Insight into the Mechanism of Reproductive Dysfunction Caused by Neonicotinoid Pesticides

Nobuhiko Hoshi,* a Tetsushi Hirano, a Takuya Omotehara, a Junko Tokumoto, a Yuria Umemura, a Youhei Mantani, b Takashi Tanida, c Katsuhioko Warita, d Yoshiaki Tabuchi, e Toshifumi Yokoyama, a and Hiroshi Kitagawa b

*Laboratory of Molecular Morphology, Department of Animal Science, Graduate School of Agricultural Science, Kobe University; a Laboratory of Histophysiology, Department of Animal Science, Graduate School of Agricultural Science, Kobe University; 1–1 Rokkodai-cho, Nada-ku, Kobe 657–8501, Japan; b Department of Anatomy and Neurobiology, Faculty of Medicine, Kagawa University; 1750–1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761–0793, Japan; and c Division of Molecular Genetics Research, Life Science Research Center, University of Toyama; 2630 Sugitani, Toyama 930–0194, Japan.

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Neonicotinoids, which were developed in the 1990s as an insecticide having selective toxicity, were later found to cause reproductive abnormalities in experimental animals. In Japan there is an attempt to preserve endangered animals, including the Japanese crested ibis, and there is a question of whether neonicotinoids affect the reproduction of this bird, since they are used in its habitat. Hence, we investigated whether the daily oral administration of the neonicotinoid clothianidin (CTD) has any deleterious effects on the reproductive function of mature male only or both young male and female quails as experimental animals. Vacuolization and the number of germ cells having fragmented DNA in seminiferous tubules, as well as the number and size of vacuoles in hepatocytes, increased dose-dependently. The ovaries showed abnormal histology in the granulosa cells, which produce progesterone. There were significant differences in egg-laying rates and embryo weights between the groups. Glutathione Peroxidase 4 (GPx4) and Manganese Superoxide Dismutase (Mn-SOD), which protect the organism from oxidative damage, showed a dose-dependent decrease. Thus, it is possible neonicotinoids affect the bird’s reproductive system through oxidative stress, reflecting an imbalance between the production of reactive oxygen species (ROS) and a biological system’s ability to readily detoxify the reactive intermediates or easily repair the resulting damage. Responding to our study, Sado Island has since succeeded in breeding Japanese crested ibis in the wild without the use of neonicotinoids.

Key words neonicotinoid; clothianidin; oxidative stress; reproductive dysfunction; bird; Japanese crested ibis

1. INTRODUCTION

It has been known that many environmental chemicals derived from industrial products exert estrogen-like activity by binding to estrogen receptors (ERs), and these are known as endocrine disrupting chemicals (EDCs). They have been demonstrated to interfere with the synthesis, secretion, transport, binding, action or elimination of natural hormones in the body that are responsible for development, behavior, fertility and the maintenance of homeostasis.1)

We revealed that synthetic estrogen diethylstilbestrol (DES) and endogenous estrogen 17β-estradiol (E2), induce a reduction in P450sc mRNA expression in Leydig cells in inverse proportion to the dose, and that treatment with cAMP can restore the reduction in P450sc mRNA expression caused by estrogenic agents.2,3) P450sc and the steroidogenic acute regulatory protein (StAR), which play important roles in the early stages of steroidogenesis, are cAMP-responsive genes; however, these 2 genes seem to show different expression patterns against estrogenic agents.4,5) The permanent male reproductive disorder induced by neonatal exposure to DES may be more likely to result from dysfunction of the hypothalamic-pituitary axis than from dysfunction of the lower reproductive organs.5) Furthermore, DES may induce alterations in the histone modification of the steroidogenic gene in Leydig cells, and natural estrogen and synthetic estrogen compounds such as DES can induce reproductive disorders through different molecular mechanisms.6,7)

We also showed consistent and characteristic differences in gene expression patterns between DES- and E2-treated Leydig cells. The reduced expression of apoptotic cell death pathways and DNA repair capability of the DES-exposed Leydig cells imply that DES promotes carcinogenic processes more strongly than does E2. These findings suggest that the deleterious effects of DES and E2 on Leydig cells differ more substantially than previously suspected, and that the molecular events induced by synthetic and natural estrogens are significantly different, even though those hormones bind to the same receptors. We have confirmed that direct paternal exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) in the absence of...
maternal exposure might be associated with decreases in the male/female sex ratio of offspring at birth without altering litter size, a finding that is consistent with the data gathered from instances of human TCDD exposure in Seveso, Italy.8) Possible mechanisms through which TCDD might decrease the fertility potential of Y-bearing gametes before conception are discussed.8) Moreover, fetal exposure to TCDD caused stable upregulation of tyrosine hydroxylase (TH) via a novel binding motif of aryl hydrocarbon receptor (AhR), AhR-responsive element III (AHRE-III), which we identified via the signaling pathway and overgrowth of TH-immunoreactive (ir) neurons in the midbrain, implying possible involvement in the etiology of neurodevelopmental disorders such as attention-deficit/hyperactivity disorder (ADHD).9,10)

Thus, our data could serve as a basis for future follow-up investigations in the field of EDCs.

2. THE LATEST INFORMATION ABOUT NEONICOTINOID PESTICIDES IN JAPAN AND WESTERN COUNTRIES

Disruption of the endocrine system can occur in various ways. Neonicotinoids, which were developed in the 1990s, are a class of neuro-active insecticides chemically similar to nicotine. They were the most widely used insecticides because they showed reduced toxicity compared to previously used organophosphate and carbamate insecticides, according to their selective toxicity. However, some of their breakdown products are toxic, and most neonicotinoids are water-soluble and break down slowly in the environment so that they can be taken up by the plant and provide protection from insects as the plant grows. The use of some members of this class has been restricted in some countries due to evidence of a connection to honeybee colony collapse disorder (CCD), which led to the disappearance of over 30% of bees in America in 2007–2008. In response to growing concerns about the impact of neonicotinoids on CCD, the European Commission in 2012 asked the European Food Safety Authority (EFSA) to study the safety of three neonicotinoids. Recently published evidence, in three papers in Science and one paper in Nature in 2012, suggest that neonicotinoids may impact bees’ ability to forage for nectar, learn and remember where flowers are located, and possibly impair their ability to find their way home to the nest or hive, with even low levels of neonicotinoid contamination.

A study by Italian researchers, published by the Proceedings of the National Academy of Sciences of the United States of America on October 21, 2013, demonstrated that neonicotinoids disrupt the innate immune systems of bees, making them susceptible to viral infections to which the bees are normally resistant. EFSA decided to restrict the use of imidacloprid, clothianidin, and thiamethoxam for two years, beginning December 1, 2013. In March 2013, the American Bird Conservancy published a review of 200 studies on neonicotinoids, including industry research obtained through the U.S. Freedom of Information Act, calling for a ban on neonicotinoid use as seed treatment because of its toxicity to birds, aquatic invertebrates, and other wildlife. Also in March 2013, the U.S. Environmental Protection Agency (EPA) was sued by a coalition of beekeepers, as well as conservation and sustainable agriculture advocates, who accused the agency of performing inadequate toxicity evaluations and allowing registration of the pesticides to stand on insufficient industry studies.

In Japan, there is an attempt to preserve endangered animals such as the Japanese crested ibis (Nipponia nippon). However, clothianidin (CTD), a neonicotinoid, is used in habitats of this bird: rice fields and farms. Since their release into the wild began in 2008, these birds have laid eggs in the three years since, but the eggs were unfertilized. In the case of the oriental stork (Ciconia boyciana), it is thought that chicks did not hatch because spermatogenesis in the male oriental stork was inhibited by residual agricultural chemicals in weatherfish. And in the Netherlands, it has been reported that many species of birds have been decreasing since the 1990s, when the use of neonicotinoids began there; indeed, some species there have become extinct. Thus, there is a concern that neonicotinoids also affect the reproduction of birds.

3. THE MECHANISM OF REPRODUCTIVE DYSFUNCTION CAUSED BY NEONICOTINOID PESTICIDES IN BIRD

We administered CTD to mature male quails, a typical animal model of birds, by daily oral administration to investigate whether neonicotinoids have any deleterious effect on their reproductive function.

Experiment 1 (The effect of CTD on the reproductive capacity in adult male quails)

The adult male quails were randomly divided into four groups of 6 or 7 quails each, treated orally with 0, 0.02, 1 or 50 mg CTD/kg body weight (control, CTD0.02, CTD1 and CTD50). After that the males bred with untreated females to estimate the egg weights, rates of fertilization and normal development; their testes, liver and spleen were examined histologically. The number and size of vacuoles in hepatocytes (Fig. 1), and vacuolization and the number of germ cells having fragmented DNA in seminiferous tubules increased dose-dependently (Figs. 2, 3). There were no significant differences in egg weights and fertilization rates between the groups, but some eggs of the CTD1 and CTD50 groups failed to develop, and embryonic length decreased dose-dependently. The embryos from females that bred with the treated males were shorter and weighed less than embryos from control females, or embryonic development was more likely to stop altogether. It is reported that neonicotinoid causes DNA fragmentation of rat sperm through oxidative stress, and the injection of DNA-fragmented sperm into human egg cells inhibits embryo development after the eight-cell stage. In addition, it is known that injury to sperm DNA after the second meiosis is not repaired, and that DNA-fragmented sperm are found in human ejaculate. Although the ratios of abnormal sperm in humans and in experimental animals were reported to be 9.3–15.5% and 0.9–1.4%, respectively, and that of DNA-fragmented sperm in human ejaculate was reported to be 20–70%, the ratios of DNA-fragmented sperm in wild animals or in endangered animals may be higher than in humans or experimental animals. As the number of DNA-fragmented sperm increases, the fertilization rate of the sperm is thought to rise, so DNA fragmentation of sperm might affect embryo development. In light of this, CTD may inhibit or delay embryo development (growth retardation) by the fragmentation of sperm DNA through oxidative stress.12)

Thus, it was found that CTD affected the reproduction of
the male quail through the fragmentation of germ cells and the inhibition or delay of embryonic development.

Experiment 2 (The effect of CTD on the reproductive capacity in young quails)

Male and female quails (4 weeks of age) were orally administered 0, 0.01, 0.1, 1 or 10 mg CTD/kg body weight (control, CTD0.01, CTD0.1, CTD1 and CTD10: one-fifth of the dose in the experiment 1) for 6 weeks. After that the males bred with treated females to estimate the egg weights, and rates of fertilization and normal development; the testes, ovaries, liver and spleen were examined histologically. Vacuolization and the number of germ cells having fragmented DNA in seminiferous tubules (Figs. 4A, B, 5), and the number and size of vacuoles in hepatocytes increasing dose-dependently were also observed. Glutathione Peroxidase 4 (GPx4) and Manganese Superoxide Dismutase (Mn-SOD), which protect the organism from oxidative damage, showed a dose-dependent decrease (Fig. 6). In the treated group, the ovaries showed dose-dependent abnormal histology, such as pyknosis in the granulosa cells (Figs. 4C, D).

Neonicotinoids, which have been reported to affect mammals through oxidative stress, reflect an imbalance between the production of reactive oxygen species (ROS) and a biological system’s ability to readily detoxify the reactive intermediates, to easily repair the resulting damage. Its relationship to sperm damage has been studied since 1943. Spermatozoa are particularly susceptible to oxidative stress-induced damage, because their cell membranes contain large quantities of polyunsaturated fatty acids, and their cytoplasm contains low concentrations of scavenging enzymes. ROS can damage DNA by causing deletions, mutations and other lethal genetic effects. It can also damage proteins and sugars. In these consequences, ROS is thought to cause decreasing sperm motility and to harm fertilization ability, male fertility and embryo development.

Histological changes in the liver, with large areas of the degeneration in the administration groups in the present study, are similar to the results of neonicotinoid administration in chickens (thiacloprid, imidacloprid) and in rats (thiamethoxam). The liver is a vital organ with a wide range of functions, including detoxification. Because of this function, liver is also prone to many diseases. As the liver’s metabolic function is activated, it produces many more ROSs, which oxidize lipids of the hepatocellular membrane, and these oxidized lipids become lipid peroxide. As a result, polysomes were left out of the endoplasmic reticulum in hepatocytes, which then lost the ability to synthesize lipoprotein and to release endocytosed fatty acid; finally, lipid droplets in hepatocytes increased. Since similar effects were observed in the present study, we can infer that neonicotinoids probably affect the liver through oxidative stress.

In male rats, neonicotinoids decreased sperm motility and epididymal sperm concentration, and increased the abnormal sperm rate. We found that it decreased the number of germ cells and increased DNA-fragmented germ cells in seminiferous epithelium in a dose-dependent manner, but we also found normal spermatogenesis in the treatment groups and little effect on the egg weights and fertilization rates in females bred.
with the treated males. Thus, it is thought that CTD does not significantly decrease reproductive function, but may affect the reproductive system potentially by decreasing germ cells by apoptosis. In light of this, CTD may inhibit or delay embryo development (growth retardation) by the fragmentation of sperm and granulosa cell DNA through oxidative stress.

Although there was little impact on the rate of fertilization and normal development, a decline in egg-laying rates, and pyknotic altered granulosa cells were observed. The impaired granulosa cells lead to the prevention of normal oocyte maturation, as the granulosa cells, which participate in progesterone production and normal egg maturation, are closely associated with the developing oocyte in the ovary. The reduced secretion of LH (luteinizing hormone) and FSH (follicle-stimulating hormone) level and glutathione (GSH), SOD, catalase (CAT) and GPx, and an increase in lipoperoxidation were reported in the imidacloprid neonicotinoid treated female rat. This strongly indicates that these sources of ovarian damage through oxidative stress reflect an imbalance between the increase in lipoperoxidation and a decrease in antioxidants. In order to achieve oocyte maturation, it is well known that it is necessary that the egg yolk store a large amount of yolk protein and neutral fat. However, because of the identification of the lipid droplet deposition to hepatic cells in our study, it was considered that the delay or prevention of oogenesis took place due to the shortage of neutral fat in the egg yolk, which carries over to the ovary, and the result was that the rate of laying-eggs fell (Fig. 7).

This suggests the possibility that CTD affects female reproduction through oxidative stress, directly or indirectly, in oocyte or granulosa cells.

4. CONCLUSION

The present results indicate the possibility that neonicotinoids severely affect the reproductive function of quails through an increase in oxidative stress, especially in highly-

Fig. 4. Representative Histology of the Testis (A: Control) (B: CTD10) and the Ovary (C: Control) (D: CTD10) in Young Quails

The vacuolization in the seminiferous epithelia and diffusely-arranged germ cells in (B) are more apparent than in Fig. 2. Intercellular spaces and pyknotic nucleus in the granulosa cells around the oocyte are more common in the CTD10 (D) than Control (C). HE staining. Bar=50µm.

Fig. 5. The Number of Single-Stranded DNA (ssDNA) Positive Germ Cells in Seminiferous Epithelia of CTD0.1, 1 and 10 Are Significantly Larger than Those of Control (C)

Values represent the mean±S.D. Significance was set at p<0.05.

Fig. 6. The Immunoreactivity for Glutathione Peroxidase 4 (GPx4) (A: Control) (B: CTD10), and Manganese Superoxide Dismutase (Mn-SOD) (C: Control) (D: CTD10)

The immunoreactivity of GPx4 and Mn-SOD is markedly reduced in the treatment groups (B, D) compared to the control groups (A, C) in a dose-dependent decrease. Nuclear staining by hematoxylin. Bar=50µm.

Fig. 7. Effect of Treatment with CTD on Egg-Laying Rate (%)

The rate of CTD10 is significantly lower than that of Control. Values represent the mean±S.D. *Significance was set at p<0.05.
susceptible individuals (Fig. 8). Further analyses are necessary to investigate the mechanisms by which neonicotinoids lead to oxidative stress.

REFERENCES


