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Quantification of the tissue distribution and accumulation of the neonicotinoid pesticide clothianidin and its metabolites in maternal and fetal mice

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ABSTRACT

Neonicotinoids (NNs) are commonly used pesticides that have a selective agonistic action on insect nicotinic acetylcholine receptors. Recent evidence has shown that NNs have adverse effects in the next generation of mammals, but it remains unclear how NNs transferred from dams to fetuses are distributed and accumulated in fetal tissues. Here, we aimed to clarify the tissue distribution and accumulation properties of the NN clothianidin (CLO) and its 6 metabolites in 7 tissues and blood in both dams and fetuses of mice administered CLO for a single day or for 9 consecutive days. The results showed that the total concentrations of CLO-related compounds in the brain and kidney were higher in fetuses than in dams, whereas in the liver, heart, and blood they were lower in fetuses. The multi-day administration increased the total levels in heart and blood only in the fetuses of the single administration group. In addition, dimethyl metabolites of CLO showed fetus/dam ratios >1 in some tissues, suggesting that fetuses have higher accumulation property and are thus at higher risks of exposure to CLO-related compounds than dams. These findings revealed differences in the tissue-specific distribution patterns of CLO and its metabolites between dams and fetuses, providing new insights into the assessment of the developmental toxicity of NNs.

1. Introduction

Neonicotinoids (NNs) have a nicotine-like structure, exhibit a selective agonistic action on insect nicotinic acetylcholine receptors (nAChRs), and strongly stimulate the central nervous system to exert insecticidal effects (Ihara et al., 2003). NNs are systemic water-soluble pesticides that are transported throughout the plant and protect it from harmful insects for a long period. The receptors of vertebrates have different compositions and structures than those of insects, and the affinity of NNs for nAChRs is much higher in insects than in vertebrates (Tomizawa and Casida, 2005). In mice, most NNs are immediately absorbed and excreted into the urine with an in vivo half-life of a few hours. In rodent models, however, numerous studies have revealed the adverse effects of NNs at no-observed-adverse-effect level (NOAEL) levels on physiological functions such as reproductive (Hoshi et al., 2014), immune (Onaru et al., 2020), metabolic (Yan et al., 2020;

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Nimako et al., 2021), and cognitive–emotional functions (Hirano et al., 2015, 2018; Yoneda et al., 2018) possibly through the generation of the oxidative stress and activation of nAChRs.

Epidemiological studies showed that NNs were passively transported from mother to fetus through the placenta (Zhang et al., 2022) and detected in the urine of children as well as adults (Ueyama et al., 2015; Harada et al., 2016; Ikenaka et al., 2019), which calls attention to the transgenerational effects of NNs. The residue standard of pesticides is based on the acceptable daily intake (ADI) calculated from toxicological data obtained mainly from adult animals, but fetuses are more sensitive than adults to potentially toxic chemicals (Charnley and Putzrath, 2001; Mori, 2004). Prenatal and postnatal exposure to NNs causes behavioral changes in mice; the neonicotinoid acetamiprid (ACE) increased sexual and aggressive behaviors in male offspring (Sano et al., 2016) and a later neonicotinoid, clothianidin (CLO), induced anxiety-like behavior in juvenile-aged mice and increased locomotor activity in mature mice (Maeda et al., 2021). We previously demonstrated that CLO is rapidly transferred from dams to fetuses through the placenta in pregnant ICR mice (Ohno et al., 2020) and that CLO and a metabolite, dimethyl-CLO, are concentrated in breast milk during lactation periods (Shoda et al., 2023a). However, the whole picture of tissue distribution or accumulation patterns through maternal–fetal transfer of CLO and its metabolites has not yet been clarified.

The toxic potency and efficacy of NNs depend on the structural characteristics of the whole molecules (Tomizawa and Casida, 2005), and various metabolites are produced by changes in the molecular structure caused by metabolism. Compared to the parent compounds, some metabolites of NNs persisted for a longer time in mammalian tissues (Ford and Casida, 2006a, 2006b) and showed increased affinity for mammalian nAChRs (Casida, 2011). Further information on the metabolic fate of NNs and its metabolites is necessary in order to consider the risks of NNs in the next generation. In this study, we hypothesized that there were fetus-dam differences in distribution and accumulation patterns of NNs. We aimed to clarify the body concentration of CLO and its metabolites in maternal and fetal tissues and blood by simultaneous LC/MS/MS analyses using mice model to obtain data for risk assessment of developmental effects of NNs in humans.

2. Material and methods

2.1. Experimental animals and procedure

Pregnant ICR mice (14–16 weeks old, > 25 g body weight, embryonic day 3.5) were purchased from Japan SLC (Hamamatsu, Shizuoka, Japan). All mice were maintained in individual (40.5 × 20.5 × 18.5 cm) ventilated cages (Sealsafe Plus Mouse; Tecniplast, Buguggiate, Italy) under controlled temperature (23 ± 2 °C) and humidity (50 ± 10%) on a 14-h light/10-h dark cycle at the Kobe University Life-Science Laboratory with ad libitum access to a pellet diet (DC-8; Clea Japan, Tokyo, Japan) and water. The experimental design is illustrated in Fig. 1. We administered CLO (purity: 95%, extracted from Dantotsu® Sumitomo Chemical Co., Tokyo, Japan; Hirano et al., 2015) or vehicle (0.5% carboxymethylcellulose, 7.5 mL/kg) to pregnant ICR mice by oral gavage, and the treatment group was divided into a single-day administration group and a multi-day (9 days) administration group. After the weighing mice, the former received 65 mg/kg/day of CLO once at E18.5 of pregnancy, and the latter received 65 mg/kg/day of CLO once a day for 9 days from E10.5 to E18.5. Based on our previous paper report (Ohno et al., 2020), 6 h after the final administration, the blood samples of fetuses and dams were collected under isoflurane anesthesia. After the euthanasia by exsanguination, the brain, liver, kidney, spleen, adrenal gland, thymus, heart, and blood samples were collected. In all groups (n = 6 mice in each group), the administration concentration was set to 65 mg/kg/day with reference to the no-observed-adverse-effect level (NOAEL) of 65.1 mg/kg from a 78-wk dietary carcinogenicity study in female mice (Food and Agriculture Organization of the United Nations, 2016; Uneme et al., 2006), and four fetuses were randomly selected from each dam (6 dams and corresponding 24 fetuses in total). The groups were defined as the dams 1-day, dams 9-day, fetuses 1-day, and fetuses 9-day groups. This study was approved by the Institutional Animal Care and Use Committee (Permission #26–05-07) and was carried out according to the Kobe University Animal Experimentation Regulations.

2.2. Chemicals

The CLO standard (purity: 99.9%) was purchased from Fluka (Buchs, Switzerland). The CLO-d3 (purity: >97.0%) was purchased from Hayashi Junyaku (Osaka, Japan). The 1-methyl-3-nitroguanidine (MNG) (purity: >98.0%) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Desmethyl-CLO (dm-CLO), desmethyl-desnitro-CLO (dm-dn-CLO), desmethyl-CLO-urea (dm-CLO-urea), desnitro-CLO (dn-CLO), and CLO-urea were synthesized at Toho University.

2.3. Extraction of neonicotinoids from tissue and blood samples

Here, 100 μL of 100-ppb CLO-d3 was placed in a 1.5-ml tube as an internal standard substance. After the addition of 50–100 mg of the tissue sample and thorough mixing, 1 ml of 1% formic acid containing 100% acetonitrile was added, and 20 zirconia beads having a diameter of 2 mm were added and shaken at 30 times/s for 3 to 10 min. Then, after centrifugation at 10,000 G for 10 min, the supernatant was transferred to a test tube (fraction 1). Formic acid was added to the same 1.5 ml tube as above, shaken with zirconia beads, and centrifuged for 10 min. The resulting supernatant (fraction 2) was transferred to the test tube containing the supernatant (fraction 1 + fraction 2). An InertSep PSA solid-phase extraction cartridge (PSA) (GL Science, Tokyo, Japan) and the samples were conditioned by the addition of 1 ml of a 1:1 mixture of acetonitrile (ACN) and dichloromethane (DCM). The samples

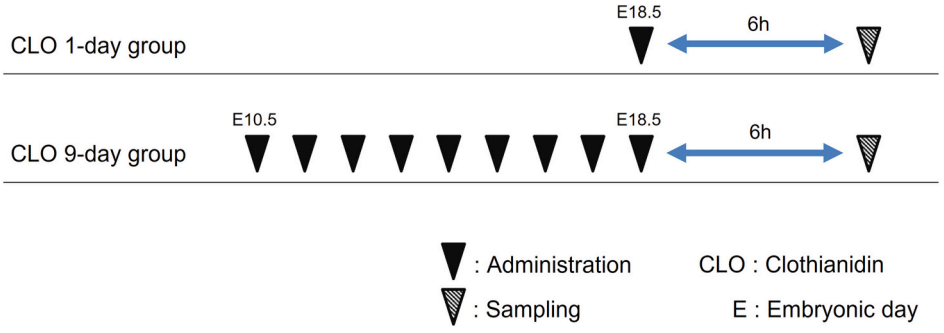

Fig. 1. Scheme showing the overall experimental design of the present study. Dams received a single administration of 65 mg/kg of clothianidin (CLO) at E18.5 (CLO 1-day group) or were administered CLO daily for 9 days from E10.5 to E18.5 (CLO 9-day group). Tissues and blood samples were collected 6 h after the last CLO administration in each group.

were passed through the PSA cartridges, and the eluents were collected in new test tubes using a 1:1 mixture of ACN and DCM (3 ml). The collected eluents were evaporated to dryness using a Speed Vac. The samples were then reconstituted with 200 μL of 3% ACN aqueous solution containing 10 ppb Cotinine-d3.

An LC-ESI/MS/MS (Agilent 6495B; Agilent Technologies, Santa Clara, CA, USA) system equipped with Kinetex Biphenyl (2.1 mm ID × 100 mm, ϕ 2.6 μm) (Phenomenex, Inc., Torrance, CA, USA) was used for the sample analysis. Solvents A and B, used for the HPLC analysis, consisted of 0.1% formic acid +10 mM ammonium acetate water solution and 0.1% formic acid +10 mM ammonium acetate methanol solution, respectively. The gradient was programmed as follows: for $t = 0$ to 1 min, 5% B (isocratic); for $t = 6$ min, 95% B (gradient); and for $t = 6$ to 8 min (gradient), 95% B (isocratic). The column oven temperature and flow rate were 60 °C and 0.5 mL/min, respectively. Target compounds were detected by multiple-reaction monitoring (MRM) in positive ionization mode as described in Table 1. The recovery rates of clothianidin and its metabolites were in the range of 62.1 ± 7.1% (MNG) to 92.3 ± 8.5% (dm-CLO). In addition, the reproducibility of the analysis system was confirmed by single or multiple analysts, with a relative standard deviation (RSD) of 10% for all compounds.

2.4. Data analysis

Statistical analyses were performed with GraphPad Prism software version 8.4.3 (GraphPad Software Inc., San Diego, CA, USA). The normality of data was analyzed by Shapiro-Wilk test. Two-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to analyze the effects between groups. The results were considered significant when the p-value was <0.05.

3. Results

3.1. Total concentrations of CLO and its metabolites in tissue and blood samples

CLO is known to be metabolized by several pathways in the mammalian body. In the present study, we detected CLO and its six metabolites from samples in both dams and fetuses. A major metabolite of CLO, desmethyl-CLO (dm-CLO), is formed by demethylation of the methyl group of CLO and is further metabolized through the reduction of the nitro group, resulting in the formation of desmethyl-desnitro-CLO (dm-dn-CLO), followed by cleavage of the imino group to form dm-CLO-urea. Thus, CLO also forms desnitro-CLO (dn-CLO) by reduction of the nitro group, which is further metabolized to CLO-urea by cleavage of the imino group. A 1-methyl-3-nitroguanidine (MNG) is formed from CLO by cleavage of the thiazolyl chlorine substituent (Fig. 2).

We investigated the distribution and accumulation of the total amount of CLO and its metabolites in the tissues and blood of both dams and fetuses exposed to CLO for a single day and for 9 days. We detected all CLO and 6 metabolites at detectable levels in 7 tissues and blood from dams and fetuses. The total concentration of CLO and its metabolites was significantly higher in the brain [$F(1, 56) = 85.57$, $p < 0.01$] and kidney [$F(1, 56) = 22.60$, $p < 0.01$] tissues of fetuses compared to those of dams (Fig. 3A and C), whereas it was significantly lower in the liver [$F(1, 56) = 67.90$, $p < 0.01$], heart [$F(1, 56) = 23.71$, $p < 0.01$] and blood [$F(1, 36) = 134.6$, $p < 0.01$] of fetuses than dams (Fig. 3B, G and H). The two-way ANOVA also showed significant main effects of the administration period in heart [$F(1, 36) = 12.49$, $p < 0.01$] and blood [$F(1, 56) = 17.50$, $p < 0.01$]. It was also observed that the total concentration of CLO and its metabolites was higher in the 9-day group than in the 1-day group in fetuses ($p < 0.01$). In the adrenal gland, the main effect of the administration period was close to significant [$F(1, 55) = 3.318$, $p = 0.0740$]. There were no main effects in thymus and spleen (Fig. 3D, E and F), and no significant interaction between dams and fetuses was observed in relation to the administration periods.

3.2. Tissue and blood concentrations of each CLO and its metabolites

We then examined the distribution and accumulation patterns of each CLO and its metabolites in 7 tissues and blood in dams and fetuses. As shown in Fig. 4A, the concentration of the parent compound, CLO, was higher in the brain [$F(1, 56) = 62.81$, $p < 0.01$] and kidney [$F(1, 56) = 18.32$, $p < 0.01$] of fetuses than dams, whereas it was lower in the liver [$F(1, 56) = 56.12$, $p < 0.01$], spleen [$F(1, 56) = 6.534$, $p < 0.05$], heart [$F(1, 36) = 7.522$, $p < 0.01$], and blood [$F(1, 56) = 57.49$, $p < 0.01$] of fetuses than dams. In all tissue and blood samples, there were no significant main effects related to the comparison between dams and fetuses, and no interactions were observed. As for dm-CLO (Fig. 4B), the concentrations in brain [$F(1, 56) = 51.49$, $p < 0.01$], kidney [$F(1, 56) = 34.50$, $p < 0.01$], and thymus [$F(1, 56) = 5.337$, $p < 0.05$] were higher in fetuses compared to dams, whereas they were lower in fetuses in liver [$F(1, 56) = 51.04$, $p < 0.01$], heart [$F(1, 36) = 35.18$, $p < 0.01$], and blood [$F(1, 56) = 122.1$, $p < 0.01$]. The main effect of the administration period was significant in the heart [$F(1,36) = 29.62$, $p < 0.01$] and blood [$F(1, 56) = 21.68$, $p < 0.01$], and the 9-day group showed higher concentrations than the 1-day group in both dams ($p < 0.05$) and fetuses ($p < 0.01$). No significant interaction was observed in any tissue or blood samples. As shown in Fig. 4C, the concentration of dm-dn-CLO in fetuses was lower than in dams in most samples, including those of the liver [$F(1, 56) = 145.0$, $p < 0.01$], kidney [$F(1, 56) = 34.69$, $p < 0.01$], thymus [$F(1, 56) = 49.92$, $p < 0.01$], spleen [$F(1, 55) = 37.20$, $p < 0.01$], heart [$F(1, 36) = 44.96$, $p < 0.01$], and blood [$F(1, 56) = 155.4$, $p < 0.01$], but it was greater in the brain [$F(1, 56) = 6.084$, $p < 0.05$] and adrenal gland [$F(1, 55) = 30.72$, $p < 0.01$] of fetuses compared to dams. A significant main effect of the administration period was observed in the adrenal gland [$F(1, 55) = 77.61$, $p < 0.01$] and heart [$F(1, 36) = 19.11$, $p < 0.01$]. A significant interaction between dams and fetuses, considering the administration period, was observed in the adrenal gland [$F(1, 55) = 39.06$, $p < 0.01$], and the post hoc analyses showed that the 9-day administration increased the accumulation of dm-dn-CLO in the adrenal gland in both dams and fetuses compared to that in the 1-day administration group ($p < 0.01$). Concerning dm-CLO-urea, fetuses showed lower concentrations than dams in the liver [$F(1, 56) = 53.87$, $p < 0.01$], spleen [$F(1, 55) = 1856$, $p < 0.01$], heart [$F(1, 36) = 30.22$, $p < 0.01$], and blood [$F(1, 56) = 37.00$, $p < 0.01$], but a higher concentration in the adrenal gland [$F(1, 55) = 13.44$, $p < 0.01$] (Fig. 4D). No marked main effect of administration period was observed, nor was any interaction found. The concentrations of dn-CLO were consistently lower in fetuses than dams in the liver [F (1,

Table 1
Multiple-reaction monitoring (MRM), reference ions, retention times (RT), recovery rates, and limits of quantification (LOQ) of target chemicals.

Chemical	MRM	Reference ion	RT	Recovery rate (% ± SD)	LOQ (ng/ml)
Clothianidin	250.02 > 132.00	169.1	3.9	86.3 ± 6.4	0.5
Clothianidin-d3	253.04 > 132.10	171.9	3.9	86.8 ± 7.2	–
dm-Clothianidin	236.00 > 132.00	113.1	2.9	92.3 ± 8.5	1.0
dm-Clothianidin-urea	192.00 > 132.10	86.1	1.8	77.1 ± 7.6	5.0
Clothianidin-urea	206.02 > 86.2	175.0	2.6	82.5 ± 11.0	0.5
dn-Clothianidin	205.03 > 132.1	45.1	1.3	72.2 ± 6.3	0.5
dm-dn-Clothianidin	191.02 > 132.1	45.2	1.2	74.4 ± 7.2	1.0
MNG	119.10 > 73.02	57.2	1.0	62.1 ± 7.1	0.5

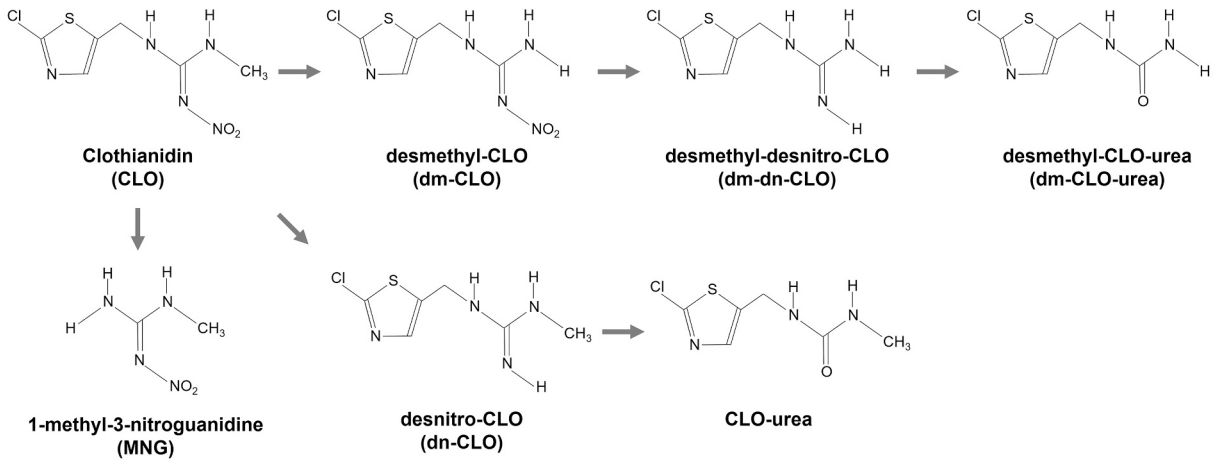

Fig. 2. Partial metabolic pathway of CLO in mice. The parent compound (CLO) undergoes metabolic transformation into several categories of metabolites, including desmethylated metabolites (desmethyl-CLO or dm-CLO, desmethyl-desnitro-CLO or dm-dn-CLO, and desmethyl-CLO-urea or dm-CLO-urea), desnitro metabolites (desnitro-CLO or dn-CLO and CLO-urea), and 1-methyl-3-nitroguanidine (MNG).

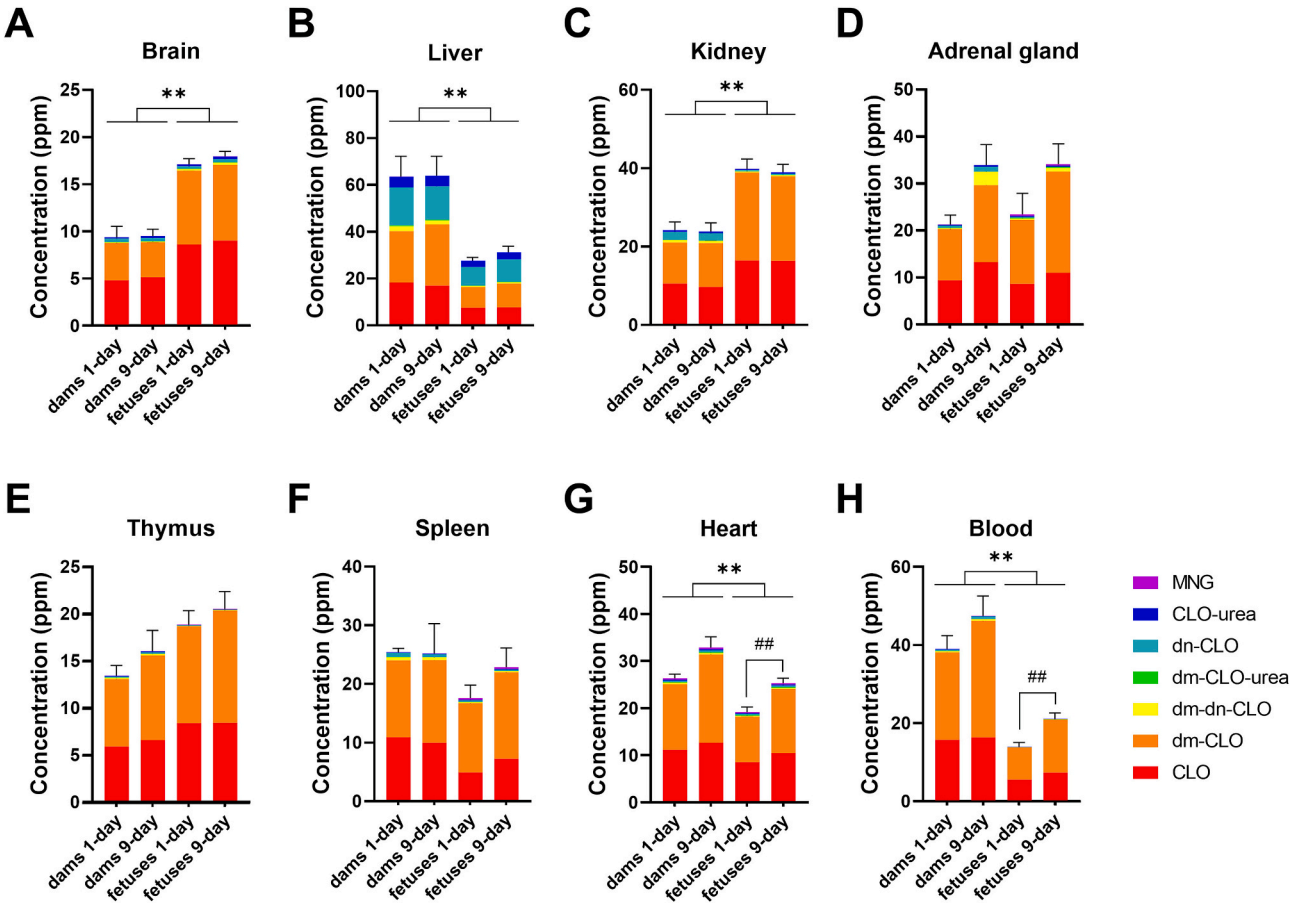

Fig. 3. The cumulative quantity and composition of CLO and its metabolites in the tissues and blood of dams and fetuses. Stacked graphs show the sum of the mean concentrations of 7 CLO compounds in the (A) brain, (B) liver, (C) kidney, (D) adrenal gland, (E) thymus, (F) spleen, (G) heart, and (H) blood of dams and fetuses. All data are shown as means ± SE (dams: $N = 6$; fetuses: $N = 24$), $*p < 0.05$, $**p < 0.01$ vs. other groups (two-way ANOVA followed by Tukey's post-hoc test).

56) = 30.30, $p < 0.01$], kidney [F (1, 56) = 149.8, $p < 0.01$], adrenal gland [F (1, 55) = 60.22, $p < 0.01$], thymus [F (1, 56) = 71.78, $p < 0.01$], spleen [F (1, 55) = 18.54, $p < 0.01$], and blood [F (1, 56) = 55.41, $p < 0.01$] (Fig. 4E). The two-way ANOVA also revealed significant main effects of administration period in the adrenal gland [F (1, 55) = 11.27, $p < 0.01$], heart [F (1, 36) = 6.996, $p < 0.05$], and blood [F (1, 56) = 5.552, $p < 0.05$] a in addition to significant interaction in the adrenal gland [F (1, 55) = 13.54, $p < 0.01$] and blood [F (1, 56) = 5.494, $p < 0.05$]. Furthermore, the 9-day administration significantly increased the dn-CLO concentration in the adrenal gland compared to the 1-day administration group only in dams ($p < 0.01$). Fig. 4F shows that the concentrations of CLO-urea in fetuses were lower than in dams in most tissues, including the liver [F (1, 56) = 14.31, $p < 0.01$], kidney [F (1, 56) = 5.571, $p < 0.05$], thymus [F (1, 56) = 33.13, $p < 0.01$], heart [F (1,

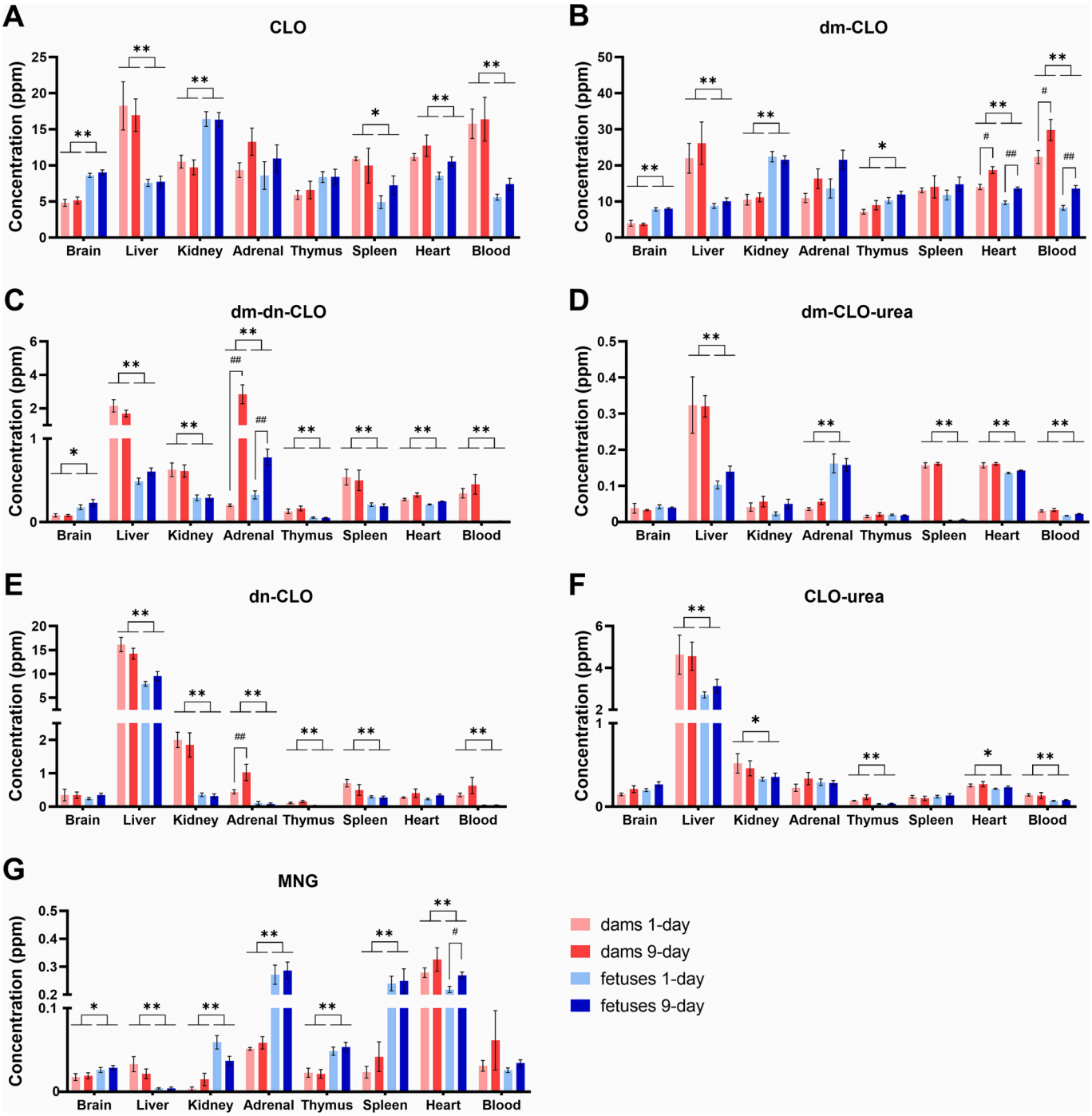

Fig. 4. The concentrations of CLO and its metabolites in different tissues and blood of dams and fetuses. The graphs display the concentrations of individual CLO compounds and their metabolites across 7 tissues and blood samples in both dams and fetuses. All data are presented as means ± standard error (SE) (dams: N = 6; fetuses: N = 24), *$p < 0.05$, **$p < 0.01$ vs. other groups (two-way ANOVA followed by Tukey's post-hoc test).

36) = 5.897, $p < 0.05$], and blood [F (1, 56) = 28.36, $p < 0.01$]. A significant main effect of administration period was observed only in the thymus [F (1, 56) = 4.667, $p < 0.05$], and there was no significant interaction in any tissues or in blood. The concentrations of MNG in fetuses were greater than in dams in the brain [F (1, 56) = 4.465, $p < 0.05$], kidney [F (1, 56) = 15.38, $p < 0.01$] adrenal gland [F (1, 55) = 23.82, $p < 0.01$], thymus [F (1, 56) = 13.80, $p < 0.01$], and spleen [F (1, 55) = 16.85, $p < 0.01$], whereas they were lower in the liver [F (1, 56) = 55.33, $p < 0.01$] and heart [F (1, 36) = 9.521, $p < 0.01$]. There were main effects of administration period in the heart [F (1, 36) = 6.722, $p < 0.05$] and blood [F (1, 56) = 4.087, $p < 0.05$], but no significant interaction was observed in any tissues or in blood. The post hoc analyses showed that the concentration of MNG in fetuses in the 9-day administration group was significantly higher than that in the 1-day administration group ($p < 0.05$).

3.3. *Relative concentrations of CLO and its metabolites in maternal and fetal tissues*

To elucidate the variations in the accumulation properties of CLO and its metabolites between dams and fetuses, the fetus/dam ratio was calculated in each tissue and blood sample. As a result, the fetus/dam

ratios of the parent compound, CLO, in the brain, kidney, and thymus and those of dm-CLO in the brain, kidney, adrenal gland, thymus, spleen, and heart, were >1 in both the 1-day and 9-day administration groups (Fig. 5A and B). As shown in Fig. 5C, the ratio of dm-dn-CLO in the brain was above 1 in both administration periods. The adrenal gland showed a value above 1 in the 1-day administration group, although it was <1 in the 9-day administration group. As for dm-CLO-urea, the tissues with fetus/dam ratios above 1 were the brain, adrenal gland, and thymus in both administration groups (Fig. 5D). In dn-CLO, none of the tissue or blood samples showed a fetus/dam ratio of >1 (Fig. 5E). As shown in Fig. 5F, the fetus/dam ratios of CLO-urea were >1 in the brain and spleen in both administration periods and were above 1 in the adrenal gland only in the 1-day administration group. For MNG, the brain, kidney, adrenal gland, spleen, and thymus showed fetus/dam ratios >1 (Fig. 5G).

4. Discussion

In the present study, we aimed to investigate differences in the distribution and accumulation of CLO and its metabolites between fetal and maternal tissues. A previous study demonstrated the kinetics of CLO and dm-CLO only in adult mouse brain and liver tissues (Ford and Casida, 2006a, 2006b). We previously reported that CLO and its metabolites in mouse blood are rapidly transferred to the fetus (Ohno et al., 2020). In the present study, high-precision and high-sensitivity analyses using LC/MS/MS techniques enabled the detection of CLO and several CLO metabolites not only in the brain and liver but also in the kidney, spleen, adrenal gland, and thymus. Our results further clarified, for the first time, the distribution and accumulation of CLO and its metabolites as they are transferred from the dam to fetal tissues. This provides valuable foundational data for assessing the developmental toxicity of CLO.

In the present study, our data revealed that there were tissue-specific differences between dams and fetuses in the total concentration of CLO and metabolites. Although the liver, heart, and blood showed dam-dominated distribution, the brain and kidney showed fetal domination. On the other hand, there were differences in the total amounts of CLO-related compounds between the administration periods only in the heart and blood; these differences included increased total concentrations of CLO and its metabolites in the 9-day administration group compared to the 1-day administration group only in fetuses. These results indicated that CLO and its metabolites tend not to accumulate in major tissues as a result of consecutive administration and that the fetus may take longer than the dam to excrete these chemicals from the body. It was also noteworthy that the total concentrations of CLO and its metabolites in tissues were more clearly different between fetuses and dams than between the administration periods.

Another significant discovery in this study was the variation in distribution and accumulation patterns among individual CLO compounds and their metabolites. The primary metabolite of CLO, dm-CLO, accounted for most of the dynamic state in the mammalian body along with the parent compound, consistent with previous reports in mice and humans (Ohno et al., 2020; Wrobel et al., 2022). In this study, the 9-day administration significantly increased the concentrations of dm-CLO in heart and blood compared to the 1-day administration in both dams and fetuses, whereas the concentrations of the parent compound were not altered. Furthermore, most tissues (brain, kidney, adrenal gland, thymus, spleen, and heart) showed fetus/dam ratios of dm-CLO >1. This suggests that dm-CLO could be a pivotal compound with the highest potential for accumulation in the fetus. For other metabolites, several tissues showed higher concentrations than blood, and it was observed that downstream metabolites were more prone to accumulate in specific tissues compared to CLO and dm-CLO. Although the blood concentration ratios of dm-dn-CLO were >1 in the liver and adrenal gland, a similar observation was made for the subsequent metabolite, dm-CLO-urea, not

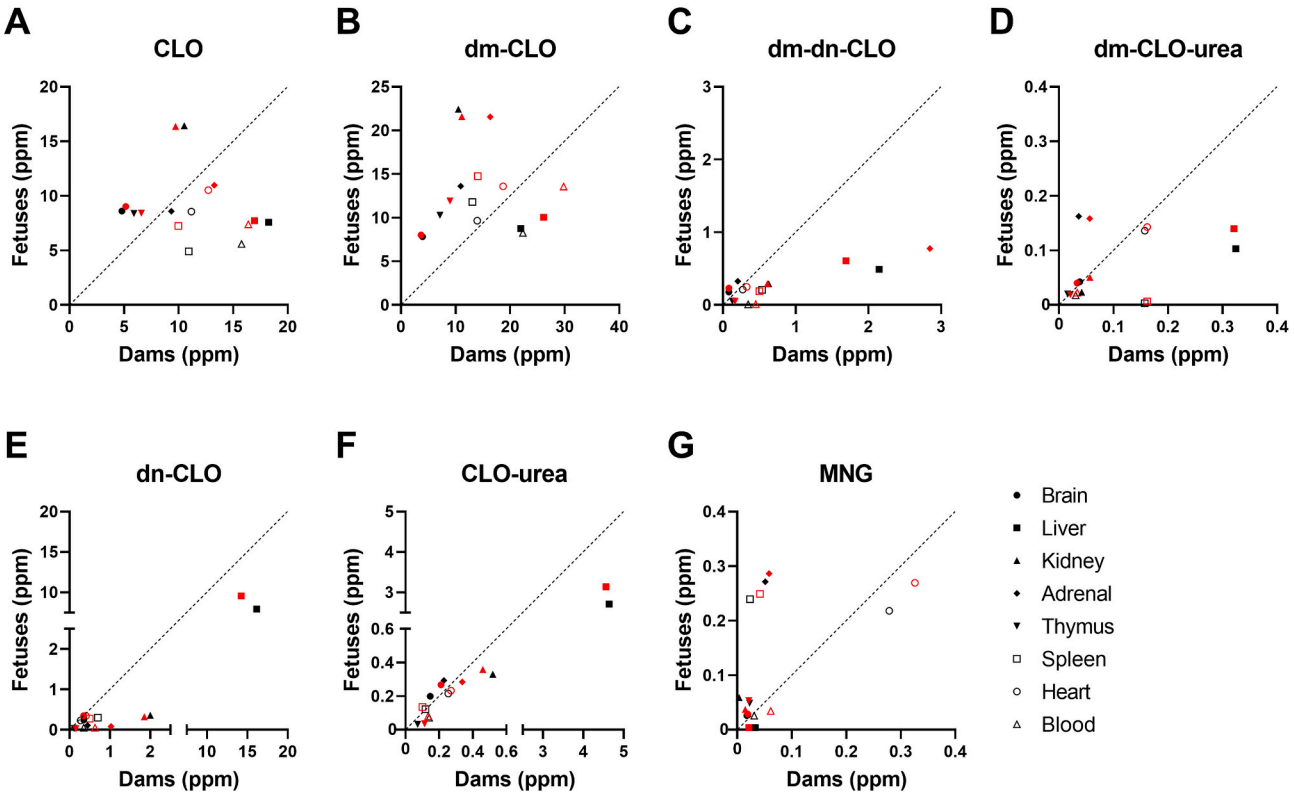

Fig. 5. Maternal–fetal ratio of CLO and its metabolite concentrations in tissues and blood. The horizontal and vertical axes represent the blood concentrations of dams and fetuses, respectively. Points above the dotted line (y = x) indicate a fetus-dominated accumulation relative to dams. Numbers of samples: dams = 6, fetuses = 24.

only in these tissues but also in the spleen and heart. These results suggest that the metabolism of dm-dn-CLO to dm-CLO-urea may occur in different parts of the body. Regarding dn-CLO and CLO-urea, these two metabolites were detected almost exclusively in the liver of both dams and fetuses, and the fetus/dam ratio was <1 in most tissues. The CYP3A family in the liver metabolizes CLO-related compounds (Casida, 2011; Kanne et al., 2005; Schulz-Jander and Casida, 2002; Shi et al., 2009), and dn-CLO and CLO-urea are located downstream of the metabolic pathway of CLO. Although the CYP3A family is known to be poorly expressed in the liver of fetal mice (Kitaoka et al., 2018), these results strongly indicate that fetal mice have the metabolic capacity to convert CLO to dn-CLO and CLO-urea in the liver. On the other hand, our data revealed that MNG had a peculiar distribution pattern compared to other metabolites; lower levels were observed in the liver in both dams and fetuses. In dams, only the heart showed a higher concentration than blood, whereas in the fetus, 5 tissues (kidney, adrenal gland, thymus, spleen, and heart) had MNG concentrations exceeding that in blood. The metabolic factors responsible for the conversion of CLO to MNG were not fully elucidated. However, it was observed that MNG was the second metabolite with a fetus/dam ratio above 1 in tissues. These results demonstrated for the first time the metabolite-specific patterns of distribution and accumulation in dams and fetuses, which may be helpful in elucidating the unexpected adverse effects of NNs in mammals.

It should also be noted that the adrenal gland exhibited distinctive accumulation properties for several metabolites, setting it apart from other tissues. The total concentration of CLO and its metabolites in the adrenal gland tended to increase in the 9-day administration group relative to that in the 1-day administration group. Furthermore, the 9-day administration yielded significantly higher concentrations of dm-CLO, dm-dn-CLO, and dn-CLO compared to the 1-day administration, and the fetuses showed markedly higher concentrations of dm-CLO-urea and MNG than the dams. Only a few previous studies have demonstrated the functional effects of NNs in the mammalian adrenal gland. In one such study, the neonicotinoid imidacloprid was found to enhance nicotine-elevated adrenaline secretion in PC12D rat adrenal chromaffin cells (Kawahata and Yamakuni, 2018). Additionally, other NNs, such as ACE and CLO, were shown to induce adrenaline release in a slice model of the rat adrenal gland (Park et al., 2021). These effects were strongly inhibited by an α3-containing nAChRs antagonist, hexamethonium. The reason for the accumulation potential of CLO metabolites in the adrenal gland remains unresolved, and further work should be undertaken to investigate the toxicity of NNs in the adrenal gland.

There is a growing body of evidence that has raised concerns about whether exposure to NNs during either the prenatal or postnatal period may have adverse effects on the developing brain. In the present study, the 6 most abundant CLO-related compounds (CLO, dm-CLO, dm-dnCLO, dm-CLO-urea, CLO-urea, and MNG) in all tissues showed fetus/dam ratios >1 in the brain. While in dams the overall levels of CLO-related compounds in the brain and kidney were lower than those in the blood, in fetuses the concentrations in the brain and kidney were either comparable to or higher than those in the blood. These results suggested that these compounds reach the fetal brain and kidney, which have an immature blood–brain barrier and filtration barrier, more readily than they reach these organs in the dams. Epidemiological studies showed that the maternal urinary concentration of dm-CLO is associated with neurodevelopmental scores (Wang et al., 2023), and several NNs detected in urine samples could be potential risk factors for chronic kidney disease in Sri Lanka (Taira et al., 2021). Given that pre- and postnatal exposure to CLO induced age- and developmental stage-dependent behavioral changes in mice (Maeda et al., 2021; Shoda et al., 2023b) and that subchronic exposure to CLO caused changes in more biochemical parameters in the infant rat kidney than in the adult rat kidney (Ozsahin et al., 2014), these two tissues could be at higher risk of the developmental effects of CLO. The limitation of this study is that the fetuses were randomly selected without regard to sex, therefore, it remains a challenge for future studies to investigate the sex differences in the developmental effects of NNs.

5. Conclusion

The present study is the first to quantitatively clarify the distribution and accumulation of CLO and its metabolites in multiple maternal and fetal tissues. The results revealed that tissue-specific distribution patterns differed between dams and fetuses. Furthermore, it was observed that several metabolites of CLO exhibited a greater propensity for accumulation in fetal tissues, including the brain and kidney, than for accumulation in these tissues in dams. This finding contributes to our understanding of the potential risks associated with developmental exposure to NNs. Considering that several NN metabolites have higher affinity for mammalian nAChRs than the parent compound, further research is needed to elucidate their contribution to the developmental toxicity of NNs.

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CRediT authorship contribution statement

Tetsushi Hirano: Writing – original draft, Visualization, Supervision, Funding acquisition, Data curation, Conceptualization. **Shuji Ohno:** Writing – review & editing, Writing – original draft, Methodology, Investigation. **Yoshinori Ikenaka:** Writing – review & editing, Methodology, Funding acquisition, Data curation. **Kanoko Onaru:** Writing – review & editing, Investigation. **Shizuka Kubo:** Writing – review & editing, Investigation. **Yuka Miyata:** Writing – review & editing, Investigation. **Mizuki Maeda:** Writing – review & editing, Investigation. **Youhei Mantani:** Writing – review & editing. **Toshifumi Yokoyama:** Writing – review & editing. **Collins Nimako:** Writing – review & editing, Validation, Methodology. **Yared Beyene Yohannes:** Writing – review & editing, Validation, Methodology. **Shouta M.M. Nakayama:** Writing – review & editing, Methodology. **Mayumi Ishizuka:** Writing – review & editing, Methodology. **Nobuhiko Hoshi:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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