The Japanese Society of Toxicology

Fundamental Toxicological Sciences

URL : http://www.fundtoxicolsci.org/index_e.html

Toxicomics Report

Effects of lithium on developmental toxicity, teratogenicity and transcriptome in medaka embryos

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(Received January 18, 2019; Accepted January 25, 2019)

ABSTRACT — In this study, we assessed embryonic developmental toxicity and teratogenicity of lithium (Li) on medaka (*Oryzias latipes*) and predict the molecular mechanisms of their effects using a nanosecond pulsed electric field (nsPEF) technique and bioinformatics analysis. The microscopic observation revealed that the 1 mg/L LiCl treatment causes the most severe deformation effects, such as thrombus, heart hypertrophy, deformation of eyes, and growth retardation to embryos. The RNA-seq analysis identified 2,483 up- and down-regulated genes, such as histogenesis and organ growth related genes, in 2 day post-fertilization embryos after treatment with nsPEF and 1 mg/L LiCl. In addition, bioinformatic analyses showed that LiCl affects several aspects of gene ontology, such as molecular functions and cellular components, and some pathways, such as spliceosome, cell cycle, selenocompound metabolism, TGF-β signaling, and RNA degradation. The upregulation of *GSK3B* (signal transduction and cell growth), *BAX* (apoptosis), and *MAP3K8* (cell death, arrest of cell cycle, and inflammation) genes were also observed in embryos exposed to LiCl. Our results suggest that the incorporation of Li compounds into medaka eggs using nsPEF shows adverse effects to the development and teratogenicity, and that these toxic effects may be affected by the alterations of certain gene expression in medaka embryos.

Key words: Lithium, Development, Transcriptome, Nanosecond pulsed electric field, *Oryzias latipes*

INTRODUCTION

Lithium (Li) is one of the alkali metal elements, and has been ubiquitously detected in various environmental samples including soil, surface water, and seawater and various food samples including grains, dairy products, fish, shellfish, meat, and vegetables (Leblanc *et al.*, 2005; Schrauzer, 2002). Li has been known to be effective for treating mood disorders such as bipolar disorder over several decades (Schrauzer, 2002). However, a previous study has indicated that Li causes side effects, such as hypothyroidism, cardiac arrhythmia, and convulsions

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(Chan *et al.*, 2000). Despite the use and disposal of ubiquitous Li-ion batteries, no data are available on the prescribed upper limit of toxicological reference value for Li (Leblanc *et al.*, 2005).

The embryos are highly sensitive to chemical exposure in animals, with the result that developmental toxicity and teratogenicity are the important endpoints in adverse effects. The fish embryo toxicity (FET) test, using non-mammalian vertebrates, such as zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*), is recommended as an alternative method for investigating the developmental toxicity and teratogenicity of chemicals (Embry *et al*., 2010). Our previous study, using a nanosecond pulsed electric field (nsPEF) technique, has demonstrated that intracellular incorporation of 17β-estradiol causes the developmental toxicity and teratogenicity in medaka embryos (Yamaguchi *et al.*, 2018). However, to our knowledge, the potential molecular mechanisms of Li on developmental toxicity and teratogenicity in animals, particularly the model fish species, are not fully understood, although a recent review reported the pharmacological and toxicological effects of Li on zebrafish (Siebel *et al.*, 2014).

In this study, we assessed the developmental toxicity and teratogenicity of Li compounds, such as lithium chloride (LiCl) and lithium carbonate (Li_2CO_3), incorporated by using nsPEF technique, on medaka embryos. In addition, we analyzed the gene expression profiles in the Li-incorporated medaka embryos using RNA-sequencing and performed gene ontology (GO) and pathway analyses to understand the potential molecular mechanisms of Li on embryonic developmental toxicity and teratogenicity in medaka.

MATERIALS AND METHODS

Test chemicals

LiCl (CAS no. 7447-41-8, purity > 99.0%) and Li_2CO_3 (CAS no. 554-13-2, purity > 99.0%) were obtained from FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan. These reagents were dissolved in Milli-Q water for preparing the stock solutions of test chemicals.

Animals

Medaka (d-rR strain) had been kept for more than 10 years in our laboratory and fed a diet of *Artemia nauplii* once daily. The glass tanks maintained a 16:8 hr light:dark photoperiod and a temperature of 25 ± 1°C. Eggs (< 6 hr after fertilization) spawned from each female fish were carefully collected and used for the Li exposure.

Exposure and toxicological evaluation

The Li incorporation into eggs using nsPEF was performed as previously described (Tominaga *et al.*, 2010; Kono *et al.*, 2015; Yamaguchi *et al.*, 2018). Briefly, eggs (quadruplicates of n = 24-36 per group) were subjected to nsPEF treatment in an isotonic solution in the presence of LiCl (0.01, 0.1, and 1 mg/L) and Li_2CO_3 (0.01, 0.1, 1, and 10 mg/L) solution prepared by diluting the stock solution in an embryo culture medium (0.1% NaCl, 0.003% KCl, 0.004% $CaCl_2$, and 0.0163% $MgSO_4$, pH = 7.3). After applying PEFs, the eggs were soaked in the same Li solutions for 2 hr, and then washed with the isotonic solution. Each egg was placed separately in a 500-mL container having the isotonic solution using a 96 multi-well plate.

During the 14-d observation period, embryos were maintained at 25 ± 1°C and the isotonic solution in the wells was changed daily. Developmental abnormalities and mortality were observed microscopically using a digital microscope VHX-900F (Keyence, Osaka, Japan) and recorded daily. Developmental delays and morphological abnormalities were defined as hyperemic edema, thrombus, defective heart formation, heart hypertrophy, defective angiogenesis, and defects of the head and eyeball (Yamaguchi *et al.*, 2018).

Gene expression analysis

Total RNA was isolated from the 2 day post-fertilization (dpf) embryos after nsPEF and LiCl (1 mg/L) treatments (pooled from n = 50 eggs per group) using the RNeasy Mini Kit (Qiagen, Hilden, Germany) as previously described (Uchida *et al.*, 2015). The quantity and purity of the total RNA were examined using a Q5000 spectrophotometer (Tomy Seiko Co., Ltd., Tokyo, Japan) and electrophoretically using the RNA 6000 Nano LabChip Kit employing an Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). RNA samples with RNA Integrity Number (RIN, as defined by Agilent Technologies) values higher than 9.3 were used for mRNA purification. The mRNA purified from total RNA using the Oligotex-dT30 <Super> mRNA Purification Kit (TAKARA BIO Inc., Shiga, Japan). The cDNA Library was prepared from mRNA (1 μg) using the Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. The constructed library underwent PCR amplification, purification and size selection (200 bp). The selected PCR products were used for emulsion PCR using Ion PGM Hi-Q OT2 Kit (Thermo Fisher Scientific). The emulsion PCR products were sequenced on an Ion PGM with an Ion 318 Chip kit v2 (200 bp, 5M reads) platform (Thermo Fisher Scientific).

Bioinformatics analyses were carried out using CLC Genomics Workbench software version 10.1.1 (Qiagen, Hilden, Germany). For assembly, the reads were uploaded into CLC Genomics as FASTA files and assembled using default parameters. All assembled contigs were compared with medaka sequences in NCBI *Oryzias latipes* mRNA database for functional annotation by using standalone Blastn (within the CLC Genomics tool). For gene expression analysis, the expression level of each gene in the control and LiCl-treated samples was calculated and normalized to Reads per Kilobase per Million (RPKM). Two-fold change in the RPKM values of the corresponding transcripts between control and LiCl-treated group was regarded as the differential expression. The genes that were abundant or scarce in the LiCl-treated group were screened for functional categorization of differentially expressed genes (DEGs). Up-regulation and down-regulation were defined as a gene expression ratio of exposed to control of greater than 3 and less than 1/3, respectively. Moreover, for genes whose total of RPKM values of control and LiCl-treated was less than 10, these were cut off as a false DEGs. For gene set enrichment analysis (GSEA), gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed by using DAVID database, and genes were categorized based on enrichment of GO terms into biological process, molecular function, and cellular component.

RESULTS

Effects of Li compounds on developmental toxicity and teratogenicity of embryos

In the pilot experiments, we initially attempted to measure LiCl uptake in medaka embryos treated with nsPEF and LiCl solution (10 mg/L) using microwave plasma-atomic emission spectroscopy (MP-AES). Results revealed that concentrations of LiCl in eggs were 1.09 µg/g with nsPEF treatment and 0.19 µg/g without nsPEF treatment (data not shown).

The occurrence rates of normal embryogenesis were more than 70% in the control groups without pulse [CTL(-)] and with pulse [CTL(+)], and no significant differences were observed between both groups (Fig. 1). However, the normal rates in embryos exposed to 0.01, 0.1, and 1 mg/L LiCl were decreased relative to the controls, but no significant differences were observed because of the large variation (Fig. 1). Among the LiCl treatment groups, the 1 mg/L treatment had the most severe developmental effects on embryos, resulting in a normal rate of 45.2% (Fig. 1). The normal rates in embryos exposed to Li_2CO_3 (0.01-10 mg/L) were also decreased relative to the controls, but no significant differences were observed because of the large variation (Fig. 1). The rates of normal embryogenesis by exposure to Li_2CO_3 were 54.1% for 0.01 mg/L, 59.7% 0.1 mg/L, 57.4% for 1 mg/L, and 54.2% for 10 mg/L (Fig. 1).

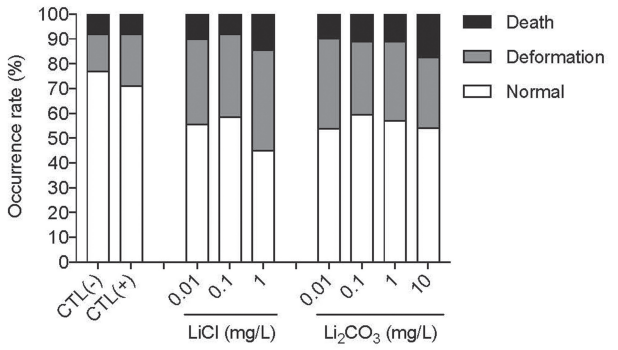

Fig. 1. Occurrence rate (%) of typical normal (black bar), deformation (thrombus, heart hypertrophy, deformation of eyes, and growth retardation) (gray bar), and death (white bar) embryos treated with pulse and Li compounds (quadruplicates of n = 24-36 per group). CTL(-), no nsPEF controls; CTL(+), nsPEF positive controls.

The occurrence rates of embryonic deformation and death in the control groups without pulse [CTL(-)] and with pulse [CTL(+)] were lower than those in all Li compound treatment groups (Fig. 1). However, in all Li compound treatment groups, typical deformations, such as thrombus (Fig. 2C), heart hypertrophy (Fig. 2D), deformation of eyes (Fig. 2E), and growth retardation (Fig. 2F) were detected by using a microscopic observation, and their rates were increased compared to controls (Fig. 1). Among the Li treatment groups, the rates of deformation (40.7%) and death (14.1%) individual in embryos were the highest in the 1 mg/L LiCl treatment group (Fig. 1). These abnormal individuals could not finally swim up.

Gene expression analysis using RNA-seq

The RNA-seq analysis identified 2,483 up- and down-regulated genes in two dpf embryos after treatment with nsPEF and 1 mg/L LiCl. Among these genes, several histogenesis and organ growth related genes, such as paired box gene and homeobox genes, were up-regulated (Table 1).

The GO enrichment analysis revealed that molecular function, such as nucleotide binding, ATP binding, RNA binding, and nucleic acid binding, and cellular components, such as nucleus, were significantly enriched in

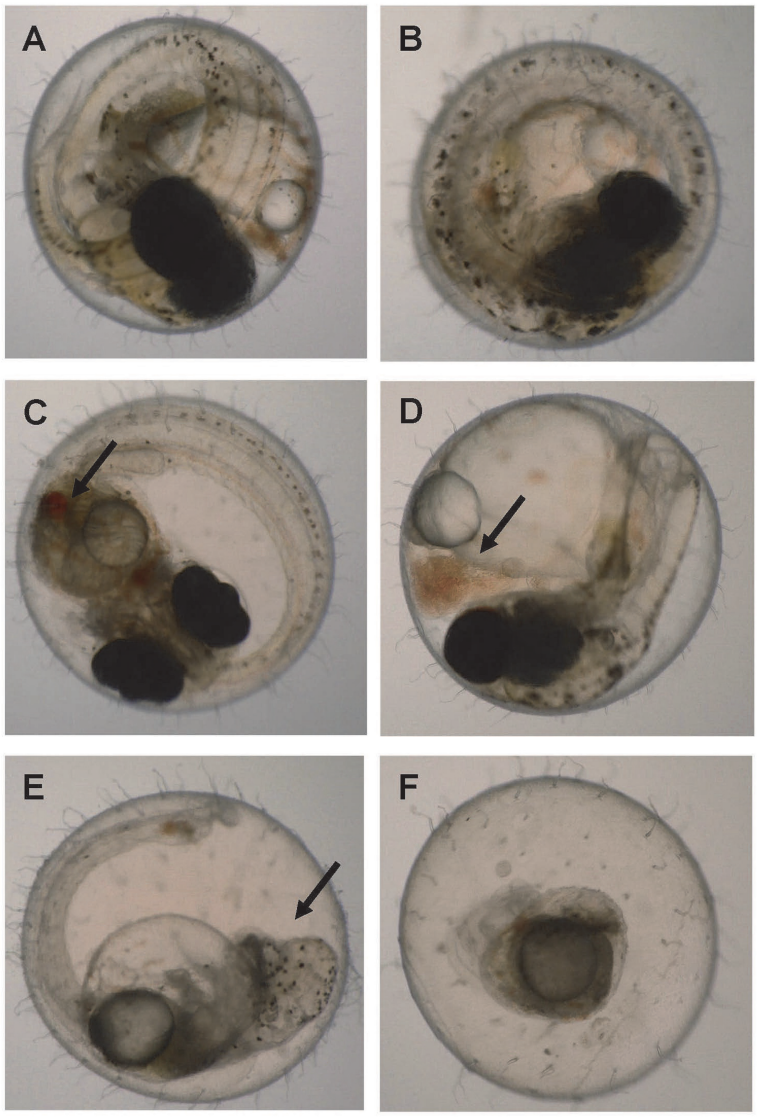

Fig. 2. Photograph of typical normal [A, CTL(-) and B, CTL(+)] and abnormal development, such as thrombus (C), heart hypertrophy (D), deformation of eyes (E), and growth retardation (F) of 2 dpf embryos treated with pulse and 1 mg/L LiCl.

embryos treated with 1 mg/L LiCl (Table 2). The GSEA of KEGG pathways revealed the effects of LiCl on spliceosome, cell cycle, selenocompound metabolism, TGF-β signaling, and RNA degradation (Table 2). The alteration of gene expression of *glycogen synthase kinase 3β* (*GSK3B*), *apoptosis regulator BCL associated X* (*BAX*), and *mitogen-activated protein kinase kinase kinase 8* (*MAP3K8*) were found by LiCl exposure (data not shown).

DISCUSSION

To investigate the potential molecular mechanisms of Li compounds on developmental toxicity and teratogenicity in medaka embryos, this study analyzed the gene expression profiles in the LiCl-incorporated embryos using RNA-seq and performed the GO and pathway analyses. Our FET test revealed that the 1 mg/L LiCl treatment had the most severe effects on embryos, resulting in high rates of deformation (40.7%) and death (14.1%)

Table 1. Up-regulated genes in medaka embryos treated with nsPEF and 1 mg/L LiCl.

Accession	Description	Fold change
XM_011487584.1	ventral anterior homeobox 2	10.0
XM_004082308.2	orthodenticle homeobox 2	8.0
XM_011473216.1	homeobox-containing protein 1-like	7.0
XM_011478722.1	homeobox protein Hox-B3a-like	5.5
XM_004077828.2	homeobox protein Hox-A11b	5.0
XM_011485656.1	POU class 3 homeobox 1	4.5
XM_004079227.2	paired box protein Pax-3	4.3
XM_011488831.1	zinc finger homeobox 4	4.1
XM_004069902.2	developing brain homeobox 2	4.0
XM_004080636.2	mesenchyme homeobox 1	4.0

Table 2. Gene ontology (GO) enrichment and pathway analyses of differentially expressed genes in medaka embryos treated with nsPEF and 1 mg/L LiCl.

		ID	Term	Count	%	P value	Benjamini
GO	MF	GO:0000166	Nucleotide binding	39	1.72	1.65×10^{-6}	5.14×10^{-4}
		GO:0005524	ATP binding	115	5.07	8.45×10^{-6}	1.31×10^{-3}
		GO:0003723	RNA binding	31	1.37	9.40×10^{-5}	9.70×10^{-3}
		GO:0003676	Nucleic acid binding	48	2.12	8.11×10^{-4}	6.11×10^{-2}
	CC	GO:0005634	Nucleus	102	4.50	2.00×10^{-5}	2.86×10^{-3}
Pathway		ola03040	Spliceosome	36	1.59	7.17×10^{-6}	1.02×10^{-3}
		ola04110	Cell cycle	33	1.46	4.54×10^{-4}	3.17×10^{-2}
		ola00450	Selenocompound metabolism	8	0.35	8.70×10^{-4}	4.04×10^{-2}
		ola04350	TGF-β signaling pathway	25	1.10	1.99×10^{-3}	6.82×10^{-2}
		ola03018	RNA degradation	21	0.93	2.07×10^{-3}	5.73×10^{-2}

MF, Molecular function; CC, Cellular component.

individual. Several studies have demonstrated the toxic effects of Li compound on the early life stage of fish. The lethal concentration (LC_{50}) of LiCl was 8.7 mg/L in 26-day fathead minnow (*Pimephales promelas*) larvae and 9.2-62 mg/L in white cloud mountain minnow (*Tanichthys albonubes*) (Long *et al.*, 1998; Aral and Vecchio-Sadus, 2008). Another study has reported the teratogenicity of LiCl on 72-hr zebrafish embryos (Selderslaghs *et al.*, 2009). These results suggest that LiCl cause toxic effects such as acute toxicity and developmental toxicity and teratogenicity on the early life stage of fish, including embryos and larvae.

Using a RNA-seq analysis, we identified the LiCl-responsive genes such as several histogenesis and organ growth related genes including paired box gene and homeobox genes in embryos. The DEGs were also involved in several GO such as molecular functions and cellular component. From the GSEA of KEGG pathways analysis, we found that LiCl may affect some binding functions in the nucleus and also affect spliceosome, cell cycle, selenocompound metabolism, TGF-β signaling, and RNA degradation. Moreover, our FET test demonstrated that typical deformations, such as thrombus, heart hypertrophy, deformation of eyes, and growth retardation were detected by using a microscopic observation. These results suggest that these altered gene expressions may be involved in developmental toxicity and teratogenicity of LiCl in medaka embryos. Furthermore, the present study clarified that the upregulation of *GSK3B* (signal transduction and cell growth), *BAX* (apoptosis), and *MAP3K8* (cell death, arrest of cell cycle and inflammation) genes were also observed in embryos exposed to LiCl. A previous study has indicated that the inhibition of GSK3 and inositol monophosphatase are mostly attributed to the effects of Li on bipolar disorders (De Sarno *et al.*, 2002). These results suggest that certain GSK3B responses to Li compound may be similar between mammals and fish.

Our results suggest that the incorporation of Li compounds into medaka eggs using nsPEF shows adverse effects on the development and teratogenicity involving gene expression of medaka embryos, and these results are in line with our previous study (Tominaga *et al.*, 2010). The nsPEF incorporation technique can import chemicals within very narrow time windows in the early embryon-

ic stages. The nsPEF method for chemical incorporation has a great advantage when compared to long-term developmental toxicity and teratogenicity assays. Our findings suggest that the nsPEF technique using medaka embryos is a powerful tool for assessing embryonic developmental toxicity and teratogenicity caused by Li compounds, and that nsPEF assisted in the prediction of various molecular mechanisms.

ACKNOWLEDGMENTS

This work was supported by Grants-in-Aid for Scientific Research (B) (no. 16H02990) from the Japan Society for the Promotion of Sciences, Japan, and the Matching Planner Program (MP27215668058) from Japan Science and Technology Agency. The authors would like to thank Richard S. Lavin for his critical reading of this manuscript.

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

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