FULL PAPER

Toxicology

Prenatal and early postnatal NOAEL-dose clothianidin exposure leads to a reduction of germ cells in juvenile male mice

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ABSTRACT. Neonicotinoids are pesticides used worldwide. They bind to insect nicotinic acetylcholine receptors (nAChRs) with high affinity. We previously reported that clothianidin (CTD), one of the latest neonicotinoids, reduced antioxidant expression and induced germ cell death in the adult testis of vertebrates. Here, we investigated the male reproductive toxicity of prenatal and early postnatal exposure to CTD, because it is likely that developmental exposure more severely affects the testis compared to adults due to the absence of the blood-testis barrier. Pregnant C57BL/6 mice were given water gel blended with CTD (0, 10 or 50 mg/kg/day; no-observed-adverse-effect-level [NOAEL for mice]: 47.2 mg/kg/day) between gestational day 1 and 14 days post-partum. We then examined the testes of male offspring at postnatal day 14. The testis weights and the numbers of germ cells per seminiferous tubule were decreased in the CTD-50 group, and abnormal tubules containing no germ cells appeared. Nevertheless, the apoptotic cell number and proliferative activity were not significantly different between the control and CTD-exposed groups. There were no significant differences in the androgen-related parameters, such as the Leydig cell volume per testis, the Sertoli cell number and the tubule diameter. The present study is the first demonstration that *in utero* and lactational exposures to CTD at around the NOAEL for mice reduce the germ cell number, but our findings suggest that these exposures do not affect steroidogenesis in Leydig cells during prenatal or early postnatal life.

KEY WORDS: blood-testis barrier, clothianidin, germ cell, neonicotinoid, prenatal and early postnatal exposure

Neonicotinoids were developed in the 1990s and have been used worldwide as an alternative to organophosphate pesticides. They are chemically similar to nicotine, and they act as agonists to the nicotinic acetylcholine receptors (nAChRs) of insects. Mammals also have nAChRs, but neonicotinoids exhibit lower toxicity to them compared to insects because the composition of insect and mammal nAChRs differ. However, an *in vitro* study showed that neonicotinoids stimulated mammalian neurons through nAChRs similarly to nicotine [15], and indeed, several *in vivo* studies reported the neurobehavioral effects of neonicotinoids [6, 23, 25].

Neonicotinoids also affect the reproductive system of vertebrates due to oxidative stress, which reflects an imbalance between antioxidants and reactive oxygen species and can cause cell death. Several studies revealed that neonicotinoids acted as pro-oxidants and induced oxidative stress [5, 12]. We demonstrated that CTD exposure decreased antioxidant levels and enhanced germ cell death in adult quails and adult mice [6, 7, 26]. It is also noteworthy that oxidative stress in Leydig cells inhibits steroidogenesis [4], and in fact, neonicotinoids have been reported to reduce the level of testosterone in adult lizards [3] and adult rats [1].

Testosterone is the major androgen produced by Leydig cells, and it is essential not only for spermatogenesis and sexual behavior in adult mammals but also for reproductive development during prenatal and early postnatal life. For example, disruption of androgen signaling in the fetus resulted in birth defects of the male reproductive system, such as cryptorchidism [11]. In

addition, fetal androgen exposure is required for the proliferation of Sertoli cells [22], whose number is fixed at around postnatal day (PND) 14; they never proliferate after that [29]. The *in utero* and/or lactational exposure to a neonicotinoid, which is expected to inhibit androgen production during development, may thus affect the testicular growth of the offspring. There are no studies assessing the reproductive effects of neonicotinoid exposure during prenatal and postnatal development, however.

Germ cells are protected by the blood-testis barrier (BTB or Sertoli cell barrier), which prevents cytotoxic agents from passing into the seminiferous tubules [2]. The BTB is not formed until PND 16 [30]. The germ cells in the fetal and juvenile testes without the BTB may thus be easily affected by neonicotinoids. Here, we investigated the effects of *in utero* and lactational exposure to the neonicotinoid clothianidin (CTD) on the major cellular components of the testis (*i.e.*, germ, Sertoli and Leydig cells) at PND 14 (juvenile mice).

MATERIALS AND METHODS

Animals

Adult male and female C57BL/6NCrSlc mice were purchased from Japan SLC (Hamamatsu, Japan) for breeding stock. 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 12-hr light/dark cycle at the Kobe University Life-Science Laboratory with *ad libitum* access to water and a pellet diet (DC-8; Clea Japan, Tokyo, Japan). This study was approved by the Institutional Animal Care and Use Committee (Permission #24-10-03) and carried out according to the Kobe University Animal Experimental Regulations.

CTD purification and HPLC analysis

CTD was isolated from Dantotsu® (containing 16% of CTD; Sumitomo Chemical, Tokyo, Japan), donated by Sado City (Niigata, Japan) as described in our earlier report [6]. Briefly, Dantotsu® was vortexed with ten volumes of distilled water, and it then settled for more than 2 days. The supernatant was discarded. This procedure was repeated five times until the white precipitate was obtained; this was dried in air at room temperature (RT). The results of high-performance liquid chromatography (HPLC) confirmed that the purity of the isolated CTD was >90% by weight.

CTD administration

Female mice in proestrus were mated 1:1 with males overnight, and females that had a vaginal plug on the following morning were designated as being at embryonic day (E) 0.5. The dams were divided into three groups: CTD-0 (Control, n=5 dams, 6 pups), CTD-10 (10 mg/kg/day, n=3 dams, 4 pups) and CTD-50 (50 mg/kg/day, n=3 dams, 6 pups). These concentrations were determined based on the no-observed-adverse-effect-level (NOAEL) for mice (47.2 mg/kg/day [28]).

To eliminate the risk of adverse effects associated with gavage, the dams were given MediGel (MediGel® Sucrarose, ClearH$_2$O, Portland, ME, U.S.A.) with or without CTD as a substitute for filtered water from E 0.5 to PND 14 of age of the offspring. Dams ingested the gel actively, and the concentration of 50 mg/kg/day CTD was reported not to affect the amount of the gel intake [6]. Each litter was randomly selected to a maximum of six pups on PND 2 to standardize the amount of milk, and a litter whose size was three or less was removed from the experiment for the same reason; the pup number of CTD-10 did not reach six. One or two animals per litter were used in order to avoid litter bias.

The amounts of isolated CTD added to the aqueous gel for the respective exposure groups were calculated from the CTD purity (90%), daily gel intake (5 g/day/mouse), total gel weight (60 g: excluding the cup weight) and average body weight (20 g: weighed at the beginning of the experiment). After different amounts of CTD separately dissolved in 600 μl of DMSO (1% volume of gel) were injected into the cups of the gel, they were shaken vigorously to mix the CTD evenly in the gel.

Tissue preparation

All mice at PND 14 were deeply anesthetized with isoflurane and perfused intracardially with ice-cold 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde. The testes were weighed and immersed with the same fixative solution overnight at 4°C. The testes were dehydrated through a graded series of ethanol followed by xylene and embedded in paraffin. Four 4-μm-thick cross-sections were taken from near the center of each testis with intervals at the tenth sections and mounted on a slide glass (Platinum Pro; Matsunami Glass, Kishiwada, Japan).

Immunohistochemistry

The sections were deparaffinized and rehydrated. Antigen retrieval was performed by heating the sections at 121°C for 20 min in 10 mM sodium citrate (pH 6.0). The endogenous peroxidase activity was then blocked with immersion in absolute methanol and 0.5% H$_2$O$_2$ for 30 min respectively at RT.

After non-specific binding sites were blocked by treatment with Blocking One Histo (Nacalai Tesque, Kyoto, Japan), primary antibodies were incubated overnight at 4°C. The concentrations and sources of the primary antibodies were as follows: rabbit anti-Adrenal 4 Binding Protein/Steroidogenic Factor 1 (Ad4BP/SF-1; 1:32,000; a kind gift from Dr. Morohashi, K. [18]), rabbit anti-Cleaved Caspase 3 (C.Caspase 3; 1:1,000; Abcam, Cambridge, U.K.) and rabbit anti-phospho-Histone H3 (pHH3; 1:2,000; Upstate, Lake Placid, NY, U.S.A.). The sections were washed in phosphate-buffered saline with 0.05% Tween-20 (PBST, pH 7.4), prior to incubation with the secondary antibody conjugated to horseradish peroxidase (Dako Envision+ system, Peroxidase;

Dako, Glostrup, Denmark) for 60 min at RT. Peroxidase activity was detected with 3,3-diaminobenzidine tetrachloride solution (EnVision+ kit/HRP[DAB], Dako). Finally, the sections were counterstained with hematoxylin, dehydrated with absolute ethanol, cleared by xylene and coverslipped with Eukitt (O. Kindler, Freiburg, Germany).

For double staining, the sections were incubated to the first primary antibody: goat anti-3β-hydroxysteroid dehydrogenase (3β-HSD; 1:600; Santa Cruz Biotechnology, Dallas, TX, U.S.A.). After incubation with the secondary antibody conjugated to alkaline phosphatase (Abcam), phosphatase activity was detected with Dako Fuchsin Substrate-Chromogen System (Dako). The reacted antibodies were removed by heating the sections as described above, and the sections were then incubated with Blocking One Histo followed by the second primary antibody: rabbit anti-Ad4BP/SF-1 (1:6,000). After incubation with the secondary antibody (Dako Envision+ system), the immunoreactivities were detected with Deep Space Black Chromogen Kit (Biocare Medical, Concord, CA, U.S.A.) which produces a dark gray to black stain in the presence of horseradish peroxidase. The sections were washed with distilled water and coverslipped with Aquatex (Merck, Darmstadt, Germany).

The images were captured using an Eclipse E600 microscope (Nikon, Tokyo, Japan) equipped with a DS-Fi1c camera (Nikon).

Immunofluorescence

The sections were deparaffinized and rehydrated, and then heated as described above for antigen retrieval. After incubation with Blocking One Histo, goat anti-GATA4 (1:200, Santa Cruz Biotechnology) antibody and rabbit anti-pHH3 (1:200) antibody were applied. The sections were washed in PBST and incubated with a mixture of donkey anti-rabbit IgG A488-conjugated antibody (1:400, Molecular Probes, Eugene, OR, U.S.A.) and donkey anti-goat IgG A568-conjugated antibody (1:400, Abcam) for 60 min at RT. After being washed in PBST, the sections were coverslipped with Fluorsave (Calbiochem, San Diego, CA, U.S.A.). Immunofluorescence was detected using an Eclipse E600 microscope.

Immunohistoplanimetry

The numbers of Sertoli cells and germ cells were quantified by counting Ad4BP/SF-1-positive and -negative cells in seminiferous tubules, respectively. We randomly chose 10 nearly round seminiferous tubules which were not degenerated. We measured the diameters of these tubules.

To calculate the frequency of cell death and the rate of proliferation, we examined the sets of sections immunostained for C.Caspase 3 or pHH3. We measured the C.Caspase 3-positive cell numbers located in the seminiferous tubules, the pHH3-positive area and the cross-sectional area excluding lymphatic space by using ImageJ (National Institutes of Health, Bethesda, MD, U.S.A.), and then, we calculated the number of C.Caspase 3-positive cells/area (cells/mm^2) and pHH3-positive area/area (%area).

Stereological analysis of Leydig cell volume per testis

The total Leydig cell volume per testis was determined by stereology as described [24]. The sets of sections immunostained for 3β-HSD were examined. The total 3β-HSD-positive area of the sections was measured by ImageJ and was expressed as a percentage of the cross-sectional area excluding the lymphatic space. The Leydig cell volume per testis of each animal was calculated by multiplication of the percentage value by the testis volume (=weight).

Statistical analysis

The statistical analyses were performed with Excel Statistics 2012 for Windows (SSRI ver. 1.00, Tokyo, Japan). Data were analyzed by one-way analysis of variance (ANOVA) and the post hoc Dunnett's test. The results were considered significant, if the *P*-value was <0.05.

RESULTS

Litter sizes, body and testis weights

No significant differences in litter sizes of dams and body weights of pups were found between the control group and the CTD-administered groups (Fig. 1A and 1B), and few deaths of pups were seen among all groups. The average testis weight of the CTD-50 mice was lower than that of the control, and the *P*-value approached significance (*P*=0.06, Fig. 1B).

Sertoli cell and germ cell numbers

Ad4BP/SF-1 was expressed by Sertoli cells in seminiferous tubules and Leydig cells in the interstitium (Fig. 2A–2C). No changes were detected in the Sertoli cell numbers (Fig. 1E). On the other hand, there appeared to be fewer germ cells in the CTD-50 group compared to the control group, and in fact, the number of germ cells per seminiferous tubule was significantly reduced in the CTD-50 group (*P*<0.05, Fig. 2F). This decreased number did not correlate with the body weights (r=0.11, *P*=0.68) or the testis weights (r=0.33, *P*=0.21). One of the CTD-50 mice also had seriously degenerated seminiferous tubules (Fig. 2D). These tubules contained no germ cells and an apparently greater number of Sertoli cells than the normal tubules around them.

Seminiferous tubule diameters

The diameters of the seminiferous tubules did not show any significant difference between the control and treatment groups (Fig. 2G).
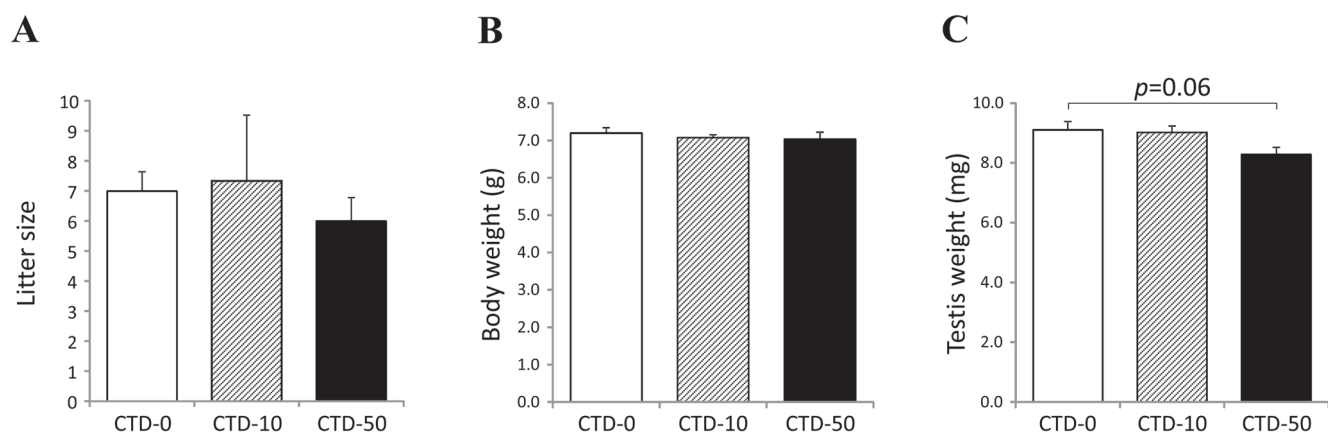
Fig. 1. Effect of treatment with CTD on litter sizes (A), body weights of pups (B) and testis weights (C). There are no significant between-group differences in litter sizes (A) and body weights of pups (B). The testis weight of the CTD-50 group is lower than that of the control (C). Values are the mean ± SE of 3–6 mice per group.
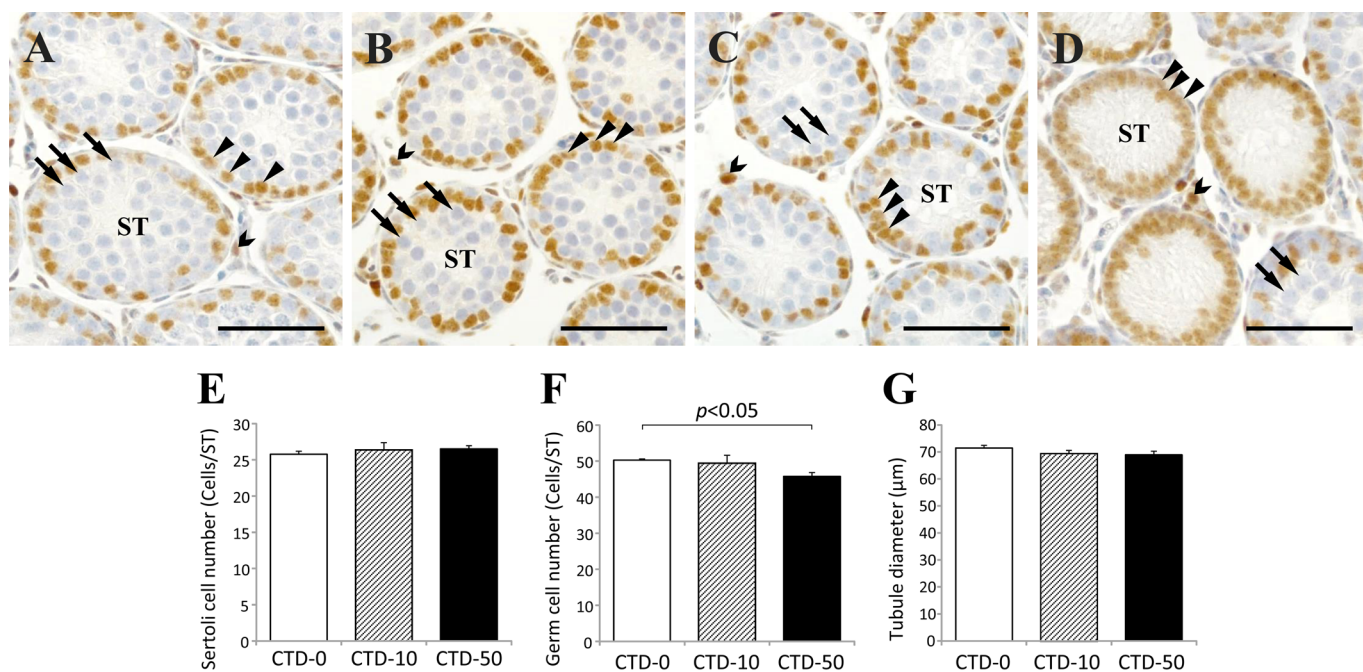

Fig. 2. Representative immunohistochemistry for Ad4BP/SF-1 of the testis in the mice of the CTD-0 (A), CTD-10 (B) and CTD-50 (C, D) groups. The immunoreactivity is detected in Sertoli cells (arrowheads) in seminiferous tubules (ST) and Leydig cells (chevrons) in the interstitium, although the germ cells (arrows) are negative. Abnormal seminiferous tubules containing no germ cells and many Sertoli cells are observed in one of the CTD-50 mice (D). There are no changes between the groups in the Sertoli cell number (E), although the germ cell number per tubule of the CTD-50 mice is significantly decreased compared to that of the control ($P<0.05$, F). The diameters of the seminiferous tubules do not differ significantly among the groups (G). Values are mean ± SE of 4–6 mice per group. Bar=50 μm.

Frequency of cell death

Almost all of the cells that were positive for C.Caspase 3, a critical executioner of apoptosis, were present in seminiferous tubules (Fig. 3A–3C). The C.Caspase 3-positive cell numbers did not differ between the control and CTD-exposed groups (Fig. 3D), and no correlation was found between the C.Caspase 3-positive cell and germ cell numbers (r=−0.17, $P=0.53$).

Rate of proliferation

The cells that were positively immunostained for pHH3, a mitosis marker, were located mainly in the seminiferous tubules and rare in the interstitium (Fig. 4A–4C). Our quantitative analysis of the cell proliferation rate revealed no significances between the control and treatment groups (Fig. 4D), and there was no correlation between the rates of proliferation and the germ cell numbers
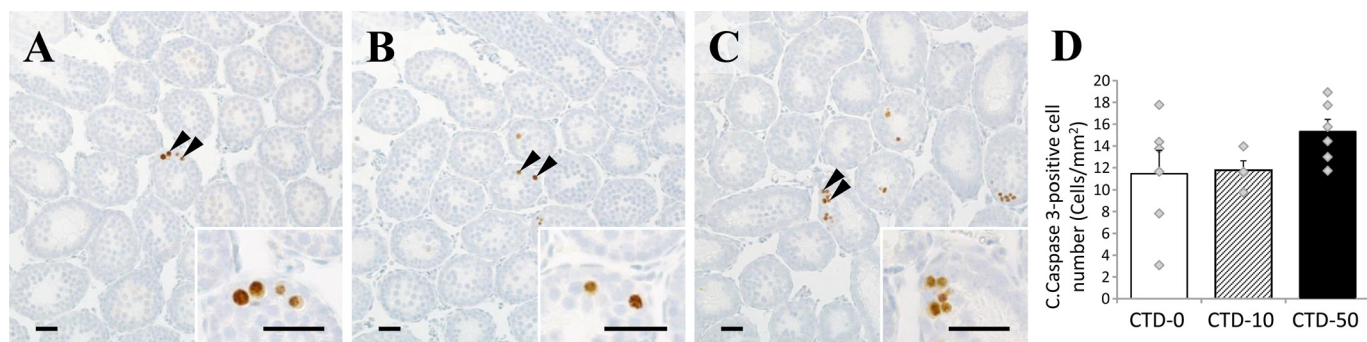
Fig. 3. Representative immunohistochemistry for C.Caspase 3 of the testis in the mice of the CTD-0 (A), CTD-10 (B) and CTD-50 (C) groups. All positive cells are present in seminiferous tubules (arrowheads). The positive cell numbers of the exposure groups are not significantly different from that of the control (D). Values are mean ± SE of 4–6 mice per group. Bar=30 μm.
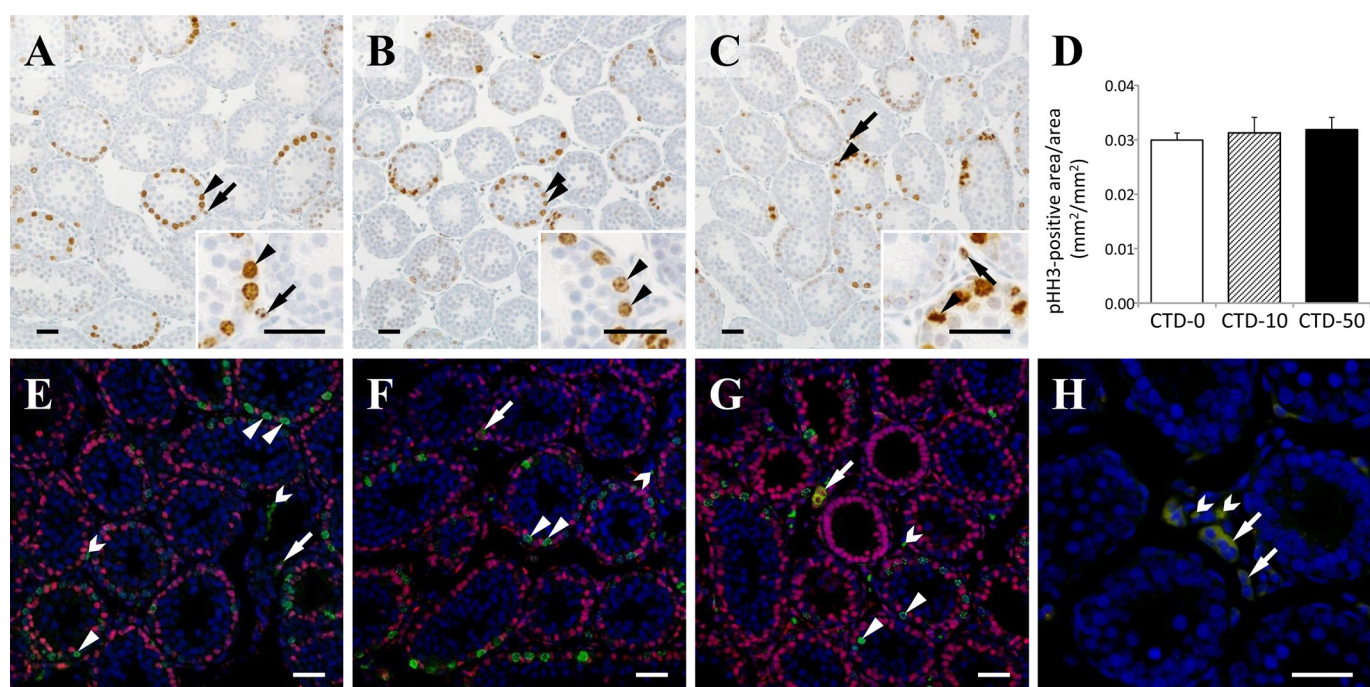

Fig. 4. Representative immunohistochemistry for pHH3 of the testis in the mice of the CTD-0 (A), CTD-10 (B) and CTD-50 (C) groups. The positive cells are located mostly in seminiferous tubules (arrowheads) and rare in the interstitium (arrows). No differences are detected in the positive area per area (D). Double staining for pHH3 (green) and GATA4 (red) shows that all pHH3-positive cells (arrowheads) are negative for GATA4 in the mice of the CTD-0 (E), CTD-10 (F) and CTD-50 (G) groups. Autofluorescence is detected in Leydig cells (arrows) and erythrocytes (chevrons) in all sections including the negative control (H). Values are mean ± SE of 4–6 mice per group. Bar=30 μm.

(r=0.10, *P*=0.72). In all groups, GATA4 reactivity was detected in Sertoli cell nuclei, and the Sertoli cells that were positive for GATA4 never showed the expression of pHH3 (Fig. 4E–4G). The negative control sections (in which PBST was used as a substitute for the primary antibodies) showed autofluorescence in the Leydig cells and erythrocytes (Fig. 4H).

Leydig cell volumes

All clusters of Ad4BP/SF-1-positive cells in the interstitium were positive for 3β-HSD, a marker of Leydig cells, whereas 3β-HSD signals were detected in a few of the single round-shaped cells that were positive for Ad4BP/SF-1 (Fig. 5A–5C). Spindle-shaped cells positive for Ad4BP/SF-1 in the interstitium did not show 3β-HSD expression. Leydig cell volumes in the CTD-exposed groups were not changed compared to those in the control group (Fig. 5D).

DISCUSSION

In the present study, *in utero* and lactational exposure to CTD at doses around the NOAEL for mice reduced the number of germ
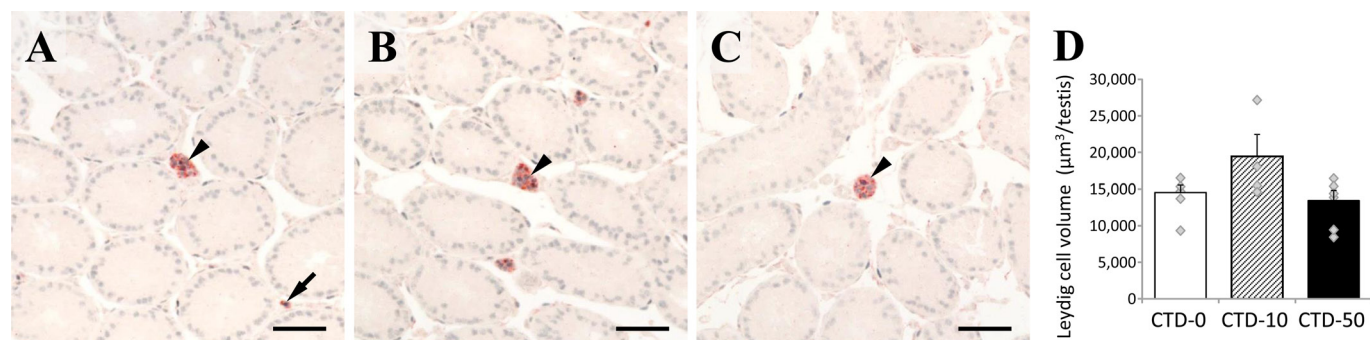
Fig. 5. Representative immunohistochemistry for 3β-HSD (red, a Leydig cell marker) and Ad4BP/SF-1 (Black) of the testis in the mice of the CTD-0 (A), CTD-10 (B) and CTD-50 (C) groups. The clusters of Ad4BP/SF-1 positive-cells in the interstitium show the expression of 3β-HSD (arrowheads). Some of the single cells in the interstitium are also double-positive (arrows). The 3β-HSD-positive Leydig cell volume per testis of the CTD-10 mice is higher than that of the control, but there are no significant differences between groups. Values are mean ± SE of 4–6 mice per group. Bar=50 μm.

cells, and abnormal tubules containing no germ cells appeared in one of the CTD-50 mice. We did not observe such effects in the CTD-50 group in our previous study in which adult mice were exposed to CTD for 4 weeks. This suggests that the germ cells in the fetal and juvenile testes, which are not protected by the BTB are more vulnerable to CTD compared to those in the adults and that a small group of mice with high sensitivity can suffer from the germ cell loss.

The amount of CTD transferred to offspring via the placenta and breast milk has not been reported, but two studies revealed that maternal exposure to CTD or acetamiprid, another neonicotinoid, altered behavior in the offspring [23, 25], implying that CTD passes through the placenta and/or breast milk and affects the growth and development of the offspring. This is consistent with our present finding that maternal CTD exposure reduced the number of germ cells.

One of the CTD-50 mice had seriously degenerated tubules. We found no correlation between the germ cell number and each parameter, such as the body weight, indicating that the decreased germ cell number resulted from individual difference of sensitivity rather than that of milk intake (*i.e.*, CTD intake). The difference of inbred mice may be partly attributed to environmental factors including stress [6], but the details remain to be elucidated.

We showed previously that in adult mice, the same abnormal tubules appeared following CTD exposure for 4 weeks [6], and chronic exposure to CTD induced germ cell death through oxidative stress in adult quails [26], and we thus speculate that CTD-triggered cell death is responsible for the germ cell loss observed at PND 14 in the present study. Nevertheless, the C.Caspase 3-positive cell numbers were not changed in the treated groups and the rate of proliferation did not decrease, indicating that germ cell death occurred at an earlier stage, or possibly, the initiation of spermatogenesis was delayed. After birth, spermatogenesis begins and the germ cell population expands exponentially with time during puberty [29], and thus, the delay of the initiation of spermatogenesis might have resulted in the decreased number of germ cells.

Because each Sertoli cell supports a limited number of germ cells [19], the magnitude of sperm production critically depends on the number of Sertoli cells, whose proliferative activity gradually declines after birth and ceases between PND 14 and 17 [29]. The Sertoli cell proliferation during fetal life is regulated largely by androgens. For example, the Sertoli cell number in androgen receptor (AR) knockout mice was reduced to approx. 50% of the control value at birth, although only interstitial cells and peritubular myoid cells expressed AR in the feral testis [10]. These cells are thought to respond to androgen signaling and to mediate Sertoli cell proliferation [20]. In the present study, we observed no changes in the Sertoli cell number at PND 14, suggesting that the androgen levels during fetal and postnatal development were not affected by CTD. The normal tubule diameters of the exposed groups indicate the same possibility, because the development of the tubules is also dependent on androgens [22].

We did not observe any adverse effects of CTD on Leydig cells at PND 14, suggesting that Leydig cell steroidogenesis was not inhibited. This is consistent with the lack of change in the Sertori cell numbers. In this study, we measured not a percentage of the 3β-HSD-positive area in the total area but rather the total Leydig cell volume per testis by stereology, because the rate does not always reflect the absolute volume of Leydig cells. AR knockout mice show apparent Leydig cell hyperplasia/hypertrophy in histology, but the total Leydig cell number in the testis is decreased due to a marked reduction in testis size [21]. Moreover, the Leydig cell volume could also be responsible for androgen production [9, 14]. Bal *et al*. [1] reported that adult rats chronically exposed to CTD showed lower testosterone levels than the controls. The apparent disparity may be accounted for by the steroidogenic activities of Leydig cells. Testosterone levels decline after birth to a nadir at around PND 10–20 before increasing up to adulthood, indicating that the activity of the Leydig cells at PND 14 was low [20]. The Leydig cells in the adult testis actively produce testosterone [20], which itself generates reactive oxygen species [27]. Thus, CTD-induced oxidative stress may damage the Leydig cells in the adult testis but not those at PND 14. However, we do not deny the possibility that CTD does not affect Leydig cells because Bal *et al*. [1] treated the rats with Dantotsu which contained not only CTD but also repellents and the other agents.

Two studies showed that neonicotinoids stimulated mammalian nAChRs [15, 16]. These nAChRs have several subtypes including α4β2 and α7, and the α4β2 receptors are expected to be weakly expressed in the Leydig cells [17]. While *in vitro* studies

demonstrated that nicotine decreased the testosterone production of Leydig cells [13, 31], nicotine was reported to act as pro-oxidants [8]. Therefore, whether nAChR signaling inhibits androgen biosynthesis or not is controversial.

In conclusion, this study is the first to reveal that maternal CTD exposure at around the NOAEL for mice affected the number of germ cells in juvenile mice, and it induced a depletion of germ cells in a highly susceptible individual mouse. Interestingly, the results also showed no change in the androgen-related parameters, *i.e.*, the Sertoli cell number, the tubule diameter and the Leydig cell volume. These results suggest the involvement of CTD-induced oxidative stress rather than nAChR agonist effects. Further analyses are necessary to determine whether CTD affects Leydig cells in the fetal testes (which actively produce androgen) and to investigate the mechanisms by which maternal CTD reduces the germ cell number.

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