FULL PAPER *Toxicology*

The combined effect of clothianidin and environmental stress on the behavioral and reproductive function in male mice

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ABSTRACT. Neonicotinoids, some of the most widely used pesticides in the world, act as agonists to the nicotinic acetylcholine receptors (nAChRs) of insects, resulting in death from abnormal excitability. Neonicotinoids unexpectedly became a major topic as a compelling cause of honeybee colony collapse disorder, which is damaging crop production that requires pollination worldwide. Mammal nAChRs appear to have a certain affinity for neonicotinoids with lower levels than those of insects; there is thus rising concern about unpredictable adverse effects of neonicotinoids on vertebrates. We hypothesized that the effects of neonicotinoids would be enhanced under a chronic stressed condition, which is known to alter the expression of targets of neonicotinoids, *i.e.*, neuronal nAChRs. We performed immunohistochemical and behavioral analyses in male mice actively administered a neonicotinoid, clothianidin (CTD; 0, 10, 50 and 250 mg/kg/day), for 4 weeks under an unpredictable chronic stress procedure. Vacuolated seminiferous epithelia and a decrease in the immunoreactivity of the antioxidant enzyme glutathione peroxidase 4 were observed in the testes of the CTD+stress mice. In an open field test, although the locomotor activities were not affected, the anxiety-like behaviors of the mice were elevated by both CTD and stress. The present study demonstrates that the behavioral and reproductive effects of CTD become more serious in combination with environmental stress, which may reflect our actual situation of multiple exposure.

KEY WORDS: anxiety-like behavior, clothianidin, environmental stress, neonicotinoid, oxidative stress

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Neonicotinoids, which were developed in the 1990s, have been some of the most widely used pesticides, accounting for over 25% of the world market share with rapidly increasing sales. They act as agonists to the nicotinic acetylcholine receptors (nAChRs) of insects with much higher affinity than to those of mammals, resulting in death of insects from abnormal excitability in their cholinergic nervous system. However, with the recent expanding use of neonicotinoids in farming, forestry and the building industry, an unanticipated phenomenon called honeybee colony collapse disorder (CCD) has inflicted great damage on crop production in many parts of the world [30]. Some neonicotinoids were proved to disturb the foraging and homing behaviors of honeybees, weakening their colonies [12, 13, 41]. Neonicotinoids have thus attracted much attention as a strongly suspected agent of CCD.

In experimental animals, neonicotinoids were immediately absorbed and largely excreted into the urine with 2–6 hr half-life *in vivo* [9]. An *in vitro* study showed that mammalian nAChRs also have some affinity for neonicotinoids, although at much lower levels compared to insect nAChRs [38]. In neonatal rats, several neonicotinoids cause excitation of cerebellar neurons through their nAChRs [20], suggesting further unpredictable adverse effects of neonicotinoids on vertebrates. According to those reports, the European Union (EU) issued a decision in 2013 to ban the use of three neonicotinoids for a fixed period of two years as a precautionary principle. Contrary to this, in Japan, the residue standards for neonicotinoids, which are several or hundreds of times higher than the corresponding standards in Europe and the United States, are being relaxed by the

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Ministry of Health, Labour and Welfare. An increasing number of research studies demonstrate that neonicotinoids are definitely harmful to mammals, birds, fishes and insects, and there is an urgent need for decisive information to judge the risk of neonicotinoids.

In particular, clothianidin (CTD), the sixth-developed neonicotinoid, is a white crystalline compound with the molecular weight of 249.68 and the lowest water solubility (0.327 g/*l*) among the neonicotinoids. A research about metabolic fate of neonicotinoids revealed that CTD was comparatively persistent in mammal brain [10]. CTD also accelerates the responses to acetylcholine in HEK cells expressing human nAChRs α4β2 [22], implying an excitatory effect of CTD on mammalian brains. The reproductive function also seems to be a target of CTD; this neonicotinoid reduced the weights of reproductive organs and semen quality parameters of immature and mature male rats [3, 4]. We recently reported that low-dose CTD affects avian reproductive functions through oxidative stress, and this effect could be more severe in birds that have higher sensitivities [15, 36].

As described above, the typical targets of neonicotinoids are nAChRs, which are expressed throughout the body, especially in postsynaptic neuromuscular junctions, autonomic ganglions and the central nervous system for cholinergic neurotransmission by acetylcholine. These ion channel-linked receptors, are a circular homo- or hetero-pentamer composed of dozens of subunits determining their physiological properties. In mammal brain, α7 homomer and α4β2 heteromer play important roles as regulators of intraneuronal signaling and neurotransmitter releases. Phenotypes of knockout models indicated that nAChRs are involved in emotional and anxiety-like behaviors, along with some psychiatric disorders [6, 29].

It recently became apparent that the expressions of nAChRs are influenced by environmental stress. For example, immobilize stress decreases the expression of nAChRs α7 in the hippocampus of mature rats [16], whereas, prenatal stress in rats increases the expression of nAChRs in the hippocampus of their adult offspring [33]. We thus hypothesized that environmental stress altered the sensitivity to neonicotinoids by modifying the cholinergic system.

The purpose of the present study was to investigate the effect of chronic combined exposure to CTD and environmental stress. The oral administration method is known to add extraordinary stress to experimental animals with a transient secretion of glucocorticoids [5]. To assess the effects of CTD and environmental stress separately, we chose a non-invasive CTD administration method using commercial oral rehydration gels. We conducted an open field test to detect behavioral changes following this combined exposure, and histological and immunohistochemical analyses were performed focusing on the expression of the antioxidant enzymes in testes.

MATERIALS AND METHODS

Experimental animals: Male C57BL/6NCrSlc mice (8 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). All mice were maintained in 40.5 × 20.5 × 18.5 cm individual 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 a pellet diet (DC-8; Clea Japan, Tokyo, Japan) and filtered water. 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 Regulation.

CTD purification and HPLC analysis: Water-soluble Dantotsu® (involving 16% of CTD; Sumitomo Chemical Co., Tokyo, Japan), donated by Sado City (Niigata, Japan), was washed with 10 times the amount of distilled water to remove the surfactant activating and granulating agents. After being left to stand for at least 48 hr, the supernatant was removed. This step was repeated five times, and then, the white precipitate was collected and air-dried naturally for a week. The content rate of CTD in the white precipitate was measured by a LaChrom high-performance liquid chromatography (HPLC) system (interface L-7000, pump L-7100, auto sampler L-7200, column oven L-7350 and UV-VIS detector L-7420; Hitachi, Tokyo, Japan) using a Capcell Pak C18 UG120 column (5 μm particles, 4.6 × 250 mm; Shiseido, Tokyo, Japan).

CTD standard (>99.8%; Wako Chemical, Osaka, Japan) and the obtained white powder were completely dissolved in dimethyl sulfoxide (DMSO) and then serial-diluted with a mobile phase consisting of 55% acetonitrile in 50 mM potassium phosphate buffer (pH 3.0), followed by filtration with a 0.20 μm syringe driven filter unit (Millex-LG; Millipore, Billerica, MA, U.S.A.). The column maintained at 40°C was eluted with the mobile phase at a flow rate of 1.0 m*l*/min. After the column was equilibrated, 10 μl of the samples was injected into the HPLC system. We monitored the resultant chromatograph at the wavelength of 260 nm and then calculated the CTD content of the obtained white powder from the calibration curve created by the peak areas and heights of the CTD standard. A single peak was observed in the samples from the white precipitate with the same retention time as that of the CTD standard. A linear calibration curve (R^2=0.999) created from samples serial-diluted with the CTD standard showed that our purification makes the content rate of CTD increased from 14–16% to 93–97% by weight.

CTD administration and stress exposure: All mice were allowed to acclimate to their home cages for a week prior to the initiation of experiments. We divided the mice into eight groups (n=5 mice in each cage): CTD-0 (Control), CTD-10 (10 mg/kg/day), CTD-50 (50 mg/kg/day) and CTD-250 (250 mg/kg/day) with the presence or absence of stress exposure. As a substitute for filtered water for the mice, we used the MediGel® Sucrarose 2 oz cup (ClearH_2O, Portland, ME, U.S.A.), which is a flavored thermoreversible hydration gel matrix. The amounts of the purified CTD for the respective administration groups were calculated from the CTD purity (95%), daily gel intake (5 g/day/mouse), total gel weight (60

g; excluding the package weight) and average mouse weight (24 g; weighed at initiation of experiments). These amounts of CTD were completely dissolved in 600 μl DMSO (1% volume of a gel) and injected into the gels and then double-boiled at 60°C followed by shaking to ensure that the CTD was diffused well. For the CTD-0 group, the same volume of DMSO without the purified CTD was injected into the gels. All gels were strapped with cable ties under the grate of the cage lid to prevent contamination with the beddings and the excretions. All gels were weighed daily to estimate the amounts of the putative CTD exposure. In the four stress groups, the mice were subjected to an unpredictable chronic stress procedure as described in our earlier report [14] with some modifications. Briefly, the following six stressors were used: 5 min forced swimming in water at room temperature (RT), 24 hr food and water deprivation, continuous overnight illumination, 30 min horizontal cage shaking (80 rpm), 24 hr switching of cagemates (being housed with another mouse) and 24 hr wet bedding. To maximize the unpredictability of this paradigm, the mice were randomly exposed to two mild stressors per day at varying times for 4 weeks.

Behavioral analysis: On the last day of the 4 week experimental period, an open field test was conducted during the light phase to evaluate the locomotor activity and the anxiety-like behavior of the mice. Briefly, the mouse was placed on the corner of an open field (60 × 60 × 30 cm) with LED illumination. All of the mouse's activities were recorded by a video camera for the subsequent 10 min, and we then analyzed the total distance traveled and the time spent in the center zone (30 × 30 cm) using SMART video tracking software V3.0 (San Diego Instruments, San Diego, CA, U.S.A.).

Tissue preparation: On the day after the completion of the 4 weeks of combined exposure to CTD and stress, all mice were deeply anesthetized with diethyl ether and transcardially perfused with 0.9% normal saline, followed by perfusion with ice-cold 4% paraformaldehyde in phosphate buffer. The testes were excised, weighed and postfixed with the same fixative overnight at 4°C. The testes were dehydrated through a graded series of ethanol followed by xylene and embedded in paraffin. Serial sections of testes were then cut at 4 μm thickness on a sliding microtome (SM2000R; Leica Microsystems, Wetzlar, Germany) and mounted on slide glasses (Platinum Pro; Matsunami Glass Ind., Kishiwada, Japan). All sections were stored at −30°C until use for the following steps.

Histological and immunohistochemical analyses: For the general histological analysis, testis sections were stained with hematoxylin and eosin (HE; Merck KGaA, Darmstadt, Germany) after their deparaffinization and hydration, following the manufacturer's instructions. To detect antioxidant enzymes in the testes, we performed the following immunohistochemistry protocol. The sections were immersed in absolute methanol and 0.5% H_2O_2 for 30 min, respectively, at RT to quench the endogenous peroxidase activity. They were then incubated with Blocking OneHisto (Nacalai Tesque, Inc., Kyoto, Japan) for 1 hr at RT for protein blocking and then incubated with the rabbit polyclonal anti-GPx4 antibody (Item No. 10005258; Cayman Chemicals, Ann Arbor, MI, U.S.A.) diluted 1:8,000 in phosphate buffered saline with 0.05% Tween-20 (PBST; pH 7.4) for 18 hr at 4°C.

After being washed with PBST, the sections were reacted with goat anti-rabbit immunoglobulins conjugated to peroxidase-labeled dextran polymer in tris (hydroxymethyl) aminomethane-HCl buffer (EnVision+; Dako, Glostrup, Denmark) for 1 hr at RT. Immunoreactivity was then detected by incubation with 3,3′-diaminobenzidine solution (EnVision+ kit/HRP[DAB], Dako). The sections were then rinsed with distilled water and counterstained lightly with hematoxylin solution for 1 min. Next, the sections were placed in a graded series of ethanol, dehydrated with absolute ethanol, cleared by xylene and coverslipped with Eukitt (O. Kindler GmbH, Freiburg, Germany).

Statistical analysis: Statistical analyses were performed with Excel Statistics 2012 (SSRI version 1.00, Tokyo, Japan). In the behavioral analyses, outliers more distant than 1.5 interquartile ranges from the upper or lower quartile were omitted. All data were analyzed by two-way ANOVA (CTD ×stress) followed by the Tukey-Kramer's post hoc test. The results were considered significant when the *P*-value was less than 0.05.

RESULTS

Gel intake, body and testis weights: The daily gel consumption, body weights and testes weights are shown in Fig. 1, and all measured values are summarized in Table 1. CTD significantly suppressed the daily gel intake in all CTD administration groups compared to the non-CTD administration groups (Fig. 1A). Although the daily intake was significantly inhibited in the stress groups, there was little difference if the values in the days of food deprivation in our stress protocol were omitted (Table 1). As a result, the body weights of mice reflected the same tendency of the gel intake. The body weights were suppressed in all stress groups relative to the non-stressed groups at the same dose of CTD (Fig. 1B). The body weights of the CTD-250 groups were significantly lower than those of the other three dose groups. On the other hand, the absolute weights of the testes were decreased by the stress procedure in the CTD-0, CTD-50 and CTD-250 groups (Fig. 1C). No interaction effects were detected in any of the measured values.

Open field test: The representative trajectory maps (Fig. 2A) revealed that the mice with the combined CTD exposure and stress procedure tended to walk alongside the walls. CTD and the stress procedure had insignificant impacts on the locomotor activities measured by the total distances traveled (Fig. 2B). By contrast, the anxiety-like behaviors that avoid being in the center zone were elevated by the stress procedure and were significantly different between the CTD-0 groups (Fig. 2C). CTD also enhanced the anxiety-like behaviors in a dose-dependent manner, and in the non-stressed condition, the difference was significant in the CTD-10 and CTD-250 groups compared to the CTD-0 group.

Histological and immunohistochemical findings: The

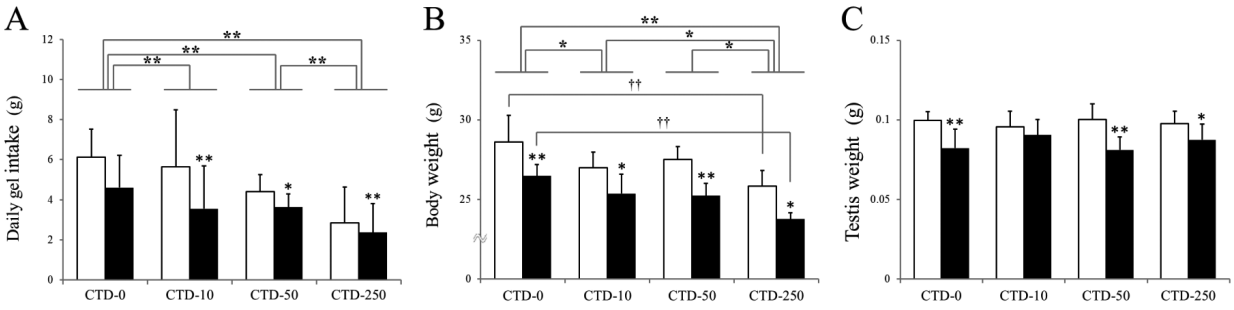

Fig. 1. Effects of combined exposure on the daily gel intake (A), body weights (B) and testis weights (C) in the non-stressed (open column) and stressed groups (closed column). The two-way ANOVA showed significant main effects for the CTD and stress (P<0.01), but the interaction effects were not significant in gel intake and body weights. (A) CTD dose-dependently inhibited the gel intake, and the inhibition was significant in all CTD-administration groups compared to the CTD-0 groups. The stress procedure significantly inhibited the gel intake in each CTD-administration group. (B) The body weights of the CTD-250 stress and non-stressed groups were significantly lower than those of the other dose groups, in addition to the significant difference between the CTD-0 and CTD-10 groups. The post hoc analyses also revealed that there were significant differences between the CTD-0 and CTD-250 stress and non-stressed groups. (C) The two-way ANOVA showed a significant main effect for stress (P<0.01), but the interaction effect was not significant in the absolute weights of testes. The stress procedure significantly suppressed the absolute testes weights in all stress groups, except the CTD-10 group. Values are mean ± SD (n=5 mice each). *P<0.05, **P<0.01.

Table 1. Body weight, gel intake, putative CTD exposure and testis weights

	Groups							
	CTD-0	CTD-0 + Stress	CTD-10	CTD-10 + Stress	CTD-50	CTD-50 + Stress	CTD-250	CTD-250 + Stress
Body weight (g)	28.62 ± 1.66	26.48 ± 0.72	26.98 ± 0.99	25.36 ± 1.22	27.50 ± 0.82	25.24 ± 0.78	25.82 ± 0.99	23.76 ± 0.40
Gel intake / day (g)	6.13 ± 1.37	5.64 ± 2.80 [6.66 ± 2.56]	4.41 ± 0.84	2.85 ± 1.77 [3.73 ± 1.59]	4.60 ± 1.59	3.55 ± 2.09 [4.65 ± 1.79]	3.64 ± 0.64	2.38 ± 1.42 [3.16 ± 1.09]
Putative exposure (mg/kg/day)	0	0	8.82 ± 1.68	5.70 ± 3.54 [7.46 ± 3.18]	46.0 ± 15.9	35.5 ± 20.9 [46.5 ± 17.9]	182 ± 32	119 ± 71 [158 ± 54.5]
Testis weight (mg)	99.6 ± 5.5	88.2 ± 11.8	95.7 ± 9.6	90.5 ± 9.7	100.3 ± 9.8	81.1 ± 8.2	97.5 ± 7.8	87.5 ± 9.7

Values are the mean ± SD (n=5 mice each). The values in square brackets are the averages after the removal of values in the deprivation days from the calculation.

general histological analyses of the testes by HE staining are shown in Fig. 3. Although there were seminiferous tubules densely arranged with germ cells and sperms in the testes of the control group, several testicular signs of toxicity were observed in combined exposure groups. Multinucleated giant cells were occasionally observed in the seminiferous tubules of the mice in the stress groups. In addition, variably sized vacuolizations were scattered at the bottom of seminiferous tubules of the CTD-administered groups. In particular, seriously degenerated seminiferous tubules, from which almost all germ cells had dropped off, were found in the CTD-250 groups. There was no marked histological difference between the Leydig cells in the testicular interstitial tissues of any of the experimental groups.

The results of the immunohistochemical analyses of the testes visualizing GPx4 are shown in Fig. 4. In the control group, strong GPx4 immunoreactivity was detected in the sperm, and diffuse GPx4 immunoreactivity was seen in the cytoplasm of spermatids. The intensity of GPx4 immunoreactivity in the sperm was decreased by stress, and the cytoplasmic immunoreactivity in the spermatids was dose-dependently decreased by CTD. In addition, abnormal immunoreactivity of GPx4 in Sertoli cells was detected in the combined exposure groups, and it became strong in degenerated seminiferous tubules in the CTD-50+stress group and the CTD-250 groups.

DISCUSSION

We verified the combined effects of CTD and stress on the reproductive and behavioral function in mature male mice by a non-invasive administration method. There are several advantages of the gels we used; compared to baking pellets, the gels can uniformly deliver the test articles to animals without exposing them to high temperature. The gels could not completely cloak the bad palatability of CTD as reported earlier using similar gels mixed with an aromatase inhibitor, anastrozole [27]; however, the gel consumption observed in combined exposure groups was enough for maintaining the health of the mice. In our preliminary experiments, we initially suspected that the CTD avoidance effect was attributable to any accessory constituents of the commercial

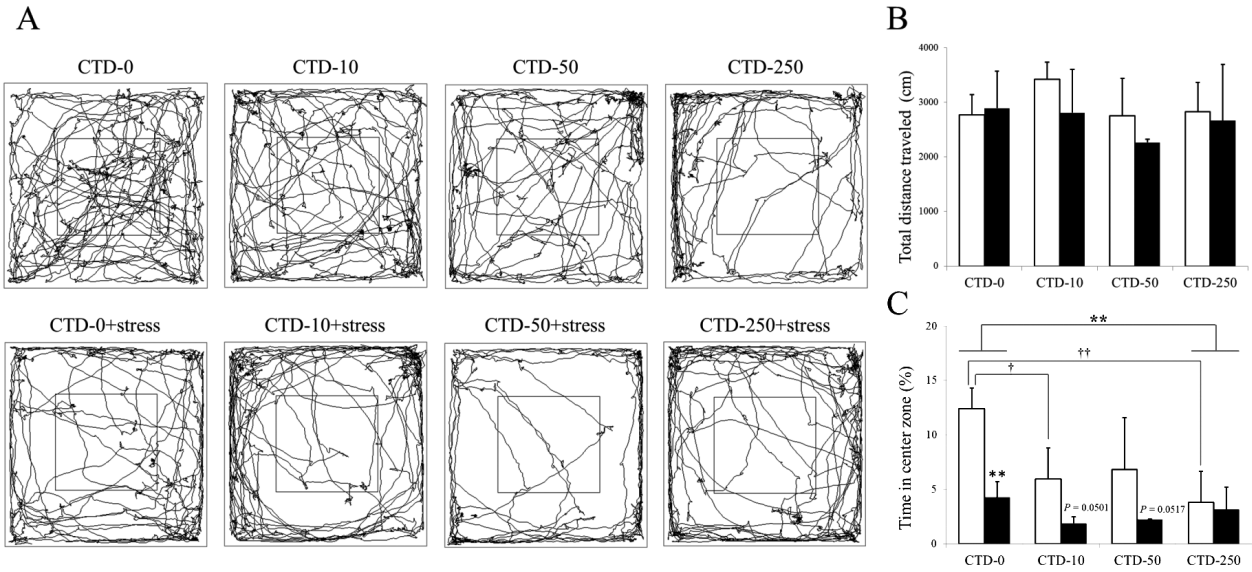

Fig. 2. Behavioral effect of combined exposure of CTD and stress in the open field activity in the non-stressed and stressed groups. (A) A representative trajectory map of the mice as illustrated by the video tracking software. The exploratory behaviors in the center zone (30 × 30 cm) of the open field (60 × 60 cm) were dose-dependently suppressed by CTD compared to the non-administration groups. (B) Total distances traveled in the open field of the non-stressed (open columns) and stressed groups (closed columns). No marked difference was detected by two-way ANOVA in the total distances traveled. (C) Time spent in the center zone in the open field of the non-stressed (open columns) and stressed groups (closed columns). Two-way ANOVA showed significant main effects for CTD (P<0.05) and stress (P<0.01), but the interaction effect was not significant. CTD significantly inhibited the times spent in the center zone in the CTD-250 groups compared to the non-CTD administration groups. A significant anxiogenic effect of the stress procedure was observed between the CTD-0 groups. In the non-stressed condition, there were significant differences in the CTD-10 and CTD-50 groups compared to the CTD-0 group. Values are mean ± SD (n=5 mice each). *P<0.05, **P<0.01.

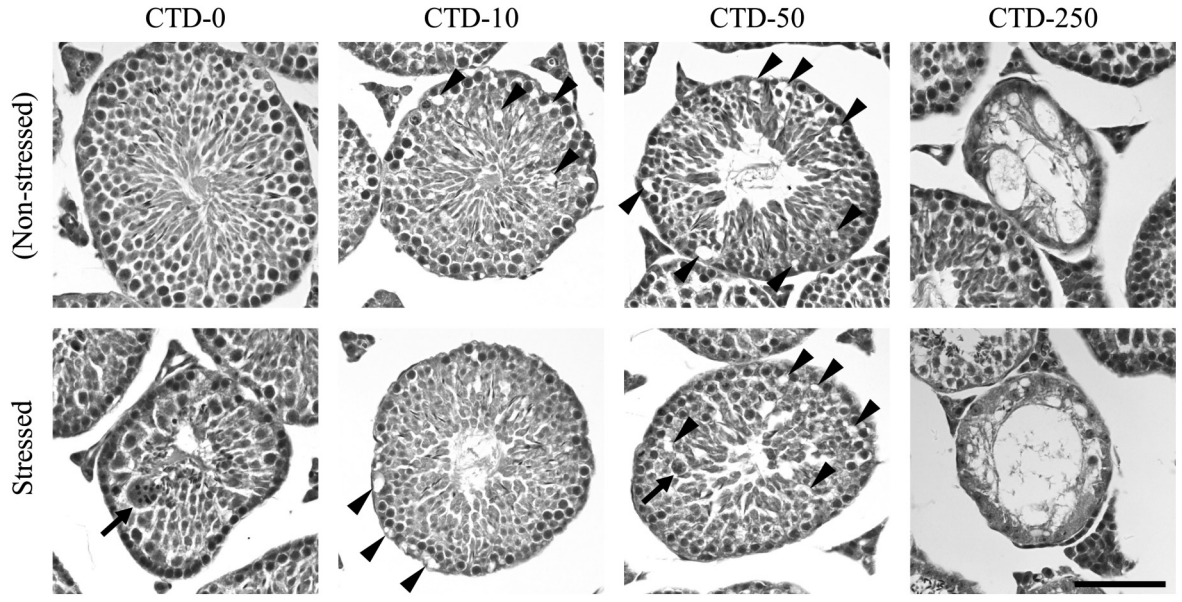

Fig. 3. Representative histology of the testis in the non-stressed and stressed groups. The seminiferous tubules of the control group showed robust spermatogenesis with densely stacked germ cells. Multinucleated giant cells (arrow) were occasionally observed in the testes of the CTD-0+stress groups. In the CTD groups, dose-dependent vacuoled degeneration (arrowhead) of seminiferous tubules was observed. Degenerated seminiferous tubules only composed of Sertoli cells were present in the CTD-250 groups. Bar=100 μm.

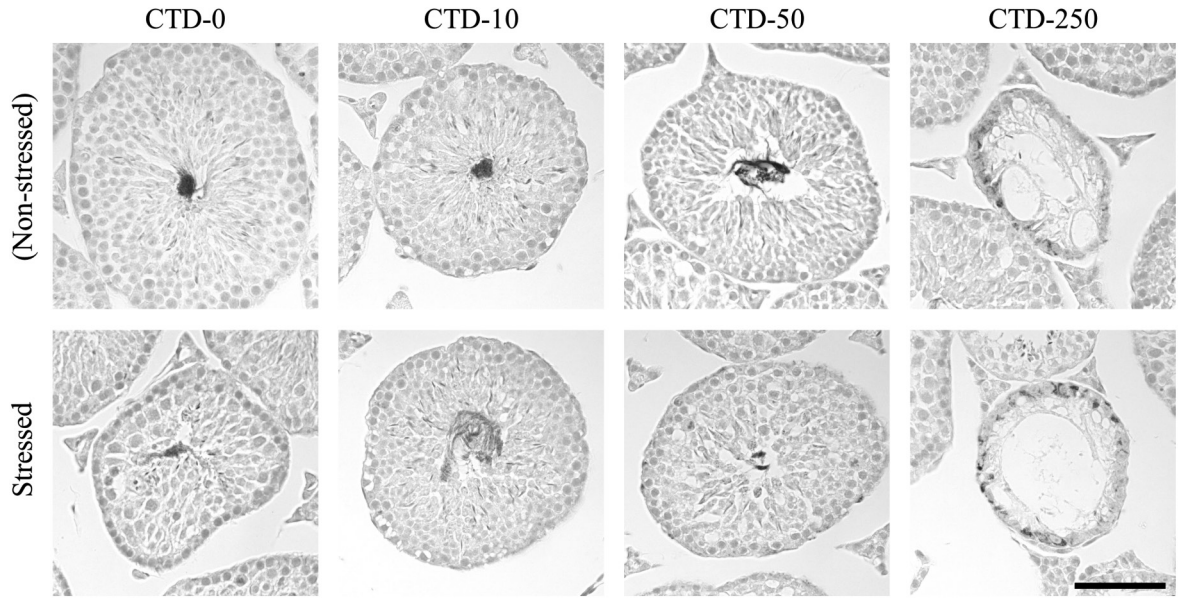

Fig. 4. Representative immunohistochemistry for GPx4 of the testis in the non-stressed and stressed groups. GPx4 immunoreactivities were detected in sperms and spermatids in the testes of the control group. In the testes of the stressed groups, reduced immunoreactivity of GPx4 in sperm was observed. In the CTD groups, spermatids showed weakened immunoreactivity of GPx4. Sertoli cells ectopically expressed GPx4 in degenerated seminiferous tubules of the CTD-50+stress group and the CTD-250 groups. Bar=100 μm.

pesticide containing CTD, but CTD itself appeared to have some repellent effects on mice. In agricultural fields, CTD-containing pesticides are used at dilutions from 1:20 to 1:4,000 for treating seeds and plants. Our purification protocol and the water solubility of CTD imply that the white precipitate resulting from the recommended concentration of CTD products is particularly dangerous for pesticide users.

In the present study, both CTD and stress concurrently affected the behavioral functioning of mature male mice. The unpredictable stress model, which is often used in the assessment of antidepressant medications, evidently raised the anxiety level in the mice. CTD dose-dependently induced anxiety alone, but there was little difference in this measure among the combined exposure groups. To the best of our knowledge, the behavioral effects of CTD in mature mice have not been previously reported.

There are only a few reports about the behavioral effects of neonicotinoids on mammals. Several parameters of behavioral developmental tests that evaluated the sense of equilibrium, coordinated movement and muscular power of offspring were changed by maternal exposure to low-dose CTD, with a sex difference [34, 35]. Thiamethoxam (TMX, 100 mg/kg/day) significantly reduced the locomotor activity of rats in an open field test and induced anxiety in an elevated plus-maze test while suppressing acetylcholinesterase (AChE) activity in three brain regions [31]. Considering that TMX is metabolized to CTD in mammals, our findings regarding the anxiogenic effects of CTD sufficiently agree with the findings of these prior studies.

Although the mechanisms of the behavioral effects of neonicotinoids remain unclear, cholinergic systems and downstream neurotransmitters are undoubtedly involved. An *in vivo* study revealed that CTD directly injected into the rat brain evoked a striatal dopamine surge through nAChRs [8]. Early types of neonicotinoids caused a subsequent desensitization of nAChRs as well as immediate neuronal excitation [20]. The behavioral consequences of chronic nicotine intake are also attributed to both the upregulation and desensitization of nAChRs [28]. The anxiety-like behaviors in several behavioral tests are part of the phenotype of nAChRs subunit α4 knockout mice [32], implying that the anxiogenic effect of CTD observed in the present study was presumably triggered by the chronic desensitization of nAChRs.

The *CHRNA7* coding α7 subunit of nAChRs contains a glucocorticoid response element [21], which may explain the decreasing nAChRs α7 expression by chronic immobilization stress [16]. These observations may support the hypothesis that the behavioral effects of CTD and stress partly share a common mechanism mediating weakened cholinergic activities, such as the desensitization of nAChRs, the suppressed level of AChE and the disturbance of the release of downstream neurotransmitters. In the CTD-250+stress groups, the stress procedure and the CTD-induced feeding suppression presumably fully suppressed the cholinergic activities, which made the anxiogenic effect of CTD invisible.

On the other hand, we found that the chronic exposure to CTD and the stress influenced the testes in different ways. The stress procedure significantly decreased the testicular weight in addition to causing the emergence of the multinucleated giant cells, and both of these are common toxicant observations in testes produced by an abnormal meiotic division of germ

cells. CTD did not show an impact on the testicular weight, whereas it dose-dependently degenerated the seminiferous epithelia. In particular, the Sertoli cell-only seminiferous tubules found in the CTD-250 groups were unexplainable by CTD-induced chronic nutritional deprivation. In the clinical field, Sertoli cell-only syndrome is known to result from partial deletions of the azoospermia factor region on the Y-chromosome, which is one of the markers of male infertility. From the toxicological perspective, germ cells are vulnerable to damage from exogenous chemicals, and they are easily phagocytized by Sertoli cells. Several endocrine-disrupting compounds (EDCs) including diethylstilbestrol and flutamide induce such a destruction of seminiferous tubules [1, 11, 40]. An increasing number of vacuolizations in seminiferous epithelia are also caused by the absence of germ cells, suggesting that they are the first target of CTD in the testes through the blood-testis barrier. This corresponds to the CTD dose-dependent increase of single-stranded DNA immunopositive germ cells observed in the testes of quails [15, 36].

GPx4 (also called phospholipid-hydroperoxide glutathione peroxidase as a member of the glutathione peroxidase family) is an antioxidant enzyme expressed in every mammalian organ. There are three types of transcripts from the same *GPx4* gene: mitochondrial, non-mitochondrial and nuclear types were identified. The mitochondrial and non-mitochondrial types in particular are expressed in spermatocytes, and GPx4 is the principal antioxidant enzymes in the testes with 30 times greater expression in the testes compared to other organs. Notably, in sperm, GPx4 is responsible for the abilities to scavenge approx. one-half of the hydrogen peroxide and 90% of the phospholipid hydroperoxides [18].

Testis-specific GPx4 knockout mice showed a phenotype of infertility with degenerated testicular tissue and morphological aberration of sperm [17]. The antigen of the polyclonal antibody used in this study is a common sequence among the three types of mouse GPx4, and thus, we could not classify the types of GPx4 immunoreactivity. The weakened GPx4 immunoreactivity in the lumen of seminiferous tubules presumably resulted from the decreases in the number and quality of the sperm.

Neonicotinoids are known to have strong oxidizing properties that affect the liver and the reproductive and central nervous systems [7, 19]. The present report is the first to describe the localized decrease of antioxidant enzymes by CTD in the testes of a mammal. Similarly, oxidative effects of psychological stress via stress hormones have been reported [23, 42]. Although a constant level of reactive oxygen species is necessary for the proliferation of spermatogonial stem cells [25], the present results also showed that oxidative mechanisms partly explain how stress impacts the reproductive function cooperatively with CTD.

Although there are no reports of plentiful expressions of GPx4 in Sertoli cells, strong ectopic expression of antioxidant enzymes was observed in Sertoli cells in degenerated seminiferous tubules in the CTD-250 groups. Because GPx4 mRNA was not expressed in Sertoli cell in rat [24], this unexpected finding may have originated from phagocyted germ cells damaged by CTD. Baek *et al*. [2] investigated whether GPx4 mRNA in the testes was altered by several EDCs, implying the measurements of oxidative conditions provide an indication of the testicular toxicity by environmental factors.

In the present study, although no remarkable interaction effect was detected, we investigated whether CTD and stress affected the behavioral and reproductive functions additively rather than synergistically in adult mice. The endogenous nAChRs agonist Lynx1 was recently reported to serve as a molecular break system for brain plasticity [26]. The developmental effects of neonicotinoids in juvenile and fetal periods remain to be determined. Neonicotinoids and their metabolites are detected at high frequency in urine samples from Japanese people [39]. Human exposure to CTD at a high concentration as high as that of the present CTD-250 groups does not occur naturally, but the precipitates that result from the low water solubility of CTD are very dangerous. In addition, a low concentration of CTD could become harmful under a stressed condition, such as fasting.

The finding that one of the metabolites of imidacloprid (IMI) seriously disturbs MAPK/ERK signaling compared to IMI itself [37] suggests that the safety coefficient (×100) for calculating the acceptable daily intake is not satisfactory. Taking the results of the present study as an example, it is clear that further research is needed to identify the toxicant mechanisms of environmental chemicals in order to protect humans and animals with high sensitivities under stressed conditions.

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