significance from the control were determined using two-way ANOVA analysis with Tukey's *post hoc* test. Difference between groups was considered statistically significant when p-value < 0.05. Statistical analysis and Graphs were performed by Prims 6 (GraphPad software, San Diego, CA).

Results and discussion

Toxicity of endosulfan to zebrafish embryos

A distinct phenomenon was observed in the endosulfan-treated groups (Figure 1(a)), characterized by curved spines. This finding increased in a concentration-dependent manner in endosulfan-exposed zebrafish embryos (Figure 1(a) and (b)). Larvae with curved spines were grouped by the degree of spine curvature into types 1, 2, and 3 – Type 1 larvae exhibited less spine curvature, without dramatic changes to their tails; Type 2 larvae had severely curved spines and extremely shortened tails; Type 3 larvae were characterized by extreme curvature of the spine and severely horizontally shortened tails (Figure 1(a)). As the concentration of endosulfan increased, Type 1 embryos dramatically increased in number, as shown in Figure 1(b). The percentage of Type 1 deformities reached 50% when the concentration of endosulfan was 1500 μg L^{-1}. Type 2 larvae were also found at endosulfan concentrations of 200 μg L^{-1}, and were enhanced by approximately 20% until the endosulfan concentration reached 1500 μg L^{-1}. Type 3 larvae were similar to Type 2. Therefore, 50% of the population was observed to have curvature of the spine at an endosulfan concentration of 400 μg L^{-1}.

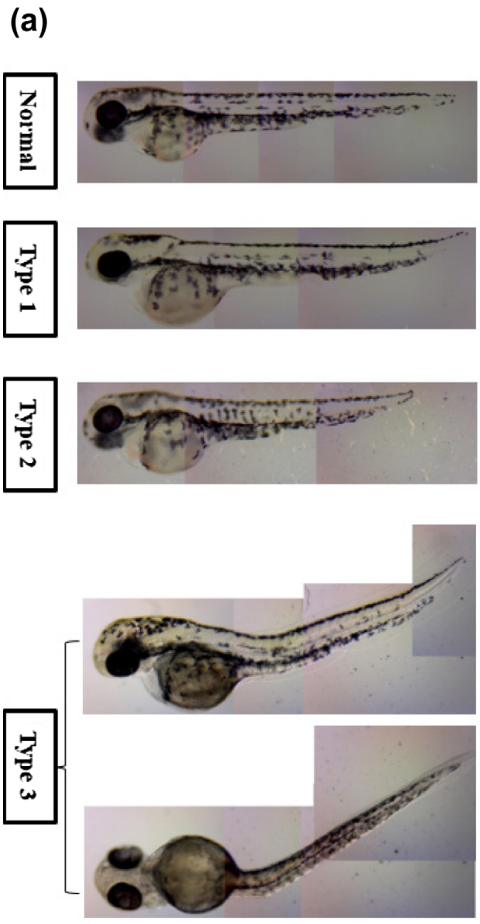
After the embryo toxicity test at ranges from 0 to 1500 μg L^{-1} of endosulfan, no more than 50% mortality was observed as the concentration reached 1500 μg L^{-1} (Figure 1(c)). No mortality was observed in control (acetone-treated) zebrafish embryos during the experiment. In addition to these findings, no mortality was observed in embryos until the concentration of endosulfan reached 600 μg L^{-1}. As our experimental data showed, endosulfan was at most weakly toxic to zebrafish embryos.[32]

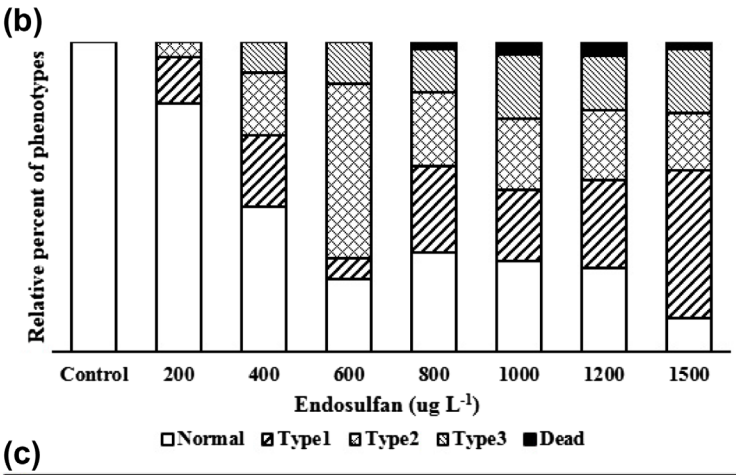
Biochemical changes in zebrafish embryos after endosulfan exposure

Zebrafish embryos exposed to endosulfan at different concentrations showed significant enhancement at two concentrations, 400 and 600 μg L^{-1}, and afterwards, there was a significant reduction of AChE activity until the concentration reached 1500 μg L^{-1} (Figure 2). A similar pattern of enhanced AChE activity in zebrafish adults was observed after chlorpyrifos exposure at 600 μg L^{-1}, which was then reduced to half of that of the control group at 1000 μg L^{-1}. Silva et al. [33] also found that AChE activity of zebrafish was inhibited *in vivo* by endosulfan and methidathion. Therefore, AChE activity in zebrafish is inhibited by endosulfan.

GST activities were significantly enhanced at certain concentrations of endosulfan in zebrafish embryos (Figure 2). This finding is similar to that of Dong et al. [23], who showed significantly higher GST activities in endosulfan-treated groups than control groups. The roles of GSTs include conjugating endosulfan and other metabolites, and removing them from biological organisms, as has been shown in *Planorbarius corneus* and *Aporrectodea caliginosa*, which discard chlorpyrifos by inducing GST expression.[34,35] Therefore, the induction of GST activity after zebrafish embryos are exposed to endosulfan may indicate enhanced conjugation reactions to remove endosulfan from fish.

CE activity in zebrafish embryos exposed to endosulfan increased at a concentration of 600 μg L^{-1} (Figure 2). These results are related to the role of CE after xenobiotics penetrate into living organisms. As CEs are responsible for sequestering xenobiotics, its expression or activity





(c)

Concentrations of Endosulfan (ug L^{-1})								
	Control	200	400	600	800	1000	1200	1500
Normal	78	16	14	7	16	14	12	5
Type1		3	7	2	14	11	13	23
Type2		1	6	17	12	11	10	9
Type3			3	4	7	10	8	10
Dead					1	2	2	1

Figure 1. Physiological changes of zebrafish larvae (a) after exposure to endosulfan and (b) the percentage of change according to type. The number of tested zebrafish larvae and those paralyzed are shown in Figure (c). Ten larvae was exposed to the endosulfan solution triplicates. Morphological malformations: normal, 3-dpf larvae of *D. rerio* with well-developed tail and body structures; Type 1, 3-dpf larvae of *D. rerio* exposed to endosulfan with deformed spines; Type 2, 3-dpf larvae of *D. rerio* exposed to endosulfan with a curved spine and shortened tail; Type 3, 3-dpf larvae of *D. rerio* exposed to endosulfan with a curved spine and horizontally shortened tail (Type 3).

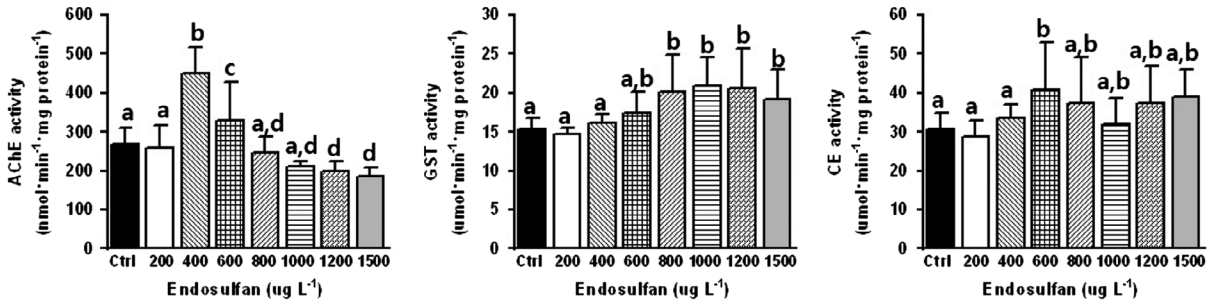


Figure 2. Acetylcholinesterase (AChE), carboxylesterase (CE), and GST activities in *D. rerio* embryos after exposure to different concentrations of endosulfan. AChE and CE activities are expressed as μmoles of acetylthiocholine iodide or α-naphthyl acetate hydrolyzed min^{-1}·mg^{-1}·protein. GST activity is expressed as μmoles of conjugated 1-chloro-2,4-dinitrobenzene min^{-1}·mg^{-1}·protein. Different letter indicates statistically different from the control ($p < 0.05$).

is generally increased to facilitate their removal from tissues, as our results exhibited. Apart from endosulfan, the sequestration of organophosphate (OP) insecticides after binding to CEs frequently occurs in animals.[21] CEs are phosphorylated by OP insecticides, and their hydrolytic activities are easily lost.[36]

qPCR analysis of gene expression in zebrafish embryos after CHL exposure

Twelve genes in zebrafish embryos were analyzed using real-time qPCR to determine alterations in gene expression levels after endosulfan treatment (Figure 3). Among them, *acacb* and *fasn* were up-regulated after exposure

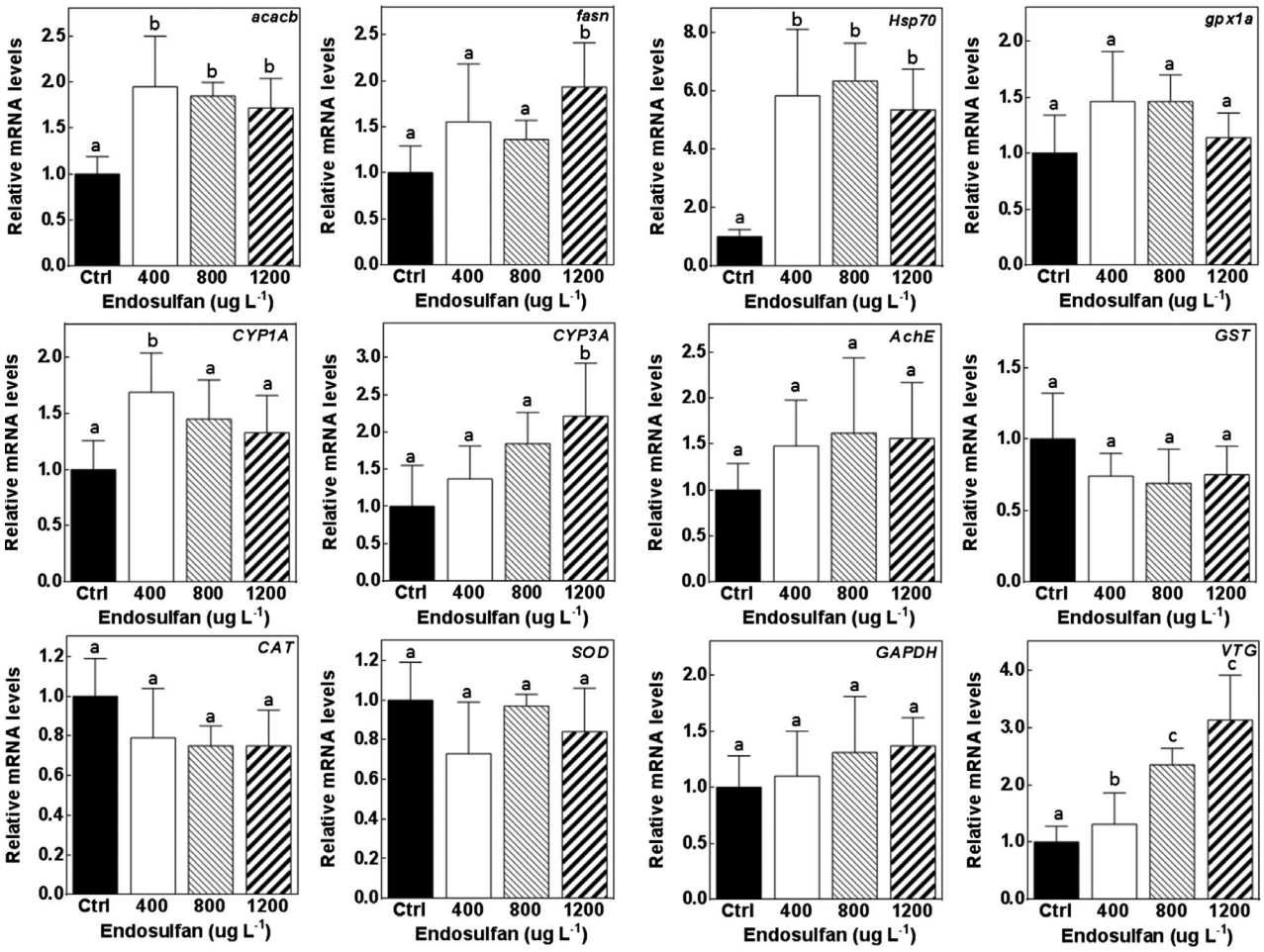


Figure 3. qPCR results for embryos treated with endosulfan for 3 days. The three different concentrations used in this study were: 400, 800, and 1200 μg L^{-1}. Two-way ANOVA analysis with Tukey's *post hoc* test was conducted to determine significant deviation from the control (*p-value < 0.05). *acacb*, acetyl-CoA carboxylase beta; *fasn*, fatty acid synthase; *Hsp70*, heat shock protein 70; *gpx*, glutathione peroxidase; *CYP1A*, cytochrome P4501A; *CYP3A*, cytochrome P4503A; *AChE*, acetylcholinesterase; *GST*, glutathione *S*-transferase; *CAT*, catalase; *SOD*, superoxide dismutase; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; *VTG*, vitellogenin.

to endosulfan in zebrafish embryos (Figure 3). The *acacb* gene expresses acetyl-CoA carboxylase beta protein in cells, and this protein plays an important role in the carboxylation of acetyl-CoA to form malonyl-CoA. This reaction is a rate-limiting step in fatty acid biosynthesis. The *fasn* gene encodes fatty acid synthase, which synthesizes fatty acids from acetyl-CoA and malonyl-CoA. Therefore, the up-regulation of these two genes in zebrafish embryos is responsible for changing fatty acid biosynthesis after endosulfan exposure. This alteration was first reported in relation to endosulfan toxicity in fishes.

The *Hsp70* gene was significantly up-regulated as embryos were exposed to endosulfan (Figure 3). Similar up-regulation of Hsp70 proteins was also observed in tilapia (*Oreochromis mossambicus*).[37] Other insecticides, including chlorpyrifos, also increased *Hsp70* expression in fish.[38] We also observed that *Hsp70*, a stressor gene, was significantly elevated by exposing zebrafish to endosulfan.

The genes *CYP1A* and *3A* were significantly up-regulated at endosulfan concentrations of 400 and 1200 μg L^{-1}, respectively. Cytochrome P450-dependent monooxygenases are primary proteins that remove xenobiotics. Endosulfan has been known to induce the activity of cytochrome P450s, such as aminopyrine *N*-demethylase and erythromycin *N*-demethylase, in zebrafish livers.[23] The activity of cytochrome P450-dependent monooxygenases, such as 7-ethoxyresorufin-*O*-deacetylase, significantly increased in the livers of endosulfan-exposed Atlantic salmon (*Salmo salar*).[39] Therefore, the induction of *CYP1A* and *3A* genes may be related to enhanced P450 monooxygenase activities in zebrafish. These findings were also observed in fish exposed to other insecticides, including chlorpyrifos.[40]

On the other hand, the vitellogenin (*VTG*) gene was also enhanced in a concentration-dependent manner at an endosulfan concentration of 1200 μg L^{-1} (Figure 3). This is consistent with the results of Chow et al. [41], who found increased expression of the *vtg1* gene after 96 h exposure to 10, 25, 50 and 75% of the EC_{50} value of zebrafish embryos.[41] Endosulfan also induced increased vitellogenin levels in the livers of male Japanese medaka (*O. latipes*), confirming estrogenic disturbance.[14]

However, endosulfan treatment did not alter the expression of other genes, including acetylcholinesterase (AChE), a cytosolic sulfotransferase gene (*SULT1 ST4*), and a catalase gene (*CAT*), in zebrafish embryos (Figure 3).

Conclusions

Herein, the acute toxicity of endosulfan to *D. rerio* embryos for 96 h was assessed, and lethal effects of endosulfan to zebrafish embryos were not detected until the concentration reached 1200 μg L^{-1}. Endosulfan induced abnormal development in zebrafish, with significant spine curvature and shortened tails in zebrafish larvae. Endosulfan enhanced carboxylesterase and GST activity in endosulfan-exposed embryos. Conversely, endosulfan exposure decreased AChE activity in zebrafish embryos. Molecular biological assessments found that *CYP450*, *1A*, and *3A* genes were elevated in order to degrade endosulfan in embryos, and a vitellogenin gene was also enhanced in a concentration-dependent manner. An acetyl-CoA carboxylase gene (*acacb*) and a fatty acid synthase gene (*fasn*) were induced by exposure to endosulfan in zebrafish embryos. These two genes are involved in the biosynthesis of fatty acids in zebrafish, a finding reported for the first time.

Disclosure statement

No potential conflict of interest was reported by the authors.

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