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# Androgen Receptor Plays a Vital Role in Carbendazim-Induced Epilepsy in Multiple-Generations of Rats

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## Abstract

Male and female parent rats were treated with carbendazim at 6.25, 12.5, 25, and 50 mg/kg bw/day, flutamide at 2.5 mg/kg bw/day, or in combination at the same doses. The mated female rats with a vaginal plug continued drug treatments according to their assigned group for a further 21 days. For F1 adult rats, due to embryo lethality carbendazim doses were adjusted to 1.56, 3.13, 6.25, and 12.5 mg/kg bw/day; and in combination to 6.25 and 12.5 mg/kg bw/day. For F2 adult rats, carbendazim doses were further adjusted to 0.39, 0.78, 1.56, 3.13, and 6.25 mg/kg bw/day; and in combination to 0.78, 1.56, and 3.13 mg/kg bw/day. This study showed that carbendazim induced endocrine-disrupting activity including changes in anogenital distance, offspring weight, vaginal opening, preputial separation, epilepsy, and reproductive organs. For F2 offspring, carbendazim at 1.56 mg/kg bw/day induced epilepsy in 5% of males, and carbendazim at 6.25 mg/kg bw/day induced epilepsy in 3% of male and 10% of female offspring. For F3 offspring, carbendazim at 0.78 and 6.25 mg/kg bw/day respectively induced epilepsy in 13% and 20% of males and in 15% and 6% of females. Furthermore, flutamide induced epilepsy in 10% of female offspring. Our data indicate that carbendazim reduced the weights of the brain, pituitary gland, testis, and penis as well as penis width and length in male offspring while epididymis weight of male rats increased in F2 offspring but decreased in F3 offspring. Based on the above findings, we infer that androgen receptors play a vital role in epilepsy in rats.

***Keywords:*** *Carbendazim; Flutamide; Reproductive and Developmental Neurotoxicity; Epilepsy; Rat*

## Introduction

Carbendazim (methyl 2-benzimidazolecarbamate) and benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate] were both used as commercial fungicides from 1972 to now. Benomyl is rapidly converted into carbendazim in the environment and in mammals (Figure 1). These two widely used fungicides have been reported to induce reproductive and developmental toxicity. Following treatment of male rats with carbendazim, sloughing of the seminiferous epithelium and severe atrophy and occlusions of seminiferous tubules in the testis were identified [1-3]. Treatment with carbendazim *in utero* reduced litter size and resulted in irreversible infertility, embryonic death, and growth retardation in rat offspring [4-6]. Furthermore, benomyl induced reproductive toxicity in rats, the consequences of

which included detachment and sloughing of germ cells, occlusion of efferent ductules in the testis, and a reduction of tissue weight and sperm count in the epididymis [7-9]. The endocrine-disrupting activity of pesticides has also been well studied [10,11]. For example, *in utero* exposure to the androgen receptor antagonist flutamide (Figure 1) or linuron led to a reduction of anogenital distance (AGD) and the retention of nipples in male rat offspring. Changes in AGD are a marker of endocrine disruptions, which are also associated with malformations of androgen receptor-dependent organs and tissues, including the testis and epididymis [12-14]. Both pre- and post-natal exposure to flutamide and linuron reduced the weights of the seminal vesicle and *levator ani bulbocavernosus* (LABC) muscle in male rats [14,15]. In female rats, treatment with the androgen receptor agonist testosterone propionate *in utero* increased AGD at weaning and in adulthood, decreased the number of areolas and nipples, and manifested the presence of prostate tissue [16]. *In utero* treatment with the androgen antagonist vinclozolin and the estrogen agonist methoxychlor led to a testis phenotype in subsequent male F1 to F4 generations that included reduced spermatogenic capacity and infertility [17]. Based on the above findings, we hypothesize that endocrine disruption plays an important role in reproductive and developmental toxicity.

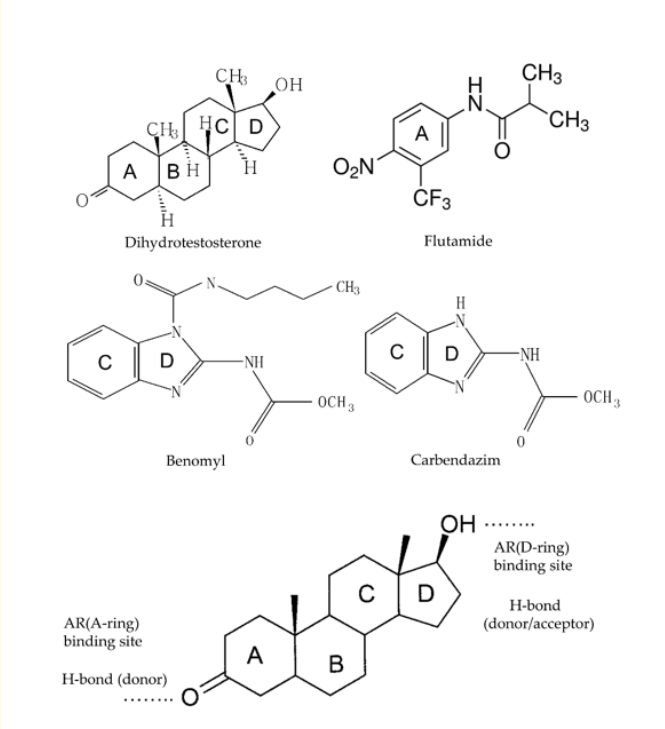


***Figure 1***

Previous research has revealed the possibility of endocrine-disrupting activity induced by carbendazim, though the findings are inconsistent. Subchronic treatment with carbendazim raised serum testosterone levels and the concentrations of androgen-binding proteins in interstitial and seminiferous tubule fluid, indicating androgenic activity in rats [1]. Male and female rats treated with carbendazim for 28 days before mating led to androgenic effects in female offspring, such as the absence of vagina and the presence of seminal vesicles [18]. Moreover, in the human ovarian granulose-like tumor cell line KGN, carbendazim and benomyl increased not only the mRNA levels

but also the activity of aromatase, suggesting that carbendazim and benomyl exhibit estrogenic activity *in vitro* [19]. In contrast, carbendazim did not exhibit agonistic activity but did exhibit antagonistic activity in human estrogen receptor α and androgen receptor reporter gene assays [20]. Our series of studies have shown that carbendazim could induce reproductive and developmental toxicity as well as endocrine-disrupting activity, primarily in parents and F1 offspring [18,21-25]. We first reported that flutamide not only could antagonize the effects induced by carbendazim, but it could also mitigate carbendazim-induced spinal and bulbar muscular atrophy in multiple generations of rats [25]. Therefore, the current literature necessitates a full investigation in order to elucidate the effects of reproductive and developmental toxicity as well as endocrine-disrupting activity induced by carbendazim in multiple generations of rats, especially developmental neurotoxicity, such as spinal and bulbar muscular atrophy.

## Aim of the Study

This study aimed to clarify whether exposure to carbendazim before mating and *in utero* could induce reproductive and developmental toxicity as well as endocrine-disrupting activity, including developmental neurotoxicity and androgen-associated endpoints. We hypothesized that in multiple generations of rats, carbendazim could induce androgenic endpoints and developmental neurotoxicity and that co-treatment with flutamide could be either antagonistic or synergistic. In this study, male and female rats were treated with carbendazim, flutamide, or in combination before mating and *in utero* in parents, F1, and F2 generations. Androgen-dependent reproductive and development endpoints as well as clinical observations, especially in developmental neurotoxicity, in parent, F1, F2, and F3 male and female rats were measured and recorded.

## Materials and Methods

### Animals

Animal use was approved by the Institutional Animal Care and Use Committee (IACUC) of the Taiwan Agricultural Chemicals and Toxic Substances Research Institute (20-TACTRI-IACUC-13). Six-week-old male (38) and female (38) Wistar rats were purchased from the Bio-LASCO (Taipei, Taiwan, ROC). Rats were housed in a specific pathogen-free animal facility in the Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Taichung, Taiwan. Animal rooms were maintained under a 12-hour-light and dark cycle, 23 ± 2°C, and 50 ± 10% relative humidity. Rats had access *ad libitum* to deionized water and rodent chow (LabDiet® 5001, PMI Nutrition International, LLC, B, MO, USA). Upon arrival, rats were quarantined for at least one week and released on the basis of adequate body weight and being free of clinical signs of disease or injury. The allocation of the rats to treatment groups was done by body weight randomization to ensure unbiased weight distribution across groups. Individual rats and offspring were housed in polycarbonate cages on Laboratory Animal Bedding (TCP Chipsi Heimtier Steu, Germany) until weaning postnatal day (PND) 21, upon which the test animals were group-housed up to 5 per cage by sex and treatment until necropsy or selection as a mating parent (F1) on PND 56. For parent rats (P) at approximately eight weeks of age, three to four male and female rats in each group were first treated for 28 days before mating; they were then mated within each treatment group for up to 14 days. Gestation day (GD) 0 was defined as the day that a vaginal plug or sperm was found in the vagina of mated females. Female rats with a plug continued the same treatment for a further 21 days, which represents the gestational period (GD 0 - GD 20). For F1 and F2 adult rats, at about eight weeks of age, three to four male and female rats in each group were mated within each treatment group for up to 14 days as parent rats were processed. Parent, F1, F2, and F3 rats were euthanized by Zoletil at 3 mg/kg bw/day and subjected to detailed postmortem examination.

### Treatment

Carbendazim was obtained from Sinon Co. (Taichung, Taiwan). All other chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise noted. Carbendazim or flutamide was suspended in corn oil and administered to rats orally one daily by gavage in a volume of 2.5 mL/kg bw/day. For parent rats, from 28 days before mating, three male and three to four female rats per dose were treated with carbendazim at 6.25, 12.5, 25, and 50 mg/kg bw/day, flutamide at 2.5 mg/kg bw/day, or in combination at the same doses. After

treatment for 28 days, male and female rats were mated within each treatment group. The female rats with a vaginal plug continued the same treatment for a further 21 days (representing gestational period). For F1 adult rats, due to the embryolethality induced by carbendazim, treatment doses were reduced as follows: carbendazim at 1.56, 3.13, 6.25, and 12.5 mg/kg bw/day; flutamide remained at 2.5 mg/kg bw/day; and in combination: flutamide remained at 2.5 mg/kg bw/day and carbendazim at 6.25 and 12.5 mg/kg bw/day. Among the treatments, the dose for F1 offspring of parents who had received carbendazim at 6.25 mg/kg bw/day was subsequently treated with carbendazim at 1.56 and 3.3 mg/kg bw/day, and those whose parents had received carbendazim + flutamide respectively at 25 + 2.5 mg/kg bw/day were subsequently treated with carbendazim + flutamide respectively at 12.5 + 2.5 mg/kg bw/day. All other treatments for F1 rats before mating and *in utero* were the same as their parent rats. For F2 adult rats, in order to investigate endocrine-disrupting activity, the doses were further reduced as follows: carbendazim to 0.39, 0.78, 1.56, 3.13, and 6.25 mg/kg bw/day; flutamide remained at 2.5 mg/kg bw/day; and in combination: flutamide remained at 2.5 mg/kg bw/day and carbendazim to 0.78, 1.56, and 3.13 mg/kg bw/day. Among the treatments for F2 male rats, those whose parents had received carbendazim at 1.56 mg/kg bw/day were subsequently treated with carbendazim at 0.39 mg/kg bw/day, and those whose parents had received carbendazim + flutamide respectively at 6.25 + 2.5 mg/kg bw/day were subsequently treated with carbendazim + flutamide respectively at 0.78 + 2.5 mg/kg bw/day. Male and female rats were examined daily for clinical signs of toxicity. Body weight and food consumption were also monitored daily throughout the treatment period. Offspring were weaned at PND 21 and fed with rodent chow for up to 8 weeks old.

### Androgen-dependent reproductive development endpoints

Androgen-dependent reproductive endpoints included signs of clinical toxicity, AGD, male and female pup weight, retention of areolae and/or nipples, malformations of external genitalia, testicular descent, preputial separation (PPS), vaginal opening (VO), as well as organ weight and malformations on PND 56. The number of pups in each litter was counted and examined for signs of clinical toxicity on PND 0. On PND 21, pups were tail-marked for identification and subsequent evaluations. AGD (at PND 2, 21, 33, and 44) as well as the body weights (at PND 1, 7, 14, 21, 33, and 56) of live male and female offspring were measured. The age of completion of PPS in male offspring during PND 40 and 50 as well as the age of onset of VO in female offspring during PND 30 and 45 were determined. Gross morphology of reproductive organs, nipple retention, abnormal testis and epididymis, hypospadias, underdevelopment of prostate or/and seminal vesicle, absence of prostate or/and seminal vesicle, presence of bladder stone, and underdevelopment of LABC muscle in male offspring were recorded on PND 56.

### Clinical observations

A previous finding reported that benomyl and carbendazim could induce disruptions to androgen receptors that led to spinal and bulbar muscular atrophy in multiple generations of rats [24]. Thus, we recorded videos of abnormality during the period of clinical observations, in which all rats were observed for clinical signs of toxicity related to chemical treatment at least once daily.

### Necropsy of parent, F1, F2, and F3 rats

For parent, F1, F2, and F3 adult rats, male and female rats on F1 PND 170, PND 140, PND 170 and PND 200 were sacrificed, respectively. Male and female rats were anesthetized with Zoletil® 3 mg/kg bw/day, and trunk blood was subsequently collected. Following blood collection, the ventral surface of the test subject was shaved to count the number of nipples. The external genitalia, including the prepuce and penis in male offspring and the vagina in female offspring, were visually inspected. Gross internal examination of the reproductive tract included inspection of the testes, epididymides, prostate, seminal vesicles, LABC muscle, scrotum, and penis in male rats and the uterus and ovary in female rats. The brain, pituitary gland, liver, kidneys, adrenal glands in male and female rats were grossly examined and weighted. Body and organ weights were recorded, which included testes, epididymides, prostate, seminal vesicles, LABC muscle, scrotum, penis, uterus and ovary, brain, pituitary gland, liver, kidneys, and adrenals. Tissues were fixed in 10% neutral buffered formalin and then processed, sectioned, and stained with hematoxylin and eosin.

### Statistical analysis

All data are expressed as mean ± SD. Data were subjected to analysis of variance (ANOVA) followed by the Student's *t*-test with the level of significance set at $p < 0.05$.

## Results and Discussion

### Effects of carbendazim, flutamide, or combination treatment before mating in males and females as well as during gestation period in females on adult body weights of parent, F1, F2, and F3

Treatment with carbendazim, flutamide, or in combination resulted in no significant effects on the body weight of female and male adult parents, F1, F2, and F3 rats on days 1, 8, 15, 22, or 28 before mating (Table 1 and 2). Similarly, the three treatments during gestational period also had no significant effects on the body weight of female parents, F1, and F2 rats on day 0, 5, 10, 15, and 20 (Table 3). Note that there was no significant difference between control and all treatment groups in terms of food consumption (Data not shown).

| Treatments | Dose (mg/kg bw/day) | No. female | 28-day treatement[1)] | | | | |
|---|---|---|---|---|---|---|---|
| **P[2)]** | | | **1** | **8** | **15** | **22** | **28** |
| Control | | 3 | 209.6 ± 5.2 | 225.2 ± 14.3 | 232.0 ± 15.7 | 243.5 ± 17.0 | 251.6 ± 16.5 |
| Carbendazim | 6.25 | 4 | 213.7 ± 6.2 | 232.5 ± 8.9 | 246.7 ± 18.6 | 260.9 ± 32.4 | 265.6 ± 28.8 |
| | 12.5 | 4 | 195.7 ± 16.7 | 210.4 ± 19.2 | 213.2 ± 36.5 | 233.8 ± 26.0 | 238.5 ± 23.6 |
| | 25 | 4 | 215.7 ± 8.7 | 232.6 ± 9.3 | 243.3 ± 5.5 | 254.4 ± 8.6 | 264.7 ± 11.9 |
| | 50 | 4 | 221.4 ± 20.5 | 238.9 ± 30.0 | 247.4 ± 27.0 | 261.6 ± 28.8 | 267.1 ± 33.8 |
| Flutamide | 2.5 | 3 | 207.6 ± 11.0 | 231.4 ± 17.0 | 239.0 ± 33.7 | 259.9 ± 30.9 | 271.7 ± 33.2 |
| Flutamide + Carbendazim | 2.5+6.25 | 4 | 210.7 ± 17.2 | 229.6 ± 18.9 | 247.1 ± 25.9 | 259.7 ± 28.1 | 266.7 ± 25.4 |
| | 2.5+12.5 | 4 | 211.8 ± 14.6 | 225.9 ± 18.4 | 234.6 ± 21.7 | 241.6 ± 16.0 | 254.9 ± 14.9 |
| | 2.5+25 | 4 | 207.9 ± 12.4 | 224.6 ± 17.2 | 243.1 ± 13.6 | 252.5 ± 17.8 | 262.7 ± 20.0 |
| | 2.5+50 | 4 | 206.3 ± 15.5 | 225.0 ± 23.1 | 237.2 ± 22.6 | 249.8 ± 24.2 | 257.4 ± 27.7 |
| **F1[3)]** | | | | | | | |
| Control | | 3 | 222.1 ± 16.6 | 233.5 ± 15.9 | 239.9 ± 20.8 | 243.2 ± 24.2 | 254.9 ± 16.6 |
| Carbendazim | 1.56 | 4 | 228.2 ± 5.2 | 240.8 ± 15.6 | 257.0 ± 9.9 | 262.2 ± 17.1 | 264.8 ± 12.5 |
| | 3.13 | 4 | 221.5 ± 14.7 | 241.0 ± 15.5 | 256.5 ± 21.0 | 265.0 ± 22.1 | 266.7 ± 20.0 |
| | 6.25 | 4 | 219.5 ± 14.5 | 240.4 ± 11.7 | 252.8 ± 20.5 | 259.5 ± 18.8 | 266.2 ± 18.7 |
| | 12.5 | 3 | 238.4 ± 13.3 | 256.3 ± 13.5 | 269.8 ± 17.3 | 276.8 ± 17.0 | 285.5 ± 20.8 |
| Flutamide | 2.5 | 3 | 247.3 ± 24.2 | 262.3 ± 30.2 | 267.1 ± 25.2 | 280.7 ± 35.7 | 282.6 ± 31.9 |
| Flutamide + Carbendazim | 2.5+6.25 | 3 | 235.3 ± 26.5 | 250.0 ± 28.5 | 258.2 ± 33.1 | 272.5 ± 40.1 | 275.7 ± 34.8 |
| | 2.5+12.5 | 4 | 225.5 ± 14.9 | 237.4 ± 13.4 | 252.1 ± 17.1 | 257.3 ± 19.1 | 258.0 ± 13.8 |
| **F2[4)]** | | | | | | | |
| Control | | 3 | 241.2 ± 16.1 | 246.0 ± 19.1 | 259.3 ± 17.5 | 264.5 ± 27.3 | 267.4 ± 23.2 |
| Carbendazim | 0.39 | 3 | 270.3 ± 12.6 | 265.5 ± 13.2 | 279.6 ± 11.7 | 282.9 ± 12.8 | 282.4 ± 4.2 |
| | 0.78 | 3 | 268.7 ± 9.0 | 272.5 ± 19.1 | 279.1 ± 22.3 | 281.9 ± 25.3 | 290.2 ± 25.2 |
| | 1.56 | 3 | 254.1 ± 15.5 | 258.2 ± 22.2 | 272.0 ± 21.7 | 274.7 ± 23.6 | 276.4 ± 27.6 |
| | 3.13 | 2 | 236.9 ± 17.4 | 234.2 ± 16.3 | 251.9 ± 19.4 | 255.3 ± 18.2 | 257.0 ± 11.2 |
| | 6.25 | 4 | 259.1 ± 20.3 | 267.5 ± 21.2 | 279.8 ± 18.2 | 284.3 ± 18.1 | 295.5 ± 29.0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Flutamide | 2.5 | 3 | 260.7 ± 21.5 | 268.2 ± 23.8 | 278.4 ± 27.7 | 289.3 ± 24.4 | 295.0 ± 25.2 |
| Flutamide + Carbendazim | 2.5+0.78 | 2 | 252.5 ± 35.1 | 258.1 ± 33.4 | 270.5 ± 35.6 | 267.8 ± 31.0 | 269.3 ± 27.7 |
| | 2.5+1.56 | 2 | 265.2 ± 45.5 | 272.8 ± 48.6 | 284.8 ± 48.7 | 283.4 ± 43.7 | 293.6 ± 51.4 |
| | 2.5+3.13 | 2 | 279.9 ± 9.3 | 282.4 ± 16.5 | 290.9 ± 24.6 | 288.9 ± 6.6 | 299.8 ± 18.8 |

***Table 1:*** *Effects of exposure to carbendazim, flutamide, and in combination for 28 days on body weight in multiple generations of female Wistar rats.*

[1)] *NS: Not significant compared to control rats.*

[2)] *P: Parent male and female rats.*

[3)] *F1: First generation of rats.*

[4)] *F2: Second generation of rats.*

| **Treatments (mg/kg bw/day)** | **Dose (mg/kg bw/day)** | **No. male** | **28-day treatment[1)]** | | | | |
|---|---|---|---|---|---|---|---|
| **P[2)]** | | | **1** | **8** | **15** | **22** | **28** |
| Control | | 3 | 307.0 ± 20.5 | 368.1 ± 15.3 | 402.1 ± 17.9 | 426.8 ± 15.7 | 447.0 ± 15.6 |
| Carbendazim | 6.25 | 4 | 297.9 ± 12.9 | 364.3 ± 6.7 | 402.9 ± 6.1 | 432.8 ± 3.9 | 445.4 ± 1.6 |
| | 12.5 | 4 | 311.6 ± 9.8 | 378.1 ± 7.1 | 409.7 ± 13.9 | 442.2 ± 8.0 | 461.5 ± 7.5 |
| | 25 | 4 | 308.7 ± 7.4 | 374.6 ± 4.5 | 406.6 ± 5.0 | 433.7 ± 3.7 | 451.3 ± 2.6 |
| | 50 | 4 | 306.7 ± 4.6 | 371.6 ± 10.4 | 402.1 ± 19.0 | 430.2 ± 19.6 | 444.6 ± 26.1 |
| Flutamide | 2.5 | 3 | 307.0 ± 12.7 | 369.1 ± 17.1 | 398.0 ± 18.9 | 429.8 ± 27.1 | 446.2 ± 30.4 |
| Flutamide + Carbendazim | 2.5+6.25 | 4 | 300.0 ± 21.9 | 359.8 ± 23.2 | 387.2 ± 21.8 | 415.5 ± 27.8 | 430.0 ± 24.8 |
| | 2.5+12.5 | 4 | 320.5 ± 10.0 | 382.5 ± 11.5 | 415.6 ± 13.6 | 440.1 ± 8.1 | 464.4 ± 8.8 |
| | 2.5+25 | 4 | 308.8 ± 18.6 | 360.2 ± 25.7 | 391.9 ± 35.8 | 420.1 ± 36.1 | 437.6 ± 36.3 |
| | 2.5+50 | 4 | 312.8 ± 10.4 | 373.4 ± 8.2 | 405.9 ± 9.1 | 429.2 ± 8.7 | 446.2 ± 8.7 |
| **F1[3)]** | | | | | | | |
| Control | | 3 | 355.0 ± 37.3 | 384.1 ± 33.3 | 410.8 ± 31.5 | 429.5 ± 26.4 | 447.8 ± 34.7 |
| Carbendazim | 1.56 | 4 | 338.4 ± 43.3 | 378.3 ± 43.4 | 403.1 ± 42.2 | 424.5 ± 45.5 | 442.1 ± 46.6 |
| | 3.13 | 4 | 340.5 ± 21.3 | 375.7 ± 32.2 | 401.1 ± 34.3 | 420.2 ± 41.8 | 435.1 ± 47.7 |
| | 6.25 | 4 | 352.5 ± 18.5 | 393.1 ± 26.4 | 422.5 ± 28.1 | 448.8 ± 33.2 | 468.1 ± 39.6 |
| | 12.5 | 3 | 346.0 ± 34.7 | 388.0 ± 40.9 | 416.2 ± 41.6 | 428.9 ± 47.7 | 446.6 ± 49.1 |
| Flutamide | 2.5 | 3 | 361.7 ± 21.4 | 390.7 ± 23.6 | 418.1 ± 18.5 | 438.1 ± 20.8 | 453.6 ± 23.3 |
| Flutamide + Carbendazim | 2.5+6.25 | 3 | 360.4 ± 7.1 | 397.1 ± 19.5 | 420.0 ± 33.9 | 441.4 ± 37.7 | 462.8 ± 45.5 |
| | 2.5+12.5 | 4 | 339.3 ± 36.5 | 367.0 ± 37.3 | 391.2 ± 42.9 | 406.9 ± 47.7 | 418.1 ± 47.7 |
| **F2[4)]** | | | | | | | |
| Control | | 3 | 422.9 ± 39.3 | 443.5 ± 47.6 | 464.5 ± 50.2 | 481.9 ± 50.4 | 498.7 ± 51.4 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Carbendazim | 0.39 | 3 | 425.2 ± 35.0 | 449.1 ± 28.5 | 473.7 ± 31.8 | 489.1 ± 30.1 | 501.4 ± 24.7 |
| | 0.78 | 3 | 404.8 ± 60.3 | 430.9 ± 55.1 | 456.2 ± 49.4 | 480.9 ± 44.8 | 493.6 ± 49.7 |
| | 1.56 | 3 | 443.3 ± 9.4 | 461.8 ± 14.0 | 486.1 ± 14.4 | 505.6 ± 14.7 | 517.1 ± 11.9 |
| | 3.13 | 2 | 429.9 ± 12.4 | 466.0 ± 15.6 | 488.7 ± 19.8 | 513.6 ± 18.2 | 529.7 ± 13.4 |
| | 6.25 | 4 | 435.1 ± 27.0 | 455.7 ± 34.1 | 478.9 ± 37.4 | 492.4 ± 34.7 | 508.1 ± 34.3 |
| Flutamide | 2.5 | 3 | 432.8 ± 8.3 | 444.5 ± 9.2 | 469.9 ± 11.2 | 484.8 ± 15.6 | 499.6 ± 21.1 |
| Flutamide + Carbendazim | 2.5+0.78 | 2 | 468.6 ± 50.3 | 488.5 ± 59.1 | 507.6 ± 61.8 | 522.3 ± 58.5 | 531.8 ± 66.1 |
| | 2.5+1.56 | 2 | 428.0 ± 20.5 | 440.6 ± 25.2 | 460.1 ± 20.9 | 465.1 ± 20.2 | 479.6 ± 34.5 |
| | 2.5+3.13 | 2 | 415.3 ± 23.1 | 431.3 ± 28.9 | 445.9 ± 28.9 | 451.9 ± 34.2 | 458.8 ± 22.9 |

***Table 2:*** *Effects of exposure to carbendazim, flutamide, and in combination for 28 days on body weight in multiple generations of male Wistar rats.*

*[1)]NS: Not significant compared to control rats.*

*[2)]P: Parent male and female rats.*

*[3)]F1: First generation of rats.*

*[4)]F2: Second generation of rats.*

| Treatments (mg/kg bw/day) | Dose (mg/kg bw/day) | No. of litters | Day of pregnancy | | | | |
|---|---|---|---|---|---|---|---|
| **P** | | | **0** | **5** | **10** | **15** | **20** |
| Control | | 3 | 253.3 ± 16.0 | 280.5 ± 11.1 | 303.7 ± 11.1 | 332.6 ± 11.9 | 407.2 ± 13.9 |
| Carbendazim | 6.25 | 4 | 278.6 ± 27.7 | 302.7 ± 33.0 | 324.0 ± 33.3 | 352.1 ± 25.8 | 419.2 ± 53.4 |
| | 12.5 | 4 | 244.1 ± 24.0 | 266.6 ± 23.9 | 288.7 ± 26.3 | 316.2 ± 37.2 | 384.0 ± 70.4 |
| | 25 | 4 | 275.2 ± 9.9 | 291.9 ± 8.6 | 315.1 ± 11.6 | 332.8 ± 11.7 | 354.2 ± 20.6* |
| | 50 | 4 | 277.1 ± 27.5 | 296.5 ± 35.2 | 311.5 ± 41.9 | 310.8 ± 45.6 | 313.8 ± 42.9* |
| Flutamide | 2.5 | 3 | 278.7 ± 35.2 | 297.7 ± 41.0 | 317.7 ± 46.1 | 346.5 ± 44.1 | 418.1 ± 43.8 |
| Flutamide + Carbendazim | 2.5+6.25 | 4 | 273.8 ± 25.4 | 299.6 ± 34.1 | 318.1 ± 35.7 | 348.5 ± 36.6 | 432.1 ± 39.5 |
| | 2.5+12.5 | 4 | 265.8 ± 17.5 | 284.9 ± 13.3 | 305.8 ± 12.2 | 333.4 ± 14.3 | 402.0 ± 28.0 |
| | 2.5+25 | 4 | 269.6 ± 16.6 | 290.4 ± 18.2 | 304.5 ± 19.2 | 327.3 ± 20.5 | 356.9 ± 15.9** |
| | 2.5+50 | 4 | 264.3 ± 30.6 | 282.4 ± 33.1 | 296.8 ± 39.4 | 321.1 ± 42.0 | 390.2 ± 54.3 |
| **F1** | | | | | | | |
| Control | | 3 | 259.7 ± 19.5 | 282.2 ± 15.3 | 306.9 ± 18.4 | 340.5 ± 14.6 | 422.4 ± 11.4 |
| Carbendazim | 1.56 | 4 | 269.7 ± 20.9 | 291.3 ± 16.3 | 314.2 ± 15.5 | 335.4 ± 35.2 | 393.2 ± 82.5 |
| | 3.13 | 4 | 285.3 ± 29.4 | 314.1 ± 32.2 | 331.1 ± 31.5 | 336.6 ± 54.8 | 379.2 ± 105.6 |
| | 6.25 | 4 | 277.1 ± 36.4 | 301.1 ± 31.9 | 325.6 ± 34.2 | 358.1 ± 35.9 | 443.0 ± 34.0 |
| | 12.5 | 3 | 290.1 ± 19.0 | 308.2 ± 21.4 | 331.8 ± 17.5 | 364.5 ± 18.3 | 443.0 ± 22.7 |
| Flutamide | 2.5 | 3 | 294.7 ± 30.2 | 320.1 ± 28.5 | 342.0 ± 23.9 | 367.9 ± 25.4 | 443.5 ± 26.5 |
| Flutamide + Carbendazim | 2.5+6.25 | 3 | 296.8 ± 36.1 | 318.7 ± 41.2 | 343.6 ± 50.4 | 374.5 ± 46.7 | 471.1 ± 60.0 |
| | 2.5+12.5 | 4 | 274.9 ± 27.4 | 297.0 ± 32.2 | 314.7 ± 27.6 | 321.0 ± 23.3 | 354.2 ± 54.3 |

| F2 | | | | | | | |
|---|---|---|---|---|---|---|---|
| Control | | 3 | 273.0 ± 23.6 | 301.0 ± 16.2 | 314.7 ± 11.7 | 343.2 ± 15.0 | 419.9 ± 12.3 |
| Carbendazim | 0.39 | 3 | 296.8 ± 9.0 | 315.8 ± 13.7 | 341.1 ± 15.5 | 371.1 ± 24.3 | 465.5 ± 31.6 |
| | 0.78 | 3 | 314.5 ± 43.6 | 325.1 ± 33.2 | 346.0 ± 40.4 | 375.5 ± 35.3 | 463.6 ± 56.2 |
| | 1.56 | 3 | 286.9 ± 19.0 | 311.3 ± 21.3 | 325.5 ± 24.2 | 359.5 ± 25.7 | 440.2 ± 35.2 |
| | 3.13 | 2 | 272.8 ± 8.4 | 298.4 ± 9.0 | 333.1 ± 8.2 | 366.6 ± 8.7 | 446.6 ± 9.5 |
| | 6.25 | 4 | 302.4 ± 20.3 | 322.5 ± 22.0 | 345.7 ± 23.9 | 373.7 ± 21.3 | 453.4 ± 25.1 |
| Flutamide | 2.5 | 3 | 295.6 ± 34.5 | 315.5 ± 29.0 | 330.5 ± 34.6 | 335.1 ± 53.7 | 367.5 ± 103.1 |
| Flutamide + Carbendazim | 2.5+0.78 | 2 | 273.6 ± 17.2 | 295.1 ± 29.4 | 316.9 ± 32.4 | 336.0 ± 25.0 | 412.7 ± 0.9 |
| | 2.5+1.56 | 2 | 294.2 ± 62.0 | 320.4 ± 60.6 | 336.5 ± 61.9 | 322.9 ± 57.7 | 323.0 ± 58.1 |
| | 2.5+3.13 | 2 | 308.0 ± 16.9 | 335.0 ± 29.8 | 353.8 ± 47.7 | 369.9 ± 77.1 | 411.5 ± 144.2 |

***Table 3:*** *Effects of in utero exposure to carbendazim, flutamide, and in combination on body weight in multiple generations of female Wistar rats.*

*[1)]p-value = $^{*} \leq 0.05$, $^{**} \leq 0.01$, $^{***} \leq 0.005$; vs. control.*

*[2)]p-value = $^{a} \leq 0.05$ vs. carbendazim.*

*[3)]p-value = $^{b} \leq 0.05$ vs. flutamide.*

### Effects of carbendazim, flutamide, or in combination on AGD

For F1 female offspring, treatment with carbendazim at 6.25 mg/kg bw/day but not at 12.5 mg/kg bw/day significantly extended AGD on PND 2 compared to control. Treatment with carbendazim at 25 and 50 mg/kg bw/day resulted in embryolethality, in which no F1 female offspring were born. There were no effects on AGD in F1 offspring following treatment with flutamide alone. Co-treatment with flutamide plus carbendazim at 6.25, 12.5, 25, or 50 mg/kg bw/day resulted in significantly extended AGD on PND 2 compared to controls; a similar finding was observed following co-treatment with flutamide and carbendazim at 12.5 mg/kg bw/day on PND 33. In contrast, co-treatment with flutamide and carbendazim at 6.25 and 12.5 mg/kg bw/day significantly shortened AGD on PND 21 compared to controls. Compared to flutamide treatment alone, shortened AGD was observed on PND 2 and 21 following co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day and on PND 2 with flutamide and carbendazim at 12.5 mg/kg bw/day on PND 2; however, extended AGD was identified on PND 33 with flutamide and carbendazim at 12.5 mg/kg bw/day co-treatment. Compared to carbendazim treatment alone, shortened AGD was found on PND 21 and 44 following co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day but extended AGD was found on PND 33 with flutamide and carbendazim at 12.5 mg/kg bw/day co-treatment.

For F2 female offspring, compared to controls, significantly shortened AGD was identified on PND 21 and 33 following treatment with carbendazim at 1.56 mg/kg bw/day, on PND 33 with carbendazim at 3.13 mg/kg bw/day, and on PND 21 with carbendazim at 12.5 mg/kg bw/day; however, extended AGD was respectively identified on PND 44 and PND 2 following treatment with carbendazim at 1.56 and 12.5 mg/kg bw/day. Compared to controls, flutamide treatment significantly extended AGD on PND 2 and 44 but shortened AGD on PND 21, and co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day extended AGD on PND 33, but co-treatment with flutamide and carbendazim at 12.5 mg/kg bw/day shortened AGD on PND 21. Compared to flutamide alone, co-treatment with flutamide and carbendazim at 6.25 and 12.5 mg/kg bw/day significantly shortened AGD on PND 2 and 44, respectively; however, co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day significantly extended AGD on PND 21.

For F3 female offspring, treatment with carbendazim at 0.39 mg/kg bw/day significantly shortened AGD on PND 21, compared with controls. Similarly, significantly shortened AGD was also found on PND 33 following treatment with carbendazim 0.78 and 1.56 mg/kg bw/day as well as on PND 44 and PND 33 with carbendazim treatment respectively at 3.13 and 6.25 mg/kg bw/day. On the contrary, compared to controls, treatment with carbendazim at 3.13 mg/kg bw/day significantly extended AGD on PND 2 but significantly shortened AGD on PND 44; and treatment with carbendazim at 6.25 mg/kg bw/day extended AGD on PND 2 but shortened AGD on PND 33 and 44. Compared to controls, flutamide significantly shortened AGD on PND 44. Co-treatment with flutamide and carbendazim at 1.56 mg/kg bw/day did not result in any pregnancy; thus, no female offspring were born from this group (Table 4).

| Treatments (mg/kg bw/day) | Dose (mg/kg bw/day) | No. of litters | PND | | | |
|---|---|---|---|---|---|---|
| **F1** | | | **2** | **21** | **33** | **44** |
| Control | | 3 | 1.18 ± 0.38 | 8.92 ± 2.72 | 11.87 ± 0.96 | 14.77 ± 1.67 |
| Carbendazim | 6.25 | 4 | 1.42 ± 0.18$^{*}$ | 9.17 ± 1.45 | 11.54 ± 0.79 | 15.09 ± 1.34 |
| | 12.5 | 4 | 1.15 ± 0.31 | 8.55 ± 2.37 | 11.43 ± 2.16 | 14.42 ± 1.73 |
| | 25 | 4 | -$^{c}$ | -$^{c}$ | -$^{c}$ | -$^{c}$ |
| | 50 | 4 | -$^{c}$ | -$^{c}$ | -$^{c}$ | -$^{c}$ |
| Flutamide | 2.5 | 3 | 1.71 ± 0.34 | 9.65 ± 1.64 | 11.78 ± 0.94 | 14.51 ± 1.04 |
| Flutamide + Carbendazim | 2.5+6.25 | 4 | 1.43 ± 0.34$^{*a}$ | 7.23 ± 0.86$^{*ab}$ | 11.49 ± 2.13 | 14.25 ± 1.05$^{b}$ |
| | 2.5+12.5 | 4 | 1.64 ± 0.42$^{*a}$ | 7.88 ± 1.57$^{*}$ | 12.62 ± 1.30$^{*ab}$ | 14.26 ± 1.47 |
| | 2.5+25 | 4 | 1.52 ± 0.58$^{*}$ | 10.01 ± 3.23 | 12.07 ± 1.26 | 14.65 ± 0.80 |
| | 2.5+50 | 4 | 1.52 ± 0.13$^{*}$ | 10.17 ± 0.80 | 12.37 ± 1.29 | 13.91 ± 0.94 |
| **F2** | | | | | | |
| Control | | 3 | 1.43 ± 0.24 | 9.28 ± 1.00 | 14.01 ± 2.60 | 14.34 ± 1.49 |
| Carbendazim | 1.56 | 4 | 1.69 ± 0.60 | 7.93 ± 1.43$^{*}$ | 12.62 ± 2.21$^{*}$ | 16.75 ± 2.41$^{*}$ |
| | 3.13 | 4 | 1.50 ± 0.18 | 8.82 ± 0.58 | 11.93 ± 1.01$^{*}$ | 14.06 ± 1.03 |
| | 6.25 | 4 | 1.36 ± 0.16 | 8.95 ± 1.01 | 13.64 ± 1.48 | 14.94 ± 1.20 |
| | 12.5 | 3 | 1.75 ± 0.66$^{*}$ | 8.03 ± 0.75$^{*}$ | 14.74 ± 0.95 | 15.32 ± 2.45 |
| Flutamide | 2.5 | 3 | 2.31 ± 0.85$^{*}$ | 8.07 ± 0.80$^{*}$ | 14.87 ± 1.53 | 17.86 ± 3.47$^{*}$ |
| Flutamide + Carbendazim | 2.5+6.25 | 3 | 1.33 ± 0.36$^{a}$ | 9.97 ± 3.07$^{a}$ | 15.80 ± 0.51$^{*}$ | 14.65 ± 0.90$^{a}$ |
| | 2.5+12.5 | 4 | 1.53 ± 0.16$^{a}$ | 8.23 ± 1.27$^{*}$ | 15.14 ± 0.99 | 13.59 ± 0.73$^{a}$ |
| **F3** | | | | | | |
| Control | | 3 | 1.57 ± 0.25 | 8.63 ± 1.00 | 17.96 ± 4.11 | 18.99 ± 2.19 |
| Carbendazim | 0.39 | 3 | 1.50 ± 0.27 | 7.52 ± 1.54$^{*}$ | 16.60 ± 4.12 | 17.08 ± 4.06 |
| | 0.78 | 3 | 1.70 ± 0.18 | 7.96 ± 1.45 | 15.47 ± 1.19$^{*}$ | 18.51 ± 1.40 |
| | 1.56 | 3 | 1.95 ± 0.56$^{*}$ | 8.80 ± 0.75 | 14.36 ± 4.49$^{*}$ | 16.80 ± 5.28 |
| | 3.13 | 2 | 2.44 ± 0.16$^{*}$ | 9.29 ± 0.60 | 14.20 ± 5.37 | 15.05 ± 5.75$^{*}$ |
| | 6.25 | 4 | 2.03 ± 0.63$^{*}$ | 8.16 ± 1.38 | 15.01 ± 1.55$^{*}$ | 17.39 ± 1.26$^{*}$ |
| Flutamide | 2.5 | 3 | 1.67 ± 0.30 | 8.15 ± 1.14 | 14.63 ± 0.92$^{*}$ | 17.00 ± 1.03$^{*}$ |

| | | | | | | |
|---|---|---|---|---|---|---|
| Flutamide + Carbendazim | 2.5+0.78 | 2 | 1.74 ± 0.24 | 8.87 ± 0.96 | 15.31 ± 1.61 | 18.46 ± 0.31[a] |
| | 2.5+1.56 | 2 | -[d] | -[d] | -[d] | -[d] |
| | 2.5+3.13 | 2 | 2.88 ± 0.28 | 9.93 ± 0.67 | 15.28 ± 1.33 | 17.70 ± 1.76 |

***Table 4:*** *Effects of in utero exposure to carbendazim, flutamide, and in combination on AGD of female offspring in multiple generations of Wistar rats.*

*[1)]p-value = [*] ≤ 0.05, [**] ≤ 0.01, [***] ≤ 0.005; vs. control.*

*[2)]p-value = [a] ≤ 0.05 vs. flutamide.*

*[3)]p-value = [b] ≤ 0.05 vs. carbendazim.*

*[c]Embryolethality.*

*[d]No pregnancy.*

For F1 male offspring, compared to controls, treatment with carbendazim at 6.25 mg/kg bw/day significantly extended AGD on PND 2 and 44, and treatment at 12.5 mg/kg bw/day also significantly extended AGD on PND 44; however, flutamide alone shortened AGD on PND 21. Treatment with carbendazim at 25 and 50 mg/kg bw/day resulted in embryolethality; thus, no F1 male offspring were born from these groups. Compared to controls, co-treatment with flutamide and carbendazim at 6.25, 12.5, and 25 mg/kg bw/day respectively shortened AGD on PND 21, 33, and 2. Compared to flutamide alone, co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day shortened AGD on PND 21 and 33. Compared to carbendazim at the same doses, co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day shortened AGD on PND 21, 33, and 44, and co-treatment with flutamide and carbendazim at 12.5 mg/kg bw/day shortened AGD on PND 33 and 44.

For F2 male offspring, compared to controls, treatment with carbendazim at 1.56 mg/kg bw/day shortened AGD on PND 21 and 33, and treatment with carbendazim at 3.13 mg/kg bw/day shortened AGD on PND 2; however, extended AGD was found on PND 2 with carbendazim treatment at 12.5 mg/kg bw/day. Compared to controls, flutamide alone shortened AGD on PND 21; shortened AGD was also found on PND 2, 21, 33, and 44 following co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day and on PND 2 and 21 following co-treatment with flutamide and carbendazim at 12.5 mg/kg bw/day. Compared to carbendazim alone at the same doses, shortened AGD was observed on PND 2, 21, and 44 following co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day as well as on PND 2 and 33 following co-treatment with flutamide and carbendazim at 12.5 mg/kg bw/day.

For F3 male offspring, compared to controls, treatment with carbendazim at 3.13 and 6.25 mg/kg bw/day extended AGD on PND 2; however, treatment with carbendazim at 3.13 and 6.25 mg/kg bw/day shortened AGD on PND 33 and 44. Similarly, flutamide alone shortened AGD on PND 2, 21, 33 and 44, and co-treatment with flutamide and carbendazim at 0.78 mg/kg bw/day shortened AGD on PND 33. In contrast, compared to flutamide alone, co-treatment with flutamide and carbendazim at 0.78 mg/kg bw/day extended AGD on PND 2, 21, 33, and 44. Compared to carbendazim alone at the same doses, co-treatment with flutamide and carbendazim at 0.78 mg/kg bw/day shortened AGD on PND 33. Note that co-treatment with flutamide and carbendazim at 1.56 mg/kg bw/day did not result in any pregnant females; therefore, no male offspring were born (Table 5).

### Effects of carbendazim, flutamide, and in combination on weight of offspring

For F1 female offspring, compared to controls, pup weight was reduced on PND 2, 7, 14, 21, 33, and 56 following treatment with carbendazim at 6.25 mg/kg bw/day as well as on PND 7, 14, and 21 with carbendazim at 12.5 mg/kg bw/day; however, flutamide alone

| Treatments (mg/kg bw/day) | Dose (mg/kg bw/day) | No. of litters | PND | | | |
|---|---|---|---|---|---|---|
| **F1** | | | **2** | **21** | **33** | **44** |
| Control | | 3 | 2.64 ± 0.40 | 15.72 ± 4.06 | 26.10 ± 4.05 | 30.22 ± 4.03 |
| Carbendazim | 6.25 | 4 | 2.90 ± 0.35* | 14.56 ± 1.64 | 26.50 ± 1.28 | 33.19 ± 3.59* |
| | 12.5 | 4 | 2.78 ± 0.49 | 13.27 ± 2.74 | 26.28 ± 2.28 | 33.96 ± 2.86* |
| | 25 | 4 | - | - | - | - |
| | 50 | 4 | - | - | - | - |
| Flutamide | 2.5 | 3 | 2.98 ± 0.85 | 13.84 ± 2.06* | 24.95 ± 1.93 | 29.91 ± 4.89 |
| Flutamide + Carbendazim | 2.5+6.25 | 4 | 2.65 ± 0.62 | 11.20 ± 1.04*ab | 23.11 ± 2.13*ab | 29.47 ± 2.51b |
| | 2.5+12.5 | 4 | 2.73 ± 0.44 | 12.40 ± 2.39* | 24.45 ± 2.11b | 29.64 ± 2.59b |
| | 2.5+25 | 4 | 2.46 ± 0.42a | 14.91 ± 2.41 | 24.88 ± 2.69 | 30.11 ± 3.34 |
| | 2.5+50 | 4 | 2.46 ± 0.20 | 13.59 ± 0.51 | 24.36 ± 1.46 | 25.14 ± 2.76 |
| **F2** | | | | | | |
| Control | | 3 | 3.19 ± 0.33 | 14.52 ± 1.31 | 27.41 ± 1.90 | 34.55 ± 5.05 |
| Carbendazim | 1.56 | 4 | 3.45 ± 0.64 | 12.99 ± 1.89* | 25.16 ± 3.37* | 35.22 ± 3.05 |
| | 3.13 | 4 | 2.83 ± 0.38* | 14.36 ± 0.83 | 27.65 ± 2.58 | 36.45 ± 1.49 |
| | 6.25 | 4 | 3.11 ± 0.34 | 14.27 ± 1.29 | 27.66 ± 2.13 | 35.56 ± 3.19 |
| | 12.5 | 3 | 3.48 ± 0.72 | 14.03 ± 1.25 | 29.13 ± 1.28* | 34.54 ± 3.56 |
| Flutamide | 2.5 | 3 | 3.41 ± 0.71 | 13.29 ± 1.71* | 26.70 ± 1.58 | 32.19 ± 3.68 |
| Flutamide + Carbendazim | 2.5+6.25 | 3 | 2.19 ± 0.29*ab | 12.44 ± 1.89*b | 26.32 ± 1.93* | 31.19 ± 3.76*b |
| | 2.5+12.5 | 4 | 2.82 ± 0.36*ab | 13.38 ± 1.02* | 27.36 ± 0.88b | 34.54 ± 2.62 |
| **F3** | | | | | | |
| Control | | 3 | 2.93 ± 0.47 | 14.37 ± 1.58 | 30.04 ± 2.19 | 38.25 ± 2.71 |
| Carbendazim | 0.39 | 3 | 2.96 ± 0.43 | 13.57 ± 1.92 | 31.78 ± 2.35 | 38.97 ± 2.15 |
| | 0.78 | 3 | 3.09 ± 0.34 | 14.60 ± 1.97 | 31.30 ± 4.09 | 38.38 ± 2.93 |
| | 1.56 | 3 | 3.04 ± 0.44 | 15.11 ± 1.34 | 30.39 ± 2.83 | 37.80 ± 2.66 |
| | 3.13 | 2 | 4.30 ± 0.36* | 14.16 ± 1.00 | 27.79 ± 1.63* | 31.39 ± 3.66* |
| | 6.25 | 4 | 3.48 ± 0.78* | 14.30 ± 2.18 | 29.51 ± 3.01* | 36.12 ± 3.26* |
| Flutamide | 2.5 | 3 | 2.51 ± 0.26* | 11.90 ± 1.13* | 25.07 ± 2.57* | 30.00 ± 2.94* |
| Flutamide + Carbendazim | 2.5+0.78 | 2 | 3.02 ± 0.43a | 14.52 ± 1.64a | 27.91 ± 1.90*ab | 37.93 ± 2.12a |
| | 2.5+1.56 | 2 | - c | - c | - c | - c |
| | 2.5+3.13 | 2 | 3.97 ± 0.44 | 15.37 ± 1.19 | 28.00 ± 1.38 | 30.50 ± 2.41 |

***Table 5:*** *Effects of in utero exposure to carbendazim, flutamide, and in combination on AGD of male offspring in multiple generations of Wistar rats.*

*1)p-value = * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.005; vs. control.*

*2)p-value = a ≤ 0.05 vs. flutamide.*

*3)p-value = b ≤ 0.05 vs. carbendazim.*

*cNo pregnancy.*

reduced pup weight on PND 2 but increased pup weight on PND 56. Compared to controls, co-treatment with flutamide and carbendazim at 6.25 and 25 mg/kg bw/day respectively decreased pup weight on PND 14 and PND 2; co-treatment with flutamide and carbendazim at 50 mg/kg bw/day also decreased pup weight on PND 2, 7, and 56. Compared to flutamide treatment alone, co-treatment with flutamide and carbendazim at 12.5 mg/kg bw/day increased pup weight on PND 2 and 7. In contrast, co-treatment with flutamide and carbendazim at 12.5 and 25 mg/kg bw/day decreased pup weight on PND 56, compared to flutamide alone; similarly, co-treatment with flutamide and carbendazim at 50 mg/kg bw/day decreased pup weight on PND 2, 7, 33, and 56. Compared to carbendazim treatment at the same doses, co-treatment with flutamide and carbendazim at 6.25 and 12.5 mg/kg bw/day increased pup weight on PND 2, 7, 21, and 33.

For F2 female offspring, compared to controls, treatment with carbendazim at 1.56, 6.25, and 12.5 mg/kg bw/day as well as co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day increased pup weight on PND 56; in contrast, treatment with flutamide alone or carbendazim alone at 3.13 mg/kg bw/day decreased pup weight on PND 2. Compared to flutamide alone, co-treatment with flutamide and carbendazim at 12.5 mg/kg bw/day increased pup weight on PND 2; however, compared to carbendazim at the same dose, co-treatment with flutamide and carbendazim at 12.5 mg/kg bw/day decreased pup weight on PND 56.

For F3 female offspring, treatment with carbendazim reduced pup weight compared to controls as follow: on PND 7, 14, and 21 at 0.39 mg/kg bw/day; on PND 21 and 33 at 0.78 mg/kg bw/day; on PND 14 and 21 at 1.56 mg/kg bw/day; and on PND 14, 21, and 33 at 3.13 and 6.25 mg/kg bw/day. On the contrary, compared to control, treatment with carbendazim at 1.56 mg/kg bw/day increased pup weight on PND 56; similarly, increased pup weight was observed on PND 56 following co-treatment with flutamide and carbendazim at 0.78 mg/kg bw/day and on PND 21 and 56 following co-treatment with flutamide and carbendazim at 3.13 mg/kg bw/day. Compared to flutamide alone, there were increases in pup weight on PND 7, 14, 21, and 33 following co-treatment with flutamide and carbendazim at 0.78 mg/kg bw/day as well as on PND 7, 14, 21, and 33 following co-treatment with flutamide and carbendazim at 3.13 mg/kg bw/day. Compared to the same doses of carbendazim, there was also an increase in pup weight on PND 21 and 33 following co-treatment with flutamide and carbendazim at 0.78 mg/kg bw/day and on PND 7, 14, 21, 33, and 56 following co-treatment with flutamide and carbendazim at 3.13 mg/kg bw/day. Co-treatment with flutamide and carbendazim at 1.56 mg/kg bw/day resulted in no pregnant females; therefore, no female offspring were born from this group (Table 6).

| Treatments | Dose (mg/kg bw/day) | No. of litters | PND | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 2 | 7 | 14 | 21 | 33 | 56 |
| **F1** | | | | | | | | |
| Control | | 3 | 6.6 ± 0.3 | 14.4 ± 1.5 | 28.4 ± 3.8 | 41.3 ± 6.6 | 104.2 ± 14.2 | 191.8 ± 13.4 |
| Carbendazim | 6.25 | 4 | 5.3 ± 0.6$^{*}$ | 12.3 ± 1.5$^{*}$ | 24.2 ± 3.3$^{*}$ | 34.9 ± 5.1$^{*}$ | 98.6 ± 9.1 | 183.3 ± 13.0$^{*}$ |
| | 12.5 | 4 | 6.6 ± 0.7 | 13.1 ± 1.3$^{*}$ | 25.8 ± 2.2$^{*}$ | 37.4 ± 4.7$^{*}$ | 101.8 ± 8.7 | 195.2 ± 18.5 |
| | 25 | 4 | -$^{c}$ | -$^{c}$ | -$^{c}$ | -$^{c}$ | -$^{c}$ | -$^{c}$ |
| | 50 | 4 | -$^{c}$ | -$^{c}$ | -$^{c}$ | -$^{c}$ | -$^{c}$ | -$^{c}$ |
| Flutamide | 2.5 | 3 | 6.4 ± 0.5$^{*}$ | 13.8 ± 1.9 | 26.7 ± 4.1 | 40.5 ± 8.0 | 109.8 ± 10.6 | 206.3 ± 14.3$^{*}$ |
| Flutamide + Carbendazim | 2.5+6.25 | 4 | 6.4 ± 0.7$^{b}$ | 13.4 ± 2.2$^{b}$ | 25.5 ± 3.2$^{*}$ | 39.1 ± 6.8$^{b}$ | 105.7 ± 11.7$^{b}$ | 195.2 ± 19.7 |
| | 2.5+12.5 | 4 | 7.0 ± 0.7$^{*ab}$ | 14.9 ± 1.6$^{ab}$ | 28.9 ± 4.2$^{b}$ | 43.2 ± 7.2$^{b}$ | 110.8 ± 14.1$^{b}$ | 195.7 ± 17.0$^{a}$ |
| | 2.5+25 | 4 | 6.1 ± 0.5$^{*}$ | 13.8 ± 1.4 | 27.9 ± 2.9 | 40.4 ± 5.1 | 104.7 ± 11.1 | 193.2 ± 12.5$^{a}$ |
| | 2.5+50 | 4 | 5.0 ± 1.0$^{*a}$ | 12.2 ± 1.9$^{*}$ | 27.3 ± 3.0 | 40.5 ± 4.7 | 100.1 ± 10.1$^{a}$ | 176.1 ± 16.2$^{*a}$ |
| **F2** | | | | | | | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Control | | 3 | 6.9 ± 0.4 | 13.6 ± 1.8 | 26.6 ± 3.1 | 39.2 ± 6.0 | 105.5 ± 17.2 | 190.3 ± 25.8 |
| Carbendazim | 1.56 | 4 | 6.8 ± 0.9* | 13.4 ± 2.2 | 25.4 ± 4.8 | 36.0 ± 8.5 | 102.3 ± 18.0 | 205.2 ± 17.2* |
| | 3.13 | 4 | 6.2 ± 0.5* | 13.8 ± 0.8 | 26.6 ± 1.4 | 38.2 ± 3.3 | 105.3 ± 6.8 | 203.9 ± 12.7 |
| | 6.25 | 4 | 6.9 ± 0.6 | 14.2 ± 1.7 | 27.5 ± 3.0 | 40.1 ± 5.3 | 111.3 ± 9.8 | 209.2 ± 14.4* |
| | 12.5 | 3 | 7.1 ± 0.5 | 13.3 ± 1.5 | 25.8 ± 4.2 | 38.3 ± 7.4 | 106.2 ± 13.6 | 209.9 ± 19.4* |
| Flutamide | 2.5 | 3 | 6.1 ± 0.5* | 13.3 ± 1.5 | 27.8 ± 2.9 | 40.6 ± 5.1 | 107.8 ± 13.6 | 205.0 ± 26.1 |
| Flutamide + Carbendazim | 2.5+6.25 | 3 | 6.8 ± 1.1 | 13.6 ± 2.3 | 27.2 ± 4.0 | 40.7 ± 6.6 | 113.4 ± 11.0 | 220.1 ± 20.1* |
| | 2.5+12.5 | 4 | 7.1 ± 0.7[a] | 13.0 ± 2.1 | 23.6 ± 5.5[a] | 35.3 ± 8.0 | 99.8 ± 14.8 | 192.7 ± 13.6[b] |
| **F3** | | | | | | | | |
| Control | | 3 | 6.9 ± 1.2 | 13.9 ± 1.2 | 29.4 ± 2.6 | 42.6 ± 3.8 | 114.1 ± 8.4 | 201.4 ± 10.1 |
| Carbendazim | 0.39 | 3 | 6.4 ± 0.7 | 12.7 ± 2.1* | 24.7 ± 4.2* | 36.0 ± 7.2* | 107.3 ± 16.7 | 202.4 ± 18.8 |
| | 0.78 | 3 | 6.3 ± 0.8 | 13.3 ± 1.9 | 27.1 ± 4.6 | 37.2 ± 6.8* | 103.5 ± 16.5* | 212.0 ± 20.8 |
| | 1.56 | 3 | 6.8 ± 0.7 | 13.8 ± 1.4 | 27.0 ± 2.5* | 39.9 ± 4.0* | 113.7 ± 7.7 | 213.3 ± 12.7* |
| | 3.13 | 2 | 6.9 ± 0.4 | 12.9 ± 1.2 | 24.7 ± 2.8* | 35.8 ± 2.3* | 103.5 ± 5.1* | 191.9 ± 12.7 |
| | 6.25 | 4 | 6.4 ± 0.8 | 12.8 ± 2.0 | 25.3 ± 4.6* | 37.1 ± 7.9* | 102.7 ± 16.8* | 198.7 ± 21.1 |
| Flutamide | 2.5 | 3 | 6.6 ± 0.7 | 12.4 ± 2.0 | 23.4 ± 3.9 | 35.9 ± 6.4 | 99.6 ± 16.0 | 198.6 ± 29.8 |
| Flutamide + Carbendazim | 2.5+0.78 | 2 | 6.8 ± 0.9 | 14.8 ± 2.2[a] | 30.0 ± 6.0[a] | 46.4 ± 9.8[ab] | 126.5 ± 17.9[ab] | 227.1 ± 11.8* |
| | 2.5+1.56 | 2 | -[d] | -[d] | -[d] | -[d] | -[d] | -[d] |
| | 2.5+3.13 | 2 | 7.1 ± 0.5 | 14.6 ± 0.6[ab] | 30.1 ± 3.0[ab] | 46.3 ± 3.2*[ab] | 119.1 ± 5.3[ab] | 211.9 ± 10.3*[b] |

***Table 6:*** *Effects of in utero exposure to carbendazim, flutamide, and in combination on body weight of female offspring in multiple generations of Wistar rats.*

*[1)]p-value = * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.005; vs. control.*

*[2)]p-value = [a] ≤ 0.05 vs. flutamide.*

*[3)]p-value = [b] ≤ 0.05 vs. carbendazim.*

*[c]Embryolethality.*

*[d]No pregnancy.*

For F1 male offspring, a reduction in pup weight was found on PND 2, 7, 14, and 21 following treatment with carbendazim at 6.25 mg/kg bw/day and on PND 2 following treatment with carbendazim at 12.5 mg/kg bw/day, compared to controls. No male offspring were born with carbendazim treatment at 25 and 50 mg/kg bw/day due to embryolethality. Flutamide alone decreased pup weight on PND 2 but increased pup weight on PND 56, compared to controls. Co-treatment with flutamide and carbendazim at 12.5 mg/kg bw/day increased pup weight on PND 33 and 56 compared to controls; however, there was a reduction in pup weight on PND 2 following co-treatment with flutamide and carbendazim at 25 mg/kg bw/day and on PND 2 and 7 following co-treatment with flutamide and carbendazim at 50 mg/kg bw/day. Compared to flutamide, pup weight was reduced on PND 56 and 2 following co-treatment with flutamide and carbendazim respectively at 6.25 and 50 mg/kg bw/day; however pup weight increased on PND 2 following co-treatment with flutamide and carbendazim 12.5 mg/kg bw/day. Compared to the same doses of carbendazim, co-treatment with flutamide and carbendazim increased pup weight on the following days and doses of carbendazim as follows: on PND 2, 7, 14, 21, and 33 with 6.25 and 12.5 mg/kg bw/day; and on PND 56 with 12.5 mg/kg bw/day.

For F2 male offspring, compared to control, decreased pup weight was found on PND 7, 14, 21, and 33 following carbendazim treatment at 1.56 mg/kg bw/day, on PND 2 following carbendazim treatment at 3.13 mg/kg bw/day, and on PND 14 following carbendazim treatment at 12.5 mg/kg bw/day; however, there was an increase in pup weight on PND 56 with carbendazim treatment at 6.25 mg/kg bw/day. Compared to controls, co-treatment with flutamide and carbendazim at 6.25 and 12.5 mg/kg bw/day decreased pup weight on PND 14. Compared to flutamide alone, co-treatment with flutamide and carbendazim at 6.25 and 12.5 mg/kg bw/day decreased pup weight on PND 14 and 21.

Fore F3 male offspring, compared to controls, decreased pup weight was found after treatment with the following doses of carbendazim: on PND 14 and 21 at 0.39 mg/kg bw/day; on PND 7, 14, 21, and 33 at 3.13 mg/kg bw/day, and on PND 21 and 33 at 6.25 mg/kg bw/day. However, carbendazim at 1.56 mg/kg bw/day increased pup weight on PND 56. Increases in pup weight were also found on PND 56 following co-treatment with flutamide and carbendazim at 0.78 mg/kg bw/day and on PND 7, 33, and 56 following co-treatment with flutamide and carbendazim at 3.13 mg/kg bw/day, compared to controls. Compared to flutamide alone, increased pup weight was observed on PND 7, 14, 21, 33, and 56 following co-treatment with flutamide and carbendazim at 0.78 and 3.13 mg/kg bw/day and on PND 2 following co-treatment with flutamide and carbendazim at 0.78 mg/kg bw/day. Compared to the same doses of carbendazim, there was also an increase in pup weight on PND 2 following co-treatment with flutamide and carbendazim at 0.78 mg/kg bw/day and on PND 7, 14, 21, 33, and 56 following co-treatment with flutamide and carbendazim at 3.13 mg/kg bw/day. No male offspring were born from co-treatments with flutamide and carbendazim at 1.56 mg/kg bw/day as no females became pregnant (Table 7).

| Treatments | Dose (mg/kg bw/day) | No. of litters | PND | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 2 | 7 | 14 | 21 | 33 | 56 |
| **F1** | | | | | | | | |
| Control | | 3 | 6.9 ± 0.5 | 14.5 ± 1.7 | 28.4 ± 4.1 | 42.1 ± 6.5 | 114.1 ± 22.1 | 265.1 ± 36.5 |
| Carbendazim | 6.25 | 4 | 5.9 ± 1.0$^{*}$ | 12.8 ± 1.8$^{*}$ | 25.5 ± 3.2$^{*}$ | 36.1 ± 5.4$^{*}$ | 113.2 ± 13.4 | 270.6 ± 15.5 |
| | 12.5 | 4 | 6.5 ± 0.6$^{*}$ | 14.2 ± 0.9 | 26.1 ± 3.5 | 39.3 ± 3.2 | 115.4 ± 9.3 | 276.4 ± 19.7 |
| | 25 | 4 | -$^{c}$ | -$^{c}$ | -$^{c}$ | -$^{c}$ | -$^{c}$ | -$^{c}$ |
| | 50 | 4 | -$^{c}$ | -$^{c}$ | -$^{c}$ | -$^{c}$ | -$^{c}$ | -$^{c}$ |
| Flutamide | 2.5 | 3 | 6.5 ± 0.6$^{*}$ | 14.2 ± 1.9 | 27.6 ± 4.0 | 42.1 ± 7.1 | 120.7 ± 14.3 | 296.2 ± 25.7$^{*}$ |
| Flutamide + Carbendazim | 2.5+6.25 | 4 | 6.7 ± 0.8$^{b}$ | 15.0 ± 2.5$^{b}$ | 27.8 ± 3.9$^{b}$ | 43.5 ± 7.6$^{b}$ | 122.9 ± 12.7$^{b}$ | 270.6 ± 16.5$^{a}$ |
| | 2.5+12.5 | 4 | 7.2 ± 0.8$^{ab}$ | 15.5 ± 2.0$^{b}$ | 29.5 ± 4.4$^{b}$ | 46.2 ± 8.5$^{b}$ | 129.7 ± 19.6$^{*b}$ | 302.5 ± 47.7$^{*b}$ |
| | 2.5+25 | 4 | 6.5 ± 0.7$^{*}$ | 14.4 ± 1.1 | 28.0 ± 4.7 | 42.0 ± 8.2 | 117.8 ± 12.9 | 283.5 ± 29.4 |
| | 2.5+50 | 4 | 5.3 ± 0.5$^{*a}$ | 12.3 ± 1.6$^{*}$ | 27.0 ± 2.6 | 40.8 ± 4.7 | 113.6 ± 11.6 | 274.6 ± 15.0 |
| **F2** | | | | | | | | |
| Control | | 3 | 7.3 ± 0.5 | 15.0 ± 1.5 | 29.1 ± 2.4 | 42.1 ± 4.1 | 125.9 ± 9.3 | 305.1 ± 21.3 |
| Carbendazim | 1.56 | 4 | 7.0 ± 0.7 | 12.7 ± 1.7$^{*}$ | 24.3 ± 3.5$^{*}$ | 35.8 ± 7.1$^{*}$ | 113.0 ± 17.5$^{*}$ | 301.2 ± 28.8 |
| | 3.13 | 4 | 6.8 ± 0.5$^{*}$ | 14.0 ± 1.4 | 28.0 ± 2.1 | 41.2 ± 5.2 | 121.0 ± 10.6 | 321.2 ± 22.1 |
| | 6.25 | 4 | 7.3 ± 0.7 | 14.0 ± 2.4 | 27.9 ± 2.7 | 41.4 ± 5.7 | 122.5 ± 15.1 | 319.4 ± 20.9$^{*}$ |
| | 12.5 | 3 | 7.6 ± 0.6 | 14.6 ± 1.1 | 27.3 ± 2.5$^{*}$ | 40.9 ± 5.2 | 121.9 ± 11.4 | 312.1 ± 19.4 |
| Flutamide | 2.5 | 3 | 7.4 ± 1.0 | 15.3 ± 2.1 | 30.2 ± 4.1 | 44.9 ± 5.9 | 125.1 ± 14.7 | 305.3 ± 29.0 |
| Flutamide + Carbendazim | 2.5+6.25 | 3 | 7.1 ± 1.0 | 14.2 ± 2.3 | 26.5 ± 4.1$^{*a}$ | 38.7 ± 6.5$^{a}$ | 120.5 ± 13.1 | 312.3 ± 27.2 |
| | 2.5+12.5 | 4 | 7.6 ± 0.4 | 14.4 ± 1.1 | 26.8 ± 2.1$^{*a}$ | 40.0 ± 4.9$^{a}$ | 121.4 ± 8.2 | 308.1 ± 10.9 |
| **F3** | | | | | | | | |
| Control | | 3 | 7.0 ± 0.9 | 14.1 ± 1.2 | 29.6 ± 3.0 | 44.4 ± 5.2 | 126.2 ± 11.8 | 311.4 ± 20.2 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Carbendazim | 0.39 | 3 | 6.9 ± 0.8 | 13.7 ± 2.3 | 26.6 ± 3.8* | 38.8 ± 6.3* | 121.4 ± 15.2 | 316.5 ± 26.5 |
| | 0.78 | 3 | 6.9 ± 0.7 | 14.9 ± 2.3 | 29.1 ± 4.6 | 44.0 ± 7.7 | 126.4 ± 17.4 | 317.3 ± 25.1 |
| | 1.56 | 3 | 7.0 ± 0.7 | 14.6 ± 1.3 | 28.9 ± 2.0 | 43.1 ± 3.3 | 127.8 ± 9.3 | 326.0 ± 15.7* |
| | 3.13 | 2 | 6.9 ± 0.3 | 13.1 ± 0.9* | 24.6 ± 0.9* | 36.5 ± 2.4* | 112.2 ± 4.0* | 301.3 ± 12.3 |
| | 6.25 | 4 | 6.9 ± 0.9 | 13.9 ± 2.5 | 26.6 ± 5.9 | 38.7 ± 9.4* | 115.9 ± 17.0* | 304.5 ± 27.7 |
| Flutamide | 2.5 | 3 | 6.9 ± 0.9 | 12.8 ± 2.1 | 23.3 ± 3.2 | 36.3 ± 6.1 | 106.11 ± 14.5 | 266.6 ± 29.5 |
| Flutamide + Carbendazim | 2.5+0.78 | 2 | 7.4 ± 0.7ab | 15.3 ± 2.3a | 30.6 ± 5.7a | 47.8 ± 10.5a | 134.2 ± 21.5a | 331.2 ± 31.5*a |
| | 2.5+1.56 | 2 | - d | - d | - d | - d | - d | - d |
| | 2.5+3.13 | 2 | 7.3 ± 0.9 | 15.1 ± 0.7*ab | 30.3 ± 1.6ab | 47.1 ± 1.9ab | 139.2 ± 4.8*ab | 340.8 ± 10.4*ab |

***Table 7:*** *Effects of in utero exposure to carbendazim, flutamide, and in combination on body weight of male offspring in multiple generations of Wistar rats.*

*1)p-value = * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.005; vs. control.*

*2)p-value = a ≤ 0.05 vs. flutamide.*

*3)p-value = b ≤ 0.05 vs. carbendazim.*

*cEmbryolethality.*

*dNo pregnancy.*

### Effects of carbendazim, flutamide, and in combination on vaginal opening in female rats

For F1 female offspring, treatment with carbendazim at 6.25 and 12.5 mg/kg bw/day did not change VO compared to controls. Due to the embryolethality, no female offspring were born from carbendazim treatment at 25 and 50 mg/kg bw/day. Flutamide treatment also did not change VO. In contrast, co-treatment with flutamide and carbendazim at 12.5 mg/kg bw/day shortened VO compared to carbendazim alone at the same dose.

For F2 female offspring, treatment with carbendazim at 1.56, 3.13, 6.25, and 12.5 mg/kg bw/day did not change the VO compared to controls; flutamide also did not change VO. Co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day shortened VO compared to carbendazim alone at the same dose.

For F3 female offspring, treatment with carbendazim at 0.39, 0.78, 1.56, 3.13, and 6.25 mg/kg bw/day did not change VO compared to control; flutamide also did not change VO. Co-treatment with flutamide and carbendazim at 3.13 mg/kg bw/day shortened VO compared to controls, flutamide alone, and carbendazim alone at the same dose. No females became pregnant following co-treatment with flutamide and carbendazim at 1.56 mg/kg bw/day; thus, no female offspring were born (Table 8).

### Effects of carbendazim, flutamide, and in combination on preputial separation in male rats

For F1 male offspring, treatment with carbendazim at 6.25 and 12.5 mg/kg bw/day did not change PPS compared to controls. No male offspring were born following carbendazim treatment at 25 and 50 mg/kg bw/day due to the embryolethality. Flutamide treatment alone did not change PPS. Co-treatment with flutamide and carbendazim at 6.25, 12.5, and 50 mg/kg bw/day shortened PPS compared to flutamide alone. Similarly, co-treatment with flutamide and carbendazim at 12.5 mg/kg bw/day shortened PPS compared to carbendazim treatment alone at the same dose.

For F2 male offspring, compared to controls, extended PPS was found following treatment with carbendazim at 3.13 and 6.25 mg/kg bw/day, treatment with flutamide alone, and co-treatment with flutamide and carbendazim at 6.25 and 12.5 mg/kg bw/day. Compared to flutamide alone, co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day shortened PPS. Compared to carbendazim at the same dose, co-treatment with flutamide and carbendazim at 12.5 mg/kg bw/day extended PPS.

For F3 male offspring, compared to control, treatment with carbendazim at 0.78 and 3.13 mg/kg bw/day and co-treatment with flutamide and carbendazim at 3.13 mg/kg bw/day shortened PPS, whereas treatment with flutamide alone extended PPS. Compared to flutamide alone, co-treatment with flutamide and carbendazim at 0.78 and 3.13 mg/kg bw/day shortened PPS. Note that no females became pregnant following co-treatment with flutamide and carbendazim at 1.56 mg/kg bw/day; therefore, there were no male offspring (Table 8).

| Treatments | Dose (mg/kg bw/day) | No. of litters | Number of offspring | | VO | PPS |
|---|---|---|---|---|---|---|
| | | | Male | Female | | |
| **F1** | | | | | | |
| Control | | 3 | 18 | 22 | 34.4 ± 2.5 | 35.9 ± 1.8 |
| Carbendazim | 6.25 | 4 | 29 | 35 | 34.6 ± 2.1 | 36.5 ± 0.9 |
| | 12.5 | 4 | 20 | 23 | 35.1 ± 1.5 | 37.0 ± 0.9 |
| | 25 | 4 | 0 | 0 | - [c] | - [c] |
| | 50 | 4 | 0 | 0 | - [c] | - [c] |
| Flutamide | 2.5 | 3 | 15 | 24 | 34.2 ± 2.0 | 36.6 ± 1.2 |
| Flutamide + Carbendazim | 2.5+6.25 | 4 | 22 | 20 | 34.0 ± 2.5 | 34.8 ± 2.1[a] |
| | 2.5+12.5 | 4 | 23 | 32 | 33.1 ± 3.2[b] | 35.4 ± 1.4[ab] |
| | 2.5+25 | 4 | 24 | 25 | 34.7 ± 2.0 | 36.7 ± 1.8 |
| | 2.5+50 | 4 | 3 | 8 | 34.0 ± 2.3 | 34.3 ± 2.1[a] |
| **F2** | | | | | | |
| Control | | 3 | 20 | 20 | 34.4 ± 7.1 | 41.4 ± 1.0 |
| Carbendazim | 1.56 | 4 | 20 | 29 | 33.6 ± 2.1 | 42.7 ± 3.2 |
| | 3.13 | 4 | 6 | 9 | 33.0 ± 1.9 | 43.0 ± 1.1[*] |
| | 6.25 | 4 | 29 | 30 | 34.8 ± 1.5 | 42.5 ± 2.0[*] |
| | 12.5 | 3 | 28 | 17 | 33.7 ± 1.7 | 40.6 ± 1.7 |
| Flutamide | 2.5 | 3 | 20 | 16 | 34.1 ± 3.0 | 43.4 ± 2.4[*] |
| Flutamide + Carbendazim | 2.5+6.25 | 3 | 20 | 12 | 33.8 ± 1.0[b] | 42.0 ± 1.0[*a] |
| | 2.5+12.5 | 4 | 11 | 9 | 33.7 ± 1.8 | 42.4 ± 1.6[*b] |
| **F3** | | | | | | |
| Control | | 3 | 17 | 17 | 34.4 ± 1.9 | 42.4 ± 1.8 |
| Carbendazim | 0.39 | 3 | 26 | 24 | 35.5 ± 2.9 | 41.8 ± 1.1 |
| | 0.78 | 3 | 16 | 13 | 34.7 ± 1.5 | 40.8 ± 1.8[*] |
| | 1.56 | 3 | 18 | 23 | 34.7 ± 1.6 | 41.9 ± 0.8 |
| | 3.13 | 2 | 9 | 8 | 34.8 ± 1.5 | 40.6 ± 1.4[*] |
| | 6.25 | 4 | 50 | 33 | 35.6 ± 2.1 | 42.6 ± 1.6 |
| Flutamide | 2.5 | 3 | 21 | 11 | 36.0 ± 2.7 | 43.6 ± 1.7[*] |
| Flutamide + Carbendazim | 2.5+0.78 | 2 | 18 | 4 | 33.0 ± 1.2 | 41.5 ± 1.3[a] |
| | 2.5+1.56 | 2 | 0 | 0 | - [d] | - [d] |
| | 2.5+3.13 | 2 | 8 | 7 | 31.9 ± 1.2[*ab] | 40.6 ± 1.4[*a] |

***Table 8:*** *Effects of in utero exposure to carbendazim, flutamide, and in combination on vaginal opening (VO) and preputial aseparation (PPS) in multiple generations of Wistar rats.*

*[1)]p-value = [*] ≤ 0.05, [**] ≤ 0.01, [***] ≤ 0.005; vs. control.*

*[2)]p-value = [a] ≤ 0.05 vs. flutamide.*

*[3)]p-value = [b] ≤ 0.05 vs. carbendazim.*

*[c]Embryolethality.*

*[d]No pregnancy.*

### Effects of carbendazim, flutamide, and in combination on epilepsy in male and female rats

For F1 offspring, treatment with carbendazim at 6.25 and 12.5 mg/kg bw/day did not affect the number of live male and female offspring compared to controls whereas treatment with carbendazim at 25 and 50 mg/kg bw/day produced no live pups due to embryolethality. Flutamide and co-treatment with flutamide and carbendazim at 6.25 and 12.5 mg/kg bw/day did not affect the number of live offspring compared to control; however, compared to flutamide alone, co-treatment with flutamide and carbendazim at 50 mg/kg bw/day reduced the number of live male offspring. In contrast, co-treatment with flutamide and carbendazim at 25 mg/kg bw/day increased the number of live male and female offspring compared to the same dose of carbendazim alone. The number of live male and female offspring was not affected after treatment with the following: F2 offspring (carbendazim at 1.56, 3.13, 6.25, and 12.5 mg/kg bw/day; flutamide alone; or in combination); and F3 offspring (carbendazim at 0.39, 0.78, 1.56, 3.13, and 6.25 mg/kg bw/day; flutamide alone; or in combination). Co-treatment with flutamide and carbendazim at 1.56 mg/kg bw/day produced no offspring as females did not become pregnant.

For F1 offspring, treatment with carbendazim at 6.25, 12.5, 25, and 50 mg/kg bw/day, flutamide alone, and co-treatment with flutamide and carbendazim at 6.25, 12.5, 25, and 50 mg/kg bw/day resulted in no incidence of epilepsy. For F2 offspring, treatment with carbendazim at 1.56 mg/kg bw/day induced epilepsy in 5% of male offspring, and treatment with carbendazim at 6.25 mg/kg bw/day induced epilepsy in 3% of male and 10% of female offspring. Neither flutamide treatment alone nor co-treatment with flutamide and carbendazim at 6.25 and 12.5 mg/kg bw/day induced epilepsy in male or female offspring. For F3 offspring, treatment with carbendazim at 0.78 and 6.25 mg/kg bw/day respectively induced epilepsy in 13% and 15% of male offspring and in 20% and 6% of female offspring. Moreover, flutamide alone induced epilepsy in 10% of female offspring (Table 9) (Video for epilepsy).


| Treatments | Dose (mg/kg bw/day) | No. of litters | Incidence of epilepsy with group basis [d] | | No. of offspring | | Group mean of absolute and incidence of epilepsy | |
|---|---|---|---|---|---|---|---|---|
| | | | Male | Female | Male | Female | Absolute | % |
| **F1** | | | | | | | | |
| Control | | 3 | 0 (0/18) | 0 (0/22) | 6.0 ± 3.6 | 7.3 ± 2.1 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Carbendazim | 6.25 | 4 | 0 (0/29) | 0 (0/35) | 7.3 ± 0.4 | 8.8 ± 2.3 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| | 12.5 | 4 | 0 (0/20) | 0 (0/23) | 5.0 ± 3.6 | 5.8 ± 4.3 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| | 25 | 4 | 0 (0/0) | 0 (0/0) | 0.0 ± 0.0** | 0.0 ± 0.0** | 0.0 ± 0.0 | 0.0 ± 0.0 |
| | 50 | 4 | 0 (0/0) | 0 (0/0) | 0.0 ± 0.0** | 0.0 ± 0.0** | 0.0 ± 0.0 | 0.0 ± 0.0 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Flutamide | 2.5 | 3 | 0 (0/15) | 0 (0/24) | 5.0 ± 1.0 | 8.0 ± 3.6 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Flutamide + Carbendazim | 2.5 + 6.25 | 4 | 0 (0/22) | 0 (0/20) | 7.3 ± 1.5 | 6.7 ± 1.5 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| | 2.5 + 12.5 | 4 | 0 (0/23) | 0 (0/32) | 5.8 ± 1.5 | 8.0 ± 1.8 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| | 2.5 + 25 | 4 | 0 (0/24) | 0 (0/25) | 6.0 ± 2.2 [b] | 6.3 ± 2.4 [b] | 0.0 ± 0.0 | 0.0 ± 0.0 |
| | 2.5 + 50 | 4 | 0 (0/3) | 0 (0/8) | 0.8 ± 1.5 [a] | 2.0 ± 4.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| **F2** | | | | | | | | |
| Control | | 3 | 0 (0/20) | 0 (0/20) | 6.7 ± 1.5 | 6.7 ± 1.5 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Carbendazim | 1.56 | 4 | 5 (1/20) | 0 (0/29) | 6.7 ± 4.0 | 9.7 ± 2.1 | 0.3 ± 0.6 | 1.7 ± 2.9 |
| | 3.13 | 4 | 0 (0/6) | 0 (0/9) | 3.0 ± 4.2 | 4.5 ± 6.4 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| | 6.25 | 4 | 3 (1/29) | 10 (3/30) | 7.3 ± 1.5 | 7.5 ± 1.3 | 1.0 ± 2.0 | 5.9 ± 11.8 |
| | 12.5 | 3 | 0 (0/28) | 0 (0/17) | 9.3 ± 1.5 | 5.7 ± 1.2 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Flutamide | 2.5 | 3 | 0 (0/20) | 0 (0/16) | 6.7 ± 1.5 | 5.3 ± 2.1 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Flutamide + Carbendazim | 2.5 + 6.25 | 3 | 0 (0/20) | 0 (0/12) | 10.0 ± 2.8 | 6.0 ± 2.8 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| | 2.5 + 12.5 | 4 | 0 (0/11) | 0 (0/9) | 5.5 ± 4.9 | 4.5 ± 4.9 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| **F3** | | | | | | | | |
| Control | | 3 | 0 (0/17) | 0 (0/17) | 5.7 ± 2.5 | 5.7 ± 1.5 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| Carbendazim | 0.39 | 3 | 0 (0/26) | 0 (0/24) | 8.7 ± 1.5 | 8.0 ± 2.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| | 0.78 | 3 | 13 (2/16) | 15 (2/13) | 8.0 ± 2.8 | 6.5 ± 2.1 | 2.0 ± 1.4 | 12.9 ± 5.4 |
| | 1.56 | 3 | 0 (0/18) | 0 (0/23) | 6.0 ± 2.0 | 7.7 ± 3.1 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| | 3.13 | 2 | 0 (0/9) | 0 (0/8) | 9.0 ± 0.0 | 8.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| | 6.25 | 6 | 20 (10/50) | 6 (2/33) | 8.3 ± 2.9 | 5.5 ± 3.2 | 2.0 ± 2.4 | 15.5 ± 14.9 |
| Flutamide | 2.5 | 3 | 0 (0/21) | 9 (1/11) | 10.5 ± 2.1 | 5.5 ± 2.1 | 0.5 ± 0.7 | 3.1 ± 4.4 |
| Flutamide + Carbendazim | 2.5 + 0.78 | 2 | 0 (0/18) | 0 (0/4) | 9.0 ± 5.7 | 2.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| | 2.5 + 1.56 | 2 | 0 (0) | 0 (0) | - [c] | - [c] | - [c] | - [c] |
| | 2.5 + 3.13 | 2 | 0 (0/8) | 0 (0/7) | 8.0 ± 0.0 | 7.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |

***Table 9:*** *Incidence of epilepsy induced by carbendazim, flutamide, and in combination in multiple generations of Wistar rats.*

*[1)]p-value = [*] ≤ 0.05, [**] ≤ 0.01, [***] ≤ 0.005; vs. control.*

*[2)]p-value = [a]≤ 0.05 vs. flutamide.*

*[3)]p-value = [b] ≤ 0.05 vs. carbendazim.*

*[c]No pregnancy.*

*[d]Incidence of epilepsy with group basis (%, number/sex total No.).*

## Effects of carbendazim, flutamide, and in combination on reproductive organs in female rats

For parent female rats, compared to controls, treatment with carbendazim at 6.25, 12.5, 25, and 50 mg/kg bw/day did not affect the relative organ weights of the liver, kidney, adrenal, uterus, and right ovary; however, treatment with carbendazim at 6.25 and 50 mg/kg bw/day increased left ovary weight. Flutamide alone did not change any organ weights. Compared to control, co-treatment with flutamide and carbendazim at 12.5 and 50 mg/kg bw/day increased relative left ovary weight while flutamide and carbendazim at 25 mg/kg bw/day reduced relative adrenal weight. Compared to the same dose of carbendazim alone, co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day decreased the relative weight of the liver and kidney.

For F1 female offspring, treatment with at 1.56, 3.13, 6.25, and 12.5 mg/kg bw/day did not affect the relative organ weight of the brain, pituitary gland, liver, kidney, adrenal, uterus, and left and right ovary compared to control; however, carbendazim at 3.13 mg/kg bw/day did have increased body weight. Flutamide alone also increased body weight compared to controls. Compared to flutamide alone, co-treatment with flutamide and carbendazim at 6.25, 25, and 50 mg/kg bw/day reduced body weight, and co-treatment with flutamide and carbendazim at 12.5 mg/kg bw/day reduced relative liver weight. Compared to carbendazim at the same dose, co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day reduced the relative organ weight of the liver and kidney, and co-treatment with flutamide and carbendazim at 12.5 mg/kg bw/day reduced pituitary gland weight.

For F2 female offspring, compared to controls, treatment with carbendazim at 0.39, 0.78, 1.56, 3.13, 6.25, and 12.5 mg/kg bw/day increased body weight but decreased not only relative brain weight but also relative liver, kidney, adrenal, uterus and left ovary weights (the 0.78 mg/kg bw/day dose did not affect the weight of the latter organs). Flutamide alone increased body weight but decreased the relative weight of the liver, kidney, adrenal, and left ovary compared to controls. Co-treatment with flutamide and carbendazim at 0.78, 1.56, 3.13, 6.25, and 12.5 mg/kg bw/day increased body weight compared to controls. Compared to flutamide alone, co-treatment with flutamide and carbendazim at 3.13 and 6.25 mg/kg bw/day increased body weight. In contrast, compared to carbendazim at the same dose, co-treatment with flutamide and carbendazim at 12.5 mg/kg bw/day decreased body weight. Compared to controls, co-treatment with flutamide and carbendazim at 0.78, 1.56, 3.13, and 6.25 mg/kg bw/day reduced relative brain weight as well as relative liver weight (in addition to the 12.5 mg/kg bw/day dose for the liver). Similarly, co-treatment with flutamide and carbendazim at 0.78, 3.13, 6.25, and 12.5 mg/kg bw/day reduced relative kidney weight compared to controls. Compared to the same dose of carbendazim alone, co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day reduced relative kidney weight.

For F3 female offspring, compared to controls, treatment with carbendazim at 0.39, 0.78, 1.56, and 6.25 mg/kg bw/day increased body weight; treatment with carbendazim at 0.39, 0.78, 1.56, and 6.25 mg/kg bw/day reduced relative brain; treatment with carbendazim at 0.39, 0.78, 1.56, and 3.13 mg/kg bw/day reduced relative liver weight; and treatment with flutamide alone reduced relative brain weight. Compared to the same dose of carbendazim, co-treatment with flutamide and carbendazim at 0.78 mg/kg bw/day reduced body weight but increased the relative weight of the brain and liver. Compared to controls, co-treatment with flutamide and carbendazim at 3.13 mg/kg bw/day reduced relative uterus weight (Table 10).

| Treatments | Dose (mg/kg bw/day) | N | Body weight (g) | Brain | Pituitary gland | Liver | Kidney | Adrenal | Uterus | Left ovary | Right ovary |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Organ weight/body weight ratio (g/g × 100) | | | | | | | |
| **P** | | | | | | | | | | | |
| Control | | 3 | 320.4 ± 14.5 | - [c] | - [c] | 3.82 ± 0.31 | 0.72 ± 0.05 | 0.04 ± 0.01 | 0.14 ± 0.10 | 0.02 ± 0.00 | 0.02 ± 0.00 |
| Carbendazim | 6.25 | 4 | 318.3 ± 24.0 | - [c] | - [c] | 4.19 ± 0.09 | 0.82 ± 0.02 | 0.03 ± 0.00 | 0.33 ± 0.14 | 0.03 ± 0.01* | 0.03 ± 0.01 |
| | 12.5 | 4 | 297.2 ± 46.4 | - [c] | - [c] | 4.33 ± 0.29 | 0.80 ± 0.08 | 0.05 ± 0.04 | 0.29 ± 0.19 | 0.02 ± 0.01 | 0.03 ± 0.01 |
| | 25 | 4 | 323.0 ± 10.2 | - [c] | - [c] | 3.58 ± 0.58 | 0.68 ± 0.06 | 0.03 ± 0.01 | 0.22 ± 0.07 | 0.03 ± 0.00 | 0.03 ± 0.01 |
| | 50 | 4 | 312.9 ± 31.9 | - [c] | - [c] | 3.78 ± 0.10 | 0.71 ± 0.04 | 0.03 ± 0.00 | 0.18 ± 0.04 | 0.03 ± 0.00* | 0.03 ± 0.00 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Flutamide | 2.5 | 3 | 311.3 ± 34.5 | -[c] | -[c] | 3.67 ± 0.33 | 0.67 ± 0.03 | 0.03 ± 0.01 | 0.20 ± 0.03 | 0.03 ± 0.01 | 0.02 ± 0.01 |
| Flutamide + Carbendazim | 2.5+6.25 | 4 | 359.8 ± 77.8 | -[c] | -[c] | 3.30 ± 0.61[b] | 0.62 ± 0.08[b] | 0.03 ± 0.01 | 0.23 ± 0.09 | 0.03 ± 0.01 | 0.02 ± 0.00 |
| | 2.5+12.5 | 4 | 318.4 ± 36.8 | -[c] | -[c] | 4.07 ± 0.30 | 0.76 ± 0.09 | 0.03 ± 0.00 | 0.22 ± 0.05 | 0.03 ± 0.01* | 0.03 ± 0.01 |
| | 2.5+25 | 4 | 316.8 ± 12.6 | -[c] | -[c] | 3.87 ± 0.15 | 0.69 ± 0.07 | 0.03 ± 0.00* | 0.27 ± 0.07 | 0.02 ± 0.00 | 0.09 ± 0.13 |
| | 2.5+50 | 4 | 291.2 ± 61.1 | -[c] | -[c] | 3.85 ± 0.61 | 0.74 ± 0.11 | 0.03 ± 0.01 | 0.22 ± 0.04 | 0.03 ± 0.01* | 0.03 ± 0.01 |
| **F1** | | | | | | | | | | | |
| Control | | 17 | 283.5 ± 30.2 | 0.70 ± 0.07 | 0.005 ± 0.002 | 3.96 ± 0.40 | 0.75 ± 0.06 | 0.03 ± 0.03 | 0.25 ± 0.09 | 0.03 ± 0.00 | 0.03 ± 0.01 |
| Carbendazim | 1.56 | 4 | 331.9 ± 13.8 | 0.59 ± 0.06 | 0.004 ± 0.001 | 4.30 ± 0.60 | 0.72 ± 0.02 | 0.03 ± 0.00 | 0.19 ± 0.01 | 0.03 ± 0.00 | 0.03 ± 0.00 |
| | 3.13 | 4 | 353.2 ± 61.1* | 0.63 ± 0.03 | 0.003 ± 0.000 | 3.63 ± 0.07 | 0.67 ± 0.06 | 0.03 ± 0.00 | 0.22 ± 0.00 | 0.03 ± 0.00 | 0.03 ± 0.00 |
| | 6.25 | 27 | 281.3 ± 26.9 | 0.65 ± 0.23 | 0.005 ± 0.003 | 4.01 ± 0.65 | 0.75 ± 0.10 | 0.06 ± 0.11 | 0.25 ± 0.07 | 0.03 ± 0.01 | 0.03 ± 0.01 |
| | 12.5 | 23 | 272.7 ± 44.0 | 0.74 ± 0.09 | 0.007 ± 0.002 | 4.15 ± 0.53 | 0.78 ± 0.05 | 0.03 ± 0.01 | 0.25 ± 0.08 | 0.04 ± 0.05 | 0.03 ± 0.01 |
| Flutamide | 2.5 | 24 | 301.8 ± 28.3* | 0.51 ± 0.33 | 0.003 ± 0.002 | 3.79 ± 0.26 | 0.70 ± 0.05 | 0.03 ± 0.00 | 0.24 ± 0.07 | 0.03 ± 0.00 | 0.03 ± 0.00 |
| Flutamide + Carbendazim | 2.5+6.25 | 20 | 284.4 ± 30.4[a] | 0.58 ± 0.29 | 0.004 ± 0.003 | 3.68 ± 0.24[b] | 0.66 ± 0.03[b] | 0.03 ± 0.01 | 0.24 ± 0.06 | 0.03 ± 0.01 | 0.03 ± 0.00 |
| | 2.5+12.5 | 32 | 313.0 ± 92.6 | 0.54 ± 0.33 | 0.005 ± 0.003[b] | 3.43 ± 0.87[a] | 0.71 ± 0.20 | 0.03 ± 0.01 | 0.28 ± 0.12 | 0.03 ± 0.01 | 0.03 ± 0.01 |
| | 2.5+25 | 25 | 272.7 ± 23.1[a] | 0.51 ± 0.32 | 0.005 ± 0.003 | 3.79 ± 0.33 | 0.71 ± 0.05 | 0.03 ± 0.00 | 0.24 ± 0.08 | 0.03 ± 0.00 | 0.03 ± 0.00 |
| | 2.5+50 | 8 | 258.6 ± 14.4[a] | 0.41 ± 0.34 | 0.003 ± 0.003 | 3.78 ± 0.29 | 0.70 ± 0.04 | 0.03 ± 0.00 | 0.28 ± 0.07 | 0.03 ± 0.00 | 0.03 ± 0.00 |
| **F2** | | | | | | | | | | | |
| Control | | 3 | 214.6 ± 14.2 | 0.89 ± 0.10 | 0.007 ± 0.003 | 4.36 ± 0.08 | 0.77 ± 0.02 | 0.04 ± 0.01 | 0.34 ± 0.21 | 0.04 ± 0.00 | 0.03 ± 0.01 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Carbendazim | 0.39 | 9 | 284.1 ± 28.9* | 0.59 ± 0.03* | 0.005 ± 0.002 | 3.45 ± 0.42* | 0.74 ± 0.01 | 0.03 ± 0.01 | 0.21 ± 0.04 | 0.03 ± 0.00 | 0.03 ± 0.00 |
| | 0.78 | 3 | 329.4 ± 24.8* | 0.65 ± 0.00* | 0.006 ± 0.005 | 4.37 ± 0.29 | 0.82 ± 0.06 | 0.03 ± 0.00 | 0.20 ± 0.02 | 0.03 ± 0.01 | 0.03 ± 0.01 |
| | 1.56 | 12 | 290.9 ± 32.7* | 0.71 ± 0.07* | 0.005 ± 0.002 | 3.70 ± 0.19* | 0.76 ± 0.07 | 0.03 ± 0.00* | 0.20 ± 0.04* | 0.03 ± 0.01 | 0.03 ± 0.00 |
| | 3.13 | 6 | 299.2 ± 31.5* | 0.68 ± 0.06* | 0.004 ± 0.002 | 3.70 ± 0.33* | 0.70 ± 0.02* | 0.03 ± 0.00 | 0.28 ± 0.06 | 0.04 ± 0.01 | 0.03 ± 0.01 |
| | 6.25 | 25 | 326.0 ± 37.5* | 0.52 ± 0.24* | 0.004 ± 0.002 | 3.73 ± 0.37 | 0.73 ± 0.05 | 0.03 ± 0.01* | 0.22 ± 0.06 | 0.03 ± 0.01 | 0.03 ± 0.00 |
| | 12.5 | 13 | 326.0 ± 20.4* | 0.66 ± 0.16* | 0.005 ± 0.002 | 3.72 ± 0.30* | 0.63 ± 0.19 | 0.04 ± 0.06 | 0.21 ± 0.05 | 0.03 ± 0.00* | 0.03 ± 0.00 |
| Flutamide | 2.5 | 16 | 281.6 ± 36.6* | 0.67 ± 0.21 | 0.005 ± 0.002 | 3.75 ± 0.39* | 0.70 ± 0.06* | 0.03 ± 0.01* | 0.30 ± 0.23 | 0.03 ± 0.00* | 0.03 ± 0.02 |
| Flutamide + Carbendazim | 2.5+0.78 | 2 | 325.2 ± 28.6* | 0.66 ± 0.02* | 0.005 ± 0.003 | 3.75 ± 0.08* | 0.65 ± 0.03* | 0.03 ± 0.00 | 0.18 ± 0.06 | 0.03 ± 0.00 | 0.02 ± 0.00 |
| | 2.5+1.56 | 2 | 338.6 ± 52.1* | 0.64 ± 0.09* | 0.003 ± 0.000 | 3.61 ± 0.31* | 0.64 ± 0.10 | 0.03 ± 0.01 | 0.16 ± 0.02 | 0.04 ± 0.00 | 0.03 ± 0.00 |
| | 2.5+3.13 | 2 | 354.9 ± 31.0*a | 0.61 ± 0.01* | 0.003 ± 0.000 | 3.41 ± 0.22* | 0.67 ± 0.03* | 0.03 ± 0.00 | 0.24 ± 0.03 | 0.03 ± 0.01 | 0.03 ± 0.01 |
| | 2.5+6.25 | 7 | 323.4 ± 22.2*a | 0.65 ± 0.02* | 0.004 ± 0.001 | 3.57 ± 0.33* | 0.66 ± 0.04*b | 0.03 ± 0.00 | 0.23 ± 0.03 | 0.03 ± 0.00 | 0.03 ± 0.00 |
| | 2.5+12.5 | 9 | 278.9 ± 12.0*b | 0.45 ± 0.34 | 0.003 ± 0.003 | 3.51 ± 0.12* | 0.64 ± 0.05* | 0.03 ± 0.01 | 0.25 ± 0.13 | 0.03 ± 0.01 | 0.03 ± 0.00 |
| **F3** | | | | | | | | | | | |
| Control | | 3 | 214.6 ± 14.2 | 0.89 ± 0.10 | 0.007 ± 0.003 | 4.36 ± 0.08 | 0.77 ± 0.02 | 0.04 ± 0.01 | 0.34 ± 0.21 | 0.04 ± 0.00 | 0.03 ± 0.01 |
| Carbendazim | 0.39 | 9 | 284.1 ± 28.9* | 0.59 ± 0.03* | 0.005 ± 0.002 | 3.45 ± 0.42* | 0.74 ± 0.01 | 0.03 ± 0.01 | 0.21 ± 0.04 | 0.03 ± 0.00 | 0.03 ± 0.00 |
| | 0.78 | 14 | 340.9 ± 24.5* | 0.57 ± 0.03* | 0.005 ± 0.001 | 3.30 ± 0.21* | 0.69 ± 0.07 | 0.02 ± 0.00* | 0.28 ± 0.18 | 0.03 ± 0.00 | 0.02 ± 0.00 |
| | 1.56 | 10 | 284.7 ± 27.7* | 0.71 ± 0.07* | 0.005 ± 0.002 | 3.70 ± 0.19* | 0.76 ± 0.07 | 0.03 ± 0.00* | 0.20 ± 0.04 | 0.03 ± 0.01 | 0.03 ± 0.00 |
| | 3.13 | 3 | 263.3 ± 28.1 | 0.79 ± 0.06 | 0.005 ± 0.002 | 3.70 ± 0.14* | 0.75 ± 0.05 | 0.03 ± 0.01 | 0.29 ± 0.09 | 0.03 ± 0.00 | 0.03 ± 0.00 |
| | 6.25 | 26 | 315.3 ± 28.8* | 0.65 ± 0.06* | 0.005 ± 0.002 | 3.41 ± 0.89 | 0.69 ± 0.14 | 0.04 ± 0.04 | 0.31 ± 0.20 | 0.03 ± 0.01 | 0.03 ± 0.01 |
| Flutamide | 2.5 | 3 | 293.6 ± 58.5 | 0.67 ± 0.10* | 0.004 ± 0.001 | 3.60 ± 0.46 | 0.70 ± 0.10 | 0.03 ± 0.01 | 0.16 ± 0.01 | 0.03 ± 0.01 | 0.02 ± 0.00 |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Flutamide+ Carbendazim | 2.5+0.78 | 3 | 246.6 ± 18.1[b] | 0.80 ± 0.07[b] | 0.004 ± 0.000 | 4.31 ± 0.31[b] | 0.76 ± 0.05 | 0.04 ± 0.00 | 0.19 ± 0.04 | 0.03 ± 0.00 | 0.03 ± 0.00 |
| | 2.5+3.13 | 3 | 229.2 ± 8.3 | 0.84 ± 0.05 | 0.004 ± 0.000 | 4.24 ± 0.33 | 0.82 ± 0.11 | 0.04 ± 0.00 | 0.21 ± 0.02[*] | 0.03 ± 0.01 | 0.03 ± 0.00 |

***Table 10:*** *Effects of before-mating and in utero exposure to carbendazim, flutamide, and in combination on relative organ weights in multiple generations of female Wistar rats.*

*[1)]p-value = [*] ≤ 0.05, [**] ≤ 0.01, [***] ≤ 0.005; vs. control.*

*[2)]p-value = [a] ≤ 0.05 vs. flutamide.*

*[3)]p-value = [b] ≤ 0.05 vs. carbendazim.*

*[c]Not measured.*

## Effects of carbendazim, flutamide, and in combination on reproductive organs in male rats

For parent male rats, treatment with carbendazim at 6.25, 12.5, 25, and 50 mg/kg bw/day or flutamide alone did not affect the relative body weight and organ weights of the liver, kidney, adrenal, left and right testis, and left and right epididymis compared to controls. Similarly, co-treatment with flutamide and carbendazim at 6.25, 12.5, 25, and 50 mg/kg bw/day did not affect the relative weights of the above organs; note that treatment with flutamide and carbendazim at 25 mg/kg bw/day increased body weight compared to controls and decreased relative right testis weight compared to carbendazim alone at the same dose.

For F1 male offspring, treatment with carbendazim at 1.56, 3.13, 6.25, and 12.5 mg/kg bw/day did not affect the relative organ weights of the brain, pituitary gland, liver, kidney, adrenal, left and right testis, and left and right epididymis compared to controls; however, increased body weight was observed at the 1.56 and 3.13 mg/kg bw/day doses. Flutamide alone did not affect body weight or the relative weights of the above organs compared to controls. Compared to flutamide alone, co-treatment with flutamide and carbendazim at 6.25, 25, and 50 mg/kg bw/day reduced body weight; co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day increased the relative organ weights of the brain, pituitary gland, kidney, and adrenal; and co-treatment with flutamide and carbendazim at 25 mg/kg bw/day increased the relative weights of the brain, kidney, left and right testis.

For F2 male offspring, compared to controls, treatment with carbendazim at 0.39, 0.78, 1.56, 3.13, 6.25, and 12.5 mg/kg bw/day increased body weight but decreased relative brain weight. Furthermore, treatment with carbendazim at 1.56 and 6.25 mg/kg bw/day reduced relative pituitary gland weight; treatment with carbendazim at 0.39, 3.13, and 6.25 mg/kg bw/day reduced relative kidney weight; treatment with carbendazim at 0.39, 0.78, 1.56, 3.13, 6.25, and 12.5 mg/kg bw/day reduced relative liver weight; treatment with carbendazim at 0.39, 0.78, 1.56, 3.13, 6.25, and 12.5 mg/kg bw/day reduced relative weights of the left and right testis; treatment with carbendazim at 0.78 mg/kg bw/day reduced relative left epididymis; and treatment with carbendazim at 0.78, 3.13, 6.25, and 12.5 mg/kg bw/day reduced relative right epididymis weight. Flutamide alone did not affect body weight and relative organ weights compared to controls. Co-treatment with flutamide and carbendazim at 0.78, 1.56, 3.13, and 6.25 mg/kg bw/day increased body weight compared to controls whereas co-treatment with flutamide and carbendazim at 12.5 mg/kg bw/day decreased body weight compared to the same dose of carbendazim. Compared to flutamide alone, co-treatment with flutamide and carbendazim at 0.78 mg/kg bw/day increased body weight. Relative brain weight decreased following co-treatment with flutamide and carbendazim at 0.78 and 1.56 mg/kg bw/day compared to controls but increased following co-treatment with flutamide and carbendazim compared to the same doses of carbendazim. Compared to controls, co-treatment with flutamide and carbendazim at 0.78, 1.56, 3.13, and 6.25 mg/kg bw/day decreased the relative

organ weights of the liver, kidney, and left and right testis, and co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day decreased the relative weight of the right epididymis. In contrast, co-treatment with flutamide and carbendazim at 12.5 mg/kg bw/day increased the relative weights of the pituitary gland, adrenal, and left and right testis compared to the same dose of carbendazim alone.

For F3 male offspring, compared to controls, treatment with carbendazim at 0.39, 0.78, 1.56, 3.13, 6.25, and 12.5 mg/kg bw/day increased body weight but reduced the relative organ weights of the brain, liver, kidney, adrenal, left and right testis; treatment with carbendazim at 1.56, 3.13, 6.25, and 12.5 mg/kg bw/day reduced relative pituitary weight; and treatment with carbendazim at 0.39, 0.78, 1.56, 3.13, 6.25, and 12.5 mg/kg bw/day increased the relative weight of the right epididymis whereas treatment with flutamide increased the relative weight of right epididymis. Co-treatment with flutamide and carbendazim at 0.78 mg/kg bw/day respectively reduced relative weights of the left testis and left epididymis compared to controls and flutamide. Compared to the same doses of carbendazim alone, co-treatment with flutamide and carbendazim at 0.78 and 3.13 mg/kg bw/day reduced body weight; however, co-treatment with flutamide and carbendazim at 0.78 mg/kg bw/day increased the relative organ weights of the brain, liver, adrenal, and left testis (Table 11).

| Treatments | Dose (mg/kg bw/day) | N | Body weight (g) | Brain | Pituitary gland | Liver | Kidney | Adrenal | Left testis | Right testis | LE | RE |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Organ weight/body weight ratio (g/g × 100) | | | | | | | | |
| **P** | | | | | | | | | | | | |
| Control | | 3 | 617.7 ± 23.2 | -[c] | -[c] | 3.15 ± 0.11 | 0.64 ± 0.04 | 0.01 ± 0.00 | 0.37 ± 0.11 | 0.35 ± 0.10 | 0.13 ± 0.02 | 0.13 ± 0.01 |
| Carbendazim | 6.25 | 3 | 602.1 ± 30.2 | -[c] | -[c] | 3.14 ± 0.39 | 0.65 ± 0.06 | 0.01 ± 0.00 | 0.33 ± 0.02 | 0.32 ± 0.02 | 0.11 ± 0.01 | 0.11 ± 0.02 |
| | 12.5 | 3 | 639.3 ± 15.3 | -[c] | -[c] | 3.25 ± 0.27 | 0.60 ± 0.04 | 0.01 ± 0.00 | 0.32 ± 0.03 | 0.32 ± 0.04 | 0.15 ± 0.05 | 0.11 ± 0.01 |
| | 25 | 3 | 604.4 ± 16.4 | -[c] | -[c] | 2.99 ± 0.16 | 0.59 ± 0.01 | 0.01 ± 0.00 | 0.33 ± 0.02 | 0.34 ± 0.01 | 0.12 ± 0.01 | 0.12 ± 0.01 |
| | 50 | 3 | 638.9 ± 47.7 | -[c] | -[c] | 2.20 ± 1.90 | 0.46 ± 0.24 | 0.01 ± 0.00 | 0.35 ± 0.28 | 0.24 ± 0.14 | 0.12 ± 0.05 | 0.11 ± 0.06 |
| Flutamide | 2.5 | 3 | 638.1 ± 30.1 | -[c] | -[c] | 2.34 ± 1.51 | 0.37 ± 0.31 | 0.08 ± 0.12 | 0.30 ± 0.01 | 0.31 ± 0.03 | 0.12 ± 0.01 | 0.12 ± 0.01 |
| Flutamide + Carbendazim | 2.5+6.25 | 3 | 616.0 ± 50.4 | -[c] | -[c] | 2.94 ± 0.13 | 0.55 ± 0.06 | 0.01 ± 0.00 | 0.30 ± 0.04 | 0.31 ± 0.04 | 0.11 ± 0.02 | 0.08 ± 0.07 |
| | 2.5+12.5 | 3 | 588.4 ± 57.9 | -[c] | -[c] | 3.15 ± 0.21 | 0.61 ± 0.07 | 0.01 ± 0.00 | 0.35 ± 0.05 | 0.35 ± 0.06 | 0.13 ± 0.02 | 0.13 ± 0.02 |
| | 2.5+25 | 3 | 655.6 ± 18.4[*] | -[c] | -[c] | 3.12 ± 0.14 | 0.56 ± 0.06 | 0.01 ± 0.00 | 0.30 ± 0.00 | 0.31 ± 0.00[b] | 0.11 ± 0.01 | 0.11 ± 0.01 |
| | 2.5+50 | 3 | 606.0 ± 23.1 | -[c] | -[c] | 3.24 ± 0.22 | 0.61 ± 0.02 | 0.01 ± 0.00 | 0.34 ± 0.04 | 0.33 ± 0.05 | 0.11 ± 0.02 | 0.12 ± 0.01 |
| **F1** | | | | | | | | | | | | |
| Control | | 14 | 431.3 ± 71.3 | 0.49 ± 0.08 | 0.003 ± 0.001 | 3.73 ± 0.31 | 0.67 ± 0.17 | 0.02 ± 0.00 | 0.42 ± 0.06 | 0.41 ± 0.08 | 0.14 ± 0.02 | 0.14 ± 0.03 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Carbendazim | 1.56 | 4 | 533.1 ± 48.5* | 0.43 ± 0.04 | 0.003 ± 0.001 | 3.47 ± 0.23 | 0.69 ± 0.04 | 0.02 ± 0.00 | 0.36 ± 0.03 | 0.36 ± 0.03 | 0.15 ± 0.01 | 0.15 ± 0.02 |
| | 3.13 | 4 | 550.8 ± 64.0* | 0.38 ± 0.04 | 0.001 ± 0.001 | 3.28 ± 0.29 | 0.67 ± 0.08 | 0.01 ± 0.00 | 0.27 ± 0.17 | 0.26 ± 0.16 | 0.12 ± 0.05 | 0.12 ± 0.06 |
| | 6.25 | 21 | 431.7 ± 37.7 | 0.48 ± 0.05 | 0.003 ± 0.001 | 3.76 ± 0.34 | 0.74 ± 0.07 | 0.01 ± 0.00 | 0.42 ± 0.04 | 0.41 ± 0.04 | 0.14 ± 0.02 | 0.14 ± 0.02 |
| | 12.5 | 20 | 439.7 ± 58.5 | 0.48 ± 0.06 | 0.004 ± 0.001 | 3.73 ± 0.59 | 0.73 ± 0.06 | 0.02 ± 0.00 | 0.40 ± 0.07 | 0.40 ± 0.07 | 0.14 ± 0.02 | 0.14 ± 0.02 |
| Flutamide | 2.5 | 15 | 490.9 ± 48.7 | 0.46 ± 0.05 | 0.003 ± 0.001 | 3.67 ± 0.25 | 0.68 ± 0.05 | 0.02 ± 0.00 | 0.38 ± 0.05 | 0.36 ± 0.09 | 0.14 ± 0.02 | 0.14 ± 0.03 |
| Flutamide + Carbendazim | 2.5+6.25 | 21 | 438.8 ± 55.0[a] | 0.50 ± 0.04[a] | 0.004 ± 0.001[a] | 3.71 ± 0.41 | 0.73 ± 0.07[a] | 0.02 ± 0.00[b] | 0.40 ± 0.04 | 0.39 ± 0.08 | 0.15 ± 0.02 | 0.14 ± 0.02 |
| | 2.5+12.5 | 23 | 464.8 ± 90.2 | 0.49 ± 0.09 | 0.003 ± 0.001 | 3.67 ± 0.38 | 0.75 ± 0.09[a] | 0.01 ± 0.00 | 0.39 ± 0.11 | 0.38 ± 0.10 | 0.14 ± 0.03 | 0.14 ± 0.03 |
| | 2.5+25 | 23 | 420.2 ± 43.4[a] | 0.47 ± 0.05[a] | 0.003 ± 0.002 | 3.94 ± 0.29 | 0.74 ± 0.04[a] | 0.01 ± 0.00 | 0.42 ± 0.06[a] | 0.41 ± 0.06[a] | 0.14 ± 0.02 | 0.13 ± 0.02 |
| | 2.5+50 | 3 | 400.3 ± 23.9[a] | 0.44 ± 0.01 | 0.002 ± 0.002 | 3.82 ± 0.20 | 0.72 ± 0.03 | 0.02 ± 0.00 | 0.36 ± 0.01 | 0.36 ± 0.02 | 0.12 ± 0.02 | 0.11 ± 0.02 |
| F2 | | | | | | | | | | | | |
| Control | | 17 | 453.4 ± 32.2 | 0.46 ± 0.04 | 0.003 ± 0.001 | 3.95 ± 0.25 | 0.74 ± 0.04 | 0.03 ± 0.06 | 0.41 ± 0.03 | 0.42 ± 0.03 | 0.14 ± 0.01 | 0.15 ± 0.01 |
| Carbendazim | 0.39 | 3 | 578.9 ± 12.5* | 0.35 ± 0.04* | 0.002 ± 0.001 | 3.43 ± 0.34* | 0.65 ± 0.05* | 0.01 ± 0.00 | 0.33 ± 0.05* | 0.32 ± 0.05* | 0.13 ± 0.02 | 0.14 ± 0.03 |
| | 0.78 | 3 | 575.2 ± 26.3* | 0.30 ± 0.12* | 0.002 ± 0.001 | 3.19 ± 0.21* | 0.69 ± 0.09 | 0.01 ± 0.00 | 0.33 ± 0.05* | 0.32 ± 0.05* | 0.12 ± 0.02* | 0.12 ± 0.01* |
| | 1.56 | 11 | 570.3 ± 54.4* | 0.39 ± 0.04* | 0.002 ± 0.001* | 3.44 ± 0.29* | 0.71 ± 0.05 | 0.01 ± 0.00 | 0.35 ± 0.04* | 0.35 ± 0.04* | 0.14 ± 0.02 | 0.14 ± 0.02 |
| | 3.13 | 8 | 562.7 ± 32.5* | 0.39 ± 0.02* | 0.002 ± 0.001 | 3.29 ± 0.29* | 0.64 ± 0.06* | 0.01 ± 0.00 | 0.34 ± 0.04* | 0.32 ± 0.04* | 0.14 ± 0.02 | 0.13 ± 0.02* |
| | 6.25 | 28 | 542.0 ± 58.1* | 0.31 ± 0.18* | 0.002 ± 0.001* | 3.58 ± 0.21* | 0.68 ± 0.05* | 0.01 ± 0.00 | 0.37 ± 0.04* | 0.36 ± 0.05* | 0.13 ± 0.02 | 0.13 ± 0.02* |
| | 12.5 | 21 | 509.3 ± 39.4* | 0.41 ± 0.15* | 0.002 ± 0.001 | 3.74 ± 0.26* | 0.72 ± 0.07 | 0.01 ± 0.00 | 0.38 ± 0.05* | 0.37 ± 0.04* | 0.13 ± 0.02 | 0.13 ± 0.02* |
| Flutamide | 2.5 | 20 | 463.4 ± 41.5 | 0.40 ± 0.15 | 0.003 ± 0.001 | 3.82 ± 0.43 | 0.71 ± 0.07 | 0.02 ± 0.00 | 0.42 ± 0.05 | 0.41 ± 0.05 | 0.15 ± 0.02 | 0.15 ± 0.02 |
| Flutamide + Carbendazim | 2.5+0.78 | 2 | 625.0 ± 72.8*[a] | 0.39 ± 0.01* | 0.002 ± 0.001 | 3.01 ± 0.03* | 0.54 ± 0.01* | 0.01 ± 0.00 | 0.32 ± 0.01*[a] | 0.33 ± 0.01*[a] | 0.12 ± 0.02 | 0.13 ± 0.02 |
| | 2.5+1.56 | 2 | 563.3 ± 27.6* | 0.41 ± 0.02* | 0.003 ± 0.001 | 3.30 ± 0.03* | 0.62 ± 0.01*[b] | 0.01 ± 0.00 | 0.32 ± 0.05*[a] | 0.32 ± 0.05*[a] | 0.13 ± 0.00 | 0.13 ± 0.00 |
| | 2.5+3.13 | 2 | 532.8 ± 59.4* | 0.43 ± 0.06 | 0.003 ± 0.001 | 3.42 ± 0.07* | 0.66 ± 0.03* | 0.01 ± 0.00 | 0.22 ± 0.25*[a] | 0.37 ± 0.04 | 0.14 ± 0.01 | 0.14 ± 0.02 |
| | 2.5+6.25 | 13 | 504.1 ± 53.3* | 0.44 ± 0.04[b] | 0.002 ± 0.001 | 3.55 ± 0.19* | 0.66 ± 0.03* | 0.01 ± 0.00 | 0.38 ± 0.02*[a] | 0.37 ± 0.03*[a] | 0.14 ± 0.02 | 0.13 ± 0.01* |
| | 2.5+12.5 | 9 | 459.9 ± 25.2[b] | 0.45 ± 0.03 | 0.003 ± 0.001[b] | 3.90 ± 0.18 | 0.80 ± 0.09 | 0.02 ± 0.00[b] | 0.39 ± 0.03[b] | 0.40 ± 0.02[b] | 0.13 ± 0.02 | 0.13 ± 0.01 |

| F3 | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | | 3 | 355.4 ± 23.9 | 0.56 ± 0.03 | 0.005 ± 0.002 | 4.60 ± 0.53 | 0.87 ± 0.06 | 0.02 ± 0.00 | 0.47 ± 0.01 | 0.49 ± 0.02 | 0.12 ± 0.02 | 0.10 ± 0.00 |
| Carbendazim | 0.39 | 9 | 501.9 ± 32.1* | 0.44 ± 0.04* | 0.003 ± 0.001 | 3.55 ± 0.16* | 0.75 ± 0.04* | 0.01 ± 0.00* | 0.39 ± 0.03* | 0.39 ± 0.04* | 0.15 ± 0.02 | 0.14 ± 0.02* |
| | 0.78 | 16 | 579.3 ± 46.8* | 0.39 ± 0.04* | 0.004 ± 0.001 | 3.29 ± 0.34* | 0.71 ± 0.05* | 0.01 ± 0.00* | 0.34 ± 0.05* | 0.34 ± 0.06* | 0.15 ± 0.03 | 0.14 ± 0.03* |
| | 1.56 | 9 | 512.8 ± 37.6* | 0.43 ± 0.03* | 0.003 ± 0.001* | 3.68 ± 0.16* | 0.75 ± 0.04* | 0.01 ± 0.00* | 0.37 ± 0.03* | 0.37 ± 0.03* | 0.14 ± 0.02 | 0.14 ± 0.01* |
| | 3.13 | 3 | 489.2 ± 14.6* | 0.45 ± 0.02* | 0.003 ± 0.001 | 3.19 ± 0.17* | 0.68 ± 0.05* | 0.01 ± 0.00* | 0.35 ± 0.03* | 0.35 ± 0.02* | 0.13 ± 0.02 | 0.13 ± 0.00* |
| | 6.25 | 32 | 615.5 ± 65.4* | 0.36 ± 0.04* | 0.002 ± 0.001* | 3.30 ± 0.69* | 0.64 ± 0.11* | 0.01 ± 0.00* | 0.30 ± 0.05* | 0.29 ± 0.06* | 0.12 ± 0.02 | 0.12 ± 0.02* |
| | 12.5 | 21 | 509.3 ± 39.4* | 0.41 ± 0.15* | 0.002 ± 0.001* | 3.74 ± 0.26* | 0.72 ± 0.07* | 0.01 ± 0.00* | 0.38 ± 0.05* | 0.37 ± 0.04* | 0.13 ± 0.02 | 0.13 ± 0.02* |
| Flutamide | 2.5 | 3 | 444.8 ± 108.3 | 0.50 ± 0.13 | 0.002 ± 0.001 | 3.90 ± 0.95 | 0.76 ± 0.13 | 0.02 ± 0.00 | 0.43 ± 0.12 | 0.42 ± 0.11 | 0.13 ± 0.00 | 0.14 ± 0.02* |
| Flutamide+ Carbendazim | 2.5+0.78 | 3 | 404.0 ± 21.9 [b] | 0.53 ± 0.03[b] | 0.002 ± 0.000 | 4.44 ± 0.18[b] | 0.74 ± 0.06 | 0.02 ± 0.00[b] | 0.43 ± 0.01*[b] | 0.32 ± 0.24 | 0.11 ± 0.01[a] | 0.12 ± 0.01 |
| | 2.5+3.13 | 3 | 356.9 ± 68.3[b] | 0.61 ± 0.12 | 0.004 ± 0.001 | 4.86 ± 1.24 | 0.81 ± 0.17 | 0.02 ± 0.00[b] | 0.45 ± 0.10 | 0.45 ± 0.11 | 0.12 ± 0.02 | 0.13 ± 0.04 |

***Table 11:*** *Effects of before-mating and in utero exposure to carbendazim, flutamide, and in combination on relative organ weights in multiple generations of male Wistar rats.*

*[1)]p-value = * ≤ 0.05, ** ≤ 0.01, *** ≤ 0.005; vs. control.*

*[2)]p-value = [a] ≤ 0.05 vs. flutamide.*

*[3)]p-value = [b] ≤ 0.05 vs. carbendazim; [c]Not measured; LE: Left Epididymis; RE: Right Epididymis.*

For parent male rats, treatment with carbendazim at 6.25, 12.5, 25, and 50 mg/kg bw/day did not affect the relative organ weight of the prostate, seminal vesicle, LABC muscle, scrotum, or penis compared to controls; however, treatment with carbendazim at 12.5 and 25 mg/kg bw/day shortened relative penis length. Co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day decreased the relative weight of the seminal vesicle, and co-treatment with flutamide and carbendazim at 25 and 50 mg/kg bw/day decreased relative penis length compared to control. Co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day decreased relative seminal vesicle weight but increased relative penis width compared to the same doses of carbendazim alone.

For F1 male offspring, compared to control, treatment with carbendazim at 1.56, 3.13, 6.25, and 12.5 mg/kg bw/day affected neither the relative organ weights of the prostate, seminal vesicle, LABC muscle, scrotum, and penis nor relative penis length and width; treatment with flutamide alone did not affect the relative weights of the same organs above. Compared to controls, co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day increased relative seminal vesicle weight but decreased penis weight; however, co-treatment with flutamide and carbendazim at 12.5 mg/kg bw/day decreased relative seminal vesicle weight and relative penis width.

Compared to flutamide alone, co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day decreased relative seminal vesicle weight, co-treatment with flutamide and carbendazim at 50 mg/kg bw/day decreased relative scrotum weight, co-treatment with flutamide and carbendazim at 6.25, 25, and 50 mg/kg bw/day increased relative penis length and width, and co-treatment with flutamide and carbendazim at 25 mg/kg bw/day increased the relative weights of the pituitary gland and LABC muscle. Compared to the same doses of carbendazim alone, co-treatment with flutamide and carbendazim at 6.25 and 12.5 mg/kg bw/day decreased the relative weights of seminal vesicle and LABC muscle, co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day decreased penis weight, and co-treatment with flutamide and carbendazim at 12.5 mg/kg bw/day decreased penis length.

For F2 male offspring, compared to controls, treatment with carbendazim at 0.39, 0.78, 1.56, 3.13, 6.25, and 12.5 mg/kg bw/day did not affect the relative weights of the prostate, seminal vesicle, LABC muscle, scrotum, and penis but did decrease relative penis length and width. Compared to controls, flutamide did not affect the relative weights of the above organs and tissues, co-treatment with flutamide and carbendazim at 0.78, 1.56, 3.13, and 6.25 mg/kg bw/day decreased relative penis length and width, co-treatment with flutamide and carbendazim at 0.78 mg/kg bw/day decreased relative penis weight, and co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day decreased the relative weights of the LABC muscle and scrotum. Compared with carbendazim treatment alone at the same doses, treatment with flutamide and carbendazim at 50 mg/kg bw/day increased relative penis width, co-treatment with flutamide and carbendazim at 6.25 and 12.5 mg/kg bw/day decreased relative prostate weight, and co-treatment with flutamide and carbendazim at 6.25 mg/kg bw/day decreased relative LABC muscle weight.

For F3 male offspring, compared to controls, treatment with carbendazim at 0.39, 0.78, 1.56, 3.13, 6.25, and 12.5 mg/kg bw/day decreased relative penis length and width, co-treatment with flutamide and carbendazim at 0.39, 1.56, and 12.5 mg/kg bw/day reduced relative penis length, co-treatment with flutamide and carbendazim at 12.5 mg/kg bw/day decreased relative scrotum weight, and flutamide treatment alone reduced relative scrotum weight. Compared to controls and flutamide alone, co-treatment with flutamide and carbendazim at 0.78 mg/kg bw/day reduced relative seminal vesicle weight. Compared to the same doses of carbendazim alone, co-treatment with flutamide and carbendazim at 0.78 and 3.13 mg/kg bw/day increased relative penis length, and co-treatment with flutamide and carbendazim at 0.78 mg/kg bw/day increased relative penis width (Table 12).

| Treatments | Dose (mg/kg bw/day) | N | Prostate | Seminal vesicle | LABC | Scrotum | Penis | Penis length | Penis width |
|---|---|---|---|---|---|---|---|---|---|
| | | | Organ weight/body weight ratio (g/g × 100) | | | | | | |
| P | | | | | | | | | |
| Control | | 3 | 0.15 ± 0.05 | 0.51 ± 0.02 | 0.29 ± 0.04 | 0.32 ± 0.11 | 0.07 ± 0.00 | 1.47 ± 0.20 | 0.79 ± 0.09 |
| Carbendazim | 6.25 | 3 | 0.11 ± 0.00 | 0.52 ± 0.02 | 0.28 ± 0.01 | 0.30 ± 0.02 | 0.07 ± 0.01 | 1.05 ± 0.23 | 0.86 ± 0.15 |
| | 12.5 | 3 | 0.12 ± 0.01 | 0.46 ± 0.05 | 0.28 ± 0.04 | 0.25 ± 0.03 | 0.06 ± 0.01 | 0.95 ± 0.05* | 0.73 ± 0.01 |
| | 25 | 3 | 0.13 ± 0.02 | 0.50 ± 0.04 | 0.30 ± 0.03 | 0.27 ± 0.04 | 0.07 ± 0.01 | 0.98 ± 0.05* | 0.77 ± 0.14 |
| | 50 | 3 | 0.18 ± 0.11 | 0.38 ± 0.10 | 0.30 ± 0.01 | 0.27 ± 0.04 | 0.06 ± 0.01 | 1.31 ± 0.36 | 0.64 ± 0.02 |
| Flutamide | 2.5 | 3 | 0.14 ± 0.01 | 0.48 ± 0.12 | 0.27 ± 0.04 | 0.33 ± 0.06 | 0.06 ± 0.00 | 1.03 ± 0.10* | 0.75 ± 0.16 |
| Flutamide + Carbendazim | 2.5+6.25 | 3 | 0.13 ± 0.03 | 0.42 ± 0.05*b | 0.31 ± 0.05 | 0.29 ± 0.05 | 0.07 ± 0.01 | 1.32 ± 0.28 | 0.89 ± 0.17 |
| | 2.5+12.5 | 3 | 0.14 ± 0.06 | 0.34 ± 0.26 | 0.22 ± 0.13 | 0.27 ± 0.08 | 0.34 ± 0.46 | 0.85 ± 0.36 | 0.85 ± 0.35 |
| | 2.5+25 | 3 | 0.11 ± 0.03 | 0.41 ± 0.11 | 0.31 ± 0.04 | 0.26 ± 0.01 | 0.06 ± 0.00 | 1.00 ± 0.06* | 0.66 ± 0.02 |
| | 2.5+50 | 3 | 0.19 ± 0.09 | 0.43 ± 0.14 | 0.35 ± 0.06 | 0.31 ± 0.05 | 0.07 ± 0.01 | 0.99 ± 0.04* | 0.71 ± 0.03b |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **F1** | | | | | | | | | |
| Control | | 14 | 0.15 ± 0.03 | 0.50 ± 0.08 | 0.35 ± 0.10 | 0.39 ± 0.14 | 0.08 ± 0.02 | 1.92 ± 0.34 | 1.26 ± 0.23 |
| Carbendazim | 1.56 | 4 | 0.16 ± 0.02 | 0.57 ± 0.06 | 0.38 ± 0.05 | 0.28 ± 0.02 | 0.07 ± 0.01 | 1.50 ± 0.39 | 0.96 ± 0.14 |
| | 3.13 | 4 | 0.12 ± 0.04 | 0.52 ± 0.11 | 0.33 ± 0.07 | 0.26 ± 0.03 | 0.06 ± 0.01 | 1.35 ± 0.13 | 0.88 ± 0.03 |
| | 6.25 | 21 | 0.14 ± 0.02 | 0.48 ± 0.10 | 0.34 ± 0.05 | 0.34 ± 0.08 | 0.08 ± 0.01 | 1.86 ± 0.26 | 1.18 ± 0.16 |
| | 12.5 | 20 | 0.17 ± 0.09 | 0.40 ± 0.09 | 0.33 ± 0.04 | 0.36 ± 0.12 | 0.09 ± 0.08 | 1.86 ± 0.22 | 1.15 ± 0.20 |
| Flutamide | 2.5 | 15 | 0.13 ± 0.05 | 0.45 ± 0.10 | 0.30 ± 0.05 | 0.36 ± 0.08 | 0.06 ± 0.01 | 1.69 ± 0.17 | 1.02 ± 0.19 |
| Flutamide + Carbendazim | 2.5+6.25 | 21 | 0.14 ± 0.08 | 0.37 ± 0.11$^{*ab}$ | 0.28 ± 0.09$^{b}$ | 0.34 ± 0.07 | 0.06 ± 0.01$^{*b}$ | 1.82 ± 0.23$^{a}$ | 1.22 ± 0.25$^{a}$ |
| | 2.5+12.5 | 23 | 0.14 ± 0.07 | 0.37 ± 0.15$^{*b}$ | 0.29 ± 0.09$^{b}$ | 0.35 ± 0.11 | 0.22 ± 0.53 | 1.60 ± 0.32$^{b}$ | 1.04 ± 0.36$^{*}$ |
| | 2.5+25 | 23 | 0.14 ± 0.03$^{a}$ | 0.44 ± 0.10 | 0.32 ± 0.05$^{a}$ | 0.30 ± 0.04 | 0.07 ± 0.01 | 1.95 ± 0.16$^{a}$ | 1.23 ± 0.16$^{a}$ |
| | 2.5+50 | 3 | 0.10 ± 0.02 | 0.41 ± 0.11 | 0.26 ± 0.07 | 0.23 ± 0.08$^{a}$ | 0.07 ± 0.01 | 1.96 ± 0.21$^{a}$ | 1.48 ± 0.32$^{a}$ |
| **F2** | | | | | | | | | |
| Control | | 17 | 0.13 ± 0.02 | 0.43 ± 0.15 | 0.34 ± 0.05 | 0.27 ± 0.04 | 0.08 ± 0.01 | 1.99 ± 0.26 | 1.04 ± 0.09 |
| Carbendazim | 0.39 | 3 | 0.11 ± 0.04 | 0.49 ± 0.07 | 0.33 ± 0.10 | 0.28 ± 0.03 | 0.07 ± 0.01 | 1.51 ± 0.14$^{*}$ | 0.83 ± 0.03$^{*}$ |
| | 0.78 | 3 | 0.14 ± 0.01 | 0.44 ± 0.08 | 0.37 ± 0.05 | 0.28 ± 0.02 | 0.06 ± 0.00 | 1.18 ± 0.18$^{*}$ | 0.75 ± 0.06$^{*}$ |
| | 1.56 | 11 | 0.14 ± 0.04 | 0.48 ± 0.08 | 0.35 ± 0.05 | 0.29 ± 0.06 | 0.07 ± 0.01 | 1.45 ± 0.17$^{*}$ | 0.80 ± 0.13$^{*}$ |
| | 3.13 | 8 | 0.12 ± 0.03 | 0.54 ± 0.06 | 0.33 ± 0.08 | 0.26 ± 0.03 | 0.07 ± 0.01 | 1.46 ± 0.15$^{*}$ | 0.82 ± 0.04$^{*}$ |
| | 6.25 | 28 | 0.13 ± 0.02 | 0.46 ± 0.08 | 0.33 ± 0.05 | 0.26 ± 0.05 | 0.07 ± 0.01 | 1.56 ± 0.14$^{*}$ | 0.88 ± 0.11$^{*}$ |
| | 12.5 | 21 | 0.12 ± 0.02 | 0.47 ± 0.09 | 0.29 ± 0.03 | 0.24 ± 0.04 | 0.07 ± 0.01 | 1.61 ± 0.20$^{*}$ | 0.92 ± 0.12$^{*}$ |
| Flutamide | 2.5 | 20 | 0.11 ± 0.04 | 0.39 ± 0.09 | 0.29 ± 0.06 | 0.23 ± 0.05 | 0.07 ± 0.01 | 1.83 ± 0.25 | 0.98 ± 0.12 |
| Flutamide + Carbendazim | 2.5+0.78 | 2 | 0.12 ± 0.06 | 0.37 ± 0.00 | 0.27 ± 0.05 | 0.24 ± 0.02 | 0.05 ± 0.02$^{*}$ | 1.20 ± 0.09$^{*}$ | 0.70 ± 0.03$^{*}$ |
| | 2.5+1.56 | 2 | 0.12 ± 0.05 | 0.43 ± 0.02 | 0.28 ± 0.04 | 0.23 ± 0.02 | 0.07 ± 0.01 | 1.19 ± 0.07$^{*}$ | 0.76 ± 0.03$^{*}$ |
| | 2.5+3.13 | 2 | 0.14 ± 0.08 | 0.48 ± 0.01 | 0.36 ± 0.01 | 0.32 ± 0.03 | 0.05 ± 0.01 | 1.46 ± 0.38$^{*}$ | 0.88 ± 0.03$^{*}$ |
| | 2.5+6.25 | 13 | 0.10 ± 0.05$^{b}$ | 0.47 ± 0.08 | 0.27 ± 0.02$^{*b}$ | 0.24 ± 0.03$^{*}$ | 0.07 ± 0.01 | 1.62 ± 0.25$^{*}$ | 0.88 ± 0.18$^{*}$ |
| | 2.5+12.5 | 9 | 0.11 ± 0.04$^{b}$ | 0.47 ± 0.03 | 0.28 ± 0.04 | 0.27 ± 0.03 | 0.07 ± 0.00 | 1.68 ± 0.03 | 1.04 ± 0.07$^{b}$ |
| **F3** | | | | | | | | | |
| Control | | 3 | 0.09 ± 0.03 | 0.37 ± 0.11 | 0.33 ± 0.09 | 0.36 ± 0.02 | 0.10 ± 0.04 | 2.41 ± 0.36 | 1.33 ± 0.15 |
| Carbendazim | 0.39 | 9 | 0.12 ± 0.03 | 0.46 ± 0.08 | 0.34 ± 0.04 | 0.32 ± 0.04 | 0.07 ± 0.01$^{*}$ | 1.45 ± 0.13$^{*}$ | 0.92 ± 0.12$^{*}$ |
| | 0.78 | 16 | 0.14 ± 0.06 | 0.48 ± 0.14 | 0.36 ± 0.04 | 0.33 ± 0.05 | 0.08 ± 0.03 | 1.51 ± 0.15$^{*}$ | 0.73 ± 0.09$^{*}$ |
| | 1.56 | 9 | 0.11 ± 0.02 | 0.40 ± 0.06 | 0.39 ± 0.13 | 0.30 ± 0.05 | 0.07 ± 0.01$^{*}$ | 1.52 ± 0.17$^{*}$ | 0.90 ± 0.09$^{*}$ |
| | 3.13 | 3 | 0.13 ± 0.08 | 0.44 ± 0.14 | 0.35 ± 0.02 | 0.33 ± 0.08 | 0.07 ± 0.01 | 1.52 ± 0.13$^{*}$ | 0.92 ± 0.09$^{*}$ |
| | 6.25 | 32 | 0.12 ± 0.03 | 0.48 ± 0.12 | 0.33 ± 0.07 | 0.29 ± 0.06 | 0.11 ± 0.24 | 1.33 ± 0.20$^{*}$ | 0.73 ± 0.11$^{*}$ |
| | 12.5 | 21 | 0.12 ± 0.02 | 0.47 ± 0.09 | 0.29 ± 0.03 | 0.24 ± 0.04$^{*}$ | 0.07 ± 0.01$^{*}$ | 1.61 ± 0.20$^{*}$ | 0.92 ± 0.12$^{*}$ |
| Flutamide | 2.5 | 3 | 0.11 ± 0.04 | 0.52 ± 0.10 | 0.32 ± 0.07 | 0.30 ± 0.03$^{*}$ | 0.07 ± 0.01 | 1.73 ± 0.36 | 1.10 ± 0.31 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Flutamide+ Carbendazim | 2.5+0.78 | 3 | 0.10 ± 0.02 | 0.31 ± 0.07[a] | 0.32 ± 0.03 | 0.33 ± 0.04 | 0.07 ± 0.01 | 1.90 ± 0.05[b] | 1.11 ± 0.13[b] |
| | 2.5+3.13 | 3 | 0.09 ± 0.04 | 0.39 ± 0.11 | 0.40 ± 0.15 | 0.37 ± 0.03 | 0.09 ± 0.05 | 2.07 ± 0.25[b] | 1.23 ± 0.33 |

***Table 12:*** *Effects of before-mating and in utero exposure to carbendazim, flutamide, and in combination on relative accessory sexual organ weights, length, and width in multiple generations of male Wistar rats.*

*[1)]p-value = [*] ≤ 0.05, [**] ≤ 0.01, [***] ≤ 0.005; vs. control.*

*[2)]p-value = [a] ≤ 0.05 vs. flutamide; [3)]p-value = [b] ≤ 0.05 vs. carbendazim; LABC: Levator Ani Bulbocavernosus Muscle.*

This study demonstrated that treatment with carbendazim, flutamide, and in combination exhibited antagonistic and synergistic effects on endocrine-disrupting activity in multiple generations of rats. These endocrine-disrupting activities were supported by findings at specific endpoints, including AGD, offspring weight, VO, PPS, epilepsy, organ weights and reproductive tissues.

In one of our previous studies, we found that carbendazim increased AGD in F1 male rats and that flutamide shortened AGD in F1 male and female rats [21]; however, the results of the current study from carbendazim or flutamide treatment on AGD in multiple generations revealed different phenotypes across genders and generations, i.e., extension and shortening of AGD in F1, F2, and F3 male and female offspring. For female offspring, basically carbendazim and flutamide decreased AGD of PND 2 to 44 in female offspring of F2 and F3 while carbendazim increased it on PND2 in F1 female offspring. For male offspring, carbendazim increased AGD in F1 on PND 2 and 44 but increased and decreased it in F2 and F3 on PND 2 to 44. Flutamide decreased it all the time in male offspring. The exactly reasons of different effect of carbendazim on AGD on PND between F1, F2 and F3 remains unclear. The possible answer might be the interaction of androgen receptor and carbendazim.

Based on the above findings, carbendazim tended to extend AGD in early PND but shortened AGD in late PND in F1, F2, and F3 female offspring, whereas flutamide shortened AGD. In F1, F2, and F3 male offspring, however, carbendazim treatment extended and shortened AGD whereas flutamide shortened AGD. Thus, the mechanisms of AGD disruption differ between carbendazim and flutamide (i.e. antiandrogens). Treatment with flutamide, linuron, procymidone, and vinclozolin *in utero* has been shown to reduce AGD in male offspring rats postnatally [14,26-28]. The exact underlying mechanisms of AGD alterations induced by carbendazim in F1, F2, and F3 male and female offspring remain unclear.

Though pup weight does not appear to be related to endocrine-disrupting activity, the effect of carbendazim on fetal toxicity could be responsible for reproductive and developmental toxicity in multiple generations of rats. As we had previously shown that the weight of VO and PPS was affected by carbendazim [21], it is possible that pup weight could be linked to endocrine-disrupting activity. Overall carbendazim decreased pup weight on PND 2 to 33 in female and male offspring. The possible reason might be related to androgenic activity induced by carbendazim.

The male and female puberty indicators, respectively PPS and VO, are important determiners of endocrine-disrupting activity. Due to the successive measurement of pup weight, we detect the VO and PPS without body weight at VO or PPS. For F1, F2, and F3 female offspring, treatment with carbendazim or flutamide alone did not change VO, the results of which are inconsistent with those from a previous study [21]. In terms of VO and PPS, although carbendazim treatment affected female rats to a small degree, the effects were not as great as those on male rats. For F2 male offspring, extended PPS was found following treatment with carbendazim at 3.13 and 6.25 mg/kg bw/day as well as with flutamide. For F3 male offspring, treatment with carbendazim at 0.78 and 3.13 mg/kg bw/day shortened PPS; however, flutamide extended PPS. Our findings on carbendazim treatment are consistent with those from our previous study [21].

This discrepancy between the results from flutamide treatment in the present study and our previous report could be due to disrupted endocrine-disrupting activity.

We previously reported that carbendazim could induce spinal and bulbar muscular atrophy in multiple generations of rats and that flutamide could antagonize these effects [25]. The present study showed that the incidence of epilepsy induced by carbendazim is a striking consequence resulting from the endocrine-disrupting activity in multiple generations of rats. In the present study carbendazim induced epilepsy in F2 and F3 offspring especially in male rats. We have reported that carbendazim might induced spinal and bulbar muscular atrophy in multiple generations of rats [25]. Just because of the incidence of developmental neurotoxicity we carried out this study. Now in the present study we found that carbendazim might induce epilepsy via androgenic activity. We inferred that the mechanism of spinal and bulbar muscular atrophy and epilepsy might be related to androgenic activity induced by carbendazim. Flutamide an androgen receptor antagonist also induced one female offspring in F3. Treatment in combination with carbendazim and flutamide blocked the incidence of epilepsy induced by carbendazim alone. This explained the antagonistic effect of carbendazim and flutamide on developmental neurotoxicity. As we know this is the first report on epilepsy induced by pesticide carbendazim we inferred that the underlying mechanisms is quite related to androgen receptor based on our previous study [18,21-25].

To the best of our knowledge, this is the first study to identify the induction of epilepsy by carbendazim. Indeed, there is no dose-response in the incidence of epilepsy induced by carbendazim. Here, we found that endocrine-disrupting activity exhibited a non-montonic dose-response effect. Prenatal developmental toxicity also exhibited three characteristics in rats: dose specificity, phase specificity, and drug specificity. In terms of the endocrine-disrupting activity of reproductive and developmental toxicity, a non-montonic dose-response often occurs. A dose of 6.25 mg/kg bw/day is the specific dose for inducing epilepsy and spinal and bulbar muscular atrophy. We identified that this dose induced significant effects on this endpoint. Here, clinic signs can be shown in our video of epilepsy. These criteria for epilepsy are referred to with the Racine Score [29]. Epilepsy in the present study met the criteria of phase 5 in the Racine Score. Note that we did not measure testosterone or estrogen concentrations in rat serum.

Though the sample size of 3 - 4 pairs of male and female rats is perhaps small, the results of this study demonstrate that enough offspring were produced to be statistically analyzed, as shown in the tables. Furthermore, the following two points could explain that a sample size of 3 - 4 in this study was sufficient for statistical analysis. First, the number of offspring and thus sample size from each maternal was small (due to embryolethality). Generally, each maternal rat can give birth to about 8 - 12 offspring, and possibly more without embryolethality. Second, reproductive toxicity is a specific mode of action. In other words, a pesticide causing reproductive toxicity is a symbolic endpoint even if it is not statistically significant - it could be seen as biologically significant by comparison with control data. We also chose a smaller sample size for this study in order to meet the principle of reduction, refinement, and replacement in animal studies. Furthermore, multiple generations in reproduction experiments produce a lot of offspring in the F1, F2, and F3 offspring, which require an immense amount of work to manage. I would like to emphasize that the number of F1 generation offspring was enough to run statistical analysis even though the litter size was 3 - 4. Note that we avoided brother-sister mating in this study. Although a preliminary histopathological examination of the rats suffering from epilepsy revealed no significant changes in male and female rats (Data not shown), a further study will focus on the immunohistochemistry within brain cells of male and female rats.

A review of the literature found that the effects of androgenic compounds can both induce or protect from epilepsy. Reports on epilepsy mainly focused on androgenic and antiandrogenic activity. In terms of inducing epilepsy, $GABA_A$ agonist muscimol induced proconvulsant effects while flutamide mitigated it [30]. Antiepileptic drugs and steroids can modulate the expression of androgen and estrogen receptors in epilepsy [31]. Sex-dependent differences also will be reported in muscimol-induced proconvulsant [32]. Androgens with testosterone, estradiol ($E_2$) and 5α-dihydrotestosterone (DHT) can promote the neuroprotective seizures [33], while flutamide increased the neuroprotective effects [34]. Flutamide blocked pentylenetetrazole-induced clonic seizures and elevated the threshold of pentylenetetrazole- or bicuculline-induced clonic seizures [35].

In terms of protecting from epilepsy, testosterone reduced pentylenetetrazole-induced ictal activity in wildtype mice but not in mice with type I 5α-reductase deficiency [36]. Androstanediol, a $GABA_A$ receptor modulator, is a neurosteroid mediator of testosterone actions on neuronal excitability and seizure susceptibility and may play a key role in men with epilepsy, especially when androgen levels decline during aging [37]. In the murine hippocampus, the anti-epileptic drug phenytoin enhanced androgen metabolism and androgen receptor expression [38]. Present in high abundance, androsterone and etiocholanolone have anticonvulsant properties and could serve as endogenous modulators of seizure susceptibility [39]. Although the specific reasons for the opposing effects of androgen receptor agonists on epilepsy remain unclear and the underlying mechanisms of inducing epilepsy require further investigation, the inferences from our results are logical. Based on our previous studies, we conclude that carbendazim exhibits androgenic activity [28, 21-25], though not as potent as testosterone or dihydrotestosterone. In the present study, carbendazim induced androgen receptor activity due perhaps to the presence of C and D rings that are homologous in dihydrotestosterone (Figure 1) [24-25,40], which resulted in epilepsy, especially in male offspring rats. Endocrine-disrupting activity depends on the similarities and differences among chemical structures of compounds, such as the endogenous androgen ligand testosterone. We infer that the androgen receptor plays a vital role in epilepsy and that the blockade of androgen receptors could ameliorate the incidence of epilepsy.

In terms of organ and tissue weights, we previously reported that carbendazim decreased the reproductive tissue and organ weights in male rats [21]. Basically, carbendazim did not affect reproductive organ and tissue weight in female offspring but reduced them in male offspring. In the present study carbendazim reduced relative brain weight in F2 and F3 of male and female offspring. Also, carbendazim reduced relative weights of pituitary gland, testis, epididymis, but shortened relative penis length and width in F2 and F3 of male offspring. The significant effect on brain weight might be related to the epilepsy incidence though there is no histopathological change. Generally speaking, our data showed that carbendazim reduced the weights of the brain, pituitary gland, testis, and penis, as well as penis width and length in male offspring; epididymis weight of male rats decreased in F3 offspring but increased in F2 offspring. All of these findings were consistent with those from our previous report [21].

## Conclusion

In the current study, brain weights in female or male rats were reduced, which remains the only evidence associating changes in the brain with epilepsy. Histopathological analysis has so far revealed no obvious changes in morphology. Nonetheless, our finding associating the pesticide carbendazim with epilepsy is worthy of attention, especially in endocrine-disrupting activity. Reproductive and developmental toxicity linked to endocrine-disrupting activity is well known; however, endocrine-disrupting activity in developmental neurotoxicity such as epilepsy is novel. The underlying mechanism of carbendazim-induced epilepsy will open the door to new studies focusing on endocrine-disrupting activity and developmental neurotoxicity in mammals as a result of the use of pesticides. Based on our current findings, we believe that the androgen receptor plays a vital role in epilepsy in rats and that developmental neurotoxicity-related endpoints require much needed investigation.

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## Conflict of Interest

The authors have no conflicts of interest to declare.

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