Effects of *in utero* and lactational exposure to the no-observed-adverse-effect level (NOAEL) dose of the neonicotinoid clothianidin on the reproductive organs of female mice

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Running head: CLO AFFECTS F1 FEMALE REPRODUCTION

**ABSTRACT**

Recently, developmental exposure to clothianidin (CLO) has been shown to cause reproductive toxicity in male mice, but the effects in female mice remain to be clarified. Pregnant C57BL/6N mice were given a no-observed-adverse-effect-level (NOAEL) dose of CLO until weaning. We then examined ovaries of 3- or 10-week-old female offspring. In the CLO-administered group, morphological changes, a decrease in the immunoreactivity of the antioxidant enzyme glutathione peroxidase 4 (GPx4), and activation of genes in the steroid hormone biosynthesis pathway were observed in 3-week-old mice, and decreases of GPx4 immunoreactivity, 17OH-progesterone and corticosterone levels were observed in 10-week-old mice, along with high rates of infanticide and severe neglect, providing new evidence that developmental exposure to CLO affects juvenile and adult mice differently.

**KEY WORDS**: clothianidin, developmental stage, DOHaD (developmental origins of health and diseases), female mice, *in utero* and lactational exposure
Neonicotinoid pesticides (NNs) were developed in the 1980s and are chemically similar to nicotine. NNs exhibit insecticidal effects by binding to the nicotinic acetylcholine receptors (nAChRs) of insects and triggering a response in neuronal cells [3, 24, 25]. NNs bind more readily to insect nAChRs than to mammalian nAChRs [38]. However, recent studies have reported reproductive toxicity [15] and neurobehavioral effects [16, 34, 35, 45] in mammals. In terms of reproductive toxicity, it has been shown that the NN clothianidin (CLO), causes the DNA fragmentation of germ cells and the inhibition of embryonic development in mature quail [18, 19, 37]. Another NN, imidaclopid, has been reported to cause significant changes in ovarian morphology and in follicle stimulating hormone (FSH), luteinizing hormone (LH), and progesterone levels in female rats [21] and to decrease testosterone levels in male rats [12]. We recently reported that CLO passes rapidly through the placental barrier in mice [27]. It has also been shown that in utero and lactational exposure to CLO decrease the numbers of germ cells in male mice [44]. Fetuses are vulnerable to chemicals, and there is concern about the effect of prenatal exposure on the health of future generations. In fact, developmental exposure to NNs induces abnormalities in behavior [4, 31, 36] and delayed sexual maturation [32] in mice. However, there are currently few animal research models for elucidating the role of prenatal and perinatal exposure to NNs in the development of diseases in adult females.

Oxidative stress is an imbalance between the oxidation and antioxidation reactions in the body, resulting in an oxidative state, and oxidative damage causes base damage, as well as strand breaks in DNA, and is thought to be one of the mechanisms by which NNs induce cytotoxicity [8]. In other studies, the oxidative stress of NNs was also found to cause cytoplasmic clumping of follicles [21] and DNA fragmentation of sperm in the reproductive systems of mammals [15] and birds [19, 37].

Hence, in utero and lactational exposure to NNs can have effects on ovarian development through oxidative stress, but it is currently not known how NNs affect the female mammalian genital organs in the next generation. Therefore, we investigated the effects of no-observed-adverse-effect-level (NOAEL) NN exposure during the embryonic and juvenile stages on developmental plasticity and female reproductive potential in the adult stage.

C57BL/6NCrSlc pregnant mice were purchased from Japan SLC (Hamamatsu, Japan) and maintained as described elsewhere [27]. This study was approved by the Institutional Animal Care and Use Committee (Permission #26-05-07) and carried out according to the Kobe University Animal Experimental Regulations. The dams were divided into two groups: a CLO-0 group administered 0 mg CLO/kg body weight/day (18 dams and 36 pups) and a CLO-65 group administered 65 mg CLO/kg body weight/day (17 dams and 33 pups). The administration concentration was set with reference to the NOAEL (ICR female mice: 65.1 mg CLO/kg/day [10, 39]). To eliminate the risk of adverse effects associated with gavage, the dams were given soft gel (MediGel® Sucrarose; ClearH2O, Portland, ME, U.S.A.) with and without CLO (extracted from Dantotsu®; Sumitomo Chemical Co., Tokyo, Japan, [15]) as a substitute for filtered water from gestational day 1.5 to postnatal day (PND) 21. Dams ingested the gel actively, and the CLO concentration of 65 mg/kg/day did not affect the amount of gel intake. Each litter was randomly culled to a maximum of six pups on PND 2 to standardize the amount
of milk, and litters of three pups or fewer were removed from the experiment for the same reason. One or two pups per litter were used in order to avoid a litter bias. The amount of isolated CLO added to the aqueous solution was calculated as follows: the CLO purity (95%), daily gel intake and average body weight (6 g/day and 22 g for early pregnancy, 6.5 g/day and 30 g for late pregnancy, 16 g/day and 29 g for the first week of lactation, and 20 g/day for the second and third weeks of lactation), total gel weight (60 g: excluding the cup weight). The calculated amount of CLO was dissolved in 600 µl of DMSO (1% volume of gel) and injected into the gel cups, and which were then shaken vigorously to mix the CLO evenly in the gel.

On PND 21 of age of the offspring, blood was collected from the dams and the pups under anesthesia with isoflurane. After the animals were euthanized, the ovaries and blood were collected. The right ovaries were weighed and fixed in 4% paraformaldehyde in phosphate buffer at 4°C for 3 hr or 6 hr, respectively. They were then dehydrated through a graded series of ethanol followed by xylene and embedded in paraffin. The left ovaries were placed in a mixture of Buffer RA1 and TCEP (NucleoSpin plus RNA isolation kit), frozen with liquid nitrogen, and stored in a −80°C freezer. Samples were collected from the 10-week-old mice in the same way, and some of the F1 animals were mated within the group when they reached 10 weeks of age and evaluated for fertility and parental care.

The tissue samples were sectioned at 4-µm thickness by a sliding microtome (SM2000R; Leica Microsystems, Wetzlar, Hesse, Germany), and mounted on a glass slide precoated with 0.2% 3-aminopropyltriethoxysilane (Shin-Etsu Chemical Co., Tokyo, Japan). To detect glutathione peroxidase 4 (GPx4) and manganese superoxide dismutase (MnSOD) immunoreactivity in the ovary, we used the following antibodies: rabbit monoclonal anti-GPx4 (1:50; ab125066; Abcam, Cambridge, UK) and rabbit monoclonal anti-MnSOD (1:300; ab68155; Abcam). The immunoreactivities of the primary antibodies were examined with 3,3-diaminobenzidine tetrahydrochloride solution (EnVision®+ kit/HRP [DAB], Dako, Glostrup, Denmark). Immunostaining of all sections was performed as previously described [28].

For the analysis of steroid hormones (17OH-progesterone and Corticosterone), LC-ESI/MS/MS was used as described elsewhere [46]. In brief, Steroid hormones and its deuterated or 13C labeled internal standard were detected with multiple reaction monitoring (MRM; 331.2 > 109.1 and 334.0 > 100.2 for 17OH-progesterone and 17OH-progesterone-13C3, and 347.2 > 121.2 and 351.4 > 121.2 for Corticosterone and Corticosterone D4) in a positive ion mode using a 6495 B Triple Quadrupole LC-MS/MS system from Agilent Technologies (Santa Clara, CA, U.S.A.). Chromatography separation was achieved by using Kinetex® Biphenyl column (1.7 µm, 150 × 2.1 mm; Phenomenex, Torrance, CA, U.S.A.) and a gradient elution as a flow rate of 0.3 ml/min with the mobile phase A as 0.2 mmol/L NH₄F containing distilled water, and B as 0.2 mmol/L NH₄F containing Acetonitrile. The gradient program was 0-0.5 min 50% B (isocratic), 0.5-10 min 95% B (gradient), and 10-12 min 95% B (isocratic).

Total RNA of the left ovaries of 3- and 10-week-old mice was extracted using a NucleoSpin XS
RNA isolation kit (Macherey-Nagel GmbH & Co., Düren, Germany). The effects of CLO on the gene expression profiles in the ovaries of 3-week-old mice were analyzed by the GeneChip system and Clariom S mouse array (Affymetrix, Santa Clara, CA, U.S.A.) as described elsewhere [14]. The raw intensity data were normalized using GeneSpring GX 14.9 software (Agilent Technologies) and analyzed using Ingenuity Pathways Analysis (IPA) tools (Ingenuity Systems, Mountain View, CA, USA). The microarray data (.CEL files) were deposited in a public database (Gene Expression Omnibus, accession number: GSE164099).

The changes in gene expression were measured by the quantitative reverse transcription polymerase chain reaction (qRT-PCR) as described previously [14] using specific primers (Supplementary Table 1) on a StepOnePlus (Life Technologies Japan Inc., Tokyo, Japan). The thermal cycling consisted of initial degeneration of 95°C for 30 sec, followed by 40 cycles of denaturing at 95°C for 5 sec and elongation at 60°C for 30 sec. The copy numbers of genes were calculated using standard curves, and normalized with the housekeeping genes β-actin (Actb). All samples were measured in duplicate and the specificity of the PCR products was confirmed by melting curves.

Statistical analysis was performed with Excel statistics 2012 (Version 1.00; SSRI, Tokyo, Japan). Organ weights and hormone data were analyzed by two-way ANOVA followed by Tukey-Kramer post hoc test. Body weights, the number of pups, sex ratio and qRT-PCR data were analyzed by Welch’s t-test, and postnatal days to vaginal opening data were analyzed by Mann-Whitney U-test. Infanticide and neglect data were analyzed by chi-square test. The results were considered significant when the P-value was less than 0.05.

In the 3-week-old mice, the ovary weight (Fig. 1B) and size (Fig. 1C and 1D) of the CLO-65 group were clearly lower than those of the CLO-0 group. There was no significant effect of CLO on the sex ratio of the pups or postnatal days to vaginal opening, but there was a trend toward smaller litter size in the CLO-65 group (P = 0.064) (Supplementary Table 2), and no significant effect of CLO on the body weights of all groups, or ovary weights of 10-week-old mice (Fig. 1A and 1B). The general histological analyses of ovaries by HE staining revealed a clear increase in the intercellular spaces between granulosa cells in the 3-week-old CLO-65 group, but there were no changes in the number of follicles or their composition in either the 3- or 10-week-old CLO-65 group compared to the respective control group (Fig. 1E and 1F, Supplementary Fig. 1A and 1B). Yanai et al. [44] reported that prenatal and postnatal exposure to CLO decreased the testicular weights and number of germ cells in childhood, but in our study there was no significant effect of CLO on the stages of follicle development or the constitution of follicles. Therefore, exposure to CLO may affect cells other than follicles in the fetal ovary.

The results of the immunohistochemical analyses of the ovary visualizing GPx4 and MnSOD are shown in Fig. 2. We assessed the intensity of GPx4 and MnSOD immunoreactivity as an index of oxidative stress in the ovary. Some degree of GPx4 immunoreactivity was observed in the nucleus and cytoplasm of oocytes, granulosa cells, stromal cells, and theca cells in all groups, as well as in the lutein cells of 10-week-old mice (Fig. 2A–D). The intensity of GPx4 immunoreactivity in the
granulosa cells, theca cells and lutein cells was decreased by CLO. Some degree of MnSOD immunoreactivity was observed in the cytoplasm of oocytes, granulosa cells, stromal cells, and theca cells in all groups, as well as in the lutein cells of the 10-week-old mice (Fig. 2E–H). There was no effect of CLO on the intensity in any of the groups. Several previous studies have demonstrated that NNs induce oxidative stress [8, 21], which reflects an imbalance between reactive oxygen species (ROS) and antioxidants and can cause cell death. GPx is an antioxidant enzyme that typically uses glutathione (GSH) as a reductant [20]. SOD is an antioxidant enzyme, and MnSOD, a type of SOD, is found in the mitochondria. We previously reported that subchronic administration of CLO caused a decrease in GPx4 and MnSOD in mature quail [37]. In addition, it has been shown that GPx4 uses GSH and that substitution of thiazole ring chlorine by GSH is one of the major metabolic pathways of CLO [20, 39]. Therefore, our present results suggest that GSH may be consumed by CLO administration, and GPx4 activity may also be decreased due to the decrease in GSH. Since GPx4 is involved in the major metabolic pathway of CLO, it is possible that GPx4 is more susceptible to CLO administration than MnSOD.

17-Hydroxyprogesterone (17-OH progesterone) and corticosterone were detected in the blood of pups (Table 1). There was a significant main effect of CLO and age with a significant interaction on 17-OH progesterone \[F(1, 45) = 16.12, P < 0.001; F(1, 45) = 6.391, P < 0.05; F(1, 45) = 4.837, P < 0.05\]. There was also a significant main effect of CLO and age with a significant interaction on corticosterone \[F(1, 45) = 8.900, P < 0.005; F(1, 45) = 12.01, P < 0.005; F(1, 45) = 4.450, P < 0.05\]. Both hormones were significantly decreased in the 10-week-old CLO-65 group compared to the CLO-0 group. These results showed that in utero and lactational exposure to NNs decreased the levels of hormones related to estrogen and progesterone in adults. A previous study revealed that prenatal and neonatal nicotine exposure changes serum progesterone and estradiol levels in 6-month-old female rats, despite inducing no changes in 4-month-old female rats, suggesting that nicotine disrupts the hypothalamic-pituitary-ovarian function, resulting in ovarian reprogramming and age-related reduced fertility [17]. However, there was no obvious histological effect of CLO on 10-week-old ovaries in our study, which may be because 17OH-progesterone and corticosterone mainly affected the adrenal glands. 17OH-progesterone is produced by the adrenal glands and gonads, and corticosterone by the adrenal glands. NNs have been reported to induce functional impairment in the hypothalamic-pituitary-adrenal axis in rats [1], and to increase adrenaline secretion in PC12D cells [22]. These findings of prenatal effects manifesting in adulthood led to the hypothesis of the developmental origins of health and diseases (DOHaD), and it is assumed that the same thing happened with CLO, which binds to nAChR as well as nicotine.

Infanticide (n = 2) and severe neglect (n = 1) were observed in the CLO-65 group of the F1 generation, but these effects were not among the pregnancy outcomes in any groups of the F0 generation or in the CLO-0 group of the F1 animals (Fig. 3). Common laboratory mouse strains have been selected for low maternal aggression to increase breeding success, and thus infanticidal mothers are rarely observed under normal husbandry conditions [23, 42]. A previous study reported that in
uterine and lactational exposures to bisphenol S cause moderate neglect, such as poor cleaning of pups as a pregnancy outcome in the F0 generation, and infanticide and severe neglect as pregnancy outcomes in the F1 generation [7]. Another study reported that environmental tobacco smoke decreases oxytocin in the plasma of rats during the lactation period [26]. Oxytocin binds to RAGE (receptor for advanced glycation end-products) and crosses the blood-brain barrier, and it has been reported that RAGE-knockout mice fail to nurture their pups [43]. Moreover, Sairenji et al. [30] revealed that maternal prolactin could be a key factor for generating nurturing behavior in offspring by activating neural circuits required for the expression of nurturing behaviors. Thus, the effects observed in the F1 animals in our study may have been due to their direct exposure to CLO, poor care from mothers with low oxytocin and prolactin levels, or a combination of these factors.

We identified 99 genes (62 up-regulated and 37 down-regulated) that showed 1.5-fold or greater differential expression between the CLO-65 group and CLO-0 group. The top 20 biological functions related to the up-regulated genes are summarized in Table 2. CLO increased the expression of genes associated with reproductive functions with annotations of “Quantity of gonadal cells”, “Quantity of germ cells”, “Quantity of gonads” and “Quantity of ovarian follicles”. The top 10 canonical pathways that were differentially activated or suppressed by the up-regulated genes are shown in Fig. 4A. The affected pathways included those for estrogen-dependent breast cancer signaling, estrogen biosynthesis and GnRH signaling relating to steroid hormone biosynthesis. Networks describing the relationships between a subset of genes and their neighboring genes are presented in Fig. 4B. Canonical pathways associated with the steroid hormone biosynthesis pathway were identified, but it was not clear whether these pathways were activated or suppressed. However, gene sets related to “Quantity of gonadal cells”, “Quantity of germ cells”, “Quantity of gonads” and “Quantity of ovarian follicles” were significantly enriched in the up-regulated genes, suggesting that steroid hormone biosynthesis pathways were activated. Wakabayashi et al. [41] revealed that estrogen administration caused a decrease in ovarian weight and a reduction in ovarian size in rats, but did not change the number of follicles, leading them to conclude that estrogen suppressed the secretion of gonadotropins from the pituitary gland. Hence, the present study suggests that CLO may have activated the estrogen biosynthetic pathway in prepubertal mice, causing a decrease in ovarian weight and size reduction at 3 weeks of age.

We chose four genes that were found to be significant in the network analyses—i.e., cytochrome P450 family 19 subfamily A member 1 (Cyp19a1), inhibin subunit beta A (InhA), progesterone receptor (Pgr) and aldo-keto reductase family 1, member B7 (Akr1b7)—for independent verification by qRT-PCR. We found that the expressions of InhA, Pgr and Akr1b7 were significantly activated, and that the activation of Cyp19a1 expression approached significance (Cyp19a1: \( P = 0.074 \); Fig. 5A) in the 3-week-old CLO-65 group, matching the pattern of the microarray results. In 10-week-old mice, there was a downward trend in the gene expression of Cyp19a1 by CLO (\( P = 0.092 \); Fig. 5B). Exposure of Hs578t cells to NNs resulted in an increase of CYP19 expression and aromatase catalytic activity [6], and such effects have been shown to result in increased biosynthesis of estrogens [5, 9].
NNs cause reproductive toxicity in humans by increasing estrone and estradiol production and inhibiting estriol production [5, 13]. Moreover, we showed that CLO decreased the GPx4 immunoreactivity in granulosa cells, theca cells and lutein cells of 3-week-old mice. Cyp19a1 is expressed in lutein cells [29], InhA in granulosa cells [33], Akr1b7 in theca cells [2], and Pgr in granulosa cells [40] and lutein cells [11]. Thus, in 3-week-old ovaries of the present study, it was suggested that CLO may induce oxidative stress in those cells, and increase the expressions of Cyp19a1, InhA, Pgr, and Akr1b7, or that CLO may act directly to increase Cyp19a1 expression. These effects, in turn, could activate the estrogen biosynthetic pathway. In 10-week-old ovaries, the expression of the Cyp19a1 showed a rather downward trend, contrary to that of 3-week-old ovaries, suggesting that in utero and lactational exposure may have effects in adulthood.

Our present study showed that developmental exposure to NOAEL-dose CLO has different effects on reproduction and developmental plasticity between juvenile and adult animals. This implies that CLO exposure in the fetal and lactation stages is subsequently manifested differently in adult animals, and proper evaluation of these effects will contribute to an improvement of toxicity testing of NNs.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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FIGURE LEGENDS

Fig. 1
Effect of clothianidin (CLO) on body weight (A) and ovary weight (B) in F1. General histology of 3-week-old ovaries (C–F) and representative examples of HE staining are shown. A: There was no effect of CLO. Data represent the means ± SD of each group and circles show the means of each group (n = 20 in each). B: The ovary weights were significantly lower in the CLO-65 group of 3-week-old mice. Data represent means ± SD of each group and circles show the values for individual mice (n = 5–16 in each). C, D: Size-reduction ovaries were observed in the CLO-65 group. E, F: Intercellular spaces between granulosa cells (arrows) were observed in the CLO-65 group. Scale bars = 500 μm (C, D). Scale bars = 100 μm (E, F). *P < 0.05 vs. the CLO-0 group.

Fig. 2
Immunohistochemistry of 3-week-old (A, B, E, F) and 10-week-old (C, D, G, H) ovaries. Representative examples of immunohistochemistry for GPx4 (glutathione peroxidase 4; A–D) and MnSOD (manganese superoxide dismutase; E–H) are shown. A–D: The intensity of GPx4 immunoreactivity was decreased in the CLO-65 group. E–H: There was no effect of CLO on the intensity of MnSOD immunoreactivity. Scale bars = 100 μm.

Fig. 3
Effects of clothianidin (CLO) on infanticide and severe neglect. In the CLO-administered group of generation F1, 3 out of 9 animals exhibited infanticide and severe neglect behaviors, whereas no animals exhibited these behaviors in the F0 generation (CLO-0: n = 13; CLO-65: n = 15) or in the CLO-0 group of the F1 generation (n = 4).

Fig. 4
Canonical pathways, and networks of up-regulated genes (62 genes) altered by exposure of clothianidin (CLO) identified by ingenuity pathway analysis (IPA) software. A: Signaling pathways activated or suppressed. Solid squares indicate pathways related to the steroid hormone biosynthesis pathway. B: A gene network map illustrating the interactions of up-regulated genes (red colored) and other molecules (n = 2 in each).

Fig. 5
Validation of microarray results using qRT-PCR in 3- (A) and 10-week-old mice (B). Gene expression levels of Cytochrome P450 family 19 subfamily A member 1 (Cyp19a1), inhibin subunit beta A (InhA), progesterone receptor (Pgr) and aldo-keto reductase family 1, member B7 (Akr1b7) were calculated relative to the housekeeping genes, β-actin (Actb), by comparison to the control group. Data represent means ± SD of each group (n = 4–5 in each). *P < 0.05 vs. the CLO-0 group.
Table 1
Clothianidin (CLO)-induced alterations at plasma levels of 17OH-progesterone and corticosterone

|                      | 3-week-old | 10-week-old |
|----------------------|------------|-------------|
|                      | CLO-0      | CLO-65      | CLO-0       | CLO-65      |
| 17OH-progesterone (ppb) | 7.88 ± 0.75 | 6.23 ± 0.36 | 7.58 ± 1.68 | 1.91 ± 1.06* |
| Corticosterone (ppb) | 445.93 ± 37.34 | 411.31 ± 17.29 | 392.21 ± 44.57 | 190.41 ± 62.78* |

Mean ± SE (n = 6–22 in each)  
*: P < 0.01 vs. the CLO-0 group
Table 2
Top 20 of biological functions of 62 up-regulated genes

| Functions Annotation                  | p -Value  | Predicted Activation State | Activation z-score | Number of molecules |
|---------------------------------------|-----------|----------------------------|--------------------|---------------------|
| Quantity of gonadal cells             | 6.20E-05  | Increased                  | 2.198              | 5                   |
| Fertility                             | 2.16E-03  | Increased                  | 2.189              | 5                   |
| Litter size                           | 8.56E-04  |                           | 1.982              | 4                   |
| Quantity of germ cells                | 6.52E-04  |                           | 1.951              | 4                   |
| Quantity of gonads                    | 1.34E-05  |                           | 1.864              | 6                   |
| Development of neurons                | 7.02E-05  |                           | 1.761              | 10                  |
| Neuritogenesis                        | 2.61E-04  |                           | 1.516              | 8                   |
| Long-term potentiation                | 4.23E-04  |                           | 1.4                | 5                   |
| Quantity of bone                      | 4.07E-04  |                           | 1.091              | 5                   |
| Differentiation of bone cells         | 4.13E-04  |                           | 1.067              | 6                   |
| Necrosis of epithelial tissue         | 5.44E-03  |                           | 1                  | 6                   |
| Apoptosis of epithelial cells         | 5.58E-03  |                           | 1                  | 4                   |
| Quantity of ovarian follicles         | 1.61E-06  |                           | 0.927              | 5                   |
| Differentiation of connective tissue cells | 2.67E-04 |                           | 0.713              | 8                   |
| Quantity of osteoclasts               | 1.98E-04  |                           | 0.577              | 4                   |
| Sprouting                             | 5.20E-03  |                           | 0.44               | 5                   |
| Quantity of connective tissue         | 1.83E-04  |                           | 0.13               | 8                   |
| Quantity of protein in blood          | 2.06E-03  |                           | -0.003             | 6                   |
| Quantity of cytokines                 | 3.35E-04  |                           | -0.09              | 5                   |
| Concentration of hormone              | 5.18E-04  |                           | -0.095             | 6                   |

activation z-score; >2.0 or < -2.0 is significantly predictive
Fig. 1

A

B

CLO-0  CLO-65

C

D

E

F
Fig. 2

CLO-0

3-week-old

GPx4

CLO-65

10-week-old

MnSOD

3-week-old

10-week-old
Fig. 3

Infanticide or severe neglect (%)

|        | CLO-0 | CLO-65 |
|--------|-------|--------|
| F0     | 0/13  | 0/15   |
| F1     | 0/4   |        | 3/9    |
Fig. 4

A

B

Morphology of reproductive system

- Enzyme
- Growth factor
- Kinase
- Ligand-dependent Nuclear Receptor
- Peptidase
- Transmembrane Receptor
- Transporter
- Other
- Function
  - Direct relationship
  - Indirect Relationship
Fig. 5

A

$p = 0.074$

Relative gene expression (normalized by Actb)

| Gene   | CLO-0 | CLO-65 |
|--------|-------|--------|
| Cyp19a1|       |        |
| INH    |       |        |
| Pgr    |       |        |
| Akr1b7 |       |        |

B

$p = 0.092$

Relative gene expression (normalized by Actb)

| Gene   | CLO-0 | CLO-65 |
|--------|-------|--------|
| Cyp19a1|       |        |
| INH    |       |        |
| Pgr    |       |        |
| Akr1b7 |       |        |

* : $p < 0.05$
Supplemental Fig. 1

General histology of 10-week-old ovaries. Representative examples of HE staining. A, B: There was no effect of CLO.

Scale bars = 100 μm.
Supplemental Table 1

Primer sequences used in quantitative reverse transcription PCR

| Gene Symbol               | Gene ID   | Direction | Primer sequence (5'-3')           |
|---------------------------|-----------|-----------|-----------------------------------|
| Actb (actin, beta)        | NM_007393.5 | forward   | ggctgtattcccctccatcg              |
|                           |           | reverse   | ccagttgtaacatgtgcatgt            |
| Cyp19a1 (cytochrome P450, family 19, subfamily a, polypeptide 1) | NM_001348171.1 | forward   | agectgtggtggacagttgg            |
|                           |           | reverse   | actcgagcctgtgcattc                |
| Inhba (inhibin beta-A)    | NM_008380.2 | forward   | acagacacctcggagatcatcag           |
|                           |           | reverse   | gatgggtcttgggtgctgtg              |
| Pgr (progesterone receptor)| NM_008829.2 | forward   | gatgtagacgtgatggttgtggg           |
|                           |           | reverse   | catgtagaaggcagcgagtag             |
| Akr17b7 (aldo-keto reductase family 1, member B7) | NM_009731.2 | forward   | cttgggtatctcagactggacatc         |
|                           |           | reverse   | aagaatgctgaccacagacttg           |
Supplemental Table 2

Effects of clothianidin (CLO) on the litter size of mothers, sex ratio and age of vaginal opening

|                                | CLO-0    | CLO-65   | p-value |
|--------------------------------|----------|----------|---------|
| Litter size                    | 9.18±0.94| 8.25±1.23| 0.064   |
| Sex ratio (male/total)         | 42.29±16.25| 50.46±14.81| 0.863   |
| Postnatal days to vaginal opening | 31.80±0.98| 30.89±0.10| 0.176   |

Mean ± SD (n = 5–13 in each)