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Quantitative elucidation of the transfer of the neonicotinoid pesticide clothianidin to the breast milk in mice

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Highlights

- CLO is metabolized maternally in mice and rapidly transferred in breast milk.
- CLO and metabolites are more highly concentrated in breast milk than maternal blood.
- Most of CLO and its metabolites were eliminated from the mother's body within 24 h.
- Infants are sensitive to chemicals, so adverse CLO effects in infants are a concern.

ABSTRACT

Neonicotinoid pesticides (NNs) have been reported to have neurobehavioral effects on offspring after fetal and lactational exposure. In this study, clothianidin (CLO), an NN, was administered orally as a single dose (6.5 mg/kg: 1/10 of the no-observed-adverse-effect level in the current Pesticide Evaluation Report) to 10-day post-partum ICR mice, and CLO and its metabolite desmethyl-CLO (dm-CLO) were quantified using liquid chromatography-electrospray ionization/tandem mass spectrometry (LC-ESI/MS/MS) after collecting maternal breast milk and blood samples over time (1, 3, 6, 9, 12, and 24 h after administration). CLO and dm-CLO were detected in the breast milk at 1 h after the administration, and their concentrations were significantly higher than those in blood at all time points. The concentrations of CLO and dm-CLO in the breast milk were at their highest levels at 1 and 3 h, respectively, and then decreased over time to become almost undetectable at 24 h after the administration. These results show that CLO is metabolized in the mother's body and is rapidly transferred to and concentrated in the breast milk. Since CLO concentrations in breast milk are higher than those in the blood, there is concern about the effects of CLO during lactation.

KEY WORDS: breast milk, clothianidin, fetomaternal transfer, maternal blood, metabolites, neonicotinoid

1. Introduction

Neonicotinoid pesticides (NNs) are nicotinic analogues that target insect-type nicotinic acetylcholine receptors (nAChRs). Until relatively recently, they had been widely adopted worldwide as alternatives to organophosphorus pesticides. The NNs are characterized by penetration, persistence, and selective toxicity, and seven are currently in wide use: imidacloprid (IMI), nitenpyram, acetamiprid (ACE), thiamethoxam (TMX), thiacloprid, clothianidin (CLO), and dinotefuran (DIN) (Bass et al., 2015; Tomizawa and Casida 2003). As competitive modulators of nAChRs, they exhibit insecticidal activity by continuously exciting and disturbing neurons, and their affinity for nAChRs is tens to hundreds of times higher than that of mammals (Tomizawa and Casida 2005).

However, since the 1990s, when the use of NNs began, there have been reports of mass deaths and disappearances of honeybees in some regions of the European Union (EU) and the United States. This phenomenon has been called "colony collapse disorder," and NNs have been suggested to be involved (Henry et al., 2012; Whitehorm et al., 2012; Gill et al., 2012). As a result, in 2013 the EU placed a moratorium on three kinds of neonicotinoids (IMI, TMX, and CLO), forbidding their use in flowering crops that appeal to honeybees and other pollinating insects, and in 2018 the EU decided to ban the outdoor use of controversial neonicotinoid pesticides altogether, based on the threat they pose to pollinators. The U.S. Environmental Protection Agency (EPA) began regulating new or expanded outdoor use in 2015 (U.S. Environmental Protection Agency, 2015). In Japan, however, rather than tightening regulations, the Ministry of Health, Labor and Welfare relaxed food residue standards for CLO and ACE in 2015, TMX in 2016, and IMI in 2017, and the use of NNs continues. Despite the above-mentioned change in their worldwide use, Japan continue to permit the use of NNs in agriculture and forestry, household pest control, pet care, gardening, and many other applications, making them a familiar part of daily life.

In recent years, NNs have been shown to have adverse effects on reproduction (Tokumoto et al., 2013; Hoshi et al., 2014; Kitauchi et al., 2021), thymus and intestinal microflora (Onaru et al., 2020), and neurobehavior (Hirai et al., 2022; Hirano et al., 2015, 2018, 2021; Nishi et al., 2022; Takada et al., 2018, 2020; Yoneda et al., 2018) due to exposure to non-toxic doses in birds and mammals, and it has been confirmed that NNs affect higher vertebrates. Along with organophosphorus and pyrethroid compounds, NNs were detected in the urine of Japanese adults, children and neonates (Ichikawa et al., 2019; Ikenaka et al., 2019; Oya et al., 2021; Ueyama et al., 2015), and CLO and its metabolites, which are NNs, were found to be rapidly transferred from mother to fetus (Ohno et al., 2020), indicating that NNs are routinely transferred between mothers and children. Although the no-observed-adverse-effect level (NOAEL) for NNs was established based on toxicity studies using adult animals, fetuses and infants are also considered to be vulnerable to chemicals because of their underdeveloped blood-brain barrier (Charnly

et al., 2001), and thus the direct application of the NOAEL of NNs to adult animals may affect the development of fetuses and infants. In fact, the exposure of perinatal mice to the NOAEL of NNs has been reported to cause germ cell abnormalities in offspring (Yanai et al., 2017) and neurodevelopmental toxicity (Hoshi, 2021; Nakayama et al., 2018; Maeda et al., 2021; Sano et al., 2016), indicating that NNs exert effects on the next generation.

Breast milk is a major source of nutrition for newborns, as it contains fats, proteins, carbohydrates, minerals, and vitamins, as well as the antibodies and lymphocytes necessary to protect the infant from infection and inflammation, and contributes to the healthy development of the immune system and intestinal microflora. For example, colostrum, which is secreted during the first few days after delivery, contains infection-fighting substances such as IgA, complement, lactoferrin, and macrophages, which protect the infant from infection. There are reports that breastfed infants have lower mortality rates than artificially fed infants, and that the incidence of acute infectious diseases such as allergies, insulin-dependent diabetes, and Campylobacter enteritis is reduced in breastfed infants (Ito and Lee 2003; Ip et al., 2007).

However, if a mother ingests a chemical substance, it may be transferred to the breast milk and affect the infant. In fact, it has been reported that anxiolytics, antiepileptics, antipyretics, anti-inflammatory drugs, and nicotine are transferred to human breast milk and affect infants (Tyson et al. 1937; Vorherr 1974; Ito and Lee 2003; Napierala et al. 2016). In addition, persistent organic pollutants, such as dioxins and organochlorine pesticides, including dichlorodiphenyltrichloroethane (DDT), polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs), have been detected in breast milk (van den Berg et al., 2017), suggesting that they affect growth hormones and thyroid hormones related to infant neurodevelopment (Kao et al., 2019). NNs, which are currently among the most used pesticides, have also been detected in human breast milk (Chen et al., 2020), suggesting that they may have been correlated with a documented suppression of neurological development in nursing infants.

We herein quantitatively evaluated the amount of CLO, which has been reported to have developmental neurotoxicity, and its metabolites transferred to breast milk over time in mice.

2. Materials and methods

2.1. Experimental animals and procedure

Pregnant ICR mice were purchased from Japan SLC (Hamamatsu, 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), humidity (50 ± 10%) and ventilation (cage: 75 times/h) 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) and water. Thirteen mother mice were used in the experiments.

2.2. CLO administration

To determine the single dose, the intake was calculated as 7.5 mL/kg body weight. CLO was dissolved in dimethyl sulfoxide (DMSO, FUJIFILM Wako Pure Chemical Co. Osaka, Japan) at a dose of 1% of the total amount, and then dissolved in 0.5% carboxymethyl cellulose (FUJIFILM Wako Pure Chemical Co.) to obtain a dose concentration of 6.51 mg/kg/day. The dose concentration was based on the non-toxic dose (65.1 mg/kg body weight) in ICR female mice in general pharmacological studies (Food and Agriculture Organization of the United Nations, 2016; Uneme et al., 2006). The CLO solution was administered as a single gavage dose to dams on postpartum day 10-11 (Fig. 1).

2.3. Quantitative analysis

2.3.1. Breast milk collection and blood sampling

At 1, 3, 6, 9, 12, and 24 h after the CLO administration, breast milk and blood samples were collected from 13 dams and stored at –80°C until analysis (Fig. 1). For the milk collection, a one-handed milking device for mice and rats (KN-591; Natsume Seisakusho, Tokyo) was used with some modifications (the teat contact area was changed to a 10 μL tip) (Fig. 2). The milk was collected immediately after administration of oxytocin (9-24 U/100 g body weight) under general anesthesia. The method of the milk collection was based on a previous study (Kawakami et al., 2015). Blood was collected from the tail vein of dams.

2.3.2. Sample extraction

From 1 to 5 μL of breast milk was measured, and then 2 mL of distilled water (D.W.), and 50 μL of CLO-d3 (100 ppb), as an internal standard, were added and the mixture was transferred to a 15 mL tube. Three mL of sodium acetate buffer (0.5 g/3 mL) and 1 mL of acetonitrile containing 1% formic acid were added, and the solution was mixed for 5 min. After that, 3.5 g of $MgSO_4$ was added and the solution was mixed for 10 min, followed by centrifugation at 10,000G for 5 min. After centrifugation, 70 μL of the supernatant was passed through a GL Science MonoSpin Phospholipid cartridge. One hundred and fifty μL of D.W. containing 1% formic acid was added to the transit solution, and was transferred to an HPLC vial for LC/MS analysis.

2.3.3. Liquid chromatography-electrospray ionization/tandem mass spectrometry (LC-ESI/MS/MS) analysis

The LC-ESI/MS/MS analysis was performed based on Ohno et al. (2020). In brief, A 6495B Triple

Quadrupole Electrospray ionization (ESI)-LC/MS system from Agilent Technologies (Santa Clara, CA, USA) was used for the measurements. For HPLC analysis, Kinetex® Biphenyl (particle size 2.7 μm, 100×3.0 mm; Phenomenex, Torrance, CA) was used as the analytical column. For solvent A, 0.1% formic acid + 10 mM ammonium acetate D.W. solution was used, and for solvent B, 0.1% formic acid + 10 mM ammonium acetate methanol solution was used. Gradient elution was programmed as follows: for t = 0~1 min, 5% B (isocratic); for t = 12 min, 95% B (gradient); and for t = 12~13 min (gradient), 95% B (isocratic). The column temperature and flow rate were set at 50 °C and 0.7 mL/min, respectively, and the target compounds were detected by multiple reaction monitoring (MRM) in positive ionization mode.

The quantification analysis was performed using the internal standard method with CLO-d3 as the internal standard.

Ohno et al. (2020) targeted six metabolites. However, in this study, more advanced pretreatment was required because breast milk, which contains more fat-soluble foreign substances than plasma, was targeted. Therefore, only CLO and the major metabolite, dm-CLO, were targeted for quantification because the recovery of the other metabolites was as low as about 20%. The recovery of CLO and dm-CLO were good, at 100% and 70%, respectively. The recovery rates of CLO and CLO metabolites were 95% CLO, 99% CLO-d3, 40% CLO-urea, 70% dm-CLO, 33% desmethyl-CLO-urea (dm-CLO-urea), and 20% desnitro-CLO (dn-CLO) at 50 ppb, and 100% CLO, 100% CLO-d3, 40% CLO-urea, 70% dm-CLO, 36% dm-CLO-urea, and 20% dn-CLO at 100 ppb. The limits of detection were 5 ppb for CLO and 10 ppb for dm-CLO, respectively.

2.4. Statistical analysis

Statistical analyses were performed with Excel Statistics 2012 (version 1.00; SSRI, Tokyo). The ratio of milk concentration to blood concentration at 1, 3, 6, and 9 h CLO and dm-CLO were analyzed using Welch's *t*-test. The results were considered significant when the *P*-value was less than 0.05.

3. Results

3.1. Kinetics and detected amounts of CLO compounds

3.1.1. CLO

CLO was detected in the breast milk and blood at 1, 3, 6, and 9 h after the CLO administration; at 12 and 24 h, CLO was below the detection limit or milk could not be collected (Table 1, Fig. 3A).

CLO levels in the breast milk were highest at 1 h after the administration in 8 of the 9 cases, with values of 3,428.8 ng/mL in dam 1, 2,299.7 ng/mL in dam 2, 3,249.2 ng/mL in dam 3, 1,474.5 ng/mL in dam 4, 2,974.6 ng/mL in dam 5, 2,170.8 ng/mL in dam 6, 2,409.9 ng/mL in dam 7 and 3,302.3 ng/mL

in dam 8. The doses decreased gradually at 3, 6, and 9 h after the administration, and no CLO was detected at 12 and 24 h. However, the CLO concentration increased by 21.4 ng/mL at 6 to 9 h after the administration in dam 7.

Maternal blood CLO concentrations were highest at 1 h after the administration in 8 of 9 animals, with values of 1,519.6 ng/mL in dam 1, 2,546.1 ng/mL in dam 2, 2,924.7 ng/mL in dam 3, 1,769.8 ng/mL in dam 5, 1,014.0 ng/mL in dam 6, 1,640.5 ng/mL in dam 7, 1,722.2 ng/mL in dam 8 and 736.6 ng/mL in dam 9. The doses decreased gradually at 3, 6, and 9 h after the administration, and no CLO was detected at 12 and 24 h.

The CLO concentration in the breast milk was significantly higher than that in the blood at 1 and 3 h after the administration (Fig. 3A).

3.1.2. dm-CLO

In the breast milk and maternal blood, dm-CLO, the metabolite of CLO, was detected at 1, 3, 6, and 9 h after the administration; at 12 and 24 h, dm-CLO was below the detection limit or milk could not be collected in most samples (Table 2, Fig. 3B).

The concentration of dm-CLO in the breast milk increased gradually at 1, 3, and 6 h after administration in dam 4, and decreased gradually at 1, 3, 6, and 9 h after the administration in dam 8, but was highest at 3 h after the administration in 7 of 9 dams, with values of 975.6 ng/mL in dam 1, 1,144.5 ng/mL in dam 2, 1,133.5 ng/mL in dam 3, 925.7 ng/mL in dam 5, 1,370.7 ng/mL in dam 6, 1,860.9 ng/mL in dam 7, and 1,196.6 ng/mL in dam 9. The dm-CLO levels decreased gradually at 6, 9 and 12 h after the administration, and no dm-CLO was detected at 24 h.

Concentrations of dm-CLO in the maternal blood were highest in 4 of the 9 dams at 1 h after the administration, with values of 768.6 ng/mL in dam 3, 230.1 ng/mL in dam 6, 521.3 ng/mL in dam 7, and 535.7 ng/mL in dam 8. The dose gradually decreased at 3, 6, and 9 h after the administration. Five of the nine dams had the highest doses at 3 h after the administration, with values of 282.7 ng/mL in dam 1, 678.3 ng/mL in dam 2, 520.6 ng/mL in dam 4, 376.7 ng/mL in dam 5, and 217.2 ng/mL in dam 9. The dose decreased gradually at 6 and 9 h after the administration. No dm-CLO was detected in the blood at 12 and 24 h after the administration.

At 1, 3, 6, and 9 h after the administration, concentrations of dm-CLO in the breast milk were significantly higher than those in the maternal blood (Fig. 3B).

3.2. Trends in CLO and dm-CLO concentrations in the breast milk and blood

In the breast milk, the highest concentration of CLO (2,663 ± 191 ng/mL) was observed at 1 h after the administration, and the highest dm-CLO concentration (1,084 ± 126 ng/mL) was observed at 3 h after

the administration. The CLO concentration remained higher than the dm-CLO concentration until 3 h after the administration, but the dm-CLO concentration exceeded the CLO concentration from 6 h after the administration (Fig. S1). In the blood, the CLO concentration was highest (1,656 ± 225 ng/mL) at 1 h after the administration, and the dm-CLO concentration was highest (397 ± 73 ng/mL) at 1 h after the administration. CLO concentrations remained higher than dm-CLO concentrations until 3 h after the administration, but dm-CLO concentrations were higher than CLO concentrations at 6 and 9 h after the administration (Fig. S1).

3.3. Ratio of CLO and dm-CLO concentrations in the breast milk to those in the blood

The ratio of CLO concentration in the breast milk to that in the blood was greater than 1 in all cases except for dam 2 at 1 h after the administration, dam 4 at 3 h after the administration, and dams 10-13 at 9 h after the administration (Table S1). The hourly distribution was also skewed toward the milk side rather than toward the y = x slope (Fig. 4A). The ratio of dm-CLO concentration in milk to that in the blood was greater than 1 in all cases except for dams 2 and 4 at 1 h after the administration and dam 4 at 3 h after the administration (Table S2). The distribution of dm-CLO in milk was more skewed toward the milk side than toward the slope of y = x (Fig. 4B).

4. Discussion

The present study demonstrated that CLO and its metabolites (dm-CLO) were transferred to breast milk very rapidly and that their concentrations in milk were significantly higher than their concentrations in maternal blood.

As a preliminary experiment, the NOAEL dose of CLO was administered to dams, and breast milk and blood samples were collected. As a result, the data variability was very large in the administered groups, so this study was conducted at 1/10 of the NOAEL (Table S3 and S4). Since no CLO or its metabolites were detected in the vehicle-treated control group, the so-called negative control was omitted for the purpose of this study.

The breast milk was collected at about 1 to 2 weeks after parturition, when milk secretion in mice is at its maximum (Hanrahan and Eisen 1970; Knight et al., 1986), in order to secure the milking volume. It has been reported that gentle finger massage around the mammary gland promotes milk evacuation and results in successful milking (Muranishi et al., 2016). Therefore, the milking tube part of the milking device (KN-591; Natsume Seisakusho, Tokyo) was modified to suction the entire teat using a 10 μL tip and silicone tube (Fig. 2). In addition, since milking without oxytocin was extremely difficult, oxytocin was injected just before milking to stimulate milk secretion. Oxytocin is a non-peptide hormone. Generally, oxytocin is released from the axon terminals of neurons in the posterior lobe of the pituitary

gland into the bloodstream when the stimulus of sucking the teats is transmitted to magnocellular neurons at the hypothalamic periventricular and supraoptic nuclei (Leng et al., 2015). The myoepithelial cells around the nipple contract, and the milk stored in the mammary gland is discharged into a primary duct beneath the nipple. Oxytocin also regulates other physiological functions, such as sexual behavior, feeding regulation, and thermoregulation (Baskerville and Douglas 2008; Leng et al. 2008; Yuan et al., 2020). It has been reported that medium- to long-term administration of oxytocin does not affect breast milk composition (Ballou et al., 2013; Nostrand et al., 1991), and in this experiment, oxytocin administration was incorporated into the milking procedure because the milk supply and confirmation of CLO transfer were of paramount importance.

CLO is known to be metabolized into several metabolites in the mammalian body (Ohno et al., 2020). In the present analysis, metabolites other than dm-CLO, the major metabolite of CLO, were below the detection limit, indicating that CLO is present in blood and breast milk mostly in the form of CLO or dm-CLO. In other words, CLO may be metabolized to CLO or dm-CLO in the blood and then transferred to breast milk, where it is excreted while maintaining the state of CLO or dm-CLO. In fact, Ford and Casida (2006) showed that CLO is immediately excreted in the urine in the form of CLO or dm-CLO, with a half-life of several tens of minutes. On the other hand, a previous study using a 10-times-higher dose (=NOAEL dose) of CLO than the current dose showed that when CLO was administered to mothers, CLO and CLO metabolites [dm-CLO, desmethyl-desnitro-CLO (dm-dn-CLO), dm-CLO-urea, dn-CLO, CLO-urea, and 1-methyl-3-nitroguanidine (MNG)] were detected in the samples (Ohno et al., 2020). However, dm-dn-CLO, dn-CLO, CLO-urea, and MNG were detected in much lower amounts than CLO and dm-CLO. Moreover, this experiment required a higher sample dilution rate to analyze breast milk, which has a higher lipid content. Therefore, the recovery rate of metabolites other than dm-CLO was as low as about 20%, suggesting that the metabolites, if present, may be too small to be detected in this experiment, which was conducted at 1/10 of the NOAEL dose. In other words, the CLO dosage, sample dilution rate, and recovery rates of metabolites differed from those in the method of Ohno et al. (2020), which was one reason why metabolites other than dm-CLO were not detected in our experiments, suggesting that other metabolites, although in small amounts, may in fact be present. As for the metabolite desnitro, which was not detected in this experiment, in the case of IMI and ACE, the binding of desnitro-IMI (dn-IMI) and desnitro-ACE (dn-ACE) to nAChR is reversed between insects and mammals, resulting in higher toxicity in mammals. In addition, the nitroguanidine derivatives of IMI, TMX, CLO, and DIN bind more strongly to α4β2 nAChRs than to Drosophila nAChRs (Kanne et al., 2005), which suggests that some metabolites of CLO may be more toxic to mammals. Furthermore, CLO is also a partial agonist of mammalian neuronal α7 nAChRs and has high affinity for them (Cartereau et al., 2018; Xiang et al., 2020), raising concerns about α7 nAChRs-mediated effects on

neurodevelopment, the immune system, and the reproductive system. In short, if CLO is present in breast milk in the form of a metabolite such as desnitro, the adverse effects on infants who ingest it are likely to be greater than the adverse effects in those who ingest the parent compound.

In the present study, CLO concentrations in both the breast milk and blood reached their highest levels at 1 h after the administration, were halved or lower at 3 h, and were below the detection limits at 12 and 24 h. The concentration of dm-CLO in the milk reached its highest level at 3 h after the administration and in the blood at 1 h after the administration, was halved at 6 h, and was at trace levels or below the detection limit at 12 and 24 h. In other words, the CLO concentrations in breast milk and blood peaked at or before 1 h after the administration and were halved at 3 h, and most of the CLO was excreted from the body within 24 h. The dm-CLO concentrations in the milk peaked at 3 h and those in the blood peaked at 1 h after the administration, and the dm-CLO concentrations in both milk and blood were halved at 6 h and mostly excreted within 24 h. Regarding the hemodynamics of CLO, previous studies have shown that the CLO blood concentration decreased with time from 1 h after administration and the dm-CLO blood concentration increased from 1 to 3 h after administration (Ohno et al., 2020), which is consistent with these findings.

In our present experiments, it was shown that the CLO and dm-CLO concentrations were significantly higher in the breast milk than in the blood. In regard to nicotine, nicotine concentrations have been shown to be higher in breast milk than in blood. This is mainly because nicotine, when administered via drinking water, is distributed into breast milk, which contains high lipid content and is more acidic (Matta et al., 2007). Although the cause of the high CLO concentrations in breast milk is not clear, the fact that both CLO and dm-CLO concentrations were higher in the breast milk than in the blood is an important new finding, suggesting that CLO may be accumulated or concentrated during the production of breast milk. This was also confirmed in our present experiments by measuring the ratio of CLO concentration in the breast milk to CLO concentration in the blood; the results were shown as a scatter plot for each time point after administration, and indicated that the tendency for CLO concentration to increase in the breast milk does not change with time. Moreover, the kinetics of CLO and dm-CLO in the milk and blood were very similar, suggesting that the metabolism and accumulation of CLO in the blood and milk are comparable. For dm-CLO only, the time of maximum blood concentration varied between 1 and 3 h after the administration, which may have been due to individual differences in the metabolic rate of each animal.

In a previous study, it was reported that neurogenesis in the hippocampal dentate gyrus was impaired in mice exposed to ACE and IMI at the postnatal age of 12-26 days (Nakayama et al., 2019), suggesting that postnatal exposure to NN impairs neurodevelopment. It was also found that urinary detection and concentration of N-desmethylacetamiprid (DMAP), a metabolite of ACE, were higher in low-birth-

weight infants (SGA) than in normal infants (AGA) (Ichikawa et al., 2019), suggesting that NN exposure via breast milk may affect neonatal neurodevelopment. In the present study, the CLO and dm-CLO concentrations were higher in the breast milk than in the blood. This suggests that CLO exposure per body weight differs between mothers and infants with different sensitivities, and that we should pay attention to NN exposure in fetuses and infants, since both are vulnerable to chemicals. In humans, infants are more sensitive to drugs due to the immaturity of their enzyme and drug systems, and differences in the number and affinity of drug receptors, so that they cannot absorb and metabolize drugs in the same way as adults. In fact, neonates have a longer gastric emptying time, and immature pancreatic and biliary functions, bacterial flora, and transport-enzyme activities, all of which are thought to affect drug absorption. In addition, CYPs, which are necessary enzymes for liver metabolism, and renal functions, such as tubular secretion and glomerular filtration, are immature during the first months of life and mature to adult levels within a few months (Matalova, et al., 2016). These facts indicate that the half-life of drugs is longer in neonates, and drugs in breast milk may accumulate and reach high concentrations in infants. In the past, simulations of chemical exposure to lactating children have shown that the half-life is the most important factor in determining the infant's systemic exposure to chemicals via breast milk (Verner et al., 2017). This means that the half-lives of CLOs and their metabolites obtained in this study are longer in infants, and the impairment of infants' health due to their accumulation is of concern.

5. Conclusion

The present study is the first report to demonstrate that CLO is metabolized maternally in mice and is transferred and concentrated in breast milk very rapidly. CLO and its metabolites were found to be more concentrated in the milk than in the blood, and most of them were eliminated from the mother's body within 24 h. Since the sensitivity of infants to chemicals is greater than that of their mothers, the developmentally adverse effects of CLO on infants are a matter of concern. In the future, it will be necessary to study the exposure of infants to CLOs through breast milk in more detail.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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FIGURE LEGENDS

Fig. 1 Experimental schedule

CLO was orally administered to ICR mice on postpartum day 10 or 11 (n=13). Breast milk and blood samples were collected at 1, 3, 6, 9, 12, and 24 h after the administration.

Fig. 2 Milking device

(A) The milking device was a one-handed milking device for mice and rats (KN-591; Natsume Seisakusho, Tokyo), which consists of a pressure adjustment pipe, sampling tube, exhaust pipe, and milking pipe. The milking pipe was replaced with the straight part of a 10 μL tip, a pipette connecting part of a 10 μL tip (110-207C; WATSON Co., Tokyo), and a silicon tube (10 mm long, 4 mm in outer diameter, 2 mm in inner diameter). The exhaust pipe was connected to an aspirator and milk was collected from a 10 μL tip in contact with the teat into a sampling tube. The milk collection method was based on that in a previous study (Kawakami et al., 2015).

(B) The 10 μL tip was approximately 20 mm in length at the pipette-connecting part and at the straight part. The colors of the parts in (A) correspond to those in (B).

Fig. 3 CLO and dm-CLO concentrations in breast milk and blood

(A) The CLO concentration in milk was highest at 1 h after the administration and was gradually decreased at 3, 6, and 9 h. The blood CLO concentration was highest at 1 h after the administration and was gradually decreased at 3, 6, and 9 h. The CLO concentration in the milk and blood was below the detection limit at 12 and 24 h. CLO concentrations in the milk were significantly higher than those in the blood at 1 and 3 h after the administration. The values are expressed as mean ± SE. The numbers of samples were as follows: 1 h (milk, blood = 9), 3 h (milk, blood = 9), 6 h (milk = 4, blood = 6), 9 h (milk = 7, blood = 9), 12 h (milk = 5, blood = 6), and 24 h (milk = 3, blood = 12). $^{**}P < 0.01$.

(B) The milk dm-CLO concentrations were highest at 3 h after the administration and were gradually decreased at 6, 9, and 12 h. Dm-CLO concentrations in the blood were highest at 1 h or 3 h after the administration and were gradually decreased at 6 and 9 h after the administration. The milk dm-CLO concentrations were below the detection limit at 24 h and the blood dm-CLO concentrations were below the detection limit at 12 and 24 h after the administration. The concentrations of dm-CLO in the milk were significantly higher than those of dm-CLO in the blood at 1, 3, 6, and 9 h after the administration. The values are expressed as mean ± SE. The numbers of samples were as follows: 1 h (milk, blood = 9), 3 h (milk, blood = 9), 6 h (milk = 5, blood = 6), 9 h (milk = 7, blood = 9), 12 h (milk = 5, blood = 6), and 24 h (milk = 5, blood = 12); $^{*}P < 0.05$, $^{**}P < 0.01$.

Fig. 4 Ratio of CLO and dm-CLO concentrations in the breast milk to those in the blood

(A) The distribution of CLO concentrations in the milk relative to CLO concentrations in the blood showed that CLO concentrations in the milk were higher. The numbers of samples were as follows: 1 h = 9, 3 h = 9, 6 h = 4, and 9 h =4.

(B) The distribution of dm-CLO concentrations in the milk relative to dm-CLO concentrations in the blood showed that dm-CLO concentrations in the milk were higher. The numbers of samples were as follows: 1 h = 9, 3 h = 9, 6 h = 5, and 9 h = 7.

Fig. S1 Changes in CLO and dm-CLO concentrations in the breast milk and blood

In the milk, the highest CLO concentration (2,663 ± 191 ng/mL) was observed at 1 h after the administration, and the highest dm-CLO concentration (1,084 ± 126 ng/mL) was observed at 3 h after the administration. In the blood, the CLO concentration was highest at 1 h after the administration (1,656 ± 225 ng/mL), and the dm-CLO concentration was highest at 1 h after the administration (397 ± 73 ng/mL). The values are expressed as mean ± SE.

Fig. 1

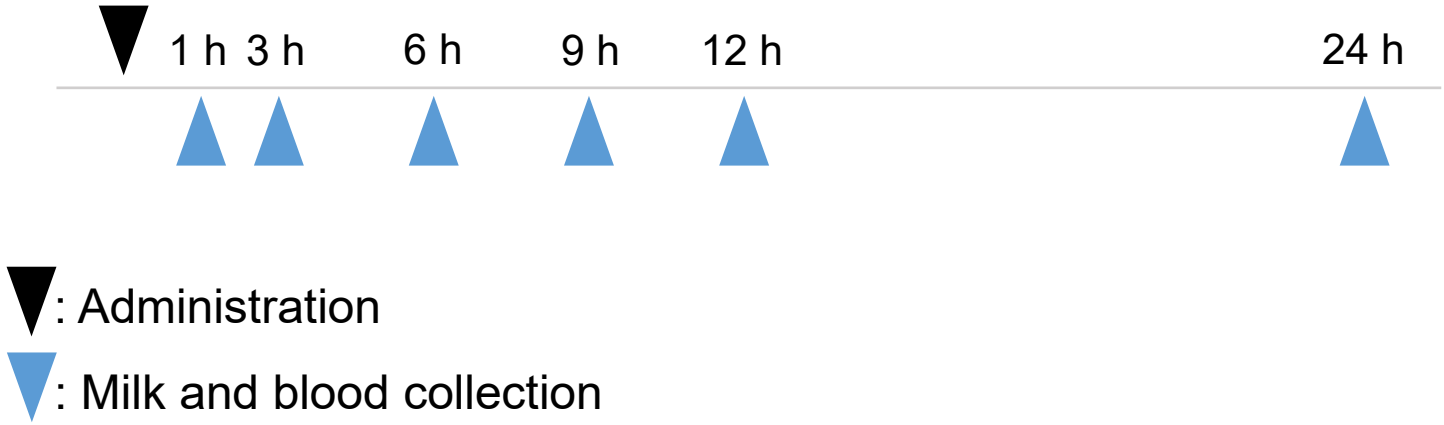

1 h
3 h
6 h
9 h
12 h
24 h
: Administration
: Milk and blood collection

Fig. 2

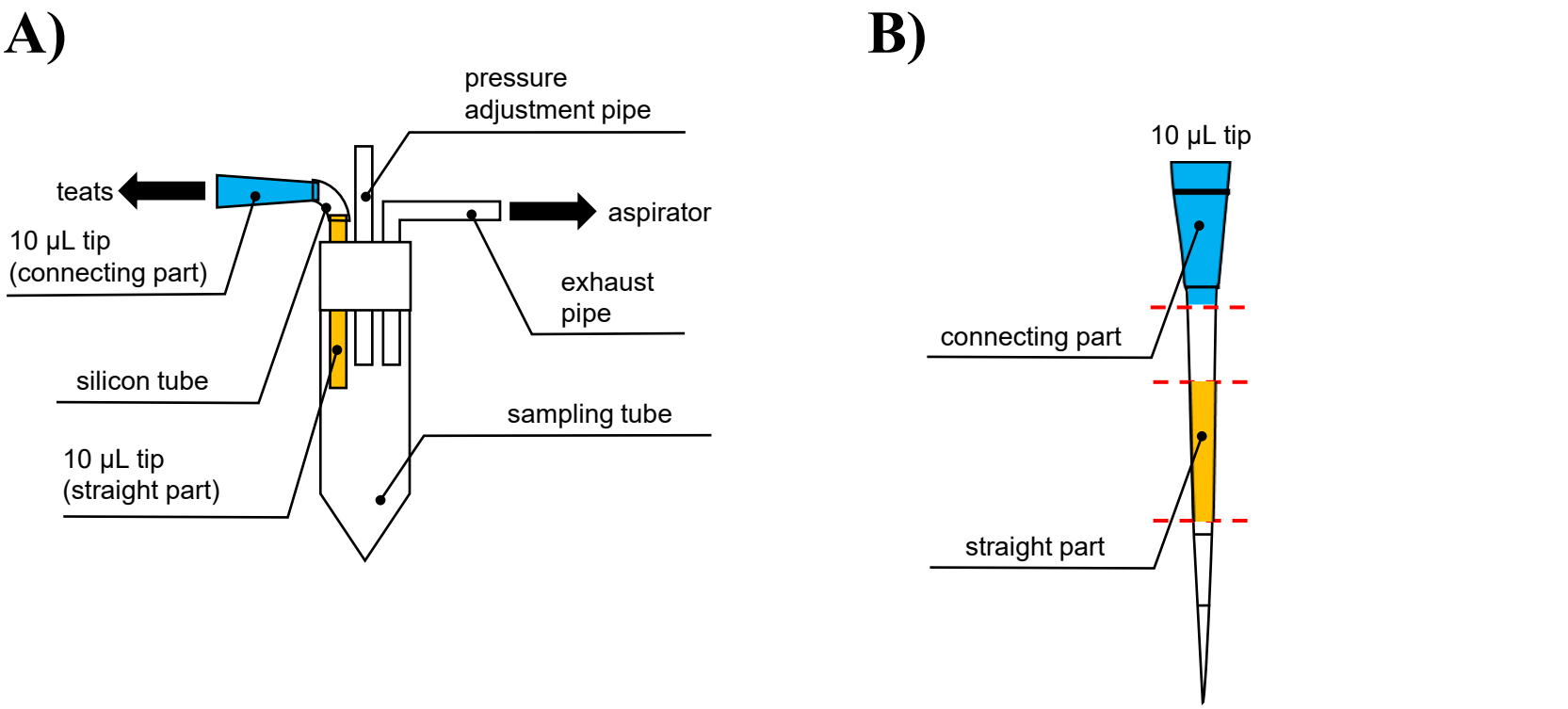
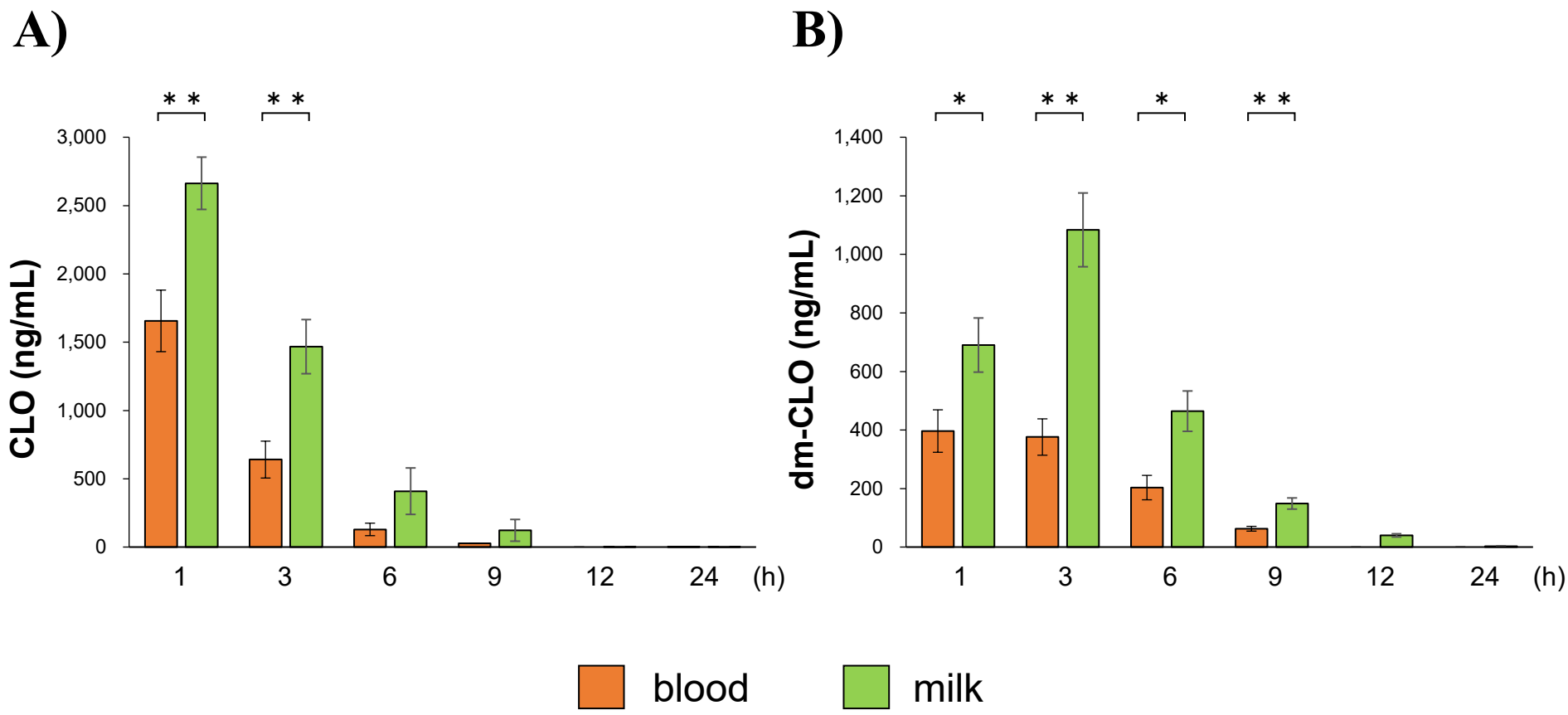

A)
pressure
adjustment pipe
teats
aspirator
10 µL tip
(connecting part)
exhaust
pipe
silicon tube
sampling tube
10 µL tip
(straight part)
B)
10 µL tip
connecting part
straight part

Fig. 3


A)
B)
CLO (ng/mL)
dm-CLO (ng/mL)
3,000
2,500
2,000
1,500
1,000
500
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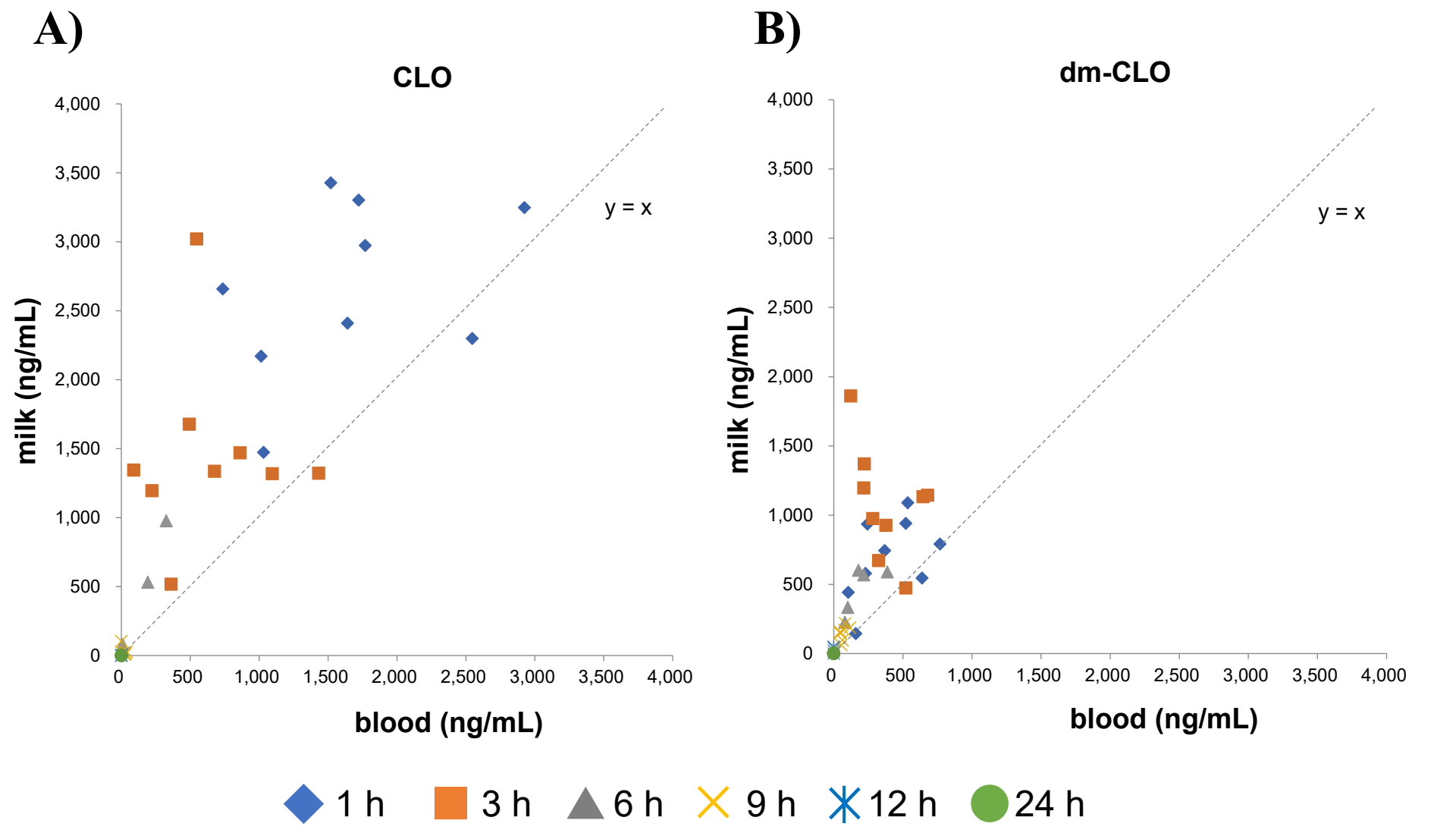
1,200
1,000
800
600
400
200
0
* *
*
1
3
6
9
12
24
(h)
blood
milk

Fig. 4


A)
CLO
milk (ng/mL)
blood (ng/mL)
y = x
0
500
1,000
1,500
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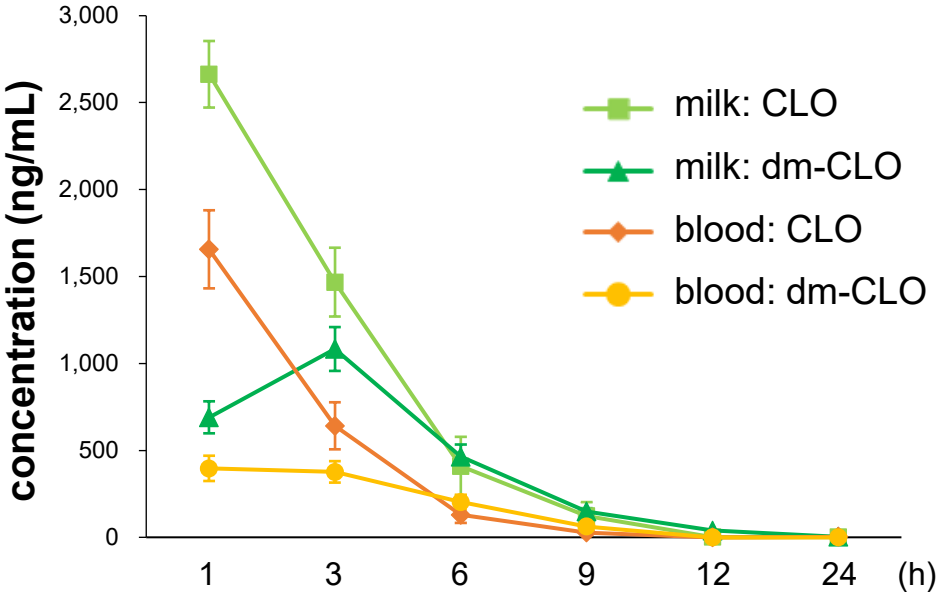
4,000
B)
dm-CLO
milk (ng/mL)
blood (ng/mL)
y = x
1 h
3 h
6 h
9 h
12 h
24 h

Fig. S1


3,000
2,500
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concentration (ng/mL)
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9
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24
(h)
milk: CLO
milk: dm-CLO
blood: CLO
blood: dm-CLO

Table 1 CLO concentration in the maternal milk and blood

dam	1 h	3 h	6 h	9 h	12 h	24 h
1	3428.8	1678.8	—	—	—	—
	1519.6	492.7	74.5	—	—	< LOD
2	2299.7	1318.6	—	—	—	—
	2546.1	1095.8	—	23.2	—	< LOD
3	3249.2	1471.2	532.9	—	—	—
	2924.7	861.2	192.7	—	—	—
4	1474.5	1322.7	977	668.9	—	—
	1032.4	1431.3	326.3	—	—	< LOD
5	2974.6	1336	—	—	—	—
	1769.8	676.4	166.2	19.2	—	< LOD
6	2170.8	1195.6	—	—	—	—
	1014	222.5	—	< LOD	—	< LOD
7	2409.9	1344.9	82.5	103.9	< LOD	—
	1640.5	89.6	9.9	< LOD	< LOD	< LOD
8	3302.3	518	44.9	25.5	—	—
	1722.2	360.5	9.3	< LOD	< LOD	< LOD
9	2659.3	3022.2	—	—	—	—
	736.6	545.3	—	—	—	< LOD
10	—	—	—	28.9	< LOD	< LOD
	—	—	—	38.4	< LOD	< LOD
11	—	—	—	10.3	< LOD	—
	—	—	—	27.2	< LOD	< LOD
12	—	—	—	18	< LOD	< LOD
	—	—	—	32.3	< LOD	< LOD
13	—	—	—	7.6	< LOD	< LOD
	—	—	—	26.8	< LOD	< LOD

— : Not examined or samples not taken; LOD: Limit of detection;

Upper cell: Breast milk; Lower cell: Maternal blood (ng/mL).

Table 2 dm-CLO concentration in the maternal milk and blood

dam	1 h	3 h	6 h	9 h	12 h	24 h
1	935.2	975.6	—	—	—	—
	243.1	282.7	253.3	—	—	< LOD
2	546.3	1144.5	—	—	—	—
	638.6	678.3	—	55.7	—	< LOD
3	790.9	1133.5	590.6	—	—	—
	768.6	646.2	388.1	—	—	—
4	143.7	473.6	568.5	—	—	—
	158.5	520.6	216.4	—	—	< LOD
5	744.5	925.7	602.5	62.1	—	—
	368.6	376.7	179.4	54	—	< LOD
6	580	1370.7	—	—	—	—
	230.1	219.8	—	29.3	—	< LOD
7	940.2	1860.9	334	155.9	< LOD	< LOD
	521.3	122	101.8	47.6	< LOD	< LOD
8	1089.8	672.2	227.2	148.4	—	—
	535.7	324	80.5	44.2	< LOD	< LOD
9	443	1196.6	—	—	—	< LOD
	104.6	217.2	—	—	—	< LOD
10	—	—	—	187.8	49.7	< LOD
	—	—	—	117.6	< LOD	< LOD
11	—	—	—	173	48	—
	—	—	—	74.8	< LOD	< LOD
12	—	—	—	218.7	40.3	< LOD
	—	—	—	81.5	< LOD	< LOD
13	—	—	—	96.6	22.2	< LOD
	—	—	—	62.6	< LOD	< LOD

— : Not examined or samples not taken; LOD: Limit of detection;

Upper cell: Breast milk; Lower cell: Maternal blood (ng/mL).

Table S1 CLO ratio of the maternal milk vs. blood

dam	1 h	3 h	6 h	9 h	12 h	24 h
1	2.3	3.4	—	—	—	—
2	0.9	1.2	—	—	—	—
3	1.1	1.7	2.8	—	—	—
4	1.4	0.9	3.0	—	—	—
5	1.7	2.0	—	—	—	—
6	2.1	5.4	—	—	—	—
7	1.5	15.0	8.3	—	—	—
8	1.9	1.4	4.8	—	—	—
9	3.6	5.5	—	—	—	—
10	—	—	—	0.8	—	—
11	—	—	—	0.4	—	—
12	—	—	—	0.6	—	—
13	—	—	—	0.3	—	—

— : Not examined or samples not taken

Table S2. dm-CLO ratio of the maternal milk vs. blood

dam	1 h	3 h	6 h	9 h	12 h	24 h
1	3.8	3.5	—	—	—	—
2	0.9	1.7	—	—	—	—
3	1.0	1.8	1.5	—	—	—
4	0.9	0.9	2.6	—	—	—
5	2.0	2.5	3.4	1.2	—	—
6	2.5	6.2	—	—	—	—
7	1.8	15.3	3.3	3.3	—	—
8	2.0	2.1	2.8	3.4	—	—
9	4.2	5.5	—	—	—	—
10	—	—	—	1.6	—	—
11	—	—	—	2.3	—	—
12	—	—	—	2.7	—	—
13	—	—	—	1.5	—	—

— : Not examined or samples not taken

Table S3 CLO concentration in the maternal milk and blood after administration of NOAEL doses of CLO

dam	1 h	6 h	12 h	24 h
CLO-1	31919.6	5517.0	151.6	2069.7
	10922.3	< LOD	—	< LOD
CLO-2	35045.3	1247.3	< LOD	< LOD
	9353.6	507.3	< LOD	< LOD
CLO-3	44963.2	2644.0	< LOD	< LOD
	13900.1	700.5	< LOD	< LOD
CLO-4	147255.7	26980.2	2969.8	< LOD
	9054.8	7439.3	753.2	< LOD
CLO-5	49791.8	4493.3	59.7	< LOD
	14700.3	1562.3	31.5	< LOD
Ctrl-1	< LOD	< LOD	< LOD	—
	< LOD	< LOD	< LOD	< LOD
Ctrl-2	< LOD	< LOD	—	—
	< LOD	< LOD	—	< LOD
Ctrl-3	< LOD	< LOD	—	—
	< LOD	< LOD	—	< LOD
Ctrl-4	< LOD	< LOD	< LOD	< LOD
	< LOD	< LOD	< LOD	< LOD
Ctrl-5	< LOD	< LOD	< LOD	—
	< LOD	< LOD	< LOD	< LOD

－ : Not examined or samples not taken; LOD: Limit of detection;

Upper cell: Breast milk; Lower cell: Maternal blood (ng/mL).

Table S4 dm-CLO concentration in the maternal milk and blood after administration of NOAEL doses of CLO

dam	1 h	6 h	12 h	24 h
CLO-1	20273.8	13545.0	4267.6	1487.7
	5956.0	855.7	—	< LOD
CLO-2	35611.8	3857.2	605.1	< LOD
	9736.3	1989.7	< LOD	< LOD
CLO-3	30322.4	16262.8	1608.0	< LOD
	10954.7	5136.3	761.0	< LOD
CLO-4	157056.6	37224.2	10363.5	186.8
	7731.8	10767.5	2869.4	< LOD
CLO-5	44303.9	15088.1	3007.8	< LOD
	13115.4	< LOD	934.2	< LOD
Ctrl-1	< LOD	< LOD	< LOD	—
	< LOD	< LOD	< LOD	< LOD
Ctrl-2	< LOD	< LOD	—	—
	< LOD	< LOD	—	< LOD
Ctrl-3	< LOD	< LOD	—	—
	< LOD	< LOD	—	< LOD
Ctrl-4	< LOD	< LOD	< LOD	< LOD
	< LOD	< LOD	< LOD	< LOD
Ctrl-5	< LOD	< LOD	< LOD	—
	< LOD	< LOD	< LOD	< LOD

— : Not examined or samples not taken; LOD: Limit of detection;

Upper cell: Breast milk; Lower cell: Maternal blood (ng/mL).