



Original Article

Electroporation of thalidomide to medaka (*Oryzias latipes*) embryo for evaluation of developmental toxicity

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ABSTRACT — The evaluation of developmental toxicity requires new methods that provide an alternative to animal use. In this study, we evaluated the effects of thalidomide exposure [upon nanosecond pulsed electric field (nsPEF) treatment] on the embryonic development of, teratogenic effects in, and gene expression levels in medaka embryos, to determine whether the adverse effects of thalidomide can be detected using medaka. Incorporation of thalidomide into medaka embryos led to malformations in individuals. The results obtained by microinjecting thalidomide into zebrafish embryos could be reproduced by medaka embryo electroporation. Furthermore, the teratogenic mechanism of thalidomide was confirmed at the genetic level. Medaka eggs, in addition to zebrafish, can be used to assess the effects of thalidomide. As the nsPEF method can efficiently incorporate of test chemicals into medaka eggs, it is suggested to have great potential for use as a high-throughput animal alternative evaluation system.

Key words: Embryonic developmental toxicity, Teratogenicity, Thalidomide, Medaka

INTRODUCTION

The developmental toxicity tests are essential for evaluating the safety of chemical substances. In general, higher animals, such as mammals, are used to assess the developmental toxicity of chemical substances; however, it is necessary to reduce the number of such tests, from the viewpoint of animal protection. Small model fish are attracting attention as alternative organisms because they have high genetic homology with humans and a developmental process that can be easily observed. Among them, medaka (*Oryzias latipes*) and zebrafish (*Danio rerio*) have been used as model organisms in many studies not only for evaluation of developmental toxicity, but also for drug discovery (Scholz *et al.*, 2008, 2018; Beedie *et al.*,

2016; Liu *et al.*, 2018; Cassar *et al.*, 2020). In addition, these organisms are easy to breed and handle; thus, the cost of testing is low, making them ideal for evaluating many chemical substances. Recently, many evaluations of chemical substances and contaminated water have been carried out, but the presence of a relatively high concentration of the test chemical in real-life scenarios raises a concern regarding the reliability of such evaluations. This is because the exposure method is primarily immersion, so not only is the exposure amount low, but the individual difference in the uptake amount is large. Therefore, the use of microinjection as the exposure method has been attempted (Mikami *et al.*, 2019), but skill level required for this method is high, owing to which high throughput cannot be achieved.

We previously developed an electroporation method using a nanosecond pulsed electric field (nsPEF) to facilitate the introduction of substances into medaka eggs. We attempted to assess teratogenicity and embryonic developmental toxicity; moreover, we determined some endpoints that included not only survival, growth, and developmental abnormalities but also changes in the gene expression levels for the assessment of toxicity of 17 β -estradiol, lithium, and benzo(a)pyrene using medaka and nsPEF treatment (Yamaguchi *et al.*, 2018, 2020; Tominaga *et al.*, 2019).

Thalidomide was prescribed as a sleeping pill around the world until approximately 1960. However, when taken by pregnant women during the early stages of pregnancy, it was found to induce severe birth defects and was withdrawn from usage. Despite this disaster associated with thalidomide usage, it is considered clinically effective for the treatment of patients with tumors because it can inhibit the expression of tumor necrosis factor α , inhibit angiogenesis, and regulate the expression of several cytokines (Gao *et al.*, 2020).

In this study, we evaluated the effects of thalidomide exposure (upon nsPEF treatment) on the embryonic development of, teratogenic effects in, and gene expression levels in medaka embryos, to determine whether the adverse effects of thalidomide can be detected using medaka.

MATERIALS AND METHODS

Test chemicals

Thalidomide (Cas No.:50-35-1, purity > 99%) was purchased from Fujifilm Wako Pure Chemical Industries Ltd. (Osaka, Japan). All other reagents used were of special grade.

Animals

Medaka (NIES-R strain) were obtained from the National Institute of Environment and had been housed in glass tanks for five years in our laboratory. The medaka were fed a diet of *Artemia nauplii* and commercial feed (Hikari, Tokyo, Japan) at least twice daily. The tanks maintained a 16:8 hr light:dark photoperiod and a temperature of 25 \pm 1°C. For prioritizing the protection of animals, the fish in this study were used to the minimum extent possible.

Chemical incorporation into medaka fertilized eggs using nsPEF and morphology observations

Chemical incorporation and subsequent observation were performed as described by Yamaguchi *et al.* (2020). Fertilized medaka eggs from various breeding pairs were

collected within 5 hr of fertilization and then randomly selected for treatment. Individual medaka eggs were subjected to nsPEF treatment in an isotonic solution in the presence of 0 to 1 mM thalidomide (n = 12–24 per group). The eggs were then soaked in the same solution for 2 hr. After treatment, they were washed thoroughly with the isotonic solution, and eggs were placed individually in a 200- μ L isotonic solution, in a 96-well plate. During the observation period, the embryos were maintained at 25 \pm 1°C, and the isotonic solution in the wells was changed daily. Developmental abnormalities, mortality, time to hatching, and hatchability were observed microscopically, using a digital microscope (VHX-900F; Keyence, Osaka, Japan), and recorded daily. Lethality of the medaka eggs was calculated using the observational results obtained 24 hr after pulse treatment. Developmental delays and morphological abnormalities were defined as hyperemic edema, defective heart formation, defective angiogenesis, and defects in the head and eyeball.

Bone stain analysis using calcein

Du *et al.* (2001) developed a simple method to fluorescently stain bone structures in live zebrafish embryos. We performed this method in medaka larvae, with slight modifications. After hatching, the medaka larvae were immersed in 0.001% calcein solution containing 0.05% tricaine methanesulfonate for 15 min. The samples were then washed with an isotonic solution and observed under a fluorescence microscope.

Gene expression analysis via quantitative real-time polymerase chain reaction (qRT-PCR)

We performed gene expression analyses on medaka larvae, after exposure to 1 mM thalidomide, using qRT-PCR. Medaka embryos were cultured, and microscopic observations were performed daily for 10 days after nsPEF treatment. Five individuals in the thalidomide-treated and control groups were collected and soaked in RNAlater. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA samples were reverse transcribed with RevaTraAce (Toyobo, Osaka, Japan) using oligo dT primers. Then, qPCR was carried out by using the Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) and Mx3000p Real-Time QPCR System (Agilent Technologies). The primers for fibroblast growth factor 8 (FGF8), atonal bHLH transcription factor 1 (Atoh1), and ribosomal protein L7 (RPL-7) are shown in Table 1. To quantify the expression levels of genes in the embryos, PCR amplification was performed in 20- μ L reaction mixtures incu-

Table 1. Primer sequences for FGF8, Atoh1, and RPL-7 genes.

Gene Name	Accession #		Primer Sequences
FGF8	XP_004076956	forward	AACGCCCACTACAACGACTG
		reverse	CGGGTGCGTTTAGTCCTTG
Atoh1	XP_004072375	forward	ACTGCTCTTACAACGAGGCG
		reverse	CGAACTTTGAGCGAGGGAGA
RPL-7	DQ118296	forward	CGCCAGATCTTCAACGGTGTAT
		reverse	AGGCTCAGCCAATCCTCAGCAT

bated at 95°C for 10 min, followed by 35 PCR cycles at 94°C for 30 sec, 62°C for 30 sec, and 72°C for 1 min. Relative expression levels were obtained, and the expression level of each gene was normalized to that of RPL-7 (Zhang and Hu, 2007).

Statistical analysis

Each experiment was repeated at least three times, and the data obtained were analyzed using one-way analysis of variance.

RESULTS AND DISCUSSION

As an alternative to the teratogenicity test, a test system using small fish eggs, such as those of zebrafish and medaka, has been proposed. Substance introduction efficiency, a technical aspect of this assay, affects its sensitivity. Microinjection has also been used, but the technical skillset required to perform this technique has hindered the achievement of high throughput. Multiple studies on zebrafish have reported about the ease of introduction of test substances via immersion, owing to the lack of a hard chorion shell. However, long-term immersion is required in a high-concentration solution, owing to which the window of exposure becomes unclear. Notably, nsPEF treatment is a potent method for incorporation of chemical compounds into medaka eggs (Tominaga *et al.*, 2010, 2019; Yamaguchi *et al.*, 2018, 2020). Using this method, chemical substances that are difficult to introduce via osmosis alone can be easily introduced inside medaka eggs with shells. In this study, thalidomide was used as the test substance to evaluate the effectiveness of using this method on medaka eggs as a teratogenicity test.

When thalidomide was incorporated into medaka embryos, malformed individuals were observed. Typical malformations included large curvature of the spine and defects of pectoral fins (Figs. 1B, C). Fins are structurally different from the limbs in mammals and chicks; however, the molecular pathways are evolutionarily conserved. The results of fluorescent bone staining by calcein revealed that there were no defects in the spine, but mal-

formations were observed due to insufficient bone formation (Fig. 1E). In zebrafish, thalidomide inhibits the development of pectoral fins because it inhibits chondrogenic differentiation in pectoral fins (Siamwala *et al.*, 2012; Asatsuma-Okumura *et al.*, 2019).

No significant lethality or malformation was observed in the control and nsPEF treatment groups. In contrast, significant lethality and malformation were observed in individuals treated with 10 µM thalidomide. The effect was concentration-dependent, increasing with increasing concentrations from 10 to 100 µM (Fig. 2). Mikami *et al.* (2019) reported that approximately 20% and 30% teratogenicity was observed when 20 nL of 30 and 80 µM thalidomide was microinjected into zebrafish eggs, respectively. Considering the high incidence of malformations in the control group in zebrafish, our results could be shown to be almost the same level. About 20% of the individuals were affected by the incorporation of thalidomide, exhibiting malformations and deaths. It has been reported that R-thalidomide shows no teratogenicity in experiments involving mice and that S-thalidomide shows approximately 30% teratogenicity, owing to the racemization and self-disproportionation of enantiomers after dissolution (Blaschke *et al.*, 1979; Tokunaga *et al.*, 2018).

Thalidomide binds to cereblon, which is a component of E3 ubiquitin ligase complex, and changes its substrate specificity to cause ubiquitination of p63, thereby promoting the degradation of p63. Then, FGF8 and Atoh1 levels are reduced, leading to malformations (Asatsuma-Okumura *et al.*, 2019). Therefore, we designed primers for the homologous genes of FGF8a and Atoh1a in medaka and measured their expression levels using qRT-PCR. FGF8a and Atoh1 gene expression was significantly reduced by thalidomide incorporation (Fig. 3). These results confirmed that decreased FGF8a and Atoh1a levels caused malformations in medaka. The thalidomide-treated samples in this study were a mixture of those with and without malformations. The association between malformations and the expression levels of these genes needs to be investigated in future studies.

In the current study, we evaluated the teratogenicity

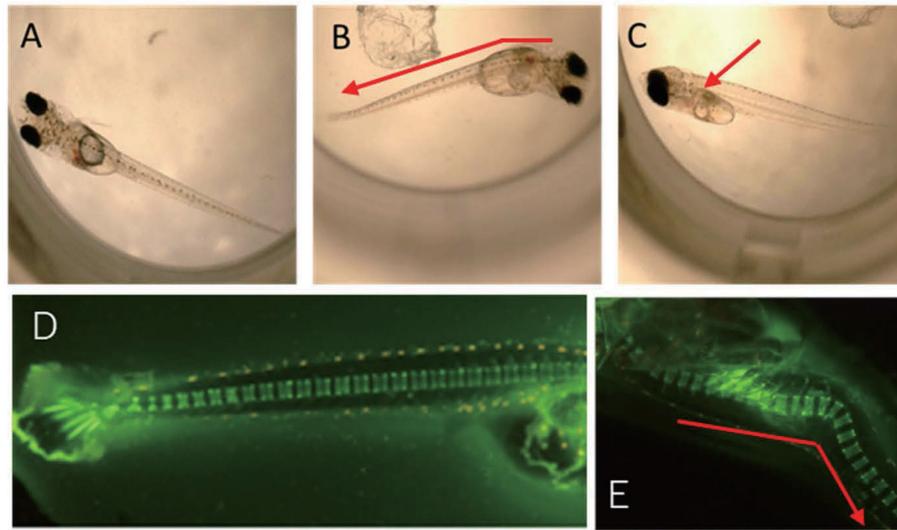


Fig. 1. Photograph of typical normal fry (A, D, CTL) and abnormal development, such as curvature of the spine (B and E) and the defect of pectoral fins (C). Optical microscopy image (A, B and C), fluorescent image (D and E).

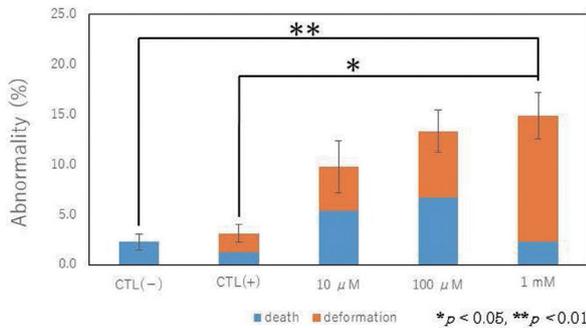


Fig. 2. Occurrence rate (%) of malformations (gray bar) and death (black bar) among nsPEF-treated embryos with thalidomide incorporated (triplicates of $n = 12-24$ per group). CTL(-), no nsPEF control; CTL(+), nsPEF positive control. * $p < 0.05$, ** $p < 0.01$.

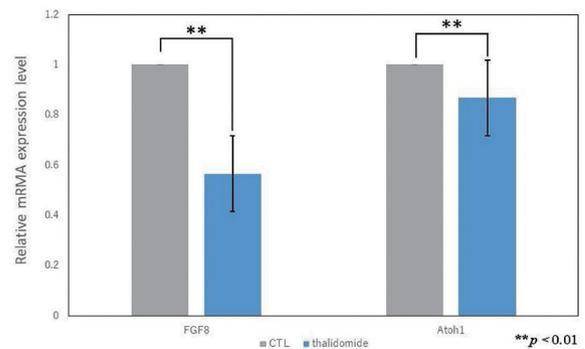


Fig. 3. Analysis of gene expression of FGF8 and Atoh1 associated with thalidomide incorporation. Data are presented as the mean and standard deviation (SD) of three independent experiments. ** $p < 0.01$.

of thalidomide via a developmental toxicity test system using medaka embryos and the nsPEF method. Therefore, in addition to zebrafish (as established via previous studies), medaka eggs can be used to assess the effects of thalidomide; moreover, the corresponding mechanism of action is reproducible. The nsPEF method, involving the efficient incorporation of test chemicals into medaka eggs, has great potential for use as a high-throughput animal alternative evaluation system.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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