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Mecoprop-p interrupts the development of zebrafish via apoptosis and vascular damage

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ABSTRACT Mecoprop-p, a chlorophenoxy herbicide, has been widely used since the 1980s. Due to its high water solubility, it could be detected in the aquatic environment, as it has already been detected in the surface water or groundwater in several countries. The toxicity of other chlorophenoxy herbicides has been reported; however, there are few studies on the toxicity of mecoprop-p, one of the chlorophenoxy herbicides, on aquatic organisms. Here, we investigated the toxic effects of mecoprop-p using zebrafish. After mecoprop-p exposure, we observed that the zebrafish larvae eyes did not form normally, heart edema was generated, and the body length was shortened. The number of cells undergoing apoptosis also increased in the anterior part including head, heart, and yolk sac of the mecoprop-p-treated zebrafish compared to the untreated controls. Moreover, cardiovascular structures, including the heart and aortic arches, were also malformed after exposure to mecoprop-p. Therefore, our results suggest that mecoprop-p could cause abnormal development in zebrafish larvae and there is also a high possibility that mecoprop-p would be toxic to other aquatic organisms.

Keywords: abnormal development, apoptosis, cardiovascular toxicity, mecoprop-p, zebrafish

INTRODUCTION

To regulate the growth of broad leaf weeds in crop fields, chlorophenoxy herbicides, including dicamba, mecoprop, mecorpop-p, and 4-chloro-2-methylphenoxyacetic acid, are widely used (Sanchis et al., 2013; Mottier et al., 2014). The toxicities of several chlorophenoxy herbicides have been reported despite their widespread use. For example, 2,4-dichlorophenoxyacetic acid can cause cerebrovascular impairments in rats by damaging the structure of the plasma membrane (Elo et al., 1988; Bradberry et al., 2000). Mecoprop-p, another chlorophenoxy herbicide, is a synthetic auxin that has been widely used to control weed growth since the 1980s (Périllon et al., 2021). It is known to be poorly absorbed in the soil but has a high water solubility of 250 mg/L at 20℃ (European Food Safety Authority [EFSA] et al., 2017; Périllon et al., 2021). Therefore, mecoprop-p will likely flow from the soil into the aquatic environment (EFSA et al., 2017; Périllon et al., 2021). Mecoprop-p was detected in the surface

water in several countries; in particular, up to 103 μg/L of mecoprop-p was detected in aquatic environments in Canada (Périllon et al., 2021). However, few studies have been conducted on the toxicity of mecoprop-p to aquatic organisms.

Zebrafish is a widely used animal model for toxicological studies (Kimmel et al., 1995; Zhang et al., 2003). They can lay approximately 200 to 300 eggs per week and undergo rapid embryogenesis up to 5 days after fertilization (Kimmel et al., 1995; Zhang et al., 2003). Since the embryos of zebrafish are transparent, it is easy to observe their morphology and the development of their organs (He et al., 2014; Park et al., 2020). Because of these advantages, zebrafish are an optimal animal model for toxicology studies that can also predict aquatic toxicity (An et al., 2021; Ha et al., 2021; Lee et al., 2021; Park et al., 2021).

In this study, zebrafish embryos were used to evaluate the toxicity of mecoprop-p. Specifically, we investigated the viability of zebrafish and the abnormal formation of their organs after exposure to mecoprop-p. Moreover, we also verified the increase in the number of apoptotic cells and the extent of cardiovascular structure damage in zebrafish. These results indicate that mecoprop-p confers developmental toxicity to zebrafish and there is also a high possibility that mecoprop-p would be toxic to other aquatic organisms as zebrafish is considered as toxicological model that can reflect the toxicity aspects of other fish (Su et al., 2021).

MATERIALS AND METHODS

Zebrafish breeding and embryo acquisition

Wild-type (AB strain) zebrafish and *flk1:eGFP* transgenic zebrafish were obtained from the Zebrafish Organogenesis Mutant Bank (KNRRCZOMB, Kyungpook National University, Republic of Korea). The fish were maintained in a water tank at 28℃ with a 14-h/10-h light cycle. Also, the pH of the water was maintained between 6.9 to 7.5, UV-filtered and circulated by water filters. To acquire zebrafish embryos, pairs of females and males were placed in a breeding box that is separated the female and mael zebrafish by placing a partition in the middle and kept in the dark for 13 h. Afterward, light stimulation was provided for proper breeding, and an embryo medium (1X Danieau's solution) was used to wash the embryos. To make 30X Danieau's solution, 1740 mM NaCl, 21 mM KCl, 12 mM magnesium sulfate heptahydrate, 18 mM calcium nitrate tetrahydrate, and 150 mM HEPES were added in 1 L of distilled water. Then, it was diluted to 1X Danieau's solution using distilled water to use as an embryo medium. After washing, the embryos were kept at 28℃. Animal experiments were conducted according to approved guidelines and regulations of the Institutional Animal Care and Use Committee at the Animal Ethics Committee in Korea University.

Mecoprop-p treatment

Mecoprop-p (Cat. No. 36773, Sigma Aldrich, USA) was dissolved in DMSO to make a 100 mg/L stock solution. A 0.03% 1-phenyl-2-thiourea embryo medium that disturbs pigmentation and facilitates observation was utilized to dilute the mecoprop-p stock solution to 25 and 50 mg/L concentrations. The negative controls were treated with 0.12% DMSO. We treated mecoprop-p to zebrafish larvae at 8 hpf, gastrula stage (García-Cambero et al., 2019). And the treatment was maintained until 96 hpf of zebrafish larvae. For each dose, 30 embryos were treated with different concentrations of mecoprop-p solutions and replaced treat solutions every day until 96 h after treatment. In each experiment, 12 zebrafish larvae were analyzed.

Observation of morphological abnormalities in zebrafish larvae after mecoprop-p treatment

We used a Leica DM 2500 microscope (Leica, Germany) to identify the morphological abnormalities in zebrafish larvae after mecoprop-p treatment. The eye size, body length, and the presence of heart edema were analyzed using ImageJ software (NIH, Bethesda, MD, USA). The area of eyes and body length from head to end of tail fins were measured. The heart rate (beats per minute) was measured manually.

Determination of apoptotic cell number via acridine orange staining

We used acridine orange (AO; Cat No. A3568, Life Technologies, USA) to measure the number of apoptotic cells in zebrafish larvae (Tucker and Lardelli, 2007). After 96 h of mecoprop-p treatment, the zebrafish larvae were incubated at 28℃ with 5 μg/mL acridine orange for 1 h. Afterward, the zebrafish larvae were washed twice with 1 mL tricaine and anesthetized, placed on glass slides, and observed on an upright fluorescence microscope (Zeiss Axio

Imager, M1; ZEISS, Oberkochen, Germany). The number of apoptotic cells was counted using ImageJ software.

Analysis of vascular damage in transgenic flk1:eGFP zebrafish larvae after mecoprop-p treatment

The vasculature was confirmed using a transgenic zebrafish model wherein enhanced green fluorescent protein was tagged to the endothelial receptor, flk1 (Choi et al., 2007). After 96 h of mecoprop-p treatment, the zebrafish larvae were washed with 1 mL tricaine to remove the treat solution and anesthetize. The anesthetized zebrafish larvae were arranged on 3 % methylcellulose and observed under an upright fluorescence microscope. The microscopy images obtained were analyzed using ImageJ software to confirm the density of the aortic arches and the distance between the sinus venous (SV) and bulbus arteriosus (BA).

Statistical analysis

One-way analysis of variance (ANOVA) was performed using SAS software (SAS Institute, Cary, NC, USA) to confirm the significance of the differences in the obtained data. p values < 0.05 were considered statistically significant. All data from experiments are expressed as means and standard deviations.

RESULTS

Mecoprop-p caused morphological abnormalities during early development in zebrafish larvae

To evaluate the toxicity of mecoprop-p, we first identified the viability of zebrafish larvae exposed to 0, 25, and 50 mg/L mecoprop-p. There was no significant change in zebrafish larvae viability upon treatment with 25 mg/L and 50 mg/L mecoprop-p, with 100% and 96.6% viability, respectively (Fig. 1A). However, morphological abnormalities were detected upon mecoprop-p exposure (Fig. 1B). Eye size decreased by 76.1% and 53.3% upon treatment with 25 mg/L and 50 mg/L, respectively (Fig. 1C). Moreover, the body length of zebrafish larvae was reduced by 98.0% and 86.7% upon treatment with 25 mg/L and 50 mg/L mecoprop-p, respectively (Fig. 1D). Heart edema increased significantly at 187.2% and 332.9% after treatment with 25 mg/L and 50 mg/L mecoprop-p, respectively (Fig. 1E).

Mecoprop-p increased the incidence of uncontrolled apoptosis in the anterior region of zebrafish larvae

To further investigate the mechanisms underlying the developmental abnormalities after mecoprop-p treatment, we compared the number of apoptotic cells in the zebrafish larvae treated with different doses of mecoprop-

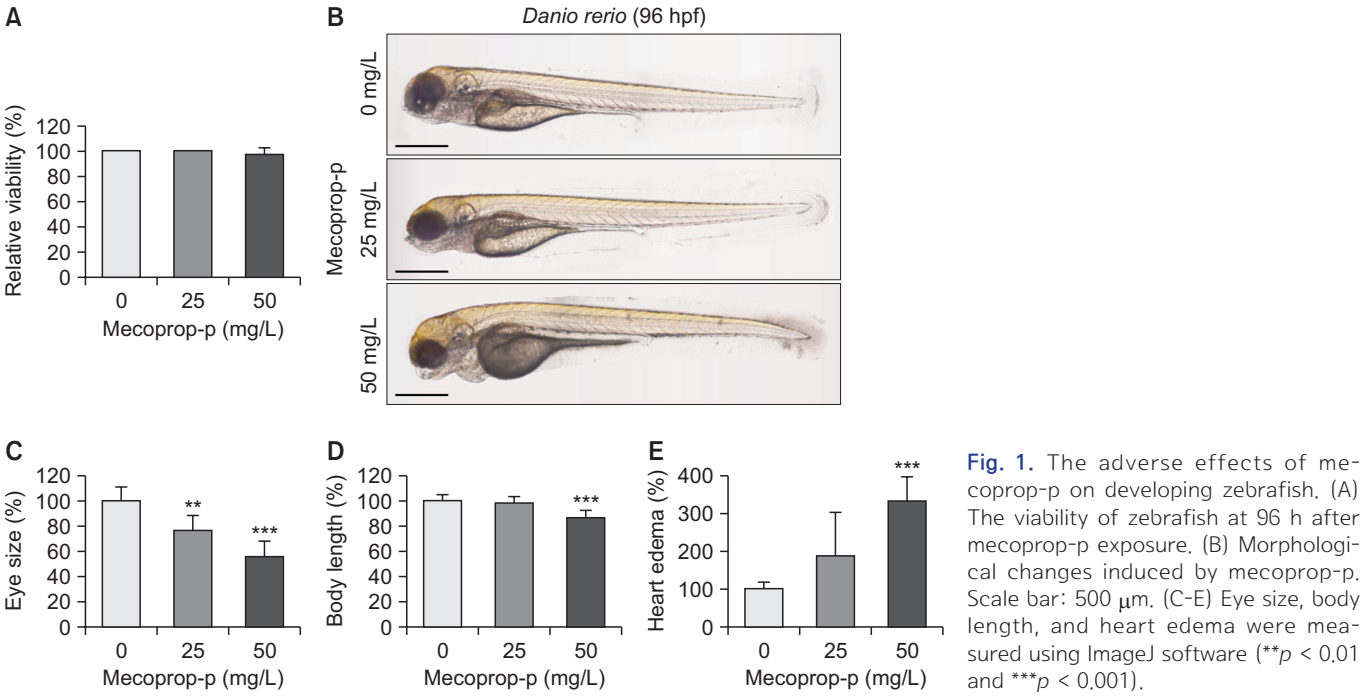

Fig. 1. The adverse effects of mecoprop-p on developing zebrafish. (A) The viability of zebrafish at 96 h after mecoprop-p exposure. (B) Morphological changes induced by mecoprop-p. Scale bar: 500 μm. (C-E) Eye size, body length, and heart edema were measured using ImageJ software (**p < 0.01 and ***p < 0.001).

p (0, 25, 50 mg/L) by staining with acridine orange. The number of apoptotic cells increased in the anterior part including eyes, ears, heart, and yolk sac of the zebrafish larvae after mecoprop-p exposure, as shown by the green fluorescence images stained with acridine orange (Fig. 2A). Specifically, the number of apoptotic cells dramatically increased by 199.8% upon treatment with 25 mg/L mecoprop-p and 260.7% after treatment with 50 mg/L mecoprop-p (Fig. 2B).

Mecoprop-p exposure induced cardiovascular abnormalities

As the size of the heart edema and extent of apoptosis increased in the anterior part including eyes, ears, heart, and yolk sac of the zebrafish larvae, we further evaluated cardiovascular structure formation using a transgenic *flk1:eGFP* zebrafish model. Structural abnormalities in the heart and aortic arches such as increased SV-BA distance and decreased relative densities of aortic arches were observed following mecoprop-p treatment (Fig. 3A). The

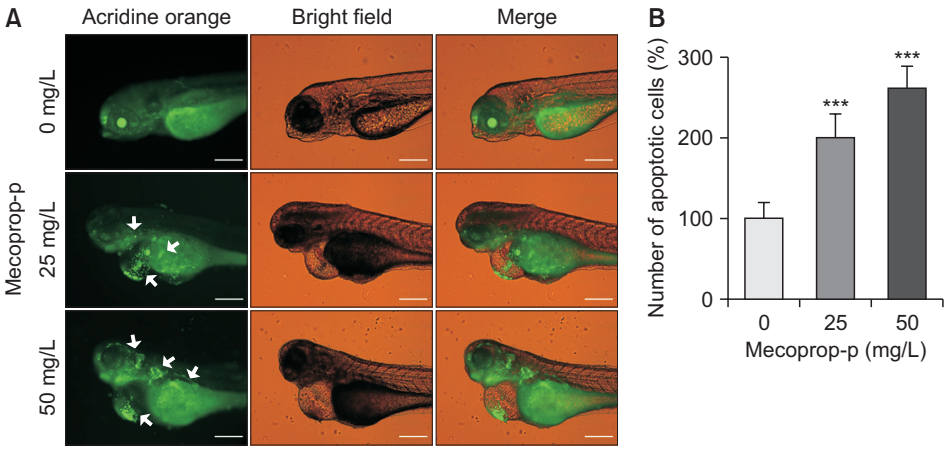

Fig. 2. Apoptotic cell death induced by mecoprop-p in zebrafish larvae was confirmed via acridine orange staining. (A) Apoptotic cells are indicated by green dots were indicated by the white arrows. Scale bar: 300 μm. (B) The number of apoptotic cells was calculated using ImageJ software (***$p < 0.001$).

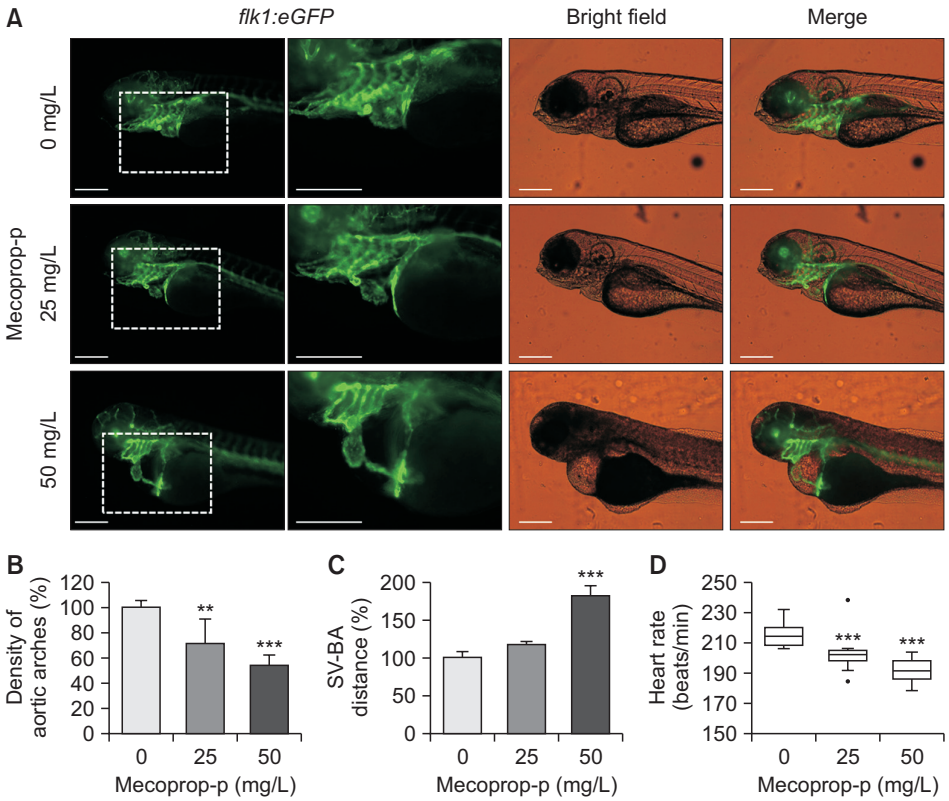

Fig. 3. The structure of the heart and blood vessels that were damaged by mecoprop-p. (A) Transgenic *flk1:eGFP* zebrafish models were observed using an upright fluorescence microscope. The structural abnormalities in the heart and aortic arches are shown. The second panels are extended images of white boxes. Scale bar: 300 μm. (B and C) The density of the aortic arches and the distance between the SV and BA were calculated using ImageJ software. (D) The heart rate per minute was measured manually (**$p < 0.01$ and ***$p < 0.001$).

density of aortic arches decreased by 71.2% and 53.8% after treatment with 25 mg/L and 50 mg/L mecoprop-p, respectively (Fig. 3B). The distance between the SV and BA increased by 117.4% and 181.8% after treatment with 25 mg/L and 50 mg/L mecoprop-p, respectively (Fig. 3C). Furthermore, the heart rate of the zebrafish larvae decreased by 92.3% and 88.6% after treatment with 25 mg/L and 50 mg/L mecoprop-p, respectively (Fig. 3D).

DISCUSSION

Mecoprop-p is a chlorophenoxy herbicide widely used since the 1980s (Périllon et al., 2021). It is known that mecoprop-p is poorly absorbed in the soil but has high water solubility (EFSA et al., 2017; Périllon et al., 2021). Such characteristics make mecoprop-p highly likely to be detected in the aquatic environment (EFSA et al., 2017; Périllon et al., 2021). Mecoprop-p has previously been detected in the groundwater or urban water in several countries such as Canada, the UK, and Ireland (Idowu et al., 2014; Périllon et al., 2021). However, despite these reports, there have been few studies on the toxicity of mecoprop-p to aquatic organisms, especially developmental toxicity. In this study, we confirmed the developmental toxicity of mecoprop-p using zebrafish larvae. We identified morphological changes such as heart edema, decreased eye size, and body length, and an increase in the number of apoptotic cells at the anterior part of zebrafish larvae including eyes, ears, heart and yolk sac upon mecoprop-p exposure. Moreover, cardiac vascular abnormalities like increased SV-BA distance and decreased density of aortic arches were observed after mecoprop-p treatment using *flk1:eGFP* transgenic zebrafish models.

First, we identified the survival rate of zebrafish larvae exposed to 0, 25, or 50 mg/L mecoprop-p. There was no significant difference in the survival rate of zebrafish larvae, but morphological abnormalities were observed in several organs. Eye size and body length tended to decrease after mecoprop-p exposure, typical symptoms of a developmental disorder (McCollum et al., 2011). Mecoprop-p also increased pericardiac edema, a representative indicator of cardiac toxicity (Zakaria et al., 2018). Actually, substances that are known to be cardiotoxic, like carbaryl and TCDD, commonly cause pericardiac edema and this suggested that mecoprop-p could have cardiovascular toxicity (Chen, 2013). As a result of exposure to mecoprop-p, the small size of eye, the shorten body length, and the heart edema were caused in the zebrafish larvae.

Next, we investigated the number of apoptotic cells in zebrafish larvae after mecoprop-p exposure. Apoptosis in zebrafish is controlled by several proteins like Bcl2, Bid, and caspase 9 (Youle and Strasser, 2008; Chowdhury et al., 2008; Eimon and Ashkenazi, 2010). The proper control of apoptosis is important during normal development (Eimon and Ashkenazi, 2010), and the timing of apoptosis regulation during development is also different for each organ. For example, apoptosis is maximal at 36 hpf in the eyes and at 20 hpf in the tail. In the brain region, apoptotic cells were clustered between 24 and 60 hpf (Cole and Ross, 2001). Therefore, it is important to regulate apoptosis according to the normal developmental process (Voss and Strasser, 2020). However, we observed that the number of apoptotic cells increased significantly in the heart, ears, eyes, and yolk sac compared to the vehicle-treated groups at 96 h after mecoprop-p treatment. This result indicated that unregulated apoptosis due to mecoprop-p exposure could cause abnormal development in zebrafish larvae.

Pericardiac edema, one of the symptoms of cardiac toxicity, was observed upon mecoprop-p treatment (Chen, 2013; Zakaria et al., 2018). Therefore, we further investigated the structure of the heart and vasculature using a transgenic *flk1:eGFP* zebrafish model. In zebrafish, the heart develops at an early stage (Bakkers 2011; Zakaria et al., 2018). As heart development occurs in an early stage, blood flows normally and the blood vessels already develop (Burggren, 2013; Park et al., 2021). We also confirmed the abnormalities in the zebrafish larvae heart by measuring the distance between the SV and BA, which are used to evaluate cardiotoxicity and cardiac circulation (Cui et al., 2016; Lu et al., 2022). The distance between the SV and BA dramatically increased, and the heart rate decreased in the mecoprop-p-treated zebrafish larvae. The aortic arches, which are vascular structures located close to the heart, play important roles in blood flow and circulation from the heart (Isogai et al., 2001). They deliver deoxygenated or oxygenated blood to the brain and lungs (Crucke and Huysseune, 2013). We observed that the aortic arches were malformed after mecoprop-p exposure, as their relative density decreased in the mecoprop-p-treated groups compared to the vehicle-treated groups.

These results indicate that mecoprop-p conferred cardiac toxicity as it induced malformations in the heart and vasculature, which play important roles in normal zebrafish development.

CONCLUSION

This study investigated the toxicity of mecoprop-p using zebrafish models. Mecoprop-p was shown to induce morphological abnormalities, such as decreased eye size and body length, and caused heart edema. Mecoprop-p also increased the number of apoptotic cells in the anterior part of the zebrafish and damaged the structures of the heart and aortic arches, which are important for normal blood flow. The results of this study indicated that since mecoprop-p could induce developmental toxicity in zebrafish by increasing apoptosis and inducing cardiovascular malformation, it might also be toxic to other aquatic organisms.

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