Original Article

Cardiotoxicity induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure through lactation in mice

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ABSTRACT — Dioxins are a group of structurally related chemicals that persist in the environment. Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most toxic congener, is a suspected risk factor for cardiac diseases in humans. TCDD induces signs of cardiotoxicity in various animals. Mouse models of TCDD exposure suggest cardiotoxicity phenotypes develop differently depending on the timing and time-course of exposure. In order to clarify and characterize the TCDD-induced cardiotoxicity in the developing period, we utilized mouse pups exposed to TCDD. One day after delivery, groups of nursing C57BL/6J dams were orally administered TCDD at a dose of 0 (Control), 20 (TCDD-20), or 80 µg/kg (TCDD-80) body weight (BW). On postnatal days (PNDs) 7 and 21, pups' hearts were examined by histological and gene expression analyses. The TCDD-80 group was found to have a left ventricular remodeling on PND 7, and to develop heart hypertrophy on PND 21. It was accompanied by fibrosis and increased expression of associated genes, such as those for atrial natriuretic peptide (ANP), β-myosin heavy chain (β-MHC), and endothelin-1 (ET-1). These results revealed that TCDD directly induces cardiotoxicity in the postnatal period represented by progressive hypertrophy in which ANP, β-MHC, and ET-1 have potentials to mediate the cardiac hypertrophy and heart failure.

Key words: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), Cardiotoxicity, Mouse, Lactational exposure

INTRODUCTION

Polychlorinated dibenzo-dioxins, polychlorinated dibenzo-furans (PCDFs), and dioxin-like polychlorinated biphenyls (PCBs) are a group of structurally related chemicals that are persistent in the environment as a result of resistance to degradation (Metelkova *et al.*, 2019; Verta *et al.*, 2007; Negri *et al.*, 2006). Among this group of chemicals, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic congener (Van den Berg *et al.*, 2006; van Ede *et al.*, 2016) and is regarded as the prototypical compound.

An elaborate review of epidemiological studies indicated that dioxin exposure in humans is associated with mortality from both ischemic heart disease and all cardiovascular disease, the stronger association being with the former (Humblet *et al.*, 2008). In a more recent meta-analysis paper, mortalities by accidental food poisoning by dioxin-like PCBs and PCDFs have been analyzed for

victims of the Yusho event in Japan in 1968 (Kuratsune *et al.*, 1996) and those of the Yucheng event in Taiwan in 1979 (Guo *et al.*, 2004). In both events, the victims were exposed to high doses of PCBs/PCDFs. The study suggests elevated mortality not only from cancers, but also from heart disease (Li *et al.*, 2015). A recent report on the follow-up study of chemical industry workers accidentally intoxicated with TCDD revealed an abnormally high proportion of coronary heart disease in the subject population (Pelcl *et al.*, 2018). These studies indicate that dioxins are risk factors for cardiotoxicity in humans.

Cardiotoxicity induced by TCDD exposure has also been reported in various animals, including fish, birds, and rodents (Kopf and Walker, 2009). Zebrafish models typically exhibit reduced blood flow, altered heart looping, and reduced heart size and contraction rate (King-Heiden *et al.*, 2012). Chick embryo models exhibit extensive cardiac dilation, thinner ventricle walls (Fujisawa *et al.*, 2014). Interestingly, rodent models of

TCDD exposure indicate that the timing of exposure is critical for developing a specific cardiac toxicity phenotype. Repeated administration of TCDD to adult mice induced systemic hypertension, an increasing tendency in heart weight, and concentric left ventricular hypertrophy (Kopf *et al.*, 2008). However, a single dose of TCDD administered to dams on gestational day (GD) 14.5 suppressed cardiomyocyte proliferation and reduced the relative heart weight in fetuses on GD 17.5 (Thackaberry *et al.*, 2005), whereas it induced cardiac hypertrophy in the offspring on postnatal day (PND) 21 (Thackaberry *et al.*, 2005; Lin *et al.*, 2001). In addition, *in utero* and lactational exposure to TCDD was found to enhance sensitivity to angiotensin II (Aragon *et al.*, 2008). These complicated responses of cardiovascular system to TCDD exposure are thought to be influenced by several factors, such as timing of the exposure during the developmental period and the time-course after the exposure. Of note, since TCDD dosing to pregnant dams results in TCDD exposure not only in the fetal period but also in the lactational period (Nau *et al.*, 1982), it is difficult to define the critical window of toxicity. In the present study, we aimed to characterize TCDD-induced postnatal cardiotoxicity and its molecular mechanisms using mouse pups lactationally exposed to TCDD.

MATERIALS AND METHODS

Reagents and chemicals

TCDD (purity $\geq 99.1\%$) was purchased from Accu-Standard (New Haven, CT, USA) and dissolved in corn oil (Wako Pure Chemicals, Osaka, Japan) containing 2% n-nonane (Wako Pure Chemicals). Other reagents were of analytical grade and purchased from Wako Pure Chemicals, unless otherwise stated.

Animals and treatment

The experimental protocols for the animal experiments were approved by the Animal Care and Use Committee of the University of Tokyo, and all animal experiments were performed in accordance with the relevant guidelines of the University of Tokyo.

Pregnant C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan). Parturition was checked twice a day, and the day of birth was designated as PND 0. On PND 1, dams were administered TCDD via gavage at a dose of 0 (Control: 3 and 5 dams for experiments on PNDs 7 and 21, respectively), 20 (TCDD-20: 3 and 5 dams on PNDs 7 and 21, respectively), or 80 (TCDD-80: 5 dams each on PNDs 7 and 21) $\mu\text{g/kg}$ body weight (BW). The doses were empirically determined in which

cardiotoxicity was observed. In the present study, male pups were examined, and the number of pups per dose group ranged from 5 to 9; from those, 2 per group were chosen at random and sacrificed on PND 7, and a second set of 2 randomly chosen pups per group were sacrificed on PND 21. For each group, one heart per time point was fixed in 10% neutral buffered formalin for histological analysis, and another heart was snap-frozen in liquid nitrogen and stored at -80°C until RNA extraction.

Histopathology

The fixed tissues were immersed in 20% sucrose solution overnight for cryoprotection, embedded in O.C.T. compound (Sakura Finetek Japan, Tokyo, Japan), and then frozen on an aluminum block that was submerged in liquid nitrogen in advance. The sliced heart sections (5- μm thickness) of PND 7 and PND 21 mice were stained with hematoxylin and eosin (Muto Pure Chemicals, Tokyo, Japan) and Masson trichrome (Muto Pure Chemicals).

RNA extraction and quantitative RT-PCR

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA) and RNeasy Mini kit (Qiagen, Hilden, Germany). These RNA samples were reverse transcribed using the Prime Script RT-PCR Kit (Takara Bio, Shiga, Japan) with oligo-dT and dN₆ primers. Gene expression level was determined by quantitative RT-PCR (qRT-PCR) using a Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan) and a LightCycler System (Roche Molecular Biochemicals, Indianapolis, IN, USA). The sequences of primers used were listed in Table 1. Melting curve analyses were performed for every PCR to verify amplification specificity. All quantitative data were calculated by dividing the copy number of the target by the weight of total RNA.

Statistical analysis

Data were statistically analyzed using one-way analysis of variance (ANOVA), followed by Dunnett post hoc test; or using the Kruskal-Wallis test, followed by Dunn test. Data are shown as mean $\pm$ SEM, and $p < 0.05$ was considered statistically significant.

RESULTS

Increased heart-to-BW ratio in lactationally TCDD-exposed mice

BW of the TCDD-20 and TCDD-80 groups was significantly lower than that of the Control group on PND 7 (Table 2: 81.7% and 74.7% of the Control group, respectively). On PND 21, BW of the TCDD-20 group tended

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Table 1. Sequences of primers used for qPCR.

Target gene		Primer sequence (5' to 3')
ANP	Forward	GGG GGT AGG ATT GAC AGG A
	Reverse	GAC ACA CCA CAA GGG CTT A
β -MHC	Forward	GGA GGG CAT TGA GTG GAC C
	Reverse	CTC CTC CTC AAG GAT GGA CA
MMP-9	Forward	CAT TCG CGT GGA TAA GGA GT
	Reverse	TCA CAC GCC AGA AGA ATT TG
MMP-13	Forward	AAA GAT TAT CCC CGC CTC AT
	Reverse	TGG GCC CAT TGA AAA AGT AG
IL-1 β	Forward	CAA GGA GAA CCA AGC AAC GA
	Reverse	GCC GTC TTT CAT TAC ACA GGA
TNF- α	Forward	CAC CAC CAT CAA GCA CTC AA
	Reverse	GAC AGA GGC AAC CTG ACC AC
ET-1	Forward	TCC AAG AAA GGA AAA CCC TGT
	Reverse	TTG TGC GTC AAC TTC TGG TC

Table 2. Body and heart weights of pups exposed to TCDD by lactation¹.

		TCDD (μ g/kg)		
		0	20	80
Body weight (g)				
	PND 7	3.99 $\pm$ 0.11	3.26 $\pm$ 0.18*	2.98 $\pm$ 0.16**
	PND 21	8.20 $\pm$ 0.37	6.66 $\pm$ 0.68	4.73 $\pm$ 0.30**
Heart weight (g)				
	PND 7	0.024 $\pm$ 0.000	0.019 $\pm$ 0.002	0.019 $\pm$ 0.002
	PND 21	0.059 $\pm$ 0.001	0.053 $\pm$ 0.004	0.042 $\pm$ 0.003**
Heart-to-body weight (%)				
	PND 7	0.61 $\pm$ 0.01	0.59 $\pm$ 0.05	0.64 $\pm$ 0.03
	PND 21	0.73 $\pm$ 0.05	0.80 $\pm$ 0.04	0.89 $\pm$ 0.02*

¹Dams were administered TCDD via gavage at a dose of 0, 20, or 80 μ g/kg BW on PND 1. The average BW, heart weight, and heart-to-BW were calculated on a litter basis. Values are means $\pm$ SEM (n = 3–5). Asterisks indicate significant difference from the corresponding control group (*, $p < 0.05$; **, $p < 0.01$) by non-repeated ANOVA with Dunnett post hoc test.

to be lower and that of TCDD-80 group was significantly lower than that of the Control group (Table 2: 81.2% and 57.7% of the Control group, respectively). Heart weight of the two TCDD-exposed groups tended to be lower than that of the Control group on PND 7, with a significant difference for the TCDD-80 group on PND 21 (Table 2). The heart-to-BW ratio was not significantly different between the TCDD-exposed groups and the Control group on PND 7, but was significantly increased in the TCDD-80 group on PND 21 (Table 2).

Increased gene expression of cardiac hypertrophy markers in lactationally TCDD-exposed mice

The abundances of mRNAs of atrial natriuretic peptide (ANP) and β -myosin heavy chain (β -MHC), markers increased in the gene expression during cardiac hyper-

trophy and heart failure (Sergeeva and Christoffels, 2013; Reiser *et al.*, 2001), were not significantly different between the TCDD-exposed groups and the Control group on PND 7 (Fig. 1). On PND 21, mRNA abundance of ANP in the TCDD-80 group was significantly higher than that in the Control group, and that in the TCDD-20 was at an intermediate level between those in the Control and TCDD-80 groups. On the other hand, β -MHC mRNA abundance on PND 21 was increased in the TCDD-80 group compared to the Control group.

Left ventricular pathology in lactationally TCDD-exposed mice

Macroscopic and histological examinations revealed that hearts from five of six pups in the TCDD-80 group showed a rounded overall shape with a decreased cavity as well as left ventricular wall and septum thickening

Fig. 1. ANP (A) and β -MHC (B) mRNA abundances in the heart of pups lactationally exposed to TCDD. Dams were administered TCDD at a dose of 0, 20, or 80 $\mu\text{g}/\text{kg}$ BW on PND 1, and pups were sacrificed on PND 7 or PND 21 ($n = 3$ and $n = 5$, respectively). Because of large variations, statistical analysis was performed using a Kruskal-Wallis test, followed by Dunn's multiple comparison test. Asterisks indicate significant difference from the corresponding control group (*, $p < 0.05$).

on PND 7 (Fig. 2C). None of the hearts in the Control group showed these changes (Fig. 2A), and hearts in the TCDD-20 group (Fig. 2B) showed intermediate morphology between those in the TCDD-80 and Control groups. By PND 21, the morphology of the left ventricle was drastically changed; the majority of mice in the TCDD-80 group had hearts with dilated left ventricular cavity and thinner walls compared with those in the Control group (Fig. 2D). The TCDD-20 group exhibited changes approximately intermediate between the TCDD-80 and the Control groups on PNDs 7 and 21. Masson trichrome stain-

ing revealed no marked differences in fibrosis between the Control and TCDD-20 groups (Fig. 2E and F). However, increased fibrosis surrounding the myocytes in the left ventricular wall was observed in the TCDD-80 group (Fig. 2G).

Increased gene expression of matrix metalloproteinases in lactationally TCDD-exposed mice

To examine expression of matrix metalloproteinases (MMPs), enzymes that degrade collagen in the extracellular matrix, cardiac MMP-9 and MMP-13 mRNAs were quantified. On PND 7, the abundances of MMP-9 and MMP-13 mRNAs were increased in the TCDD-20 and TCDD-80 groups in a dose-dependent manner (Fig. 3A and B). On PND 21, MMP-9 mRNA abundances in the TCDD-exposed groups were not significantly different from the Control group. On the other hand, mRNA abundance of MMP-13 was significantly increased in the TCDD-20, but not in the TCDD-80 group (Fig. 3).

Induced gene expression of IL-1 β and TNF- α in lactationally TCDD-exposed mice

Abundances of mRNAs of IL-1 β and TNF- α , cytokines that have the potential to increase MMP activity (Siwik *et al.*, 2000), were also examined. IL-1 β mRNA abundance was significantly induced by TCDD exposure in a dose-dependent manner on PND 7 (Fig. 4A), but was not increased on PND 21. On the other hand, TNF- α mRNA abundance was not significantly altered at either time point by TCDD exposure (Fig. 4B).

Endothelin-1 induction by lactational TCDD exposure

We next examined the expression of endothelin-1 (ET-1), a potent inducer of MMPs and an important regulator of cardiac functions (Hathaway *et al.*, 2015). ET-1 mRNA abundance was unchanged on PND 7 in the TCDD-20 and TCDD-80 groups compared with the Control group, but was significantly upregulated on PND 21 in the TCDD-80 group (Fig. 5A). We also tested whether there was a correlation between cardiac ET-1 expression and ANP and/or β -MHC in PND 21 pups. There was a moderate correlation between the expression of ET-1 and ANP, a high correlation between ET-1 and β -MHC ($r = 0.643$ and $r = 0.904$, $n = 5$, respectively) (Fig. 5B and C).

DISCUSSION

Our present study provided clues to understand TCDD-induced developmental stage-dependent abnor-

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Fig. 2. Representative histological (A, B, C, E, F, and G) and stereoscopic microscope photographs (D) of whole heart (A, B, C, and D) or left ventricular wall (E, F, and G) on PND 7 and PND 21. Dams were administered TCDD at a dose of 0 µg/kg BW (A, D left, E), 20 µg/kg BW (B, F), or 80 µg/kg BW (C, D right, and G) on PND 1. Pups were examined on PND 7 (A, B, and C) or PND 21 (D, E, F, and G). Tissue slices were stained with hematoxylin and eosin (A, B, and C) or Masson trichrome (E, F, and G). Bar = 1.0 mm for A–C; and bar = 100 µm for E–G. (D) Dotted lines and arrows show left ventricle and left ventricular wall, respectively.

malinity in the heart. It has been reported that TCDD-exposed pregnant mice carried fetuses with reduced heart-to-BW ratio (Thackaberry *et al.*, 2005), but that pups born to these dams had significantly increased heart-to-BW ratios measured at weaning and in adulthood (Lin *et al.*, 2001; Thackaberry *et al.*, 2005). Since TCDD is a

lipophilic compound that is prone to persist in the body of animals (Aragon *et al.*, 2008), the increased heart-to-BW in the postnatal period after the prenatal TCDD exposure may be caused by persisting TCDD in the body. We confirmed that TCDD administration to dams in the postnatal period increased the heart-to-BW ratio (Table 2). The

Fig. 3. MMP-9 (A) and MMP-13 (B) mRNA abundances in the heart of pups lactationally exposed to TCDD. Asterisks indicate significant differences from the corresponding control group (**, $p < 0.01$) by non-repeated ANOVA with Dunnett post hoc test.

Fig. 4. IL-1 β (A) and TNF- α (B) mRNA abundances in the heart of pups lactationally exposed to TCDD. Asterisks indicate significant differences from the corresponding control group (*, $p < 0.05$; **, $p < 0.01$) analyzed by non-repeated ANOVA with Dunnett post hoc test.

results could partly support the above raised possibility and indicate that TCDD exposure poses a risk to the heart in both pre- and postnatal periods, and that TCDD induces apparently opposite effects in terms of heart-to-BW ratio depending on the developmental stages. However, the increase in heart-to-BW by postnatal TCDD exposure is accompanied by lowered BW (Table 2) while that by prenatal exposure is not (Thackaberry *et al.*, 2005), which raises the possibility that TCDD-induced systemic toxicity significantly affect the heart-to-BW ratio increase by the postnatal exposure. In addition, the prenatal dose to dams (3–24 $\mu\text{g/kg}$), which can increase the heart-to-BW ratio in adulthood (Thackaberry *et al.*, 2005; Lin *et al.*, 2001), is much lower than the postnatal dose to dams

(80 $\mu\text{g/kg}$) in the present study. Therefore, prenatal period is thought to be more susceptible to TCDD-induced cardiac hypertrophy than postnatal period.

In humans who were accidentally exposed to TCDD by a herbicide explosion in Seveso, Italy, 1976, Consonni *et al.* (Consonni *et al.*, 2008) reported increased mortality from ischemic heart disease (IHD) among male residents ($n = 7$ deaths, RR = 2.5; 95% CI: 1.2, 5.2) and increased mortality from hypertension among female residents ($n = 4$ deaths, RR = 2.6; 95% CI: 1.0, 6.9). Although the mechanistic association of the experimental results and the epidemiologic cohort is not clear, TCDD has a potential to induce cardiotoxicity during the developmental period. According to the developmental ori-

Fig. 5. ET-1 (A) mRNA levels in the heart of pups lactationally exposed to TCDD and their correlation with the cardiac hypertrophy markers ANP (B) and β-MHC (C) on PND 21. Asterisks indicate significant differences from the corresponding control group (*, $p < 0.05$) by non-repeated ANOVA with Dunnett post hoc test.

gins of health and disease (DOHaD) paradigm, fetuses raised under nutrition deficient conditions may develop non-communicable diseases, such as cardiovascular disease and diabetes, later in adulthood, and this paradigm has been extended to cover prenatal exposure to various chemicals, including dioxins (see reviews Tohyama, 2019; Heindel *et al.*, 2017). An increase in the heart-to-BW ratio found in previous studies (Lin *et al.*, 2001; Thackaberry *et al.*, 2005) and this study may be explained by the DOHaD paradigm, in which epigenetic alterations will affect the health status.

Histological examinations revealed left ventricular hypertrophy in TCDD-exposed pups on PND 7, when the relative heart weight was not yet increased. The hypertrophy was similar to that of concentric remodeling in that there was a thickened left ventricle without an increase in mass (Gaasch and Zile, 2011; Oktay *et al.*, 2016). On PND 21, the abnormality had progressed to a relative heart weight increase and fibrosis indicative of an eccentric remodeling pattern (increased mass and chamber with thinner walls). The transition between concentric and eccentric types of left ventricular hypertrophy and the prognostic outcome of each type are currently unclear (Oktay *et al.*, 2016). The TCDD-exposed murine heart may be a useful model to explore mechanisms of transition from concentric to eccentric left ventricular hypertrophy. Since it has been reported previously that chronic exposure of TCDD in adult mice induced left ventricular hypertrophy (Kopf *et al.*, 2008), the heart is thought to be a target organ of TCDD exposure throughout the developmental stages.

An increase in TCDD-induced relative heart weight was accompanied by an increase in the expression of the cardiac hypertrophy marker genes ANP and β-MHC in the TCDD-80 group on PND 21, but not on PND 7 (Table 2 and Fig. 1). The increase in these cardiac hypertrophy markers was in parallel with an increase in ET-1 (Fig. 5). On the other hand, mRNA abundance of an inflammatory cytokine IL-1β, which has a role in inducing cardiac myocyte growth (Palmer *et al.*, 1995; Xu *et al.*, 2015), was increased as early as PND 7, but became similar by PND 21 (Figs. 4). These findings suggest that the pathogenesis of left ventricular hypertrophy observed on PND 7 had a mechanism distinct from that on PND 21. Among potential factors playing a role in TCDD-induced cardiac hypertrophy on PND 21, ET-1 was increased in the expression and thought to have a potential to be a candidate (Fig. 5). A previous study using hypertensive rats revealed that ET-1 is minimally expressed in left ventricular hypertrophy stage, but is significantly elevated in later stage when relative heart weight was increased

(Iwanaga *et al.*, 1998). In addition, treatment of an antagonist against ET-1 receptors suppressed cardiac hypertrophy (Moser *et al.*, 2002) and improved left ventricular functions and survival rate (Iwanaga *et al.*, 1998). Taken together, ET-1 may play roles in both cardiac structural and functional abnormalities after TCDD exposure. However, the potential effect of ET-1 to induce MMPs were not likely since MMP -9 and -13 were increased on PND 7 whereas ET-1 was induced on PND 21. As to the left ventricular hypertrophy observed on PND 7, the increased expression of MMPs may be a molecular basis of the pathogenesis since overexpression of MMP-9 has been reported to increase the expression of inflammatory cytokines and left ventricular mass (Toba *et al.*, 2017). Further investigation is required to elucidate the roles of ET-1, MMP -9 and -13, and IL-1 β in TCDD-induced cardiac hypertrophy.

In conclusion, the present study revealed that TCDD directly induces cardiotoxicity in the postnatal period represented by left ventricular hypertrophy of the concentric remodeling type that transitioned to the eccentric type in later stages. ANP, β -MHC, and ET-1 showed upregulated gene expression and may mediate cardiac hypertrophy and heart failure. Comparison of doses required to induce cardiotoxicity revealed that prenatal period is more susceptible to TCDD-induced cardiotoxicity than postnatal period.

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

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