Original Article

The Derivative of Diesel Exhaust Particles, 4-Nitro-3-Phenylphenol, Exerts Both Androgenic and Anti-Androgenic Effect in Castrated Immature Male Rats

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Abstract

This study investigated the effect of 4-nitro-3-phenylphenol (PNMPP) on testosterone-implanted castrated rats for 7 days. Exp I: The rats were injected with PNMPP at high doses (0.1, 1 and 10 mg/kg). Exp II: The rats were injected with low doses (0.001 and 0.01 mg/kg) or flutamide (4 mg/kg). The results show that at 10 mg/kg, PNMPP induced a significant increase in serum testosterone concentration, whereas treatment at 0.1, 1 and 10 mg/kg slightly increased adrenal gland weight ($P > 0.5$). Liver weight in the rats treated with 10 mg/kg was insignificantly decreased, which might reduce enzyme elimination. Moreover, serum corticosterone concentration in the rat treated with 10 mg/kg showed a decrease insignificantly ($P > 0.5$). Adrenal gland weight increased insignificantly ($P > 0.5$). PNMPP might mimic androgens effect on the hypothalamus-pituitary-adrenal feedback loop to glucocorticoid, resulting in decreased corticosterone concentration. PNMPP treatment at 10 mg/kg induced significant decreases in serum FSH and LH concentrations and had a trend toward a decrease when treated with 0.1 and 1.0 mg/kg. We presumed that PNMPP treatment at 10 mg/kg disturbed the hypothalamus-pituitary axis by decreasing gonadotropin concentrations. Furthermore, prolactin (PRL) concentration increased in the rats in a dose-dependent manner. This result supports the effect of PNMPP on hypothalamus-pituitary axis by directly increasing PRL concentration. The weight of reproductive organs and kidneys in rats treated with high doses also showed androgenic effect by increasing significantly. The lower doses showed anti-androgenic effect by inducing a significant increase in serum FSH concentration in a dose-dependent manner and an insignificant increase in serum LH concentration of the rats, similar to flutamide effect. These substances furthermore reduced organ weight. We concluded that PNMPP possessed biphasic effects; it was androgenic at high doses and anti-androgenic at low doses.

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Introduction

Diesel exhaust (DE) and diesel exhaust particles (DEP) containing numerous organic compounds such as nitroaromatic hydrocarbons, polyaromatic hydrocarbons, aliphatic hydrocarbons, etc. cause many health problems.[1-4] There also have been reports about the effects of DE and DEP on the reproductive function.[5-10] 4-Nitro-3-phenylphenol (PNMPP) and 3-methyl-4-nitrophenol (PNMC) from DEP showed estrogenic activity by increasing uterine weight and increasing myometrial contractility in immature ovariectomized rats.[11] PNMC showed direct effects on the adrenal gland to reduce corticosterone release, but increased adrenocorticotropic hormone (ACTH).[12] Exposure of P-nitrophenol (PNP), a nitrophenol derivative of diesel exhaust particles, induced condensed nuclei, vacuolated cytoplasm and a decrease in testicular cell viability and spermatogonial cell number.[13] Long-term exposure of PNP quails caused decreasing plasma LH concentration as well induced testicular histopathological changes to include significant testicular atrophy.[11] PNMC increased testosterone concentration and decreased follicle-stimulating hormone (FSH) and luteinizing hormone (LH) concentrations in castrated immature rats.[15] Nanoparticle-rich diesel exhaust (NR-DE) exposure to adult mice increased the expression of several genes that are involved in testicular cholesterol synthesis.[8] From these lines of evidence, the current study focused on the effect of substance isolated from DE and DEP on the reproductive system.

Our previous study reported that PNMPP exhibits

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both the androgen agonist action, by suppressing the effect of GnRH and then testosterone concentration in pituitary cell culture, and androgen antagonist action, by increasing testosterone concentration in Leydig cell culture.[16] We hypothesized that at different doses, PNMPP might have both androgenic and anti-androgenic activity. This study investigated the effects of various doses of PNMPP on androgen-dependent organs and related hormones by using the Hershberger assay.

Materials and Methods

Chemicals

4-Nitro-3-phenylphenol (PNMPP) isolated from DEP was synthesized by the method described previously.[2]

Animals

Immature male Wistar-Imamichi rats were purchased from Imamichi Institute for Animal Reproduction, Ibaraki, Japan. They were maintained under conditions of controlled lighting (14L:10D, lights-on at 0500 h), temperature (22 ± 2°C) and humidity (50 ± 5%). Food (CE-2 commercial diet; Japan Clea Co., Tokyo, Japan) and water were available *ad libitum*. All procedures were carried out in accordance with the guidelines established by Tokyo University of Agriculture and Technology, for use of laboratory animals.

Experimental procedure

The rats, 28 days of age, were castrated 7 days before the study. At the onset of the study, the rats were implanted with silastic tubes containing crystalline testosterone (Dow Corning, Midland, USA). To reduce stress from the experimental procedure, all rats were trained by handling for 5 days prior to the onset of the experiment.

Hershberger assay

Experiment I. High doses of PNMPP for androgenic activity assay

After implantation of a silastic tube containing crystalline testosterone, the rats were randomly divided into four groups (n = 7/group), each received a daily subcutaneous (s.c.) injection for 7 days: Control group received s.c. vehicle (PBS containing 0.05% Tween 80); treatment groups received s.c. PNMPP (0.1, 1 or 10 mg/kg).

Experiment II. Low doses of PNMPP for anti-androgenic activity assay

After implantation of a silastic tube containing crystalline testosterone, the rats were also randomly divided into four groups (n = 7/group), each received daily s.c. injection for 7 days with either vehicle (control group), PNMPP (0.01 or 0.001 mg/kg) (treatment groups), or 4.0 mg/kg flutamide, an androgen receptor antagonist, as a positive control group.

The rats from both experiments were decapitated at 24 h after the last injection. Blood samples were collected immediately and separated for serum with refrigerated centrifugation at 1,700 x g for 30 minutes. Serum samples were stored at -20°C until FSH, LH, prolactin (PRL), testosterone and corticosterone assays. Pituitaries were removed and instantly collected for pituitary FSH, LH and PRL assays. The seminal vesicles plus coagulating glands, ventral prostate, levator ani muscle plus bulbocarvernosus muscle, Cowper's gland, glans penis, spleen, liver, kidneys and the adrenal glands were removed and weighed. Body weight was recorded daily throughout the study period.

Radioimmunoassay (RIA)

Serum FSH, LH and PRL concentrations were measured using NIDDK RIA kits (Torrance, CA, USA) for rat FSH, LH and PRL. Iodinated preparations were rat FSH-I-5, LH-I-5 and PRL-I-5. The antisera used were anti-rat FSH-S-11, anti-rat LH-S-11 and anti-rat PRL-S-9. Results were expressed as rat FSH RP-2, rat LH RP-3 and PRLRP-3. The intra- and inter-assay coefficients of variations were 4.8 and 11.4% for FSH, 5.4 and 6.9% for LH, and 3.4 and 5.2% for PRL, respectively.

Serum testosterone and corticosterone concentrations were measured using double-antibody RIA systems with ^{125}I-labeled radioligands.[17-18] Antisera against testosterone (GDN250) and corticosterone (GDN377) provided by Dr. G. D. Niswender (Colorado State University, Fort Collins, CO, USA) were used. The intra- and inter-assay coefficients of variations were 5.9 and 5.8% for testosterone and 9.8 and 17.5% for corticosterone, respectively.

Statistical analyses

The data were expressed as mean ± SE. One-way analysis of variance (ANOVA) was used to compare the mean among groups. Post hoc multiple comparison analyses were performed with the Least Significant Difference (LSD) test when the *F* ratio for the ANOVA was significant at $P < 0.01$ and 0.05.

Results

Effect of PNMPP on organ weight

The relative organ weights were adjusted to the ratio of organ weight per body weight. By comparing with the control, a 10 mg/kg of PNMPP induced a significant increase in weights of Cowper's glands ($P < 0.05$) and levator ani muscle ($P < 0.01$). Kidney weight increased in the rats treated with 1.0 mg/kg ($P < 0.01$) (Table 1).

In the rats treated with low doses, there was a significant decrease in kidney weight of the rats treated with 0.001 and 0.01 mg/kg ($P < 0.05$) and a decrease in adrenal weight of the rats treated with 0.01 mg/kg (Table 2). Flutamide induced a decrease in weight of reproductive organs (prostate glands, seminal vesicle plus coagulating glands, levator ani muscle, Cowper's glands and penis) and kidney ($P < 0.01$).

Table 1 The relative weight of reproductive and non-reproductive organs (unit) of the rats treated subcutaneously with PNMPP (0.1, 1 and 10 mg/kg/day) for 7 days compared with the control.

	Control	PNMPP		
		0.1 mg/kg	1.0 mg/kg	10 mg/kg
N	7	7	7	7
Relative organ weights				
Prostate	0.518 ± 0.04	0.465 ± 0.08	0.434 ± 0.04	0.444 ± 0.06
Seminal vesicle	0.745 ± 0.07	0.765 ± 0.05	0.875 ± 0.08	0.854 ± 0.10
Levator ani muscle	1.102 ± 0.03	1.154 ± 0.02	1.202 ± 0.03	1.312 ± 0.08**
Cowper's glands	0.118 ± 0.04	0.129 ± 0.01	0.132 ± 0.01	0.140 ± 0.01
Penis	0.415 ± 0.02	0.448 ± 0.03	0.415 ± 0.03	0.452 ± 0.02
Liver	42.50 ± 0.85	43.54 ± 0.88	44.71 ± 1.14	42.22 ± 0.82
Kidneys	9.28 ± 0.08	9.79 ± 0.16	10.10 ± 0.22**	9.63 ± 0.22
Spleen	3.171 ± 0.10	3.291 ± 0.21	3.340 ± 0.14	3.163 ± 0.17
Adrenal glands	0.294 ± 0.02	0.382 ± 0.04	0.314 ± 0.01	0.311 ± 0.01

Data represent mean ± SEM; *, ** indicate $P < 0.05$ and < 0.01, respectively.

Table 2 The relative weight of reproductive and non-reproductive organs (unit) of the rats treated subcutaneously with PNMPP (0.001 and 0.01 mg/kg/day) and flutamide for 7 days (4 mg/kg) compared with the control.

	Control	PNMPP		Flutamide
		0.001 mg/kg	0.01 mg/kg	
N	7	7	7	7
Relative organ weights				
Prostate	0.391 ± 0.04	0.266 ± 0.04**	0.330 ± 0.03	0.084 ± 0.01**
Seminal vesicle	0.691 ± 0.06	0.508 ± 0.07	0.533 ± 0.05	0.130 ± 0.01**
Levator ani muscle	1.031 ± 0.06	0.907 ± 0.07	0.988 ± 0.05	0.689 ± 0.04
Cowper's glands	0.070 ± 0.01	0.071 ± 0.01	0.074 ± 0.00	0.027 ± 0.01**
Penis	0.502 ± 0.05	0.366 ± 0.01*	0.457 ± 0.03	0.241 ± 0.01**
Liver	46.31 ± 0.91	45.41 ± 1.11	45.89 ± 0.88	47.81 ± 0.78
Kidneys	10.825 ± 0.28	10.122 ± 0.18*	10.178 ± 0.12	9.799 ± 0.13**
Spleen	3.253 ± 0.16	2.844 ± 0.17	3.060 ± 0.14	3.038 ± 0.09
Adrenal glands	0.260 ± 0.02	0.241 ± 0.01	0.210 ± 0.01	0.250 ± 0.01

Data represent mean ± SEM; *, ** indicate $P < 0.05$ and < 0.01, respectively.

Effect of high doses of PNMPP (0.1, 1.0 and 10 mg/kg) on hormonal concentrations

Serum FSH and LH concentrations decreased significantly ($P < 0.05$) in the rats treated with 10 mg/kg of PNMPP and had a trend toward decrease in the rats of 0.1 and 1.0 groups ($P > 0.05$), when compared with the control (Figure 1E and F). Nonetheless, there was no significant difference in pituitary FSH and LH concentrations between all PNMPP treatment groups (0.1, 1.0 and 10 mg/kg) and control (Figure 1A and B). Serum and pituitary PRL concentrations increased significantly in the rats of 10 mg/kg group ($P < 0.05$) and had a trend toward increase in 0.1 and 1.0 mg/kg groups ($P > 0.05$) (Figure 1C and G).

Serum testosterone concentration increased significantly in the rats of 10 mg/kg group ($P < 0.05$) (Figure 1D). Serum corticosterone concentrations had an insignificant decrease in the rats of 1 and 10 mg/kg by comparing with the control ($P > 0.05$) (Figure 1H).

Effect of low doses of PNMPP (0.001 and 0.01 mg/kg) and flutamide on hormonal concentrations

In PNMPP treatment, serum FSH concentrations increased significantly ($P < 0.01$) (Figure 2E), but there was no significant difference in pituitary FSH in all groups compared with the control (Figure 2A). Pituitary and serum LH concentrations in both groups, however, did not significantly differ from the control ($P > 0.5$) (Figure 2B and F).

PRL concentration increased significantly in serum of the rats treated with 0.001 and 0.01 mg/kg PNMPP ($P < 0.01$ and 0.05, respectively) and in pituitary of the rats treated with 0.01 mg/kg groups ($P < 0.05$) (Figure 2G and C). Flutamide treatment induced a significant increase in serum FSH, LH and PRL concentrations and also pituitary PRL and LH concentrations (Figure 2C, E, F and G).

There were no significant differences in serum testosterone and corticosterone concentrations in either treatment with PNMPP (0.001 and 0.01 mg/kg groups) or flutamide as compared to control (Figure 2D and H).

Discussion

In the present study, PNMPP treatment at 10 mg/kg to testosterone-implanted castrated immature rats induced a significant increase in serum testosterone concentrations ($P<0.05$) when compared with control group. This present study coincides with the prior studies of NR-DE in male rats. After exposure to NR-DE, adult rats also had a significantly increased in testosterone concentration.[19-20] NR-DE has been reported to disturb reproduction by increasing testosterone production, steroidogenic acute regulatory protein (StAR)- and cytochrome P450 side-chain cleavage (P450scc)-mRNA, in addition to increasing the expression of gene regulat-

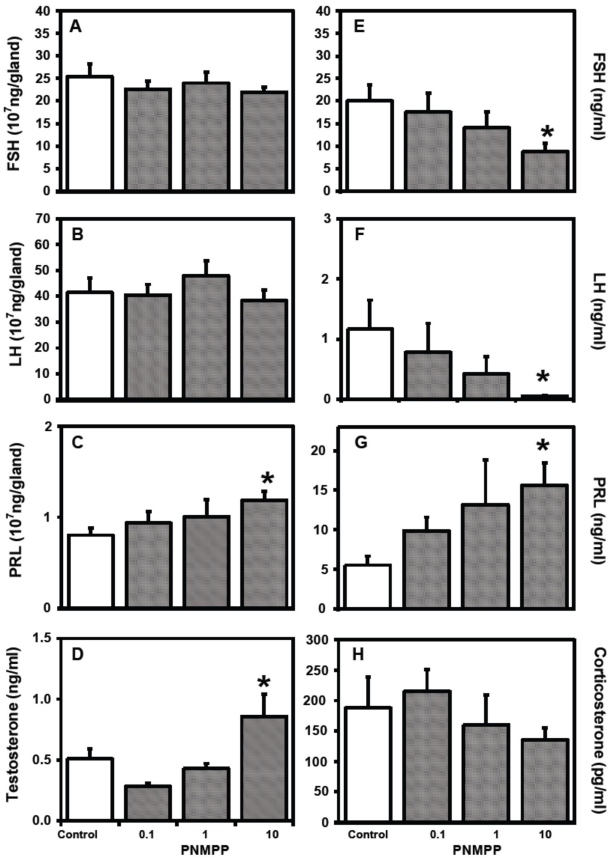

Figure 1 Pituitary and serum FSH (A, E), LH (B, F), and PRL (C, G) concentrations in addition to serum testosterone (D) and corticosterone (H) concentrations in the rats treated subcutaneously with PNMPP (0.1, 1 or 10 mg/kg/day) compared with the control. Data represent mean ± SE; *, **P < 0.05 and < 0.01, respectively.

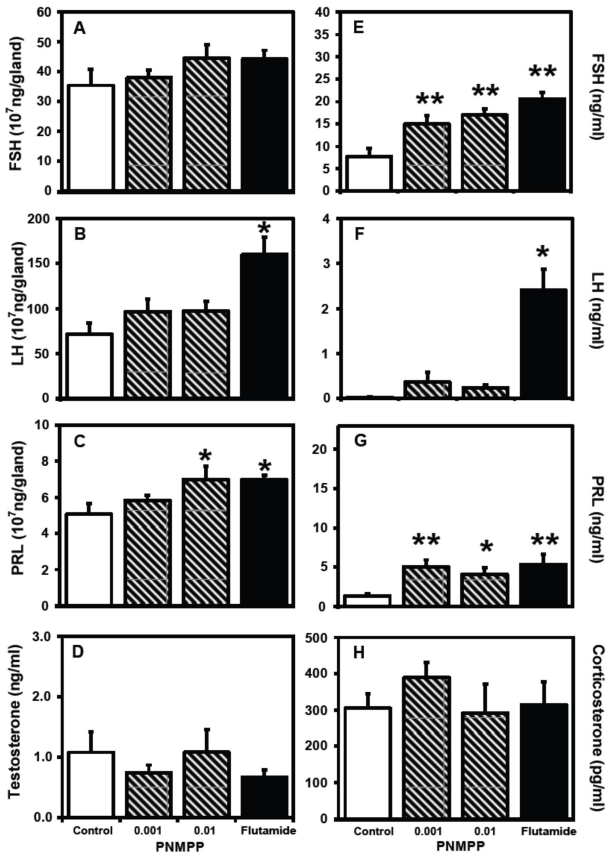

Figure 2 Pituitary and serum FSH (A, E), LH (B, F), and PRL (C, G) concentrations in addition to serum testosterone (D) and corticosterone (H) in the rats treated subcutaneously with PNMPP (0.001 or 0.01 mg/kg/day) and flutamide (4 mg/kg) as a positive control compared with the control. Data represent mean ± SE; *, **P < 0.05 and < 0.01, respectively.

ing cholesterol synthesis in male mice and rats.[8,21] PNMC treatment to the castrated male rats induced a significant increase in plasma testosterone concentration as well.[15] Urinary excretion of 17-ketosteroid, which are among the metabolism of androgen from both adrenal gland and gonad in both sexes, was significantly elevated, which is related to the increasing adrenal gland weight in female rats exposed to DE.[19]

In the present study, 0.1, 1 and 10 mg/kg of PNMPP slightly increased adrenal gland weight when compared with the control (P > 0.5). The present study assumed that PNMPP could increase testosterone production from the adrenal gland in the castrated rats.

Moreover, the increase in testosterone concentration in the rats might be caused from the effect of PNMPP on enzyme elimination released from the liver. Liver weight in rats treated 10 mg/kg of PNMPP non-significantly decreased, whereas testosterone concentrations significantly increased. As found in PNMC treatment, liver weight was decreased significantly whereas testosterone concentration in serum was increased significantly. This previous result suggested that PNMC manifest role on reducing hepatic P450 enzymes to metabolite hormone and induced accumulation of serum testosterone released from the implanted tube in circulation.[15] Accordingly, we may assume that 10 mg/kg of PNMPP reduced metabolic deactivation in the rat liver and increased serum testosterone concentration.

Treatment with 10 mg/kg PNMPP induced a trend toward non-significant decrease in corticosterone concentration in serum as compared with the control ($P > 0.5$), but adrenal gland weight increased. PNMPP might mimic androgen playing a role to stimulate the sensitivity of hypothalamus-pituitary-adrenal feedback loop to glucocorticoid by increasing the expression of glucocorticoid receptor in the pituitary, resulting in a decrease in ACTH and glucocorticoid synthesis.[22] Testosterone propionate injection to adult mice increased plasma testosterone concentration, while decreasing corticosterone concentration.[23] Then, corticosterone concentration showed a negative correlation with testosterone concentration, which is coincident with the present result. From the results of PNMPP treatment, 10 mg/kg PNMPP increased the concentration of serum testosterone by increasing adrenal gland weight and decreasing corticosterone concentration, while the liver weight was decreased.

PNMPP treatment at 10 mg/kg induced a significant decrease in serum FSH and LH concentrations and had a trend toward decreases when treated with 0.1 and 1.0 mg/kg. Testosterone plays a role to regulate the syntheses and releases of FSH and LH from the anterior pituitary. By negative feedback mechanism, testosterone decreased the secretion of either gonadotropin-releasing hormone (GnRH) or kisspeptin, a peptide hormone that plays a role to stimulate GnRH, thus resulting in decreased FSH and LH productions.[24-25] PNMC likewise increased plasma testosterone concentration and decreased plasma FSH and LH concentrations, indicating that the substance acts on the hypothalamus-pituitary axis.[15] Previous research indicated that PNMPP effects seemed to be similar to the effect of diesel exhaust particles which disturb reproductive functions.

Serum and pituitary PRL concentrations increased in the rats treated with a high dose of PNMPP in a dose-dependent manner. Previous report indicates that testosterone can increase PRL concentration by stimulating PRL synthesis and release by the lactotroph of the anterior pituitary. Testosterone can be aromatized to estradiol (E_2) and stimulates PRL release.[26] The use of dopamine agonist could not inhibit hyperprolactinemia induced by testosterone replacement. This indicates that testosterone might increase PRL by increasing estrogen.[27] The present study hypothesized that PNMPP had androgenic effect to increase PRL release. We conclude that a high dose of PNMPP (10 mg/kg) exerts androgenic effects as a result of decreasing gonadotropin and corticosterone concentrations together with increasing PRL and testosterone concentrations.

The weights of Cowper's glands and levator ani muscle as well as kidneys also increased in PNMPP-treated rats at 10 mg/kg. The weight of male reproductive organs dependent on androgen is increased in castrated immature rats implanted with testosterone. Testosterone injection significantly increased seminal vesicle masses in adult male mice.[23] Injection with PNMC induced a significant increase in the weight of seminal vesicle and Cowper's glands in immature castrated animals.[15] PNMC and PNMPP showed estrogenic effect on increasing uterine weight in immature ovariectomized rats.[11] NR-DE also insignificantly increased reproductive organ weight in adult male rats.[8]

PNMPP treatment at 0.1, 1 and 10 mg/kg induced a higher weight of kidney when compared with the control. From a previous study, testosterone treatment increases renal protein excretion and induces hypertrophy of kidneys.[28] Castration decreased mouse kidney weight which could be recovered by testosterone administration.[29] In addition, androgen receptor (AR) was detected in the epithelial cells of kidney and was diminished by castration.[30] Testosterone enathate injection to juvenile male rats increased renal mass.[31] This indicated that a high dose of PNMPP showed androgenic effects by increasing reproductive gland weight and kidney weight in castrated rats.

In contrast to high-dose (10 mg/kg) PNMPP, the lower doses (0.001 and 0.01 mg/kg) induced a significant increase in serum FSH concentration in a dose-dependent manner. Anterior pituitary FSH together with both serum and anterior pituitary LH concentrations also increased as well but not statistically significant. Treatment of flutamide, an androgen antagonist, also significantly increased serum FSH and LH concentrations as well as pituitary LH concentration in the rat. Flutamide disrupt androgen-mediated negative feedback action on gonadotropin secretion at the pituitary level and then increase gonadotropin secretion.[32-33] From an *in vitro* study, PNMPP at 10^{-5}-10^{-7} M significantly suppressed FSH and LH production from GnRH-stimulated pituitary cells when compared with control whereas PNMPP at 10^{-8}-10^{-9} did not alter FSH and LH production.[16] We assumed that PNMPP treatment at 0.001 and 0.01 mg/kg had an anti-androgenic effect by increasing serum FSH and LH concentration.

Furthermore, flutamide was reported to inhibit androgen effect on reproductive organ growth and could decrease the weight of accessory sex organs in male rats.[30,32,34] PNMPP at the low doses (0.001 and 0.01 mg/kg) behaved as an anti-androgen by reducing reproductive organ weight, including prostate gland, seminal vesicle, Levator ani muscle, Cowper's glands and penis, the same as flutamide effects. Weights of kidneys and adrenal glands significantly decreased in the rats treated with both PNMPP at the low doses (0.001 and 0.01 mg/kg) and flutamide. High doses of PNMPP treatment exhibited androgenic effect by increasing the weight of adrenal glands and kidneys directly, but low dose of PNMPP treatment exhibited anti-androgen effect by decreasing adrenal weight. These data confirm the anti-androgen effect of PNMPP at low doses, similar to flutamide.

From prior reports, androgen treatment moreover demonstrated a biphasic effect. Androgen binding protein (ABP) production and testicular weight were decreased when treated with low doses (10-100 μg/day) of testosterone propionate (TP). On the contrary, there was an increase in both testicular weight and ABP production in spite of suppressed serum FSH and LH levels at higher doses of TP.[35] Testosterone treatment to goldfish at low doses (0.2 and 2 μg) stimulated gonadotropin mRNA production; it, however, inhibited or did not stimulate at the higher dose (20 μg).[36] Likewise, the proliferation of human prostate cancer cells was decreased with dihydrotestosterone (DHT) treatment at low concentration (0.1 nM) but increased at higher concentrations (1 nM).[37] Synthetic nonmetabolizable androgen (R1881) showed a biphasic response by proliferation

of prostate cencer cells. R1881 at 1 nM or higher inhibited cell growth, while at 0.1 nM or lower stimulated cell growth.[38] We believed that a different effect of androgen (whether androgen or anti-androgen effect) might be due to a sustained blockage of AR nuclear translocation or an epigenetic inactivetion of the transcribability of androgen.[39]

These results indicate the effects of high dose PNMPP that are similar to androgen. However, the lower doses of PNMPP exhibit anti-androgenic effect similar to flutamide. Accordingly, we can conclude that PNMPP possess biphasic effects by showing both androgenic effects at high doses and anti-androgenic effects at low doses in the present study.

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Conflict of Interest

None to declare.

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