Effects of 3-Methyl-4-Nitrophenol in Diesel Exhaust Particles on the Regulation of Reproductive Function in Immature Female Japanese Quail (Coturnix japonica)

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Abstract. In a previous study, we found that 3-methyl-4-nitrophenol (PNMC), a component of diesel exhaust particles and also a degradation product of the insecticide fenitrothion, exhibits reproductive toxicity in the adult male Japanese quail. The present study investigated the toxicity of PNMC in the female Japanese quail and its ability to influence reproduction in immature females. The quail (21-day-old) were injected intramuscularly (im) with PNMC at doses 0.1, 1 or 10 mg/kg body weight daily for 3 days. There was no significant difference in body growth between the PNMC-administered and control birds. However, the weights of the oviducts were significantly lower in the PNMC-treated birds at all doses. Furthermore, the plasma concentrations of luteinizing hormone (LH) and estradiol-17β were significantly decreased with 1 and 10 mg/kg of PNMC. These findings suggest that PNMC might influence the hypothalamo-pituitary-gonadal axis with decreasing in secretion of GnRH, LH and ovarian steroid hormones and subsequently disturb growth of the reproductive organs of immature female quail. This study indicates that PNMC induces reproductive toxicity at the central level and disrupts reproductive function in the immature female quail.

Key words: 3-Methyl-4-nitrophenol (PNMC), Diesel exhaust particle, Immature Japanese quail (coturnix japonica), Luteinizing hormone (LH), Oviduct

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shown to be a vasodilator [13, 14] and to have estrogenic [15–17] and anti-androgenic activity [17, 18]. Furthermore, our previous studies showed that a single dose of PNMC induces significant testicular atrophy in the adult male Japanese quail [19] and that PNMC suppresses testicular function in the immature rat [20]. In addition to its presence in DEP, PNMC is a degradation product of the insecticide fenitrothion [21], a widely used pesticide with high potential for exposure by humans, livestock, and poultry in both rural and residential environments. Accumulation of PNMC from these sources could have serious effects on wildlife and human health via disruption of the endocrine and reproductive systems. However, the direct effects of PNMC on birds are still unclear as little experimental data has been reported.

The Japanese quail (Coturnix japonica) has been used extensively as a laboratory animal for reproductive toxicity testing. Quail are considered to be representative of terrestrial birds and are accepted models for assessing both the acute and chronic effects of pesticides and other chemicals in wild birds [22, 23]. Moreover, the ovaries and oviducts of immature female quail are very low in weight (less than 5% of those in sexually mature quail) and develop rapidly upon stimulation by ovarian steroidal hormones, estrogens, androgens, and progesterone [24]. The immature oviduct is especially sensitive to estrogens. Stimulation by estradiol induces rapid growth of the oviduct and induces protein synthesis essential for egg production [25]. For the above reasons, the present study used immature female quail to examine the in vivo effects of PNMC on reproductive function.

Material and Methods

Chemicals
PNMC was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). Estradiol-17β was purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA).

Birds
Female Japanese quail (21-day-old) were housed in metal cages in a controlled environment (lights on, 0500–1900 h; temperature, 23 ± 2 C; humidity, 50 ± 10%; air exchanged 20 times per hour). The quail were provided with food (Kanematsu quail diet, Kanematsu Agri-tech, Ibaraki, Japan) and water ad libitum. This study was conducted in accordance with the Guiding Principles in the Use of Animals in Toxicology and was approved by the Animal Care and Use Committee of the Japanese National Institute for Environmental Studies.

Administration of PNMC
Sexually immature female Japanese quail (21-day-old) were treated daily with an intramuscular (i.m.) injection of PNMC in phosphate buffered saline (PBS, pH 7.2) containing 0.05% (v/v) Tween 80 (0.1, 1 or 10 mg/kg body weight /0.2 ml) for 3 days. Birds were injected with vehicle (PBS containing 0.05% Tween 80) as a negative control and with 100 µg/kg of estradiol-17β in sesame oil as a positive control. The quail (n=9–10 per group) were weighed and euthanized by decapitation 24 h after the last injection. Following decapitation, blood samples were collected into heparinized plastic tubes and centrifuged at 1,700 × g for 15 min at 4 C. Plasma was separated and stored at −20 C until assayed for estradiol-17β and progesterone, and luteinizing hormone (LH). Livers, ovaries and oviducts were collected and weighed.

Determination of plasma concentrations of LH, estradiol-17β and progesterone
Plasma concentrations of LH, estradiol-17β and progesterone were determined by specific radioimmunoassay (RIA). Plasma concentrations of LH were measured with a USDA-ARS RIA kit (Beltsville, MD, USA) for chicken LH. The antiserum used was anti-avian LH (HAC-CH27-01RBP75). The hormone for iodination was chicken USDA-cLH-I-3. The antiserum against avian LH was kindly provided by the Biosignal Research Center (Institute for Molecular and Cellular Regulation, Gunma, Japan) [19, 27]. Plasma concentrations of estradiol-17β and progesterone (GDN 337) were determined using a double-antibody RIA system with 125I-labeled radioligands as described previously [28]. Briefly, the antiserum against estradiol-17β (GDN 244) and progesterone (GDN 337) [29] were kindly provided by Dr. G. D.
Niswender (Colorado State University, Fort Collins, CO, USA). The intra- and inter-assay coefficients of variation were 4.8 and 5.8% for estradiol-17β and 6.9 and 11.2% for progesterone, respectively.

Statistical analysis
All data are presented as means ± SEM. Statistical analysis was performed using one-way analysis of variance followed by Fisher’s protected least significant difference test (Fisher’s PLSD). The StatView 5.0 software was used for statistical analysis (SAS Institute, Cary, NC, USA). The criterion for significance was P<0.05.

Results

Effects of PNMC on organs and body weights
The body weights and weights of livers, ovaries, and oviducts of the female birds are shown in Table 1. Administration of PNMC and estradiol-17β for 3 days did not cause significant changes in body weight or weights of livers or ovaries in the immature female quail. There were significant increases (P<0.05) in the oviduct weights of the estradiol-17β-treated birds. However, there was a significant decrease (P<0.05) in the oviduct weights of all birds treated with PNMC compared with the control birds (Table 1).

Effects of PNMC on the plasma concentrations of LH, estradiol-17β, and progesterone
The plasma concentrations of LH, estradiol-17β, and progesterone of the PNMC- and estradiol-17β-treated birds are shown in Fig. 1. The plasma concentrations of LH were significantly lower (P<0.05) in the 1 and 10 mg/kg PNMC- and estradiol-17β-treated groups, compared with the control group (Fig. 1A). Furthermore, the plasma concentrations of estradiol-17β were also significantly low (P<0.05) in the groups treated with 1 and 10 mg/kg PNMC compared with the control group (Fig. 1B). On the other hand, the estradiol-17β-treated group showed significantly high levels of estradiol-17β (Fig. 1B). The plasma concentrations of progesterone showed no changes in the PNMC-treated birds (Fig. 1C), but significantly high levels of progesterone were seen in the estradiol-17β-treated birds compared with the controls (Fig. 1C).

Discussion
To our knowledge, this is the first report demonstrating that PNMC, a specific component of DEP, induces reproductive functional impairment in a female avian model. In the present study, administration of PNMC significantly decreased the plasma concentrations of LH and estradiol-17β in immature female Japanese quail. These results suggest that the PNMC in DEP probably acts on the hypothalamus-pituitary axis to reduce gonadotropin releasing hormone (GnRH) and/or LH release and that the decrease in the plasma levels of estradiol-17β may be due to a reduction in circulating LH.

We have previously reported that PNMC, a nitrophenol derivative compound isolated from DEP, possesses estrogenic activity in vivo and in vitro [15–17]. We hypothesized that PNMC would stimulate growth of the immature oviduct by direct activation of the estrogen receptor. However, the present results show a decrease in the weight of the oviduct as a result of a decrease in the plasma concentration of estradiol-17β. The plasma concentrations of LH were decreased by PNMC,
implying that PNMC has a suppressive effect on the secretion of LH from the pituitary gland. Our results show that there is a difference in sensitivity to estrogenic activity between that at the hypothalamus-pituitary level and that at the oviduct level; the hypothalamus and pituitary are far more sensitive. The weak estrogenic activity of PNMC causes suppression of LH but fails to maintain the weight of the oviduct. Therefore, the present findings suggest that PNMC possesses estrogenic activity on the hypothalamus-pituitary axis of the immature quail.

Our results have great significance in regard to environmental issues. Remarkable amounts of DEP are exhausted into the air in many countries. In Japan, 58,902 tons [30] are emitted each year, and this is an amount that cannot be ignored. One kilogram of DEP contains 28 mg of PNMC [13, 14], however, the exact concentration of PNMC in the environment remains unknown. PNMC is also known as a degradation product of fenitrothion [21], which is a widely used insecticide that is accumulating in the air, soil and water in many countries [31, 32]. According to the data submitted to the pollutant release and transfer registers, the amount of fenitrothion emitted into the environment in 2002 in Japan was approximately 1,300 tons; roughly half of this amount degrades into PNMC [33]. Rainwater in Roskilde (Denmark) contained as much as 2483 ng/l of PNMC [34]. These findings clearly indicate that PNMC is present in large amounts in the environment (from diesel exhaust and fenitrothion used on farms), including in rainwater, and may have serious effects on wildlife and human beings.

In conclusion, the present study shows that PNMC impairs reproductive function in the immature female Japanese quail probably through toxic effects on the hypothalamus and pituitary. Our findings suggest that PNMC may have suppressive effects on reproductive function in humans, domestic animals, and wildlife.

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![Plasma concentrations of LH (A), estradiol-17β (B), and progesterone (C) in immature female Japanese quail treated for 3 days with PNMC (0.1, 1 or 10 mg/kg daily, intramuscular) or estradiol-17β (100 µg/kg) (E2). Each bar represents the mean ± SEM of 9 to 10 birds per group. *P<0.05 compared with control rats (Fisher’s PLSD test).](image)
providing antiserum against chicken LH. This study was supported in part by a Grant-in-Aid for Scientific Research (Basic research C-17510052, B-1831004, P07582) from the Japan Society for the Promotion of Science and the 21st Century Center-of-Excellence Program (E-1) from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a Sasakawa Scientific Research Grant from The Japan Science Society (16–278 and 18–348).

References

22. OECD (Organization for Economic Cooperation and Development).


