Retrospective *In Utero* Exposure Assessment of PCBs Using Preserved Umbilical Cords and Its Application to Case-Control Comparison

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Abstract

Objectives: The aim of this study is to assess preserved umbilical cords as chemical exposure media to investigate *in utero* chemical exposure. Furthermore, we aim to apply preserved umbilical cords to retrospective studies of the relationship between *in utero* chemical exposure and neurodevelopment disorders.

Methods: Two sets of preserved umbilical cord samples were analyzed for exposure to polychlorinated biphenyls (PCBs): one composed of samples from 20 healthy Japanese subjects and the other set included samples from 17 autistic patients and 7 healthy siblings of theirs.

Results: The possibility of external PCBs contamination during storage of preserved umbilical cord samples was found, and due to this problem, the study design should be limited to the comparison between PCBs concentration in preserved cord samples from autistic patients and that in those from their sibling. Total PCBs concentrations in preserved cords from autistic patients and their control siblings were compared and we found no statistically significant difference between them (Wilcoxon signed rank test, $p > 0.05$).

Conclusions: The association between autism and *in utero* PCBs exposure was not clarified in this study; however, retrospective studies such as a case-control study of siblings using preserved umbilical cords can be a method of choice for examining the possible relationship between *in utero* chemical exposure and childhood disorders.

Key words: retrospective study, preserved umbilical cord, prenatal exposure, polychlorinated biphenyls, childhood disorder

Introduction

In utero exposure to environmental toxicants, such as heavy metals and organic pollutants, may sometimes cause defects in childhood and adolescence, long after an apparently normal birth (1). The long “latent period” of this kind can hamper the establishment of cause-effect relationship. For the present, the only method of investigating the association between *in utero* exposure to chemicals and childhood disorders is to carry out a prospective epidemiologic study (cohort study)

because it is usually difficult to obtain information on the exposure level of children to chemicals of interest when they were fetus (2–5). A prospective cohort study is, however, time-consuming, labor-intensive and costly, although the results obtained suffer less from various biases and are, therefore, reliable. Taking the number of chemicals that may have adverse health consequences and the number of childhood disorders into consideration, it is evident that investigations of all the potential associations between suspected chemicals and disorders by prospective cohort studies are not realistic. If retrospective study, such as a case-control study, is applicable, it will be easier to examine the possible relationship between chemical exposure and childhood disorders and to generate a hypothesis to be confirmed by subsequent prospective cohort studies. To this end, it is essential to establish a method for the retrospective assessment of *in utero* chemical exposure of subjects.

In this report, we suggested an approach to investigating *in*

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utero chemical exposure using preserved umbilical cords for case-control study and applied it to a retrospective study of the relationship between *in utero* chemical exposure and neurodevelopment disorder. Fresh umbilical cords, as well as placentas, have been used as appropriate media for the biological monitoring of chemical exposure *in utero* in epidemiologic studies (6–9). The concentrations of chemicals in these tissues of embryonic origin are considered to represent the levels of chemicals that have passed through the blood-placenta barrier and the quantities of chemicals that have been delivered to the fetus. The Japanese have a custom of preserving the umbilical cord of a newborn baby as a memento of its birth and this allows us to utilize a preserved umbilical cord as a medium to retrospectively assess *in utero* chemical exposure of donors. We have already reported the usage of preserved umbilical cords obtained from healthy Japanese adults for *in utero* PCBs exposure assessment (10). Akagi et al. (11) also utilized preserved umbilical cord samples from Minamata disease patients to estimate methylmercury exposure level *in utero*.

We applied our proposed *in utero* exposure assessment method to autistic patients to determine whether there is any relationship between autism and *in utero* PCBs exposure. Chemical exposure is suspected as one of the etiologies of autism (12–14).

Materials and Methods

Two sets of preserved umbilical cord samples were analyzed in this study: one composed of samples from 20 healthy Japanese subjects born in the 1960s–80s, 1998 and 2001, whose PCBs data were already reported in our previous study (10), and included additional data for samples supplied from two subjects born in 1927 and 1930, and analyzed for PCBs in this study. The two subjects were born before PCBs were produced and used in Japan and served as “control” for this analysis.

The other set included samples from 17 autism patients and 7 healthy siblings of theirs born in 1967–2000. This sample set included five groups of siblings: one group of four, one group of twins and three groups of two. Autistic patients were diagnosed according to the American Psychiatric Association's DSM-IV by physicians of The University of Tokyo Hospital.

All of the umbilical cords had been preserved in the usual Japanese manner before they were supplied for PCBs analysis, that is, wrapped in cotton gauze or cotton wool and placed in a wooden box. The cords were completely dried and their lengths and weights were typically 10–20 mm and 100 mg, respectively (10). The medical ethics committee of The University of Tokyo approved the study design, and cord samples were analyzed only for subjects who gave their informed consent or subjects whose parents gave their informed consent.

The procedure for sample preparation was the same as that in our previous study (10). Briefly, the cords were washed with acetone and cut into fine pieces with stainless steel scissors, levigated with pestle and mortar. Twenty-eight ^{13}C -labelled internal standards (IUPAC #3, 8, 15, 28, 31, 37, 52, 77, 81, 95, 101, 105, 114, 118, 123, 126, 153, 156, 157, 167, 169, 170, 180, 189, 194, 202, 206 and 209) were added, and followed

by 70 mL of toluene in a Soxhlet apparatus for extraction for 16 hrs. The crude extract was then purified using a multilayer column packed as follows (from the bottom): 0.5 g of silica/2 g of silica containing 2% KOH (w/w)/0.5 g of silica/7 g of silica containing 50% H_2SO_4 /0.5 g of silica/2 g of silica containing 10% AgNO_3 /1 cm of anhydrous Na_2SO_4 .

In this study, all 209 PCB isomers in the preserved umbilical cords were analyzed. Congener-specific PCB analysis was carried out using a gas chromatograph (6890 series, Hewlett Packard, USA) equipped with an HT8 column (length, 50 m; i.d., 0.22 μm ; film thickness, 0.25 μm ; SGE International) and high-resolution mass spectrometry (resolution >10000, AutoSpec-Ultima, Micromass). The concentrations of PCBs in the preserved umbilical cords were expressed on a tissue weight basis. For the external quality assurance of our PCBs analysis, a certified reference material (CRM) from the National Research Council Canada (NRCC) CARP-1 was analyzed and the results indicated that our analysis was not biased (10).

PC software SPSS version 12.0 J was employed for statistical analyses. The Wilcoxon signed rank test was used to test the significance of differences in the concentrations of total PCB and coplanar PCB (Co-PCB) in the cord samples between autistic patients and their control siblings. Stepwise multiple regression analysis was used to extract significant variable(s) that explain PCBs concentrations in the preserved cord samples. Independent variables used were age, sex and the presence/absence of autism. For the latter two variables, 0 and 1 dummy variables were used. The criteria for the entrance into, and elimination from, multiple regression function was the probability of F values ($P_{\text{in}}=0.05$ and $P_{\text{out}}=0.1$).

Results and Discussion

Figure 1 shows the plots of the total PCBs concentration (sum of the concentrations of 209 isomers) as a function of the birth year of the 20 healthy subjects. Eighteen of the plots in this figure were recapitulated from our previous study (10). The total PCBs concentrations in the samples from the subjects born in 1927 and 1930 were 3.3 and 17 ng/g, respectively. Because PCBs usage started in 1954 (lasted up to 1972) in diverse electrical appliances (e.g., transformers and capacitors) in Japan, it is expected that PCBs should not be detected in

Fig. 1 Total PCB concentration in umbilical cords as function of birth year of subjects. The horizontal lines in the figure denote the period of PCBs production and usage in Japan (1954–1972).

Table 1 Mean, standard deviation (SD), median, minimum and maximum concentrations of total PCBs and Co-PCB in preserved cord samples from case-sibling matched pairs and those of total PCBs of all autistic subjects (includes unmatched patients)

	autistic case/control	compound	mean±SD	median	min	max	statistical test
siblings	autistic cases (n=5)	total PCB (ng/g)	55±99	8	0.49	204	Wilcoxon signed-rank test
	control (n=7)		159±191	93	0.39	454	
	autistic cases (n=5)	Co-PCB (ng/g)	0.44±0.56	0.24	0.05	1.4	
	control (n=7)		0.49±0.77	0.16	0.06	2.9	
all subjects	autistic cases (n=17)	total PCB (ng/g)	51±82	13	0.49	250	—

Wilcoxon signed-rank test: $p>0.05$

the preserved umbilical cords from subjects born before 1954. Therefore, the detection of PCBs in these two samples indicates that they were externally contaminated with PCBs during storage. This contamination problem seriously hampers the utility of preserved umbilical cords as exposure assessment media. It is, however, expected that the level of PCBs contamination during storage is similar if samples were preserved under identical conditions. In Japan, the umbilical cords of newborns are usually preserved by the parents, and due to this custom, the umbilical cords of siblings are usually stored in the same place. Therefore, comparison may be applicable for the concentrations of PCBs in preserved umbilical cords between case and his/her sibling because contamination, if any, can be assumed to be similar between the two samples. Retrospective study such as case-control study of siblings using preserved umbilical cords can be a method of choice for examining the possible relationship between *in utero* chemical exposure and childhood disorders.

A preliminary investigation for the case-control study using preserved umbilical cords was performed on autistic patients to examine whether there is any relationship between autism and *in utero* PCBs exposure. The study group consisted of 4 males (80%) and 1 female (20%) for autistic cases, and 3 males (43%) and 4 females (57%) for case-matched sibling controls. The mean age (in 2005) was 18.4 years for the cases and 21.3 for sibling controls. Mean, standard deviation (SD), median, minimum and maximum concentrations of total PCBs and Co-PCB in the cord samples of autistic cases and control siblings are listed in Table 1. Co-PCB in this table includes

PCBs to which toxic equivalency factors were assigned in the 1998 World Health Organization-Toxic Equivalent Factors (WHO-TEF) scheme. Figure 2 shows the total PCB concentrations of autistic patients and their healthy siblings and the trend line of healthy subjects shown in Fig. 1. There was no significant difference between the concentrations of total PCBs in the preserved cord samples from autistic cases and their matched sibling controls (Wilcoxon signed rank test, $p>0.05$), indicating that *in utero* PCBs exposure level is not associated with autism as shown by our retrospective investigation method. This observation was similar for Co-PCB. This was further examined by including data of 12 additional autistic cases (8 males and 4 females; mean age, 18.6 yrs) and 14 controls (5 males and 9 females; mean age, 28.0 yrs) into the data set and statistically analyzed by multiple regression analysis if the presence of autism affected the concentrations of total PCBs in umbilical cords. In this case, 14 controls for unmatched autistic patients were selected from the healthy subjects born after 1965 (shown in Fig. 1). In the analysis, log-transformed PCBs concentration was used as a dependent variable and age at 2005, sex (male 0; female 1) and the presence (1)/absence (0) of autism were included as independent variables. Stepwise variable selection was employed. Age was the only variable selected as significant to explain total PCBs concentrations in umbilical cord samples ($R^2=0.486$, $p<0.001$) when the analysis was applied to the autistic and control subjects ($n=38$; autistic subjects=17, controls=21). The presence of autism was not selected as a significant variable. The presence of autism was not selected as significant either when the multiple regression analysis was applied to 44 umbilical cord samples that included 20 healthy Japanese (shown in Fig. 1), although sex was selected as a significant variable in addition to age in this case. The selection of sex in the 44-sample set may be due to the presence of one female subject with an extremely high total PCBs (5700 ng/g) and sex was not selected after the deletion of the data of this subject from the data set. Thus age, that is, birth year, not the presence of autism, was the variable that determined total PCBs levels in Japanese umbilical cords.

Despite active studies have been performed on multiple aspects, the etiology of autism is still unclear. Chemical exposure was considered one candidate etiology (12–14), however, the present study revealed no statistically significant association between PCBs concentration in preserved umbilical cords and autism. Firm conclusions cannot be drawn from the present results as to whether *in utero* PCBs exposure is not one of the etiologies of autism because of the small sample size as well as

Fig. 2 Total PCB concentration in autistic patients and their healthy siblings. Symbols indicate autistic patient (□; n=5)-matched sibling control (■; n=7) and autistic unmatched subjects (+; n=12). The line indicates the trend line of healthy subjects shown in Fig. 1.

the external contamination of preserved umbilical cord samples used as an indicator of *in utero* exposure to PCBs. However, it should be stressed that the proposed approach can be effective in retrospectively relating *in utero* chemical exposure and childhood disorders if the target chemicals are free from external contamination during storage, e.g., methylmercury (11).

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