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# Methylmercury causes epigenetic suppression of the tyrosine hydroxylase gene in an *in vitro* neuronal differentiation model

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ABSTRACT

Methylmercury (MeHg) is the causative substance of Minamata disease, which is associated with various neurological disorders such as sensory disturbance and ataxia. It has been suggested low-level dietary intake of MeHg from MeHg-containing fish during gestation adversely affects the fetus. In our study, we investigated the toxicological effects of MeHg exposure on neuronal differentiation focusing on epigenetics. We used human fetal brain-derived immortalized cells (LUHMES cells) as a human neuronal differentiation model. Cell viability, neuronal, and catecholamine markers in LUHMES cells were assessed after exposure to MeHg (0–1000 nM) for 6 days (from day 2 to day 8 of neuronal differentiation). Cell viability on day 8 was not affected by exposure to 1 nM MeHg for 6 days. mRNA levels of *AADC*, *DBH*, *TUJ1*, and *SYN1* also were unaffected by MeHg exposure. In contrast, levels of *TH*, the rate-limiting enzyme for dopamine synthesis, were significantly decreased after MeHg exposure. Acetylated histone H3, acetylated histone H3 lysine 9, and tri-methyl histone H3 lysine 9 levels at the *TH* gene promoter were not altered by MeHg exposure. However, tri-methylation of histone H3 lysine 27 levels, related to transcriptional repression, were significantly increased at the *TH* gene promotor after MeHg exposure. In summary, MeHg exposure during neuronal differentiation led to epigenetic changes that repressed *TH* gene expression. This study provides useful insights into the toxicological mechanisms underlying the effects of developmental MeHg exposure during neuronal differentiation.



## 1. Introduction

Methylmercury (MeHg) is the causative substance of Minamata disease, Minamata disease is associated with various neurological disorders such as sensory disturbance and ataxia and is the consequence of exposure to high concentration of MeHg. Recently, adverse effects of relatively low levels of MeHg have become an important issue in the toxicological study of MeHg. MeHg is accumulated in organisms at higher levels of the food chain. Humans are mainly exposed to MeHg by consumption of contaminated seafood. It has been suggested that low-level MeHg dietary intake through MeHg-rich fish during gestation has adverse effects on the fetus [1]. Importantly, the nervous system is particularly sensitive to chemical substances during development [2]. Two previous representative epidemiological studies on the risk of low-level dietary intake of MeHg on child development are a birth cohort study of Faroe Island and a Child Development Study in the Seychelles. The birth cohort study of Faroe Island showed that low levels of MeHg may cause neuropsychological dysfunctions in the domains of language, attention, and memory in childhood neurodevelopment [3]. However, no significant association of low-level MeHg intake with the child development were found in the Child Developmental Study in the Seychelles [4]. Although several epidemiological studies such as the two shown above have been conducted, no universal conclusion on the risk of low-level MeHg exposure during fetal neurodevelopment has been reached.

Previous epidemiological studies have reported that low-level prenatal MeHg exposure may be associated with the poor psychomotor development [5,6]. *In vivo* and *in vitro* studies shown that low-level MeHg exposure adversely affects the developing cranial nervous system [7,8]. Experimental studies focusing on the catecholamine system have indicated that relatively low concentrations

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of prenatal MeHg exposure may be associated with functional changes in the neurotransmitters catecholamine, rather than global morphological changes of the developing brain [9,10]. In addition, in vivo studies have shown that low-level MeHg exposure may disrupt the catecholamine system [11,12]. These studies indicates that MeHg exposure may lead to neurological disorders. However, the underlying toxicological mechanisms remain to be elucidated.

Recently, the idea of "Developmental Origin of Health and Diseases (DOHaD)" has gained increasing recognition. The DOHaD hypothesis posits that environmental conditions during the embryonic period are related to the risk of developing diseases in adulthood [13]. Therefore, it is necessary to consider not only the immediate impact of chemical exposure on the embryo or fetus, but also its possible effects in adulthood. The DOHaD hypothesis suggests that epigenetic changes, such as DNA methylation and histone modification, could be induced by environmental conditions during development. These epigenetic alterations are maintained in adulthood and increase the risk of developing diseases [14]. Epigenetics is defined as the study of the transcriptional system that do not entail changes in the DNA sequence [15]. The correct programing of epigenetic modifications on the genome during development is essential to successfully complete the ontogeny [16]. Therefore, epigenetics is an important factor when investigating the molecular mechanisms of the effects of MeHg exposure during the embryonic period. To the best of our knowledge, the influences of developmental MeHg exposure on epigenetic changes has not been investigated previously.

In the present study we investigated the effects of MeHg exposure on neuronal differentiation in terms of epigenetics, using human fetal brain-derived immortalized cells (LUHMES cells) as a human neuronal differentiation model.

## 2. Materials and methods

### 2.1. LUHMES cell culture and differentiation

The LUHMES cell line (CRL-2927) used in this study was purchased from American Type Culture Collection (ATCC, Manassas, VA USA). Culture dishes were pre-coated with 50 μg/mL poly-L-ornithine (Sigma, St. Louis, MO USA) and 1 μg/mL fibronectin (Wako, Osaka Japan) in distilled water at 37 °C for 3 h. LUHMES cells were grown in Advanced Dulbecco's modified Eagle's medium/F12 (Advanced DMEM/F12) (Invitrogen, Carlsbad, CA USA), supplemented with 1 × N-2 supplement (Invitrogen), 1 × GlutaMAX (Invitrogen) and 40 ng/mL human recombinant basic FGF (bFGF) (Wako), at 37 °C in the humidified atmosphere of a 5%-$CO_2$ incubator. LUHMES cells were differentiated following a previously published protocol [17]. LUHMES cells were seeded onto a pre-coated 100-mm dish and cultured for 24 h in differentiation medium containing Advanced DMEM/F12, 1 × N-2 supplement, 1 × GlutaMAX, 1 μg/mL doxycycline (Tokyo Chemical Industry, Tokyo Japan), 40 ng/mL Glial Cell Line-derived Neurotrophic Factor (GDNF) (Wako) and 1 mM N6, 2′-*O*-dibutyryladenosine 3′,5′-cyclic monophosphate sodium salt (dbcAMP) (Nacalai, Kyoto, Japan). After 2 days of cell differentiation, cells were trypsinized and replated onto a pre-coated 100-mm dish ($1 \times 10^7$ cells), 6-well plate ($2 \times 10^6$ cells/well), or 96-well plate ($1 \times 10^5$ cells/well). Cells were maintained in differentiation medium for 6 days.

### 2.2. Real time RT-PCR

Total RNA was extracted using PureLink RNA Mini Kit (Invitrogen). Total RNA (1 μg per sample) was reverse transcribed to cDNA by using ReverTra Ace qPCR RT Master Mix (Toyobo, Osaka, Japan). Real time RT-PCR analysis was performed using THUNDERBIRD® SYBR qPCR Mix (Toyobo) and amplified using a StepOne Real-Time PCR System (Thermo Fisher Scientific). Primers used in the real time RT-PCR analysis were: Organic cation/carnitine transporter4 (*OCT4*) (forward: 5′-GACAGGGGGAGGGGAG-3'; reverse: 5′-CTTCCCTCCAACCAGT-3′), SRY-box 2 (*SOX2*) (forward: 5′-GAAGGATAAGTACACGCTGCCCG-3'; reverse: 5′-GCTGGTCATGGAGTTGTACTGC-3′), Tubulin beta 3 class III (*TUJ1*) (forward: 5′-CGCCCCAGTATGAGGGAGAT-3'; reverse: 5′-AGTCGCCCACGTAGTTGC-3′), Synapsin I (*SYN1*) (forward: 5′-CGAGCCGCACACCGACT-3'; reverse: 5′-AACTTCCATATCCACAGAGAATCCA-3′), Tyrosine hydroxylase (*TH*) (forward: 5′-TCATCACCTGGTCACCAAGTT-3'; reverse: 5′-GGTCGCCGTGCCTGTACT-3′), Aromatic L-amino-acid decarboxylase (*AADC*) (forward: 5′-AGGAAGCCCTGGAGAGAGACA-3'; reverse: 5′- CTTGTTGCAGATAGGACCGACTT-3′), Dopamine beta-hydroxylase (*DBH*) (forward: 5′-CCACAACCCACTGGTGATAGAA-3′, reverse: 5′-TGATCCCCGCGTTGAAG-3′), and Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (forward: 5′-TGGTGAAGACGCCAGTGGA-3'; reverse: 5′-GCACCGTCAAGGCTGAGAAC-3′). The level of *GAPDH* cDNA in the sample was used as an internal control for all PCR amplification reactions.

### 2.3. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde (Nacalai, Kyoto, Japan) and permeabilized with 0.1% Triton-X. After blocking with 2% normal goat serum (Wako) in PBS at room temperature (RT) for 1 h, cells were incubated with primary antibodies, [mouse anti-TUJ1 antibody (Sigma)] (1:2000 dilution) at 4 °C overnight. After primary antibody reactions, secondary antibodies [goat anti-mouse Alexia 488 (Thermo Fisher Scientific, Waltham, MA USA)] (1:200 dilution) were added to the sample. Nuclei were stained with DAPI (Thermo Fisher Scientific) (1:2000 dilution) for 5 min. Samples were observed using a confocal imaging system (Zeiss LSM 700, Carl Zeiss, Oberkochen, Germany).

### 2.4. Cell viability

LUHMES cells were treated with MeHg (Tokyo Chemical Industry) at several concentrations (0, 1, 10, 15, 20, 25, 35, 50, 100, 1000 nM) for 6 days, from day 2–8 of cell differentiation. Cell viability was measured using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan).

### 2.5. Western blotting

Cells were scraped from the plate at day 8 of cell differentiation and lysed in 1 × Ripa buffer [25 mM Tris-HCl (pH7.5), 0.15 M NaCl, 1% NP-40, 0.1% deoxycholic acid sodium salt, 0.1 mM phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, 2 μg/mL aprotinin]. The 15 μg of protein were separated by SDS-PAGE. The bands on the poly acrylamide gel were transferred to a PVDF membrane in transfer buffer (0.3% Tris, 1.44% glycine, 20% methanol). The transfer membrane was incubated in 5% skimmed milk at RT for 60 min. The membrane was incubated with the primary antibodies [rabbit anti-TH antibody (Millipore) (1:1000 dilution) and mouse anti-β-actin antibody (Santa Cruz Biotechnology) (1:1000 dilution)] at 4 °C overnight. Then, the membrane was incubated with secondary antibodies [goat anti-rabbit antibody conjugated with HRP (Santa Cruz Biotechnology) (1:2500 dilution) or goat anti-mouse antibody conjugated with HRP (Santa Cruz Biotechnology) (1:2500 dilution)] at RT for 30 min. Finally, the membrane was incubated with ECL prime (GE Healthcare) to generate chemiluminescence from HRP. Chemiluminescence was detected by using LAS3000 mini (Fuji film, Tokyo, Japan).

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