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Antagonistic and Synergistic Effects of Carbendazim and Flutamide Exposures *In Utero* on Reproductive and Developmental Toxicity in Rats

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ABSTRACT

Carbendazim (methyl 2-benzimidazolecarbamate) and its parent compound benomyl are systemic fungicides that have reproductive and developmental toxicity in male rats. The major objectives of this study were to determine the ability of carbendazim exposure *in utero* to alter androgen-dependent development markers in rat offspring and investigate the effects of antiandrogen flutamide on the carbendazim-mediated reproductive and developmental alterations. Pregnant female rats were treated with 6.25, 12.5 or 25 mg/kg carbendazim, 25, 50 or 100 mg/kg benomyl, and 0.6, 2.5 or 10 mg/kg flutamide by gavage once daily from gestational day 0 to 20. Alternatively, another group of female rats was cotreated with 25 mg/kg carbendazim or 100 mg/kg benomyl and 0.6, 2.5, and 10 mg/kg flutamide. The various treatments decreased the survival rates of pups on postnatal day (PND) 1 and 21. In male offspring, 12.5 and 25 mg/kg carbendazim increased anogenital distance (AGD), an androgen-dependent marker, on PND 2. Treatment with benomyl also increased AGD. Cotreatment with 25 mg/kg carbendazim with 0.6, 2.5, and 10 mg/kg flutamide blocked the androgenic effect on AGD induced by carbendazim. The androgenic effects of carbendazim and benomyl on AGD were reversible on PND 22 and later. Carbendazim had no effects on other androgen-dependent markers including testis and epididymis malformations, hypospadias, nipple retention, and organ weights of seminal vesicle and *levator ani bulbocavernosus* muscle on PND 56. Surprisingly, carbendazim antagonized the antiandrogenic effects on these markers induced by flutamide cotreatment. In female offspring, carbendazim produced synergistic effects on the flutamide cotreatment-mediated increases of organs weights in liver and kidney on PND 56. Carbenazim had no marked effects on female reproductive organs. These findings show that carbendazim exposure *in utero* displays a transient and weak androgenic effect and reduces flutamide antiandrogenicity in male rats. The fungicide enhances flutamide-mediated liver and kidney weight increases in female rats. The antagonistic and synergistic carbendazim and flutamide interactions *in utero* warrant further investigations.

Key words: carbendazim, benomyl, flutamide, reproductive development, *in utero*

INTRODUCTION

Carbendazim (methyl 2-benzimidazolecarbamate) and benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate] were introduced as commercial fungicides in 1972 (Figure 1). Benomyl is rapidly converted to carbendazim in the environment and in experimental animals. The widely used fungicides have reproductive and developmental toxicity. Administration of carbendazim to male rats induced sloughing of seminiferous epithelium and severe atrophy and occlusions of seminiferous tubule in testis[1-3]. Carbendazim administration in utero decreased litter size and induced irreversible infertility, embryonic death, and growth retardation in rat offspring[4-6]. Benomyl caused reproductive effects such as detachment and sloughing of germ cells, occlusion of efferent ductules in testis, and decreases of tissue weight and sperm count in epididymis of rats[7-9].

Endocrine-disrupting active compounds including pesticides are potential reproductive and developmental toxicants[10,11]. For instance, *in utero* exposure to antiandrogens flutamide (Figure 1), linuron, and *p,p'*-DDE resulted in decrease of anogenital distance (AGD) and retention of nipples in male rat offspring. These antiandrogenic activities were associated with permanent malformations of androgen-dependent reproductive organs such as testis and epididymis[12-14]. Pre- and post-natal exposure to flutamide and linuron decreased seminal vesicle and *levator ani bulbocavernsous* muscle weights in male rats[14,15]. *In utero* administration of androgen testosterone propionate increased AGD at weaning and adulthood, reduced number of areolas and nipples, and induced the presence of prostate tissue in female rats[16]. Exposures to antiandrogen vinclozolin and estrogen methoxychlor promoted an adult testis phenotype of decreased spermtatogenic capacity and male infertility in F_1 to F_4 generations[17]. These studies

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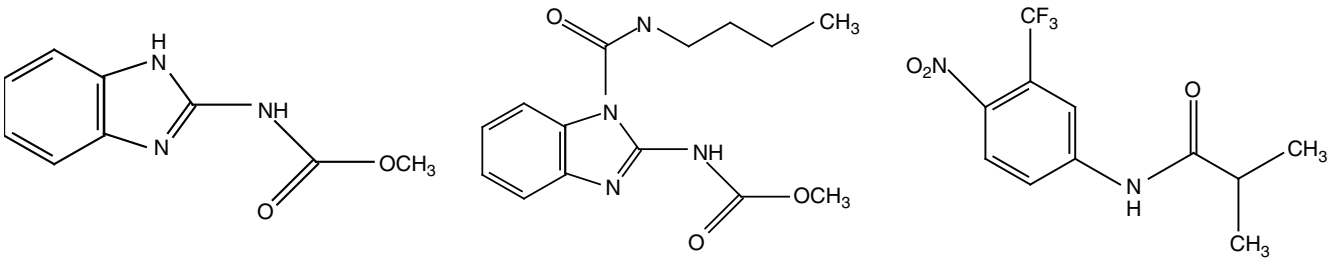

Figure 1. Chemical structures of carbendazim, benomyl and flutamide.

indicate that endocrine disruption may play a mechanistic role in developmental and reproductive toxicity and it is important to define the endocrine-disrupting activity of a xenobiotic.

The endocrine-disrupting activity of carbendazim has not been clearly defined. Treatment of male rats with carbendazim increased testosterone level and androgen binding protein concentration in the interstitial and seminiferous tubule fluid, suggesting an androgenic activity *in vivo*[1]. Premating treatment of male and female rats with carbendazim resulted in androgenic effects including absence of vagina and presence of seminal vesicles in female offspring[18]. Treatment with carbendazim and benomyl increased the activity and mRNA level of aromatase of human ovarian granulose-like tumor cell line KGN, indicating the benzimidazole fungicides were estrogenic *in vitro*[19]. Carbendazim was negative for agonistic and antagonistic activity in reporter gene assays for the human estrogen receptor α and androgen receptor[20]. Direct information regarding the endocrine-disrupting activity of carbendazim exposure *in utero* remains unavailable.

The present study investigated whether exposure to carbendazim during gestation causes reproductive effects and induces alterations of androgen-dependent developmental markers. We hypothesized that carbendazim produces androgenic effects on the developmental markers, which can be reversed by an antiandrogen, flutamide. In these regards, rats were exposed to carbendazim and flutamide *in utero* from gestation day (GD) 0 to 20 alone and in combination. Furthermore, their effects on androgen-dependent endpoints were determined in male and female offspring from postnatal day (PND) 2 to 56. For comparison purposes, a parallel study was conducted using rats treated with benomyl.

MATERIALS AND METHODS

I. *Animals*

Three-week-old male and female Sprague-Dawley rats were purchased from the National Laboratory Animal Center, Taipei, Taiwan. Rats were housed in specific-pathogen-free animal facility in 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% relatively humidity. Animal had access *ad libitum* to deionized water and rodent chow (LabDiet® 5001, PMI Nutrition International, LLC, Brentwood, MO, USA). Upon arrival, rats were quarantined for at least one week and released on the basis of adequate body weight and free from clinical signs of disease or injury. Male and female rats were mated within each treatment group for 14 days. GD 0 was defined as the day that sperm was found in vagina of the mated female. Animal allocation to treatment groups was done by body weight randomization to ensure unbiased weight distribution across groups. Individual dams and offspring were housed in polycarbonate cages on Laboratory Animal Bedding (TCP Chipsi Heimtier Steu, Germany) until weaning PND 21, at which the test subjects were group-housed, up to 5 per cage, by sex and treatment until necropsy on PND 56. Male and female offspring were euthanized by CO_2 asphyxiation and subjected to detailed postmortem examination.

II. *Treatment*

Carbendazim and benomyl (99% pure) were obtained from Sinon Co., Taichung, Taiwan. All other chemicals were obtained from Sigma (St. Louis, MO, USA) unless otherwise noted. Pesticide or flutamide was suspended in corn oil and administered to animals orally by gavage in a volume of 2.5 mL/kg body weight, once daily. From GD 0 to 20, 5 dams per dose were treated with carbendazim at 6.25, 12.5, and 25 mg/kg; benomyl at 25, 50, and 100 mg/kg; or flutamide at 0.6, 2.5, and 10 mg/kg. Alternatively, rats were cotreated with 25 mg/kg carbendazim and 0.6, 2.5, and 10 mg/kg flutamide or with 100 mg/kg benomyl and 0.6, 2.5 or 10 mg/kg flutamide. Dams were examined daily for clinical signs of toxicity. Dam body weight and food consumption were monitored daily throughout dosing and lactation. Rat offspring were weaned at PND 21 and fed up to 8-week-old. Dam organ weight was determined on PND 21. The conception rate during GD 21 and 22,

proportion of pups born alive on PND 1, proportion of pups surviving to weaning on PND 21, and sex ratio on PND 56 were calculated.

III. *Androgen-dependent Reproductive Development End Points*

The androgen-dependent reproductive end points 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, vaginal opening, and organ weight and malformation on PND 56[13,21]. Pups were counted and examined for signs of clinical toxicity on PND 0 and were individually identified by tail-labels on PND 21. AGD, and live male and female offspring weights for all pups were measured on PND 2, 22, and 42. Age of completion of preputial separation and body weight in male offspring during PND 40 and 50 were also measured. Age of onset of vaginal opening and body weight 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, absent prostate or/and seminal vesicle, bladder stone, and underdevelopment of *levator ani bulbocavernosus* muscle in male offspring were recorded on PND 56.

IV. *Necropsy of Dams*

Pups were weaned on PND 21. Dams were euthanized by CO_2 asphyxiation. Body and organ weights, liver, kidneys, adrenals, uterus, ovaries, thyroids and number of implantation sites were recorded on PND 21.

V. *Necropsy of F1 Animals*

Male and female offspring on PND 56 were euthanized by CO_2 asphyxiation and trunk blood was collected. Following blood collection, the ventral surface of the test subject was shaved for counting the number of nipples. The external genitalia, including the scrotum, prepuce, and penis in male offspring and vaginal in female offspring were visually inspected. Gross internal examination of the reproductive tract included inspection of the testes, epididymides, prostate, seminal vesicles, *levator ani bulbocavernosus* muscle, and penis. Additionally, the liver, kidneys, adrenal glands and thyroids were grossly examined and weighed. Body and

Table 1. Effects of *in utero* exposures to carbendazim, benomyl, and flutamide on live pups and sex ratio of male and female offspring on PND 1, PND 21, and PND 56

Treatment	Dose (mg/kg)	Number of litters	Proportion of live pups on PND 1	Proportion of survival pups on PND 21	Live offspring per litter on PND 56		Sex ratio of live offspring on PND 56
					Male	Female	
Control		5	0.97 ± 0.04	0.97 ± 0.05	5.8 ± 1.6	6.8 ± 2.3	0.47 ± 0.15
Carbendazim	6.25	5	0.82 ± 0.06*	0.80 ± 0.05*	4.6 ± 1.5	5.0 ± 2.9	0.48 ± 0.14
	12.5	5	0.83 ± 0.05*	0.80 ± 0.04*	5.7 ± 2.3	5.7 ± 1.5	0.49 ± 0.17
	25	5	0.73 ± 0.06*	0.70 ± 0.04*	3.8 ± 3.3	2.5 ± 1.3*	0.49 ± 0.35
Benomyl	25	5	0.80 ± 0.06*	0.79 ± 0.05*	5.6 ± 2.2	5.0 ± 4.3	0.58 ± 0.29
	50	4	0.86 ± 0.13*	0.84 ± 0.14*	6.0 ± 0.0	7.0 ± 1.4	0.46 ± 0.05
	100	5	0.00 ± 0.00	N.A.[a]	N.A.	N.A.	N.A.
Flutamide	0.6	5	0.78 ± 0.04*	0.75 ± 0.06*	7.0 ± 1.7	5.3 ± 2.3	0.57 ± 0.13
	2.5	5	0.89 ± 0.04*	0.87 ± 0.05*	6.3 ± 2.9	5.5 ± 2.6	0.54 ± 0.16
	10	5	0.91 ± 0.03*	0.88 ± 0.04*	7.0 ± 1.7	6.0 ± 2.0	0.54 ± 0.11
Carbendazim + Flutamide	25 + 0.6	5	0.80 ± 0.05*†	0.79 ± 0.06*†	6.6 ± 3.0	6.0 ± 1.2†‡	0.51 ± 0.14
	25 + 2.5	4	0.81 ± 0.05*†	0.79 ± 0.05*†	6.2 ± 2.2	3.8 ± 3.6*	0.70 ± 0.28
	25 + 10	4	0.71 ± 0.05*‡	0.70 ± 0.06*‡	6.5 ± 2.1	5.0 ± 2.8	0.57 ± 0.22
Benomyl + Flutamide	100 + 0.6	4	0.83 ± 0.10*	0.82 ± 0.08*	6.0 ± 3.9	4.5 ± 3.1	0.66 ± 0.24
	100 + 2.5	4	0.90 ± 0.07*	0.88 ± 0.05*	7.0 ± 2.6	5.0 ± 1.2	0.57 ± 0.10
	100 + 10	5	0.85 ± 0.07*	0.84 ± 0.08*	5.6 ± 3.3	3.8 ± 2.7	0.58 ± 0.24

Pregnant Sprague-Dawley rats were treated orally with carbendazim, benomyl, and flutamide at the designated doses once daily from GD 0 to 20. Control rats were treated with corn oil only. Pregnant dams were kept to delivery of the offspring at term. Rat offspring were weaned on PND 21 and fed up to 8 weeks old. Number of live pups per litter and sex ratio at 8-week-old are presented as mean ± SD for number of litters.

[a]N.A.: not available due to embryotoxicity of benomyl.

*Value is significantly different from the control value, $p < 0.05$.

†Value is significantly different from the respective value of treatment with 25 mg/kg carbendazim alone, $p < 0.05$.

‡Value is significantly different from the respective value of treatment with flutamide alone, $p < 0.05$.

organ weights including testes, epididymides, prostate, seminal vesicles with fluid, *levator ani bulbocavernosus* muscle, and penis, liver, kidneys, adrenals and thyroids were collected. Tissues were fixed in 10% neutral buffered formalin, processed, sectioned, and stained with hematoxylin and eosin.

VI. *Statistical Analysis*

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

RESULTS

Treatment with 6.25, 12.5, and 25 mg/kg carbendazim; 25, 50, and 100 mg/kg benomyl; and 0.6, 2.5, and 10 mg/kg flutamide from GD 0 to 20 had no effects on maternal food consumption, body weight, and implantation site (data not shown). On PND 1, carbendazim at the doses of 6.25, 12.5, and 25 mg/kg resulted in 15%, 14%, and 25% decreases of live pups born, respectively (Table 1). Benomyl at 25, 50, and 100 mg/kg produced 19%, 13%, and 100% decreases in live pups. Treatment with 0.6, 2.5, and 10 mg/kg flutamide caused 20%, 8%, and 6% drop, respectively. On PND 21, carbendazim, benomyl, and flutamide decreased survival rate, in manners similar to the decreases observed on PND 1. On PND 56, the numbers of live male and female rats per litter and sex ratio in the variously single treatment groups were similar to the respective controls with the exception that 25 mg/kg carbendazim decreased number of female rats by 63%. On the other hand, cotreatment of 25 mg/kg carbendazim with 0.6 and 2.5 mg/kg flutamide increased the survival rate by 10% and 11% on PND 1 and by 13% on PND 21, respectively, compared to that of treatment with 25 mg/kg carbendazim alone. Cotreatment with carbendazim and flutamide had no effects on the numbers of live male rats per litter and sex ratio on PND 56, compared to treatment with carbendzim. Cotreatment with carbendazim and 0.6 mg/kg flutamide resulted in a 140% increase of live female rats per litter, relative to treatment with carbendazim. Cotreatment with 100 mg/kg benomyl and flutamide blocked the embryotoxicity induced by treatment with benomyl. Carbendazim, benomyl, and flutamide had no marked effects on maternal organ weights of ovary, uterus, liver, kidney, adrenal, and thyroid on PND 21 (data not shown). The later studies had no results from rats cotreated with benomyl and flutamide because no comparisons could be made with the results from rats treated with benomyl alone due to embryotoxicity.

Treatment with 6.25, 12.5, and 25 mg/kg carbendazim produced no effect, 8%, and 4% increases of AGD in male rats on PND 2, respectively (Table 2). Benomyl at 25 and 50 mg/kg produced 4% and 9% increases. In contrast, treatment with the antiandrogen flutamide at 0.6, 2.5, and 10 mg/kg caused 4%, 10%, and 16% decreases. On PND 22 and 42, carbendazim and benomyl had no effects on AGD and flutamide caused dose-dependent decreases. Cotreatment with carbendazim and 0.6, 2.5, and 10 mg/kg flutamide did not produce dose-related changes of AGD, compared to treatment with carbendazim alone on PND 2. AGD of rats cotreated with carbendazim and fluamide was 43% to 77% greater than that of rats treated with flutamide alone on PND 22 and was 7% to 10% greater on PND

Table 2. Effects of *in utero* exposures to carbendazim, benomyl, and flutamide on AGD of male offspring on PND 2, PND 22, and PND 42

Treatment	Dose (mg/kg)	Number of rats	AGD (cm)		
			PND 2	PND 22	PND 42
Control		30	0.58 ± 0.05	1.82 ± 0.13	3.91 ± 0.31
Carbendazim	6.25	23	0.58 ± 0.03	1.83 ± 0.13	4.05 ± 0.31
	12.5	17	0.62 ± 0.01*	1.86 ± 0.14	3.87 ± 0.37
	25	14	0.60 ± 0.03*	1.91 ± 0.17	3.87 ± 0.26
Benomyl	25	20	0.60 ± 0.03*	1.81 ± 0.15	3.89 ± 0.18
	50	12	0.63 ± 0.04*	1.91 ± 0.19	3.92 ± 0.25
Flutamide	0.6	16	0.55 ± 0.05*	1.32 ± 0.15*	3.91 ± 0.40
	2.5	25	0.52 ± 0.01*	1.23 ± 0.13*	3.72 ± 0.35*
	10	19	0.49 ± 0.03*	1.10 ± 0.13*	3.29 ± 0.25*
Carbendazim + Flutamide	25 + 0.6	31	0.57 ± 0.08	2.05 ± 0.37‡	4.31 ± 0.30‡
	25 + 2.5	15	0.57 ± 0.03	1.75 ± 0.62‡	3.98 ± 0.44‡
	25 + 10	13	0.53 ± 0.02	1.95 ± 0.70‡	3.60 ± 0.35‡

Pregnant Sprague-Dawley rats were treated orally with carbendazim, benomyl, and flutamide at the designated doses once daily from GD 0 to 20. Control rats were treated with corn oil only. AGD of offspring was measured from PND 2 to 42. Data are presented as mean ± SD for number of rats.

*Value is significantly different from the control value, $p < 0.05$.

‡Value is significantly different from the respective value of treatment with flutamide alone, $p < 0.05$.

42. These data indicated that carbendazim reduced the antiandrogenic effects of flutamide in the cotreated rats. Treatment with 6.25, 12.5 and 25 mg/kg carbendazim delayed the age at preputial separation in male rats by 4%, 5%, and 4%, respectively, during PND 40 to 50 (Table 3). Treatment with 25 mg/kg benomyl caused an 8% delay. Flutamide at 0.6 and 2.5 mg/kg had no effects on the age of reproductive development. The antiandrogen at 10 mg/kg caused hypospadias and consequently preputial separation was not measured. Cotreatment with carbendazim and flutamide did not produce dose-related changes of preputial separation age, relative to that of treatment with carbendazim alone.

Exposure to 6.25, 12.5, and 25 mg/kg carbendazim or 25 and 50 mg/kg benomyl had no effects on nipple retention in male rats on PND 56 (Table 4). Exposure to

Table 3. Effects of *in utero* exposures to carbendazim, benomyl, and flutamide on sexual development in male offspring PND 40 to 50

Treatment	Dose (mg/kg)	Number of rats	Preputial separation	
			Age of completion (day)	Body weight (g)
Control		30	43.1 ± 1.6	158.1 ± 19.2
Carbendazim	6.25	23	44.7 ± 2.1*	190.8 ± 20.3*
	12.5	17	45.3 ± 2.2*	165.0 ± 19.1
	25	14	44.9 ± 1.6*	195.4 ± 23.8*
Benomyl	25	20	46.6 ± 2.5*	176.9 ± 20.7*
	50	12	43.0 ± 1.8	160.4 ± 11.8
Flutamide	0.6	16	43.6 ± 1.2	157.8 ± 21.0
	2.5	25	43.7 ± 0.9	164.3 ± 22.0
	10	19	N.A.[a]	N.A.
Carbendazim + Flutamide	25 + 0.6	31	45.3 ± 2.3*‡	165.6 ± 16.2†‡
	25 + 2.5	15	44.4 ± 2.1	175.5 ± 16.0*†
	25 + 10	13	45.0 ± 2.5*	179.4 ± 11.7*†

Pregnant Sprague-Dawley rats were treated orally with carbendazim, benomyl, and flutamide at the designated doses once daily from GD 0 to 20. Control rats were treated with corn oil only. Age of completion for preputial separation and body weight in male offspring following *in utero* exposure to carbendazim, benomyl, and flutamide singly or in combination were measured during PND 40 to 50. Data are presented as mean ± SD for number of rats.

[a]N.A.: not available; male offspring showed hypospadias and preputial separation was not determined.

*Value is significantly different from the control value, $p < 0.05$.

†Value is significantly different from the respective value of treatment with 25 mg/kg carbendazim alone, $p < 0.05$.

‡Value is significantly different from the respective value of treatment with flutamide alone, $p < 0.05$.

Table 4. Effects of *in utero* exposures to carbendazim, benomyl, and flutamide on nipple retention in male offspring on PND 56

Treatment	Dose (mg/kg)	Incidence of nipple retention				
		Pair of nipple				Number of nipples per pup
		0	1	2	3	
Control		30/30 (5/5)[a]	0/30 (0/5)	0/30 (0/5)	0/30 (0/5)	0
Carbendazim	6.25	23/23 (5/5)	0/23 (0/5)	0/23 (0/5)	0/23 (0/5)	0
	12.5	17/17 (5/5)	0/17 (0/5)	0/17 (0/5)	0/17 (0/5)	0
	25	14/14 (5/5)	0/14 (0/5)	0/14 (0/5)	0/14 (0/5)	0
Benomyl	25	20/20 (5/5)	0/20 (0/5)	0/20 (0/5)	0/20 (0/5)	0
	50	12/12 (4/4)	0/12 (0/4)	0/12 (0/4)	0/12 (0/4)	0
Flutamide	0.6	16/16 (5/5)	0/16 (0/5)	0/16 (0/5)	0/16 (0/5)	0
	2.5	24/25 (5/5)	0/25 (0/5)	0/25 (0/5)	1/25 (1/5)	0.24 ± 1.20
	10	2/19 (1/5)	2/19 (1/5)	15/19 (5/5)	0/19 (0/5)	3.37 ± 1.34*
Carbendazim + Flutamide	25 + 0.6	31/31 (5/5)	0/31 (0/5)	0/31 (0/5)	0/31 (0/5)	0
	25 + 2.5	15/15 (4/4)	0/15 (0/4)	0/15 (0/4)	0/15 (0/4)	0
	25 + 10	13/13 (4/4)	0/13 (0/4)	0/13 (0/4)	0/13 (0/4)	0 ‡

Pregnant Sprague-Dawley rats were treated orally with carbendazim, benomyl, and flutamide at the designated doses once daily from GD 0 to 20. Control rats were treated with corn oil only. Nipple retention of male offspring was measured on PND 56. Data are presented as mean ± SD for number of rats.

[a]Litter incidence in parenthesis.

*Value is significantly different from the control value, $p < 0.05$.

‡Value is significantly different from the respective value of treatment with flutamide alone, $p < 0.05$.

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10 mg/kg flutamide resulted in a marked increase of nipple retention, whereas the 0.6 and 2.5 mg/kg exposures had no effects. Cotreatment with carbendazim and flutamide blocked the flutamide-mediated increase of nipple retention, indicating an antagonistic interaction between the fungicide and the antiandrogen.

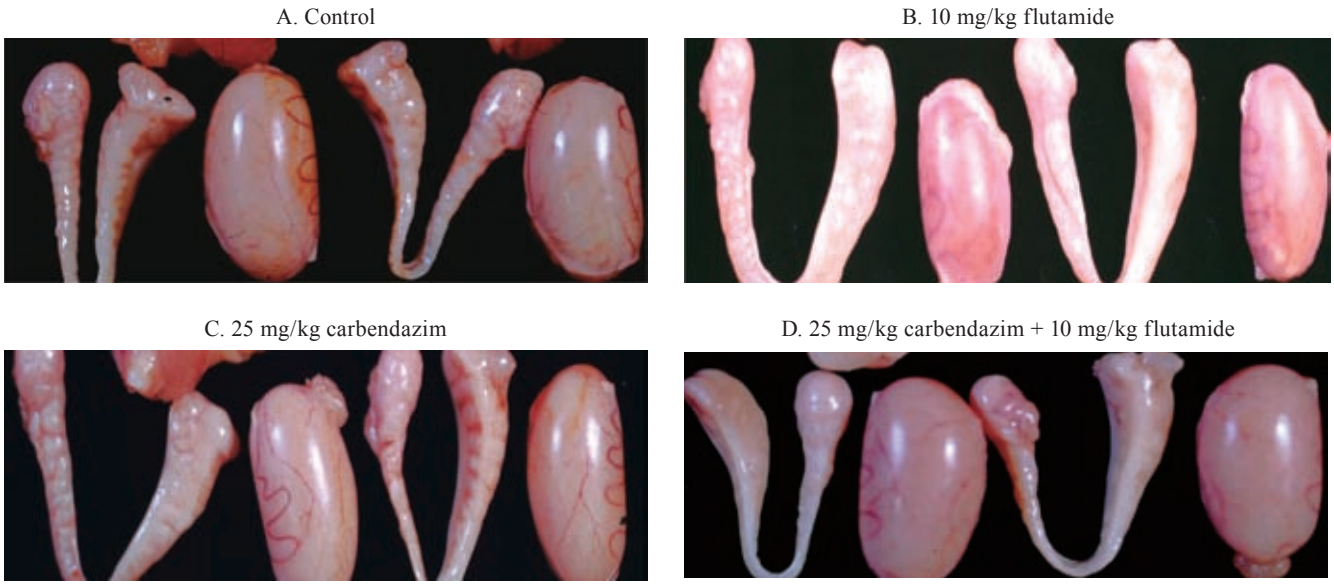

Figure 2. Effects of *in utero* exposures to carbendazim and flutamide on gross morphology of testes and epididymides in male offspring on PND 56. Pregnant female rats were administered orally with carbendazim and flutamide alone or in combination from GD 0 to 20. Control rats were treated with corn oil only. Male offspring were sacrificed on PND 56. In comparison with control (A), testis and epididymis of rats treated with 10 mg/kg flutamide (B) showed inflammation. Histopathology scores of testis and epididymis of rats treated with flutamide were higher than those of controls, rats treated with carbendazim, and rats cotreated with carbendazim and flutamide (data not shown). The sizes of testes rats treated with 25 mg/kg carbendazim (C) were smaller than those of controls. Morphology and sizes of testis and epididymis from rats cotreated with 25 mg/kg carbendazim and 10 mg/kg flutamide (D) were comparable to those of controls.

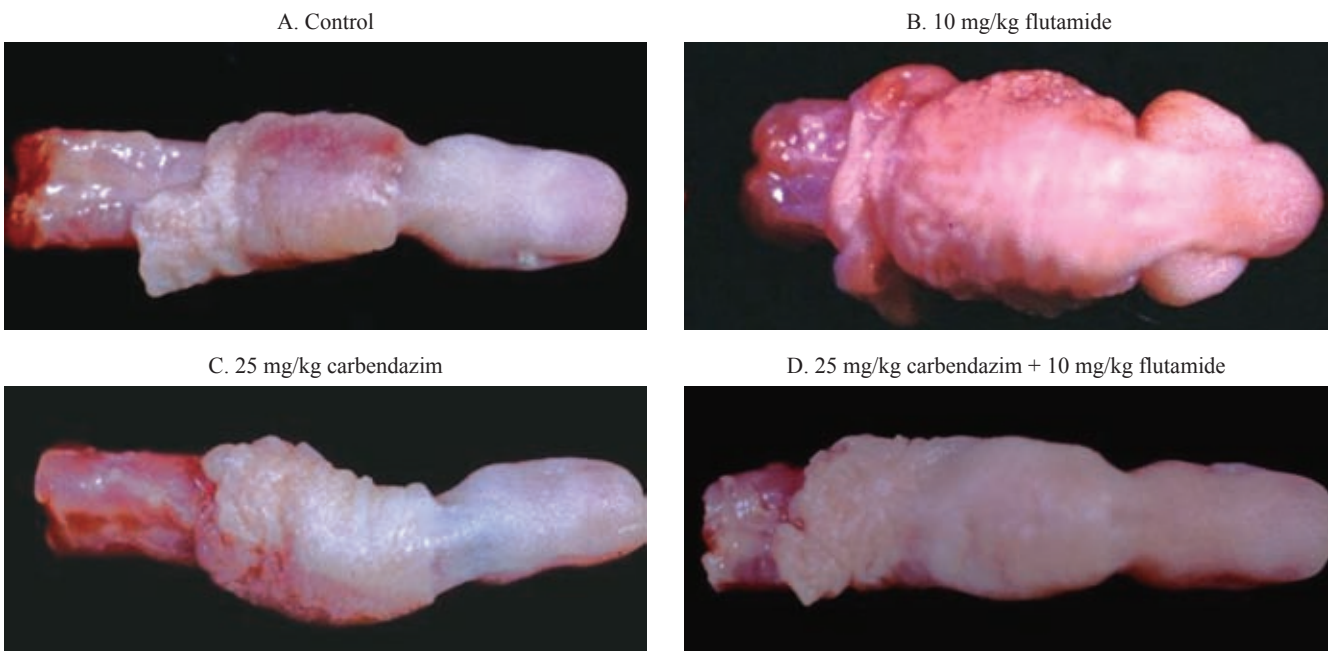

Figure 3. Effects of *in utero* exposures to carbendazim and flutamide on gross morphology of penis in male offspring on PND 56. Sprague-Dawley rats were administered orally with carbendazim and flutamide alone or in combination once daily from GD 0 to 20. Control rats were treated with corn oil only. Male offspring rat were sacrificed on PND 56. Penis of rats treated with 10 mg/kg flutamide (B) showed hypospadias, in contrast to controls (A). Morphology of penis of rats treated with 25 mg/kg carbendazim (C) was not different from that of controls. Morphology of penis of rats cotreated with 25 mg/kg carbendazim and 10 mg/kg flutamide and 25 mg/kg carbendazim (D) displayed a recovery from hypospadias induced by 10 mg/kg flutamide (B).

The gross examinations of androgen-dependent organs of male rats on PND 56 showed that exposures to 6.25, 12.5, and 25 mg/kg carbendazim or 25 and 50 mg/kg benomyl increased formations of abnormal testis and epididymis and bladder stone. However, the increases were not statistically significant (Table 5). The testis and epididymis abnormalities included size reduction, malformed morphology, and inflammation (Figure 2). Exposure to 0.6, 2.5, and 10 mg/kg flutamide caused increasing incidences of abnormal testis and

Table 5. Effects of exposures to carbendazim, benomyl, and flutamide on gross morphology of reproductive organs of male offspring on PND 56

Treatment	Dose (mg/kg)	Incidence of malformations[a]						
		A	B	C	D	E	F	G
Control		0/30 (5/5)[b]	0/30 (0/5)	0/30 (0/5)	0/30 (0/5)	0/30 (0/5)	0/30 (0/5)	0/30 (0/5)
Carbendazim	6.25	0/23 (5/5)	0/23 (0/5)	0/23 (0/5)	0/23 (0/5)	0/23 (0/5)	0/23 (0/5)	0/23 (0/5)
	12.5	2/17 (1/5)	2/17 (1/5)	0/17 (0/5)	0/17 (0/5)	0/17 (0/5)	0/17 (0/5)	0/17 (0/5)
	25	1/14 (1/5)	1/14 (1/5)	0/14 (0/5)	0/14 (0/5)	0/14 (0/5)	3/14 (2/5)	0/14 (0/5)
Benomyl	25	0/20 (0/5)	0/20 (0/5)	0/20 (0/5)	0/20 (0/5)	0/20 (0/5)	1/20 (1/5)	0/20 (0/5)
	50	1/12 (1/4)	1/12 (1/4)	0/12 (0/4)	0/12 (0/4)	0/12 (0/4)	0/12 (0/4)	0/12 (0/4)
Flutamide	0.6	3/16 (2/5)	3/16 (2/5)	0/16 (0/5)	0/16 (0/5)	0/16 (0/5)	1/16 (1/5)	0/16 (0/5)
	2.5	3/25 (2/5)	3/25 (2/5)	7/25 (3/5)*	0/25 (0/5)	0/25 (0/5)	2 /25 (1/5)	0/25 (0/5)
	10	7/19 (4/5)*	7/19 (4/5)*	18/19 (5/5)*	1/19 (1/5)	1/19 (1/5)	1/19 (1/5)	0/19 (0/5)
Carbendazim + Flutamide	25 + 0.6	5/31 (3/5)	5/31 (3/5)	0/31 (0/5)	0/31 (0/5)	0/31 (0/5)	0/31 (0/5)	0/31 (0/5)
	25 + 2.5	1/15 (1/4)	1/15 (1/4)	0/15 (0/4)‡	0/15 (0/4)	0/15 (0/4)	0/15 (0/4)	0/15 (0/4)
	25 + 10	0/13 (0/4)‡	0/13 (0/4)‡	9/13 (4/4)*‡	1/13 (1/4)	1/13 (1/4)	2/13 (1/4)	0/13 (0/4)

Pregnant Sprague-Dawley rats were treated orally with carbendazim, benomyl, and flutamide at the designated doses once daily from GD 0 to 20. Control rats were treated with corn oil only. Rat offspring were weaned on PND 21 and incidence of abnormal androgen-dependent tissue was determined on PND 56. Data are presented as mean ± SD for number of rats.

[a]Parameters of malformations: A: testis displaying small size, malformed morphology, and inflammation; B: epididymis displaying small size, malformed morphology, and inflammation; C: hypospadias in penis; D: underdeveloped prostate or/and seminal vesicle; E: absent prostate or/and seminal vesicle; F: bladder stone; G: underdeveloped *levator ani bulbocavernosus* muscle.

[b]Litter incidence in parenthesis.

*Value is significantly different from the control value, $p < 0.05$.

‡Value is significantly different from the respective value of treatment with flutamide alone, $p < 0.05$

Table 6. Effects of *in utero* exposures to carbendazim, benomyl, and flutamide on organ weights in male offspring on PND 56

Treatment	Dose (mg/kg)	N[a]	Body weight (g)	Testis	Epididymis	Kidney	Adrenal	Liver
				Organ weight/body weight ratio (g/g × 100)				
Control		30	429.2 ± 30.7	0.95 ± 0.08	0.32 ± 0.03	1.12 ± 0.09	0.02 ± 0.00	5.09 ± 0.49
Carbendazim	6.25	23	432.9 ± 31.6	0.93 ± 0.08	0.31 ± 0.03	1.12 ± 0.08	0.02 ± 0.00	5.05 ± 0.51
	12.5	17	382.5 ± 42.7*	1.00 ± 0.11	0.32 ± 0.04	1.19 ± 0.16	0.02 ± 0.00	4.35 ± 0.63*
	25	14	444.5 ± 60.9	0.88 ± 0.18	0.34 ± 0.06	1.11 ± 0.11	0.02 ± 0.00	4.58 ± 0.40*
Benomyl	25	20	431.3 ± 63.8	0.86 ± 0.09*	0.33 ± 0.03	1.05 ± 0.08*	0.02 ± 0.00	4.57 ± 0.59*
	50	12	419.9 ± 33.8	0.86 ± 0.10*	0.30 ± 0.05	1.09 ± 0.07	0.01 ± 0.00*	4.89 ± 0.32
Flutamide	0.6	16	370.6 ± 31.9*	0.93 ± 0.19	0.35 ± 0.06	1.15 ± 0.08	0.02 ± 0.00	4.77 ± 0.49
	2.5	25	377.7 ± 77.6*	0.90 ± 0.19*	0.34 ± 0.05*	1.18 ± 0.13	0.01 ± 0.00*	4.53 ± 0.42*
	10	19	414.3 ± 46.9	0.81 ± 0.24*	0.33 ± 0.04	1.18 ± 0.11	0.02 ± 0.01	4.79 ± 0.30*
Carbendazim + Flutamide	25 + 0.6	31	338.9 ± 78.3*†	1.03 ± 0.23*†	0.35 ± 0.09*†	1.15 ± 0.20	0.02 ± 0.01	4.35 ± 0.61*
	25 + 2.5	15	387.2 ± 18.3*†	1.02 ± 0.07*‡	0.33 ± 0.03	1.30 ± 0.09‡	0.02 ± 0.00‡	4.64 ± 0.18*
	25 + 10	13	337.0 ± 31.9*†‡	1.14 ± 0.28*‡	0.37 ± 0.07*†‡	1.36 ± 0.20*‡	0.02 ± 0.01	4.90 ± 0.68

Pregnant Sprague-Dawley rats were treated orally with carbendazim, benomyl, and flutamide at the designated doses once daily from GD 0 to 20. Control rats were treated with corn oil only. Offspring were weaned on PND 21 and sacrificed on PND 56. Data are presented as mean ± SD for number of rats.

[a]Number of male offspring examined.

*Value is significantly different from the control value, $p < 0.05$.

†Value is significantly different from the respective value of treatment with 25 mg/kg carbendazim alone, $p < 0.05$.

‡Value is significantly different from the respective value of treatment with flutamide alone, $p < 0.05$.

epididymis, hypospadias (Figure 3), bladder stone, and underdevelopment and absence of prostate and/or seminal vesicles. The 10 mg/kg dose group showed 37% increases of testis and epididymis malformations. Exposures to 2.5 and 10 mg/kg flutamide increased incidence of hypospadias by 28% and 95%, respectively. Cotreatment with carbendazim and 0.6, 2.5, and 10 mg/kg flutamide had no effects on organ malformations, compared to treatment with carbendazim. On the other hand, cotreatment with carbendazim and 10 mg/kg flutamide markedly reduced the incidences of testis and epididymis malformations and hypospadias induced by flutamide (Table 5, Figure 2 and Figure 3).

Treatment with carbendazim, benomyl, and flutamide did not produce dose-related changes of body weight or the organ weights of epididymis, adrenal, liver, and kidney in male rats on PND 56 (Table 6). In testis, carbendazim had no effects; 25 and 50 mg/kg benomyl

Table 7. Effects of *in utero* exposures to carbendazim, benomyl, and flutamide on weights of reproductive organs in male offspring on PND 56

Treatment	Dose (mg/kg)	N[a]	Prostate	Seminal vesicle	*Levator ani bulbocavernosus* muscle	Penis
			Organ weight/body weight ratio (g/g × 100)			
Control		30	0.09 ± 0.02	0.60 ± 0.08	0.42 ± 0.08	0.08 ± 0.01
Carbendazim	6.25	23	0.09 ± 0.02	0.59 ± 0.08	0.41 ± 0.08	0.08 ± 0.01
	12.5	17	0.08 ± 0.02	0.62 ± 0.09	0.44 ± 0.07	0.09 ± 0.01
	25	14	0.09 ± 0.02	0.66 ± 0.11	0.38 ± 0.07	0.09 ± 0.01
Benomyl	25	20	0.11 ± 0.03	0.61 ± 0.10	0.39 ± 0.07	0.08 ± 0.02
	50	12	0.10 ± 0.02	0.62 ± 0.06	0.42 ± 0.08	0.09 ± 0.01
Flutamide	0.6	16	0.09 ± 0.02	0.67 ± 0.08*	0.45 ± 0.06	0.09 ± 0.01*
	2.5	25	0.09 ± 0.03	0.58 ± 0.07	0.38 ± 0.06*	0.08 ± 0.01
	10	19	0.06 ± 0.02*	0.47 ± 0.16*	0.24 ± 0.09*	0.08 ± 0.02
Carbendazim + Flutamide	25 + 0.6	31	0.09 ± 0.04†	0.67 ± 0.17†	0.46 ± 0.09	0.09 ± 0.02†
	25 + 2.5	15	0.08 ± 0.02†	0.64 ± 0.11†	0.44 ± 0.07‡	0.09 ± 0.01‡
	25 + 10	13	0.07 ± 0.02*†	0.53 ± 0.10†	0.28 ± 0.08*†‡	0.08 ± 0.01†

Pregnant Sprague-Dawley rats were treated orally with carbendazim, benomyl, and flutamide at the designated doses once daily from GD 0 to 20. Control rats were treated with corn oil only. Offspring were weaned on PND 21 and sacrificed on PND 56. Data are presented as mean ± SD for male offspring rats.

*Value is significantly different from the control value, $p < 0.05$.

†Value is significantly different from the respective value of treatment with 25 mg/kg carbendazim alone, $p < 0.05$.

‡Value is significantly different from the respective value of treatment with flutamide alone, $p < 0.05$.

Table 8. Effects of *in utero* exposures to carbendazim, benomyl, and flutamide on AGD of female offspring on PND 2, PND 22, and PND 42

Treatment	Dose (mg/kg)	Number of rats	AGD (cm)		
			PND 2	PND 22	PND 42
Control		31	0.42 ± 0.06	1.26 ± 0.01	2.12 ± 0.01
Carbendazim	6.25	12	0.43 ± 0.05	1.02 ± 0.01	1.83 ± 0.14
	12.5	12	0.43 ± 0.06	0.94 ± 0.01	1.72 ± 0.15*
	25	12	0.45 ± 0.08	0.95 ± 0.01	1.88 ± 0.08
Benomyl	25	21	0.42 ± 0.04	0.99 ± 0.01	1.71 ± 0.14*
	50	4	0.43 ± 0.08	0.88 ± 0.01	1.81 ± 0.08
Flutamide	0.6	14	0.37 ± 0.06*	0.95 ± 0.03*	1.85 ± 0.13
	2.5	20	0.32 ± 0.05*	0.87 ± 0.01*	1.73 ± 0.14*
	10	18	0.30 ± 0.05*	0.81 ± 0.01*	1.73 ± 0.11*
Carbendazim + Flutamide	25 + 0.6	18	0.41 ± 0.08	1.06 ± 0.10*	2.44 ± 0.20*†‡
	25 + 2.5	11	0.31 ± 0.01*†	0.94 ± 0.12*	2.07 ± 0.33‡
	25 + 10	10	0.38 ± 0.08*†‡	1.12 ± 0.09*	2.07 ± 0.33‡

Pregnant Sprague-Dawley female rats were treated orally with carbendazim, benomyl, and flutamide at the designated doses once daily from GD 0 to 20. Control rats were treated with corn oil only. AGD of female offspring was measured from PND 2 to 42. Data are presented as mean ± SD for number of rats.

*Value is significantly different from the control value, $p < 0.05$.

†Value is significantly different from the respective value of treatment with 25 mg/kg carbendazim alone, $p < 0.05$.

‡Value is significantly different from the respective value of treatment with flutamide alone, $p < 0.05$.

Table 9. Effects of *in utero* exposures to carbendazim, benomyl, and flutamide on sexual development in female offspring during PND 30 and 45

Treatment	Dose (mg/kg)	Number of rats	Vaginal opening	
			Age of onset (day)	Body weight (g)
Control		31	37.2 ± 2.2	110.8 ± 26.5
Carbendazim	6.25	12	35.2 ± 1.7*	107.0 ± 11.6
	12.5	12	37.6 ± 4.8	108.0 ± 9.9
	25	12	34.2 ± 1.5*	115.8 ± 7.7
Benomyl	25	21	35.9 ± 2.4	99.4 ± 12.2*
	50	4	34.0 ± 1.1*	108.9 ± 5.6
Flutamide	0.6	14	33.9 ± 1.8*	99.0 ± 9.5*
	2.5	20	35.0 ± 3.6*	97.8 ± 8.1*
	10	18	36.6 ± 2.8	91.0 ± 25.7*
Carbendazim + Flutamide	25 + 0.6	18	35.7 ± 2.4‡	104.8 ± 12.6†
	25 + 2.5	11	36.2 ± 2.5†	107.8 ± 12.4‡
	25 + 10	10	34.8 ± 1.2*	114.7 ± 6.7‡

Age and body weight of onset for vaginal opening in the female offspring following *in utero* exposure to carbendazim, benomyl, and flutamide at the designated doses once daily from GD 0 to GD 20 were measured from PND 30 to PND 40. Data are presented as mean ± SD for number of rats.

*Value is significantly different from the control value, $p < 0.05$.

†Value is significantly different from the respective value of treatment with 25 mg/kg carbendazim alone, $p < 0.05$.

‡Value is significantly different from the respective value of treatment with flutamide alone, $p < 0.05$.

caused 10% and 9% decreases; and 0.6, 2.5, and 10 mg/kg flutamide resulted in no effect, 5%, and 14% decreases of organ weight, respectively. Cotreatment with 25 mg/kg carbendazim and 0.6, 2.5, or 10 mg/kg flutamide produced 8%, 7%, and 21% increases, respectively, relative to controls. The 0.6 mg/kg flutamide cotreatment increased testis weight by 16%, compared to that of carbendazim treatment alone. The 2.5 and 10 mg/kg flutamide cotreatment produced 22% and 41% increases, compared to those of the respective flutamide treatment alone. In epididymis, cotreatment with carbendazim and 10 mg/kg flutamide resulted in 16%, 9%, and 12% increases of tissue weight relative to controls, carbendazim, and flutamide treatment, respectively. In kidney, the 10 mg/kg flutamide cotreatment resulted in 21%, 23%, and 15% increases in organ weight, respectively.

Carbendazim and benomyl treatment did not have dose-dependent effects on organ weights of prostate, seminal vesicle, *levator ani bulbocavernosus*, and penis in male rats (Table 7). Treatment with 2.5 and 10 mg/kg flutamide decreased *levator ani bulbocavernosus* muscle weight by 10% and 44%, respectively. The flutamide treatment had no dose-related effects on the other organ weights. Cotreatment with 25 mg/kg carbendazim and 2.5 or 10 mg/kg flutamide produced 16% and 12% increases of *levator ani bulbocavernosus* muscle weight, relative to those of the respective flutamide treatment.

Exposures to 6.25, 12.5, and 25 mg/kg carbendazim or 25 and 50 mg/kg benomyl did not produce marked effects on AGD in female rats on PND 2, 22, and 42 (Table 8). Exposures to 0.6, 2.5 and 10 mg/kg flutamide decreased the reproductive development marker by 12%, 24%, and 30% on PND 2 and 24%, 31%, and 35% on PND 22, respectively. Exposures to 2.5 and 10 mg/kg flutamide caused 18% decreases of AGD on PND 42. Coexposures to carbendazim and 0.6, 2.5, and 10 mg/kg flutamide resulted in 30%, 10%, and 10% increases of AGD, compared to carbendazim exposure on PND 42, respectively. The respective coexposures produced 32%, 19%, and 20% increases of AGD, compared to flutamide exposure alone. Treatment with carbendazim, benomyl, and flutamide *in utero* did not produce dose-related changes of the age at vaginal opening during PND 30 and 45 (Table 9). Cotreatment with carbendazim and flutamide did not produce significant changes of the female reproductive development marker.

Treatment with carbendazim, benomyl, and flutamide did not alter the body weights of female rats on PND 56 (Table 10). Carbendazim had no marked or dose-related effects on the organ weights of ovary, uterus, adrenal, kidney, and liver. In contrast, treatment with 25 and 50 mg/kg benomyl increased ovary weight by 17% and 35% and kidney weight by 39% and 35%, respectively. The 25 mg/kg benomyl treatment increased liver weight by 14%. Treatment with benomyl and flutamide had no effects on uterus or adrenal weights. Flutamide at 0.6 and 2.5 mg/kg produced 35% and 22% increases of ovary weight, respectively. In liver, treatment with 0.6, 2.5, and 10 mg/kg flutamide produced 12%, 22%, and 25% increases of tissue weight, respectively. In kidney, treatment with 2.5 and 10 mg/kg flutamide led to 35% increases of organ weight, while the 0.6 mg/kg treatment had no effects. Cotreatment with carbendazim and 0.6, 2.5, and 10 mg/kg flutamide resulted in 54%, 54%, and 57% increases of liver weight, compared to controls; 58%, 57%, and 61% increases, relative to treatment with carbendazim; and

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Table 10. Effects of *in utero* exposures to carbendazim, benomyl, and flutamide on weights of reproductive organs in female offspring on PND 56

Treatment	Dose (mg/kg)	N[a]	Body weight (g)	Ovary	Uterus	Kidney	Adrenal	Liver
				Organ weight/Body weight ratio (g/g × 100)				
Control		31	253.4 ± 39.6	0.06 ± 0.02	0.31 ± 0.16	0.87 ± 0.13	0.03 ± 0.01	3.84 ± 0.53
Carbendazim	6.25	12	256.4 ± 39.1	0.06 ± 0.02	0.30 ± 0.16	0.85 ± 0.12	0.03 ± 0.01	3.79 ± 0.52
	12.5	12	257.1 ± 37.2	0.06 ± 0.02	0.32 ± 0.16	0.85 ± 0.12	0.03 ± 0.01	3.77 ± 0.48
	25	12	258.3 ± 34.0	0.06 ± 0.01	0.35 ± 0.16	0.84 ± 0.11	0.03 ± 0.01	3.75 ± 0.48
Benomyl	25	21	239.2 ± 20.7	0.07 ± 0.01*	0.29 ± 0.10	1.20 ± 0.15*	0.03 ± 0.01	4.37 ± 0.54*
	50	4	239.4 ± 3.7	0.08 ± 0.00*	0.41 ± 0.12	1.17 ± 0.10*	0.03 ± 0.01	4.23 ± 0.40
Flutamide	0.6	14	236.2 ± 26.3	0.08 ± 0.01*	0.31 ± 0.19	0.89 ± 0.07	0.03 ± 0.00	4.28 ± 0.51*
	2.5	20	248.9 ± 31.6	0.07 ± 0.01*	0.25 ± 0.07	1.17 ± 0.13*	0.03 ± 0.00	4.67 ± 0.33*
	10	18	242.8 ± 19.3	0.06 ± 0.01	0.31 ± 0.10	1.17 ± 0.11*	0.04± 0.01	4.78 ± 0.56*
Carbendazim + Flutamide	25 + 0.6	18	224.8± 15.3†	0.08 ± 0.02*†	0.28 ± 0.11†	1.29 ± 0.14*‡	0.04 ± 0.01‡	5.92 ± 0.50*†‡
	25 + 2.5	11	238.7 ± 12.9	0.08 ± 0.01*†‡	0.36 ± 0.18*†‡	1.36 ± 0.07*†‡	0.03 ± 0.01‡	5.90 ± 0.44*†‡
	25 + 10	10	234.2 ± 35.7	0.06 ± 0.01‡	0.23 ± 0.07*‡	1.32 ± 0.15*†‡	0.03 ± 0.01‡	6.02 ± 0.99*†‡

Pregnant Sprague-Dawley rats were treated orally with carbendazim, benomyl, and flutamide at the designated doses once daily from GD 0 to 20. Control rats were treated with corn oil only. Female offspring were weaned on PND 21 and sacrificed on PND 56. Data are presented as mean ± SD for number of rats.

[a]Number of female offspring.

*Value is significantly different from the control value, $p < 0.05$.

†Value is significantly different from the respective value of treatment with 25 mg/kg carbendazim alone, $p < 0.05$.

‡Value is significantly different from the respective value of treatment with flutamide alone, $p < 0.05$.

38%, 26%, and 26% increases, compared to the respective flutamide treatment. Cotreatment with carbendazim and 0.6, 2.5, and 10 mg/kg flutamide resulted in 49%, 57%, and 52% increases, relative to controls and 45%, 16%, and 13% increases, compared to the respective flutamide treatment. The 2.5 and 10 mg/kg flutamide cotreatment resulted in 61% and 56% increases of kidney weight, relative to treatment with carbendazim. These liver and kidney increases indicated synergistic carbendazim and flutamide interactions.

DISCUSSION

The present findings show that exposure to carbendazim *in utero* decreases the viability of offspring and displays androgenic activity in male rats during weaning. The endocrine-disrupting activity is supported by the following lines of evidence. Firstly, carbendazim increased AGD, an androgen-dependent development end point on PND 2. Secondly, the parent compound of carbendazim, benomyl, also increased AGD. Thirdly, the increase was sensitive to the blocking effect of an antiandrogen flutamide. The endocrine-disrupting activity of carbendazim is transient and weak because the increase in male AGD was reversible on PND 22 and 42. In addition, the fungicide had no effects on other androgen-dependent end points determined including preputial separation, nipple retention, and reproductive organ malformation. In female rats, carbendazim exposure *in utero* did not produce marked changes of vaginal opening, AGD, and female reproductive organ weights.

In utero exposure to benomyl at 100 mg/kg resulted in embryotoxicity which was blocked by co-exposures to androgen receptor antagonist flutamide at 0.6, 2.5, and 10 mg/kg. It seems unlikely that the benomyl-induced reproductive effect was entirely caused by chemical toxicity, because benzimidazole fungicides are generally regarded to have low acute and systemic toxicity. For instances, LD_{50} (orally in rats) of benomyl and carbendazim are 10 g/kg and 2-15 g/kg, respectively[22, 23]. The highest doses of benomyl and carbendazim used in the present studies were 1% and 1.6% to 12.5% of their respective LD50. The flutamide doses used in the present study were lower than those used in previous studies of endocrine-disrupting activities of the antiandrogen[14,30]. Therefore, a direct chemical interaction between benomyl and flutamide was probably not a mechanism underlying the blocking effect of flutamide on benomyl embrotoxicity. On the other hand, an androgen receptor-mediated activity may play a significant mechanistic role, because co-exposure to androgen receptor antagonist produced the blocking effect. Alternatively, the effect might be a result of other unidentified activities. For example, Kangasniemi *et al.*[24] reported that cotreatment with flutamide and a gonadotropin-releasing hormone antagonist (Nal-Glu) suppressed rat spermatogenesis to protect spermatogonial stem cells against the anticancer drug procarbazine, possibly as a consequence of altered paracrine regulation. It remains to be investigated whether hormonal regulation or other mechanisms are responsible for the blocking of benomyl embryotoxicity by flutamide.

The carbendazim-induced alteration in male AGD is in marked contrast to the alterations induced by antiandrogens. Administrations of flutamide, linuron, vinclozolin, and procymidone to female pregnant rats during gestation resulted in decreases of AGD in male rats on postnatal days[14,25-27]. The effects of carbendazim on reproductive development are different from those of testosterone propionate, an androgen receptor agonist. Subcutaneous exposure to 0.5 mg of testosterone propionate on GD 13-18 produced a transient decrease in male AGD on PND 2. Exposure to 0.5, 1, 2, and 10 mg of testosterone propionate decreased glands penis weight of male rats on PND 161-178. In female rats, 0.5 mg of testosterone propionate increased AGD at weaning and adulthood, reduced number of areolas and nipples, and removed prostate tissue in adulthood[16]. These results indicate that the reproductive development effects of carbendazim are distinct from those of prototypic antiandrogen and androgen in several aspects. It is difficult to generalize or predict the endocrine-disrupting activity of the benzimidazole fungicide.

In the present study, although the flutamide treatment by gavage on GD 0 to 21 decreased AGD and *levator ani bulbocavernosus* muscle weight, it increased nipple retention, testis and epididymis malformations, and hypospadias in male rats. These flutamide-induced alterations of androgen-dependent end points are similar to the results of McIntyre *et al*.[14] and Goto *et al*.[28] in which flutamide was administered orally at 6.25 to 50 mg/kg on GD 12 to 21 and subcutaneously at 3 to 30 mg/kg on GD 16 to 21, respectively.

A major and unexpected finding of this study is that carbendazim co-administration *in utero* can protect male rat offspring against the adverse effects of flutamide on reproductive development. This conclusion is based on the results showing that carbendazim diminished the flutamide-induced AGD reduction, nipple retention, testis inflammation, and hypospadias in male offspring. To the best of our knowledge, this study is the first to show that a xenobiotic can modulate flutamide reproductive development toxicity. The exact mechanisms underlying this compensatory effect are not clear. Flutamide is a potent androgen receptor antagonist. Treatment of male rats with carbendazim increased androgen binding protein concentration and activity in testis[1,18]. Additions of carbendazim to testis tissue extract replaced [^{3}H]hydroxytestosterone previously bound to the androgen receptor[18]. These studies suggest that the androgen receptor may possibly be a common target of action of flutamide and carbendazim. Therefore, an androgen receptor-mediated mechanism may contribute at least partly to the protective effect of carbendazim in which fungicide competes with flutamide for binding to androgen receptor to diminish the adverse effects of flutamide on androgen-dependent tissues.

The present study does not exclude the possibility that the carbendazim protective effect is a manifestation of the fungicide interacting with flutamide antagonistically via androgen-receptor independent mechanisms which are important in reproductive development such as hypothalamic-pituitary function; biosynthesis, transport, and metabolism of androgens; and DNA methylation of critical genes[17,21]. Alternatively, carbendazim may interact with cellular signaling pathway to modulate the endocrine-disrupting activity of flutamide. For instance, exposure *in utero* to the antiandrogen flutamide at 0.4, 2, or 10 mg/kg altered the expression of genes involved in tumor growth factor-β signaling pathway which increased apoptotic germ cell death and consequently hypospermatogenesis in rat testis[29]. Carbendazim might possibly modulate the tumor growth factor-β signaling pathway or other pathways in a compensatory fashion to produce the protective effect in testis and related androgen-dependent tissues. Additional studies are required to further investigate this and other possibilities.

The present study further showed that *in utero* carbendazim co-administration synergistically increased flutamide-mediated increases of liver and kidney in female rats. The mechanisms underlying the synergistic interaction are not clear. Histological studies of these tissues did not show marked hepatic or renal lesions (data not shown). Oral administration of 5 to 100 mg/kg flutamide to male rats for 15 days increased liver weight in a dose-dependent manner[30]. Yamaha *et al*.[20] reported that oral administration of 300 mg/kg benomyl to immature female rats for 3 days and castrated male rats for 10 days increased their relative liver weights. Information on carbendazim-mediated increase of liver or kidney weight apparently is not available. However, Jacobsen *et al*.[31] reported that oral treatment with a mixture of five pesticides containing carbendazim for 28 days increased liver weight in male rats. These previous studies indicated that liver is a target organ of flutamide, benomyl, and possibly carbendazim. An increase of liver or kidney weight can be a reflection of induction of hypertrophy of the target organs for xenobiotic metabolism. It will be of interest to determine the *in utero* effects of carbendazim and benomyl on liver and kidney xenobiotic-metabolizing enzymes in rat offspring.

Given carbendazim and benomyl are widely used agricultural fungicides, the results of the present study are significant to toxicology and valuable to hazard identification, which will facilitate the health risk assessment of human exposures to these fungicides. In conclusion, this report shows that carbendazim exposure *in utero* induces a minor reproductive toxicity and a transient androgenic activity in rat offspring. Carbendazim protects male rats against developmental toxicity of flutamide and enhances the increases of liver and kidney weights mediated by the antiandrogen in female rats. Additional studies are warrant to investigate the antagonistic and synergistic interactions *in utero*.

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