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Prochloraz Decreases Aromatase Activity and Serum Concentrations of E2, Testosterone, T3, and T4 in Pubertal Developmental Rats

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Abstract

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Prochloraz, an imidazole fungicide, antagonizes androgen receptors and inhibits gonadal steroidogenesis. Also, both antiandrogen and aromatase inhibition induced by prochloraz and interacting with thyroid were remained unclear. Based on the above, we hypothesized that pubertal exposure to prochloraz not only can cause reproductive and developmental toxicity but can also disrupt thyroid function in pubertal rats. To investigate the adverse effects of prochloraz on pubertal development and thyroid function, we performed a study on pubertal rats in accordance with the US EPA OCSPP Harmonized Test Guidelines Series Number 890. For female rats, treatments (corn oil; 5 mg/kg bw/day 17α-ethinyl estradiol (EE), a positive control; or 15, 50, or 150 mg/kg bw/day prochloraz) were administered daily by oral gavage between postnatal day (PND) 22 and 42. For male rats, treatments (corn oil; 0.4 mg/kg bw/day testosterone propionate (TP), a positive control; 3 mg/kg bw/day flutamide, a negative control; or 15, 50, or 150 mg/kg bw/day prochloraz) were administered daily by oral gavage between PND 23 and 53. Our results showed that in male rats, 150 mg/kg bw/day prochloraz decreased body weight (PND 32 and PND 34-53) and reduced the tissue weights of the epididymis, prostate, levator ani plus bulbocavernosus muscles, and seminal vesicle plus coagulating gland with and without fluid. However, these effects were not observed in female rats. Prochloraz did not change absolute or relative thyroid weights in pubertal male or female rats. At a dose of 150 mg/kg bw/day, prochloraz delayed the age of preputial separation in male rats but did not affect vaginal opening in female rats. Prochloraz decreased serum concentrations of triiodothyronine and thyroxine in female rats while in male rats, prochloraz decreased aromatase activity and serum concentrations of 17β-estradiol, testosterone, and thyroxine. Finally, prochloraz decreased the serum concentration

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of blood urea nitrogen in male rats but not in female rats. Our results suggest that the primary mechanisms by which prochloraz induced endocrine-disrupting activities involved (1) decreasing estrogen, androgen, and thyroid hormones and (2) inhibiting aromatase activity. No observed adverse effect level (NOAEL) pertaining to accessory glands or epididymis tissue and organ weights were noted when prochloraz was administered at 50 mg/kg bw/day. The chronic study NOAEL is 0.9 mg/kg bw/day based on a two-year feeding study with dogs. In the current study, the ratio of $NOAEL_{tissue\ weight}/NOAEL_{chronic}$ was greater than 1. Therefore, the concentration of prochloraz use was deemed safe. NOAEL for hormone effect induced by prochloraz is less than 15 mg/kg bw/day. The ratio of $NOAEL_{hormone}/NOAEL_{chronic}$ is possibly greater than 1. However, future research should seek to comprehensively elucidate the underlying mechanisms through which prochloraz decreases thyroid hormones.

Key words: thyroid hormone, prochloraz, vaginal opening, preputial separation, pubertal rats

Introduction

Both wildlife studies and laboratory animal studies have demonstrated that environmental chemicals can negatively impact reproductive development in humans [7]. Furthermore, environmental chemicals with antiandrogen effects have been shown to down-regulate male development in laboratory rats [13, 38]. The adverse effects of antiandrogenic chemicals differ according to the timing of exposure, whereby the periods of gestational and pubertal development are particularly susceptible [38, 39]. Previous reports have also shown that some chemicals inhibit androgen function via multiple mechanisms [14, 24, 49]. Understanding these mechanisms is important in evaluating the consequences of exposure on gestational and pubertal reproductive development in both humans and wildlife.

Prochloraz is an imidazole fungicide used to protect crops through the inhibition of aromatase (CYP51), which weakens fungal cell membranes [27, 48]. Prochloraz also interferes with androgen signaling via multiple mechanisms. For example, prochloraz has been found to (1) antagonize androgen receptors in transcription activation assays [2, 29, 44] and (2) reduce the weights of androgen-sensitive tissues in Rat Hershberger assays [44, 46]. In addition to being an androgen receptor antagonist, prochloraz has been reported to inhibit steroidogenesis. *In utero* exposure to prochloraz can decrease testosterone levels in fetal rats and increase progesterone levels *ex vivo* [23, 43, 49]. Furthermore, prochloraz exposure during sexual differentiation has been found to cause reproductive abnormalities in male rat

offspring, including phallus abnormalities, decreased reproductive organ weights, and increased retention of nipples/areolas [23, 29, 43]. The major mechanisms by which prochloraz impacts male reproductive development likely involve antagonistic effects against androgen receptors and/or decreased androgen levels. Although antiandrogen properties and aromatase inhibition effects have been observed following prochloraz exposure during the pubertal development period, the effects of prochloraz on thyroid function have not been well established.

The Endocrine Disrupter Screening and Testing Advisory Committee of the USEPA recommended a screening strategy to investigate endocrine-disrupting compounds that inhibit steroid biosynthesis, alter thyroid hormone function, or act as agonists/antagonists to estrogen and/or androgen receptors [40]. Pubertal female and male rat models have been designed to investigate the effects of fungicides on pubertal development, thyroid function, and hypothalamic-pituitary-thyroid function. In these models, 22- or 23-day-old weanling female and male rats were exposed to the test substance for 20 or 30 days during the pubertal development period [41, 42]. Based on previous reports which investigated the inhibition of aromatase (CYP19) activity, we hypothesized that prochloraz may disturb thyroid-related functions in pubertal male and female rats. Therefore, in the current research, we investigated the potential effects of prochloraz on thyroid and hypothalamic-pituitary-thyroid function through a study on pubertal development and thyroid function in male and female Wistar rats.

Materials and methods

1. Chemicals

This study was performed using the following substances: testosterone propionate (TP, purity ≥ 97%), flutamide (purity ≥ 97%), corn oil (0.9 g/mL), 17α-ethinyl estradiol (EE, purity ≥ 98%) (Sigma-Aldrich Co., St. Louis, MO, USA), and prochloraz (purity ≥ 97%; Sinon Co., Taichung, Taiwan, ROC).

2. Animals

Our protocol for animal use was approved by the Institutional Animal Care and Use Committee of the Taiwan Agricultural Chemicals and Toxic Substances Research Institute (13-TACTRI-IACUC-007 and 13-TACTRI-IACUC-008). Five-week-old male and female Wistar rats were purchased from BioLASCO (Taipei, Taiwan, ROC). The rats were acclimated to the laboratory environment and reared under the following conditions: temperature, 21 ± 2 °C; humidity, 40~70%;

frequency of ventilation, at least 10 times per hour; and alternating 12-h light and 12-h darkness cycles. The rats were provided with a pellet rodent diet and water *ad libitum* until they were sacrificed.

We assigned 18 male and 18 female rats to each treatment group. At 12 weeks of age, rats within each treatment group were allowed to mate over a 14-day period. Gestation day (GD) 0 was defined as the day that sperm was observed in the vagina of the female following mating. Dams were allowed to deliver their pups naturally. Any litters with fewer than eight pups (including both male and female pups) or not delivered by GD 23 were excluded from the study. Note that sufficient litter numbers were needed to ensure that (1) each treatment group comprised 7-10 female pups and 10 male pups and (2) littermates would not be placed in the same treatment group. Furthermore, populations of female and male pups that were as homogeneous as possible were obtained by eliminating an equal number of pups from the heaviest and the lightest ends of the weight distribution curve so that all animals used in the study had weights in the middle of the distribution curve. The pups were assigned to treatment groups such that the mean body weights and variances for all groups were similar. In other words, pups were allocated to treatment groups under the basis of body weight randomization to ensure unbiased weight distribution across all groups.

3. Treatment

Weights and clinical observations for both male and female rat offspring were recorded daily prior to treatment. For male offspring, treatments (control; TP 0.4 mg/kg bw/day; flutamide 3 mg/kg bw/day; prochloraz 15, 50, or 150 mg/kg bw/day) were administered by oral gavage from postnatal day (PND) 23 to PND 53. Beginning on PND 30, males were examined daily for preputial separation (PPS). On PND 53, males were dosed and then sacrificed two hours later. For female offspring, treatments (control; 17α-ethinyl estradiol [EE] 5 mg/kg bw/day; prochloraz 15, 50, or 150 mg/kg bw/day) were administered by oral gavage between PND 22 and PND 42. Females were examined daily for vaginal opening (VO). On PND 42, females were dosed and then sacrificed two hours later.

4. Clinical observations and body weights

Throughout the study period, all female and male rats were observed for clinical signs of toxicity related to chemical treatment at least once per day. On working days, all cages were checked for dead or moribund animals in the mornings and afternoons. The body weight of

each rat was recorded daily to the nearest 0.1 g prior to treatment.

5. Measurement of organ weights

Two hours after the final treatment, each rat was treated with the control (females and males); EE 5 mg/kg bw/day (females); TP 0.4 mg/kg bw/day (males); flutamide 3 mg/kg bw/day (males); or prochloraz 15, 50 or 150 mg/kg bw/day (females and males). All rats were then anesthetized with Zoletil 3 mg/kg bw/day (females and males) in the same order as they had been treated.

Uteri and ovaries were dissected and carefully trimmed of fat to avoid loss of luminal content. For this, each uterus was cut just above its junction with the cervix and at the junction of the uterine horns and ovaries. Each uterus was weighed with and without luminal content. Thyroids, livers, kidneys, pituitary glands, adrenal glands, ovaries, seminal vesicles, coagulating glands with and without fluid, prostates, levator ani plus bulbocavernosus muscles (LABC), epididymides, testes, and penes were also carefully dissected and weighed.

6. Vaginal opening

Each female animal was examined daily for VO from PND 21. On the day that VO was first detected, the age and body weight of the rat were recorded. Vaginal lavage was collected daily from the day following VO until the end of the study by repeated pipetting 0.9% saline into the vagina. The lavage fluid was applied to a clean glass slide, and the smear was viewed immediately under low magnification (×100) with a microscope. Cytology was evaluated, and the estrous cycle stage was determined using the method described by Everett et al. (1989) [13]. The appearance of a small "pinhole", vaginal thread, and complete VO were all recorded on the days that they were observed. The age upon VO (i.e., the endpoint in this analysis) was considered to be the day that complete VO was observed; a pinhole or thread did not represent complete VO, even though these observations were recorded. However, if any animal within any treatment group showed an incomplete opening (such as persistent threads or a pinhole) for more than three days, a separate analysis was conducted based on the age at which an incomplete opening was first observed. Note that even if VO otherwise appeared complete, documentation of a vaginal thread was crucial. Documenting the "initiation" of VO was also crucial, and we sought to record VO observations daily after dosing. However, regardless of whether VO observations were collected before or after dosing, they were recorded at approximately the same time each day.

7. Estrous cyclicity

From the day of VO up to and including the day of necropsy, daily vaginal smears were obtained and evaluated under a low-power light microscope for the presence of leukocytes, nucleated epithelial cells, or cornified epithelial cells. The vaginal smears were classified as diestrus (predominantly comprised of leukocytes mixed with some cornified epithelial cells), proestrus (predominantly comprised of clumps of round, nucleated epithelial cells), or estrus (predominantly comprised of cornified epithelial cells). Note that metestrus was classified as an early part of diestrus rather than a late part of estrus. The age of first vaginal estrus was recorded, and we preferred to record daily estrous cycle observations after dosing, although this was not always possible. Regardless of whether estrous cycle observations were collected before or after dosing, they were recorded at approximately the same time each day.

At the end of the study, the overall estrous pattern of each female was characterized as regular cycling (recurring 4- to 5-day cycles), irregular cycling (cycles with periods of diestrus longer than three days or periods of cornification longer than two days), or not cycling (prolonged periods of either vaginal cornification or leukocytic smears). In cases where there were too few days between VO and the end of the study for more than one cycle to be observed, classification was based on available data. Specifically, in these cases, animals were assumed to have regular cycling if the partial data fit that definition and irregular cycling if we were not able to distinguish between irregular cycling and not cycling.

8. Preputial separation

PPS, the separation of the foreskin of the penis from the glans, is an early reliable marker of pubertal progression, which normally occurs between 40 and 50 days of age, depending on the rat species (the average age of PPS is 43 days) [26]. In the present study, PPS was monitored from PND 22 to 53. All males were observed for PPS at approximately the same time each day. Partial separation (i.e., in which a thread of cartilage remained) was recorded; however, only the day of complete separation was used in data analysis. The presence of a persistent thread of tissue between the glans and prepuce was also recorded on the days that it was observed. The day of complete PPS was the endpoint used in the analysis for the age upon PPS. However, if any animal in any treatment group showed incomplete separation (including persistent threads) for more than three days, a separate analysis was conducted using the age at which partial separation was first observed. Even if PPS otherwise appeared complete,

documentation of a thread was crucial. Recording the "initiation" of PPS was also crucial, and we preferred to record daily PPS observations after dosing, though this was not always possible. Regardless of whether PPS observations were collected before or after dosing, they were recorded at approximately the same time each day.

9. Hematochemistry

Blood samples from rats treated with control (females and males); EE 5 mg/kg bw/day (females); TP 0.4 mg/kg bw/day (males); flutamide 3 mg/kg bw/day (males); and prochloraz 15, 50, or 150 mg/kg bw/day (female and male) were collected and coagulated for 30 min in an SST II tube (#367953, BD Co., Plymouth, UK). The blood samples were put on an ice bath prior to centrifugation. After coagulation, the blood was centrifuged at 3,000 g for 15 min. The serum was then transferred into siliconized microcentrifuge tubes and stored at -80 °C until use. Serum creatinine and blood urea nitrogen levels were detected with an automated clinical chemistry analyzer (DRI-CHEM 4000i, Fujifilm Co., Tokyo, Japan).

10. Hormonal measurements

In serum from rats treated with control (females and males); EE 5 mg/kg bw/day (females); TP 0.4 mg/kg bw/day (males); flutamide 3 mg/kg bw/day; and prochloraz 15, 50, and 150 mg/kg bw/day (females and males), the following were determined using a magnetic bead panel (#RPTMAG-86K, #PTHYMAG-30K, Millipore Co., St. Charles, MI, USA): levels of luteinizing hormone (LH), follicle-stimulating hormone (FSH), thyroxine (T4), triiodothyronine (T3), and thyroid-stimulating hormone (TSH).

Serum from all rat groups was assayed for testosterone (T) and estradiol (E2) levels using an EIA kit (#582701, #582251, Cayman Co.). LH, FSH, total T4, T3, and TSH levels were determined using a magnetic bead panel (#RPTMAG-86K, #PTHYMAG-30K, Millipore Co.). A cytochrome P450 19A1 ELISA kit (CSB-EL006394RA, Cusabio Co.) was used to determine aromatase levels.

11. Statistical analysis

Data were expressed as the mean ± standard deviation (SD). Data pertaining to the mean initial and necropsy body weights, mean age, body weight upon VO or PPS, organ weight, and hormone levels were analyzed for homogeneity of variance using Bartlett's test. Nonparametric analysis of variance was applied in samples found to be homogeneous. Absolute organ weights were analyzed using

analysis of covariance with body weight upon necropsy as a covariate. When a significant treatment effect was observed, Dunnett's test (control vs. treatment groups) was used to compare groups. The level of statistical significance was set a priori at $\alpha = 0.05$.

Results

1. Effects on body and organ weights

There were no clinical signs of toxicity in male and female rats from any treatment group during the study period. EE significantly decreased the body of female rats between PND 26 and 42 (Fig. 1A), while prochloraz did not. Conversely, 150 mg/kg bw/day prochloraz did decrease the body weight of male rats (Fig. 1B).

In female rats, EE significantly decreased final body weight and body weight gain by 29% and 49%, respectively. Both 50 and 150 mg/kg bw/day prochloraz increased final body weight by 12% and 8%, respectively, and increased body weight gain by 13% and 11%, respectively. In male rats, TP decreased final body weight and body weight gain by 9% and 11%, respectively, while flutamide and 50 mg/kg bw/day prochloraz did not. However, a prochloraz dose of 150 mg/kg bw/day decreased final body weight and body weight gain by 11% and 14%, respectively (Table 1). In females, EE significantly decreased liver weight by 18%, whereas 50 and 150 mg/kg bw/day prochloraz increased liver weight by 30% and 37%, respectively. EE decreased kidney and adrenal gland weights by 27% and 21%, respectively, while prochloraz did not lead to any increases in kidney or adrenal gland weights. Pituitary, ovaries, uterus wet and blotted weights were not affected by EE or prochloraz (Table 2). Prochloraz did not significantly affect kidney, pituitary, adrenal, ovaries, or uterus wet and blotted weights. Finally, we did not observe significant effects on absolute or relative thyroid weights (Table 3).

In male rats, liver weight was not affected by TP, flutamide, or prochloraz. However, while TP and flutamide did not affect kidney weight, 150 mg/kg bw/day prochloraz decreased kidney weight by 12%. TP decreased pituitary weight by 24%, while flutamide and prochloraz did not affect pituitary weight. TP also increased adrenal weight by 13%, whereas flutamide did not. Prochloraz at a dose of 150 mg/kg bw/day decreased adrenal weight by 10%. TP did not change the weight of the seminal vesicle and coagulating gland with or without fluid. Flutamide decreased the weight of the seminal vesical and coagulating gland with fluid by 25%, and doses of 50 and 150 mg/kg bw/day prochloraz respectively decreased the weight of seminal vesicle and coagulating gland with fluid by 25% and 50% in a dose-dependent manner. Flutamide and

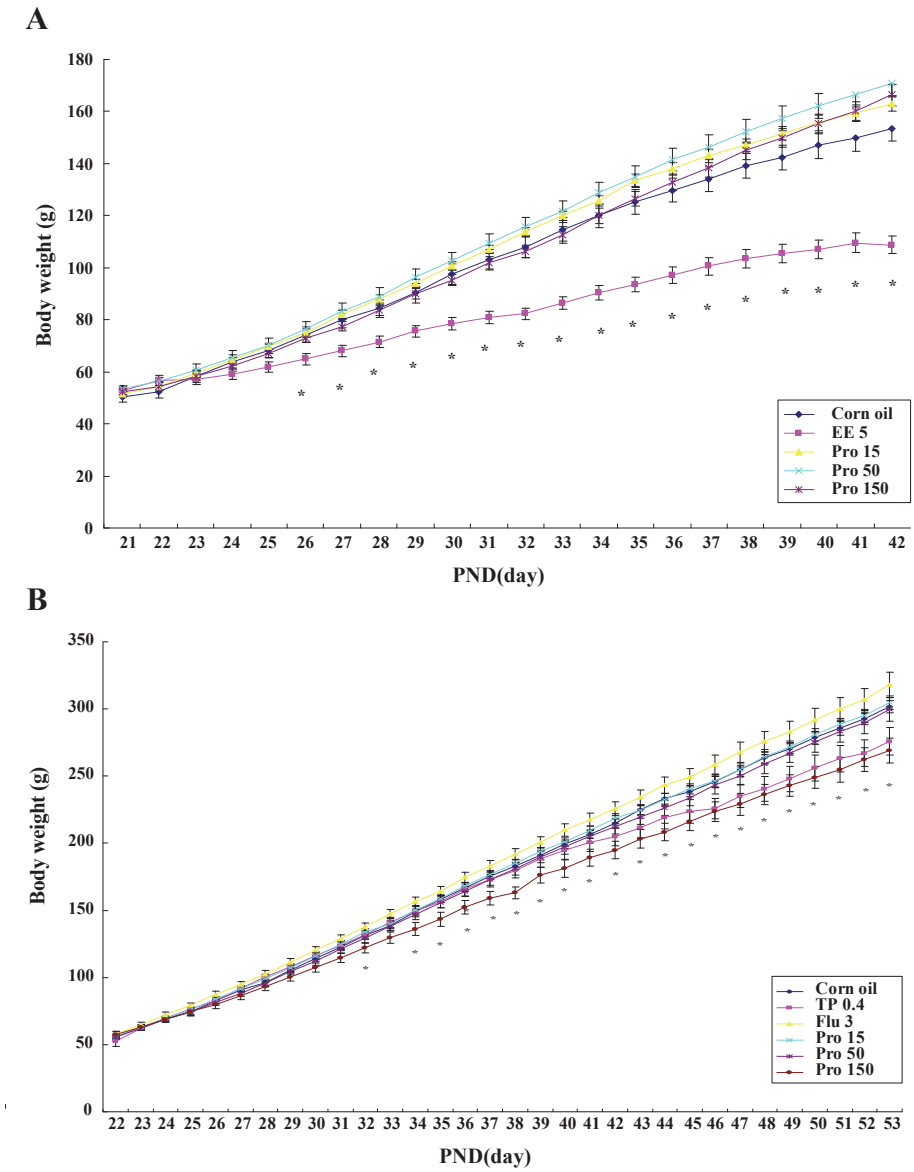

Fig. 1. Body weight changes in female Wistar rats treated with 5 mg/kg bw/day 17a-ethinyl estradiol (EE 5); male Wistar rats treated with 0.4 mg/kg bw/day testosterone propionate (TP 0.4) or 3 mg/kg bw/day flutamide (Flu 3); and female and male Wistar rats treated with 15, 50, or 150 mg/kg bw/day prochloraz (Pro 15, Pro 50, and Pro 150). Female rats were dosed daily from 21 days of age until necropsy was performed on postnatal day (PND) 42 (A). Male rats were dosed daily from 22 days of age until necropsy was performed on postnatal day (PND) 53 (B). Data are expressed as the mean ± SD of 10 animals per treatment group. The * symbol indicates that mean body weight was significantly different from that of the control, $P < 0.05$.

Table 1. General growth in female and male rats treated with various treatments

Treatments[3)]	Vehicle Control	EE[1)] 5	TP[2)] 0.4	Flutamide 3	Prochloraz 15	Prochloraz 50	Prochloraz 150
Female							
Sample size (n)	10	10			10	10	10
Initial body weight (g)	52 ± 8	57 ± 6			54 ± 6	56 ± 5	55 ± 4
Final body weight (g)	153 ± 14	109 ± 11***			163 ± 10	171 ± 16*	166 ± 13
Final body weight (%)	100	71			106	111	109
Body weight gain (g)	101 ± 11	52 ± 8***			109 ± 9	114 ±12*	112 ± 12*
Male							
Sample size (n)	10		10	10	10	10	10
Initial body weight (g)	63 ± 4		62 ± 5	65 ± 6	63 ± 5	62 ± 5	63 ± 7
Final body weight (g)	302 ± 15		276 ± 32*	318 ± 30	305 ± 15	300 ± 28	269 ± 29**
Final body weight (%)	100		91	105	101	99	89
Body weight gain (g)	239 ± 14		213 ± 30*	253 ± 26	242 ± 13	237 ± 26	206 ± 26***

1) EE: 17α-ethinyl estradiol
2) TP: Testosterone Propionate
3) Treatment dose: mg/kg bw/day
P-value = * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.005

Table 2. Organ weights of female rats at necropsy

Treatments[2)]	Vehicle control	EE[1)] 5	Prochloraz 15	Prochloraz 50	Prochloraz 150
Sample size (n)	10	10	10	10	10
Liver (g)	7.3 ± 0.8	6.0 ± 0.7***	7.9 ± 0.6	9.5 ± 1.5***	10.0 ± 1.0***
Liver/BW (%)	4.8 ± 0.2	5.5 ± 0.3***	4.9 ± 0.2	5.5 ± 0.4***	6.0 ± 0.2***
Kidney (g)	1.5 ± 0.2	1.1 ± 0.2***	1.5 ± 0.1	1.6 ± 0.2	1.5 ± 0.3
Kidney/BW (%)	0.96 ± 0.05	1.04 ± 0.14	0.90 ± 0.05*	0.91 ± 0.05*	0.87 ± 0.08*
Pituitary (mg)	9.0 ± 1.7	10.0 ± 2.0	8.1 ± 2.0	8.0 ± 0.7	7.9 ± 1.3
Pituitary/BW (%)	0.005 ± 0.001	0.009 ± 0.002***	0.005 ± 0.001*	0.005 ± 0.001***	0.005 ± 0.001**
Adrenals (mg)	47.3 ± 5.9	37.5 ± 6.3***	50.7 ± 6.4	49.3 ± 5.1	46.0 ± 6.2
Adrenals/BW (%)	0.030 ± 0.004	0.035 ± 0.008	0.031 ± 0.004	0.029 ± 0.003	0.028 ± 0.002*
Ovaries (mg)	78.1 ± 18.6	63.4 ± 16.9	79.0 ± 13.1	75.8 ± 14.0	69.9 ± 16.6
Ovaries/BW (%)	0.050 ± 0.009	0.058 ± 0.012	0.048 ± 0.006	0.045 ± 0.010	0.042 ± 0.010
Uterus, wet (mg)	329 ± 112	431 ± 130	361 ± 135	357 ± 208	311 ± 109
Uterus, wet/BW (%)	0.214 ± 0.070	0.403 ± 0.146***	0.221 ± 0.083	0.206 ± 0.107	0.188 ± 0.064
Uterus, blotted (mg)	304 ± 79	332 ± 40	314 ± 77	288 ± 82	280 ± 79
Uterus, blotted/BW (%)	0.198 ± 0.049	0.306 ± 0.039***	0.192 ± 0.045	0.169 ± 0.044	0.169 ± 0.049

1) EE: 17α-ethinyl estradiol
2) Treatment dose: mg/kg bw/day
P-value = * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.005

Table 3. Absolute and relative thyroid weights of female rats at necropsy

Treatments[2)]	Vehicle control	EE[1)] 5	Prochloraz 15	Prochloraz 50	Prochloraz 150
Sample size (n)	10	10	10	10	10
Thyroid with trachea (mg)	140 ± 15	111 ± 14	133 ± 14	132 ± 12	139 ± 17
Thyroid without trachea (mg)	28 ± 15	23 ± 16	29 ± 12	28 ± 10	27 ± 10
Thyroid/BW with trachea (%)	92 ± 10	103 ± 9	81 ± 6	78 ± 7	83 ± 9
Thyroid/BW without trachea (%)	18 ± 10	21 ± 13	18 ± 9	17 ± 6	16 ± 6

[1)] EE: 17α-ethinyl estradiol

[2)] Treatment dose: mg/kg bw/day

P-value = * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.005

150 mg/kg bw/day prochloraz decreased the weight of seminal vesicle and coagulating gland without fluid by 25% and 37%, respectively. TP and flutamide did not affect prostate weight, while 150 mg/kg bw/day prochloraz decreased prostate weight by 43%. TP did not change LABC weight, while flutamide and prochloraz decreased LABC weight by 8% and 29%, respectively. TP, flutamide, and 150 mg/kg bw/day prochloraz decreased the weight of the left epididymis by 31%, 14%, and 22%, respectively, and decreased the weight of the right epididymis by 31%, 14%, and 19%, respectively. TP decreased left testis weight by 65%, while flutamide increased left testis weight by 17%. TP decreased the right testis weight by 64%, whereas flutamide and 15 mg/kg bw/day prochloraz increased right testis weight by 9% and 10%, respectively. In summary, prochloraz decreased prostate, LABC, and epididymis weights in a dose-dependent manner; however, decreases were only significant when a high dose of prochloraz was administered (Table 4).

The absolute and relative weights of thyroid with or without trachea were not affected by TP, flutamide, or prochloraz (Table 5).

2. Effects on blood urea nitrogen and creatinine in serum

Blood urea nitrogen (BUN) and creatine were used as indicators to investigate the effects of prochloraz on the blood chemistry in pubertal rats. In female rats, EE did not change the serum concentration of BUN or creatinine, while 15, 50, and 150 mg/kg bw/day prochloraz decreased it by 26%, 19%, and 25%, respectively. EE did not change the serum concentration of creatinine, while 15 and 50 mg/kg bw/day prochloraz decreased creatinine by 33% and 33%, respectively. In male rats, TP, flutamide, and 15 or 150 mg/kg bw/day

Table 4. Organ weights of male rats at necropsy

Treatments[3)]	Vehicle control	TP[1)] 0.4	Flutamide 3	Prochloraz 15	Prochloraz 50	Prochloraz 150
Sample size (n)	10	10	10	10	10	10
Liver (g)	14.0 ± 1.4	13.0 ± 1.8	15.4 ± 2.5	14.6 ± 1.0	15.3 ± 1.8	15.6 ± 2.1
Liver/BW (%)	4.6 ± 0.3	4.7 ± 0.3	4.8 ± 0.4	4.8 ± 0.3	5.1 ± 0.3**	5.8 ± 0.3***
Kidney (g)	2.5 ± 0.2	2.3 ± 0.3	2.6 ± 0.3	2.6 ± 0.2	2.5 ± 0.3	2.2 ± 0.2*
Kidney/BW (%)	0.82 ± 0.08	0.83 ± 0.05	0.80 ± 0.04	0.85 ± 0.04	0.82 ± 0.07	0.84 ± 0.12
Pituitary (mg)	11.9 ± 1.8	9.0 ± 2.6*	12.3 ± 2.8	12.0 ± 1.7	11.4 ± 1.5	9.8 ± 2.6
Pituitary/BW (%)	0.004 ± 0.001	0.002 ± 0.001*	0.004 ± 0.001	0.004 ± 0.001	0.004 ± 0.000	0.003 ± 0.001
Adrenals (mg)	63.5 ± 6.8	71.5 ± 8.3*	66.0 ± 7.7	64.0 ± 9.1	63.0 ± 8.0	56.9 ± 6.3*
Adrenals/BW (%)	0.021 ± 0.003	0.026 ± 0.005**	0.021 ± 0.002	0.021 ± 0.003	0.021 ± 0.003	0.021 ± 0.002
Seminal vesicle + coagulating gland, with fluid (SVCGf) (mg)	761 ± 142	820 ± 154	574 ± 104**	694 ± 135	567 ± 115***	380 ± 88***
SVCGf/BW (%)	0.25 ± 0.04	0.30 ± 0.06*	0.18 ± 0.03****	0.23 ± 0.04	0.19 ± 0.03**	0.14 ± 0.04***
Seminal vesicle + Coagulating gland, without fluid (SVCGwf) (mg)	519 ± 87	568 ± 89	428 ± 51**	528 ± 91	445 ± 88	328 ± 74***
SVCGwf/BW(%)	0.17 ± 0.03	0.21 ± 0.03*	0.13 ± 0.01**	0.17 ± 0.03	0.15 ± 0.02	0.12 ± 0.03***
Prostate (mg)	195 ± 68	188 ± 37*	175 ± 62	219 ± 46	186 ± 42	112 ± 34***
Prostate/BW (%)	0.064 ± 0.020	0.068 ± 0.012	0.054 ± 0.017	0.072 ± 0.015	0.062 ± 0.011	0.041 ± 0.011**
LABC (mg) [2)]	605 ± 112	657 ± 108	554 ± 79*	587 ± 62	576 ± 99	432 ± 83***
LABC/BW (%)	0.20 ± 0.03	0.24 ± 0.02**	0.17 ± 0.01*	0.19 ± 0.02	0.19 ± 0.03	0.16 ± 0.03*
Left epididymis (LE) (mg)	197 ± 18	135 ± 31***	169 ± 19**	211 ± 17	179 ± 26	154 ± 19***
LE/BW (%)	0.065 ± 0.004	0.049 ± 0.009***	0.053 ± 0.004***	0.069 ± 0.007	0.060 ± 0.008	0.057 ± 0.006**
Right epididymis (RE) (mg)	192 ± 17	132 ± 33***	165 ± 23**	206 ± 21	182 ± 27	155 ± 18***
RE/BW (%)	0.064 ± 0.004	0.048 ± 0.010***	0.052 ± 0.005***	0.068 ± 0.008	0.061 ± 0.008	0.058 ± 0.006*
Left testis (LT) (mg)	1294 ± 78	453 ± 330***	1383 ± 107*	1355 ± 117	1324 ± 127	1277 ± 171
LT/BW (%)	0.43 ± 0.02	0.16 ± 0.11***	0.44 ± 0.03	0.44 ± 0.03	0.44 ± 0.04	0.48 ± 0.04**
Right testis (RT) (mg)	1284 ± 80	456 ± 314***	1403 ± 105*	1408 ± 150*	1309 ± 136	1283 ± 162
RT/BW (%)	0.43 ± 0.02	0.16 ± 0.10***	0.44 ± 0.03	0.46 ± 0.06	0.44 ± 0.04	0.48 ± 0.04**

[1)] TP: Testosterone Propionate

[2)] LABC: levator ani plus bulbocavernosus muscles

[3)] Treatment dose: mg/kg bw/day

P-value = * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.005

Table 5. Absolute and relative thyroid weights of male rats at necropsy

Treatments[2)]	Vehicle control	TP[1)] 0.4	Flutamide 3	Prochloraz 15	Prochloraz 50	Prochloraz 150
Sample size (n)	10	10	10	10	10	10
Thyroid with trachea (mg)	185 ± 14	172 ± 13	177 ± 12	186 ± 15	188 ± 7	172 ± 19
Thyroid without trachea (mg)	32 ± 9	35 ± 7	31 ± 11	33 ± 8	32 ± 10	28 ± 10
Thyroid/BW with trachea (%)	61 ± 6	63 ± 8	56 ± 6	61 ± 4	64 ± 7	64 ± 10
Thyroid/BW without trachea (%)	11 ± 3	13 ± 2	10 ± 4	11 ± 2	11 ± 4	10 ± 3

1) TP: Testosterone Propionate

2) Treatment dose: mg/kg bw/day

P-value = $^{*} \leq 0.05$, $^{**} \leq 0.01$, $^{***} \leq 0.005$

prochloraz did not change the serum concentration of BUN; however, a dose of 50 mg/kg bw/day prochloraz decreased the serum concentration of BUN by 15%. TP, flutamide, and 15 or 50 mg/kg bw/day prochloraz did not change the serum concentration of creatinine whereas a dose of 150 mg/kg bw/day prochloraz led to a 33% increase in the serum concentration of creatinine (Table 6).

3. Effects on vaginal opening and estrous cyclicity in female rats

The mean age and body weight of female control rats upon VO were 27.2 days and 81 g, respectively. Treatment with EE significantly advanced VO to 25.7 days of age and substantially reduced the mean body weight at the time of VO to 64.0 g. Prochloraz did not change the time of VO, which was respectively observed to be 26.3, 28.0, and 26.8 days at doses of 15, 50, and 150 mg/kg bw/day. EE significantly reduced the mean age and body weight upon VO by 6% and 21%, respectively, in comparison with control female rats, while prochloraz did not lead to any significant effects (Table 7). The estrous cycles of individual animals were evaluated from the day after VO until the end of the study. Most control rats exhibited regular cycling (data not shown).

4. Effects on preputial separation in male rats

The mean age and body weight of male control rats upon PPS were 31.7 days and 129 g, respectively. TP substantially advanced the mean age of PPS to 29.8 days and reduced the mean body weight at the time of PPS to 114 g. In contrast, flutamide significantly delayed the mean age of PPS to 36.3 days and increased the mean body weight at the time of PPS to 175 g. The mean ages (32.7, 33.4, and 35.2 days for 15, 50, and 150 mg/kg bw/day prochloraz,

Table 6. Serum concentrations of blood urea nitrogen and creatinine in female and male rats treated with various treatments

Treatments[4)]	Vehicle control	EE[1)] 5	TP[2)] 0.4	Flutamide 3	Prochloraz 15	Prochloraz 50	Prochloraz 150
Female							
Sample size (n)	10	10			10	10	10
BUN (mg/dL)[3)]	22.8 ± 2.9	23.2 ± 3.6			16.8 ± 3.2***	18.5 ± 2.1***	17.1 ± 2.9***
Creatinine (mg/dL)	0.3 ± 0.1	0.3 ± 0.1			0.2 ± 0.1*	0.2 ± 0.1*	0.3 ± 0.1
Male							
Sample size (n)	10		10	10	10	10	10
BUN (mg/dL)[3)]	18.1 ± 2.5		19.1 ± 3.1	17.5 ± 1.9	15.7 ± 2.9	15.4 ± 2.6*	16.8 ± 6.1
Creatinine (mg/dL)	0.3 ± 0.0		0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.2*

1) EE: 17α-ethinyl estradiol
2) TP: Testosterone Propionate
3) BUN: blood urea nitrogen
4) Treatment dose: mg/kg bw/day
P-value = * ≤ 0.05, ** ≤ 0.01

Table 7. Mean age and body weight at the time of vaginal opening (VO) in female rats and preputial separation (PPS) in male rats

Treatments[5)]	Vehicle control	EE[1)] 5	TP[2)] 0.4	Flutamide 3	Prochloraz 15	Prochloraz 50	Prochloraz 150
Female							
Sample size (n)	10	10			10	10	10
VO (d)[3)]	27.2 ± 1.9	25.7 ± 0.8*			26.3 ± 2.0	28.0 ± 2.8	26.8 ± 3.0
Body weight at VO (g)	81 ± 18	64 ± 7*			78 ± 15	89 ± 13	77 ± 14
Male							
Sample size (n)	10		10	10	10	10	10
PPS (d)[4)]	31.7 ± 1.5		29.8 ± 2.2*	36.3 ± 3.6***	32.7 ± 1.7	33.4 ± 3.6	35.2 ± 2.6***
Body weight at PPS (g)	129 ± 14		114 ± 14*	175 ± 26***	140 ± 22	141 ± 26	144 ± 19

1) EE: 17α-ethinyl estradiol
2) TP: Testosterone Propionate
3) VO: vaginal open
4) PPS: preputial separation
5) Treatment dose: mg/kg bw/day
P-value = * ≤ 0.05, ** ≤ 0.01

respectively) and body weights (140, 141, and 144 g for 15, 50, and 150 mg/kg bw/day prochloraz, respectively) upon PPS of rats treated with prochloraz were generally comparable to those of control rats. However, rats treated with 150 mg/kg bw/day prochloraz showed a significant increase in body weight of 11% (Table 8).

5. Effects on serum hormone concentrations in female and male rats

In female rats, EE significantly increased the serum concentration of E2 by 157%, whereas prochloraz did not lead to a significant change. EE did not affect the serum concentration of LH, while 15 mg/kg bw/day prochloraz decreased LH by 51%. EE decreased the serum concentration of FSH by 82%, while prochloraz did not lead to a significant change in the serum concentration of FSH. EE did not change the serum concentration of T3; however, 15, 50, and 150 mg/kg bw/day prochloraz decreased T3 by 40%, 39%, and 40%, respectively. EE did not affect the serum concentration of T4, while 15 and 150 mg/kg bw/day prochloraz respectively decreased T4 by 27% and 51% in a dose-dependent manner. EE increased the serum concentration of TSH by 148%, whereas prochloraz did not lead to a significant change in the serum concentration of TSH. Neither EE nor prochloraz affected the serum concentrations of T or aromatase (Table 8).

In male rats, T and flutamide did not change the serum concentration of E2, while 15, 50, and 150 mg/kg bw/day prochloraz respectively decreased E2 by 75%, 75%, and 92% in a dose-dependent manner. TP did not change the serum concentration of T, whereas flutamide increased T by 87%. Prochloraz decreased the serum concentration of T in a dose-dependent manner (a reduction of 64% and 78% at doses of 50 and 150 mg/kg bw/day, respectively). TP decreased the serum concentration of LH by 79%, while flutamide and prochloraz did not. TP decreased the serum concentration of FSH by 44%, whereas 50 mg/kg bw/day prochloraz increased FSH by 43%. Flutamide did not affect the serum concentration of FSH. TP, flutamide, and prochloraz at doses of 15, 50, and 150 mg/kg bw/day decreased the serum of concentration of aromatase by 46%, 64%, 81%, 82%, and 72%, respectively. TP, flutamide, and prochloraz did not change the serum concentration of T3 or TSH. TP increased the serum concentration of T4 by 62%, while 15, 50, and 150 mg/kg bw/day prochloraz respectively decreased the serum concentration of T4 by 58%, 69%, and 75% in a dose-dependent manner (Table 9).

Table 8. Serum hormone concentrations of female rats at necropsy

Treatments[9]	Vehicle control	EE[1] 5	Prochloraz 15	Prochloraz 50	Prochloraz 150
Sample size (n)	10	10	10	10	10
E2 (pg/mL)[2]	167 ± 136	429 ± 273*	142 ± 90	136 ± 74	119 ± 91
T (pg/mL)[3]	186 ± 93	137 ± 59	176 ± 37	141 ± 40	159 ± 69
LH (pg/mL)[4]	7006 ± 5021	3550 ± 1781	3444 ± 1037*	3775 ± 1600	4150 ± 1546
FSH (pg/mL)[5]	937 ± 432	172 ± 137***	924 ± 450	611 ± 373	1207 ± 883
Aromatase (pg/mL)	89.2 ± 35.1	86.8 ± 30.5	72.3 ± 23.4	70.4 ± 33.2	90.0 ± 21.3
T3 (pg/mL)[6]	4501 ± 1642	5425 ± 1724	2681 ± 301***	2745 ± 359***	2722 ± 517***
T4 (pg/mL)[7]	463 ± 98	503 ± 105	340 ± 88*	367 ± 121	225 ± 129***
TSH (pg/mL)[8]	904 ± 749	2246 ± 1164**	430 ± 165	516 ± 279	486 ± 285

1) EE: 17a-ethinyl estradiol
2) E2: 17ß-estradol
3) T: testosterone
4) LH: luteinizing hormone
5) FSH: follicle-stimulating hormone
6) T3: triiodothyronine
7) T4: thyroxine
8) TSH: thyroid-stimulating hormone
9) Treatment dose: mg/kg bw/day
P-value = * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.005

Table 9. Serum hormone concentrations of male rats at necropsy

Treatments[9]	Vehicle control	TP[1] 0.4	Flutamide 3	Prochloraz 15	Prochloraz 50	Prochloraz 150
Sample size (n)	10	10	10	10	10	10
E2 (pg/mL)[2]	12.2 ± 5.7	7.8 ± 5.9	14.7 ± 8.0	3.1 ± 1.6***	3.1 ± 2.0***	1.0 ± 0.6***
T (pg/mL)[3]	1308 ± 824	1255 ± 320	2440 ± 957*	469 ± 156*	589 ± 542	293 ± 210***
LH (pg/mL)[4]	1196 ± 627	256 ± 289***	2216 ± 1444	1030 ± 486	1654 ± 604	996 ± 700
FSH (pg/mL)[5]	11.5 ± 4.1	6.4 ± 4.1*	16.0 ± 5.3	13.2 ± 5.4	16.4 ± 5.8*	16.0 ± 5.4
Aromatase (pg/mL)	78.0 ± 39.5	41.8 ± 7.2*	27.8 ± 15.3*	15.0 ± 26.8***	13.9 ± 20.3***	21.7 ± 21.8***
T3 (pg/mL)[6]	3558 ± 525	3837 ± 478	3668 ± 542	3918 ± 662	3625 ± 597	3754 ± 937
T4 (pg/mL)[7]	1346 ± 547	2184 ± 834*	1736 ± 803	569 ± 99***	421 ± 85***	336 ± 58***
TSH(pg/mL)[8]	461 ± 181	492 ± 189	435 ± 248	336 ± 127	510 ± 380	369 ± 204

1) TP: Testosterone Propionate
2) E2: 17ß-estradol
3) T: testosterone
4) LH: luteinizing hormone
5) FSH: follicle-stimulating hormone
6) T3: triiodothyronine
7) T4: thyroxine
8) TSH: thyroid-stimulating hormone
9) Treatment dose: mg/kg bw/day
P-value = * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.005

Discussion

This study, performed under the US EPA OCSPP Harmonized Test Guidelines Series Number 890, investigated the effects of prochloraz on pubertal developmental and thyroid function in rats. In brief, we confirmed that prochloraz, an androgen receptor antagonist, generally decreased the tissue weights of the epididymis, prostate, LABC, and seminal vesicle plus coagulating gland with and without fluid in pubertal male rats. Prochloraz did not affect organ weights in pubertal female rats but did decrease the serum concentrations of T3 and T4 in female rats. High doses of prochloraz also delayed the age of PPS but did not affect VO in female rats. Our most important finding is that prochloraz decreased the serum concentrations of E2, T, aromatase, and T4 in male rats.

This study also revealed that prochloraz decreased tissue weights of the epididymis, prostate, LABC, and seminal vesicle plus coagulating gland with and without fluid in pubertal male rats. These results, combined with our findings that prochloraz decreased serum concentrations of E2, T, and aromatase, all indicate that aromatase [3, 9, 25, 45], T biosynthesis [1, 5, 50], steroidogenesis [6, 12, 19, 26], and antiandrogens [16, 28, 44] were inhibited. Prochloraz has been previously reported to inhibit aromatase, steroidogenesis, and antiandrogens in rats [5, 6, 43, 44, 45, 46], trout [1, 26, 34], cell cultures, and *in vitro* [12, 18, 19, 25, 45], *Xenopus laevis* [16], zebrafish [4, 9, 10, 35], New Zealand mud snail [15], and in studies involving computational prediction methods [11, 22, 47].

Our results (i.e., results showing that a high dose of prochloraz (150 mg/kg bw/day) decreased the tissue weights of the epididymis, prostate, LABC, and seminal vesicle plus coagulating gland with and without fluid in pubertal male rats) support findings by Blystone et al. (2007a) [5], in which 125 mg/kg bw/day prochloraz decreased androgen-sensitive organ weights, and findings by Vingaard et al. (2005a) [43]. Note that in the current study, doses of 15 and 50 mg/kg bw/day prochloraz did not affect VO in female pubertal rats or PPS in male pubertal rats. The mechanism by which prochloraz affects antiandrogens is the same as that of vinclozolin, procymidone, linuron, and flutamide. Some previous reports have therefore investigated the cumulative effects of mixtures involving prochloraz with vinclozolin, procymidone, linuron, flutamide, triazoles, and phthalate chemicals. Most of the cumulative effects of mixtures involved antiandrogen and endocrine-disrupting activities in a dose-additive manner [8, 17, 20, 21, 30, 31, 32, 36, 37]. Conversely, treatment with a combination of prochloraz, cyproconazole, and epoxiconazole decreased adrenal gland atrophy and gene expression of molecular

toxicology endpoints [33].

In summary, this study arrived at many of the same conclusions pertaining to antiandrogens, inhibition of aromatase, and inhibition of steroidogenesis as previous reports; however, some of the endpoints that we used were different.

Vinggaard et al. (2002) [44] reported an increase in LH and a reduction of T4 and TSH levels in castrated rats. In that study, effects on seminal vesicles, LH, T4, and TSH were also evident in intact prochloraz-exposed young adult rats. Thus, the authors reported that prochloraz (1) antagonized the peripheral androgen receptors, which resulted in a decreased growth of androgen-dependent tissues, and (2) antagonized central androgen receptors blocking the negative feedback mechanism of testosterone, which resulted in increased LH secretion from the pituitary. The current study showed that prochloraz decreased serum concentrations of T4 in male rats and serum concentrations of T3 and T4 in female rats but did not affect the serum concentration of LH. Note that the antiandrogenic effects of prochloraz observed were generally weaker but qualitatively comparable to those of flutamide. Our results and those of Vinggaard et al. (2002) [44] showed discrepancies for serum concentration of LH, about which we are unclear; however, we defer to their conclusion that differential effects on T4 and TSH levels indicate that prochloraz may impact pubertal development via multiple mechanisms (i.e., in addition to androgen receptor antagonism) *in vivo*.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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撲克拉降低青春期大鼠血清中雌素二醇、睪固酮、甲狀腺素及環化酶濃度

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摘要

呂水淵、陳敏貞、廖婧淳、陳婉心、蔡韙任。2022。撲克拉降低青春期大鼠血清中雌素二醇、睪固酮、甲狀腺素及環化酶濃度。臺灣農藥科學 12：1-25。

咪唑類 (imidazole) 殺菌劑撲克拉拮抗雄性素受體並抑制性腺類固醇生合成。同時，撲克拉拮抗雄性素受體與抑制環化酶作用與甲狀腺交互作用機制仍未明朗，基於此，本研究假說為青春期雌、雄大鼠暴露撲克拉可能會干擾生殖和發育及甲狀腺功能。為探討撲克拉可能誘發青春期大鼠生殖與發育毒性及甲狀腺功能影響，本研究根據美國環保署 (US EPA OCSPP) 測試指南系列編號 890 (series 890)，探討撲克拉對大鼠青春期發育和甲狀腺功能影響。對青春期雌大鼠，處理組別包括對照組 (玉米油)、陽性對照藥劑炔雌醇 (5 mg/kg bw/day, 17α-ethinyl estradiol, EE) 及撲克拉 (15、50 或 150 mg/kg bw/day)，胃管口服投予期間為出生後第 22 至 42 天。對青春期雄大鼠，處理組別包括對照組 (玉米油)、 陽性對照藥劑睪固酮丙酸鹽 (0.4 mg/kg bw/day, testosterone propionate, TP)、陰性對照氟他胺 (3 mg/kg bw/day, flutamide) 及撲克拉 (15、50 或 150 mg/kg bw/day)，投予期間為出生後第 23 至 53 天。結果顯示，撲克拉在高劑量 150 mg/kg bw/day 降低雄大鼠體重 (出生後第 32 及第 34 至 53 天)、附睪、前列腺、提睪肌與球海棉體肌、貯精囊和凝固腺 (含液與不含液) 等組織重，但在雌大鼠生殖相關組織則無明顯影響。撲克拉在高劑量 150 mg/kg bw/day 延遲雄大鼠陰莖與包皮分離時間(preputial separation, PPS)，但未影響雌大鼠的陰道開啓時間 (vaginal open, VO)。撲克拉降低雌大鼠血清中三碘甲狀腺素 (triiodothyronine, T3) 和甲狀腺素 (thyroxine, T4) 的濃度。同時，撲克拉降低雄大鼠血清中雌素二醇 (17β-estradiol, E2)、睪固酮 (testosterone, T)、環化酶 (aromatase) 和甲狀腺素 (T4) 的濃度；另，雄大鼠血清中尿素氮濃度下降，而雌大鼠則否。研究結果，撲克拉主

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要降低雌、雄大鼠雌性素、雄性素、甲狀腺素和抑制環化酶活性表現內分泌干擾作用。撲克拉對雄大鼠附睪與副性腺重影響無可見毒害劑量 (no observed adverse effect level, NOAEL) 為 50 mg/kg bw/day 較長期每日可接受攝食量 (acceptable daily intake, ADI) 0.01 mg/kg bw/day 所選擇之狗 2 年慢毒性 NOAEL 值 0.9 mg/kg bw/day 相比大於 1，屬安全；至於內分泌干擾作用 NOAEL 為小於 15 mg/kg bw/day，較長期狗 2 年慢毒性 NOAEL 值 0.9 mg/kg bw/day 相比可能大於 1，可能屬安全；惟降低雌、雄大鼠血清中雌性素、雄性素、甲狀腺素和抑制環化酶活性之內分泌干擾作用機制仍需進一步探討。

關鍵詞：甲狀腺素、撲克拉、 陰道開啓、 陰莖與包皮分離、青春期大鼠