Studies on Endocrine Disruptors Contained in Diesel Exhaust Particles

（ディーゼル排気微粒子中に含まれる内分泌撹乱化学物質に関する研究）

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Chapter 1. Introduction

1-1. Air pollution and diesel exhaust

Pollution is becoming an important public health problem and a political issue, due to the rapid growth in world population and the increasing world-wide migration from rural to urban areas. Recent United Nations estimates have indicated that 47% of the global population is living in urban areas. This urbanization has brought about an increasing need for transportation and hence an increase in motor vehicle generated air pollutants. There is growing international concern regarding the adverse health effects of air pollution. In Japan, more than seventy two million vehicles are officially registered (excluding motorcycles). In addition, the motor vehicle generated air pollutants, diesel exhaust particles (DEP) account for a highly significant percentage of the particles emitted in many towns and cities. Furthermore, the atmosphere in urban areas contains a large amount of vapor and solid phase pollutants. The majority of the solid phase pollutants are suspended particulate matter (SPM), which may have adverse effects on human health. Recently, the health significance of fine particles in SPM, such as particles smaller than 2.5 μm in aerodynamic diameter (PM2.5) and nanoparticles have been receiving increased attention. Most of the fine particles in urban areas are DEP (the majority of DEP are in the range of 0.02-0.5 μm). Many countries exhaust vast amounts of DEP into the atmosphere. For example, in Japan 58902 tons (39), in the United States of America 111530 tons (111), in England (UK) 37000 tons (3), and in EURO, the highest of 240000 tons (81) are emitted each year and this is an amount that can not be ignored.

DEP contain a vast number of organic constituents and is considered as containing significant environment compounds including polyaromatic hydrocarbons, nitro aromatic hydrocarbons, heterocycles, quinones, aldehydes, and aliphatic hydrocarbons (9, 17, 92, 93). The DEP have several hazardous effects on human health,

including lung cancer (37, 58), allergic rhinitis (64, 98), and bronchial asthma-like disease (61, 91). Moreover, DEP has endocrine-disrupting properties and potential adverse effects on both male and female reproductive functions. Diesel exhaust suppresses spermatogenesis in adult mice (122) and rats (109, 117). Furthermore, pregnant C57BL mice injected with DEP extracts showed significant increase in abortion rate and uterine weight (110). These *in vivo* findings show that DEP contain compounds that have modulated estrogenic and anti-androgenic activities. *In vitro* studies similarly have shown that DEP possess estrogenic, anti-estrogenic, and anti-androgenic activities (45, 59, 75, 99).

1-2. Isolation and identification of nitrophenols from diesel exhaust particles (DEP)

Although DEP contain carbon nuclei which absorb numerous and diverse chemicals, the specific compound(s) responsible for phenomena such as suppressed spermatogenesis and increased abortion rate and uterine weight remain unclear. To address this question, Dr. Akira K Suzuki and his coworkers recently isolated four nitrophenol derivatives from DEP, namely 4-nitrophenol (PNP), 2-methyl-4-nitrophenol, 3-methyl-4-nitrophenol (PNMC), and 4-nitro-3-phenylphenol from DEP and showed that they had vasodilatation, estrogenic and anti-androgenic activity (Fig. 1-1). The 1 kg DEP contains 28 mg PNMC and 15 mg PNP (62, 100). In addition to its presence in DEP, PNMC is a degradation product of the insecticide fenitrothion (34) (Fig. 1-2), which is widely used in many countries. Also, PNP is a degradation product of the insecticides parathion and methylparathion (43) (Fig. 1-3), which are not currently in use in Japan but are still used worldwide in countries such as China, as fumigants, acaricides, and pre-harvest soil and foliage treatments for a wide variety of crops, both outdoors and in greenhouses. The accumulation of PNMC and PNP in air (69), soil (67), and water (5) may have serious deleterious effects on

wildlife and human health through disturbance of endocrine and reproductive systems. Therefore, it is an important aim of the current study to clarify the effects of PNMC and PNP isolated from DEP on wildlife and human health.

1-3. Hypothalamic-Pituitary-Gonadal axis (HPG) (Fig. 1-4)

The physiology of reproduction centers on the HPG axis. The HPG axis is composed of the hypothalamus and its neural connections with the rest of the brain, the pituitary, and the testis (male) or ovary (female). The classic view of this axis is that the anterior hypothalamus and preoptic area represent the regions that are responsible for the synthesis of the peptide gonadotropin-releasing hormone (GnRH), which is the primary regulator of the pituitary gonadotrophs. Axons arising from these hypothalamic nuclei project toward the medial basal hypothalamus and into the outer zone of the median eminence (ME). From the ME, GnRH reaches the anterior pituitary via hypothalamo-hypophyseal portal system and stimulates gonadotropic cells to release luteinizing hormone (LH) and follicle stimulating hormone (FSH) into the general circulation. LH and FSH bind to specific receptors in the ovary and testis and regulate gonadal function by stimulating gametogenesis and production of steroid hormones. In the male, LH binds to specific membrane receptors on Leydig cells of the testis, which leads to generation of cAMP and other messengers that ultimately cause the secretion of androgens (testosterone). LH in combination with FSH is required for maturation of spermatozoa. FSH stimulates testicular growth and increases production of androgen-binding protein by Sertoli cells. Androgen-binding protein concentrates testosterone near the sperm, enabling normal spermatogenesis. Inhibin is a glycoprotein hormone secreted from Sertoli cells and is now known to be one of the important gonadal hormones which regulate the secretion of FSH (14). Within the testis, inhibin production is stimulated by FSH and acts by negative feedback at the level of the pituitary to decrease FSH secretion. In females, inhibin decreases

secretion particularly of the FSH of the anterior pituitary and even LH. LH stimulates ovarian production of estrogen and progesterone. LH surges midway in the estrous cycle are responsible for ovum maturation and ovulation, and sustained LH secretion stimulates the corpus luteum to produce progesterone. FSH exerts primary control over development of the ovarian follicle, and both FSH and LH are responsible for follicular secretion of estrogen. This axis normally functions in a tightly regulated manner to produce at optimal circulating steroids for development of secondary sexual characters, spermatogenesis and maintenance of fertility.

1-4. Japanese quail *(Coturnix japonica)*

The Japanese quail (*Coturnix japonica*) was originally domesticated in Japan around the 11th century as a pet song bird (118). Nowadays, this poultry is commercially raised for egg production in East Asian countries including Japan, and for meat production in Western European countries such as Spain and France. The Japanese quail as a laboratory animal began in the late 1950s, because of its adaptability to battery breeding cages, small body size, early sexual maturation, short generation interval (16-17days), regular egg laying and high egg production (200-300 eggs a year) (118), it has been used extensively in research.

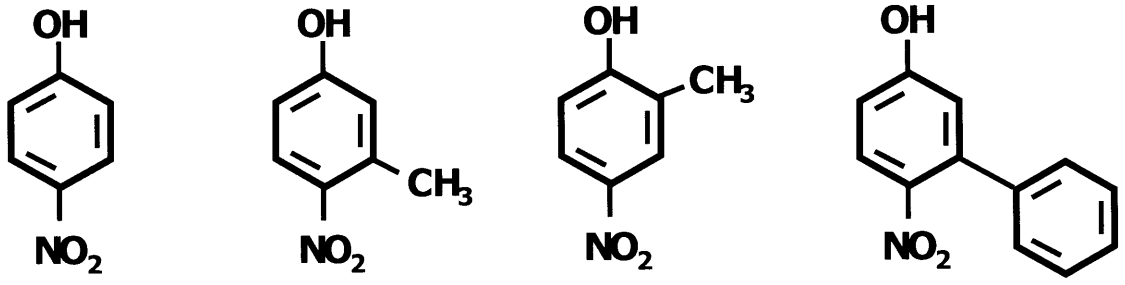
The Japanese quail can be sexed as early as three weeks of age, based on the feather color the females which can be easily identified by the slightly whiter plumage under the throat and upper breast, different from the characteristically black stippled feathers of the male (Fig. 1-5). The plumage color on the throat and breast is cinnamon or rusty brown. Males generally live longer than females. When matured, the weight of males is in the range of 100-140 grams, and they reach sexual maturity at 5 to 6 weeks of age. When males are sexually matured, a large glandular or bulbous structure appears above the cloacal opening. The cloacal gland, which is situated at the posterior end of the cloaca, is well developed in sexually mature male quails and

rudimentary in females. This gland is an androgen-dependent structure (90) and produces foam that is transferred to the female at copulation, which is hypothesized to help maintain sperm in the cloaca of the female and in this way improve egg fertilization. Growth of the cloacal gland can be induced in females by treatment with testosterone but they never reach the same size as in males (2). The cloacal gland becomes demasculinized by embryonic estrogen exposure (1) and the gland of in ovo estrogen treated males does not respond to androgens to the same extent as the gland of normal males. Male quail testes are located in the abdominal cavity behind the kidney. Furthermore, quail testes show a seasonal increase in size, which is mainly due to an increase in the size of the seminiferous tubules associated with sperm production, as well as increase in the number of interstitial cells, which are responsible for the production of testicular hormones. These changes are triggered by increasing day length under the influence of gonadotropins. The adult female Japanese quail is generally larger than the male, weighing about 120-160 grams, and they start laying eggs as early as 6 weeks of age. In the quail, as in most avian species, only the left gonad develops into the ovary. The left ovary is attached by the mesovarian ligament at the cephalic end of the left kidney. A unique feature of the avian reproduction system is the unilateral development of the ovary and oviduct in the female embryo. The right ovary grows slowly in the 8- to 10-day-old embryo, becoming a tiny rudiment in the 15 day-old embryo (12).

The Japanese quail as a laboratory animal has been extensively used in reproductive toxicity testing. Quail are considered to be representative of terrestrial birds and accepted models for assessing both the acute and chronic effects of pesticides and other chemicals in wild birds (19, 71), because spermatogenesis is well characterized (55) and the cloacal gland is a good marker of gonadal development (76).

1-5. Objectives of the present study

The present study focuses on clarifying the adverse effects induced by 3-methyl-4-nitrophenol (PNMC) and 4-nitrophenol (PNP) isolated from DEP on the reproductive function in male and female avian (quail) and mammalian (rat) animal models.



4-nitrophenol (PNP) | 3-methyl-4-nitrophenol (PNMC) | 2-methyl-4-nitrophenol | 4-nitro-3-phenylphenol

Fig. 1-1 Chemical structure of *P*-Nitrophenols isolated from DEP

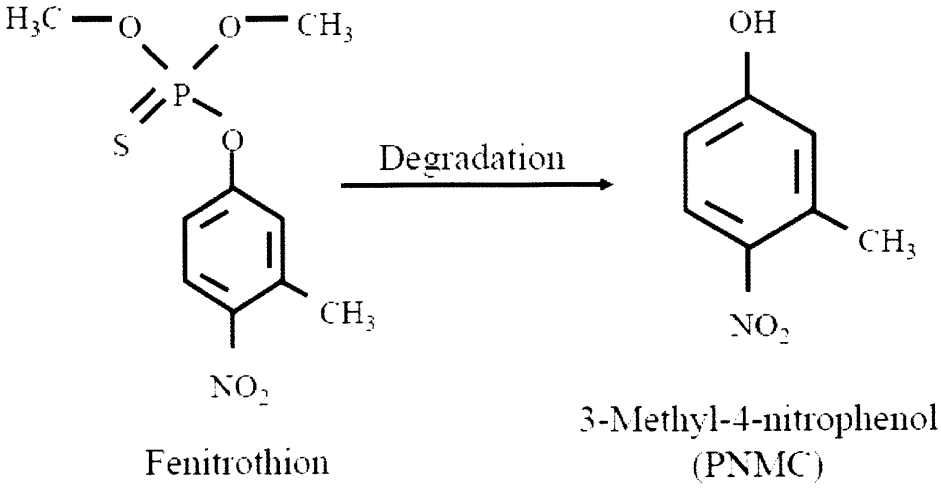


Fig. 1-2 Chemical structure of 3-methy-4-nitrophenol (4-nitro-*m*-cresol, PNMC), a component of DEP and a degradation product of the insecticide fenitrothion.

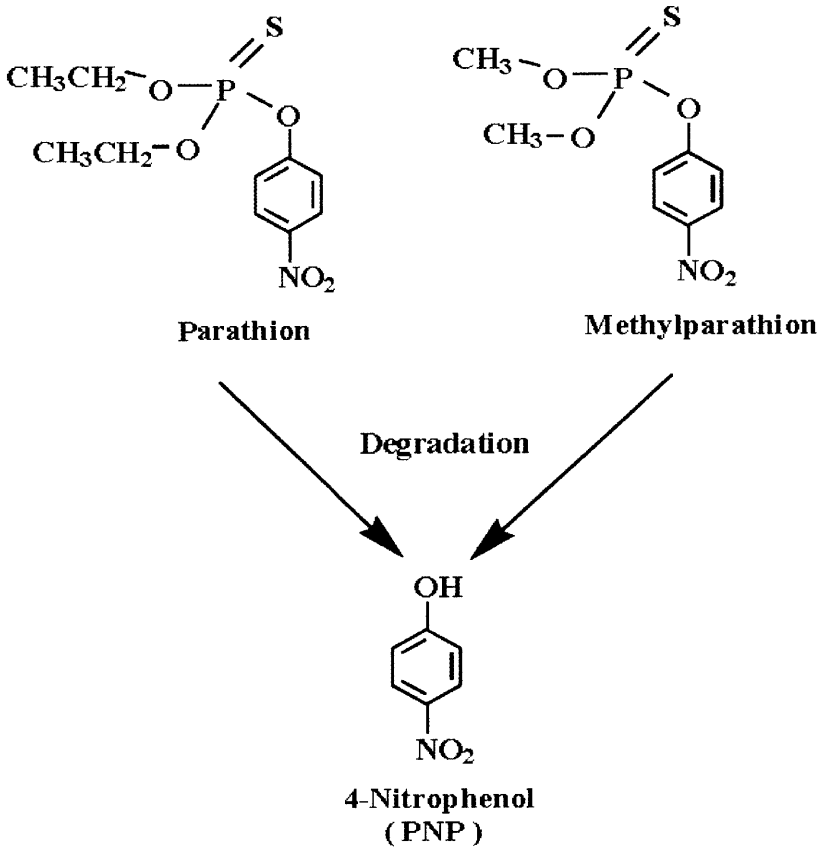


Fig. 1-3 Chemical structure of 4-nitrophenol (*p*-nitrophenol, PNP), a component of DEP and a degradation product of the insecticide parathion.

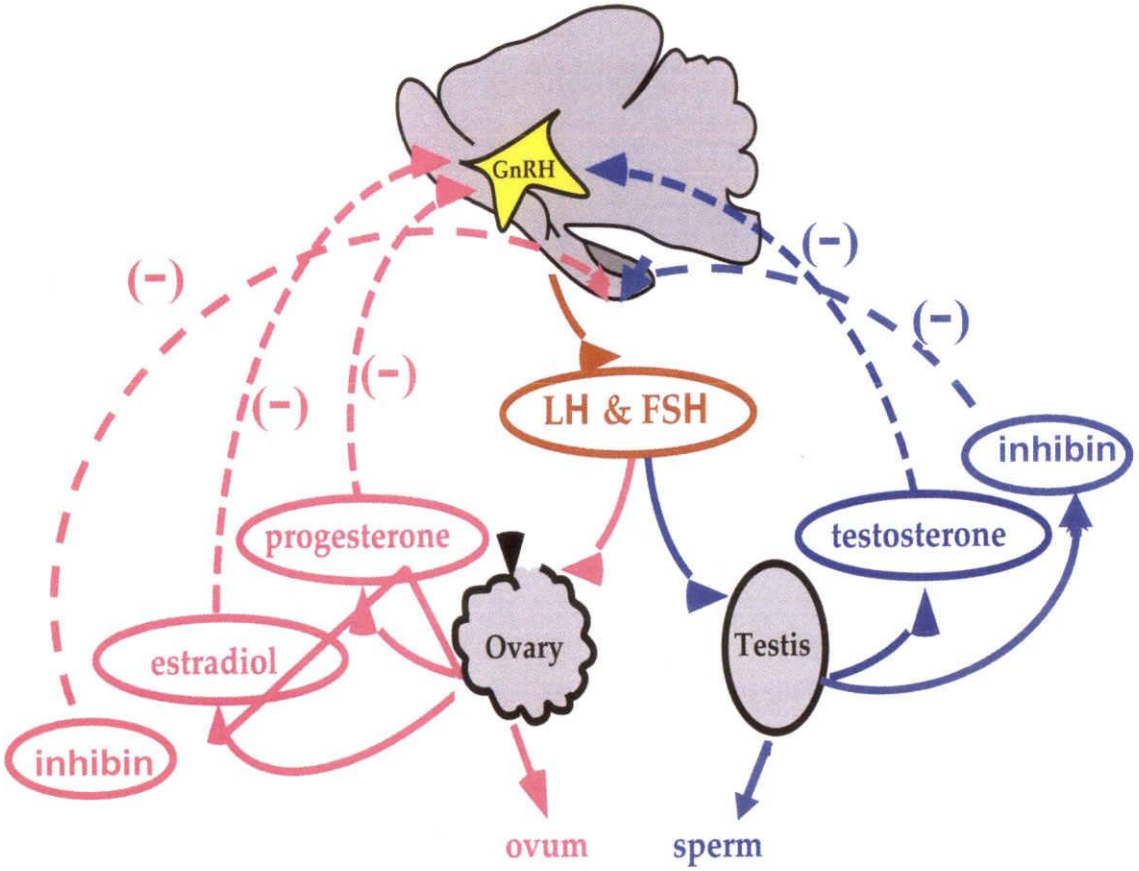


Fig. 1-4 Hypothalamic-Pituitary-Gonadal axis (HPG)

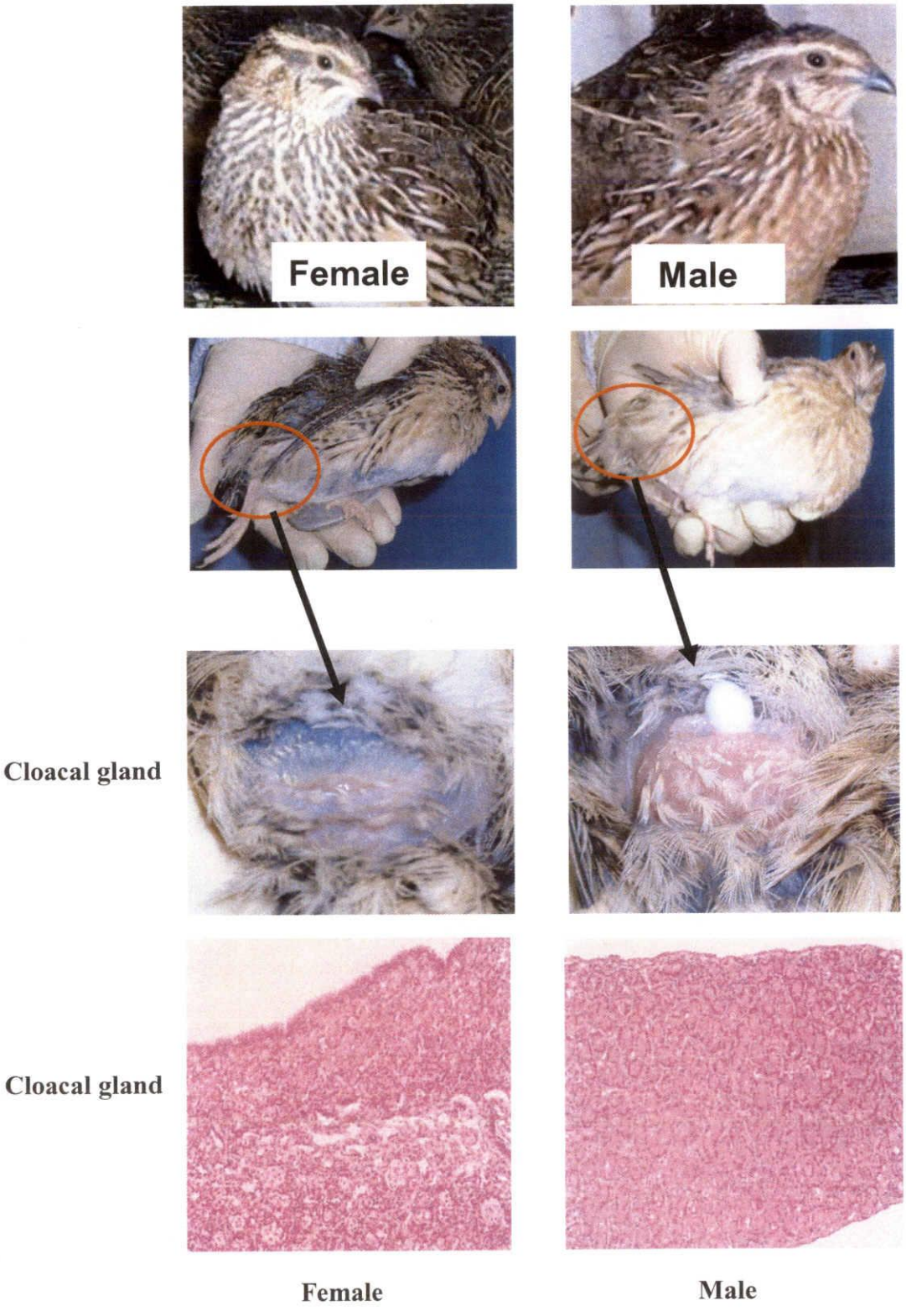


Fig. 1-5 Japanese quail *(Coturnix japonica)*

Chapter 2. General Materials and Methods

2-1. Animals

All experimental procedures were carried out in accordance with the Guiding Principles in the Use of Animals in Toxicology, and were approved by the Animal Care and Use Committee of the Japanese National Institute for Environmental Studies. The experimental animal of the present study were healthy Japanese quail (*Coturnix japonica*) and rats (Wistar-Imamichi) raised under controlled environment (lights on 0500 to 1900 h, temperature 23 ± 2 °C, humidity 50 ± 10%, and air exchanged 20 times hourly) in Japanese National Institute for Environmental Studies.

2-2. Histology

Tissue samples were immediately fixed in 4% paraformaldehyde (Sigma Chemical Co., St. Louis, MO, USA) in 0.05 M phosphate buffered saline (PBS), pH 7.4, and embedded in paraffin. The paraffin-embedded tissue blocks were serially sectioned at 6 μm thickness and placed on poly-L-lysine (Sigma Aldrich Co., St. Louis, MO, USA) coated slide glasses (Dako Japan Co., Kyoto, Japan). The preparation of tissues for histology is shown in Fig. 2-1.

2-3. Immunohistochemistry

After tissue sections were deparaffinized with xylene, they were subjected to antigen retrieval by autoclaving in 0.01M sodium citrate buffer (pH 6.0) at 121 °C for 15 min. The sections were then incubated in 0.3% H_2O_2 in methanol at room temperature for 1 h, followed by incubating with block solution of 0.5% casein-Tris saline (CTS) (0.05 M Tris-HCl with 0.15 M NaCl, pH 7.6; CTS) at 37 °C for 1 h to quench nonspecific staining. Following this, the tissue sections were incubated for 16 h at 37 °C with rabbit polyclonal first antibodies in CTS. The sections were then incubated with first antibody (1:500 or 1:1000) raised against human placental

3β-hydroxysteroid dehydrogenase (3βHSD) (16) and anti-(Tyr30)-porcine inhibin α-chain (1-30)-NH_2 (inhibin α) conjugated to rabbit serum albumin for 16 h at room temperature. The antibody of 3βHSD was kindly supplied by Dr. J. I. Mason (Edinburgh University, Edinburgh EH3 9YW, U.K.). The antibody against inhibin α subunit was anti-[Tyr^{30}] inhibin- α-chain (1-30)-NH_2 conjugated to rabbit serum albumin. The [Tyr^{30}]-inhibin- α–chain (1-30)-NH_2 was kindly provided by Dr. N. Ling, (Neuroendocrine Inc., San Dieg, USA). Then the sections were treated with 0.25% (v/v) biotinylated second antibodies (Elite ABC kit; Vector Lab. Burlingame, CA, USA) in CTS for 1 h at 37 °C, and were subsequently incubated with 2% (v/v) avidin-biotin complex (Elite ABC kit) in CTS for 30 min at room temperature. The reactions were visualized by treating with 0.025% 3,3'-diaminobenzidine tetrachloride (DAB, Sigma Chemical Co.) in 10 mM Tris-bufffered saline containing 0.01 % H_2O_2 for 1-30 min. Specificity of the antibodies was examined using normal rabbit IgG instead of first antibody. The procedures for immunohistochemistry are shown in Fig. 2-2.

2-4. Radioimmunoassay (RIA) of hormones

Plasma and conditioned media concentrations of LH and FSH were measured by using National Institute of Diabetes and Digestive and Kidney Disease (NIDDK) rat radioimmunoassay (RIA) kits (Bethesda, MD, USA) for rat LH and FSH. The antiserum used was anti-rat LH-S-11 and anti-rat FSH-S-11. The intra- and inter-assay coefficients of variation were 5.4% and 6.9% for LH, 4.3% and 10.3% for FSH, respectively. The protocol for RIA of FSH and LH is shown in Fig. 2-3.

Quails plasma concentrations of LH were measured with a USDA-ARS radioimmunoassay (RIA) kit (Beltsville, MD, USA) for chicken LH. The antiserum used was anti-avian LH (HAC-CH27-01RBP75). 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, Maryland, USA (48). The antiserum against avian LH was kindly provided by the Biosignal Research Center, Institute for Molecular and Cellular Regulation, Gunma, Japan (32). The protocol for RIA of quail LH is shown in Fig. 2-4.

Plasma and conditioned media concentrations of ACTH were measured by double-antibody RIAs using ^{125}I-labeled radioligands as described previously (108). Synthetic rat ACTH 1-39 (Sigma Chemical Co., St Louis, MO, USA) was used as the reference standard. The intra- and inter-assay coefficients of variation were 11.3 and 11.9%, respectively. The protocol for RIA of ACTH is shown in Fig. 2-3.

Plasma and testicular concentrations of ir-inhibin were measured as described previously (31). The iodinated preparation was 32-kDa bovine inhibin, and the antiserum used was rabbit antiserum against bovine inhibin (TNDH-1). Results were expressed in terms of 32 kDa bovine inhibin. The intra- and inter-assay coefficients of variation were 8.8 and 14.4%, respectively. The detailed procedures are shown in Fig. 2-5.

Plasma, testicular, and conditioned media concentrations of testosterone (102) , plasma and conditioned media concentrations of progesterone(102) and corticosterone (42) were determined by double-antibody RIA system with ^{125}I-labeled radioligands as described previously. The antiserum against testosterone (GDN 250) (29) and progesterone (GDN 337) were kindly provided by Dr. G. D. Niswender, Colorado State University (Fort Collins, CO, USA). The antiserum corticosterone was purchased from UCB-Bioproducts, Belgium. The intra- and inter-assay coefficients of variation were 6.3 and 7.2% for testosterone, 9.5 and 16.4% for corticosterone, and 6.9 and 11.2% for progesterone, respectively. The detailed procedures are shown in Fig. 2-6

and Fig. 2-7.

Plasma concentrations of free triiodothyronin (FT3) and free L-thyrocine (FT4) were measured by using AMERLEX-MAB FT3 and FT4 kits (Trinity Biotech, Bray, Co. Wicklow, Ireland). The intra- and inter-assay coefficients of variation were 7.0% and 8.5% for FT3, and 6.5% and 7.5% for FT4, respectively.

2-5. General statistics

All data are expressed as mean ± SEM (standard error of the mean). Statistical analysis was performed using one-way or two-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference test (Fisher's PLSD) or Dunnett's test. Statistical analysis was performed using the software program StatView 5.0 (SAS Institute Inc., Cary, NC, USA). A probability value of $P < 0.05$ was considered significant.

Flow chart of the tissues preparation for histology

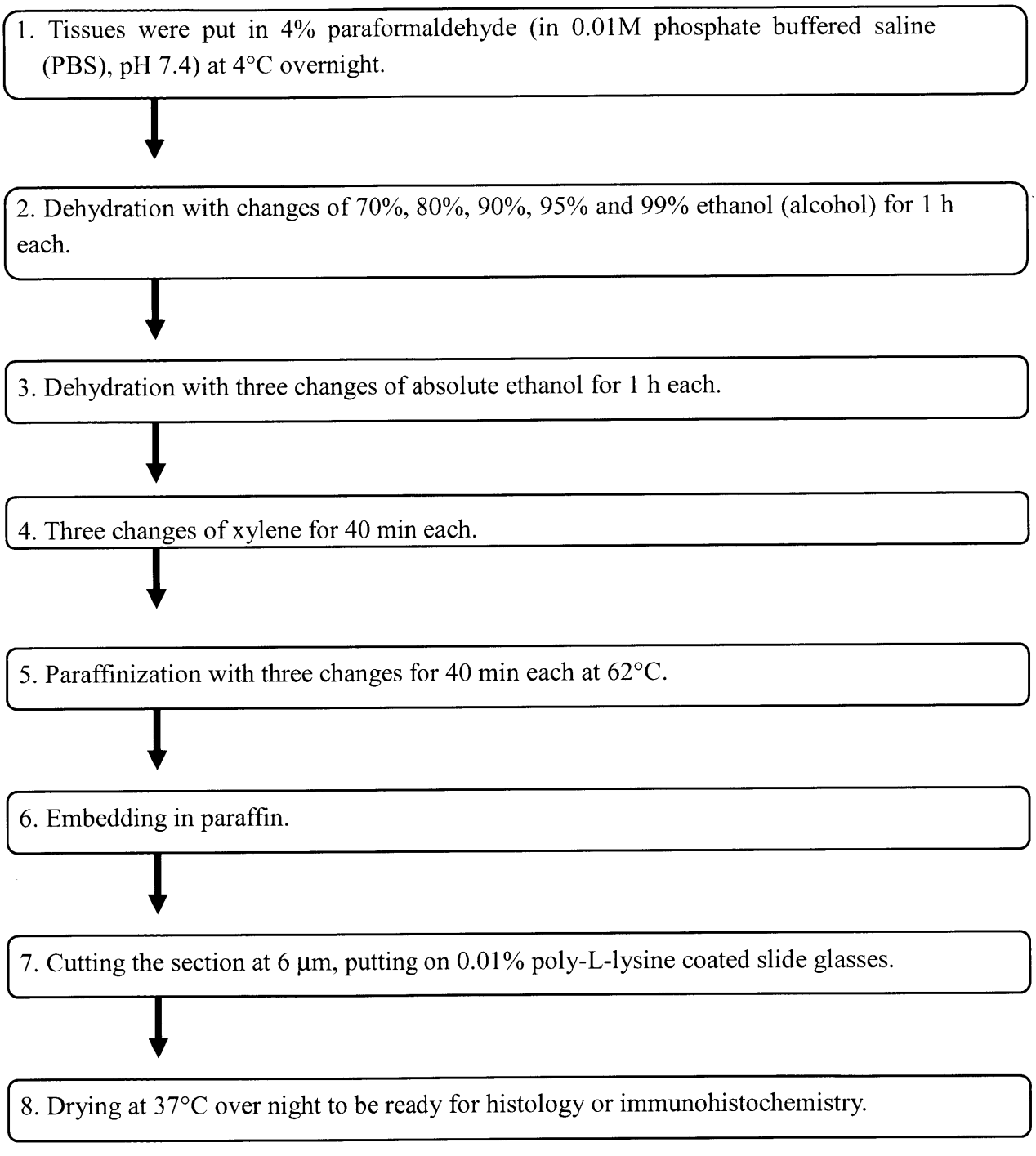


Fig. 2-1 Procedure of tissues preparation for histology.

Flow chart of immunohistochemistry

1. Deparaffinization tissue section
   (1) Deparaffinized tissue sections in xylene, three times, 10~20 min each time.
   (2) Hydrate sections in graded ethanol (alcohol) series (100%, 100%, 90%, 70%), 5 ~10 min each concentration of ethanol (alcohol).
   (3) Distilled water for 5 min to remove ethanol.

↓

2. Putting into autoclave and keep 15 min at 121°C in 0.01 M sodium citrate (pH6.0).

↓

3. Wash in distilled water three times, 5 min each time.

↓

4. Incubate sections for 60 min with 3% H_2O_2 methanol at room temperature (RT).

↓

5. Wash in 0.01 M PBS buffer three times, 5 min each time.

↓

6. Incubate sections for 30~60 min with block solution at 37°C (bottle A:10ml PBS+ blocking serum (NGS) 3 drop)

↓

7. Blot (tapping) excess solution from sections, and incubate sections with diluted primary antibodies at 37 °C for 60 min or (overnight) in a humidified chamber.

↓

8. Repeat step 5.

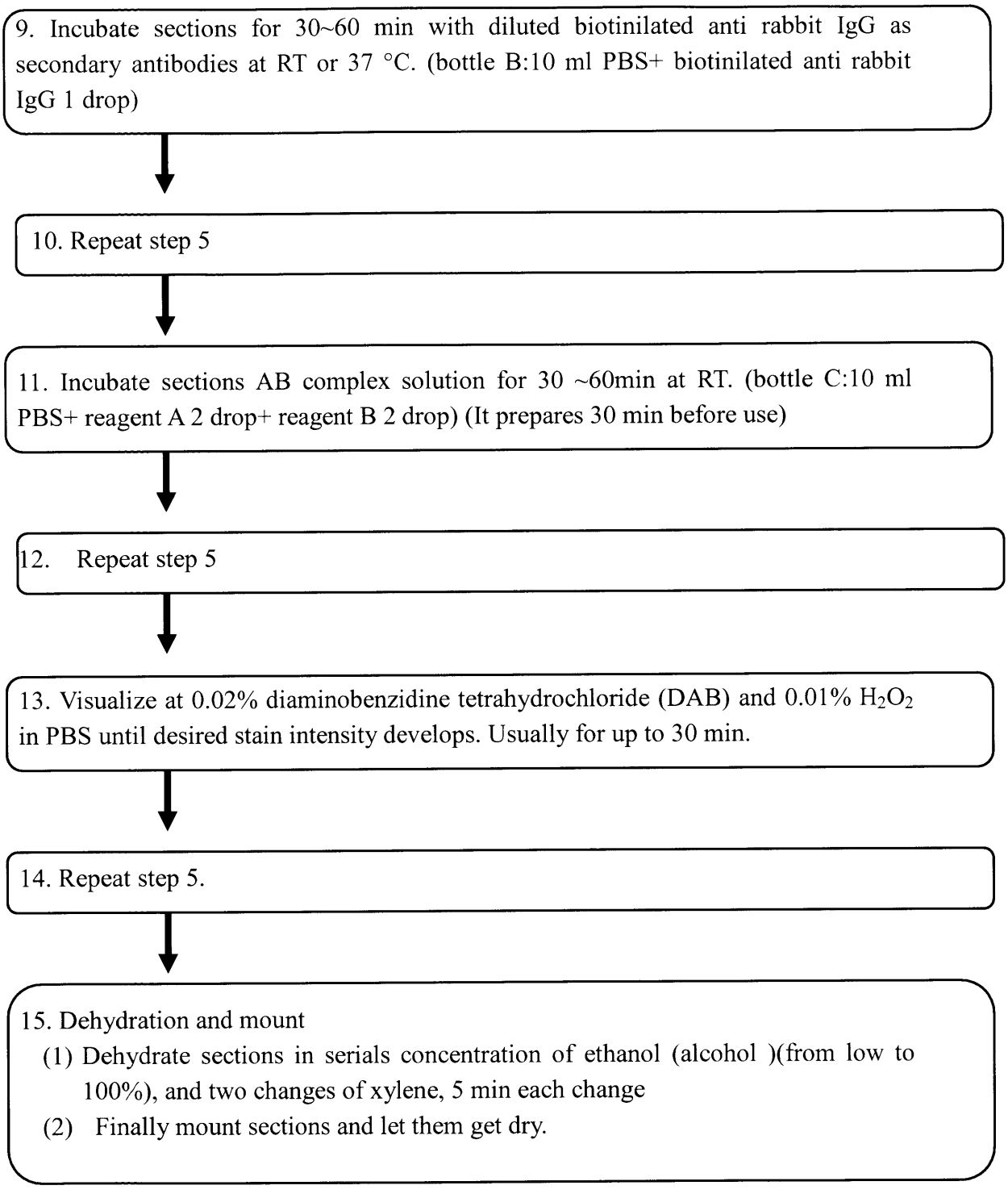


Fig. 2-2 Procedure for immunohistochemistry.

Flow chart of the RIA for rat gonadotrophins (LH, FSH) and ACTH

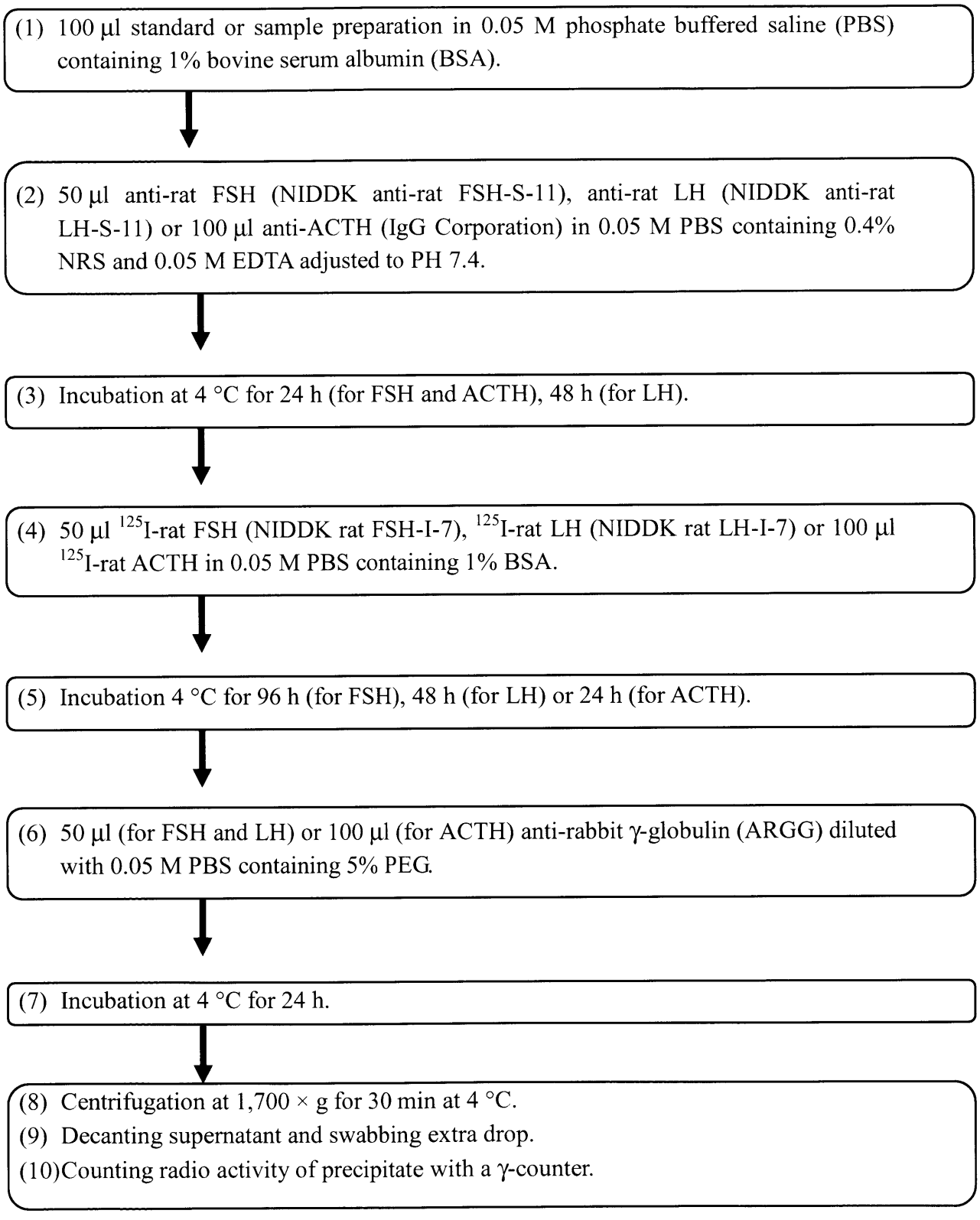


Fig. 2-3 Procedure of RIA for rat gonadotrophins (LH, FSH) and ACTH.

Flow chart of the RIA for quail LH

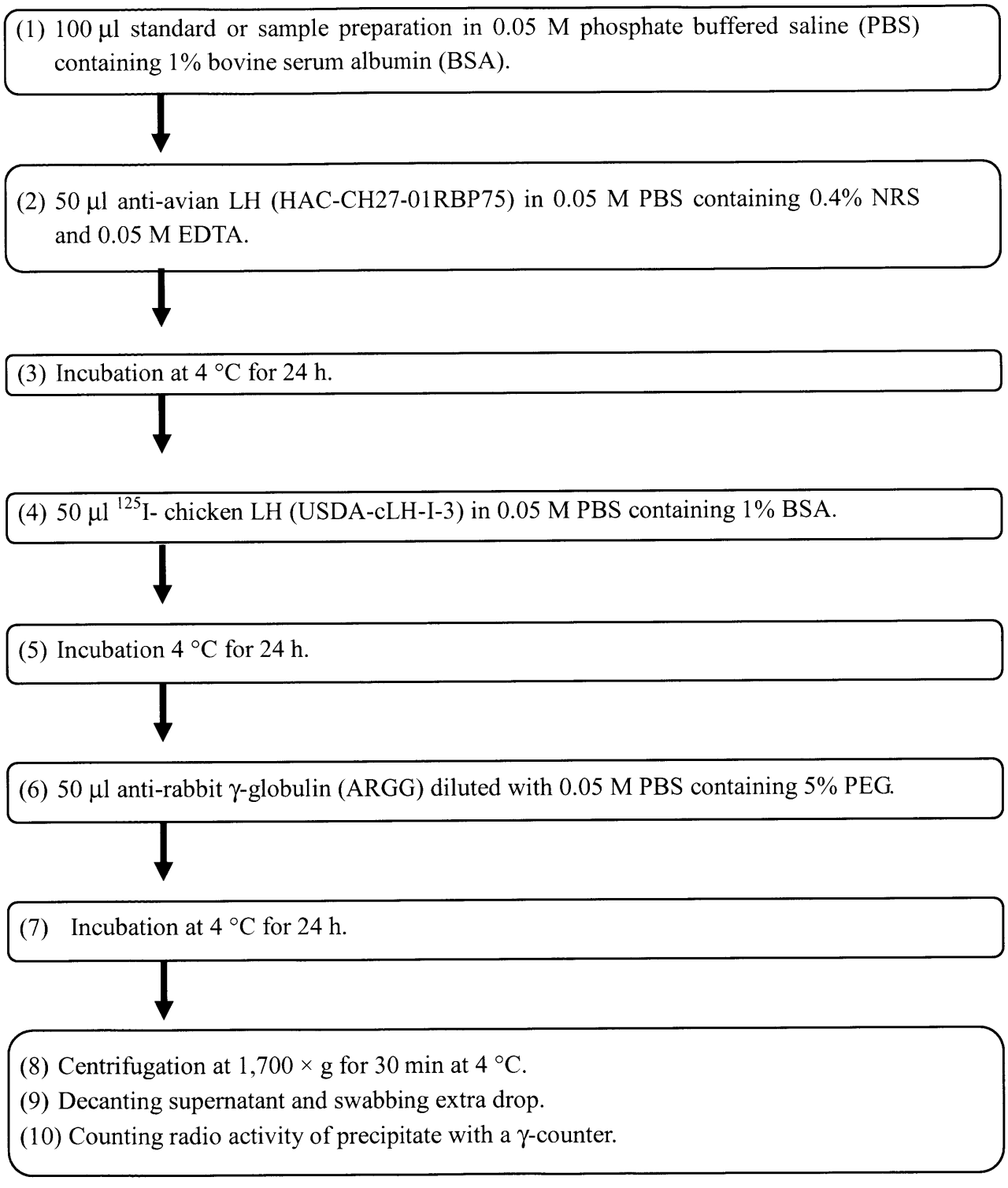


Fig. 2-4 procedure of RIA for quail LH.

Flow chart of the RIA for rat immunoreactive (ir-) inhibin

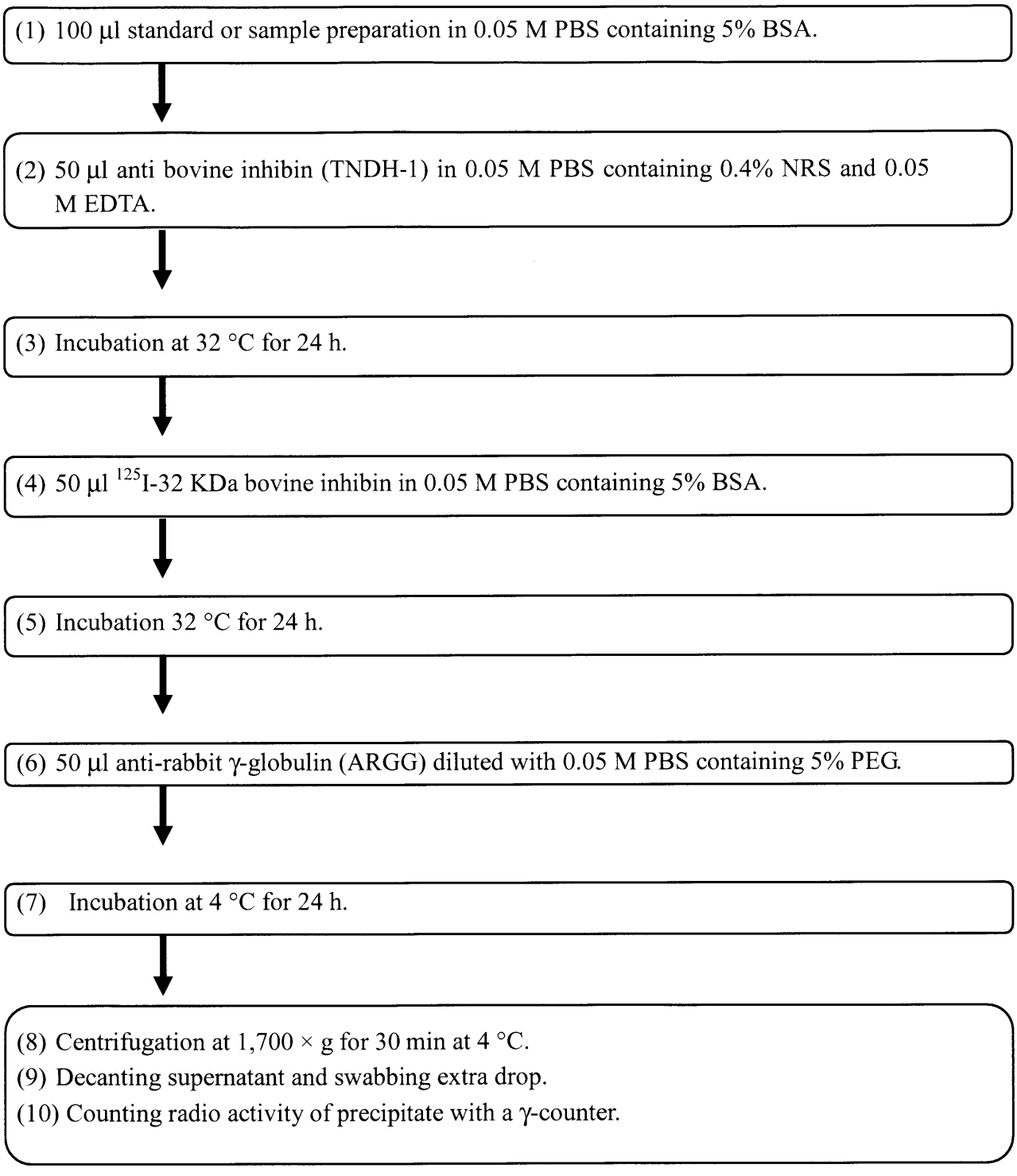


Fig. 2-5 Procedure of RIA for rat immunoreactive (ir-) inhibin.

Flow chart of the RIA for steroid hormones-1

Part 1 of flow chart: extraction

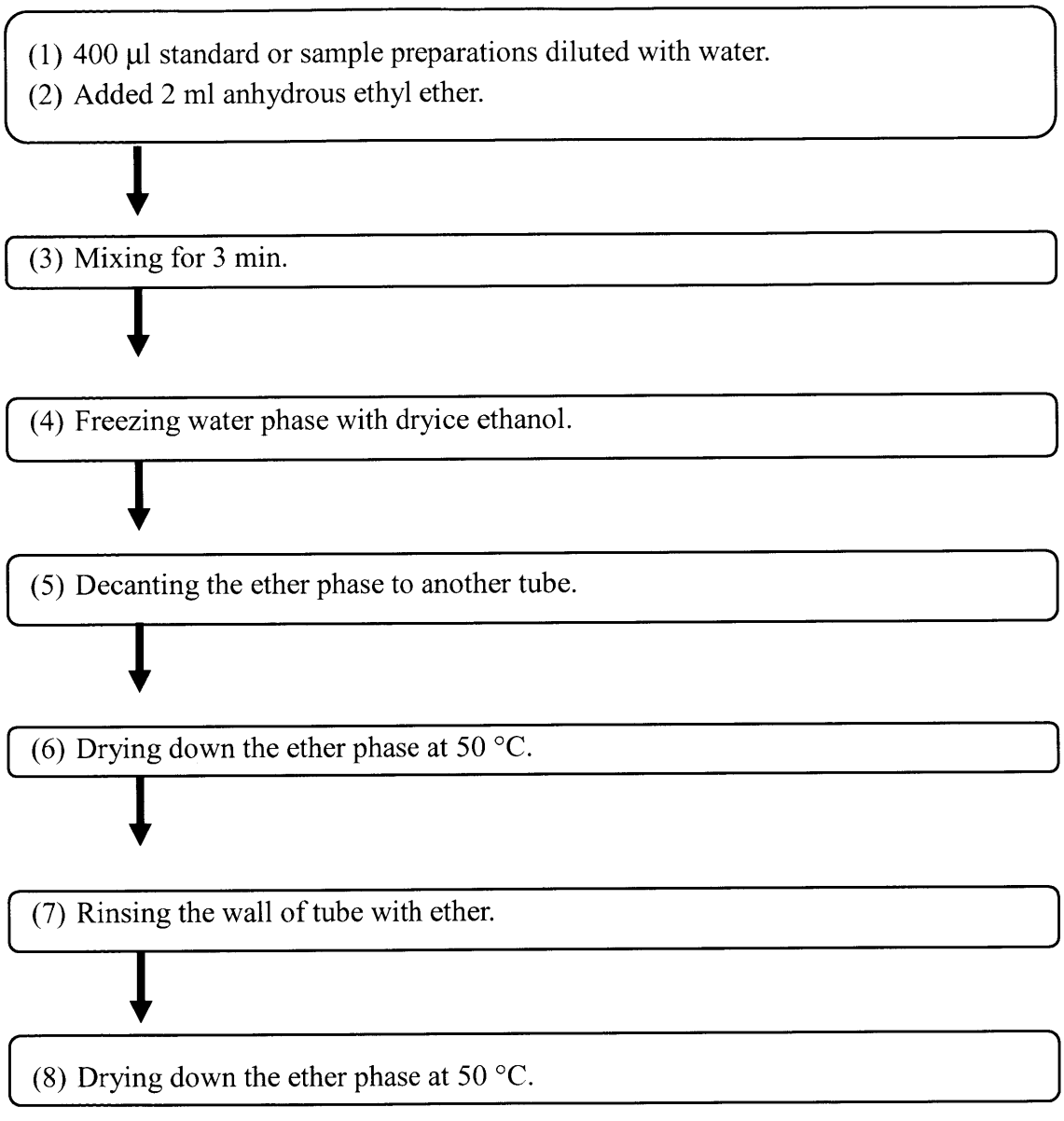


Fig. 2-6 Extraction procedure of RIA for steroid hormones.

Part 2 of flow chart: assay after extraction

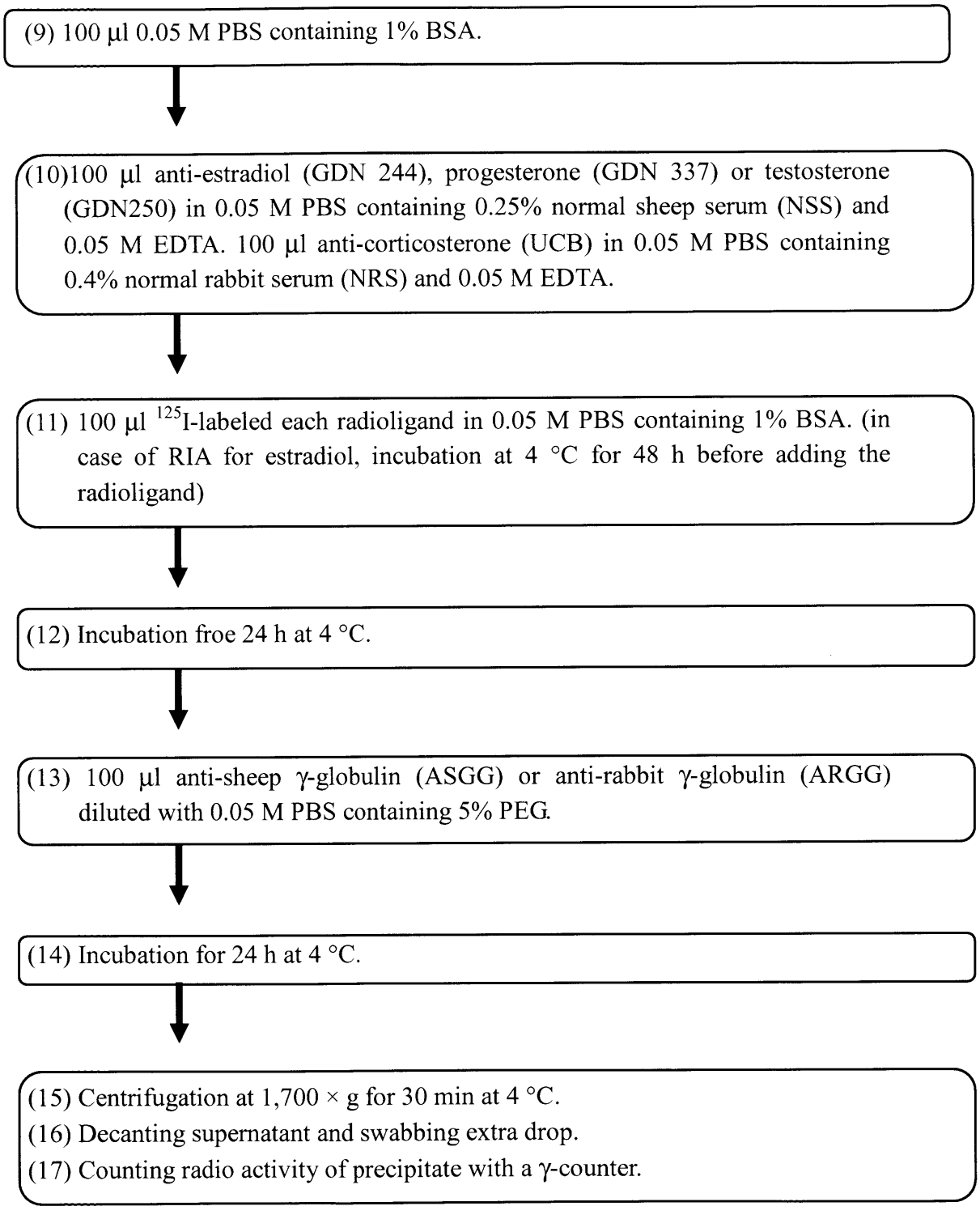


Fig. 2-7 Procedure of RIA for steroid hormones.

Chapter 3. Impairment of Testicular Function in Adult Male Japanese Quail *(Coturnix japonica)* After a Single Administration of PNMC

3-1. Background

Diesel air pollution is a significant environmental problem and has broad effects on human health, including lung cancer (37, 58), allergic rhinitis (64, 98), and bronchial asthma-like disease (61, 91). DEP, the soluble organic fraction of particulate matter from diesel exhaust, are also toxic to the male and female reproductive systems (109, 110, 117, 122). However, the specific compounds responsible for this toxicity are still unclear.

Four nitrophenols, 4-nitrophenol, 2-methyl-4-nitrophenol, 3-methyl-4-nitrophenol (PNMC), and 4-nitro-3-phenylphenol were isolated from DEP and showed that they had vasodilatory activity (62, 100), estrogenic activity (25, 26, 101), and anti-androgenic activity (101). In addition to its presence in DEP, PNMC is a degradation product of the insecticide fenitrothion, a widely used pesticide with high potential for human, livestock, and poultry exposure in both rural and residential environments. The accumulation of PNMC from these sources could have significant effects on wildlife and human health via disruptions of endocrine and reproductive systems.

Despite the potentially significant effects, the possible biological impact and basic data on the toxicity of PNMC are still unknown. To determine the basic potential endocrine and reproductive toxicities of PNMC, I used the adult male terrestrial Japanese quail (*Coturnix japonica*). The Japanese quail as a laboratory animal has been extensively used in reproductive toxicity testing. Quail are considered to be representative of terrestrial birds and accepted models for assessing both the acute and chronic effects of pesticides and other chemicals in wild birds (19, 71), because spermatogenesis is well characterized (55) and the cloacal gland is a good

marker of gonadal development (76). In the present study, I used this animal model to examine the *in vivo* effects of a single dose of PNMC and *in vitro* effects on the testicular function of adult male quail.

3-2. Material and Methods

3-2-1. Chemicals

3-Methyl-4-nitrophenol (4-nitro-*m*-cresol, PNMC) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Collagenase (type V), soybean trypsin inhibitor, Medium 199 (M199) from Sigma (Sigma Chemical Co., ST. Louis, MO, USA).

3-2-2. Animals

Japanese quail (*Coturnix japonica*) came from L selected lines, in which chicks hatch after 17 days of incubation and the birds reach sexual maturity at 6 weeks. Birds were provided with food (Kanematsu quail diet, Kanematsu Agri-tech Co. Ltd., Ibaraki, Japan) and water *ad libitum*. Six- to nine-week old male birds were housed in metal cages in a controlled environment (lights on 0500 to 1900 h, temperature 23 ± 2 °C, humidity 50 ± 10%, and air exchanged 20 times hourly).

3-2-3. Administration of PNMC

Mature male Japanese quail were treated with a single intramuscular (im) injection of PNMC (78, 103 or 135 mg/kg body weight). The doses were decided by the preliminary experiments of LD_{50} of PNMC in the adult male quail. The LD_{50} of PNMC in the adult male quail was 135 mg/kg, so the 3 lower doses including 135 mg/kg were adopted in this study. Controls were injected with vehicle alone (PBS containing 0.05% Tween 80). The quail (n=6-9 per group) were weighed and

euthanized by decapitation at 1, 2 and 4 weeks after the injection. Following decapitation, blood samples were collected in heparinized plastic tubes and centrifuged at 1700 × g for 15 min at 4 °C. Plasma was separated and stored at –20 °C until assayed for testosterone and luteinizing hormone (LH). The testes were collected and weighed, and the cloacal gland area (longest length × greatest width) was measured.

3-2-4. Regrouping according to testicular atrophy

Birds with testicular atrophy were found in all PNMC-treated groups, but none was found in the control group. Birds were separated into three atrophy groups (severe, intermediate, and mild) on the basis of testicular weight. The severe atrophy group included birds in which the weight of both testes was at least 50% (1.33 ± 0.064 g) lighter than the mean of the control group (2.66 ± 0.128 g). The intermediate group included birds with one testis weighing less than 50% of the control weight. The criterion for the mild atrophy group was one testis weight of 50% to 70% of the control weight.

3-2-5. Histopathology

Procedures of histopathology were described in general materials and methods of chapter 2.

3-2-6. Effects of PNMC on hypothalamus-pituitary function

To observe the direct effects of PNMC on the secretion of LH, birds were treated with a single im injection of small amount of PNMC (25 mg/kg) to avoid an acute toxic effect that was observed at the highest dose setting (See results). Control birds were treated with vehicle alone (PBS containing 0.05% Tween 80). Eight birds were used in both groups. Blood was collected in heparinized syringes from the jugular vein

1 and 3 h after the injection. Six hours after injection, birds were killed by decapitation and blood was collected. All blood samples were centrifuged at 1700 × g for 15 min at 4 °C, and plasma was separated and stored at –20°C until it was assayed for LH.

3-2-7. Interstitial cell preparation

Interstitial cell preparations containing Leydig cells were prepared from the testes of adult quail as previously described with minor modifications (46). The testes were immediately removed from adult quail after death by cervical dislocation and testicular cells were dispersed by treating the decapsulated testis in M 199 medium with 0.71 mg/ml sodium bicarbonate and 2.21 mg/ml HEPES containing collagenase (0.25mg/ml) and soybean trypsin inhibitor (0.025mg/ml) at 37°C for 30 min in a shaking water bath. After incubation, the supernatant, containing Leydig cells, was decanted through nylon mesh to remove debris. The cells were washed by centrifugation and resuspended in 10 ml of M199 with 1% fetal bovine serum. The viability of the cells was evaluated by means of the trypan blue exclusion test and found to be 92%. Cells (10^5 cells/well/100 μl) were cultured in 96- well culture plates at 37°C under a 95% air-5% CO_2 atmosphere. Following a 20 min equilibration period, cells were exposed for 3, 8, or 24 hr to 10^{-6}, 10^{-5}, or 10^{-4} M PNMC (100 μl) dissolved in M199. The viability of treated cells was determined by Lactate dehydrogenase (LDH) Cytotoxicity Detection Kit (Takara, Code No. MK401, Otsu, Shiga, Japan). No significant differences in LDH release activity were observed at treated PNMC cells compared with control cells (data not shown). Conditioned media were assayed for testosterone.

3-2-8. Determination of concentrations of luteinizing hormone (LH) and testosterone

The detailed RIA procedures for determination of LH and testosterone in plasma and conditioned media were described in general materials and methods of chapter 2.

3-2-9. Statistics

The statistical methods are shown in chapter 2. The acute effects of PNMC on the secretion of LH were analyzed using two-way ANOVA followed by Dunnett's test.

3-3. Results

3-3-1. Acute effect of PNMC

Of 28 birds treated with the highest dose of PNMC (135 mg /kg), 6 died within 10 min of treatment (Table 3-1). Of 26 birds in the 103 mg/kg treatment group, one bird died. The birds showed behavior such as dyspnea, opening the beak and tremor prior to death. None died in the 78 mg/kg or control groups. The surviving birds in all treatment groups grew normally, with no differences in body weights (data not shown).

3-3-2. Testicular atrophy

Results concerning with testicular atrophy are shown in Tables 1 and 2. Morphology and histology of atrophic testes are also shown in Fig. 3-1. PNMC treatment induced testicular atrophy as early as one week after treatment, but neither the severity nor the incidence of atrophy showed a dose-dependent relationship (Table 3-1). The highest rate of testicular atrophy was observed 2 weeks after treatment with PNMC in all groups. In birds receiving 78 mg/kg and 103mg/kg PNMC, 70% and 67% birds showed testicular atrophy (Table 3-1). Some birds showed significant weight decreases in both testes, but others showed an asymmetrical decrease (Table 3-2). In most cases, the right testis was significantly decreased in size, whereas the left testis was not significantly different from the controls (Table 3-2).

3-3-3. Morphology and histology of the testes and cloacal glands

Overall testicular morphology was normal in the control group (Fig. 3-1A), whereas PNMC treatment induced severe atrophy, either bilaterally or on the right side only (Figs. 3-1B, C). Control sections showed compartmentalization of germ cells in the seminiferous tubules, with spermatozoa visible in normal-sized lumen (Fig. 3-1D). In birds with testicular atrophy on one side, seminiferous tubules contained only a thin layer of spermatogenic lineage cells, and spermatids and spermatozoa were absent (Fig. 3-1E). In contrast, paired atrophic testes showed no compartmentalization of germ cells or spermatozoa, and had highly atrophic seminiferous tubules that were devoid of all cells except spermatogonia and Sertoli cells (Fig. 3-1F). Control cloacal glands had normal morphology and produced cloacal gland foam (Fig. 3-1G), whereas PNMC-treated birds with testicular atrophy had smaller cloacal glands and did not produce cloacal foam (Figs. 3-1H, I).

3-3-4. Plasma concentrations of LH and testosterone

Plasma concentrations of LH and testosterone in PNMC treated birds are shown in Fig. 3-2 and Fig. 3-3. There were no treatment-induced changes in basal levels of plasma LH at any time point with relatively large individual variation (Fig. 3-2). Plasma concentrations of testosterone were significantly lower in both the 78 and 103 mg/kg-treated groups at 1, 2, and 4 weeks after PNMC treatment (Fig. 3-3A, B), whereas the high-dose group showed a significant decrease only at 4 weeks after treatment (Fig. 3-3C).

Plasma concentrations of LH and testosterone in grouped birds by atrophy level are shown in Table 3-2. There are two types of circulating levels of LH in PNMC treated birds having various grades of atrophy testes. The first type of birds revealed that plasma concentrations of LH were significantly lower in the severe atrophy group

(1.34 ± 0.45 ng/ml) as compared with controls (4.66 ± 0.61 ng/ml) (Table 3-2). Plasma concentrations of LH in this type of birds decreased with increasing severity of testicular atrophy, and the level paralleled with testis weight. In this type of birds, plasma concentrations of testosterone were significantly lower in severe, intermediate and mild atrophy groups as compared with controls. It is also paralleled with testis weight (Table 3-2). On the other hand, the second type of birds showed that plasma concentrations of LH were higher as compared with controls though they have atrophic testes and low levels of testosterone. This type of birds was observed 1 out of 6, 2 out of 6 and 4 out of 16 in the severe, intermediate and mild atrophy group, respectively. Data were shown in parentheses in each group (Table 3-2).

3-3-5. Cloacal gland area

Cloacal gland area was significantly decreased in all atrophy groups, with the lowest value observed in the severe atrophy group. Changes in cloacal gland area were similar with plasma levels of testosterone (Table 3-2).

3-3-6. Acute effects of PNMC on secretion of LH

There was a clear time-dependent decline in plasma LH concentrations in the group treated with PNMC (Fig. 3-4). PNMC treatment (25 mg/kg) significantly reduced plasma LH concentrations ($P < 0.05$) from 1 hour after injection.

3-3-7. Dose and time dependent effects of PNMC on testosterone secretion of interstitial cells

Dose and time dependent effects of PNMC on testosterone secretion into interstitial cell cultured medium were examined (Fig. 3-5). In the cells exposed to PNMC for 4 h, the amount of testosterone secretion was almost same as in the control groups. However, significant reduction of testosterone secretion was detected in cells

treated with 10^{-6}, 10^{-5} or 10^{-4} M PNMC at 8 h and 10^{-6} and 10^{-5} M PNMC at 24 h.

3-4. Discussion

The present study clearly demonstrated that a specific component of DEP induced testicular impairment in an avian model. A single administration of PNMC in adult male Japanese quail induced acute toxicological responses as well as significant testicular atrophy and decreases in plasma concentrations of LH and testosterone.

The acute toxicological responses were observed in the birds treated with the high dose (135mg/kg), and the conditions encountered were dyspnea and tremor prior to death. From these conditions it can be speculated that PNMC causes acute toxicity and death, possibly by a blood pressure drop followed by an ischemic shock, as it has been reported that PNMC has a potent vasodilating activity (62, 100).

The present study clearly demonstrated that there are two types of responses in secretion of LH and testosterone in PNMC treated birds. The first type of PNMC treated birds showed low plasma levels of both LH and testosterone. On the other hand, the second type of birds treated with PNMC showed high levels of LH but low levels of testosterone. These results clearly indicate that the site of action of PNMC is in male quail. The first type of response suggests the direct effect of PNMC on the hypothalamus and pituitary axis to reduce secretion of LH. In this case, therefore, it is suggested that PNMC firstly act on the hypothalamus to reduce pulsatile secretion of gonadotrophin-releasing hormone (GnRH) from the hypothalamus, and then reduce pulsatile secretion of LH from anterior pituitary glands, followed by a reduction of testosterone secretion from the testis. In addition, the present study demonstrated that plasma concentrations of LH, significantly decreased from 1 hour after a single injection of PNMC. These results strongly support that PNMC may act directly on the hypothalamus–pituitary axis for reducing GnRH release from the hypothalamus, and subsequent reduce in LH release from the anterior pituitary gland. On the other hand,

the second type of response suggests the direct effect of PNMC on the testis to reduce secretion of testosterone. In this case, it is suggested that PNMC firstly act directly on testes for reduction of testosterone secretions. This reduction of testosterone induces hypersecretion of GnRH from hypothalamus and subsequently increases in secretion of LH from anterior pituitary glands. It is well known that Leydig cells play a crucial role in synthesizing testosterone and regulating the process of spermatogenesis (94). Alteration of Leydig cell function can lead to adverse effects on testicular functions (94). The present study clearly demonstrated that PNMC reduce testosterone production in cultured testicular interstitial cells. This observation is in agreement with *in vivo* study of the second type that showed higher LH levels and lower testosterone levels, which the testosterone levels were suppressed prior to the toxic effects on the pituitary that would reduce the LH levels. The present results, therefore, strongly suggest that PNMC has a direct effect on the testis in addition to the effects on the hypothalamus and the pituitary, whereas an exact reason responsible for these two different types of response are not clear at the present time.

Testicular atrophy often showed an asymmetric response, with atrophy most frequently observed in the right testes. A characteristic feature of sexual development in both female and male birds is gonadal asymmetry: the right ovary does not develop, and the right testis is often slightly smaller than the left (54). Treatment of avian embryos with an estrogenic chemical, diethylstilboestrol, induces a similar asymmetrical pattern, with greater atrophy in the right testis (80, 87). In birds, the left embryonic gonad has ambisexual potential, whereas the right gonad is exclusively masculine (80). The mechanism underlying this phenomenon requires further study.

The atrophic paired testes showed no compartmentalization of germ cells and spermatozoa, and seminiferous tubules were atrophic and almost devoid of cells except for the spermatogonia and Sertoli cells. These results suggest that circulating gonadal hormone in the treated birds were reduced with the addition of the toxic effects of

PNMC to the seminiferous tubules. The direct effect of PNMC on testes results in the decrease in spermatogenesis, leading to the reduction in the sperm production of the treated birds. In avian testes, interstitial cells (Leydig cells), as well as testicular germ cells and Sertoli cells, contain steroidogenic enzymes, which produce progesterone, androgen and estrogen (50, 83, 89). Thus, steroidogenic activities were destroyed in the treated group testes because the seminiferous epithelium was thinner and thus decreased the sperm population.

The present study showed that the cloacal gland area in birds with testicular atrophy was significantly smaller than in normal birds. The androgen-dependent cloacal gland, posterior to the cloaca, is a secondary sex characteristic unique to the genus *Coturnix japonica*. The cloacal gland contains androgen receptors and grows in response to circulating androgen (6, 40, 76, 77), so it is a widely used indicator of androgen status in the male during sexual maturation. The decrease in cloacal gland area in the treated groups may be attributed to the reduced testosterone level recorded in the present study.

Previous papers reported that PNMC, a nitrophenol derivative compound isolated from DEP has been shown to posses estrogenic activity *in vivo* and *in vitro* (25, 26, 101). A previous paper reported that an estrogenic chemical bisphenol-A reduced the weight of the combs and testes in the male chicken (27). It is well known that the growth of the comb and testes are highly promoted by testosterone and inhibited by estrogen (6). In addition, PNMC also has anti-androgenic activity *in vitro*(101). Previous reports showed that flutamide, a potent androgen antagonist, decreased accessory sex organ weight in rats *in vivo* (4, 119). It is suggested that estrogenic and anti-androgenic potency of PNMC may be involved in suppression of testicular function in the PNMC treated quail in the present study. In the present study, effect of PNMC on secretion of testosterone is not dose dependent manner. However, the present results suggest that the ratio of estrogenic and anti-androgenic potency of

PNMC may be involved in this phenomenon.

The present study is also important to the environmental perspective. Remarkable amount of DEP are exhausted into the air of many countries. In Japan 58,902 tons (39) are emitted each year and this is an amount that can not be ignored. The amount of PNMC which is included in 1 kg of DEP is 28 mg (62, 100). The environmental concentrations of PNMC are not well known since the research of the isolation of the compounds found in DEP has just been begun. In addition, PNMC is a known degradation product of the insecticide fenitrothion (11), which is used widely in many countries and is being accumulated in the air, soils and water (67, 69). According to the data submitted by the pollutant release and transfer registers (PRTR), the amount of fenitrothion emitted into the environment in 2002 in Japan was approximately 1,300 tons, and roughly half of this is degraded into PNMC (34). It was also reported that the amount of PNMC contained in the rain water in Roskilde, Denmark was as high as 2483 ng/l (5). These findings clearly indicate that PNMC exists in large amounts in the environment from diesel exhaust, fenitrothion used on farms, and in rainwater. It is difficult to directly interpret the present results of the effects of PNMC to the wildlife since the doses do not relate to the environmental concentration. However, as demonstrated in the results from this research, it is certain that PNMC has toxic effects on the reproductive system, and therefore the possibility of the large amounts of PNMC in the environment having serious effects on wildlife and human beings can not be ignored.

In conclusion, the present study clearly shows that PNMC impairs reproductive function in male Japanese quail through toxic effects on the hypothalamus, pituitary, and the testis.

Table 3-1 Testicular atrophy in adult male quail treated with PNMC in 1 week, 2 weeks and 4weeks

Compound	Group	Dose (mg/kg)	Number of birds	Number of deaths	Grade of atrophy			Rate of atrophy (%)
					Severe	Intermediate	Mild	
PNMC	1 week	0	7	0	0	0	0	0
		78	8	0	0	1	1	25
		103	8	0	0	0	2	25
		135	9	3	0	0	1	17
	2 weeks	0	8	0	0	0	0	0
		78	10	0	3	2	2	70
		103	10	1	2	1	3	67
		135	10	1	1	0	2	33
	4 weeks	0	7	0	0	0	0	0
		78	8	0	0	1	2	38
		103	8	0	0	1	2	38
		135	9	2	0	0	1	14

Table 3-2 Testis weight, cloacal gland area, testosterone and LH concentrations by atrophy group after treatment with PNMC

Treatment	Grade of atrophy	Number of animals	Right testes (g)	Left testes (g)	Combined testes (g)	Cloacal gland area (cm^2)	Testosterone concentration (ng/ml)	LH concentration (ng/ml)
Control	Normal	22	1.11±0.07	1.45±0.09	2.56±0.14	1.62±0.07	2.12±0.29	4.66±0.61
	Severe atrophy	5 (1)	0.31±0.06*** (0.01)	0.35±0.07*** (0.01)	0.66±0.12*** (0.26)	0.74±0.13*** (0.26)	0.15±0.07*** (0.10)	1.34±0.45* (14.02)
PNMC	Intermediate atrophy	4 (2)	0.47±0.05*** (0.32, 0.33)	1.27±0.15 (2.24, 2.11)	1.74±0.17* (2.56, 2.44)	1.55±0.10* (1.81, 1.49)	0.46±0.12* (0.74, 0.20)	2.33±0.89 (10.46, 12.85)
	Mild atrophy	12 (4)	0.85±0.07*** (0.96±0.12)	1.27±0.11 (1.37±0.21)	2.12±0.12* (2.32±0.13)	1.18±0.07*** (1.53±0.23)	0.48±0.14*** (1.61±0.51)	3.03±0.67 (16.18±1.83)

The values are expressed as mean ± SEM.

***$P < 0.001$, *$P < 0.05$ compared with value of control quail (ANOVA and Dunnett's test)

Numbers in parenthesis represent values of animals having high plasma concentrations of LH

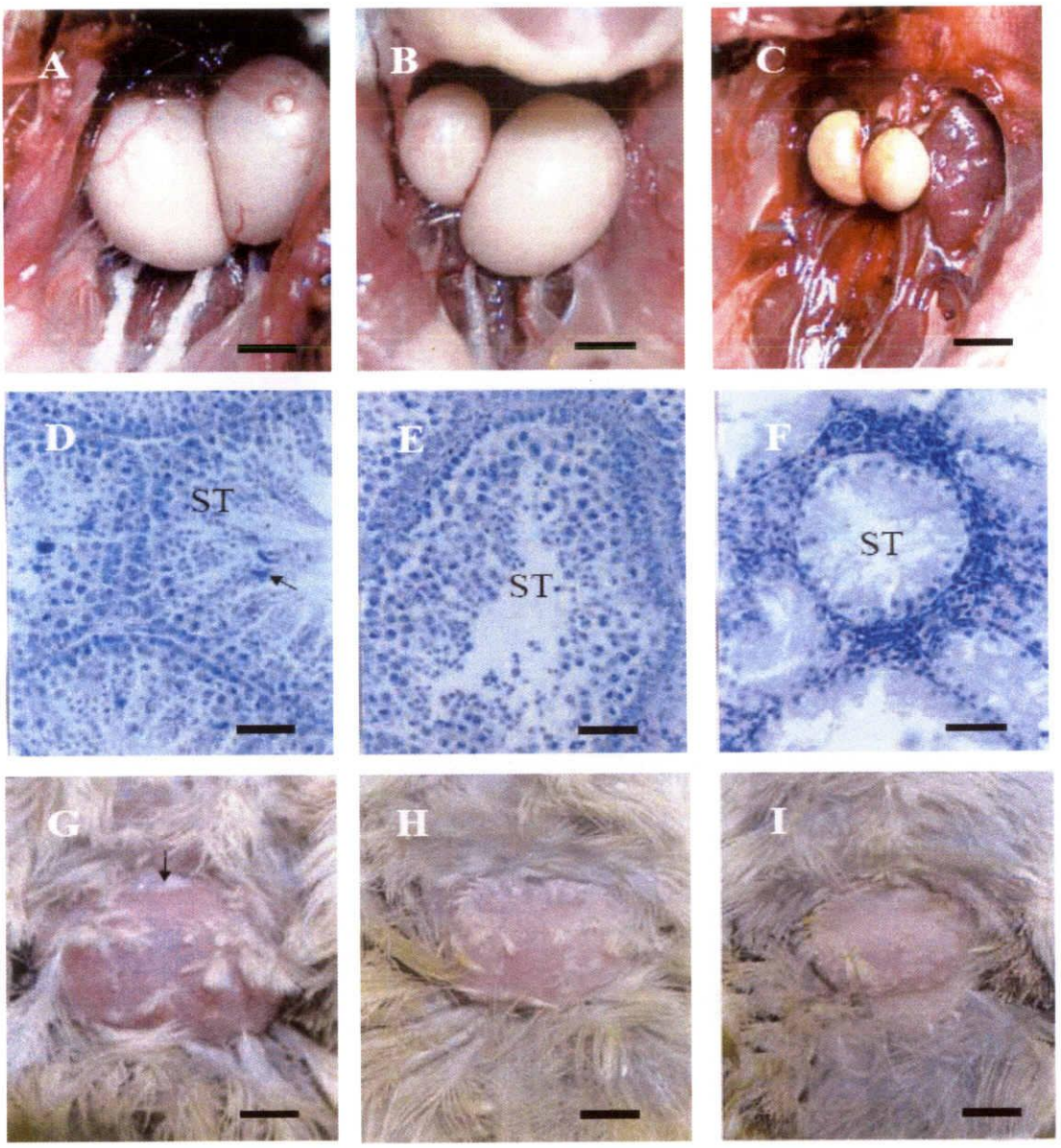


Fig. 3-1 Representative testicular and cloacal gland morphology and histology. (A) The testes of vehicle-treated (control) quail showed normal overall morphology. The testes of PNMC-treated quail showed asymmetrical (B) or bilateral (C) atrophy. (D) The testes of vehicle-treated (control) quail showed different stages of spermatogenesis and compartmentalization of germ cells in the seminiferous tubules. (E) The spermatogenic lineage showed losses of spermatids and spermatozoa in the asymmetrically atrophic testes. (F) Elimination of germ cells and the presence of only spermatogonia and Sertoli cells in bilaterally atrophic testes. (G) The cloacal gland of vehicle-treated (control) quail showed normal morphology and produced cloacal gland foam. The size of the cloacal gland was reduced and no cloacal gland foam appeared in quail with asymmetric testes (H) or bilaterally atrophic testes (I). ST = seminiferous tubules; arrows indicate sperm (D) and cloacal gland foam (G). HE stains. Bar (A-C) = 6 mm, bar (D-F) = 50 μm, bar (G-I) = 4 mm.

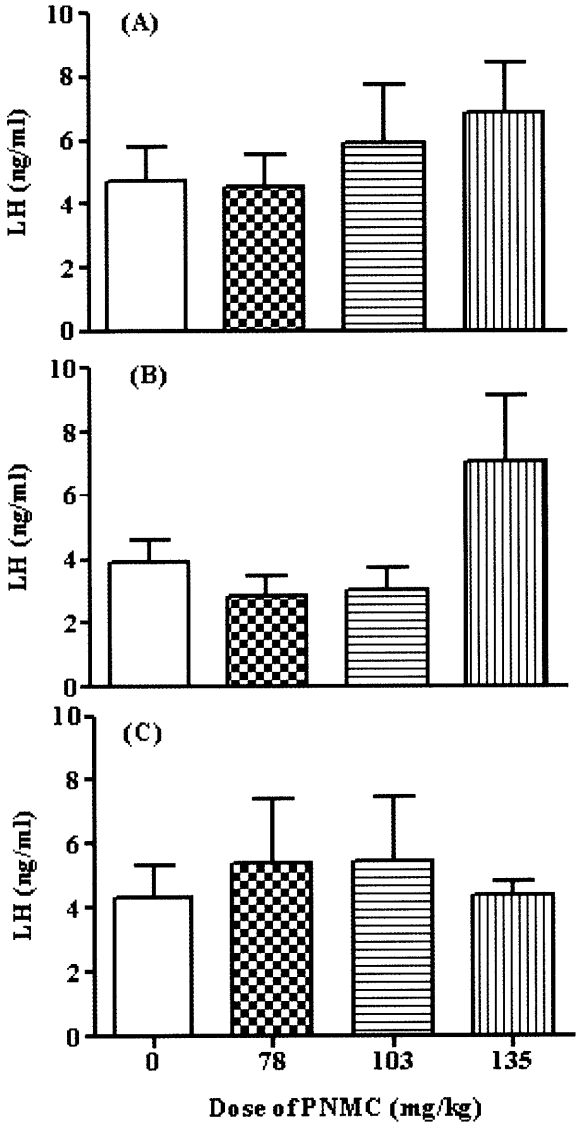


Fig. 3-2 Plasma concentrations of LH in adult male quail treated with PNMC (78, 103, and 135 mg/kg) after 1 week (A), 2 weeks (B), or 4 weeks (C). Each bar represents mean ± SEM of 6 to 9 quail per group.

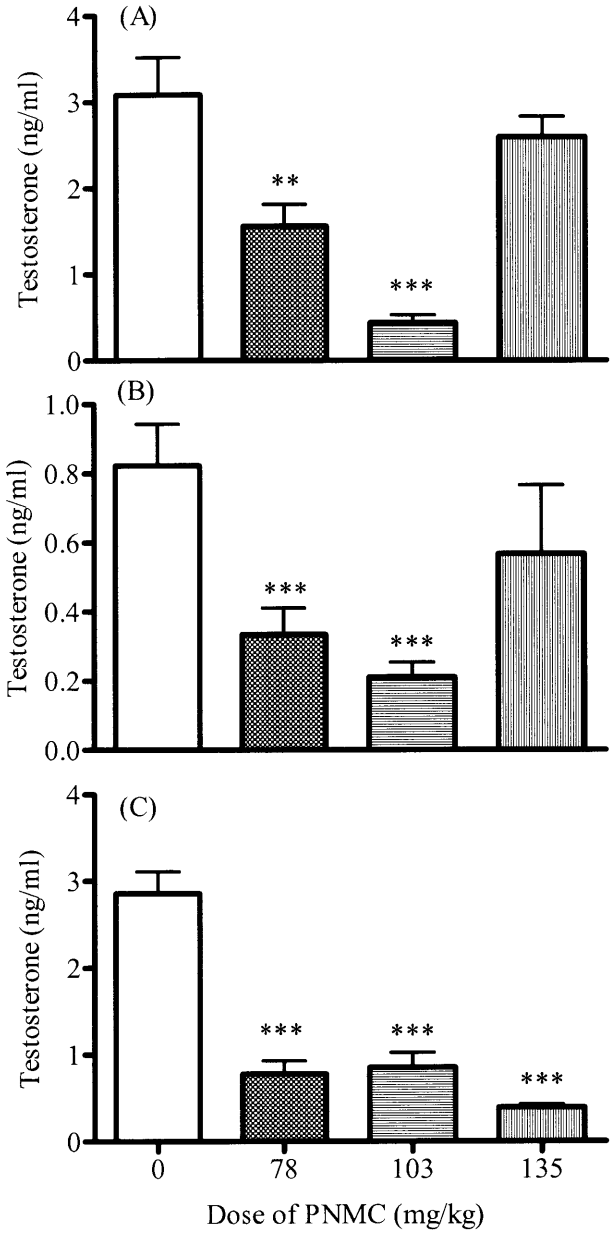


Fig. 3-3 Plasma concentrations of testosterone in adult male quail treated with PNMC (78, 103, or 135 mg/kg) after 1 week (A), 2 weeks (B), or 4 weeks (C). Each bar represents mean ± SEM of 6 to 9 quail per group. ***$P < 0.001$, **$P < 0.01$ compared with control quail (Dunnett's test).

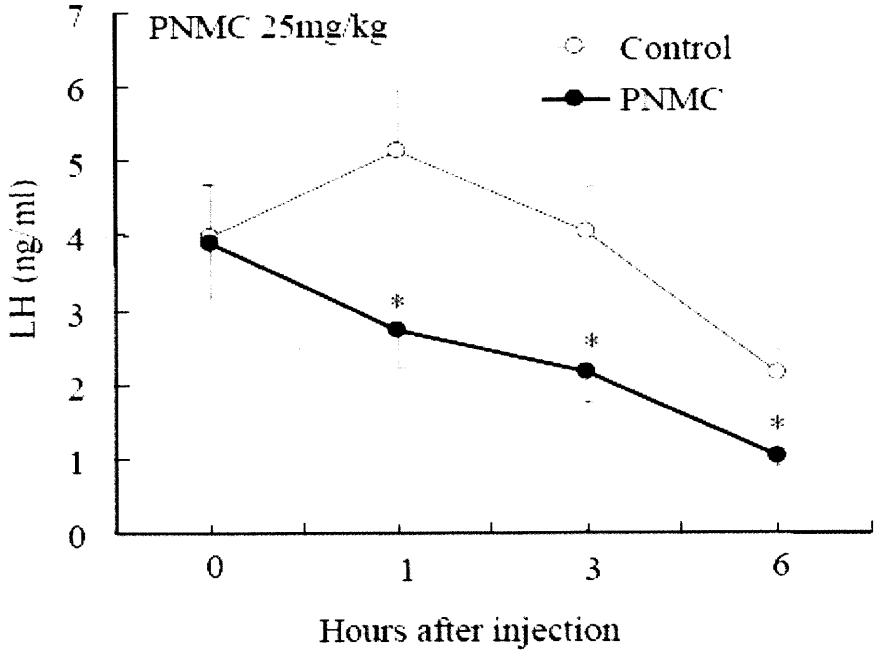


Fig. 3-4 Changes in plasma concentrations of LH in adult male quail treated with vehicle (control; ○) or PNMC (25 mg/kg; ●). Each bar represents mean ± SEM of 8 quail per group. *P < 0.05 compared with control when analyzed using two-way ANOVA followed by Dunnett's test.

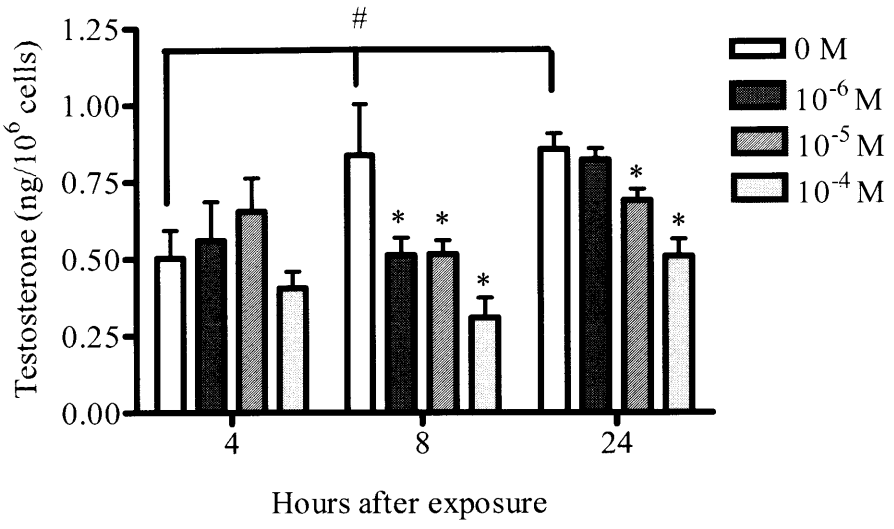


Fig. 3-5 Dose and time dependent effect of PNMC on secretion of testosterone in quail testicular interstitial cell culture. The cells were incubated for 4, 8 or 24 h in M199 containing different doses of PNMC (10^{-6}, 10^{-5} or 10^{-4} M). Each bar represents mean ± SEM (n=6). *$P < 0.05$ compared with same time control, $^{\#}P < 0.05$ compared with 4 hours control (Dunnett's test).

Chapter 4. Effects of PNMC on the Regulation of Reproductive Function in Mature and Immature Female Japanese Quail *(Coturnix japonica)*

4-1. Background

Mortality and reproductive abnormalities of wild birds caused by environmental pollutants and pesticides have been reported in endocrine disrupting chemicals. Indeed, many contaminants possess reproductive toxicity to avian species as a result of endocrine disrupting effects and adverse effects on the reproductive system such as abnormality of the reproductive organs and impairment of egg laying (23). PNMC isolated from DEP showed that they had vasodilatory (62, 100), estrogenic (25, 26, 101), and anti-androgenic activities (101). In addition, PNMC is a degradation product of the insecticide fenitrothion (11), a widely used pesticide with high potential for human beings, animal, and poultry exposure in both rural and residential environments. The accumulation of PNMC from these sources might have significant effects on wildlife and human health via disruptions of endocrine and reproductive systems.

Japanese quail as a laboratory animal has been extensively used in reproductive toxicity testing. Quail are considered to be representative of terrestrial birds and accepted models for assessing both the acute and chronic effects of pesticides and other chemicals in wild birds (19, 71). Results in the chapter 3 of the present study showed that a single administration of PNMC in adult male Japanese quail induced a marked testicular atrophy and clearly shows that PNMC impairs reproductive function in male Japanese quail through its toxic effects on hypothalamus, pituitary, and testis. Moreover, weight of the ovaries and oviducts of immature female quail are very low (less than 5% of those in mature quail) and develop rapidly upon stimulation by long photoperiods and the concomitant increases in the secretion of gonadotropins and ovarian steroidal hormones, estrogens, androgens and progesterone (21). The

immature oviduct is sensitive to estrogens. Stimulation by estradiol induces rapid growth of the oviduct and induces protein synthesis essential for egg production (78). In the present study, mature and immature female quail were used to investigate effects of PNMC on the reproductive function in avian species.

4-2.Materials and methods

4-2-1. Chemicals

PNMC (4-nitro-*m*-cresol) was purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Estradiol -17β (E_2) was purchased from Sigma Chemical Co, St Louis, Mo.

4-2-2. Animals

Japanese quail (*Coturnix japonica*) came from L selected lines, in which chicks hatch after 17 days of incubation and birds were provided with food (Kanematsu quail diet, Kanematsu Agri-tech Co. Ltd., Ibaraki, Japan) and water *ad libitum.* Three-week old and six- month old female birds were housed in metal cages in a controlled environment (lights on 0500 to 1900 h, temperature 23 ± 2 °C, humidity 50 ± 10%, and air exchanged 20 times hourly).

4-2-3. Administration of PNMC in mature female birds

Adult female Japanese quail were reared and naturally bred for one week, then separated from male, and were divided into four groups. Each group of birds was daily intramuscularly (im) injected with PNMC at dose 1, 10 or 100 mg/kg body weight for 5 days. Controls were injected with vehicle (PBS containing 0.05% Tween 80). The experimental period was separated into the pretreatment, treatment, and post-treatment periods, for each 5 days. Eggs were collected every day during the

pretreatment, treatment and post-treatment periods for each 5 days, then numbered and weighted. The collected eggs were stored at 12-13 °C for incubation. The female quail (n=6-10 per group) were weighed and killed by decapitation at the end of experiment. Following decapitation, blood samples were collected in heparinized plastic tubes and centrifuged at 1700 × g for 15 min at 4 °C. Plasma was separated and stored at –20 °C until it was assayed for estradiol-17β, progesterone, and LH. Liver, kidneys, spleens, ovary, and oviduct were collected and weighed. The eggs were incubated under controlled temperature at 37.8 °C and 60% relative humidity with a turning cycle, once an hour, and hatchability was measured at 18th day of incubation.

4-2-4. Administration of PNMC in immature female birds

Immature female Japanese quail were treated with im injection of PNMC (0.1, 1 or 10 mg/kg body weight) for 3 days. Control birds were injected with vehicle (PBS containing 0.05% Tween 80) and the other group of birds were injected with 100 μg/kg of estradiol 17β in sesame oil as a positive control. Twenty four hours after the last injection, birds (9 to 10 in each group) were weighed and euthanized by decapitation. Following decapitation, blood samples were collected in heparinized plastic tubes and centrifuged at 1700 × g for 15 min at 4 °C. Plasma was separated and stored at –20 °C until assayed for estradiol-17β, progesterone and LH. The liver, ovary, and oviduct were collected and weighed and the ovaries were immediately fixed in 4% paraformaldehyde in 0.05 M PBS for immunohistochemical observation.

4-2-5. Determination of plasma concentrations of LH, estradiol-17β, and progesterone

The detailed RIA procedures for determination of LH, estradiol-17β, and progesterone were described in general materials and methods of chapter 2.

4-2-6. Immunohistochemical localization of inhibin α-subunit and 3β-hydroxysteroid dehydrogenase (3βHSD) in ovares of immature birds

Immunohistochemistry procedure for ovarian tissues was shown in general materials and methods of chapter 2. The antibody of 3βHSD was kindly supplied by Dr. J. I. Mason (Edinburgh University, Edinburgh EH3 9YW, U.K.). The antibody against inhibin α subunit was anti-[Tyr^{30}] inhibin- α-chain (1-30)-NH_2 conjugated to rabbit serum albumin. The inhibin α subunit peptide was kindly provided by Dr. N. Ling, (Neuroendocrine Inc., San Dieg, USA).

4-2-7. Statistics

The statistical methods were shown in general materials and methods of chapter 2.

4-3. Results

4-3-1. Effects of PNMC on organs and body weights of mature female birds

Out of 10 birds treated with the highest dose of PNMC (100 mg/kg), 4 birds died within 10 min of treatment. No birds died in the 1, 10 mg/kg or in the control group. The surviving birds in all treatment groups grew normally, with no differences in body weights during the pretreatment, treatment and post-treatment periods (Table 4–1).

Daily injection of PNMC for 5 days, weights of the liver, kidneys and spleens did not change (Table 4-2). 100 mg/kg of PNMC tended to decrease weights of ovary and oviduct, but the difference were not statistically significant (Table 4-2).

4-3-2. Effects of PNMC on egg laying and hatchability of mature female birds

Egg laying rate and egg weights of treated birds with PNMC during the pretreatment, treatment and post-treatment periods were shown in Table 4-3. The egg

weights showed no changes during the pretreatment, treatment and post-treatment periods. However, egg laying rate decreased at all doses of PNMC along treatment period compared with pretreatment, and recovered during the post-treatment period, but the difference were not statistically significant (Table 4-3). There was no change in rate of hatchability at all doses of PNMC treatment groups during the experiment (Table 4-4).

4-3-3. Effects of PNMC on plasma concentrations of LH, estradiol-17β, and progesterone in mature female birds

Plasma concentrations of LH, estradiol-17β, and progesterone in PNMC treated birds were shown in Fig. 4-1. Plasma concentrations of LH were significantly lower ($P < 0.05$) in the groups treated with 10 and 100 mg/kg PNMC -treated group, compared with the control group (Fig. 4-1A). Furthermore, plasma concentrations of estradiol-17β were also significantly lower ($P < 0.05$) in the groups treated with 10 and 100 mg/kg PNMC compared with the control group (Fig. 4-1B). Plasma concentrations of progesterone did not change in PNMC-treated birds compared with the control birds (Fig. 4-1C).

4-3-4. Effects of PNMC on organs and body weights in immature female birds

Body weight, weights of the liver, ovary and oviduct of female birds are shown in Table 4-5. Administration of PNMC and estradiol-17β for 3 days did not cause toxicity in the immature female quail and there were no change in body weight, weights of the liver and ovary. Estradiol-17β -treated birds showed significant increase in oviduct weights. However, all doses of PNMC-treated birds showed significant decrease in oviduct weights compared to control birds (Table 4-5).

4-3-5. Effects of PNMC on plasma concentrations of LH, estradiol-17β, and

progesterone in immature female birds

Plasma concentrations of LH, estradiol-17β, and progesterone in PNMC and estradiol-17β treated immature female birds were shown in Fig. 4-2. Plasma concentrations of LH were significantly lowers ($P < 0.05$) in the groups treated with 1 and 10 mg/kg PNMC and estradiol-17β -treated groups compared with the control group (Fig. 4-2A). Furthermore, plasma concentrations of estradiol-17β were also significantly lower ($P < 0.05$) in the groups treated with 1 and 10 mg/kg PNMC compared with the control group (Fig. 4-2B), whereas significantly high levels of estradiol-17β were observed in the estradiol-17β-treated group (Fig. 4-2B). Plasma concentrations of progesterone showed no changes in PNMC-treated female birds (Fig. 4-2C), but significant high levels were seen in the estradiol-17β-treated birds compared to the control bird (Fig. 4-2C).

4-3-6. Immunohistochemical localization of inhibin α-subunit and 3βHSD in ovaries of immature birds

The results of immunohistochemical localization of inhibin α-subunit and 3βHSD of ovaries in PNMC-treated birds were shown in Fig.4-3. Ovarian morphology of PNMC treated birds was indistinguishable from that of the control. Immunoreactivity for 3βHSD was observed in only interstitial cells of ovaries in both control and PNMC-treated birds (Fig. 4-3C, D). Inhibin α -subunit was immunolocalized in the granulosa cells, theca cells and interstitial cells of ovaries in both control and PNMC-treated birds (Fig. 4-3E, F). There was no apparent difference in the area in the ovary that stained positive by antisera against inhibin α-subunit and 3βHSD between the control and PNMC-treated birds.

4-4. Discussion

In the present study, administration of PNMC significantly decreased plasma

concentrations of LH and estradiol-17β in mature and immature female Japanese quail. The present results suggest that PNMC in DEP acts directly on the hypothalamus-pituitary axis for reducing gonadotropin releasing hormone (GnRH) from hypothalamus, and/or LH release from pituitary glands, and that decrease in plasma levels of estradiol-17β might be due to reduction of circulating LH.

The acute toxicological response was observed in the adult birds treated with the high dose (100 mg/kg) of PNMC, and the effected birds displayed signs of dyspnea and tremor prior to death. From these conditions we speculated that PNMC causes acute toxicity and death, possibly by dropping of blood pressure followed by an ischemic shock, as it has been reported that PNMC has a potent vasodilating activity (62, 100).

The present study showed that egg laying rate of adult female quails decreased during the period of PNMC treatment compared with the pretreatment and recovered during the post-treatment period. Previous papers reported that PNMC has estrogenic activity *in vivo* and *in vitro*(25, 26, 101). Estrogen is required for normal differentiation and development of the oviduct in female birds (79) . However, exposure to excessive estrogen can cause oviduct abnormalities and impaired egg laying (30). It is suggested that the estrogenic potency of PNMC may be involved in the impairment of egg laying in the PNMC-treated adult female quail.

It was hypothesized that PNMC will stimulate growth of the immature oviduct by direct activation of the estrogen receptor. The present results did not provide evidence for PNMC exerting an estrogen-like effect on the oviduct in immature female quail, and weights of the liver (another estrogen sensitive organ in birds) were not influenced by PNMC. However, the plasma concentrations of LH were decreased by PNMC, resulting to the fact that PNMC has an activity to increase the effect of negative-feedback. This explains why the plasma concentrations of estradiol-17β decreased. These results support the hypothesis that PNMC possesses

estrogenic activity in immature quail.

Remarkable amount of DEP are exhausted into the air of many countries. In Japan, 58,902 tons (39) are emitted each year and such large amount can not be ignored. The amount of PNMC in 1 kg of diesel exhaust particles is 28 mg (62, 100). Furthermore, PNMC is a degradation product of the insecticide fenitrothion (34), which is widely used in many countries. According to the data submitted by the pollutant release and transfer registers, the amount of fenitrothion emitted into the environment in 2002 in Japan was approximately 1,300 tons, and roughly half of this is degraded into PNMC (34). In Japan the most conspicuous use of fenitrothion is spraying on small areas such as paddy fields or pinewoods as an insecticide. In one government report, the maximal concentration (5 days average) of fenitrothion was 6.5 $\mu g/m^3$ (1 hour peak; 22 $\mu g/m^3$) measured at close proximity of a spraying by automatic helicopter on pinewoods (Japan Environment agency, 1997). The value indicates that a standard adult person (60 kg BW, inhalation volume; $15m^3$) can inhale 48.8 μg of PNMC per 24 hours, about half of which is from fenitrothion. These findings clearly indicate that large amounts of PNMC exist in the environment resulted from diesel exhaust and fenitrothion.

The results of the present study, a certain that PNMC has deleterious effects on the female reproductive system through toxic effects to the hypothalamus and /or pituitary, and therefore existence of large amounts of PNMC in the environment possibly have serious effects on reproduction of wildlife and human being.

Table 4-1 Body weights of adult female quail treated with PNMC during the

Groups	Number of birds	pretreatment	treatment	Post-treatment
control	8	140.5 ± 5.6	134.1 ± 4.5	141.4 ± 4.6
1mg/kg	10	136.9 ± 2.1	137.8 ± 2.4	142.6 ± 2.6
10 mg/kg	10	137.7 ± 1.4	136.3 ± 2.7	142.6 ± 3.4
100 mg/kg	6	135.6 ± 3.8	138.3 ± 5.3	138.8 ± 2.8

pretreatment, treatment and post-treatment periods

The values are expressed as mean ± SEM.

Table 4-2 Organ weights of adult female Japanese quail tread with PNMC in the end of the experiment

	Control	PNMC treated (mg/kg)		
	0	**1**	**10**	**100**
Number of birds	10	10	10	6
Liver (g)	3.487 ± 0.089	3.825 ± 0.219	4.180 ± 0.287	3.926 ± 0.477
Kidneys (g)	1.631 ± 0.072	1.508 ± 0.069	1.627 ± 0.080	1.580 ± 0.042
Spleens (mg)	66 ± 10	64 ± 7	76 ± 6	59 ± 11
Ovary (g)	5.396 ± 0.625	6.241 ± 0.370	5.770 ± 6.241	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 ± SEM.

Table 4-3 Egg laying rate and egg weights of female Japanese quail treated with PNMC during the pretreatment, treatment and post-treatment period

	Egg weights (g)			Egg laying rate(total eggs/total days)		
Groups	Pretreatment	Treatment	Post- treatment	Pretreatment	Treatment	Post- treatment
control	8.8 ± 0.17	9.0 ± 0.18	9.2 ± 0.35	4.6 (23/5)	3.8 (19/5)	5.0 (25/5)
1 mg/kg	9.2 ± 0.19	9.8 ± 0.25	9.6 ± 0.16	6.0 (30/5)	4.0 (20/5)	6.6 (33/5)
10 mg/kg	9.5 ± 0.17	9.6 ± 0.19	9.9 ± 0.20	7.8 (39/5)	5.0 (25/5)	5.2 (26/5)
100 mg/kg	9.8 ± 0.20	9.4 ± 0.45	9.5 ± 0.49	5.2 (26/5)	2.4* (12/5)	4.2 (21/5)

The values are expressed as mean ± SEM.

*$P<0.05$ compared with the value for pretreatment and post- treatment (Chi-square for independence test)

Table 4-4 Egg hatchability of female Japanese quail treated with PNMC during the pretreatment, treatment and post-treatment period

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

Table 4-5 The organ weights of immature female quail treated with PNMC and estradiol-17β for 3 days

	Control	estradiol-17β (100μg/kg/day)	PNMC (mg/kg/day)		
			0.1	1	10
Number of animals	9	10	10	10	10
Body weight (g)	96.8 ± 3.5	96.1±1.3	96.6±1.8	91.26±1.6	93.4±2.0
Liver (g)	2.3 ± 0.1	2.3±0.1	2.6±0. 1	2.5±0.1	2.3±0.1
Ovary (mg)	69.8 ± 7.4	70.7±6.6	70.3±9.5	59.3±2.2	65.8±5.8
Oviduct (mg)	46.3 ± 4.4	79.9 ± 3.3 *	32.1 ± 2.0*	26.9 ± 1.6*	22.5 ± 1.6*

The values are expressed as mean ± SEM.
*P<0.05 compared with the value for the control group (ANOVA and Fisher's PLSD)

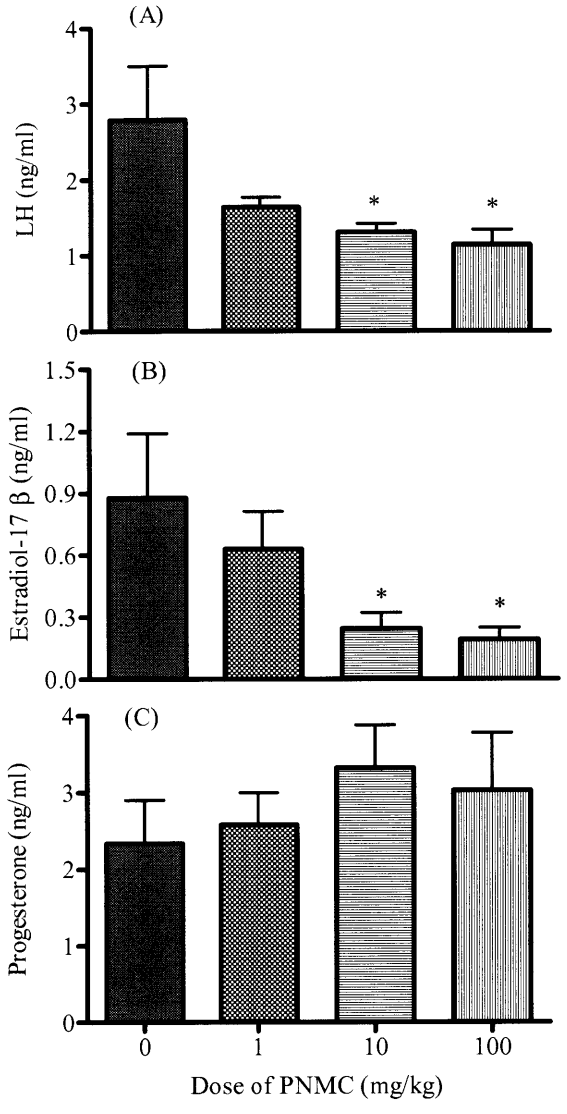


Fig. 4-1 Plasma concentrations of LH (A), estradiol-17β (B), and progesterone (C) in mature female Japanese quail treated for 5 days with PNMC (1, 10 or 100 mg/kg daily, intramuscular). Each bar represents the mean ± SEM of 6 to 10 birds per group. *P < 0.05 compared with control birds (Fisher's PLSD test).

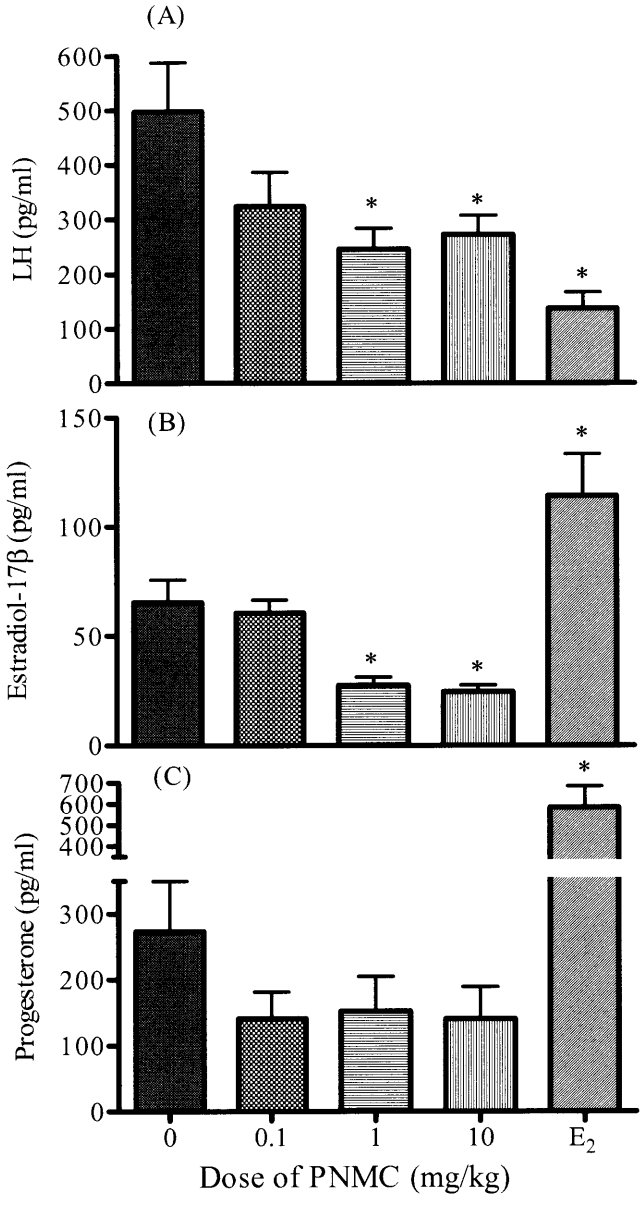


Fig. 4-2 Plasma concentrations of LH (A), estradiol-17β (B), and progesterone (C) in immature female Japanese quail treated for 3 days with PNMC (0. 1, 1 or 10 mg/kg daily, intramuscular) or estradiol-17β (100 μg/kg) . Each bar represents the mean ± SEM of 9 to 10 birds per group. *$P < 0.05$ compared with control rats (Fisher's PLSD test).

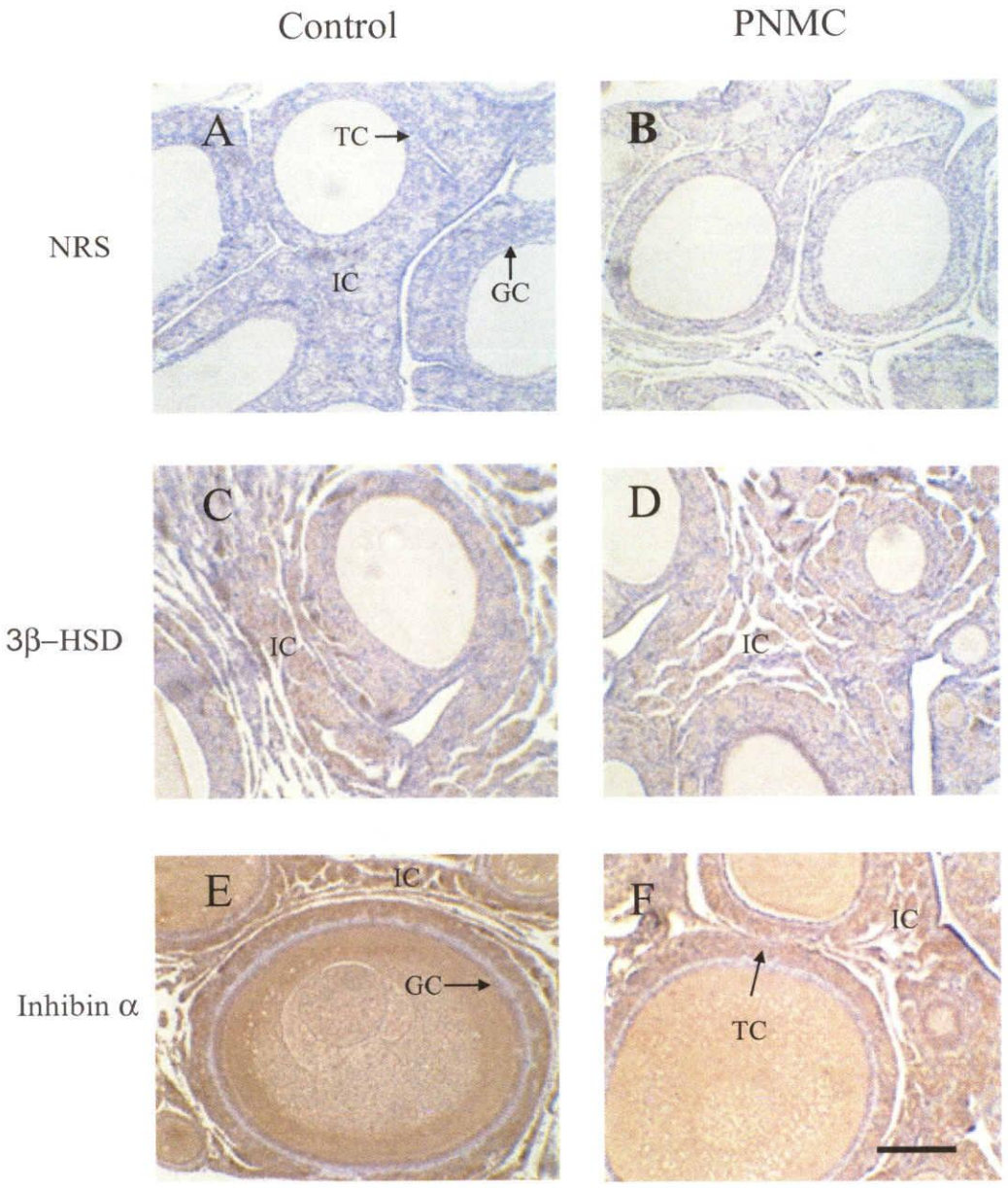


Fig. 4-3 Iimmunohistochemical locations of inhibin α subunit (INHα) and 3β-hydroxysteroid dehydrogenase (3β-HSD) in the ovary of immature female quail treated with PNMC. Left column (A, C and E) and right column (B, D and F) are for immunostaining of NRS, (INHα) and (3β-HSD) in the ovary of vehicle-treated (control) and PNMC (100 mg/kg)-treated quail. IC=interstitial cells, GC=granulose cells, TC=thecal cells, Scale bar =50 μm.

Chapter 5. Effects of PNMC on the Regulation of Testicular Function in Immature Male Rats

5-1. Background

The number of chemicals known to affect human health by disrupting normal endocrine function through interactions with hormone receptors continue to increase (13). DEP contain many kinds of compounds that are hazardous to human health: for example, compounds in DEP have been connected to lung cancer (37, 58), allergic rhinitis (64, 98), and bronchial asthma-like disease (61, 91). DEP extracts possess estrogenic, anti-estrogenic, and anti-androgenic activities (45, 59, 75, 99).

Androgens play a pivotal role in the development and maintenance of the male reproductive system (56, 84). An important endocrine- disrupting effect of DEP is their potential adverse impact on function of male reproductive-system. Diesel exhaust can suppress spermatogenesis in adult mice (122) and rats (109), and in growing rats (117). It is possible that this suppression of sperm production is caused in part by the anti-androgenic effects of DEP, since DEP contain carbon nuclei, which absorb vast numbers of chemicals. The specific compound responsible for this phenomenon remains unclear.

PNMC is a nitrophenol derivative that has been isolated from DEP. It is a vasodilator (62, 100), and it also has estrogenic activity (25, 26, 101). In addition, PNMC is a degradation product of the widely used insecticide fenitrothion (11, 34). Accumulation of PNMC from these sources has serious effects on wildlife and human health via the disruption of endocrine and reproductive systems.

It has been reported that PNMC has estrogenic activity *in vitro* and *in vivo* (25, 26, 101). A previous paper showed that some estrogenic compounds are known to also possess anti-androgenic activity (97). Therefore, the present study evaluates the anti-androgenic activity of PNMC using the recombinant yeast screen assay and

Hershberger assay. Also, there is no information of the effects of PNMC on reproductive function in intact immature male rats. In the present study immature male rats were used to examine the *in vivo* effects of PNMC on plasma and testicular concentrations of testosterone, immunoreactive (ir)-inhibin, basal LH, and FSH, and to examine *in vitro* effects on the secretion of LH and FSH from cultured anterior pituitary cells, and the secretion of testosterone from cultured interstitial Leydig cells.

5-2. Materials and methods

5-2-1. Chemicals

3-Methyl-4-nitrophenol (4-nitro-*m*-cresol, PNMC) was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Collagenase (type V), soybean trypsin inhibitor, hyaluronidase, deoxyribonuclease I (DNase I), fetal bovine serum (FBS), medium 199 (M199), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified eagle medium (DMEM), MEM non-essential amino acids, and penicillin-streptomycin were purchased from Gibco (Grand Island, NY, USA). Human chorionic gonadotropin (hCG; 2200 IU/mg) was obtained from Sankyo ZoKi Co. (Tokyo, Japan). 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) was obtained from Dojindo Laboratories (Tokyo, Japan).

5-2-2. Animals

Twenty-one-day-old male Wistar-Imamichi rats were purchased from Imamichi Institute for Animal Reproduction (Kasumigaura, Ibaraki, Japan). They were kept in a controlled environment with 12 hours light/12 hours dark; temperature, 23 ± 2 °C; humidity, 50% ± 10%; and ventilation with fresh-air changes hourly. Food (CE-2 commercial diet, Japan Clea Co., Tokyo, Japan) and water were available *ad libitum*.

5-2-3. Anti-androgenic activity of PNMC

5-2-3-1. Anti-androgenic recombinant yeast screen assay

Yeast (*Saccharomyces cerevisiae*) contains a gene for the human androgen receptor (hAR) and reporter gene *lacZ* (encoding the enzyme β-galactosidase), which were kindly provided by Prof. John P. Sumpter (Brunel University, Uxbridge, UK). This strain was developed in the Genetics Department of Glaxo Wellcome Pty. Ltd (Stevenage, Herts, UK). In the presence of a ligand such as testosterone, the androgen receptor binds to an androgen responsive element on a plasmid, thereby initiating transcription of the reporter gene *l*acZ. β-galactosidase is synthesized and secreted into the assay medium. This produces a color change from yellow to red, which was measured by absorbance at 540 nm. Anti-androgenic activity was determined by hAR-yeast screen according to the method of Sohoni and Sumpter (97), with slight modifications. To determine anti-androgenic activity, 5α-dihydrotestosterone (DHT, Sigma) was added to the assay medium of the appropriate assay at a background concentration (1.25×10^{-9} M; background DHT) that produced a sub-maximal response (approximately 65% of androgenic activity). Androgenic activity in the presence of PNMC (serially diluted form 1.0×10^{-3} M to 4.9×10^{-7} M) on the hAR-yeast plates was measured at 32 °C, 48 h and the plates were removed at 28 °C, 24 h, giving a total incubation time of 72 h. After 72 h incubation, the absorbance of color change in the medium was measured at wave lengths of both 540 and 620 nm, to allow correction for turbidity (a measure of the growth rate of the yeast cell). The background DHT (3.1×10^{-9} M; positive control of androgenic activity) and blank were also measured. Anti-androgenic activity was determined by comparing color change of PNMC against background DHT. Corrected value = chemical absorbance at 540 nm – (chemical absorbance at 620 nm – blank absorbance at 620 nm).

5-2-3-2. Hershberger assay

The Hershberger assay was performed in accordance with the current draft guidelines for the rodent Hershberger assay (95). Immature male rats aged 21 days were castrated 1 week before the experiment. At 28 days of age, each rat was weighed and implanted with a 5-mm-long silastic tube (1.57 mm ID, 3.18 mm OD; Dow Corning, Midland, MI, USA) containing crystalline testosterone. The testosterone-containing tubes were incubated in saline at 37 °C for 24 h before implantation to avoid the possibility of surge-like release after implantation (44). An implant of this size restores serum testosterone to a physiological level of approximately 1.8 ng/ml. PNMC (0.01, 0.1 of 1 mg/kg) was then administered for 5 days by subcutaneous injection. Rats in the negative control group were injected with vehicle alone (Phosphate-buffered saline; PBS containing 0.05% Tween 80), and rats in the positive control group were injected with the androgen antagonist flutamide (4 mg/kg) in sesame oil. Twenty-four hours after the last injection, rats were weighed and euthanized by decapitation. Blood samples were collected in plastic tubes containing heparin and centrifuged at 1700 × g for 15 min at 4 °C. Plasma was separated and stored at –20 °C until assay for follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone. Five androgen dependent accessory sex glands (ventral prostate, VP; seminal vesicles, SV; levator ani plus bulbocavernosus muscles, LABC; Cowper's gland, COW; and the glans penis, GP) were excised, carefully trimmed of excess adhering connective tissue and fat, and immediately weighed. The liver, kidneys, and adrenal glands were also weighed.

5-2-4 Effects of PNMC on reproductive function

5-2-4-1. Administration of PNMC

Twenty-eight-day-old rats were injected subcutaneously (s.c.) with PNMC (1, 10, or 100 mg/kg body weight) daily for 5 days. Rats injected with vehicle alone (PBS containing 0.05% Tween 80) were used as the control group. Twenty-four hours after the last injection, rats were weighed and decapitated. Blood samples were collected in plastic tubes containing heparin and were centrifuged at 1700 × g for 15 minutes at 4 °C. Plasma was separated and stored at –20 °C until assayed for LH, FSH, testosterone and ir-inhibin. The testes were removed and weighed, and the right testis was homogenized in saline at 4 °C. The supernatant was collected and stored frozen at –20 °C until assayed for testosterone and ir-inhibin. Accessory reproductive glands (VP, SV, LABC, COW and GP) were excised, carefully trimmed of excess adhering tissue and fat, and immediately weighed. The liver and kidneys were also weighed.

5-2-4-2. Preparation of anterior pituitary cells and cell cultures

The anterior pituitary cells of 28-day-old male rats were prepared. All media contained 100 U/mL penicillin and 100 μg/mL streptomycin. The methods used for the preparation of pituitary cell cultures were similar to those described previously (47). Briefly, anterior pituitaries were minced and treated for 30 minutes with 2.8 mg/mL collagenase, 0.8 mg/mL hyaluronidase, 8 mg/mL BSA, and 200 U/mL DNase in DMEM (pH 7.3) containing 50 mM HEPES and 1% MEM non-essential amino acids. The cells were then washed three times with DMEM containing 10% FBS and the supernatant cells were filtered through cell strainers (70 μm nylon; Falcon, BD Biosciences, Bedford, MA, USA). The viability of the cells was evaluated by means of the trypan blue exclusion test and found to be 90%. Cells (2×10^4 cells/well) were cultured in DMEM with 10% FBS on 96-well culture plates at 37 °C under an atmosphere of 95% air–5% CO_2. After 78 h of preculture and a change in the medium, cells were exposed for 24 h to 10^{-6}, 10^{-5}, or 10^{-4} M PNMC dissolved in DMEM. Then the medium of treated cells was changed, and the cells were exposed to

gonadotropin-releasing hormone (GnRH; 10 nM) (National Institute of Diabetes and Digestive and Kidney Disease; NIADDK) or vehicle for 4 h and then harvested. Cultured media were stored at –20 °C until assayed for LH and FSH.

5-2-4-3. Isolation and culture of testicular interstitial cells

Interstitial cell preparations containing the Leydig cells were prepared from the testes of 28-day-old rats as described by Kleinefelter et al with minor modifications (46). The testes were immediately removed, and testicular cells were dispersed by treating the decapsulated testes at 37°C for 20 minutes in M199 medium with 8 mM sodium bicarbonate and 9 mM HEPES containing collagenase (0.25 mg/mL) and soybean trypsin inhibitor (0.025 mg/mL) in a shaking water bath. After incubation, the supernatant containing Leydig cells was decanted through nylon mesh to remove debris. The cells were washed by centrifugation and resuspended in 10 mL of M199 with 1% FBS. The viability of the cells was evaluated by means of the trypan blue exclusion test and found to be 92%. Cells (10^5 cells/well) were cultured in 96-well culture plates at 37 °C under an atmosphere of 95% air–5% CO_2. Following a 20-minutes equilibration period, cells were exposed for 24 h to 10^{-6}, 10^{-5}, or 10^{-4} M PNMC with or without hCG (0.1 IU/mL) dissolved in M199. Cultured media were stored at –20 °C until assayed for testosterone.

5-2-5. Radioimmunoassay (RIA)

The detailed RIA procedures for determination of LH, FSH, immunoreactive (ir)-inhibin and testosterone were described in general materials and methods of chapter 2.

5-2-6. Statistics

The statistical methods were shown in chapter 2.

5-3. Results

5-3-1. Anti-androgenic recombinant yeast screen assay

As shown in Fig. 5-1, significant anti-androgenic activity was found with PNMC at all concentrations (from 4.9×10^{-7} M through 1.0×10^{-3} M) compared to background DHT (2.028 ± 0.013) ($P < 0.01$). The responses at high concentrations of PNMC (from 1.6×10^{-5} M through 1.0×10^{-3} M) were lower than the baseline (blank) levels.

5-3-2. Hershberger assay

Administration of PNMC or flutamide to castrated-immature, testosterone-treated male rats for 5 days had no effect on the body weight gain or on the weight of the kidneys, adrenals, or liver (Table 5-1).

The weights of the androgen-dependent accessory sex glands (VP, SV, LABC, COW, and GP) were shown in Table 5-2. Flutamide-treated rats showed significantly decreased ($P < 0.001$) weights of VP (29% of control value), SV (35% of control), LABC (62% of control), COW (40% of control), and GP (65% of control), respectively. The weight of VP was significantly reduced at PNMC doses of 0.01 mg/kg (88% of control) and 0.1 mg/kg (84% of control), respectively. The weight of SV was also reduced significantly at PNMC doses of 0.01 mg/kg (89% of control), 0.1 mg/kg (84% of control), 1 mg/kg (89% of control); and the weight of GP at the same PNMC dose rates was significantly reduced to 90%, 91%, and 86%, respectively of the control value. However, the weights of LABC and COW did not change significantly.

The plasma concentrations of FSH, LH, and testosterone in castrated-immature, testosterone-treated male rats given PNMC or flutamide were shown in Fig. 5-2. Plasma FSH and LH levels were significantly higher in the 0.1 mg/kg PNMC-treated group and the flutamide-treated group compared with those in the control group (Fig.

5-2 A, B). Plasma concentrations of testosterone in the treated groups were not significantly different from the control levels (Fig. 5-2C).

5-3-3. Effects of PNMC on organ and body weights

Body weights and liver and kidney weights were shown in Table 5-3. The rats in all treatment groups grew normally, and there were no differences in body weight, body weight gain, or kidney weight among the groups (Table 5-3). The liver weights decreased significantly in the groups treated with PNMC at 10 or 100 mg/kg compared with the control group (Table 5-3). Weights of the testes, VP, LABC, and GP (Table 5-4) were not affected by PNMC treatment. However, PNMC at 10 mg/kg significantly ($P < 0.05$) reduced the weights of the epididymis, SV, and COW compared with those of the control group (Table 5-4). The weights of SV, LABC, and COW were also lower in rats treated with PNMC at 100 mg/kg compared with the control group; however, the differences were not significant (Table 5-4).

5-3-4. Effects of PNMC on plasma and testicular hormones

Plasma concentrations of LH, FSH, testosterone, and ir-inhibin in immature rats treated with PNMC for 5 days were shown in Fig. 5-3. Plasma concentrations of LH and FSH were significantly increased ($P < 0.05$) in the group treated with PNMC at 100 mg/kg compared with the control group (Fig. 5-3A, C). Plasma concentrations of testosterone and ir-inhibin were significantly decreased ($P < 0.05$) in the group treated with PNMC at 100 mg/kg compared with the control group (Fig. 5-3B, D).

Testicular contents of testosterone and ir-inhibin in immature rats treated with PNMC for 5 days were shown in Fig. 5-4. Testicular contents of testosterone were significantly decreased in the group treated with PNMC at 100 mg/kg compared with the control group (Fig. 5-4A). Testicular contents of ir-inhibin were also significantly decreased in the groups treated with PNMC at 1 or 100 mg/kg compared with the

control group (Fig. 5-4B).

5-3-5. Effect of *in vitro* exposure of anterior pituitary cells to PNMC on LH and FSH release

To determine the direct effects of PNMC on pituitary function, anterior pituitary cells were incubated in the presence of PNMC for 24 h, and concentrations of LH and FSH in cultured media were measured. Although GnRH stimulation caused a significant increase in the concentrations of LH and FSH in the control group (Fig. 5-5A, B), the concentrations of LH and FSH did not show significant differences in any of the PNMC-treated cells (Fig. 5-5A, B).

5-3-6. Effect of *in vitro* exposure of interstitial Leydig cells to PNMC on testosterone release

To determine the direct effects of PNMC on testicular testosterone production, freshly isolated interstitial cells were incubated in the presence of PNMC with or without hCG for 24 h, and then measured concentrations of testosterone in the cultured media. Basal (unstimulated) testosterone concentrations were lower at all doses of PNMC compared with those in the control group (Fig. 5-6). The hCG stimulation caused a 4-fold increase in concentrations of testosterone in the control group. Concentrations of testosterone in the cultured media were significantly lower in all PNMC-treated groups than in the control group in both the basal and the hCG-stimulated cultures (Fig. 5-6).

5-4. Discussion

The present study clearly demonstrated by the recombinant yeast screen and Hershberger assays that PNMC showed anti-androgenic activity *in vitro* and *in vivo*.

In the recombinant yeast screen, PMNC could inhibit androgenic activity of DHT.

As the reason, PNMC acts an antagonist of human androgen receptor. The responses of PNMC at high concentrations were lower than baseline (blank). This is because the increase in β-galactosidase concentration in the blank human androgen receptor-yeast was faster than in the PNMC-treated yeast at high concentrations. It also indicates that, at high concentrations, the PNMC was toxic to the yeast cells. It is speculated that this cytotoxicity originated from the cresol skeleton of PNMC.

In the present study, immature castrated male rats were used in the 5-day Hershberger assay. A rodent 5- to 7-day Hershberger assay is one of the assays in the Tier-1 Screening Battery proposed by Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) (18). The Hershberger assay has been used for detecting androgen receptor agonists/antagonists by organ weight measurements in sexually immature rats (35). Generally, accessory sex glands and tissues are dependent upon androgen stimulation to gain and maintain weight during and after puberty. If endogenous testicular sources of androgen are removed, exogenous sources of androgens are necessary to increase or maintain the weights of these tissues (4). Several investigators have used this model successfully to assay androgen antagonists (85, 116, 120, 121). Flutamide, an androgen antagonist, had potent anti-androgenic activity at 0.4 mg/kg per day and caused a significant decrease in accessory sex organ weight. Also, the weights of ventral prostate, seminal vesicles, and glans penis were significantly decreased by PNMC treatment. Results of Hershberger assay in the present study thus clearly suggest that PNMC possess anti-androgenic activity.

The results of many experiments have suggested that castration of rats should be followed by a 1-week recovery period (4), and to ensure complete recovery, the same 7-day period between castration and initiation of treatment were used in the present study. In addition, body weight gain is a measurement mandatory for providing information on the general health and well-being of the animal. Moreover, plasma

FSH and LH levels may be additional useful endpoints in the Hershberger assay. As with flutamide, 0.1 mg/kg PNMC significantly increased plasma FSH and LH levels in rats, though plasma testosterone levels were relatively constant. The high dose of PNMC (1 mg/kg) did not show the increase in plasma concentrations of FSH and LH, though the mechanism remains unclear. These results point to the conclusion that PNMC has anti-androgenic effects on the hypothalamus-pituitary axis and the accessory sex glands. Previous 5-day Hershberger assays have also shown that plasma LH levels are significantly increased by flutamide, whereas plasma testosterone levels are similar to those of controls (119). In fact, androgen receptor antagonists such as flutamide bind to the androgen receptor and effectively block the recognition of androgens, leading to the altered development of androgen-dependent tissues (70) and plasma hormone levels (119). Another anti-androgenic compound, the synthetic non-steroidal procymidone, also increases plasma LH level in rats (36).

The Hershberger assay responds to anti-androgenic compounds acting through different mechanisms of action, so it is important to confirm any purported androgen receptor-activity with other methods. The Hershberger assay was used to demonstrate that PNMC can inhibit androgen-dependent tissue growth. The effects of PNMC on androgen-dependent tissue weights were similar to those caused by treatment with the reference anti-androgen flutamide.

Administration of PNMC significantly increased plasma concentrations of LH and FSH in intact immature male rats. In contrast, plasma concentrations of testosterone and ir-inhibin were lower in the PNMC-treated animals. Testicular contents of testosterone and ir-inhibin were also significantly decreased in the PNMC-treated rats compared with those in control rats. Testosterone and inhibin are synthesized in the Leydig and Sertoli cells in male rats (7, 82). LH and FSH are the major stimulants of testosterone and inhibin production from Leydig cells and Sertoli cells, respectively (20, 112). These findings suggest that PNMC affects testicular

function by affecting the functions of Leydig cells and Sertoli cells, and that the increased levels of plasma LH and FSH might be due to a reduction of the negative feedback regulation by testosterone and inhibin. Leydig cells are known to play a crucial role in synthesizing testosterone and regulating spermatogenesis (94). Alteration of Leydig cells function can adversely affect spermatogenesis (94). The present study demonstrated that PNMC suppress GnRH stimulated LH and FSH secretion from pituitary cells *in vitro*. In addition the present study also showed that PNMC inhibit testosterone production in cultured testicular interstitial Leydig cells These results, therefore, strongly suggest that PNMC has a direct toxicological effect on Leydig cells in the testes.

PNMC had no effect on normal growth in rats. However, PNMC caused weight reduction in the accessory reproductive organs. Although these results did not showed dose-dependant effect, but showed an inverted-U dose response, previously reported in several environmental chemicals such as bisphenol A (73). Previous papers have reported that PNMC isolated from DEP possesses estrogenic activity *in vivo* and *in vitro*(25, 26, 101). A previous paper reported that the estrogenic chemical bisphenol-A inhibits the function of accessory reproductive organs (66). Estrogens such as estradiol and diethylstilbestrol (DES) also inhibit the development of spermatogonia and the function of Leydig and Sertoli cells in the fetal rat testes (52). In addition, PNMC also shows an anti-androgenic activity *in vitro* and *in vivo* in the present study. Previous reports have shown that flutamide, a potent androgen antagonist, decreases the weight of accessory sex organs in rats (4, 119). These results suggest that both the estrogenic and the anti-androgenic potency of PNMC may be involved in the suppression of testicular function in PNMC-treated rats.

In conclusion, the present study clearly shows that PNMC in DEP have anti-androgenic activity *in vitro* and *in vivo*. Also, the present study shows that PNMC impairs the reproductive functions of male rats by toxic effects on testicular

function. The present findings suggest that PNMC may have suppression effects on reproductive function in humans, domestic animals, and wildlife.

Table 5-1 Organ weights after five days of treatment of immature castrate-T (5mm silastic implant) rats with PNMC and flutamid in Hershberger assay

	Control (T)	Flutamide (4mg/kg/day+T)	PNMC(mg/kg/day)		
			0.01+T	0.1+T	1+T
Number of animals	7	7	7	7	7
Body weight (g)	93.29±2.52	93.29±1.82	94.71±1.85	95.14±1.53	95.29±1.81
Weight gain (g)	25.11±1.73	26.14±0.72	24.91±1.26	29.04±1.27	26.61±1.50
Liver (g)	4.58±0.26	4.66±0.21	4.53±0.16	4.96±0.24	4.64±0.23
Kidneys (g)	1.03±0.03	0.97±0.03	1.06±0.02	1.10±0.02	1.09±0.03
Adrenals (mg)	27.09±0.64	25.96±0.55	28.67±1.34	30.40±2.12	27.56±0.67

The values are expressed as mean ± SEM.
T=testosterone

Table 5-2 Relative accessory sex organ weights after five days of treatment of immature castrate-T (5 mm silastic implant) implanted rats with PNMC and flutamideHershberger assay

Organ weights (mg)/ Body weight (g)×100 (mg/g×100)	Control (T)	Flutamide (4mg/kg/day+T)	PNMC(mg/kg/day)		
			0.01+T	0.1+T	1+T
VP (mg/g×100)	39.34±1.46	11.40±0.83***	34.71±1.54*	32.90±1.49**	36.39±1.31
SV (mg/g×100)	72.74±2.43	25.62±1.56***	64.75±2.74*	60.77±2.39**	64.70±3.52*
LABC (mg/g×100)	115.38±4.10	71.22±1.78***	112.89±3.75	107.61±2.09	110.80±2.59
GP (mg/g×100)	42.39±1.99	27.97±0.70***	38.10±1.17*	38.38±0.54*	36.27±1.45**
COW (mg/g×100)	6.97±0.41	2.81±0.22***	6.66±0.05	6.26±0.23	6.96±0.32

The values are expressed as mean ± SEM. (n=7).

T=testosterone

VP, ventral prostate; SV, seminal vesicle plus coagulating glands; LABC, levator ani plus bulbocavernosus muscles; COW, Cowper's gland; GP, glans penis

***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ compared with value of control group (ANOVA and Fisher's PLSD)

Table 5-3 Body weights and organ weights of immature rats treated with PNMC for 5 days

	Control	PNMC (mg/kg/day)		
	0	**1**	**10**	**100**
Number of animals	9	9	9	10
Body weight (g)	98.9 ± 1.1	100.0 ± 0.4	94.7 ± 2.0	97.1 ± 2.6
Weight gain (g)	24.5 ± 0.9	25.2 ± 0.5	23.7 ± 1.3	24.9 ± 1.9
Liver (g)	4.91 ± 0.18	4.84 ± 0.07	4.39 ± 0.13*	4.39 ±0.15*
Kidneys (g)	1.15 ± 0.04	1.17 ± 0.02	1.09 ± 0.03	1.14 ± 0.04

The values are expressed as mean ± SEM for 9 – 10 animals.

*$P < 0.05$ compared with the value for the control group (ANOVA and Fisher's PLSD)

Table 5-4 Weights of testes and accessory sex organ in immature rats treated with PNMC for 5 days

	Control	PNMC (mg/kg/day)		
Description	**0**	**1**	**10**	**100**
Testes (g)	0.70 ± 0.03	0.74 ± 0.02	0.62 ± 0.04	0.70 ± 0.05
Epididymis (g)	0.11 ± 0.01	0.11 ± 0.01	0.09 ± 0.01*	0.10 ± 0.01
VP (mg)	35.7 ± 3.2	39.0 ± 2.8	32.5 ± 2.1	37.3 ± 3.1
SV (mg)	27.1 ± 2.3	24.7 ± 1.2	21.1 ± 1.2*	23.1 ± 2.6
LABC (mg)	72.0 ± 6.0	66.5 ± 2.5	61.5 ± 3.9	64.9 ± 3.6
COW (mg)	5.6 ± 0.9	4.4 ± 0.5	3.8 ± 0.3*	4.4 ± 0.5
GP (mg)	24.3 ± 1.9	29.5 ± 1.4	25.1 ± 2.2	27.7 ± 1.0

The values are expressed as mean ± SEM for 9 – 10 animals.
VP, ventral prostate; SV, seminal vesicle plus coagulating glands; LABC, levator ani plus bulbocavernosus muscles; COW, Cowper's gland; GP, glans penis
*$P < 0.05$ compared with the value for the control group (ANOVA and Fisher's PLSD)

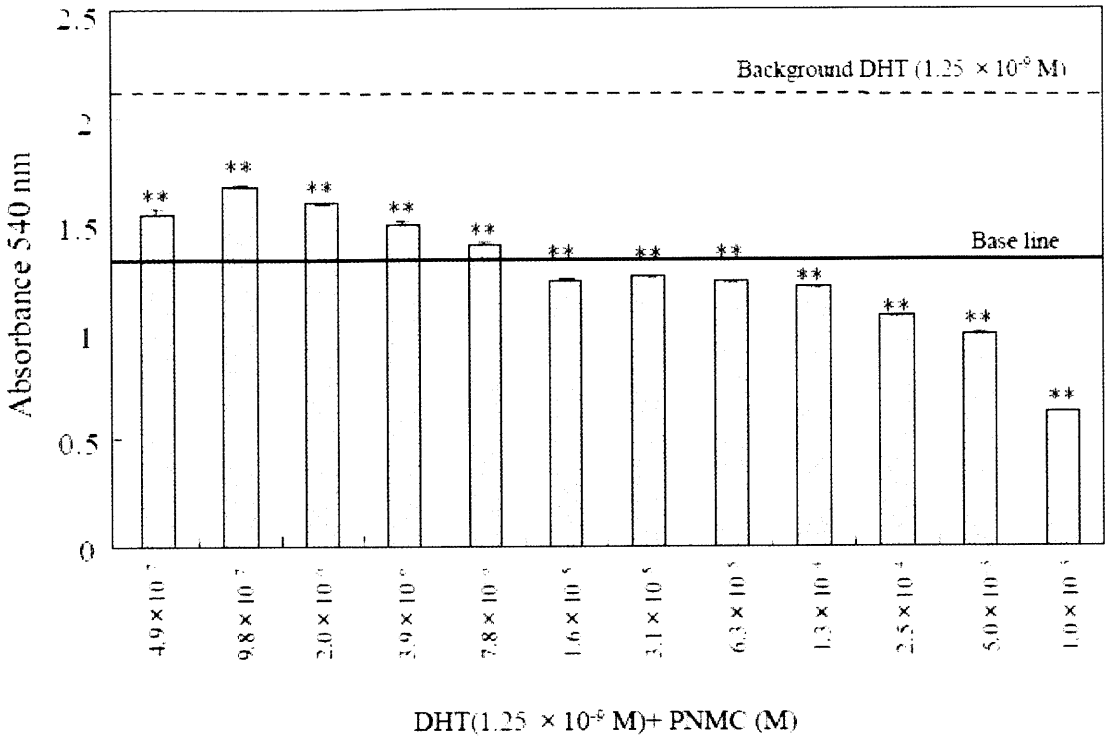


Fig. 5-1 Anti-androgenic activity of PNMC. Anti-androgenic activity was evaluated by a hAR-yeast screen. Data show means ± SEM (n=6) of activity measured by absorbance of the culture medium after 72 h of incubation. $^{**}P < 0.01$ compared with background 5α-dihydrotestosterone (DHT) level (Fisher's PLSD test).

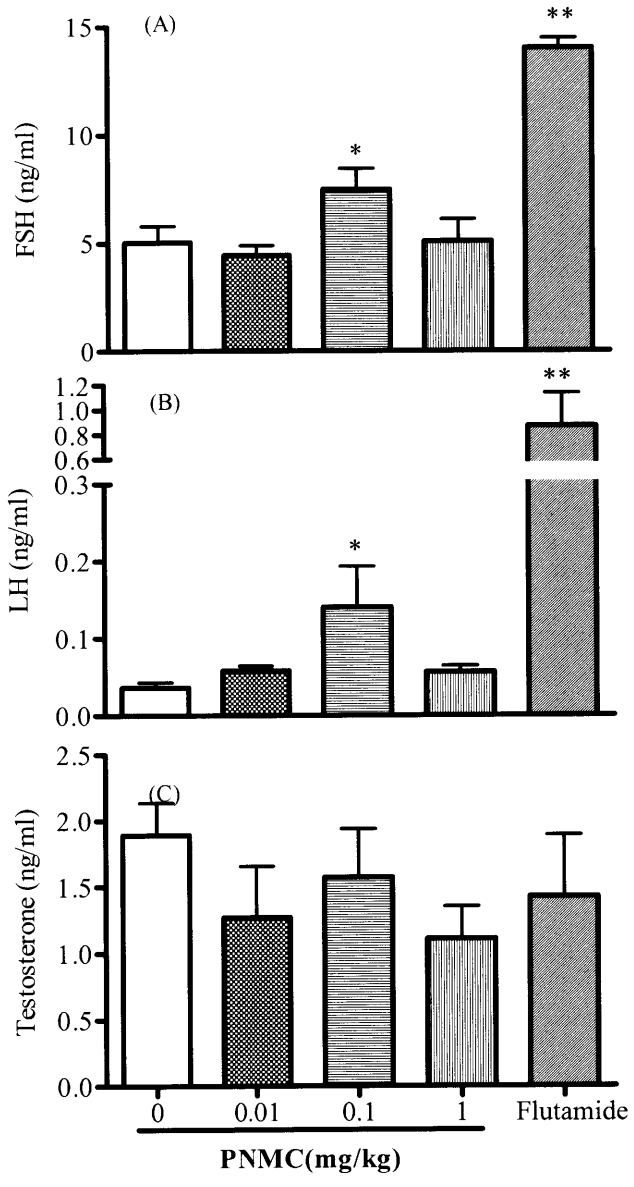


Fig. 5-2 Plasma concentrations of FSH (A), LH (B), and testosterone (C) in castrated rats treated for 5 days with testosterone (5 mm silastic implant) with or without flutamide (4 mg/kg daily, subcutaneously) or PNMC (0.01, 0.1 or 1 mg/kg daily, subcutaneously) in Hershberger assay. Each bar represents the mean ± SEM of 7 rats per group. $^{**}P < 0.01$, $^{*}P < 0.05$ compared with control rats (Fisher's PLSD test).

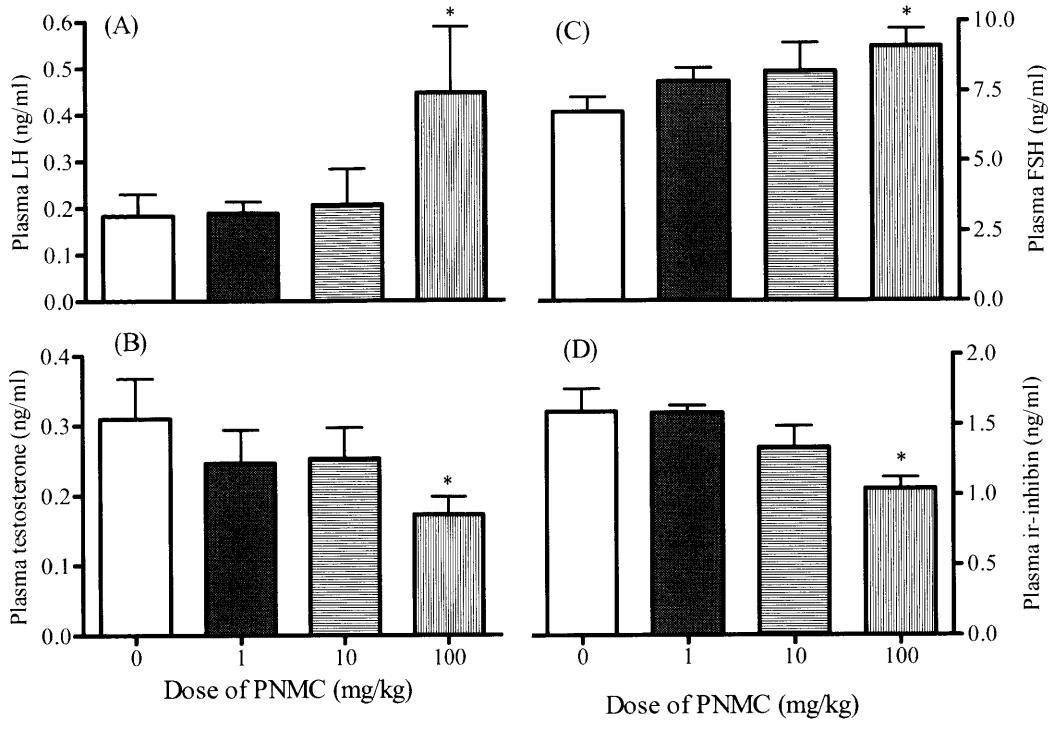


Fig. 5-3 Plasma concentrations of LH (A), testosterone (B), FSH (C), and immunoreactive (ir)-inhibin (D) in immature rats treated with PNMC at doses of 1, 10, or 100 mg/kg/day for 5 days. Each bar represents the mean ± SEM of 9 or 10 rats per group. *$P < 0.05$ compared with control rats (Fisher's PLSD).

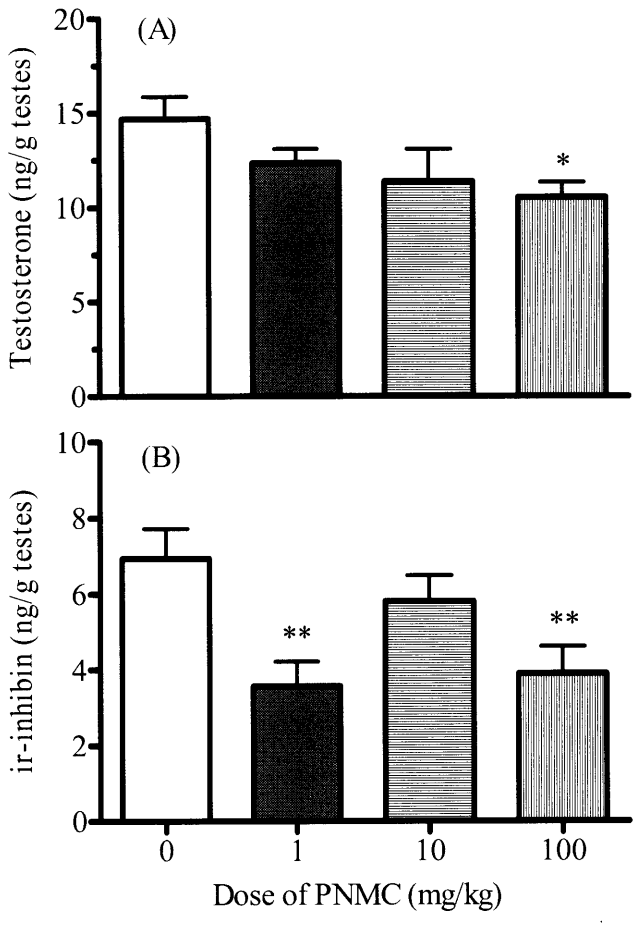


Fig. 5-4 Testicular contents of testosterone (A) and immunoreactive (ir)-inhibin (B) in immature rats treated with PNMC at doses of 1, 10, or 100 mg/kg/day for 5 days. Each bar represents the mean ± SEM of 9 or 10 rats per group. $*P < 0.05$ and $**P < 0.01$ compared with control rats (Fisher's PLSD).

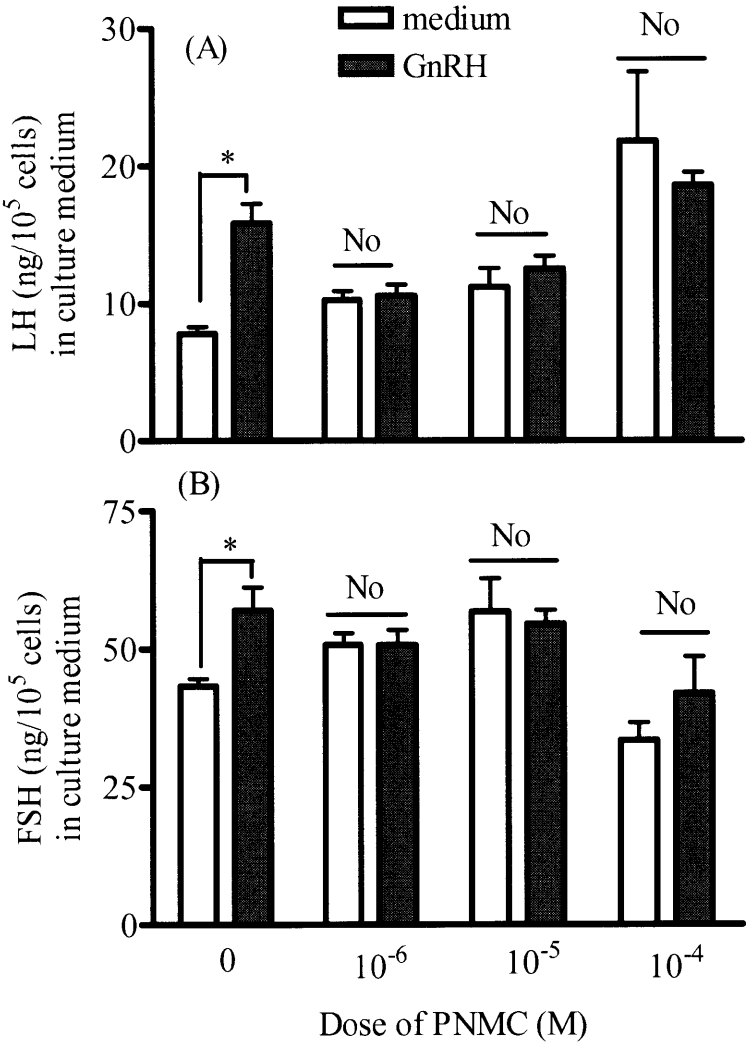


Fig. 5-5 Effect of PNMC on LH and FSH secretion in basal and GnRH-stimulated anterior pituitary cells. Anterior pituitary cells were incubated for 24 hours with 10^{-6}, 10^{-5} or 10^{-4} M PNMC either with or without GnRH (10 nM). Each bar represents the mean ± SEM of three observations. $*P < 0.05$ compared with the basal (medium) control (Fisher's PLSD).

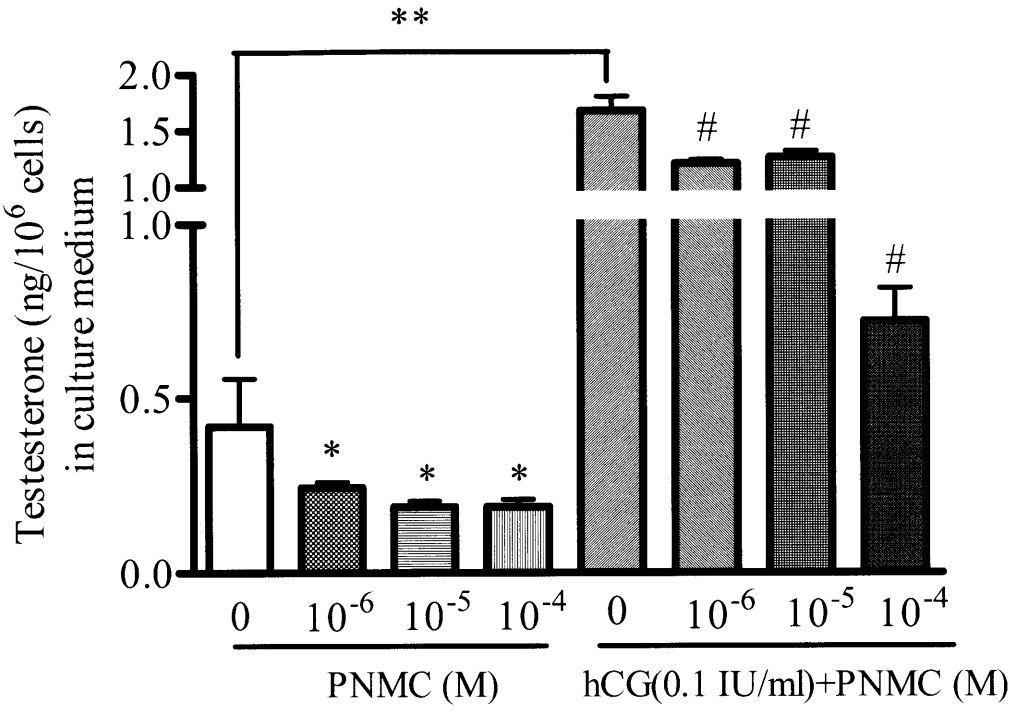


Fig. 5-6 Effect of PNMC on testosterone secretion in basal and human chorionic gonadotropin (hCG)-stimulated Leydig cells. Interstitial cells containing Leydig cells were incubated for 24 hours with 10^{-6}, 10^{-5} or 10^{-4} M PNMC either with or without hCG (0.1IU/mL). Each bar represents the mean ± SEM of three observations. *$P <$ 0.05 compared with the basal (M199) control, $^{\#}P <$ 0.05 compared with the hCG stimulation control (Fisher's PLSD).

Chapter 6. Effects of PNMC on the Suppression of Adrenocortical Function in Immature Male Rats

6-1. Background

DEP generated by motor vehicles are the main cause component of air pollutant. DEP contain thousands of compounds that have several hazardous effects on human health, including lung cancer (37, 58), allergic rhinitis (64, 98), and bronchial asthma-like disease (61, 91). Another important of feature of DEP is their endocrine-disrupting properties and potential adverse effects on both male and female reproductive functions. Diesel exhaust suppresses spermatogenesis in adult mice (122) and rats (109, 117), and pregnant C57BL mice injected with DEP extracts show significant increases in abortion rate and uterine weight (110). These *in vivo* findings show that DEP contain compounds that can modulate estrogenic and anti-androgenic activities. *In vitro* studies similarly have shown that DEP possess estrogenic, anti-estrogenic, and anti-androgenic activities (45, 59, 75, 99). However, because DEP contain carbon nuclei, which absorb numerous and diverse chemicals, the specific compound(s) responsible for these phenomena remain unclear.

PNMC, a nitrophenol derivative isolated from DEP has been shown to be a vasodilator (62, 100); it also has estrogenic (25, 26, 101) and anti-androgenic activities (101). Furthermore, the previous *in vivo* study showed that a single administration of PNMC in adult male Japanese quail induced marked testicular atrophy (53). In addition, PNMC is a degradation product of the insecticide fenitrothion (11, 34), a widely used pesticide with high potential for exposure of humans, livestock, and wild animals in both rural and residential environments. The accumulation of PNMC from these sources has serious effects on wildlife and human health via endocrine and reproductive disruptions.

It is well known that the adrenal gland is vital to health and has a role in

reproduction and development. However, previous studies have not addressed the effects of PNMC on adrenal function. In the present study, immature male rats were used to examine the *in vivo* and *in vitro* effects of PNMC on the pituitary–adrenal axis, and also evaluated the *in vivo* effect of PNMC on the thyroid gland.

6-2. Materials and methods

6-2-1. Chemicals

3-Methyl-4-nitrophenol (4-nitro-*m*-cresol; PNMC) was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Collagenase (type V), hyaluronidase, deoxyribonuclease I (DNase I), fetal bovine serum (FBS), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA), and Dulbecco's Modified Eagle medium (DMEM) and penicillin–streptomycin from Gibco (Grand Island, NY, USA). Synthetic rat adrenocorticotropic hormone (ACTH; AFPRFR 7890) was obtained from NHPP-NIDDK (Baltimore, MD, USA).

6-2-2. Animals

Immature male (21-day-old) Wistar–Imamichi rats were purchased from the Imamichi Institute for Animal Reproduction (Kasumigaura, Ibaraki, Japan). They were kept in a controlled environment (lights on, 0700–1900h; temperature, 23 ± 2 °C; humidity, 50% ± 10%; air exchanged 20 times hourly). Food (CE-2 diet, Japan Clea Co., Tokyo, Japan) and water were available *ad libitum*.

6-2-3. Administration of PNMC

PNMC (1, 10, or 100 mg/kg) was subcutaneously injected in 28-day-old rats with (in phosphate-buffered saline [PBS] containing 0.05% Tween 80) daily for 5 days. Additional rats were injected with vehicle alone as the negative-control group. At 24

h after the last injection, rats (9 per group) were weighed and decapitated. Blood samples were collected into plastic tubes containing heparin and centrifuged at 1700 × g for 15 min at 4 °C. Plasma was separated and stored at –20 °C until assayed for ACTH, progesterone, corticosterone, free triiodothyronin (FT3) and free L-thyrocine (FT4). The adrenal glands were excised, carefully trimmed of excess adhering tissue and fat, and immediately weighed.

6-2-4. Preparation of anterior pituitary cells and cell cultures

Anterior pituitary cells were prepared from 28-day-old male rats. All culture media contained 100 U/ml penicillin and 100 μg/ml streptomycin. The methods used for the preparation of pituitary cell cultures were similar to those described previously (47), with minor modifications. Briefly, anterior pituitaries were minced into DMEM with 2.8 mg/ml collagenase, 0.8 mg/ml hyaluronidase, 8 mg/ml BSA, and 200 U/ml DNase I incubated the reactions at 37°C for 30 min. The cells were then washed three times with DMEM containing 10% FBS and the supernatant cells were filtered through cell strainers (pore size 70-μm nylon mesh; Falcon, BD Biosciences, Bedford, MA, USA). The viability of the cells was evaluated by means of the trypan blue exclusion test and found to be approximately 90%.

Pituitary cells (2×10^4 cells/well) were cultured in DMEM with 10% FBS in 96-well culture plates at 37 °C under an atmosphere of 95% air – 5% CO_2. After 78 h of preculture and a change of medium, cells were exposed for 24 h to 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M PNMC dissolved in DMEM, and then the medium was collected from each well. Culture media were stored at –20 °C until assayed for ACTH.

6-2-5. Isolation and culture of primary adrenal cells

Adrenal glands were obtained from intact 28-day-old male rats (n=10). Animals were killed by decapitation, and adrenal glands were removed immediately. For

isolation of adrenal cells, whole glands were used, and the procedures used followed previously described methods (28) with minor modifications. Briefly, the isolation and dissociation of adrenal cells were performed at 37 °C for 30 min in DMEM with collagenase (2 mg/ml) and DNase I (200 U/ml) in a shaking water bath. The cells then were washed three times with DMEM containing 2% FBS, and the cells in the supernatant were filtered through cell strainers (70-μm nylon mesh; Falcon). Cells were used in suspension at a concentration of 10^5 cells/ml, in DMEM medium supplemented with 2% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The viability of the cells was evaluated by means of the trypan blue exclusion test and found to be approximately 93%.

Adrenal cells (2×10^4 cells/well) were cultured in 96-well culture plates for 24 h at 37 °C under an atmosphere of 95% air – 5% CO_2. The isolated adrenal cells were cultured for 24 h and then incubated for an additional 24 h in the present of 10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M PNMC dissolved in DMEM. After final incubation with PNMC, culture media were replaced with fresh media with 1 nM ACTH (to stimulate cells) or vehicle only, the cells were incubated for another 4 h, and then the culture medium was collected from each well. Culture media were stored at –20 °C until assayed for corticosterone and progesterone.

6-2-6. Radioimmunoassay (RIA)

The detailed RIA procedures for determination of ACTH, progesterone, corticosterone, FT3 and FT4 were described in general materials and methods of chapter 2.

6-2-7. Statistics

The statistical methods were shown in chapter 2.

6-3. Results

6-3-1. Effects of PNMC on adrenal glands weights

The rats in all treatment groups grew normally, and there were no differences in the body weight among the groups. Absolute and relative adrenal glands weights (Table 6-1) were decreased significantly ($P < 0.05$) in the groups treated with 10 and 100 mg/kg PNMC compared with the control group.

6-3-2. Effects of PNMC on plasma ACTH, corticosterone and progesterone

Plasma concentrations of ACTH increased significantly ($P < 0.05$) in the group treated with 100 mg/kg PNMC daily for 5 days compared with the control group (Fig. 6-1A). Plasma concentrations of corticosterone decreased significantly ($P < 0.01$) in all PNMC-treated groups (Fig. 6-1B), and plasma concentration of progesterone in the groups treated with 10 or 100 mg/kg PNMC were significantly low as compared with the control group ($P < 0.05$ and $P < 0.01$, respectively; Fig. 6-1C).

6-3-3. Effects of PNMC on plasma free triiodothyronin (FT3) and free L-thyrocine (FT4)

Although the plasma concentration of FT_3 did not differ between groups (Fig. 6-2A), that of FT_4 declined significantly in immature rats treated with 100 mg/kg PNMC compared with the control group ($P < 0.05$, Fig. 6-2B).

6-3-4. Effect of *in vitro* exposure of anterior pituitary cells to PNMC on ACTH production

To determine the direct effects of PNMC on ACTH secretion, anterior pituitary cells were cultured in the presence of PNMC for 24 h and then measured the concentration of ACTH in culture media. High concentrations of PNMC tended to

decrease the basal release of ACTH from pituitary cells, but the differences were not statistically significant (Fig. 6-3).

6-3-5. Effect of *in vitro* exposure of adrenal cells to PNMC on corticosterone and progesterone production

To determine the direct effects of PNMC on adrenal corticosterone and progesterone production, freshly isolated adrenal cells were cultured in the presence of PNMC for 24 h and then measured the concentration of corticosterone and progesterone in the culture media. Compared with ACTH unstimulated control values, basal (unstimulated) corticosterone concentrations were decreased in cells treated with 10^{-5} M PNMC ($P < 0.01$, Fig. 6-4A), but progesterone production was unchanged (Fig. 6-4B). Stimulation with ACTH in the absence of PNMC treatment caused a significantly increase in the concentration of corticosterone and progesterone compared with basal values ($P < 0.01$, Fig. 6-4A, B). In contrast, ACTH stimulation led to significantly ($P < 0.01$) decreased concentrations of corticosterone in the culture media of PNMC-treated cells (Fig. 6-4A). Similarly to its effect on corticosterone production, ACTH stimulation decreased progesterone secretion from all groups of PNMC-treated cells compared with ACTH-treated control cells ($P < 0.01$, Fig. 6-4B).

6-4. Discussion

The present study clearly demonstrated that a specific component of DEP directly inhibits adrenocortical function. In rats, corticosterone is the predominant adrenal steroid hormone, and its secretion is directly stimulated by ACTH secretion from corticotrophs in the anterior pituitary gland. In the presten study, administration of PNMC significantly increased plasma concentrations of ACTH in immature male rats. In contrast, plasma concentrations of corticosterone and progesterone significantly decreased in PNMC-treated animals. Furthermore, whereas PNMC tend to decreased

ACTH production in cultured anterior pituitary cells, the pollutant did inhibit corticosterone and progesterone production in cultured primary adrenal cells. This observation is in agreement with the *in vivo* part of our study that showed increased ACTH levels and decreased corticosterone and progesterone levels in PNMC-treated rats. Together the present findings demonstrate that PNMC suppresses adrenal function and that the associated increased levels of plasma ACTH might reflect reduced negative feedback regulation by corticosterone. The present results, therefore, strongly suggest that PNMC has a direct toxicologic, but noncytotoxic, effect on adrenocortical cells at the doses in the present study.

The adrenal gland is an important endocrine organ, producing and secreting gluococorticoids in response to stress, infection, and trauma, thereby supporting homeostasis of the organism. Although PNMC lacked any effect on the overall growth of rats, it reduced the weights of the adrenal glands and the plasma concentrations of corticosterone and progesterone. This decrease in adrenal weight suggests disruption of the negative feedback system involved in the hypothalamus–pituitary–adrenal axis. The *in vitro* study clearly showed that ACTH stimulation decreased secretion of corticosterone and progesterone in PNMC-treated adrenal cells. These results suggest that the inhibitory effect of PNMC on secretion of corticosterone and progesterone maybe mediated via the ACTH receptor mechanism. Previous papers reported that PNMC isolated from DEP has estrogenic activity *in vivo* and *in vitro* (25, 26, 101). Estrogen inhibits steroid production by human fetal adrenal cortical cells (24, 60, 114), and the phytoestrogen genistein decreases serum corticosterone levels in human adrenal H295R cells by inhibiting 3β-hydroxysteroid dehydrogenase and cytochrome P450 c21-hydroxylase activity (74). These results suggest that, because of its estrogenic activity PNMC may suppress the adrenal function of rats through interaction with the estrogen receptor or direct effects on steroidogenic enzymes.

It is well known that thyroid hormones are important for the growth, development and metabolism of many tissues, and they play an important role in adrenal function. Hypothyroidism has been reported to reduce adrenal weight (41) and the plasma concentration of corticosterone and to affect circadian adrenocortical rhythm (63, 103-105, 107). In the present study, PNMC-treated rats showed decreased adrenal weight, decreased plasma concentrations of corticosterone, and hypothyroidism, suggesting that the chemical depresses thyroid gland function. Thyroid hormones are also known to influence the immune system. Previous studies have reported that thyroid hormones increase the proliferation of lymphocytes (49), and mallards with methimazole-induced hypothyroidism have reduced cell-mediated immunity (22). Therefore, the decreased thyroid hormone production noted in PNMC-treated rats likely could lead to dysfunction of thyroid hormone-associated endocrine and immune systems.

The present results have great significance in regard to environmental issues. Vast amounts of DEP are emitted into the atmosphere every year. For example, in Japan alone, approximately 5.9×10^7 Kg of DEP are released annually (39), an amount we cannot afford to ignore. A kilogram of DEP contains approximately 28 mg PNMC (62, 100), but the exact concentrations of PNMC in the environment remain unknown. In addition, it is well known that fenitrothion, one of the most common insecticide worldwide, is degraded into PNMC (11, 34). Data provided by the Pollutant Release and Transfer Registers (PRTR), reveal that the amount of fenitrothion discharged into the environment in Japan in 2002 was approximately 1.3×10^6 Kg, roughly half of which was degraded into PNMC (34). In addition, the amount of PNMC in rainwater in Roskilde, Denmark, peaked at 2483 ng/l in 2000 and 2001(5), and PNMC is becoming more abundant in the air and soils (68). From these data, it is obvious that large amounts of PNMC are present in the environment because of fenitrothion use and diesel exhaust, and this condition may exert profound negative

effects on wildlife and humans.

In conclusion, the present results demonstrate that PNMC from DEP impairs adrenal and thyroid functions. PNMC affects the adrenal glands directly to reduce corticosterone secretion, and in addition it may cause suppression of immune function.

Table 6-1 Body weight and adrenal gland weights of immature male rats treated with PNMC for 5 days

	Control	PNMC (mg/kg/day)		
	0	**1**	**10**	**100**
Number of animals	9	9	9	9
Body weight (g)	97.6 ± 1.7	101.6 ± 1.1	98.0 ± 1.2	99.9 ± 1.6
Adrenal glands weight (mg)	32.6 ± 1.7	29.9 ± 1.6	28.6 ± 1.0*	28.0 ± 0.7*
Adrenal glands weight/ body weight (mg/g)	0.335 ± 0.02	0.294 ± 0.01	0.292 ± 0.01*	0.281 ± 0.01*

Values are expressed as means ± SEM.

* $P < 0.05$ compared with value for the control group (ANOVA and Fisher's PLSD)

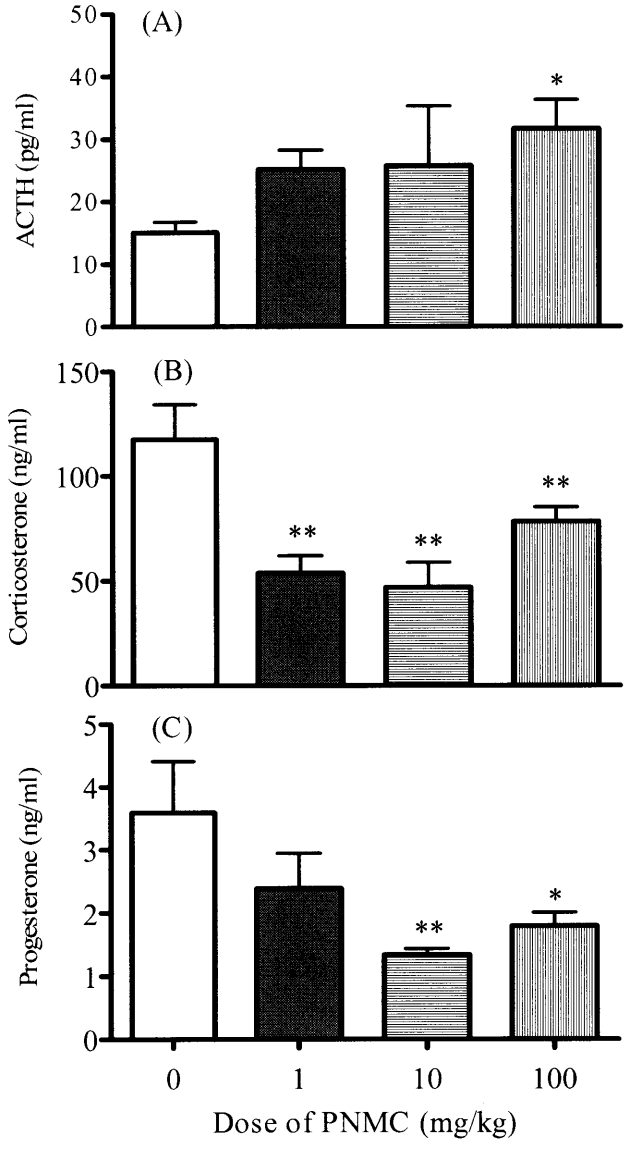


Fig. 6-1 Plasma concentrations of ACTH (A), corticosterone (B), and progesterone (C) in immature male rats treated with PNMC at doses of 1, 10 or 100 mg/kg daily for 5 days. Each bar represents the mean ± S.E.M. of 9 rats per group. * $P < 0.05$ and ** $P < 0.01$ compared with control rats (Fisher's PLSD).

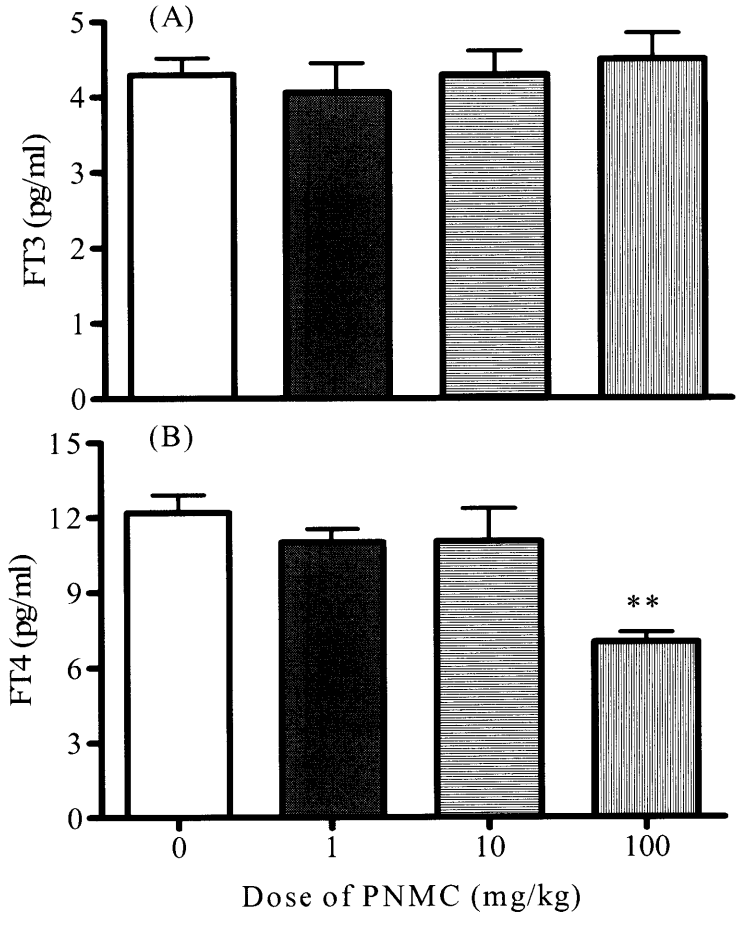


Fig. 6-2 Plasma concentrations of FT_3 (A) and FT_4 (B) in immature male rats treated with PNMC at doses of 1, 10 or 100 mg/kg daily for 5 days. Each bar represents the mean ± S.E.M. of 9 rats per group. * $P < 0.05$ compared with control rats (Fisher's PLSD).

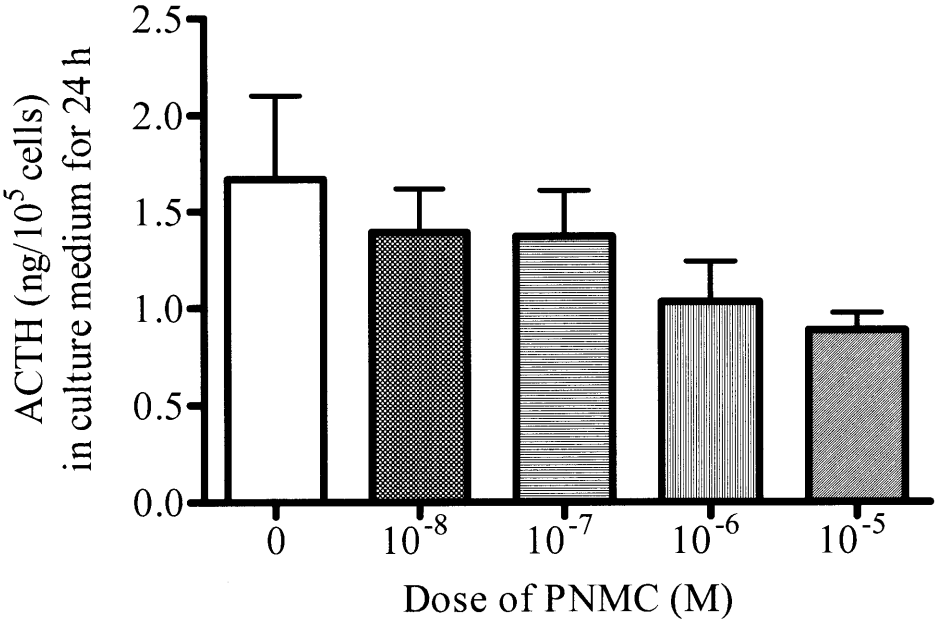


Fig. 6-3 Effect of PNMC on ACTH secretion from anterior pituitary cells. Anterior pituitary cells were incubated for 24 h with 10^{-8}, 10^{-7}, 10^{-6} or 10^{-5} M PNMC. Each bar represents the mean ± S.E.M. (n=6). The experiment was done 3 times, and a representative result is shown.

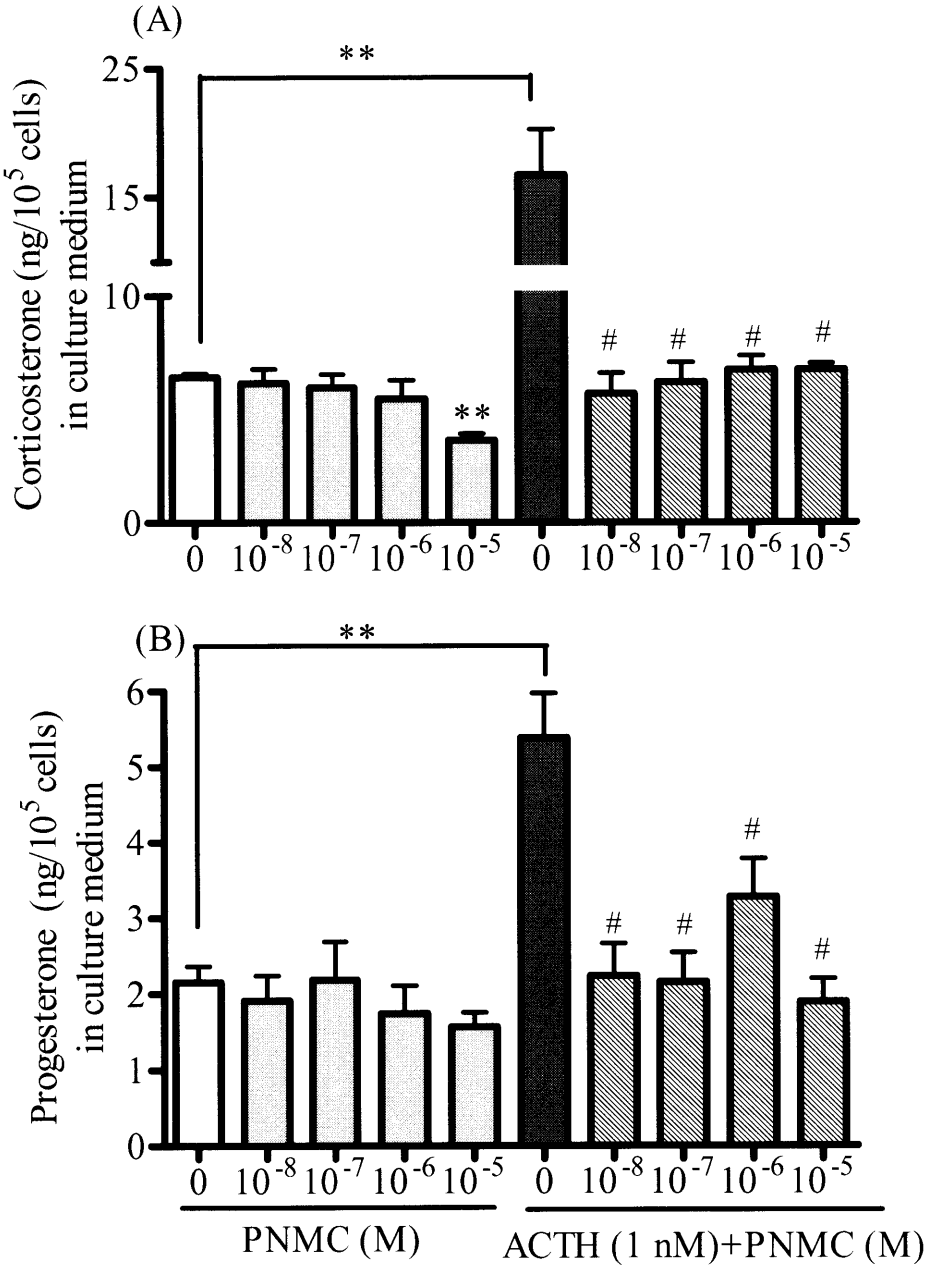


Fig. 6-4 Effect of PNMC on ACTH - unstimulated or stimulated adrenal cells on corticosterone and progesterone secretion. Adrenal cells were incubated for 24 h with 10^{-8}, 10^{-7}, 10^{-6} or 10^{-5} M PNMC either with or without ACTH (1 nM). Each bar represents the mean ± S.E.M. (n=6). The experiment was done 3 times, and a representative result is shown. ** $P < 0.01$ compared with the ACTH unstimulation control, $^{\#}P < 0.01$ compared with the ACTH stimulation control (Fisher's PLSD).

Chapter 7. Estrogenic and Anti-Androgenic Activities of PNP

7-1. Background

Air pollution is a grave problem throughout the world, and DEP are a leading contributor to this situation. DEP contain thousands of compounds that have hazardous effects on human health, including lung cancer (37, 58), allergic rhinitis (64, 98), and bronchial asthma-like disease (61, 91). Another important of feature of DEP is their endocrine- disrupting effects and potential adverse impact on both male and female reproductive function. Previous papers have reported that diesel exhaust suppresses spermatogenesis in adult mice (122) and rats (109, 117). In addition, pregnant C57BL mice injected with DEP extract showed significant increases in abortion rate and uterine weight (110). These *in-vivo* findings show that DEP contain compounds that can modulate estrogenic and anti-androgenic activities. *In- vitro* studies also have shown that DEP possess estrogenic, anti-estrogenic, and anti-androgenic activities (45, 59, 75, 99). However, the specific compound(s) responsible for these phenomena remains unclear. Recently, four nitrophenol derivatives: 4-nitrophenol (PNP), 2-methyl-4-nitrophenol, 3-methyl-4-nitrophenol, and 4-nitro-3-phenylphenol were isolated from DEP. These derivatives have vasodilatory activity (62, 100), as well as estrogenic and anti-androgenic activities *in- vitro* (101)

In 2001, the Organization for Economic Cooperation and Development (OECD) proposed the use of the rodent uterotrophic assay and Hershberger assay as rapid *in-vivo* screening assays for detecting the estrogenic and androgenic properties of suspected endocrine-disrupting chemicals, and both of these assays continue to be validated by the OECD. In the previous study, PNP, a degradation product of parathion as well as a component of DEP, had affinity with estrogen and androgen receptors and showed estrogenic and anti-androgenic activities *in vitro* in a recombinant yeast screening assay (101). In the present study, the estrogenic and

anti-androgenic activities of PNP were evaluated *in vivo* by using immature rat uterotrophic and Hershberger assays.

7-2. Materials and methods

7-2-1. Chemicals

4-nitrophenol (*p*-nitrophenol; PNP) was purchased from Tokyo Kasei Kogyo Tokyo, Japan). Estradiol-17β (E_2) and 2-methyl-N-[4-nitro-3-(trifluoromethyl) phenyl] propanamide (flutamide) were obtained from Sigma (St Louis, MO, USA).

7-2-2. Animals

Immature male and female Wistar-Imamichi rats (age, 21 days) were purchased from the Imamichi Institute for Animal Reproduction (Kasumigaura, Ibaraki, Japan). They were kept in a controlled environment under a photoperiod of 12 h light: 12 h dark (lights on, 0700 to 1900h), temperature of 23 ± 2 °C, humidity of 50% ± 10%, and air exchanged 20 times hourly. Food (CE-2 diet, Japan Clea Co., Tokyo, Japan) and water were available *ad libitum*.

7-2-3. Uterotrophic assay

The estrogenic activity of PNP *in vivo* was examined by uterotrophic assay of immature rats. Immature (25-day-old) female rats were ovariectomized and injected them subcutaneously once daily with 1, 10, or 100 mg/kg PNP for 7 days, beginning on the day of the surgery. Additional rats were injected with vehicle only (PBS containing 0.05% Tween 80) as a negative control or with 5 μg/kg estradiol-17β in sesame oil as a positive control. At 24 h after the last injection, rats (6 to 8 per group) were weighed and euthanized by decapitation. The uteri were weighed, and blood samples were collected in plastic tubes containing heparin and centrifuged at 1700 × g

for 15 min at 4 °C. Plasma was separated and stored at –20 °C until assayed to determine concentrations of FSH and LH.

7-2-4. Hershberger assay

This Hershberger assay was performed according to the current draft guidelines for the rodent Hershberger assay (72). Immature (21-day-old) male rats were castrated 1 week before the experiment. At 28 days of age, rats were implanted with silastic tubing (length, 5 mm; inner diameter, 1.57 mm; outer diameter, 3.18 mm; Dow Corning, Midland, MI, USA) containing crystalline testosterone. Testosterone-containing tubes were incubated in saline at 37 °C for 24 h before implantation to avoid a surge-like release after implantation. An implant of this size for 5 days has been shown to restore plasma testosterone to a physiologic level of approximately 1.8 ng/ml (51). The 28-day-old rats were injected subcutaneously with PNP (0.01, 0.1, or 1 mg/kg) once daily for 5 days. Additional rats were injected with vehicle only (PBS containing 0.05% Tween 80) as a negative control and with 4 mg/kg flutamide, an androgen antagonist, in sesame oil as a positive control. At 24 h after the last injection, rats (n = 8 to 9 per group) were weighed and euthanized by decapitation. Blood samples were collected in plastic tubes containing heparin and centrifuged at 1700 × g for 15 min at 4 °C. Plasma was separated and stored at –20 °C until assayed for FSH and LH concentrations. Five androgen-dependent accessory sex glands (ventral prostate, VP; seminal vesicle, SV; levator ani plus bulbocavernosus muscles, LABC; Cowper's gland, COW; and glans penis, GP) were excised from each rat, carefully trimmed of excess adhering tissue and fat, and immediately weighed. The liver, kidneys, and adrenal glands also were excised and weighed.

7-2-5. Radioimmunoassay (RIA)

The detailed RIA procedures for determination of LH, FSH and testosterone were described in general materials and methods of chapter 2.

7-2-6. Statistics

The statistical methods were shown in chapter 2.

7-3. Results

7-3-1. Immature rat uterotrophic assay

Daily injection of PNP for 7 days did not lead to decreased body weight or changes in the weights of the kidneys, adrenals, liver or pituitary (Table 7-1). In contrast, PNP induced significant (approximately 125% of control value; $P < 0.05$) increases in uterine weight in the 10 and 100 mg/kg treatment groups compared with that of the negative control (Table 7-2). Rats treated with estradiol-17β (5 μg/kg) showed even greater increased uterine weight (approximately 427%; $P < 0.01$), compared with the negative control group (Table 7-2).

7-3-2. Hershberger assay

Administration of PNP and flutamide to testosterone-implanted castrated immature male rats for 5 days had no effect on body weight gain and the weights of the adrenals and pituitary (Table 7-3). In contrast, liver weight was decreased significantly ($P < 0.01$) in the 0.1 and 1 mg/kg treatment groups and kidney weight decreased significantly ($P < 0.01$) in the 0.1 mg/kg PNP and flutamide groups compared with those of the control group (Table 7-3). Weights of the androgen-dependent accessory sex glands (VP, SV, LABC, COW, and GP) are shown in Table 4. The flutamide- treated rats showed significant ($P < 0.01$) decreases in the weights of VP (approximately 23% of control value), SV (approximately 28% of

control), LABC (approximately 60% of control), GP (approximately 58% of control), and COW (approximately 37% of control). The weight of VP was significantly ($P < 0.05$) reduced at PNP doses of 0.01, 0.1, and 1 mg/kg, to approximately 85%, 84%, and 81% of the control value, respectively. The weight of SV was reduced significantly ($P < 0.05$) only at 0.1 mg/kg PNP (approximately 86% of control). The LABC weight was significantly ($P < 0.05$) reduced at PNP doses of 0.01 mg/kg (approximately 93% of control) and 0.1 mg/kg (approximately 92% of control). The GP weight was significantly ($P < 0.05$) reduced at PNP doses of 0.1 mg/kg (approximately 89% of control) and 1 mg/kg (approximately 87% of control). The weight of COW, however, did not vary significantly for any of the three PNP-treated groups.

7-3-3. Plasma concentrations of FSH and LH

Plasma concentrations of FSH and LH in ovariectomized immature rats treated with PNP or estradiol-17β and in testosterone-supplemented, castrated, immature male rats treated with PNP or flutamide were shown in Figs. 7-1 and 7-2, respectively. In the ovariectomized immature female rats, there were no significant changes in plasma concentrations of FSH and LH in the PNP-treated groups. Plasma concentrations of FSH and LH, however, decreased significantly ($P < 0.01$) in the estradiol-17β-treated group (Fig. 7-1A, B). In the testosterone-supplemented, castrated, immature rats, plasma concentrations of FSH and LH were significantly ($P < 0.05$) higher in the 0.1 mg/kg PNP treatment group and the flutamide-treated group, compared with control values (Fig. 7-2 A, B).

7-4. Discussion

PNP exhibited estrogen-like effects on female rats, and anti-androgen-like effects on male rats. The implications of these studies can be appreciated from both

endocrinologic and environmental perspectives. The present results are in agreement with those of the previous *in vitro* study in which PNP showed estrogenic and anti-androgenic activities (101).

The ovariectomized rat model is effective for detecting estrogen agonists and antagonists (15). Uterine weight has widely been used as a sensitive parameter for evaluating estrogenic activity (86). Previous studies suggested that the uteri of 25-day-old immature rats were sensitive to estrogenic effects (25, 26). Therefore, this immature rat model was used to evaluate the estrogenic activity of PNP in the present study. Immature female rats injected with 10 or 100 mg/kg PNP showed significant increases in uterine weight. In contrast, plasma LH and FSH did not change compare with the control levels in any PNP treatment group. These findings suggest that the estrogenic activity of PNP is insufficient to suppress secretion of LH and FSH from gonadotroph. In the previous study, PNP showed affinity for human estrogen receptor α in transformed yeast (101). Therefore, it is suggested that PNP shows estrogenic activity in the uterotrophic assay via its effect on estrogen receptor α.

Immature castrated male rats were used in the 5-day Hershberger assay; 5- to 7-day rodent Hershberger assays are a component of the Tier-1 Screening Battery proposed by the Endocrine Disruptor Screening and Testing Advisory Committee (18). The Hershberger assay has been used for detecting androgen receptor agonists and antagonists through measurement of organ weight (35). Generally, accessory sex glands are dependent upon androgen stimulation to gain and maintain organ weight during and after puberty. If endogenous testicular sources of androgen are removed, exogenous sources of androgens are necessary to increase or maintain the response of reproductive tissue to testosterone (4). In the present study, PNP treatment led to significant decreases in the weights of VP, SV, LABC and GP, though these results showed a low dose effect and an inverted-U dose response, previously reported in several environmental chemicals such as bisphenol A (113). As expected, flutamide,

an androgen antagonist, showed potent anti-androgenic activity and induced significant decreases in accessory sex organ weights at a daily dose of 0.4 mg/kg.

Plasma FSH and LH levels may be useful additional endpoints in Hershberger assay, and 0.1 mg/kg PNP significantly increased plasma FSH and LH to the same levels as seen with flutamide. However, 0.01 and 1 mg/kg PNP doses had no effect on plasma FSH and LH levels. The PNP resulted in an inverted-U dose response for FSH and LH secretion. This pattern has also been well reported on several environmental chemicals such as bisphenol A and octylphenol (73, 113), though as with these others, the mechanism remains unclear. These results suggest that PNP has anti-androgenic effects on the hypothalamus-pituitary axis and the accessory reproductive glands. A previous study also reported that flutamide significantly increased plasma LH levels in a 5-day Hershberger assay, whereas plasma testosterone levels were similar to those of the control (119). In the present study, plasma LH and FSH concentration significantly increased without an increase in concentration of testosterone, indicating that the negative feedback effects are canceled by the anti-androgenic effects of PNP. In fact, flutamide can bind to the androgen receptor and effectively block the recognition of androgens, resulting in altered development of androgen-dependent tissues (70) and variations in plasma hormone levels (119). The previous study showed that PNP binds to the human androgen receptor and inhibits the androgenic activity of 5α-dihydrotestosterone in the recombinant yeast screen assay (101). The present results suggest that PNP has an anti-androgenic effect in the Hershberger assay that is mediated through the androgen receptor, and the inhibitory mechanism of androgenic activity may be related to the hypothalamus–pituitary axis and other hormones that are involved in testosterone synthesis and metabolism.

The present results also are important from an environmental perspective. Many countries exhaust vast amounts of DEP into the atmosphere, and previous reports indicated that 1 kg DEP contains 15 mg PNP (62, 100). The exact

concentration of PNP in the environment is unknown because the research of the isolation of the compounds found in DEP has just begun. PNP, one of the chemicals isolated from DEP, is also a degradation product of the insecticide parathion (43), which is used as a fumigant, acaricide, and pre-harvest soil and foliage treatment for a wide variety of crops, both outdoors and in greenhouses worldwide. Accumulation of PNP in air (69), soil, and water (5) could have serious deleterious effects on wildlife and human health through disturbance of endocrine and reproductive systems.

In conclusion, PNP shows estrogenic activity in immature female rats, as well as anti-androgenic activity (in light of the decreased weights of testosterone-stimulated accessory sex glands and increased plasma LH and FSH levels) in male rats. Therefore, the present result clearly demonstrate that PNP is an endocrine-disrupting chemical.

Table 7-1 Organ weights of ovariectomized rats treated with PNP and estradiol-17β

	Control	Estradiol-17β (5 μg/kg/day)	PNP (mg/kg/day)		
			1	10	100
Number of animals	6	8	6	7	7
Body weight (g)	89.98 ± 2.42	86.71 ± 1.01	89.47 ± 2.75	88.06 ± 1.57	86.57 ± 1.65
Weight gain (g)	32.92 ± 1.42	29.06 ± 0.86	32.93 ± 1.72	32.21 ± 0.29	30.16 ± 0.95
Liver (g)	4.37 ± 0.15	4.43 ± 0.12	4.47 ± 0.21	4.37 ± 0.09	4.07 ± 0.11
Kidneys (g)	1.00 ± 0.01	0.96 ± 0.01	0.98 ± 0.03	0.95 ± 0.03	0.98 ± 0.02
Adrenals (mg)	33.33 ± 2.17	31.00 ± 1.57	29.33 ± 1.65	31.14 ± 1.78	34.29 ± 0.87
Pituitary (mg)	2.60 ± 0.25	3.86 ± 0.26*	3.50 ± 0.50	2.43 ± 0.37	2.71 ± 0.42

Values are expressed as means ± SEM.

*$P < 0.05$ (ANOVA and Fisher's PLSD) compared with value for control group.

Table 7-2 Uterine weights of ovariectomized rats treated with PNP and estradiol-17β

	Control	Estradiol-17β (5 μg/kg/day)	PNP (mg/kg/day)		
			1	10	100
Number of animals	6	8	6	7	7
Uterine weight (mg)	66.00 ± 6.94	282.00 ± 3.45**	56.17 ± 1.83	82.50 ± 5.68*	82.33 ± 3.75*
Uterine weight/body (mg/g)	0.73 ± 0.07	3.26 ± 0.20**	0.63 ± 0.02	0.93 ± 0.07*	0.96 ± 0.05*

Values are expressed as means ± SEM.

*$P < 0.05$ (ANOVA and Fisher's PLSD) compared with value for control group.

**$P < 0.01$ (ANOVA and Fisher's PLSD) compared with value for control group.

Table 7-3 Organ weights of immature castrated rats after 5 days of treatment with testosterone (T, 5-mm silastic implant) and PNP or flutamide

	Control (T)	Flutamide (4 mg/kg/day+T)	PNP (mg/kg/day)		
			0.01+T	0.1+T	1+T
Number of animals	8	8	9	9	8
Body weight (g)	108.14 ± 2.04	105.84 ± 1.79	104.80 ± 1.74	102.96 ± 3.74	102.56 ± 1.60
Weight gain (g)	32.24 ± 1.18	29.14 ± 1.40	29.23 ± 0.84	27.26 ± 3.46	27.59 ± 0.73
Liver (g)	5.01 ± 0.12	5.18 ± 0.19	4.64 ± 0.19	4.22 ± 0.09**	4.37 ± 0.07**
Kidneys (g)	1.16 ± 0.02	1.08 ± 0.03**	1.15 ± 0.02	1.07 ± 0.02**	1.13 ± 0.02
Adrenals (mg)	39.75 ± 3.44	33.25 ± 2.50	37.44 ± 1.62	37.33 ± 2.17	38.75 ± 1.58
Pituitary (mg)	4.25 ± 0.31	3.63 ± 0.26	4.11 ± 0.20	3.89 ± 0.20	4.75 ± 0.65

Values are expressed as means ± SEM.

Control animals received testosterone only; all other animals received testosterone in addition to treatment as described in thetable.

**$P < 0.01$ (ANOVA and Fisher's PLSD) compared with value for control group.

Table 7-4 Weights of accessory sex organs of immature castrated rats treated for 5 days with testosterone (T, 5-mm silastic implant) and PNP or flutamide

	Control	Flutamide	PNP (mg/kg/day)		
	(T)	(4 mg/kg/day+T)	0.01+T	0.1+T	1+T
VP (mg)	44.29 ± 2.25	10.38 ± 0.65**	37.75 ± 1.91*	37.29 ± 2.08*	35.88 ± 2.41*
SV (mg)	74.43 ± 4.59	21.13 ± 1.70**	69.25 ± 2.46	64.29 ± 1.80*	71.75 ± 2.98
LABC (mg)	122.29 ± 3.38	73.13 ± 1.78**	113.17 ± 3.54*	112.00 ± 1.49*	120.29 ± 1.29
GP (mg)	44.17 ± 1.28	25.50 ± 1.12**	41.00 ± 1.34	39.22 ± 0.76*	39.14 ± 1.83*
COW (mg)	8.38 ± 1.29	3.13 ± 0.44**	7.22 ± 0.28	7.33 ± 0.44	7.38 ± 0.26

VP, ventral prostate; SV, seminal vesicle plus coagulating glands; LABC, levator ani plus bulbocavernosus muscles; COW, Cowper's gland; GP, glans penis.

Values are expressed as means ± SEM. Control animals received testosterone only; all other animals received testosterone in addition to treatment as described in the table.

*$P < 0.05$ (ANOVA and Fisher's PLSD) compared with value for control group.

**$P < 0.01$ (ANOVA and Fisher's PLSD) compared with value for control group.

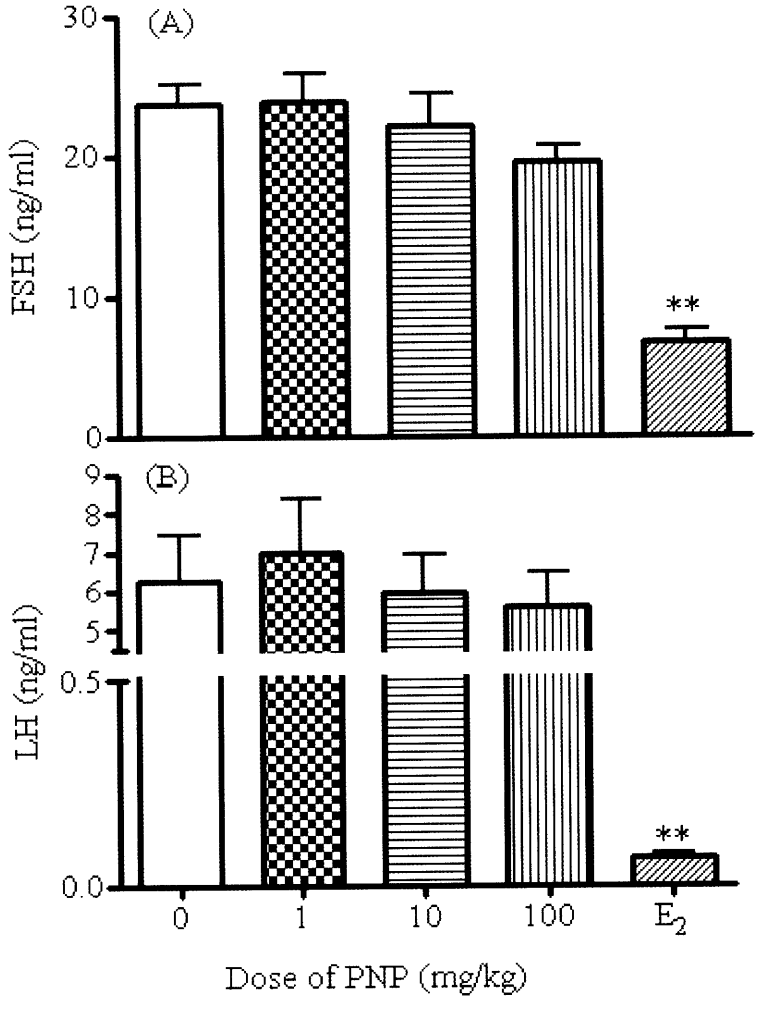


Fig. 7-1 Plasma concentrations of follicle-stimulating hormone (FSH, A) and luteinizing hormone (LH, B) in ovariectomized immature rats injected subcutaneously daily for 7 days with PNP (1, 10, or 100 mg/kg) or estradiol-17β (E_2; 5 μg/kg). Each bar represents the mean ± SEM of 6 to 8 rats per group. $^{**}P < 0.01$ compared with value for negative-control rats (Fisher's PLSD).

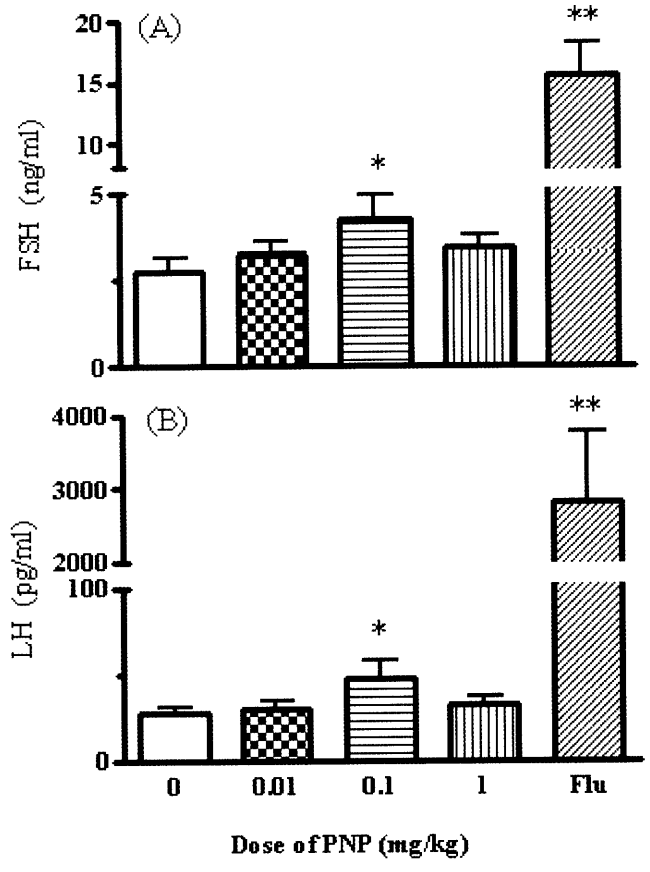


Fig. 7-2 Plasma concentrations of FSH (A) and LH (B) in castrated rats treated for 5days with testosterone (5-mm silastic implant) and PNP (0.01, 0.1, or 1 mg/kg subcutaneously once daily) or flutamide (Flu; 4 mg/kg subcutaneously once daily). Each bar represents the mean ± SEM of 8 to 9 rats per group. $^{**}P < 0.01$; $^{*}P < 0.05$ compared with value for negative-control rats (Fisher's PLSD).

Chapter 8. General discussion and summary

8-1. General discussion

In the present dissertation, the effects of nitrophenols (PNMC and PNP) isolated from DEP on reproductive function in birds and mammals were examined. The results of the present study demonstrated that PNMC directly suppresses testicular, adrenocortical and thyroid function in immature male rats.

It is well established that a functional relationship exists between the adrenals and gonads (88). The present study showed that PNMC significantly increases plasma concentrations of LH, FSH and ACTH, and decreases plasma concentrations of testosterone, ir-inhibin, corticosterone and progesterone in intact immature male rats. In addition, the *in vitro* study showed that PNMC inhibits testosterone production in cultured testicular interstitial Leydig cells and also corticosterone and progesterone production in cultured primary adrenal cells. These findings clearly demonstrate that PNMC affects testicular function by reducing the functions of Leydig cells and also Sertoli cells, and that the increased levels of plasma LH and FSH might be due to a reduction in the negative feedback regulations by testosterone and inhibin. Furthermore, the present study clearly demonstrated that PNMC suppresses adrenal function, and that the associated increase in levels of plasma ACTH might reflect reduced negative feedback regulation by corticosterone. It has been reported that a high concentration of serum corticosterone increases sensitivity to the negative feedback effects of testosterone and inhibits testicular function in rats (115). In humans, the inhibition of sexual functions has often been observed during pathological circumstances accompanied by elevated levels of circulating corticosteroids, which usually disappear after a return to the normal adrenal function (96).

It is known that thyroid hormones play an important role in adrenal and gonadal function (41, 63). It has been shown that hypothyroidism directly produces adrenal

dysfunction and hyposecretion of corticosterone by the adrenal gland (103, 106). Thyroid hormones are also known to influence the immune system. Previous studies have reported that thyroid hormones increase the proliferation of lymphocytes (49), and mallards with methimazole-induced hypothyroidism have reduced cell-mediated immunity (22). PNMC affects, and in addition may cause suppression of immune function through reduction of function of thyroid and adrenal glands. Therefore, the results of present study, which showed a decrease in thyroid hormones, could lead to dysfunction of thyroid hormone-associated endocrine and immune systems (Fig.8-1). It can be surmised that, as with mammals, PNMC has endocrine disrupting effects on birds. However, the exact mechanism by which PNMC exerts its effects has still to be resolved. Future studies are needed to elucidate its effects on endocrine function in animals.

There are many similarities among birds, mammals, and other vertebrate classes regarding endocrine systems. These vertebrate classes share similar hormones and hormone receptors, and fundamental feedback mechanisms are similar. However, important differences between birds and mammals and other vertebrate classes do exist. Birds offer unique morphological, physiological, and behavioral adaptations that are not seen in other vertebrates, including the ability to fly which requires adaptations for high metabolic rates and lower body mass, oviparity with hard-shelled eggs, a different physiological basis for gender development, and complex mating attraction behaviors. Sex determination and control of differentiation is linked to the heterogametic sex. In birds, the homogametic sex is male (ZZ), the heterogametic sex (ZW) is female. Differences in the reactions to PNMC in birds and mammals were also seen in this study. In the present study, acute toxicological responses were seen in the quail treated with the highest dose of 135 mg/kg PNMC (6 birds died), and 103 mg/kg PNMC (one bird died). However, none died in rats treated with the high dose of PNMC (100 mg/kg). 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, the preliminary experiments, demonstrated that the LD_{50} of PNMC in the adult male quail and mouse were 135 mg/kg and 490 mg/kg, respectively. These results indicate that the sensitivity of the quail is higher than the mouse. It is known that PNMC inhaled into the body is metabolized by UDP-glucuronosyltransferase (UGT) (57) and sulfotransferase (SULT) (65), and excreted in the urine. In a recent study by Professor Tamie Nasu (Department of Occupational and Environmental Health, Nagoya University), it was found that UGT activity for PNMC in the hepatic microsomes and cytosols of quails is lower compared with rats and mice (personal communication). The fact that UGT activity in quails is less than in rodents suggests that PNMC metabolism is slower or less efficient in the quail liver compared to the rodents' liver. Therefore, quails may be more inclined to accumulate PNMC within the body, and resulted in the higher toxicity than the rodents. The above findings suggest that there is a difference in reactivity between mammals and birds. Further studies are required, however, in order to understand the mechanisms underling this difference.

The present study also is important from an environmental perspective. Many countries exhaust vast amounts of DEP into the atmosphere. For example, in Japan 58902 tons (39), in the United States of America 111530 tons (111), in England (UK) 37000 tons (3), and in EURO, the highest of 240000 tons (81) are emitted in the each year. One kilogram of DEP contains approximately 28 mg PNMC and 15 mg PNP (62, 100). Therefore, the estimated total amount of PNMC and PNP comes to about 1.65 and 0.88 ton per year in Japan, respectively. On the other hand, these nitrophenol derivatives are also degradation product of insecticides. PNMC is degradation product of the insecticide fenitrothion (10, 33), which is widely used as an organophosphorus insecticide and mainly used in agriculture for controlling chewing and sucking insects on rice, cereals, fruits, vegetables, stored grains, and cotton in many countries. According to data reported by the Pollutant Release and Transfer

Registers (PRTR), the amount of fenitrothion emitted into the environment in 2002 in Japan was approximately 1300 tons, and roughly half of this amount would have been degraded into PNMC. In Japan the most conspicuous use of fenitrothion is spraying on small areas such as paddy fields or pinewoods as an insecticide. In one government report, the maximal concentration (5 days average) of fenitrothion was 6.5 $\mu g/m^3$ (1 hour peak; 22 $\mu g/m^3$) measured at close proximity of a spraying by automatic helicopter on pinewoods (38). The value indicates that a standard adult person (60 kg BW, inhalation volume; $15m^3$) can inhale 48.8 μg of PNMC per 24 hours, about half of which is from fenitrothion. Baroja et al. also reported that PNMC was detected with a concentration of 24.1 ng/m^3 at the sixth day and 54.7ng/m^3 at the second week after the spray applications with fenitrothion in forestry atmospheres in Spain (8).

PNP is also a degradation product of the insecticides parathion and methylparathion (43), which are not currently in use in Japan but are still used worldwide in countries such as China, as fumigant, acaricide, and pre-harvest soil and foliage treatment for a wide variety of crops, both outdoors and in greenhouses worldwide. A previous study also reported that rainwater in Roskilde, Denmark, contains high levels of PNMC (83 ng/l) and PNP (1037 ng/l), which were mainly due to degradation product of insecticides (5). Theses fidings indicate that nitrophenol derivatives are not only exhausted by DEP, but also exist in the environment of the spraying pesticide. Actually, nitrophenols are identified in the atmosphere, rainwater, soil and shore bottom. Especially, about 50% of degradation product of insecticides could change to nitrophenol derivatives, which could be absorbed by the respiratory organ and skins of the livings including human beings. Moreover, chances are that the livings eat the rice and the vegetables scattered with nitriphenol derivatives from insecticide. And what is worrying about is that, it influents not only on the health of human beings but also on the ecosystem by the feeding chain. Therefore, the preset study is the first indicating that degradation product of

insecticides, nitrophenol derivatives as novel environmental pollutants are accumulated in air (69), soil, and water (5) have serious deleterious effects on wildlife and human health (Fig. 8-2).

The present study has made clear that nitrophenol derivatives derived from DEP have endocrine disrupting effects on the gonadal, adrenal and thyroid functions, and suppressive effects on the reproductive and immune systems. These findings are sure to prove to be a big step in elucidating the physiological effects of DEP. It is desirable that future study will be conducted in order to elucidate the mechanisms by which these substances affect the gonadal, adrenal and thyroid glands, and also the immune system.

8-2. Summary

Air pollution poses a serious threat to the lives of all animals and plants on the earth including human beings. Especially in urban areas, diesel exhaust particles (DEP) exhausted by the diesel passenger car is the main source for air pollution. Although the effects of DEP on the respiratory, circulatory and immune systems have been pointed out for a long time, the influences on the endocrine and genital systems have been ignored. On the other hand, because there are thousands of chemical constituents in DEP and the properties of DEP are various, the studies have yielded a wide range of results. Therefore, it is necessary to elucidate the specific chemical constituents of DEP in order to clarify the specific actions of DEP. For this purpose, the effects of 3-methyl-4-nitrophenol (PNMC) and 4-nitrophenol (PNP) isolated from DEP on the reproductive function of birds (quail) and the mammals (rat) were investigated in the present study.

Ⅰ. Influence to birds

1. Effects of PNMC on reproductive function in adult male Japanese quail

Adult male Japanese quail were treated with a single dose of PNMC 78, 103, or 135 mg/kg body weight, intramuscularly, and then trunk blood and testes were collected 1, 2 or 4 weeks later. Various levels of testicular atrophy were observed in all groups treated with PNMC. Spermatogenesis, cloacal gland area, and plasma LH and testosterone concentrations were also reduced in birds with testicular atrophy. To determine the acute effect of PNMC on gonadotropin secretion from the pituitary, adult male quail were administrated a single injection of PNMC (25 mg/kg, im), and plasma concentrations of LH were measured at 1, 3, and 6 hours. This dose significantly lowered plasma levels of LH at all three time points. These results suggest that PNMC acts on the hypothalamic–pituitary axis, by reducing circulating LH within a few hours and subsequently reducing testosterone secretion. In addition, to investigate the direct effects of PNMC on the secretion of testosterone from interstitial cells in quail testes, cultured interstitial cells containing Leydig cells were exposed to PNMC (10^{-6}, 10^{-5}, or 10^{-4} M) for 3, 8, or 24 hr. These amounts of PNMC significantly reduced basal secretion of testosterone in a time- and dose-dependent manner. In conclusion, these findings suggest the direct effect of PNMC on the testis by reducing testosterone secretion. These results clearly indicate that PNMC induces reproductive toxicity at both the central and gonadal levels with disrupting testicular function in adult male quail.

2. Effects of PNMC on reproductive function in mature and immature female Japanese quail

To investigate outcomes effects of PNMC on reproductive function in female Japanese quail, the mature and immature female Japanese quail were used. Mature female quail were reared and bred naturally for one week, and then the female quail were injected intramuscularly with PNMC at doses 1, 10, or 100 mg/kg body weight daily for 5 days. The experiment was separated into the pretreatment, treatment, and

post-treatment periods for each 5 days. There was no significant change in the body weight, egg weights, and hatchability during pretreatment, treatment and post-treatment periods. However, egg laying rate showed a tender decrease in all dose of PNMC treatment period compared with pretreatment or post-treatment periods, and recover during the post-treatment period. Plasma concentrations of LH were significantly decreased in birds treated with 10 and 100 mg/kg PNMC. The immature female quail were injected intramuscularly with PNMC at doses 0.1, 1, or 10 mg/kg body weight daily for 3 days. There was no significant change in the body weight between the PNMC-treated and the control bird. However, the weights of the oviducts were significantly lower in birds treated with all doses of PNMC. Furthermore, plasma concentrations of LH and estrodiol-17β were significantly decreased in birds treated with 1 and 10 mg/kg PNMC. These results suggest that PNMC might influence the hypothalamo-pituitary-gonadal axis with decrease in LH secretion, and consequently disturb the growth of reproductive organs of mature and immature female quail, and disturb the egg laying of mature female quail. This study clearly indicates that PNMC induces endocrine malfunction at the central level and subsequently disrupts reproductive processes in the female quail.

Ⅱ. Influence to mammals

1. Anti-androgenic activity of PNMC and effects of PNMC on testicular function in immature male rats

The present study was investigated anti-androgenic activity of PNMC using an *in vitro* recombinant yeast screen assay and *in vivo* Hershberger assay. Recombinant yeast screen assay showed that PNMC possesses anti-androgenic activity at low concentrations. Furthermore, castrated 28-day-old immature male rats implanted with a 5-mm-long silastic tube containing crystalline testosterone and subcutaneously

injected with PNMC at doses 0.01, 0.1 and 1 mg/kg for 5 consecutive days showed a significant decrease in weights of the seminal vesicles, ventral prostate, and glans penis. Plasma FSH and LH levels were significantly increased in the 0.1 mg/kg PNMC treatment group. These results demonstrate that PNMC clearly has anti-androgenic activity in both *in vitro* and *in vivo*, and therefore PNMC can be considered as an endocrine-disrupting chemical. The present study, also investigated the PNMC on the reproductive functions of male immature rats. Twenty-eight-day-old rats were subcutaneously injected with PNMC (1, 10, or 100 mg/kg) daily for 5 days. Weights of the epididymis, seminal vesicle, and Cowper's gland in PNMC- treated rats were significantly decreased at dose of 10 mg/kg. Plasma concentrations of LH and FSH were significantly increased with PNMC at 100 mg/kg. However, plasma concentrations of testosterone and immunoreactive (ir)-inhibin were significantly decreased with PNMC at 100 mg/kg. Testicular contents of testosterone were significantly decreased in the PNMC treated group at 100 mg/kg compared with the control group. Furthermore, testicular contents of ir-inhibin were significantly decreased with PNMC at 1 or 100 mg/kg. To investigate the direct effects of PNMC on the secretion of LH and FSH from the anterior pituitary gland, and on the secretion of testosterone from the testes, cultured anterior pituitary and interstitial Leydig cells were exposed to PNMC (10^{-6}, 10^{-5}, or 10^{-4} M) with or without gonadotropin-releasing hormone (GnRH; 10 nM) and human chorionic gonadotropin (hCG; 0.1 IU/mL) for 24 hours. PNMC did not change basal level of FSH and LH secretion. However, PNMC significantly inhibited both basal and hCG-stimulated testosterone production. These results suggest that PNMC has a direct inhibiting effect on testicular testosterone secretion in immature rats.

2. Effects of PNMC on adrenocortical function in immature male rats

To investigate the effect of PNMC on the adrenocortical functions of immature

male rats, 28-day-old rats were subcutaneously injected with PNMC (1, 10 or 100 mg/kg) daily for 5 days. The adrenal glands weights significantly decreased in rats treated with 10 or 100 mg/kg PNMC. Plasma concentrations of ACTH were significantly increased in animals treated with 100 mg/kg PNMC. In contrast, plasma concentrations of corticosterone were significantly decreased in all PNMC-treated groups, and plasma concentrations of progesterone were also significantly decreased in rats treated with 10 or 100 mg/kg PNMC. Moreover, plasma concentrations of free L-thyrocine (FT_4) were significantly decreased in rats treated with 100 mg/kg PNMC. Treatment with PNMC (10^{-8}, 10^{-7}, 10^{-6}, or 10^{-5} M) did not change basal ACTH released from cultured anterior pituitary cells. In contrast, PNMC significantly inhibited ACTH-stimulated production of corticosterone and progesterone from cultured adrenal cells. These results clearly show that PNMC has a direct effect on the adrenal gland through reduction of corticosterone secretion, and the associated increase in plasma ACTH is probably due decreased negative feedback regulation by corticosterone in immature male rats. Furthermore, PNMC may participate in suppression of the thyroid gland function. Present findings suggest that PNMC may influence the immune system through suppression of thyroid and adrenal function.

3. Estrogenic and anti-androgenic activities of PNP

In the present study, uterotrophic and Hershberger assays were used to investigate the estrogenic and anti-androgenic activities of PNP *in vivo*. When ovariectomized immature female rats daily subcutaneously injected with 1, 10, or 100 mg/kg PNP for 7 days, a significant increase in uterine weight was seen in groups receiving 10 or 100 mg/kg PNP. Furthermore, castrated immature male rats implanted with a silastic tube (length, 5 mm) containing crystalline testosterone and daily subcutaneously injected with 0.01, 0.1, or 1 mg/kg PNP for 5 days, these animals receiving a dose of 0.1 mg/kg PNP showed a significant decrease in weight of seminal

vesicles, ventral prostate, levator ani plus bulbocavernosus muscles, as well as glans penis. Plasma FSH and LH levels did not change in female rats but were significantly increased in male rats treated with 0.1 mg/kg PNP. These results clearly demonstrated that PNP has estrogenic and anti-androgenic activities.

In conclusion, this study clarified that PNMC and PNP isolated from DEP has endocrine disrupting activity in the function of the testis, ovary, thyroid gland and adrenal gland. Additionally, PNMC and PNP are also degradation products of the insecticide fenitrothion and parathion, suggesting that the earth had been already polluted by accumulation of large amounts nitrophenols. The accumulation of nitrophenols in air, soil, and water may have serious deleterious effects on wildlife and human health through disturbance of endocrine and reproductive systems. These findings are extremely important on establishing basic data on declaring the effects of air pollutants to mammals including human beings, and to promote an effective policy to protect our environment.

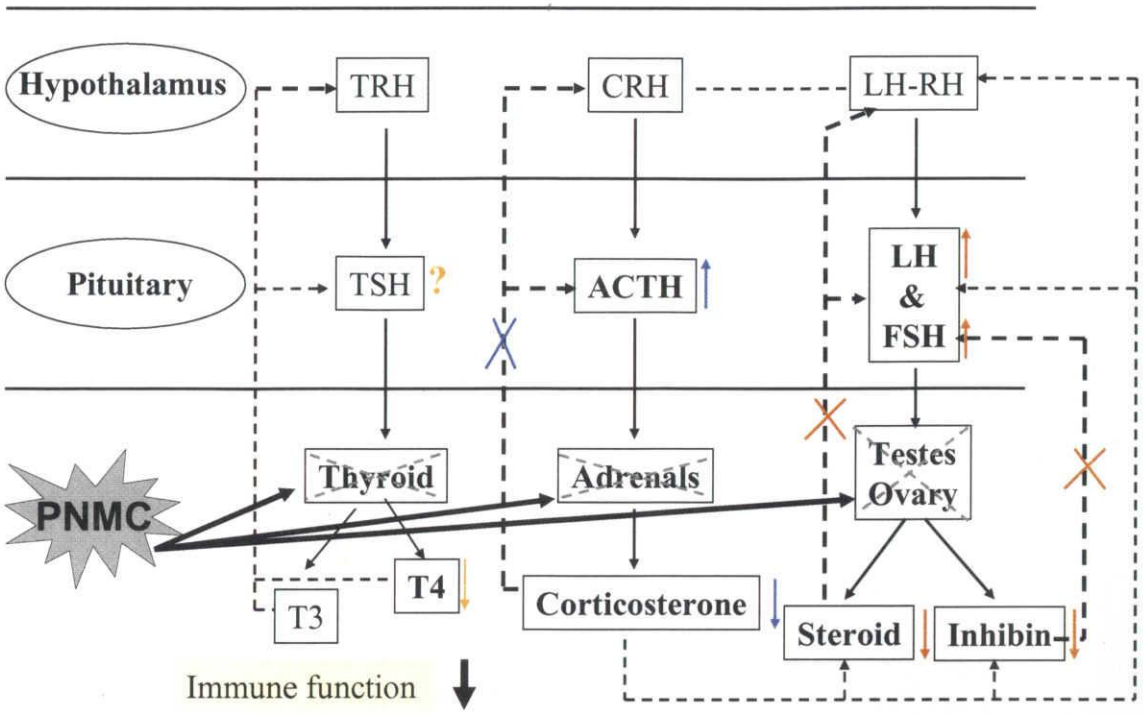


Fig. 8-1 Schematic illustration of the effects of PNMC on the suppression of adrenal, gonadal and thyroid function in rats. PNMC has the direct suppressive effects on secretion of hormones from thyroids, adrenals and gonads. PNMC has also suppressive effects on LH and FSH secretions in the pituitary gland, whereas effects on TSH and ACTH are not clear in the present time.

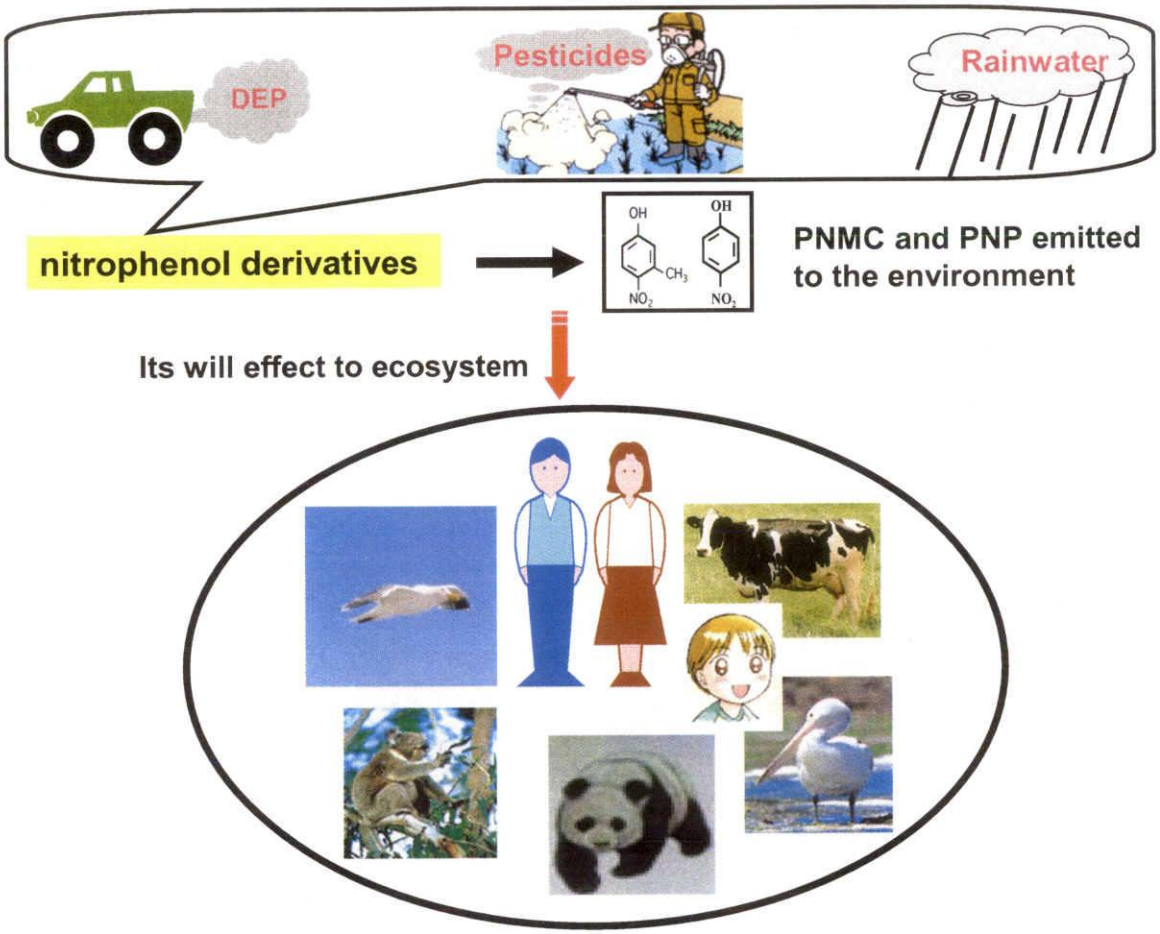


Fig. 8-2 The biological effects of nitrophenol derivatives as novel environmental pollutants on wildlife and human health. Nnitrophenols are identified in the atmosphere, rainwater, soil and shore bottom. The nitrophenol derivatives (PNMC and PNP) are not only exhausted by DEP, but also exist in the environment from the pesticide. PNMC and PNP are degradation product of the insecticide fenitrothion and parathion, and about 50% of degradation product could degraded into PNMC and PNP, respectively. These nitrophenol derivatives as novel environmental pollutants are accumulated in air, soil, and water have serious deleterious effects on wildlife and human health.

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2. **Li C**, Taneda S, Suzuki AK, Furuta C, Watanabe G, Taya K. Anti-androgenic activity of 3-methyl-4-nitrophenol in diesel exhaust particles. *Eur J Pharmacolo.* 543 (1-3): 194-199, Aug, 2006
3. **Li C**, Taneda S, Suzuki AK, Furuta C, Watanabe G, Taya K. Estrogenic and anti-androgenic activities of 4-nitrophenol in diesel exhaust particles. *Toxicol Appl Pharmacol.* 217 (1): 1-6, Nov, 2006
4. **Li C**, Taneda S, Suzuki AK, Furuta C, Watanabe G, Taya K. Effects of 3-methyl-4-nitrophenol in diesel exhaust particles on the regulation of testicular function in immature male rats. *J Androl.* 28 (2): Mar, 2007 (In press)
5. **Li C**, Takahashi S, Taneda S, Furuta C, Watanabe G, Suzuki AK, Taya K. Effects of 3-methyl-4-nitrophenol in diesel exhaust particles on the regulation of reproductive function in immature female Japanese quail *(Coturnix japonica). J Reprod Dev.* 53 (3) Jun, 2007 (In press)

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