Journal of Food and Drug Analysis

Volume 19 | Issue 4 Article 10

2011

Carbendazim-induced androgen receptor expression antagonized by flutamide in male rats

Journal of Food and Drug Analysis, Vol. 19, No. 4, 2011, Pages 418-428 藥物食品分析 第十九卷 第四期

Carbendazim-Induced Androgen Receptor Expression Antagonized by Flutamide in Male Rats

YA-HUI HSU[1], CHIUNG-WEI CHANG[1], MIN-CHEN CHEN[1], CHAO-YUN YUAN[1], JING-HUEI CHEN[2], JUI-TI MA[2], TZUU-HUEI UENG[3] AND SHUI-YUAN LU[1,2*]

[1.] Applied Toxicology Division, Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Council of Agriculture, Executive Yuan, Taichung, Taiwan, R.O.C.

[2.] Department of Biotechnology, Asia University, Taichung, Taiwan, R.O.C.

[3.] Institute of Toxicology, College of Medicine, National Taiwan University, Taipei, Taiwan, R.O.C.

(Received: November 22, 2010; Accepted: July 22, 2011)

ABSTRACT

Carbendazim, a widely used fungicide, exhibits reproductive and developmental toxicity. The objective of this study was to investigate the expression of androgen receptor induced by carbendazim and the antagonistic effect of flutamide. Rats were treated with carbendazim, flutamide or a combination of both to measure androgen receptor mRNA, immune activity and protein expression. Carbendazim treatment increased androgen receptor mRNA in a dose-dependent manner, while flutamide, an androgen receptor antagonist, blocked the androgen receptor mRNA. Co-treatment with carbendazim and various flutamide doses decreased the androgen receptor mRNA in a dose-dependent manner. Conversely, co-treatment with flutamide and various carbendazim doses increased the androgen receptor mRNA in a dose-dependent manner. Immunohistochemistry and Western blot analyses showed that carbendazim increased androgen receptor activity (especially in rat testes) in a dose-dependent manner, while flutamide decreased it. Furthermore, carbendazim or flutamide treatment for 7 days increased testosterone and follicular stimulating hormone concentrations in the serum of male rats in a dose-dependent manner, which might involve the disruption of the androgen receptor. Although we need to examine the exact mechanism of androgen receptor involved in the reproductive toxicity and endocrine-disrupting activity induced by carbendazim and its parent, benomyl, we should first discuss how to take advantage of flutamide antagonism on carbendazim-induced reproductive and endocrine-disrupting activity possibly in human. In summary, carbendazim induced androgen receptor expression in mRNA and protein levels, while flutamide antagonized this effect. This is the first report on the antagonistic effect of flutamide on the carbendazim-androgenic effect on mRNA and protein levels. The results would help to clarify the mechanism of carbendazim- and chemical-induced developmental toxicity and endocrine-disrupting activity.

Key words: Carbendazim, Flutamide, endocrine-disrupting activity, androgen receptor, male rats

INTRODUCTION

Carbendazim (methyl-2-benzimidazole carbamate, MBC) is a systemic fungicide used to kill pests[(1)]. The LD_{50} of carbendazim and its precursor, benomyl, is above 10,000 mg/kg/day in rats, which makes it a low toxicity pesticide[(2)]. Previous studies show that carbendazim and benomyl induce testicular atrophy and inhibit microtubule function[(3)], leading atrophy of semiferous[(4)] and infertility[(5)]. Carbendazim treatment induces embryolethality, craniomeningocele, exencephaly, microphthalmia and hydrocephalus[(6)]. Benomyl treatment produces exencephaly, hydrocephalus and cleft palate[(7)].

The abnormalities induced by carbendazim and benomyl occur in male and female rats. Rehnberg *et al.* reported that carbendazim treatment increases the level of androgen-binding protein in the serum, interstitial fluid and seminiferous tubule fluid in the testes[(8)]. This implies that the androgen receptor is involved in the carbendazim-induced abnormalities of reproductive tissues, such as the testes in rats. Lim and Miller later reported that carbendazim treatment did not induce severe testicular toxicity in male rats during pre-puberty, but did so during puberty and post-puberty[(3)]. This implies that androgen receptors

* Author for correspondence. Tel: +886-4-23302101 ext. 505; Fax: +886-4-23323073; E-mail: lusueyen@tactri.gov.tw

are active during puberty and post-puberty, but not in pre-puberty. The androgen receptor plays an important role in the growth of uterus in rats[9,10]. Carbendazim induces endocrine-disrupting activity in the offspring of male and female rats[11]. Carbendazim and flutamide exposure *in utero* has antagonistic and synergistic effects on reproductive and developmental toxicity in rats[12]. Yamada *et al.* indicated that functional genomics may explain the inability of benomyl to act via steroid-receptor-mediated mechanisms[13]. Flutamide and linuron anti-androgens decreased the androgen receptor mRNA of rat testes. This decrease may be related to the anti-androgenic activity induced by flutamide and linuron[14,15].

The effects of carbendazim and its precursor benomyl on mammals and birds include male reproductive toxicity, developmental toxicity and endocrine-disrupting activity. Previous reports indicate that carbendazim and benomyl effects include sloughing of germ cells, testes and epididymis toxicity, and DNA damage of testicular cells[16-31]. In addition to male toxicity, carbendazim and benomyl induce developmental toxicity in mammals, according to *in vitro* and *in vivo* tests[7,11,12,32-41].

Relatively little information is available concerning the endocrine-disrupting activity induced by carbendazim and benomyl. Some reports show that carbendazim and benomyl increase the serum and pituitary luteining hormone (LH), 17β-estradiol (E_2) and follicular stimulating hormone (FSH) [6,42]. However, the mechanism of reproductive and developmental toxicity remains unclear. Rajeswary *et al.* showed that carbendazim-induced reproductive toxicity is due to increasing oxidative stress[43]. Spencer *et al.* indicated that carbendazim and benomyl led to developmental toxicity and that it is unrelated to steroid or receptor mechanisms[36]. Previous research also shows that carbendazim and colchicine are both microtubule disruptors in rats. Markelewicz Jr. *et al.* indicated that carbendazim and 2,5-hexanedione synergistically disrupt rat spermatogenesis due to their effects on microtubules[44]. The molecular mechanism of carbendazim's endocrine-disrupting activity remains unclear. This study investigated whether the carbendazim-induced androgen receptor expression is antagonized by flutamide in the testes, epididymis and prostate of rats. This study hypothesized that carbendazim could induce androgen receptor expression in the testes, epididymis and prostate of male rats. Further, the carbendazim-induced androgen receptor expression in the testes, epididymis and prostate could be reversed by the anti-androgen, flutamide. To test the hypotheses, rats were exposed to carbendazim and flutamide separately and in combination.

MATERIALS AND METHODS

I. *Animals and Treatments*

Three-week-old male Sprague-Dawley rats were purchased from the National Laboratory Animal Center, Taipei, Taiwan. The rats were housed in a specific-pathogen-free animal facility in the Taiwan Agricultural Chemicals and Toxic Substances Research Institute (TACTRI) in Taichung. The animal rooms were maintained at a 12-hour light and dark cycle, 23 ± 2°C and 50 ± 10% relative humidity. Upon arrival, the rats were quarantined for at least 1 week and were released only when they exhibited adequate body weight gain and no clinical signs of disease or injury. Carbendazim (99% pure) was obtained from Sinon Co. (Taichung, Taiwan). Flutamide (FLU) and the other chemicals were obtained from Sigma (St. Louis, MO, USA), unless otherwise stated. The pesticide was suspended in corn oil and orally administered to five rats in each group once a day by gavage at a volume of 2.5 mL/kg body weight. Male rats (322 ± 15 g) were randomly assigned to each treatment group. In order to carry out the time- and dose-dependent tests, the protocol included two treatment-duration and dosages. The first one was as follows: The doses of carbendazim were 0, 25, 50, 100, 200, 400 and 800 mg/kg/day for 56 days. The doses of flutamide were 0, 6.25, 12.5, 25, 50 and 100 mg/kg/day for 28 days. In mixed doses, the rats were co-treated with 675 mg/kg/day of carbendazim and 0, 6.25, 12.5, 25, 50 and 100 mg/kg/day of flutamide for 28 days. The second one was as follows: The doses of carbendazim were 0, 6.25, 25, 100 and 400 mg/kg/day for 7 days, while the doses of flutamide were 0, 0.78, 3.13, 12.5 and 50 mg/kg/day for 7 days. The rats in the co-treatment group were given either 400 mg/kg of carbendazim and 0, 0.78, 3.13, 12.5 and 50 mg/kg/day of flutamide, or 50 mg/kg/day of flutamide and 0, 6.25, 25, 100 and 400 mg/kg/day of carbendazim for 7 days. All animal care and experimental procedures were approved by the Committee for Animal Experiment Management of TCATRI.

II. *Immunohistochemical (IHC) Evaluation*

The testes tissues of three groups of rats were tested: 1) 0, 25, 50, 100, 200, 400 and 800 mg/kg/day of carbendazim for 56 days; 2) 0, 6.25, 12.5, 25, 50 and 100 mg/kg/day of flutamide for 28 days; and 3) co-treatment with 675 mg/kg/day of carbendazim and 0, 6.25, 12.5, 25, 50 and 100 mg/kg/day of flutamide for 28 days. Testes from the following test groups were fixed in 10% neutral buffered formalin for one week. The tissues were then dehydrated with increasing concentrations of ethanol, cleared in toluene and embedded in paraffin. Sections were cut into 5-μm slices and deparaffinized, hydrated and treated with 0.3% H_2O_2 in PBS (pH 7.6) for 30 min to block endogenous peroxidase activity, and finally treated with a protein-blocking solution (5% goat serum diluted in phosphate-buffered saline). These steps were followed by heating the sections in a microwave oven for antigen retrieval using a 0.01 M citrate buffer solution (pH 5.5). Tissue sections were immunostained with rabbit anti-AR (N-20, Santa Cruz Biotechnology, Inc., CA, USA), which was diluted 1 : 250 in phosphate-buffered saline and 0.25% bovine serum albumin and maintained at room temperature overnight. The tissue sections were then developed with a streptavidin-HRP kit (Chemicon IHC Select® CA, USA), using diaminobenzidine as the chromogen, and

were counterstained with hematoxylin. All images were optimized by using a inverted microscope (Leica, Wetzlar GmbH, Germany). To quantify the relative amount of AR protein in the IHC, 200 nucleus stained per field in a slide, 5 field per slide, 5 slides per dose were counted. The intensity of AR protein stained in nucleus was graded as (0, negative), + (1, mild), ++ (2, moderate), +++ (3, intense), ++++ (4, more intense) or +++++ (5, very intense). The measurements were control group adjusted and the values were statistically analyzed.

III. *Reverse Transcription-Polymerase Chain Reaction (PCR)*

Testes (n = 5) from the following treatment groups were stored at -80°C for 7 days. Total RNA was extracted with an RNeasy® Mini kit (QIAGEN, TAIGEN Bioscience Corporation, Duesseldorf, Germany) according to the protocol provided by the manufacturer. For the reverse transcription (RT) reaction, 3 μL of total RNA was used from the individual rats of each group. The RT-PCR reactions in this study were carried out with SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA polymerase kits from Invitrogen (Cat. No. 12574-026) in DNA Engine® & DNA Engine Tetrad® Peltier Thermal Cyclers (PTC-200, MJ Research, Incorporated, Massachusetts 02451 USA). For AR mRNA amplification, the primers were designed to amplify a 570-bp fragment (forward, 5′-TGCTGCCTTGTTATCTAGTCTCA-3′; reverse, 5′-ACCATATGGGACTTGATTAGCAG-3′) (annealing temperature, 60°C; the number of cycles, 24, 26 and 28; product size, 570 bp). PCR was subsequently performed using an optimized protocol of between 24 and 28 cycles. Each cycle consisted of the following: 94°C, 30 s; 60°C, 30 s and 72°C, 45 s. For β-actin mRNA amplification, the primers were designed to a 359-bp fragment (forward, 5′-CTGTGCCCATCTATGAGGGTTAC-3′; reverse, 5′-AATCCACACAGAGTACTTGCGCT-3′) (annealing temperature, 60°C; the number of cycles, 24, 26 and 28; product size, 359 bp). PCR was subsequently performed using an optimized protocol of between 24 and 28 cycles. Each cycle consisted of the following: 94°C, 30 s; 60°C, 30 s and 72°C, 45 s. PCR products were resolved in a 1.2% agarose gel and stained with ethidium bromide, and DNA bands from triplicate reactions were quantified using a FOTO/Analyst® Investigator System (Fotodyne Incorporated, Hartland, WI, USA). The PCR products for β-actin served as an internal standard.

IV. *Western Blot*

A Polytron PT3100 homogenizer (Kinematica AG, Littau, Switzerland) was used to examine frozen testicular tissues from the following treatment groups. Tissues of testes from the first protocol were homogenized for a few seconds in an M-PER® Mammalian Protein Extraction Reagent (Cat. No. 78505, Pierce). The homogenates were then centrifuged at 105,000 ×g for 1 h at 4°C. The supernatants were aliquoted and stored at -86°C before use. Before western blotting, protein contents were measured by BCA protein assay (Cat. No. 23225, Pierce) with BSA as the standard. Equal amounts of protein were loaded onto each polyacrylamide gel. The antibody dilutions were 1 : 200 for the anti-AR antibody (N-20, Santa Cruz Co., CA) and 1 : 5000 for the horseradish peroxidase conjugated goat anti-rabbit IgG (AP132P, Chemicon International).

For each treatment group, five samples were analyzed in two separate blots. Total protein extracts from the testicular tissue were denatured and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 7.5% polyacrylamide. The proteins were transferred to nitrocellulose membranes. The membranes were then blocked for non-specific binding and incubated with polyclonal primary antibodies for AR (N-20, Santa Cruz Co., CA) and β-actin (AP132P, Chemicon International). After incubation with primary antibody, the membranes were incubated with horseradish peroxidase-linked anti-goat IgG secondary antibody and visualized on film exposed to enhanced chemiluminescence (Visualizer™ Western Blot Detection Kit, Millipore, MA, USA). The relative amount of protein in the resulting immunoblot bands was estimated by measuring the optical densities of the bands on exposed films using a FOTO/Analyst® Investigator System (Fotodyne Incorporated, WI, USA). The measurements were background adjusted and the values were statistically analyzed. Protein for ß-actin served as an internal standard.

V. *Hormone Analysis*

Serum luteinizing hormone (LH) (RPN 2562, Amersham, UK), follicular stimulating hormone (FSH) (RPN 2560, Amersham, UK; AE R004, Biocode, Belgium), 17β-estradiol (E_2) (Cayman Chemical., Ann Arbor, MI, USA) and testosterone (T) (Cayman Chemical., Ann Arbor, MI, USA) levels were determined using the relevant EIA systems. The serum samples collected from rats treated with 0, 6.25, 25, 100 and 400 mg/kg/day of carbendazim for 7 days and 0, 0.78, 3.13, 12.5 and 50 mg/kg/day of flutamide for 7 days were directly applied to the well in the kit and measurements were taken according to the procedure described by the manufacturer.

VI. *Statistical Analysis*

The values of AR in Western blot and RT-PCR were normalized against β-actin. All results were statistically analyzed with the *t*-test and $p < 0.05$ was considered statistically significant. The other data were expressed as mean ± SE. Data were subjected to ANOVA followed by *t*-test. The level of significance was set at $p < 0.05$.

RESULTS

I. *Effects of Carbendazim, Flutamide and Combined Treatment on Androgen Receptor Immunolabeling*

Journal of Food and Drug Analysis, Vol. 19, No. 4, 2011

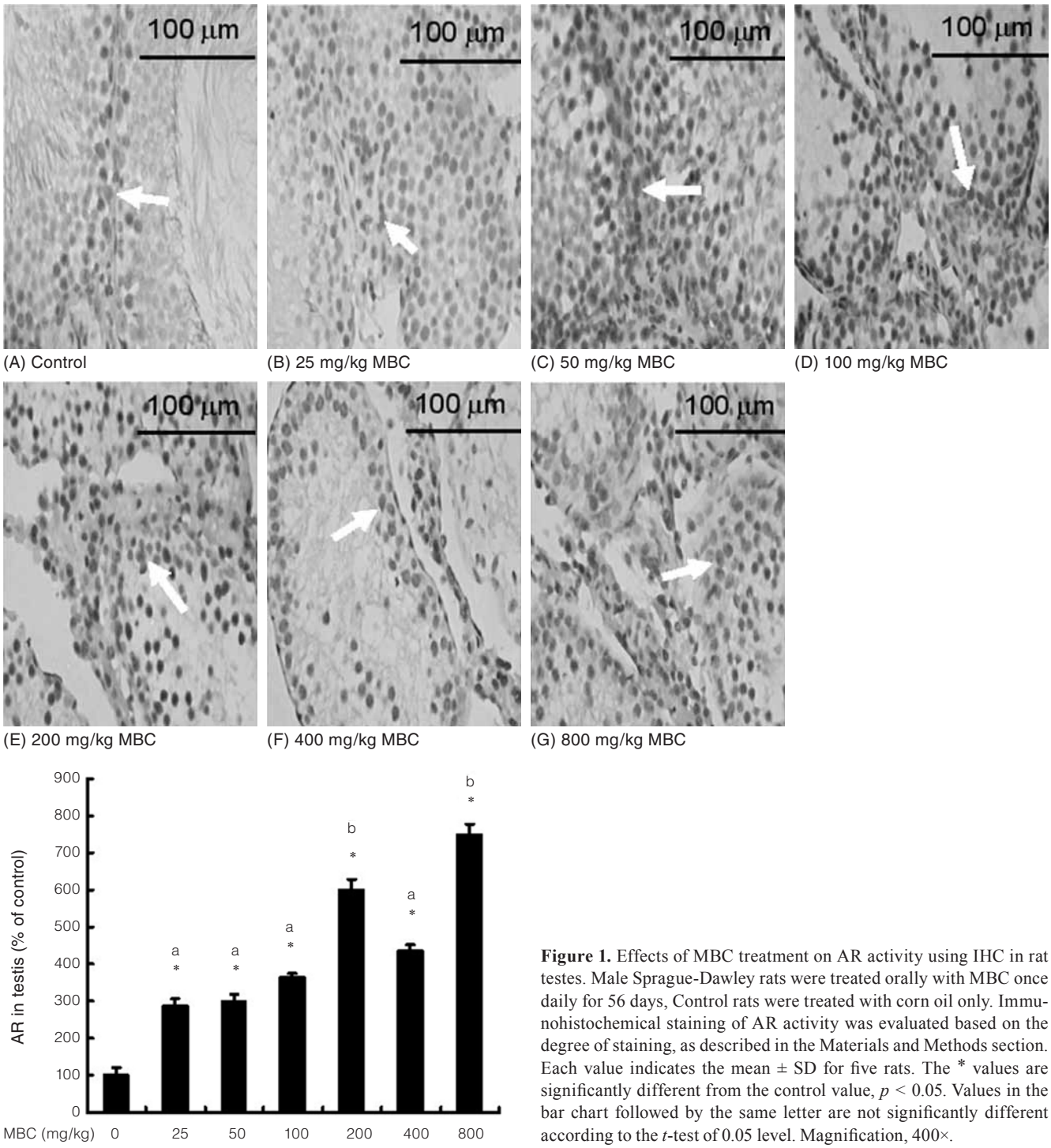

Figure 1. Effects of MBC treatment on AR activity using IHC in rat testes. Male Sprague-Dawley rats were treated orally with MBC once daily for 56 days, Control rats were treated with corn oil only. Immunohistochemical staining of AR activity was evaluated based on the degree of staining, as described in the Materials and Methods section. Each value indicates the mean ± SD for five rats. The * values are significantly different from the control value, $p < 0.05$. Values in the bar chart followed by the same letter are not significantly different according to the *t*-test of 0.05 level. Magnification, 400×.

The testes of rats treated with 25, 50, 100, 200, 400 and 800 mg/kg/day of carbendazim for 56 days exhibited significantly increased androgen receptor immunoexpression in a dose-dependent manner (Figure 1). However, treatment with 6.25, 12.5, 25, 50 and 100 mg/kg/day of flutamide for 28 days significantly decreased androgen receptor immunoexpression in a dose-dependent manner when compared to that of control (data not shwon). Co-treatment with 675 mg/kg/day of carbendazim and 6.25, 12.5, 25, 50 and 100 mg/kg/day of flutamide for 28 days significantly decreased androgen receptor immunoexpression when compared to that of carbendazim alone (data not shwon). A comparison of the androgen receptor immunoexpression induced by carbendazim, flutamide and their combination shows that 675 mg/kg/day carbendazim could reverse the effect of a gradually increasing dose of flutamide until 100 mg/kg/day. This shows that flutamide had a stronger antagonist effect than carbendazim. In addition to measuring the testis tissue,

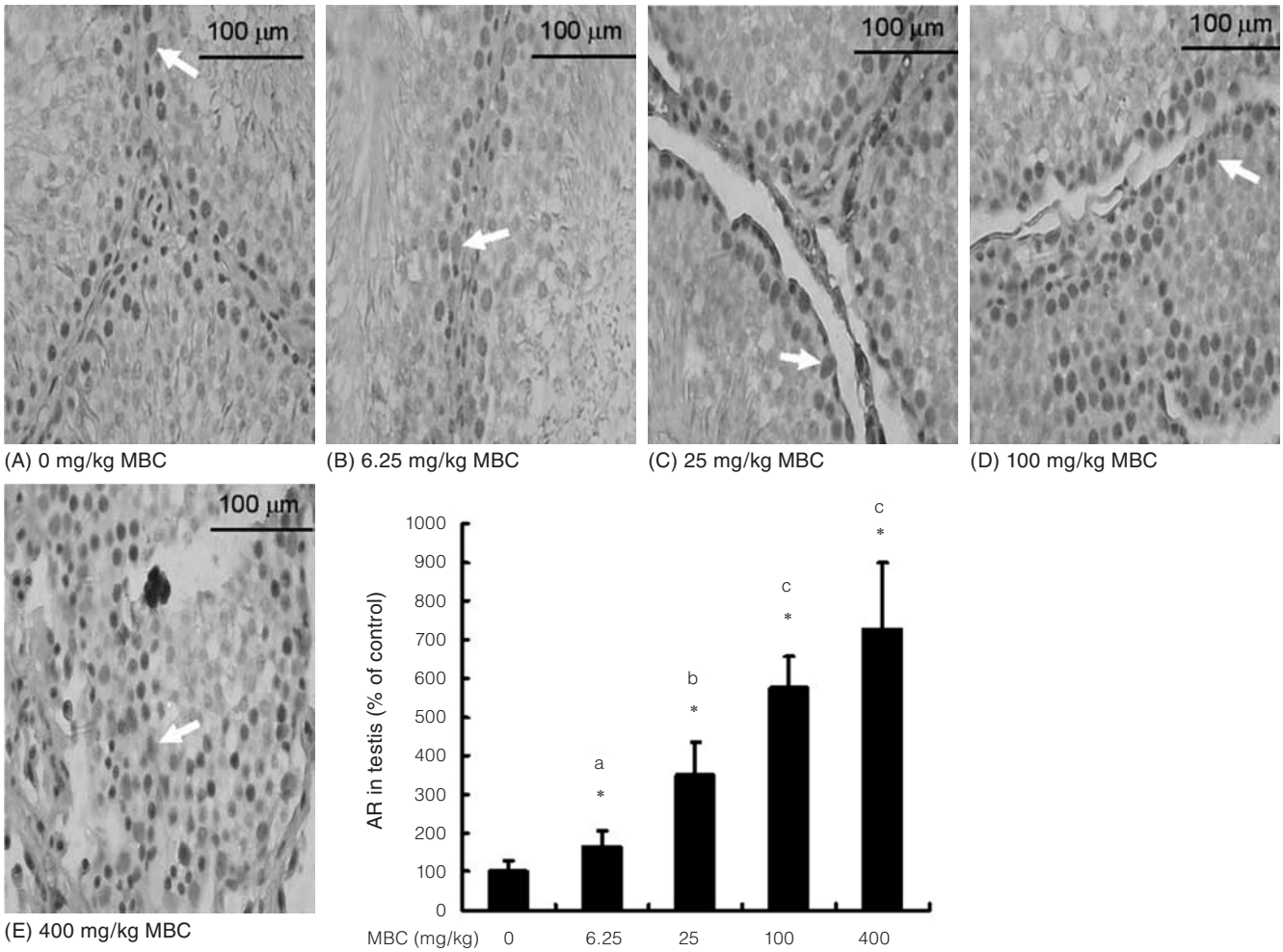

Figure 2. Effects of MBC treatment on AR activity using IHC in rat testes. Male Sprague-Dawley rats were treated orally with MBC at the doses indicated once daily for 7 days. Control rats were treated with corn oil only. Immunohistochemical staining of AR activity was evaluated based on the degree of staining, as described in the Materials and Methods section. Each value indicates the mean ± SD for five male rats. The * values are significantly different from the control value, $p < 0.05$. Values in bar chart followed by the same letter are not significantly different according to the *t*-test of 0.05 level.

we also investigated the effect of carbendazim, flutamide and in combination on tissues of epididymis and prostate in male rats. Basically carbendazim induced the androgen receptor protein in epididymis and prostate but there is no dose-dependent manner when compared to that of control group (data not shwon). Treatment with 6.25, 12.5, 25, 50 and 100 mg/kg/day of flutamide for 28 days significantly decreased the androgen receptor protein expression in epididymis without dose-dependent manner and in prostate with dose-depdenent manner when compared to that of control group (data not shwon). Co-treatment with 675 mg/kg/day of carbendazim and 6.25, 12.5, and 50 mg/kg/day of flutamide for 28 days significantly decreased androgen receptor protein in epididymis but not in 25 and 100 mg/kg/day flutamide when compared to that of carbendazim alone (data not shwon). Also, co-treatment with 675 mg/kg/day of carbendazim and 6.25, 12.5, 25, 50 and 100 mg/kg/day of flutamide for 28 days significantly decreased androgen receptor protein in prostate when compared to that of carbendazim alone (data not shwon).

To further determine the dose- and time-dependent effects of carbendazim, flutamide and their combination on androgen receptor immunoexpression in rat testes, the doses and treatment period were shortened. Treatment with 6.25, 25, 100 and 400 mg/kg/day of carbendazim for 7 days significantly induced the androgen receptor immunoexpression in a dose-dependent manner in rat testes (Figure 2). However, treatment with 0.78125, 3.125, 12.5 and 50 mg/kg/day of flutamide for 7 days significantly decreased the androgen receptor immunoexpression in rat testes (data not shwon), which is likely because flutamide reduced androgen receptor mRNA in androgen receptor activity. Co-treatment with 400 mg/kg of carbendazim and 0.78125, 3.125, 12.5 and 50 mg/kg/day of flutamide significantly decreased androgen receptor immunoexpression in a flutamide dose-dependent manner in rat testes (data not shwon). However, co-treatment

with 50 mg/kg/day of flutamide and 6.25, 25, 100 and 400 mg/kg/day of carbendazim significantly increased the androgen receptor immunoexpression in a carbendazim dose-dependent manner in rat testes (data not shown).

II. *Effects of Carbendazim, Flutamide and Their Combination on Androgen Receptor mRNA Expression in Male Rats*

Treatment with 6.25, 25, 100 and 400 mg/kg/day of carbendazim for 7 days significantly increased androgen receptor mRNA expression in a dose-dependent manner in rat testes. However, the androgen receptor expression induced by 400 mg/kg/day of carbendazim was less than that induced by a dose of 100 mg/kg/day (Figure 3A). This suggests that carbendazim is an androgen receptor agonist in rat testes. In contrast, treatment with 0.78125, 3.125, 12.5 and 50 mg/kg/day of flutamide for 7 days significantly decreased androgen receptor expression in a dose-dependent manner in rat testes (Figure 3B). As flutamide is a well-known androgen receptor antagonist, this result confirmed previous results and could be used as a quality control[14]. Co-treatment with 400 mg/kg of carbendazim and 0.78125, 3.125, 12.5 and 50 mg/kg/day of flutamide significantly decreased androgen receptor mRNA in a flutamide dose-dependent manner (Figure 3C). This suggests that flutamide antagonized carbendazim-induced androgen receptor expression. Inversely, co-treatment with 50 mg/kg of flutamide and 6.25, 25, 100 and 400 mg/kg/day of carbendazim significantly increased the androgen receptor mRNA in a carbendazim dose-dependent manner (data not shown). This suggests that a high dose of carbendazim can have an agonistic effect on the androgen receptor expression by competing with flutamide to bind to the androgen receptor.

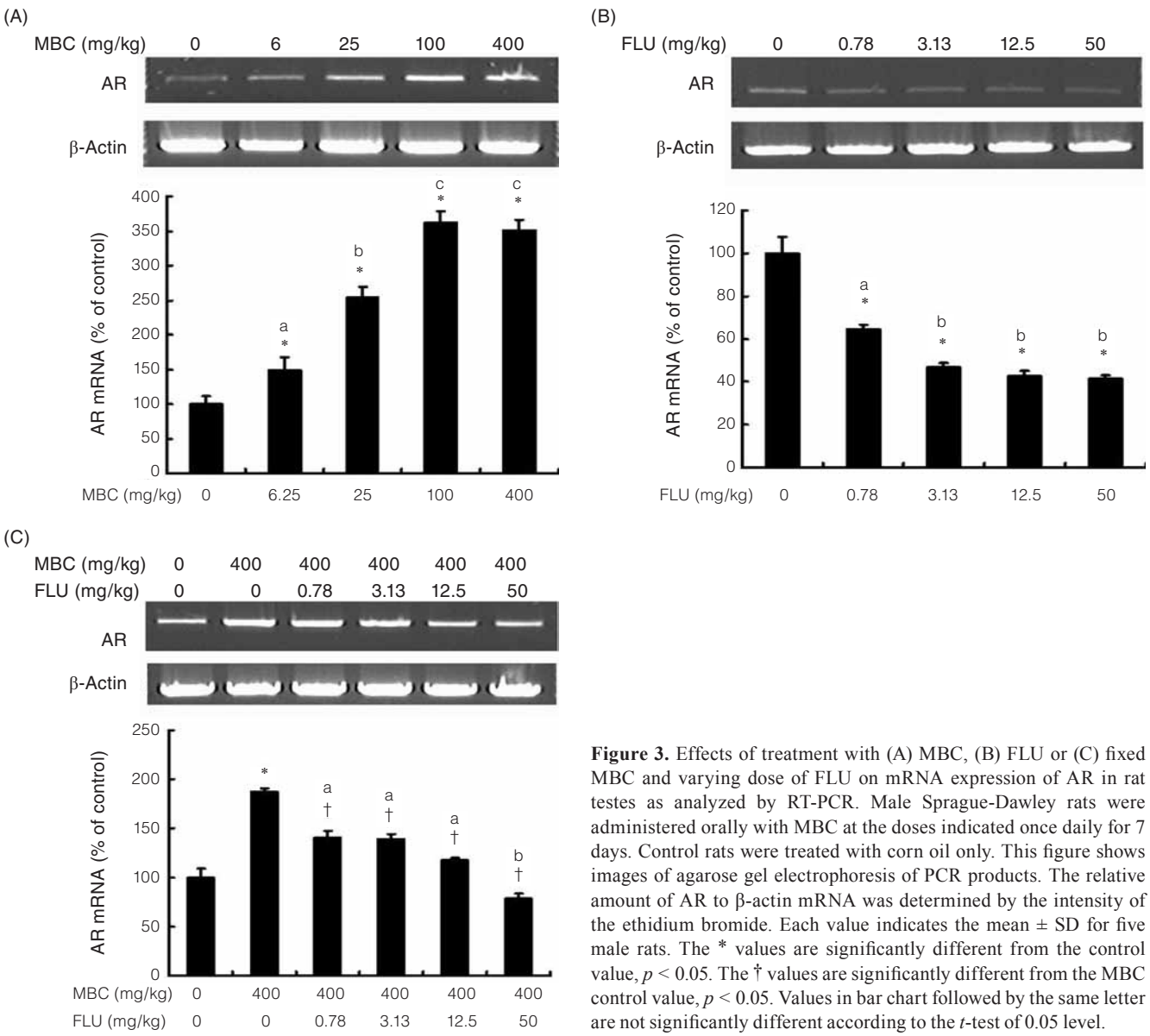

Figure 3. Effects of treatment with (A) MBC, (B) FLU or (C) fixed MBC and varying dose of FLU on mRNA expression of AR in rat testes as analyzed by RT-PCR. Male Sprague-Dawley rats were administered orally with MBC at the doses indicated once daily for 7 days. Control rats were treated with corn oil only. This figure shows images of agarose gel electrophoresis of PCR products. The relative amount of AR to β-actin mRNA was determined by the intensity of the ethidium bromide. Each value indicates the mean ± SD for five male rats. The * values are significantly different from the control value, $p < 0.05$. The † values are significantly different from the MBC control value, $p < 0.05$. Values in bar chart followed by the same letter are not significantly different according to the *t*-test of 0.05 level.

III. *Effects of Carbendazim, Flutamide and Their Combination on Androgen Receptor Protein Expression*

In addition to investigating the androgen receptor mRNA and immunoexpression, this study further analyzes the androgen receptor protein expression induced by carbendazim, flutamide and their combination. Treatment with 25, 50, 100, 200, 400 and 800 mg/kg/day of carbendazim for 56 days significantly increased androgen receptor protein in a dose-dependent manner (Figure 4A), except for the dose of 400 mg/kg/day (data not shown). At doses of 6.25, 25, 100 and 400 mg/kg/day of carbendazim for 7 days, 400 mg/kg of carbendazim showed no androgen receptor protein expression. Immunohistochemistry analysis shows that this may be due to the death of Sertoli cells. Basically, this result agrees with the androgen receptor protein (Figure 1). However, treatment with 6.25, 12.5, 25, 50 and 100 mg/kg/day of flutamide for 28 days significantly decreased androgen receptor immunoexpression in a dose-dependent manner (Figure 4B). Co-treatment with 675 mg/kg of carbendazim and 6.25, 12.5, 25, 50 and 100 mg/kg/day of flutamide for 28 days significantly decreased androgen receptor protein in a flutamide dose-dependent manner, except for the dose of 6.25 mg/kg/day (Figure 4C). A comparison of the androgen receptor protein expression induced by carbendazim, flutamide and their combination shows that carbendazim could reverse the flutamide effect in rat testes.

To further study the dose- and time-dependent effects of carbendazim, flutamide and their combination on androgen receptor protein expression in rat testis, the doses and treatment period were shortened. Treatment with 6.25, 25, 100 and 400 mg/kg/day of carbendazim for 7 days significantly increased androgen receptor protein expression in rat testes in a dose-dependent manner, except for the 400 mg/kg/day

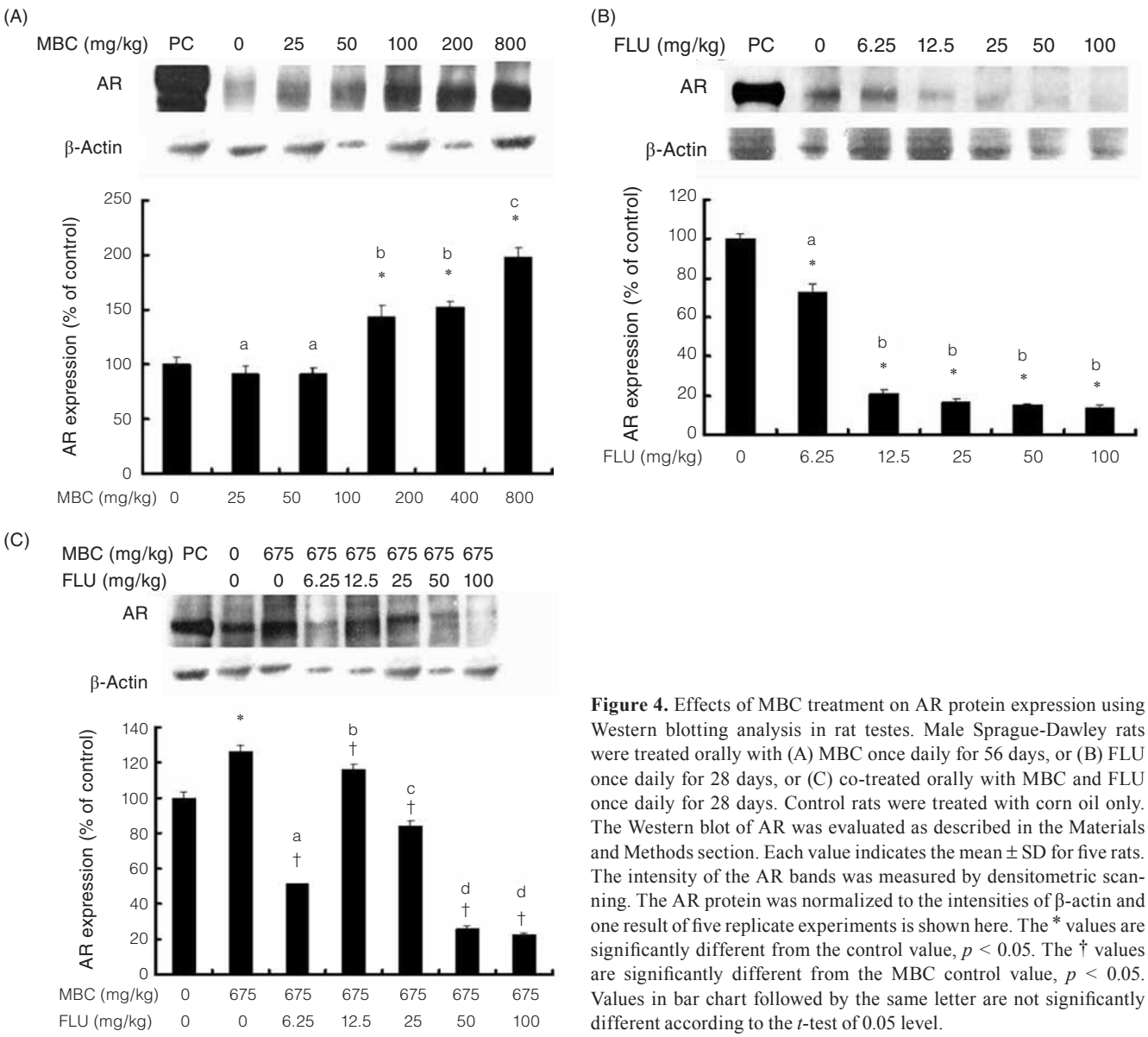

Figure 4. Effects of MBC treatment on AR protein expression using Western blotting analysis in rat testes. Male Sprague-Dawley rats were treated orally with (A) MBC once daily for 56 days, or (B) FLU once daily for 28 days, or (C) co-treated orally with MBC and FLU once daily for 28 days. Control rats were treated with corn oil only. The Western blot of AR was evaluated as described in the Materials and Methods section. Each value indicates the mean ± SD for five rats. The intensity of the AR bands was measured by densitometric scanning. The AR protein was normalized to the intensities of β-actin and one result of five replicate experiments is shown here. The * values are significantly different from the control value, $p < 0.05$. The † values are significantly different from the MBC control value, $p < 0.05$. Values in bar chart followed by the same letter are not significantly different according to the *t*-test of 0.05 level.

Journal of Food and Drug Analysis, Vol. 19, No. 4, 2011

dose (data not shown). Treatment with 400 mg/kg/day of carbendazim showed no androgen receptor protein expression, which may be due to the death of Sertoli cells in the testes. However, treatment with 0.78125, 3.125, 12.5 and 50 mg/kg/day of flutamide for 7 days significantly decreased the androgen receptor protein expression in rat testes (data not shwon). This result agrees with the androgen receptor mRNA and immunohistochemistry analysis.

IV. *Carbendazim- and Flutamide-Induced Testosterone and Follicular Stimulating Hormone Levels in Rat Serum*

Treatment with carbendazim or flutamide for 7 days increased the testosterone concentration in the serum of male rats in a dose-dependent manner (Figure 5A and 5B). Treatment with carbendazim or flutamide for 7 days increased the follicular stimulating hormone concentration in the serum of male rats in a dose-dependent manner (Figures 5C and 5D).

DISCUSSION

The results of this study show that exposure to carbendazim for 7 and 56 days induced androgen receptor gene expression in a dose-dependent manner in rat testes. The following pieces of evidence indicate carbendazim-induced androgen receptor gene expression. Firstly, carbendazim increased androgen receptor mRNA, immune activity and protein expression in a dose-dependent manner and was antagonized by flutamide. Secondly, carbendazim induced androgen receptor immune activity and protein expression in a time-dependent manner. Thirdly, carbendazim induced androgen receptor mRNA, immune activity and protein expression in the testes, epididymis and prostate in a dose-dependent manner.

The results of androgen receptor protein expression in 400 mg/kg of carbendazim for 7 days and 56 days showed unreasonable but consistent data. This may be because

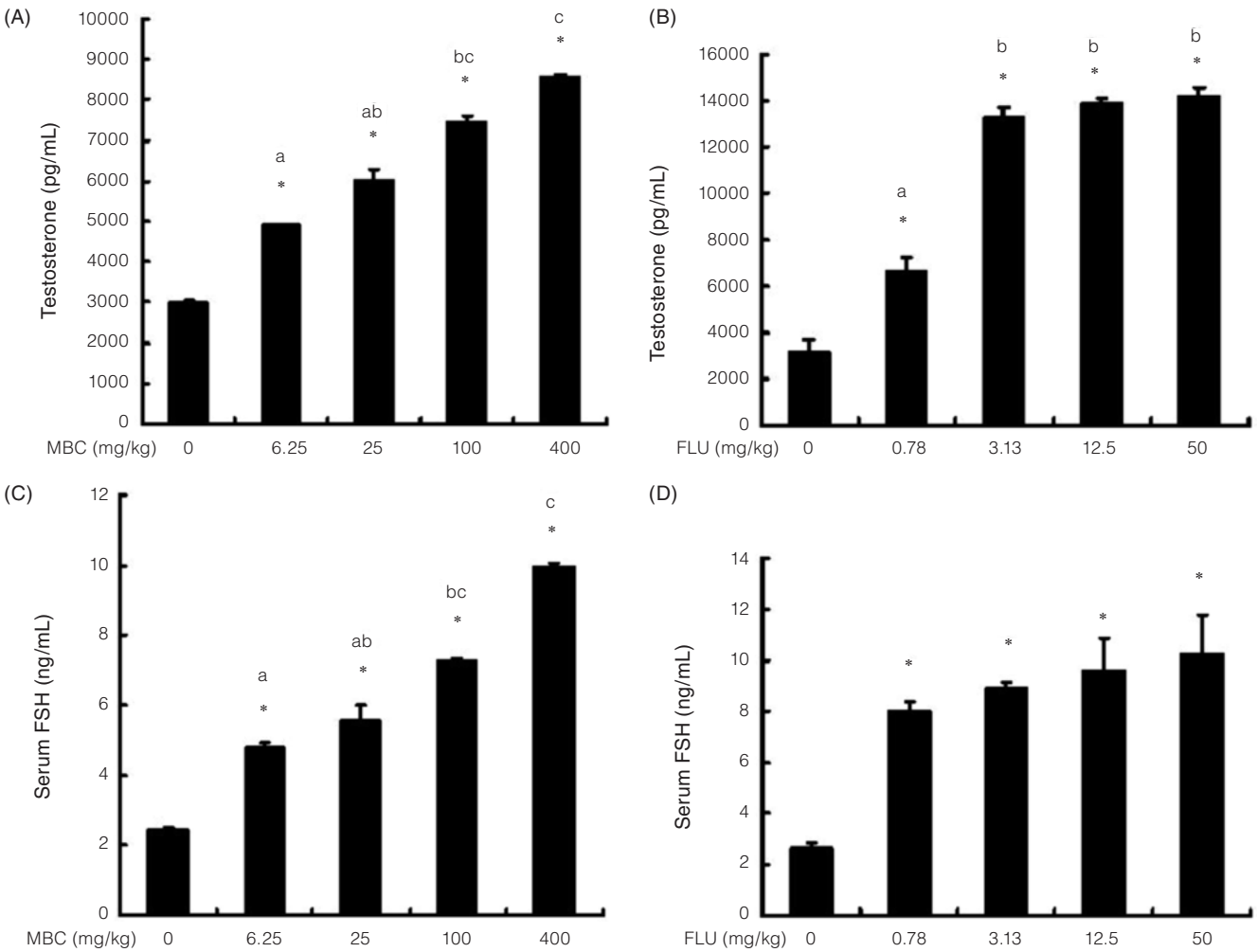

Figure 5. Effects of treatment with (A) MBC and (B) FLU on serum testosterone or (C) MBC and (D) FLU on serum FSH level in male rats. Male Sprague-Dawley rats were treated orally with MBC or FLU once daily for 7 days. Control rats were treated with corn oil only. Serum testosterone was evaluated as described in the Materials and Methods section. Each value indicates the mean ± SD for five rats. The * values are significantly different from the control value, $p < 0.05$. Values in bar chart followed by the same letter are not significantly different according to the t-test of 0.05 level.

the damage in the Sertoli cells of the testes led to a loss of androgen receptor protein expression. Reproductive and developmental toxicity exhibits drug specific, phase specific and dose specific characteristics. It is quite possible that a dose of 400 mg/kg of carbendazim damaged the Sertoli cells in the testes of rats with dose-specific activity and futher investigation is required. The other unreasonable data is that the co-treatment with 675 mg/kg/day of carbendazim and 6.25 mg/kg/day of flutamide decreased androgen receptor protein levels more than the treatment with 675 mg/kg/day of carbendazim alone. Whether the analysis process resulted in the effect or unrecovered implications still needs to be investigated.

This is the first study to show that carbendazim induced androgen receptor mRNA, immunoexpression and protein expression in rats. This result might explain the endocrine-disrupting activity in carbendazim-, flutamide- and co-treatment-induced and antagonized reproductive and developmental toxicity in rats. The endocrine-disrupting activities were as follows: Co-treatment of male rats with 675 mg/kg/day of carbendazim and 50 or 100 mg/kg/day of flutamide once daily for 28 days blocked the decrease in testicular weight induced by treatment with carbendazim alone[11]. Co-treatment prevented the loss of spermatozoa and cell morphology and the decrease in sperm concentration induced by carbendazim. We have proved that endocrine-disrupting activity in carbendazim-induced reproductive and developmental toxicity in rats[11]. Also we reported that antagonistic and synergistic effects of carbendazim and flutamide exposures in utero on reproductive and developmental toxicity in rats[12]. We inferred that AR might play an important role in carbendazim-induced reproductive and developmental toxicity and endocrine-disrupting activity. This topic requires further investigation.

The carbendazim-induced androgen receptor expression in rat testes is in marked contrast to that induced by anti-androgens. Most androgen receptor disruptors are anti-androgens, including vinclozolin, prochloraz, di(2-ethylhexyl)phthalate (DEHP), finasteride, linuron, p,p'-DDT and -DDE, and iprodione[45-48]. The significant difference between these disruptors is that AR is an agonist for carbendazim and an antagonist for those anti-androgens. Though damage such as testicular atrophy in the reproductive tissues of male rats seems to be common and similar in pathology between carbendazim and anti-androgens, the mechanism of toxicity may be quite different and requires further analysis.

The current study shows that the androgen receptor expression in the reproductive tissues of rats can be antagonized by flutamide in a dose- and time-dependent manner. Whether androgen receptor plays an important role in carbendazim-induced reproductive and developmental toxicity and endocrine-disrupting activity and how it acts in these toxicities requires further clarification.

Based on chemical structures and reproductive toxicity and endocrine-disrupting activity induced by flutmaide and carbendazim, we speculated that the principle of flutamide antagonistic on carbendazim-induced reproductive toxicity and endocrine-disrupting activity is androgen receptor affinity of the functional groups NCO and NOO, while that of carbendazim, androgen receptor agonist, is androgen receptor affinity of the functional group, NCOO and C and D ring of testosterone. The androgen receptor antagonistic pesticides, vinclozolin[49-55], procymidone[56-57], linuron[58] and iprodione[56] also shared the same functional groups NCO and NCN of androgen receptor affinity.

ACKNOWLEDGMENTS

The authors would like to acknowledge the financial assistance of the Bureau of Animal and Plant Health Inspection and Quarantine, Council of Agriculture, Executive Yuan, Taipei, Taiwan, R.O.C. through the project 96AS-14.2.2-PI-P1. The authors are grateful to Sinon Co., Taichung, Taiwan, for providing the carbendazim standard material used in this study. The authors would also like to thank Dr. Shun-Cheng Wang, Dr. Jenn-Sheng Hwang and Dr. Bih-Yuh You, who were crucial to the planning and execution of this study.

REFERENCES

1. Vettorazzi, G. 1977. State of the art of the toxicological evaluation carried out by the joint FAO/WHO expert committee on pesticide residues. III. Miscellaneous pesticides used in agriculture and public health. Residue. Rev. 66: 137-184.
2. WHO working group. 1993. Benomyl. Environmental Health Criteria 148: 135.
3. Lim, J. and Miller, M. G. 1997. Role of testis exposure levels in the insensitivity of prepubertal rats to carbendazim-induced testicular toxicity. Fundam. Appl. Toxicol. 37: 158-167.
4. Carter, S. D. and Laskey, J. W. 1982. Effect of benomyl on reproduction in the male rat. Toxicol. Lett. 11: 87-94.
5. Carter, S. D., Hess, R. A. and Laskey, J. W. 1987. The fungicide methyl 2-benzimidazole carbamate causes infertility in male Sprague-Dawley rats. Biol. Reprod. 37: 709-717.
6. Cummings, A. M., Harris, S. T. and Rehnberg, G. L. 1990. Effects of methyl benzimidazolecarbamate during early pregnancy in the rat. Fundam. Appl. Toxicol. 15: 528-535.
7. Ellis, W. G., Semple, J. L., Hoogenboom, E. R., Kavlock, R. J. and Zeman, F. J. 1987. Benomyl-induced craniocerebral anomalies in fetuses of adequately nourished and protein-deprived rats. Teratog. Carcinog. Mutagen. 7: 357-375.
8. Rehnberg, G. L., Cooper, R. L., Goldman, J. M., Gray Jr, L. E., Hein, J. F. and McElroy, W. K. 1989. Serum and testicular testosterone and androgen binding protein profiles following subchronic treatment with carbendazim. Toxicol. Appl. Pharmacol. 101: 55-61.
9. Weihua, Z., Ekman, J., Almkvistm, Å., Saji, S., Wang,

L., Warner, M. and Gustafsson, J. Å. 2002. Involvement of androgen receptor in 17β-estradiol-induced cell proliferation in rat uterus. Biol. Reprod. 67: 616-623.
10. Cheng, G., Weihua, Z., Mäkinen, S., Mäkelä, S., Saji, S., Warner, M., Gustafsson, J. Å. and Hovatta, O. 2002. A role for the androgen receptor in follicular atresia of estrogen receptor beta knockout mouse ovary. Biol. Reprod. 66: 77-84.
11. Lu, S. Y., Liao, J. W., Kuo, M. L., Wang, S. C., Hwang, J. S. and Ueng, T. H. 2004. Endocrine-disrupting activity in carbendazim-induced reproductive and developmental toxicity in rats. J. Toxicol. Environ. Health A 67: 1501-1515.
12. Lu, S. Y., Liao, J. W., Kuo, M. L., Hwang, J. S. and Ueng, T. H. 2006. Antagonistic and synergistic effects of carbendazim and flutamide exposures in utero on reproductive and developmental toxicity in rats. J. Food Drug Anal. 14: 120-132.
13. Yamada, T., Sumida, K., Saito, K., Ueda, S., Yabushita, S., Sukata, T., Kawamura, S., Okuno, Y. and Seki, T. 2005. Functional genomics may allow accurate categorization of the benzimidazole fungicide benomyl: lack of ability to act via steroid-receptor-mediated mechanisms. Toxicol. Appl. Pharmacol. 205: 11-30.
14. Ohsako, S., Kubota, K., Kurosawa, S., Takeda, K., Qing, W., Ishimura, R. and Tohyama, C. 2003. Alterations of gene expression in adult male rat testis and pituitary shortly after subacute administration of the antiandrogen flutamide. J. Reprod. Dev. 49: 275-290.
15. Turner, K. J., McIntyre, B. S., Phillips, S. L., Barlow, N. J., Bowman, C. J. and Foster, P. M. 2003. Altered gene expression during rat Wolffian duct development in response to in utero exposure to the antiandrogen linuron. Toxicol. Sci. 74: 114-128.
16. Hess, R. A., Moore, B. J., Forrer, J., Linder, R. E. and Abuel-Atta, A. A. 1991. The fungicide benomyl (methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate) causes testicular dysfunction by inducing the sloughing of germ cells and occlusion of efferent ductules. Toxicol. Sci. 17: 733-745.
17. Linder, R. E., Rehnbert, G. L., Strader, L. F. and Diggs, J. P. 1988. Evaluation of reproductive parameters in adult male Wistar rats after subchronic exposure (gavage) to benomyl. J. Toxicol. Environ. Health A 25: 285-298.
18. Akbarsha, M. A., Kadalmani, B., Girija, R. and Faridha, A. 2000. Carbendazim generates symplasts in rat spermatogenic clones. India J. Exp. Biol. 38: 1251-1254.
19. Akbarsha, M. A., Kadalmani, B., Girija, R., Faridha, A. and Hamid, K. S. 2001. Spermatotoxic effect of carbendazim. India J. Exp. Biol. 39: 921-924.
20. Gray Jr, L. E., Ostby, J., Sigmon, R., Ferrell, J., Rehnberg, G., Linder, R., Cooper, R., Goldman, J. and Laskey, J. 1988. The development of a protocol to assess reproductive effects of toxicants in the rat. Reprod. Toxicol. 2: 281-287.
21. Gray Jr, L. E., Ostby, J., Ferrell, J., Sigmon, R., Cooper, R., Linder, R., Rehnberg, G., Goldman, J. and Laskey, J. 1989. Correlation of sperma and endocrine measures with reproductive success in rodents. Prog. Clin. Biol. Res. 302: 193-206.
22. Gray Jr, L. E., Ostby, J., Linder, R., Goldman, J., Rehnberg, G. and Cooper, R. 1990. Carbendazim-induced alterations of reproductive development and function in the rat and hamster. Fundam. Appl. Toxicol. 15: 281-297.
23. Rajeswary, S., Mathew, N., Akbarsha, M. A., Kalyanasundram, M., and Kumaran, B. 2007. Protective effect of vitamin E against carbendazim-induced testicular toxicity-histopathological evidences and reduced residue levels in testis and serum. Arch. Toxicol. 81: 813-821.
24. Aire, T. A. 2005. Short-term effects of carbendazim on the gross and microscopic features of the testes of Japanese quails (Coturnix coturnix japonica). Anat. Embryol. 210: 43-49.
25. Nakai, M., Toshimori, K., Yoshinaga, K., Nasu, T. and Hess, R. A. 1998. Carbendazim-induced abnormal development of the acrosome during early phases of spermiogenesis in the rat testis. Cell Tissue Res. 294: 145-152.
26. Kadalmani, B., Girija, R., Faridha, A. and Akbarsha, M. A. 2002. Male reproductive toxic effects of carbendazim: hitherto unreported targets in testis. Indian J. Exp. Biol. 40: 40-44.
27. Linder, R. E., Strader, L. F., Slott, V. L. and Suarez, J. D. 1992. Endpoints of spermatotoxicity in the rat after short duration exposures to fourteen reproductive toxicants. Reprod. Toxicol. 6: 491-505.
28. Perreault, S. D., Linder, R. E., Strader, L. F. and Slott, V. 1989. The value of multiple endpoint data in male reproductive toxicology: revelations in the rat. Prog. Clin. Biol. Res. 302: 179-192.
29. Carter, S. D., Hein, J. F., Rehnberg, G. L. and Laskey, J. W. 1984. Effect of benomyl on the reproductive development of male rats. J. Toxicol. Environ. Health 13: 53-68.
30. Barnes, T. B., Verlangieri , A. J. and Wilson MC. 1983. Reproductive toxicity of methy-1-(butylcarbamoyl)-2-benzimidazole carbamate (benomyl) in male Wistar rats. Toxicol. 281: 103-115.
31. Bjørge. C., Brunborg, G., Wiger, R., Holme, J. A., Scholz, T., Dybing, E. and Søderlund, E. J. 1996. A comparative study of chemically induced DNA damage in isolated human and rat testicular cells. Reprod. Toxicol. 10: 509-519.
32. Hewitt, M. J., Mutch, P. and Pratten, M. K. 2005. Potential teratogenic effects of benomyl in rat embryos cultured in vitro. Reprod. Toxicol. 20: 271-280.
33. Vergieva, T. 1985. Behavioral teratology-results achieved and perspectives of development. J. Hyg. Epidemiol. Microbiol. Immunol. 29: 121-127.
34. Vergieva, T. 1998. Single day treatment- a feasible tool in revealing not dependent on maternal toxicity teratogenic potential. Adv. Exp. Med. Biol. 444: 191-199.
35. Cummings, A. M., Ebron-McCoy, M. T., Rogers, J. M., Barbee, B. D. and Harris, S. T. 1992. Developmental effects of methyl benzimidazole carbamate following

exposure during early pregnancy. Fundam. Appl. Toxicol. 18: 288-293.
36. Spencer, F., Chi, L. and Zhu, M. X. 1996. Effect of benomyl and carbendazim on steroid and molecular mechanisms in uterine decidual growth in rats. J. Appl. Toxicol. 16: 211-214.
37. Ellis, W. G., De Roos, F., Kavlock, R. J. and Zeman, F. J. 1988. Relationship of periventricular overgrowth to hydrocephalus in brains of fetal rats exposed to benomyl. Teratog. Carcinog. Mutagen. 8: 377-391.
38. Sitarek, K. 2001. Embryolethal and teratogenic effects of carbendazim in rats. Teratog. Carcinog. Mutagen. 21: 335-340.
39. Piersma, A. H., Verhoef, A. and Dortant, P. M. 1995. Evaluation of the OECD 421 reproductive toxicity screening test protocol using 1-(butylcarbamoyl)-2-benzimidazolecarbamate (benomyl). Teratog. Carcinog. Mutagen. 15: 93-100.
40. Hoogenboom, E. R., Ransdell, J. F., Ellis, W. G., Kavlock, J. R. and Zeman, F. J. 1991. Effects on the fetal rat eye of maternal benomyl exposure and protein malnutrition. Curr. Eye Res. 10: 601-612.
41. Zeman, F. J., Hoogenboom, E. R., Kavlock, R. J., and Semple, J. L. 1986. Effects on the fetus of maternal benomyl exposure in the protein-deprived rat. J. Toxicol. Environ. Health Part A 17: 405-417.
42. Goldman, J. M., Rehnberg, G. L., Cooper, R. L, Gray Jr, L. E., Hein, J. F. and McElroy, W. K. 1989. Effects of the benomyl metabolite, carbendazim on the hypothalamic-pituitary reproductive axis in the male rat. Toxicol. 57: 173-182.
43. Rajeswary, S., Kumaran, B., Ilangovan, R., Yuvaraj, S., Sridhar, M., Venkataraman, P., Srinivasan, N. and Aruldhas, M. M. 2007. Modulation of antioxidant defense system by the environmental fungicide carbendazim in Leydig cells of rats. Reprod. Toxicol. 24: 371-380.
44. Markelewicz Jr ,R. J., Hall, S. J. and Boekelheide, K. 2004. 2,5-Hexanedione and carbendazim coexposure synergistically disrupts rat spermatogenesis despite opposing molecular effects on microtubules. Toxicol. Sci. 80: 92-100.
45. Christiansen, S., Scholze, M., Dalgaard, M., Vingaard, A. M., Axelstad, M., Kortenkamp, A. and Hass, U. 2009. Synergistic disruption of external male sex organ development by a mixture of four antiandrogens. Environ. Health Perspect. 117: 1839-1846.
46. OECD. 2009. Hershberger bioassay in rats: A short-term screening assay for (anti)androgenic properties. OECD Guideline for Testing of Chemicals 441.
47. Wilson, V. S., Blystone, C. R., Hotchkiss, A. K., Rider, C. V., Gray Jr, L. E. 2008. Diverse mechanisms of anti-androgen action: impact on male rat reproductive tract development. Int. J. Androl. 31: 178-187.
48. Blystone, C. R., Lambright, C. S., Cardon, M. C., Furr, J., Rider, C. V., Hartig, P. C., Wilson, V. S. and Gray Jr, L. E. 2009. Cumulative and antagonistic effects of a mixture of the antiandrogens vinclozolin and iprodione in the pubertal male rat. Toxicol. Sci. 111: 179-188.
49. Moorman, W. J., Cheever, K. L., Skaggs, S. R., Clark, J. C., Turner, T. W., Marlow, K. L. and Schrader, S. M. 2000. Male adolescent exposure to endocrine-disrupting pesticides: vinclozolin exposure in peripubertal rabbits. Andrologia 32: 285-293.
50. Wolf, C. J., LeBlanc, G. A., Ostby, J. S. and Gray Jr, L. E. 2000. Characterization of the period ofsensitivity of fetal male sexual developmental to vinclozolin. Toxicol. Sci. 55: 151-161.
51. Monosson, E., Kelce, W. R., Lambright, C., Ostby, J. and Gray Jr, L. E. 1999. Peripubertal exposure to the antiandrogenic fungicide, vinclozolin, delays puberty, inhibits the development of androgen-dependent tissues, and alters androgen receptor function in the male rat. Toxicol. Ind. Health 15: 65-79.
52. Gray Jr, L. E., Ostby, J., Monosson, E. and Kelce, W. R. 1999. Environmental antiandrogens: low doses of the fungicide vinclozolin alter sexual differentiation of the male rat. Toxicol. Ind. Health 15: 48-64.
53. Laws, S. C., Carey, S. A., Kelce, W. R., Cooper, R. L Gray Jr, L. E. 1996. Vinclozolin does not alter progesterone receptor (PR) function in vivo despite inhibition of PR binding by its metabolites in vitro. Toxicol. 112: 173-182.
54. Wong, C., Kelce, W. R., Sar, M. Wilson, E. M. 1995. Androgen receptor antagonist versus agonist activities of the fungicide vinclozolin relative to hydroxyflutamide. J. Biol. Chem. 270: 19998-20003.
55. Gray Jr, L. E., Ostby, J. S. Kelce, W. R. 1994. Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rat. Toxicol. Appl. Pharmacol. 129: 46-52.
56. Gray Jr, L. E., Wolf, C., Lambright, C., Mann, P., Price, M., Cooper, R. L Ostby, J. 1999. Administration of potentially antiandrogenic pesticides (procymidone, linuron, iprodione, chlozolinate, p,p'-DDE, and ketoconazole) and toxic substances (dibutyl- and diethylhexyl phthalate, PCB 169, and ethane dimethane sulphonate) during sexual differentiation produces diverse profiles of reproductive malformations in the male rat. Toxicol. Ind. Health 15: 94-118.
57. Ostby, J., Kelce, W. R., Lambright, C., Wolf, C. J., Mann, P., Gray, L. E. Jr. 1999. The fungicide procymidone alters sexual differentiation in the male rat by acting as an androgen-receptor antagonist in vivo and in vitro. Toxicol. Ind. Health 15: 80-93.
58 Lambright, C., Ostby, J., Bobseine, K., Wilson, V., Hotchkiss, A. K., Mann, P. C., Gray, L. E. Jr. 2000. Cellular and molecular mechanisms of action of linuron: an antiandrogenic herbicide that produces reproductive malformations in male rats. Toxicol. Sci. 56: 389-399.