Effects of 3-Methyl-4-nitrophenol on the Reproductive Toxicity in Female Japanese Quail (*Coturnix japonica*)

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We previously showed that 3-methyl-4-nitrophenol (4-nitro-*m*-cresol, PNMC), a component of diesel exhaust particles and a degradation product of the insecticide fenitrothion, has reproductive toxicity in adult male and immature female Japanese quail (*Coturnix japonica*). Here we investigated effects of PNMC on the reproductive toxicity of mature female Japanese quail. The experiment consists of 3 periods of pretreatment, treatment, and post-treatment for 5 d each. The birds were reared, bred naturally for 1 week, and after 5 d of pretreatment, then injected intramuscularly with PNMC at doses 1, 10, or 100 mg/kg body weight daily for 5 d. Body weight, egg weight, and hatchability did not differ among the observation periods. However, at all doses of PNMC, the egg-laying rate showed a modest decrease during the treatment period, with recovery during the post-treatment period. Plasma concentrations of luteinizing hormone (LH) and estradiol-17 β , were significantly decreased ($p < 0.05$), and plasma concentrations of progesterone significantly increased ($p < 0.05$) in birds treated with 10 and 100 mg/kg PNMC. These results suggest that PNMC have acute toxicity, and inhibited LH secretion, disturbing egg-laying in mature female quail. Our findings indicate that PNMC induces endocrine malfunction at the central level and subsequently disrupts reproductive processes in mature female quails.

Key words 3-methyl-4-nitrophenol; diesel exhaust particle; reproductive toxicity; mature Japanese quail

Endocrine-disrupting chemicals in environmental pollutants and pesticides cause mortality and reproductive abnormalities in wild birds, including abnormalities of reproductive organs and impairment of egg-laying.¹⁾ 3-Methyl-4-nitrophenol (4-nitro-*m*-cresol, PNMC), a component of diesel exhaust particles (DEP), is a vasodilator^{2,3)} and has estrogenic^{4–6)} and anti-androgenic^{6,7)} activity. Furthermore, our previous studies showed that PNMC induced marked testicular atrophy in adult male Japanese quail and significantly decreased the plasma concentrations of LH and estradiol-17 β in immature female Japanese quail,^{8,9)} as well as suppressing testicular and adrenal function in immature rats.^{10,11)} In addition to its presence in DEP, PNMC is a degradation product of the insecticide fenitrothion,¹²⁾ a widely used pesticide with high exposure potential to humans, livestock, and poultry, in both rural and residential environments. The accumulation of PNMC from these sources could have profound effects on wildlife and human health through disruption of the endocrine and reproductive systems.

Japanese quail is extensively used in reproductive toxicity testing. Quail are representative of terrestrial birds and are accepted models for assessing both the acute and chronic effects of pesticides and other chemicals in wild birds.^{13,14)} Because no information concerning the effects of PNMC on reproductive toxicity in mature female birds is available, we here used mature female quail to examine the *in vivo* toxic effects of PNMC on plasma concentrations of progesterone, estradiol-17 β , basal luteinizing hormone (LH), egg-laying rate, and hatchability.

MATERIALS AND METHODS

Chemicals 3-Methyl-4-nitrophenol (4-nitro-*m*-cresol; PNMC) was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan).

Birds Japanese quail (*Coturnix japonica*) came from L (low antibody response)-selected lines,^{15,16)} of which chicks hatch after 17 d of incubation, and birds were provided with food (Kanematsu quail diet, Kanematsu Agri-tech, Ibaraki, Japan) and water *ad libitum*. Female birds (age, 6 month) were housed in metal cages in a controlled environment (lights on, 0500–1900 h; temperature, 23 $\pm$ 2 $^{\circ}$ C; humidity, 50 $\pm$ 10%; air exchanged 20 times hourly). 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.

Experimental Design Adult female Japanese quail were reared, naturally bred for 1 week, separated from male quail, and allocated into four groups. After 5 d of pretreatment, each group of birds was intramuscularly injected with PNMC at 1, 10 or 100 mg/kg body weight daily for 5 d. The LD₅₀ of PNMC in the adult male quail was 135 mg/kg.⁸⁾ Controls were injected with vehicle only (PBS containing 0.05% Tween 80). The experimental period was separated into pretreatment, treatment, and post-treatment periods, each of which was 5 d long. Eggs were collected daily during the 5-d pretreatment, treatment, and post-treatment periods, numbered, and weighed. The collected eggs were incubated under controlled conditions of 37.8 $^{\circ}$ C and 60% relative humidity

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with turning once each hour, and hatchability was measured at the 18th day of incubation. The maternal female quail ($n=6$ to 10 per group) were weighed and killed by decapitation at the morning of the end of experiment. After decapitation, blood samples were collected in heparinized plastic tubes and centrifuged at $1700 \times g$ for 15 min at 4°C . Plasma was separated and stored at -20°C until it was assayed for estradiol-17 β , progesterone, and LH. The liver, kidney, spleen, ovary, and oviduct were collected and weighed.

Radioimmunoassay Plasma concentrations of LH were measured with a USDA-ARS RIA kit (Beltsville, MD, U.S.A.) for chicken LH. The antiserum used was anti-avian LH (HAC-CH27-01RBP75). The hormone for iodination was chicken USDA-cLH-I-3. The results are expressed in terms of USDA-cLH-K-3. The intra- and inter-assay coefficients of variation were 5.2% and 11.2%, respectively. USDA-cLH-I-3 and USDA-cLH-K-3 were kindly provided by Dr. John A. Proudman (Biotechnology and Germplasm Laboratory, Animal and Natural Resources Institute, Beltsville, MD, U.S.A.).¹⁷⁾ The antiserum against avian LH was kindly provided by the Biosignal Research Center of Institute for Molecular and Cellular Regulation (Gunma, Japan).^{8,18)} Plasma concentrations of estradiol-17 β and progesterone were determined with a double-antibody RIA system using ^{125}I -labeled radioligands as described previously.¹⁹⁾ The antisera against estradiol-17 β (GDN 244) and progesterone (GDN 337)²⁰⁾ were kindly provided by Dr. G. D. Niswender (Colorado State University, Fort Collins, CO, U.S.A.). The intra- and inter-assay coefficients of variation were 4.8% and 5.8% for estradiol-17 β , and 6.9% and 11.2% for progesterone, respectively.

Statistical Analysis All data are presented as mean $\pm$ S.E.M. Statistical analysis was performed by one-way analysis of variance followed by Dunnett's multiple comparison test (StatView 5.0, SAS Institute, Cary, NC, U.S.A.). The criterion for significance was $p < 0.05$.

RESULTS

Effect of PNMC on Organs and Body Weight An acute toxicologic response occurred in highest dose of 100 mg/kg PNMC treated group, of the 10 birds treated with 100 mg/kg PNMC, 4 died within 10 min of treatment. No birds died in the 1 and 10 mg/kg of PNMC or control groups. The surviving birds in all treatment groups grew normally, with no differences in body weights during the pretreatment, treatment, and post-treatment periods (data not shown). Daily injection of PNMC for 5 d did not change weights of the liver, kidney, and spleen (Table 1). PNMC at 100 mg/kg

tended to decrease the weights of the ovary and oviduct, but not significantly (Table 1).

Effect of PNMC on Egg-Laying and Hatchability The egg-laying rate and egg weights of PNMC-treated birds during the pretreatment, treatment, and post-treatment periods are shown in Table 2. Egg weights did not change during any period, but PNMC treatment decreased the egg-laying rate

Table 1. Organ Weights of PNMC-Treated Mature Female Japanese Quail

	Dose of PNMC (mg/kg)			
	0	1	10	100
No. of birds	8	10	10	6
Liver (g)	3.487 ± 0.089	3.825 ± 0.219	4.180 ± 0.287	3.926 ± 0.477
Kidney (g)	1.631 ± 0.072	1.508 ± 0.069	1.627 ± 0.080	1.580 ± 0.042
Spleen (mg)	66 ± 10	64 ± 7	76 ± 6	59 ± 11
Ovary (g)	5.396 ± 0.625	6.241 ± 0.370	5.770 ± 0.241	4.322 ± 0.665
Oviduct (g)	6.162 ± 0.242	6.713 ± 0.404	6.790 ± 0.311	5.958 ± 0.427

The values are expressed as mean $\pm$ S.E.M.

Fig. 1. Plasma Concentrations of LH (A), Estradiol-17 β (B), and Progesterone (C) in Mature Female Japanese Quail Treated for 5 d with PNMC (0, 1, 10, or 100 mg/kg Daily, Intramuscularly)

Each bar represents the mean $\pm$ S.E.M. of 6 to 10 birds per group. * $p < 0.05$ compared with value for control quail (Dunnett's multiple comparison test).

Table 2. Egg Weight and Egg-Laying Rate of PNMC-Treated Mature Female Japanese Quail during the Pretreatment, Treatment, and Post-treatment Periods

Dose of PNMC (mg/kg)	Egg weight (g)			Egg laying rate (no. eggs/5d)		
	Pretreatment	Treatment	Post-treatment	Pretreatment	Treatment	Post-treatment
0	8.8 ± 0.17	9.0 ± 0.18	9.2 ± 0.35	0.57 ± 0.12	0.48 ± 0.10	0.63 ± 0.09
1	9.2 ± 0.19	9.8 ± 0.25	9.6 ± 0.16	0.60 ± 0.09	0.40 ± 0.11	0.66 ± 0.11
10	9.5 ± 0.17	9.6 ± 0.19	9.9 ± 0.20	0.78 ± 0.11	0.50 ± 0.05	0.52 ± 0.10
100	9.8 ± 0.20	9.4 ± 0.45	9.5 ± 0.49	0.80 ± 0.09	$0.32 \pm 0.10^*$	0.70 ± 0.10

The values are expressed as mean $\pm$ S.E.M. * $p < 0.05$ compared with the value for pretreatment and post-treatment birds (Dunnett's multiple comparison test).

Table 3. Egg Hatchability of PNMC-Treated Mature Female Japanese Quail during the Pretreatment, Treatment, and Post-treatment Period

Dose of PNMC (mg/kg)	Hatchability (%)		
	Pretreatment	Treatment	Post-treatment
0	47.8 (11/23)	57.9 (11/19)	56.0 (14/25)
1	70.0 (21/30)	65.0 (13/20)	54.5 (18/33)
10	61.5 (24/39)	64.0 (16/25)	80.8 (21/26)
100	57.7 (15/26)	58.3 (7/12)	71.4 (15/21)

The number of eggs that hatched/total number of eggs is given in parentheses.

throughout the treatment period, with recovery during the post-treatment period. And the egg-laying rates were significantly lower ($p < 0.05$) in the treatment period of group treated with 100 mg/kg PNMC compared with the value for pretreatment and post-treatment birds (Table 2). Hatchability was similar at all doses of PNMC throughout the experiment (Table 3).

Effect of PNMC on Plasma Concentrations of LH, Estradiol-17 β , and Progesterone Plasma concentrations of LH and estradiol-17 β were significantly lower ($p < 0.05$) in the groups treated with 10 and 100 mg/kg PNMC than controls (Figs. 1A, B). In contrast, plasma concentrations of progesterone were significantly increased ($p < 0.05$) in the groups treated with 10 and 100 mg/kg PNMC compared with controls (Fig. 1C).

DISCUSSION

In the present study, administration of PNMC significantly decreased plasma concentrations of LH and estradiol-17 β , significantly increased plasma concentrations of progesterone, and decreased the egg-laying rate of mature female Japanese quail. The present results suggest that PNMC may have toxic effects on the hypothalamic-pituitary axis to reduce LH secretion from the pituitary gland. The decrease in circulating levels of estradiol-17 β may be due to reduced circulating LH.

An acute toxicologic response occurred in adult birds treated with the highest dose (100 mg/kg) of PNMC, and affected birds displayed signs of dyspnea and tremor prior to death. These results suggest that PNMC causes acute toxicity and death, possibly by a rapid decrease in blood pressure followed by ischemic shock. This proposed mechanism is consistent with the reports of the potential vasodilatory activity of PNMC.^{2,3} However, none died in rats treated with the high dose of PNMC (100 mg/kg) in our previous studies. These results suggest that the sensitivity to PNMC of the quail and the rat is different and that PNMC has a high acute toxicity to the quail. In addition, it is known that PNMC inhaled by the body is metabolized by uridine diphosphate glucuronosyltransferase (UGT) and sulfotransferase,²¹ and Lee *et al.* have demonstrated that UGT activity for PNMC in quail is less than that in the rodents.²² These results suggest that PNMC metabolism is less efficient in the quail compared to the rodents, so that quails are more inclined to accumulate PNMC within the body, and in resulting with the higher toxicity than the rodents.

city than the rodents.

PNMC treatment decreased the egg-laying rate of adult female quail, but the rate recovered during the post-treatment period. Previous studies have shown that PNMC has estrogenic activity *in vivo* and *in vitro*.^{4,6} Estrogen is required for normal differentiation and development of the oviduct in female birds.²³ However, exposure to excessive estrogen can cause oviduct abnormalities and impaired egg-laying.²⁴ Furthermore, PNMC decreased plasma concentrations of LH in the present study, suggesting that negative-feedback has occurred due to PNMC. In contrast, here we found that administration of PNMC significantly increased plasma concentrations of progesterone, and PNMC treatment of H295R cells also increases progesterone levels.²⁵ We suggest that the estrogenic effects of PNMC are involved in the impairment in egg-laying of PNMC-treated adult female quail.

In conclusion, PNMC has toxic effects on the reproductive system of mature female Japanese quail through toxic effects on the hypothalamus and/or pituitary. Therefore the vast amounts of PNMC in the environment may have profound deleterious effects on the reproduction of wildlife, especially birds, and humans.

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