ASSESSMENT OF PRENATAL EXPOSURE TO NEUROTOXIC METALS (ALUMINIUM, ARSENIC, CADMIUM AND LEAD) AND UP-REGULATION OF DIOXIN-INDUCIBLE GENE, CYP1A1, IN THE NEURAL STEM CELL CULTURE

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Abstract
Although previous studies have suggested a relationship between the environmental toxicant such as heavy metals and neurodevelopmental disorders, these involvements have not been fully investigated. In this study, we measured concentrations of neurotoxic metals (aluminium, arsenic, cadmium and lead) in 6 types of human perinatal samples (maternal blood, urine, breast milk, umbilical cord blood, placenta and umbilical cord) by inductively coupled plasma mass spectrometer (ICP-MS) to assess the transplacental contamination during pregnancy. Detectable levels of metals were found in almost all samples, suggesting that fetuses are exposed to these metals during pregnancy. In addition, effects of neurotoxic metals on developing neural cells were investigated using murine neurosphere culture, which is a selective culture method for neural stem cells. As the results, exposure to 100 μg/L metals decreased size of neurospheres. Immunohistochemical analysis showed beta III tubulin positive neurons and GFAP positive astrocytes remarkably decreased in culture treated with 100 μg/L of metals. By using this culture technique, we also found exposure to metals caused up-regulation of dioxin-inducible gene, Cyp1a1, in neural stem cells. These results suggested even transient exposure to metals during early neurodevelopmental stage causes adverse effects on proliferation and differentiation of neural stem cells.

Introduction
A number of children diagnosed the neurodevelopmental disorders such as attention deficit hyperactivity disorder (ADHD), autism and learning disorder have been increasing in recent years [1]. The exact etiology is complicated and not understood completely. Environmental pollutions are suspected of causing neurological disruption in children [2,3]. Although heavy metals such as lead, cadmium, arsenic and methyl mercury are known as neurotoxic chemicals against all people, fetuses are more sensitive to these heavy metals. Many kinds of heavy metals can easily cross the placenta and invade umbilical cord blood [4]. Also, the blood-brain barrier, which protects brain parenchyma from toxic chemical in the adult brain, is not completely formed until 6 months after birth [5]. The fetal exposure to heavy metals is great concerns considering the involvement of environmental pollutions in neurodevelopmental disorders. However, we know little about the effect of heavy metals on early stage of neural development. Therefore, first aim of this study was to assess the fetal exposure levels of neurotoxic metals by measuring perinatal maternal and fetal samples.
In addition, we investigated the effect of neurotoxic metals on neural stem cells, which are the most primitive cells in the developing central nervous system. Neural stem cells are characterized by their abilities of self-renewal, proliferation and differentiation into all types of neural cells. To perform the present study, we used neurosphere method, which is a selective culture method for neural stem cells.

Materials and Methods
Sample collections and analysis for ICP-MS
All perinatal samples including maternal blood (MB), maternal urine (MU), breast milk (BM), placenta (PL), umbilical cord blood (UCB) and umbilical cord (UC) were collected from healthy female volunteers at Tokai University after the procedure of informed consent. Samples were frozen at -20^{o}C until analysis. After thawing samples, placenta and umbilical cord were dried at 80^{o}C oven and weighed. All samples were pre-treated with ultrapure nitric acid and hydrogen peroxide at room temperature and then digested with microwave. Concentrations of aluminium (Al), arsenic (As), cadmium (Cd) and lead (Pb) in resulting samples were analyzed

by ICP-MS (HP4500, Yokokawa analytical systems).

Cell isolation and Cultures

Ganglionic eminences were dissected from E14.5 (The day of appearance of the vaginal plug is E0.5.) C57BL6 mice. Tissues were collected into Ca^{2+}, Mg^{2+} free Hanks balanced salt solution (Invitrogen), and mechanically triturated by using a blue tip and yellow tip sequentially. Dissociated cells were suspended into Neurophere medium (DMEM/F-12 [Invitrogen] supplemented with EGF [Peprotech, 20 ng/ml], FGF2 [Peprotech, 20 ng/ml], and B27 [Invitrogen, 1:50]) at $1x10^5$ cells/ml, and cultured in $25cm^2$ tissue culture flasks (Iwaki) for 7 days. To evaluate effects of metals, mix solution including arsenic, cadmium and lead were added into neurosphere medium at final concentrations ranging from 50 to 200ug/L.

Proliferation and differentiation assay

To evaluate effects of metals on proliferating potential, we measured the diameter of neurospheres after 7days in culture with or without metals. Additionally, to study effects of exposure of metals on differentiation potential, neurospheres were collected and re-plated onto poly L-lysine- coated dishes in differentiation medium (DMEM/F-12 supplemented with B27). After 7 days, cells were fixed for 10 minutes with 4% paraformaldehyde. Immunocytochemistry was performed by using primary antibodies against beta-III tubulin (Tuj1, SIGMA) and glial fibriary acidic protein (GFAP, DAKO) to detect neurons and astrocytes, respectively. Appropriate fluorescent conjugated-secondary antibodies were used to visualize specific immunoreactions.

RT-PCR analysis

Total RNA was prepared from neurospheres after 7days in culture using the RNA extraction kit ISOGEN (Nippon Gene), and 1ug of RNA was transcribed using Superscript III reverse transcriptase (Invitrogen), following the manufacture's recommendations. Resulting cDNA was subjected to the PCR using Ex-Taq kit (Takara Bio). The sense and antisense primers, respectively, were as follows: GAPDH, 5'-GGTCATCATCTCCGCCCCTTC-3' and 5'-CCACCACCCTGTTGCTGTAG-3'; Cyp1a1, 5'-AACACTTCCAAGTGCAGATGCGGTC-3' and 5'-TCAAACCAGTTAGCCACTGGAGCAC-3'.

Results and Discussion

Detection of metals in maternal and fetal samples

Table 1 shows the mean concentrations and S.D. of the metals in maternal and fetal samples. Al, As and Pb were detected in almost all samples in this study. Cd was detected 5 of 7 MB, 4 of 7 MU, and 6 of 7 UCB samples. Cd was also detected all of BM, PL and UC samples. Thus, it became clear that fetuses were contaminated with multiple neurotoxic metals during pregnancy. It has been reported that concentrations of several toxic chemicals were higher in maternal than in umbilical cord blood [6,7]. However, there are no significant differences between the levels of MB and UCB in all elements investigated.

Association between maternal age and fetal samples

Previous studies have reported a positive correlation between maternal age and blood concentrations of toxic chemicals such as DDE and PCBs [8], because older women have a greater opportunity for exposures to these chemicals. 7 mothers aged 34 ± 4.2 years (range 28-41) and their children participated in this study. Correlation coefficients between maternal age and UCB are shown in Table 2. Between maternal age versus UCB, Al and Pb showed a relatively high correlation coefficient (Al: r=0.73, Pb: r=0.83).

Association between maternal and fetal samples

The association between maternal and fetal samples of toxic metals is important to evaluate the transplacental exposure of fetuses. Correlation coefficients between maternal and fetal samples are shown in Table2. Between MB versus UCB, As and Pb showed high correlation coefficient (As: r=0.83, Pb: r=0.949).

Association among maternal samples

There is the great concern that some toxic chemicals might be transferred to nursing infants via breast milk. We compared the concentrations of metals among maternal samples (Table 2). Between MB versus BM, As and Pb showed a relatively high correlation coefficient (As:r=0.51, Pb: r=0.69).

Association among fetal samples

The umbilical cord blood has been used to assess the exposure to toxic chemicals during pregnancy. However, the umbilical cord is easier to collect technically without ethical problems. The use of umbilical cords to assess the chemical exposure seems to be an ideal method. Thus, we compared the concentrations of metals among fetal samples. Correlation coefficients among fetal samples are shown in Table2. Cd showed a significant correlation between PL and UC (r=0.95). However, there was no correlation between UCB versus UC in any elements.

Adverse effects of metals on fetal neural development

In order to investigate effects of metals on fetal neural development, we used neurosphere method, in which neural stem cells can proliferate as maintaining abilities to differentiate into mature neurons and glial cells. First, the effect of metals on neural stem cell proliferation was evaluated by measuring size of neurospheres treated or untreated with metals for 7 days. At 50μg/L metals concentration, there was no significant difference as compared with control neurospheres. Exposure to 100μg/L of metals significantly reduced the size of neurospheres (Figure1 A,B). Treatment with 200μg/l of metals completely inhibited the formation of neurosphere (data not shown). Thus, it was suggested that fetal exposure to metals prevent proliferation of neural stem cells.

Table1. Concentrations of metals in maternal and fetal samples

●Aluminium

Sample ID	MB(μg/L)	MU(μg/L)	BM (μg/L)	PL(μg/g) *	UCB(μg/L)	UC(μg/g) *
1	75.6	53.7	60.3	258.0	62.4	196.0
2	61.5	48.5	49.0	277.3	37.0	228.7
3	41.0	54.5	39.5	129.3	47.0	134.0
4	53.0	53.5	50.5	208.0	48.5	178.7
5	70.0	51.6	77.2	176.0	50.4	94.7
6	91.2	54.6	50.2	237.2	57.2	89.2
7	102.0	57.0	64.8	ND	44.0	144.0
Mean±SD	70±21.23	53.7±2.68	50.5±12.45	222.6±55.01	48.5±8.37	144±51.87

●Arsenic

Sample ID	MB(μg/L)	MU(μg/L)	BM (μg/L)	PL(μg/g) *	UCB(μg/L)	UC(μg/g) *
1	17.2	58.8	5.4	47.9	13.0	196
2	5.6	87.3	6.7	38.0	8.7	229
3	17.9	18.5	4.1	44.7	16.3	134
4	14.1	43.3	4.4	29.9	12.3	179
5	11.7	31.0	3.2	19.7	11.7	95
6	13.7	103.8	9.2	25.4	10.5	89
7	11.6	91.6	5.6	29.4	12.7	144
Mean±SD	13.70±4.13	58.77±32.87	5.436±1.99	29.87±10.34	12.29±2.34	144±51.87

●Cadmium

Sample ID	MB(μg/L)	MU(μg/L)	BM (μg/L)	PL(μg/g) *	UCB(μg/L)	UC(μg/g) *
1	0.12	3.21	0.54	106.87	0.06	4.33
2	ND	ND	0.48	33.21	0.38	0.49
3	0.23	ND	0.49	34.91	0.85	1.16
4	0.42	ND	0.37	33.68	0.22	0.49
5	ND	0.09	0.40	20.23	ND	0.48
6	0.35	0.83	0.83	22.96	0.29	1.22
7	0.06	0.27	0.26	28.28	0.06	1.46
Mean±SD	0.23±0.15	0.55±1.44	0.48±0.18	33.20±30.00	0.26±0.29	1.16±1.36

●Lead

Sample ID	MB(μg/L)	MU(μg/L)	BM (μg/L)	PL(μg/g) *	UCB(μg/L)	UC(μg/g) *
1	0.4	0.4	1.8	13.5	0.2	4.8
2	2.5	1.8	2.2	12.0	2.4	7.7
3	1.7	1.4	2.1	9.8	2.3	5.9
4	2.3	1.8	2.4	7.2	2.3	6.4
5	0.9	0.9	3.6	13.5	0.6	4.9
6	8.3	3.2	0.9	9.1	8.8	4.1
7	3.5	2.7	1.4	14.7	1.2	11.3
Mean±SD	2.30±2.65	1.77±0.98	2.11±0.85	11.97±2.75	2.27±2.91	5.87±2.47

Abbreviations: MB; maternal blood, MU; maternal urine, BM; breast milk, PL; placenta, UCB; umbilical cord blood, UC; umbilical cord
* Dry weight

We next examined the effect of metals on the subsequent differentiation potential of neural stem cells derived from neurosphere culture. In control culture, cells derived from neurospheres attached onto Poly L lysine and fibronectin coated-dishes and elongated processes (Figure 1C). Immunohistochmical analysis detected beta III tubulin positive neurons and GFAP positive astrocytes in control culture (Figure 1D). On the other hand, process elongation could not be found in culture pre-treated with 100mg/L metals (Figure 1C). The number of differentiated neurons and astrocytes decreased compared to control culture (Figure 1D), suggesting prenatal transient exposure of metals impairs differentiation of neural stem cells.

Table2. Correlation coefficients (r) of metals between maternal age and MB, between maternal age and UCB, between maternal and fetal samples, among maternal samples, and among fetal samples.

	Maternal age and concentrations in MB	Maternal age and concentrations in UCB	Between maternal and fetal samples			Among maternal samples		Among fetal samples	
			MB vs. PL	MB vs. UCB	MB vs UC	MB vs. MU	MB vs. MM	UCB vs. UC	PL vs. UC
Al	0.13	0.73	0.57	0.24	0.29	0.24	0.51	0.33	0.57
As	0.31	0.17	0.36	0.83	0.37	0.5	0.23	0.05	0.4
Cd	0.3	0.02	0.13	0.3	0.08	0.04	0.38	0.25	0.95
Pb	0.46	0.63	0.51	0.94	0.06	0.9	0.69	0.34	0.13

Up-regulation of dioxin-inducible gene, Cyp1a1, in neural stem cells.

Expression of Cyp1a1 has been used as a biomarker for aryl hydrocarbon receptor (AhR) activation and a warning of dioxin-like toxicity. Recently, it has been demonstrated that Cyp1a1 is up-regulated by heavy metals such as Pb, Cu and As in several types of cells. We performed RT-PCR analysis to investigate whether Cyp1a1 expression is induced by metals in neural stem cells or not. There was no detection level of Cyp1a1 in neurospheres untreated with metals (Figure1 E). On the other hand, treatment with metals (50 and 100 μg/L) remarkably increased Cyp1a1 expression. In this study, we could not clarify whether up-regulation of Cyp1a1 in neural stem cells by exposure of metals is mediated by AhR-XRE pathway or not. To understand this mechanism, further studies are required.

In conclusion, our results demonstrated (1) fetus tissues were contaminated with multiple neurotoxic metals during pregnancy, and (2) these metals caused adverse effects on proliferation and differentiation of neural stem cells.

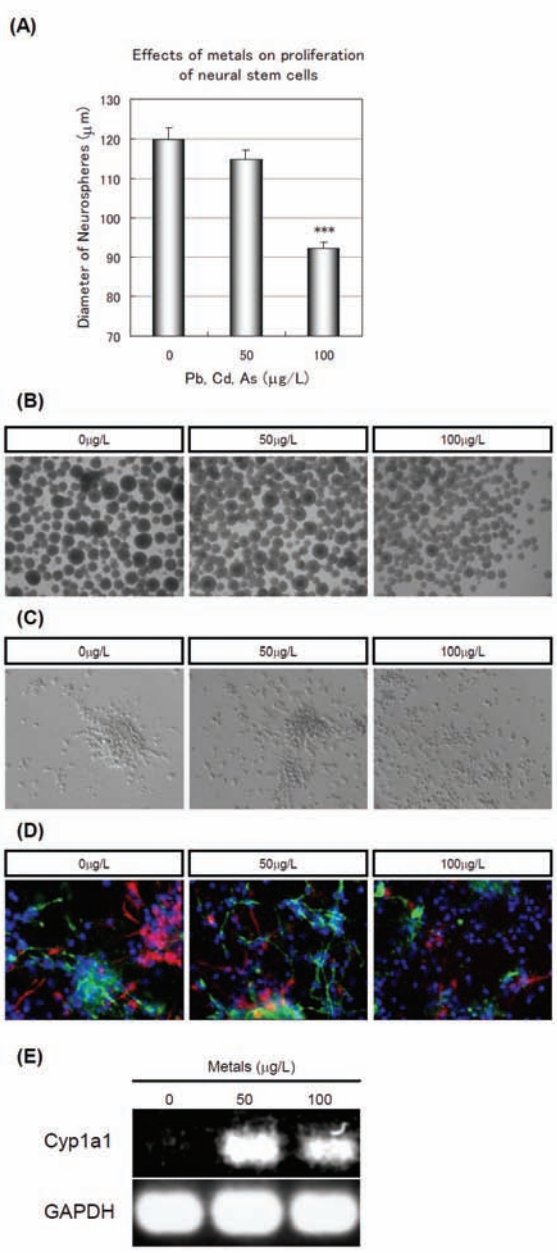

Figure 1. Effects of metals on neural stem cells. Neural stem cells were cultivated with or without Pb, Cd and As within neurosphere culture medium for 7 days. Subsequently, neurospheres were collected and transferred into differentiation medium to induce neuronal and glial maturation.
(A) Exposure of 100μg/L metals significantly decreases proliferation of neural stem cells. *** ; $P<0.001$. (B) Representative images of neurosphere cultures showing metals decrease neurosphere size.(C) Phase contrast microscopy of neurosphere-derived cells treated or untreated with metals.Exposure of 100μg/L metals inhibits cells to elongate their processes.(D) Immunostaining for neurons (anti-beta III tubulin;green) and astrocytes (anti-GFAP;red). Nucleus were stained with DAPI (blue).Exposure of 100μg/L metals prevents differentiation into neurons and astrocytes. (E) Rt-PCR analysis for detecting Cyp1a1 mRNA. Expression of Cyp1a1 is up-regulated by exposure of metals.

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