EVALUATION OF A 5-DAY HERSHEYBERGER ASSAY USING YOUNG MATURE MALE RATS: METHYLTTESTOSTERONE AND p,p'-DDE, BUT NOT FENITROTHION, EXHIBITED ANDROGENIC OR ANTIANDROGENIC ACTIVITY IN VIVO

Osamu SUNAMI, Takeshi KUNIMATSU, Tomoya YAMADA,
Setsuko YABUSHITA, Tokuo SUKATA, Kaori MIYATA, Yusuke KAMITA,
Yasuoyoshi OKUNO, Takaki SEKI, Iwao NAKATSUKA and Masatoshi MATSUO

Environmental Health Science Laboratory, Sumitomo Chemical Company, Ltd.,
3-1-98 Kasugade-naka, Konohana-ku, Osaka 554-8558, Japan

(Received May 17, 2000; Accepted August 7, 2000)

ABSTRACT — A 5-day Hersberger assay using young mature male rats to detect compounds interfering with androgen receptor (AR)-mediated mechanisms was evaluated for ability to identify p,p'-DDE (a weak AR antagonist) and methyltestosterone (MT, an AR agonist). Fenitrothion, an organophosphate pesticide, was also evaluated in this validated assay.

Castrated male Crl:CD(SD)IGS rats (1 week after castration, 11 weeks of age) were subjected to experiments. To determine a suitable value of testosterone propionate (TP) as a reference androgen for detection of antiandrogenic chemicals, castrated male rats were treated daily with TP (0, 0.06, 0.25, 1, 4, or 16 mg/kg/day, s.c.). TP produced increases in weights of ventral prostate, seminal vesicles and levator ani plus bulbocavernosus muscles. Serum androgen level measured by RIA kit (mostly TP) were elevated in a dose-related manner, while the weights of organs with 1 mg/kg/day of TP were nearly equivalent to the maximum responses (i.e., sub-maximal). One hundred mg/kg/day of p,p'-DDE significantly attenuated TP 0.1 mg/kg-induced increases in weights of seminal vesicles and muscles, and TP 1 mg/kg-induced increases in weights of ventral prostate, seminal vesicles and muscles, but did not affect the weight of these organs in either TP 16 mg/kg-treated or intact rats, demonstrating that the dose range of 0.1 - 1 mg/kg TP is suitable for reference androgen. Oral treatment with 100 mg/kg of MT increased the weights of ventral prostate, seminal vesicles and muscles as strongly as did subcutaneous injection of 1 mg/kg of TP. These findings demonstrate that the 5-day Hersberger assay using young mature as well as immature male rats is a sensitive and valid short-term screening method for the detection of chemicals interfering with AR-mediated mechanisms.

To determine whether fenitrothion interferes with AR-mediated mechanisms in vivo, fenitrothion (0, 0.75, 1.5 or 3 mg/kg/day) was administered by gavage for 5 days to castrated rats for androgenicity, or to castrated rats treated with 1 mg/kg TP for antiandrogenicity. Treatment with fenitrothion had no adverse effects on clinical signs, body weight, or liver or kidney weights, but cholinesterase activities in the brain and erythrocytes were significantly suppressed by fenitrothion to, respectively, 77-81% and 66-67% of control levels. In the antiandrogenicity experiment, serum androgen levels of TP-treated, castrated rats did not differ among groups. Treatment with 100 mg/kg of p,p'-DDE as a positive control again significantly attenuated TP-induced increases in weights of the ventral prostate and seminal vesicles, while fenitrothion had no effect on the weights of any organs. In the androgenicity experiment, treatment with 100 mg/kg of MT significantly increased weights of ventral prostate, seminal vesicles and muscles, but fenitrothion had no effects on the weights of any of these organs. These findings yield no evidence that fenitrothion interferes with AR-mediated mechanisms in vivo, consistent with the result of several toxicological bioassays.

KEY WORDS: Endocrine disruptor, Screening, Hersberger assay, Fenitrothion, p,p'-DDE, Methyltestosterone

Correspondence: Tomoya YAMADA
INTRODUCTION

The endocrine system has important functions in a multitude of physiological processes including embryogenesis, cellular differentiation, homeostasis, and carcinogenesis. Recently, public concern has emerged that certain environmental xenobiotics may cause toxicity in wildlife and humans by disrupting endocrine systems. However, in most cases, the link between such environmental chemicals with hormone-like activity and adverse effects on humans, fish, and wildlife has not been well established. For regulatory purposes, toxicological tests have been performed according to internationally accepted test guidelines. However, several recent workshops and publications have questioned whether the current test guidelines are suitable for identifying endocrine active compounds (EACs) (Ankley et al., 1997; EDSTAC, 1998; Gray et al., 1997; OECD, 1997). The need for revision of existing test guidelines and for development of new test guidelines specifically to address the potential adverse effects arising as a result of endocrine disruption has been proposed by several committees such as the Endocrine Disruptor Screening Testing and Advisory Committee (EDSTAC, 1998) and the OECD Endocrine Disruptor Testing and Assessment (EDTA) Working Group. Screening programs for endocrine disruptors are currently being implemented.

Particular public concern has arisen concerning the possibility that some of these environmental xenobiotics may affect human health by mimicking endogenous estrogens (Colborn et al., 1993). Recently, this concern has extended not only to the estrogen-hormone system, but to the androgen- and thyroid-hormone systems, as well. Several in vitro assays have been developed to identify androgens or antiandrogens on the basis of receptor-mediated mechanisms such as competitive binding and receptor/reporter gene assays. Sumpter (1998) demonstrated that fenitrothion was the firstly identified androgen agonist in the environment, based on a yeast-based in vitro assay. Fenitrothion is an organophosphorus pesticide that has been in use since 1959 (WHO, 1992). It is used in agriculture to control insects on rice, cereals, fruits, vegetables, stored grains and cotton. It is also used to control insects in forests and for fly, mosquito, and cockroach control in public health programs (WHO, 1992). Based on the toxicological data, fenitrothion has been believed not to interact with AR-mediated mechanisms. However, fenitrothion has not yet been examined in in vivo tests specifically focused on endocrine disruption.

The Hershberger assay is one of the in vivo assays proposed by EDSTAC and OECD for chemicals that have the potential to act like endogenous male sex hormones, because it has been used predominantly by the pharmaceutical industry to evaluate androgenic and antiandrogenic agonists for potential therapeutic use (Dorfman 1969a and 1969b; Hershberger et al., 1953). However, there are many variations in the protocol used for this assay (Ashby and Lefevre, 2000b; Dorfman 1969a and 1969b; Hershberger et al., 1953; Kelce et al., 1997; O’Connor et al., 1999a; Raynaud et al., 1980; Rittmaster et al., 1991; Shao et al., 1994; Snyder et al., 1989; Sunahara et al., 1987; Wakeling et al., 1981; Yamada et al., 2000). Validation and standardization are required for use of the Hershberger assay as a screening tool for EACs based on AR-mediated mechanisms. Key issues include the following: animal species, animal age, acclimatization period after castration, administration period, administration route of test chemicals and reference androgen, dose of reference androgen for antiandrogenicity, time intervals separating administrations of test chemicals and reference androgen, organs to be weighed, method of weighing, and others. The following assay conditions were used in this study: (1) young mature rats, 11 weeks old, (2) 1-week acclimatization period after castration, (3) 5-day treatment with oral gavage of test chemicals, (4) 5-day subcutaneous injection of reference androgen (testosterone propionate), (5) co-administration of test chemical and reference androgen, within 20 min, and (6) determination of weights of the ventral prostate, seminal vesicles and levator ani plus bulbocavernosus muscles. The present study focused on whether this assay can be used to detect activity of positive control, including p.p’-DDE (100 mg/kg/day) for antiandrogenicity (Ashby and Lefevre, 2000a and 2000b; Gaido et al., 1997; Kelce et al., 1995; O’Connor et al., 1999a; You et al., 1998), and methyltestosterone (100 mg/kg/day; based on this study) for androgenicity (Ashby and Lefevre, 2000b; Clark and Fast, 1996).

The objectives of the present study were to evaluate the reliability and feasibility of the 5-day Hershberger assay using young mature male rats, and to clarify whether the organophosphate pesticide fenitrothion interacts with AR-mediated mechanisms using this in vivo assay.

MATERIALS AND METHODS

Test materials

Materials were obtained from the following manu-
Hershberger assay using young mature rats.

facturers: testosterone propionate (TP) (purity: >97%, Catalog No. 207-08431) and methyltestosterone (MT) (purity: >97%, Catalog No. 134-09932), Wako Pure Chemical Industries (Osaka, Japan); 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene (p,p'-DDE) (purity: 99%, Catalog No. 12,389-7), Aldrich Chemical Company (Milwaukee, WI); fenitrothion (purity: 99.7%), Sumitomo Chemical Co. (Osaka, Japan); pulverized diet CRF-1, Oriental Yeast Co. (Tokyo, Japan); corn oil (Nakarai Tesque, Inc., Kyoto); DPC total testosterone kit, Diagnostic Products Corporation (Los Angeles, CA).

Animals and housing

All experiments were performed in accordance with The Guide for Animal Care and Use of Sumitomo Chemical Co. Ltd. Male Crl:CD(SD)IGS rats were purchased from Charles River Japan, Inc. (Shiga, Japan). They were 9 weeks of age upon arrival, and were acclimatized to the laboratory environment for 7 days before use. After the quarantine period, animals in good health (based on clinical signs and body weights) were selected for the study, and then castrated under ether anesthesia. Castration was performed via the scrotum, and chemical treatment was not commenced until 7 days later to allow for complete recovery from surgical stress (based on body weight and food consumption, animals were considered to be recovered from the operation stress within 1 week). During the experiment, rats were housed two per cage in stainless steel wire mesh cages under controlled environmental conditions, including a temperature of 24 ± 2 degree centigrade, a relative humidity of 55 ± 10%, a frequency of ventilation of more than 10 air exchanges/hr, and a 12-hr light/dark cycle (lights on, 0800-2000). Drinking water and pellet rodent diet were available ad libitum. Prior to the operation, rats were assigned to each group by the stratified randomization method using a computer program based on body weight and body weight gain during a quarantine period, so that no significant differences in mean body weight were present among the groups. At the commencement of treatment, it was confirmed that no significant difference existed in mean body weight among the groups, since variation in animal body weight may be a source of variation in weight of accessory sex glands.

Study design

For each experiment, the study design included 6 males per group, since small group size would be preferable from the viewpoint of animal welfare and suffice for routine screening; EDSTAC (1998) noted that most studies are able to detect significant effects of test chemicals with only 5 animals per group (we selected 6 animals per group based on number of animals per cage, 2). Test and reference substances were suspended or dissolved in a test vehicle (corn oil). The daily amounts of administration were 5 ml/kg BW for oral gavage and 0.5 ml/kg BW for subcutaneous injections. The amount administered for each animal was calculated based on body weights on Days 1 and 3, since daily body weight changes at this age are within 2% of body weight: in fact, body weight changes for Days 1-3, and 3-6 were within only 2.2 and 3.5%, respectively. For all experiments, clinical signs, body weight, and weights of liver and kidneys were determined as indices of systemic toxicity.

1. Experiment 1 (Optimization of dosage of reference androgen)

This experiment was performed to determine a suitable value of TP as reference androgen for assessment of antiandrogenicity of test chemicals. Castrated male rats were administered TP (doses: 0.06, 0.25, 1, 4, or 16 mg/kg/day) daily by subcutaneous injections on the dorsal surface in the morning (AM 0900-1130). Control rats were administered vehicle in the same manner. One day after the final administration, all rats were anesthetized with ether, and then euthanized by blood withdrawal from the abdominal aorta (AM 0830-1100). Collected blood was used for determination of serum androgen levels. After careful trimming to remove fat and other contiguous tissue in a uniform manner, the ventral prostate, seminal vesicles (with coagulating glands) and levator ani plus bulbocavernous muscles were weighed wet (the ventral prostate and seminal vesicles were weighed after fixation overnight in a 10% neutral-buffered formalin; we previously confirmed that this procedure did not affect wet weights of these organs and enhanced study accuracy). The ventral prostate and seminal vesicles (with coagulating glands) were weighed separately, since these organs respond to different androgens (Gray et al., 1997; Prahalada et al., 1998).

2. Experiment 2 (Evaluation of 5-day Hershberger assay for p,p'-DDE or MT)

For assessment of antiandrogenicity, p,p'-DDE 100 mg/kg/day was administered daily by gavage for 5 days to castrated male rats treated with 0.1, 1, or 16 mg/kg/day of TP, or age-matched intact rats. To prevent leakage of the TP solution administered by subcutaneous injections on the dorsal surface, p,p'-DDE was
administered first by gavage, and then TP was administered within 20 min (administration in the opposite order was confirmed to produce identical responses). The dose of \( p,p'-\text{DDE} \) was selected based on results of previous studies (Kelce et al., 1995; O'Connor et al., 1999a; You et al., 1998). For assessment of androgenicity, MT (20, 100 or 200 mg/kg/day) was administered daily by gavage for 5 days to castrated male rats. Each dose of MT was separately examined since no suitable reference dose could be found; however, each experiment was conducted with a concurrent control. To determine the magnitude of response to MT, changes in weights of the designated organs following s.c. injection of 1 mg/kg/day of TP were compared. The designated organs were weighed as described in Experiment 1.

3. Experiment 3 (Assessment of androgenic and antiandrogenic activities of fenitrothion in the validated Hershberger assay)

To determine whether fenitrothion interferes with AR-mediated mechanisms in vivo, fenitrothion (0, 0.75, 1.5 or 3 mg/kg/day) was administered by gavage for 5 days to castrated rats to determine its androgenic effects, and to castrated rats treated with 1 mg/kg TP to determine its antiandrogenic effects. The designated organs were weighed as described in Experiment 1. In the experiment for assessment of antiandrogenicity, serum androgen levels were determined. Clinical signs, body weight, food consumption, weights of liver and kidneys, and cholinesterase (ChE) activities in the brain and erythrocytes (control and highest dose groups) were also examined as indices of systemic toxicity. Three mg/kg/day of fenitrothion was selected based on results of a preliminary study in which 3 mg/kg/day of fenitrothion significantly decreased ChE activity in brain and erythrocytes, meeting the criteria for clear toxicological effects of the FAO/WHO (1999) and for maximum tolerated dose (MTD) of the US EPA (1987). The dose of MT and \( p,p'-\text{DDE} \) at 100 mg/kg/day were concurrently administered as positive controls for androgenicity and antiandrogenicity, respectively.

Blood sampling and serum androgen measurement

On the morning of the day of euthanization, rats were transferred from the animal room to a room adjacent to the necropsy room. All rats were maintained quietly for at least 1 hr prior to blood sampling (to avoid effects of transfer stress on hormone levels). Rats were euthanized by blood withdrawal from the abdominal aorta under light ether anesthesia, which had previously been confirmed not to unduly affect circulating androgen levels. Serum was prepared from a portion of the collected blood and stored at -80 degrees centigrade until analysis of serum androgen concentrations, which were measured using commercially available RIA kit. The antibody used has cross-reactivity to 11-ketotestosterone, 5α-dihydrotestosterone, TP, etc., therefore, the data were represented here as serum androgen levels. All samples were measured in duplicate in the same assay, and intra-assay coefficients of variations were 8-9%.

Assay of ChE activity

Erythrocytes were obtained by centrifuging the heparinized blood at 2120 g for 10 min, and washed with ice-cold saline by centrifugation, diluted with 2-fold saline, and assayed for ChE. The right hemisphere of the brain was weighed and homogenized with an 11.5-fold volume (v/w) of 0.1% cold Triton X-100 solution. The homogenate was centrifuged at 2120 g for 10 min, and the supernatant was assayed for ChE. ChE activities were determined using acetylthiocholine iodide as a substrate by the methods of Ellman et al. (1961) with modification, with autoanalyzers (AutoAnalyzer II (Bayer Corp., New York) for erythrocytes and AutoAnalyzer Express Plus (Bayer Corp., New York) for the brain). The assay method for ChE activity using iodide as a substrate can measure both true and pseudo-ChE in specimens from rats (Unakami et al., 1987).

Statistical analysis

All results were analyzed by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test. Testosterone data were analyzed by the Kruskal-Wallis test (Kruskal and Wallis, 1952 and 1953), followed by the Mann-Whitney U-test (Mann and Whitney, 1947), if values were below the limit of quantitation. The significance of differences from the control group was estimated at probability levels of 1 and 5%.

RESULTS

Rationale for use of young mature male rats

Fig. 1 shows the coefficients of correlation (\( R^2 \)) between body and organ weights, and coefficients of variation of each organ weight and body weight at 7 and 11 weeks (the data shown in Fig. 1 was derived from the representative control group of the previous studies: similar findings were observed in several stud-
Hershberger assay using young mature rats.

ies performed in this laboratory). A relatively high correlation between each organ weight and body weight was observed in rats at 7 weeks but not at 11 weeks. The data of rats aged 7 weeks is consistent with the previous findings: the near-linear increase in body weight after weaning and non-linear changes in relative organ weights (absolute organ weight/body weight) of the seminal vesicles and prostate were noted over at least the first two weeks after weaning (Ashby and Lefevre, 2000a). The coefficient of variation for each organ weight and body weight was also larger at 7 weeks than at 11 weeks. These findings suggest that variation in animal body weight may be a source of variation in the weight of accessory sex glands in immature rats, and that rats aged 11 weeks have fewer factors affecting assay reliability. Subsequent experiments were therefore performed with young mature rats: the experiments were performed at 11 weeks of age.

Optimization of dosage of reference androgen

This experiment was performed to optimize the conditions of treatment with TP as a reference androgen for assessment of antiandrogenic effects. Subcutaneous injections of TP increased serum androgen levels in a dose-related manner. The increase was not saturable: statistically significant increases in serum androgen levels compared to control were observed at dose levels of 0.25 mg/kg/day and higher (Fig. 2). The weights of ventral prostate, seminal vesicles, and levator ani plus bulbocavernosus muscles were also increased; the responses at 1 mg/kg/day of TP were

**Fig. 1.** Comparison of variation in organ weights between immature and young mature rats. Values are correlations between organ weight and body weight for male rats 7 or 11 weeks of age. $R^2$, correlation coefficient; CV, coefficient variation of organ weight. CVs of body weights of male rats 7 (n=10) and 11 (n=12) weeks old were 0.085 and 0.042, respectively.

**Fig. 2.** Serum androgen levels in castrated rats treated with testosterone propionate. Castrated rats were administered testosterone propionate (TP) daily by subcutaneous injection for 5 days. One day after the final injection, serum androgen levels were determined using an RIA kit. Values are means±SDs; n=6. Significantly different from control: **p<0.01.
nearly equivalent to the maximum response (i.e., submaximal) (Fig. 3). The dose-response relationships for organ weights differed from those of serum androgen level.

**Evaluation of 5-day Hershberger assay using young mature rats by treatment with p,p'-DDE or MT**

One hundred mg/kg/day of p,p'-DDE significantly attenuated TP 0.1 mg/kg/day-induced increases in weights of seminal vesicles and muscles, and TP 1 mg/kg/day-induced increases in weights of ventral prostate, seminal vesicles and muscles, but did not affect weights of these organs in either TP 16 mg/kg/day-treated or intact rats (Fig. 4). These findings demonstrate that a dose range of 0.1 - 1 mg/kg/day TP is suitable for reference androgen in assessment of antiandrogenic effects. One mg/kg/day of TP was selected for subsequent experiments, since relatively large organs make handling easy.

To determine a positive control for androgenicity, TP was orally administered for 5 days at dose levels up to 16 mg/kg/day. However, no effects were observed on any organs tested (data not shown). Treatment with MT by oral gavage at dose levels of 100 or 200 mg/kg/day (but not 20 mg/kg/day) significantly increased the weights of ventral prostate, seminal vesicles and muscles, with equivalent increases at 100 and 200 mg/kg/day (Fig. 5). Furthermore, the magnitude of responses was equivalent to that for subcutaneous injection of 1 mg/kg/day TP.

![Graph of organ weights](image1)

**Fig. 3.** Organ weights of castrated rats treated with testosterone propionate. Castrated rats were administered testosterone propionate (TP) daily by subcutaneous injection for 5 days. One day after the final injection, the animals were euthanized and the designated organs were weighed. Values are means±SDs; n=6. Significantly different from vehicle control: **p<0.01.

![Graph of organ weights](image2)

**Fig. 4.** Effects of p,p'-DDE on organ weights in intact rats and castrated rats treated with testosterone propionate. Castrated rats administered testosterone propionate (TP) daily by subcutaneous injection or intact rats were administered p,p'-DDE (100 mg/kg/day) by oral gavage for 5 days. One day after the final administration the animals were euthanized and the designated organs were weighed. Values are means±SDs; n=6. Significantly different from each control (p,p'-DDE 0 mg/kg/day): *p<0.05, **p<0.01.
Hershberger assay using young mature rats.

**In vivo assessment of androgenic and antiandrogenic activities of fenitrothion in the validated Hershberger assay**

On assessment of systemic toxicity, clinical signs, body weight, and liver and kidney weights were not affected by fenitrothion in a series of experiments, whereas 3 mg/kg/day of fenitrothion significantly suppressed ChE activities in the brain and erythrocytes to respectively 77-81% and 66-67% of control values (Tables 1 and 2).

TP injection to castrated rats significantly increased serum androgen levels compared to those in no TP-treated castrated rats; serum androgen levels did not differ among the injected groups (Table 1). Although treatment with 100 mg/kg/day of p,p' -DDE as a positive control appeared to result in higher serum androgen levels than in control, these differences were not statistically significant; this finding was consistent with that of a previous study (You et al., 1998). As shown in Fig. 6, treatment with 100 mg/kg/day of p,p' -DDE significantly attenuated TP-induced increases in weights of the ventral prostate and seminal vesicles (to 77 and 80% of control values, respectively). The weight of muscles also tended to be decreased. In contrast, fenitrothion had no significant effects on any organs.

On assessment of androgenicity, treatment with 100 mg/kg of MT as a positive control significantly increased weights of the ventral prostate, seminal vesicles and muscles; however, fenitrothion had no effects on weights of any organs (Fig. 7).

To confirm reproducibility of the above findings with fenitrothion, a series of experiments was per-

![Graphs showing organ weights and effects of treatments](https://via.placeholder.com/150)

**Fig. 5.** Effects of methyltestosterone or testosterone propionate on organ weights in castrated rats. Castrated rats were administered methyltestosterone (MT) daily by oral gavage or testosterone propionate (TP) by subcutaneous injection for 5 days. One day after the final administration, the animals were euthanized and the designated organs were weighed. Values are means±SDs; n=6. Significantly different from each control: **p<0.01.

**Fig. 6.** Effects of fenitrothion or p,p' -DDE on organ weights in castrated rats treated with testosterone propionate. Castrated rats administered testosterone propionate (TP) daily by subcutaneous injection were administered fenitrothion (F) or p,p' -DDE by oral gavage for 5 days. Doses were 0.75, 1.5, or 3 mg/kg/day for fenitrothion, and 100 mg/kg/day for p,p' -DDE. "TP 0" means castrated rats without TP treatment. One day after the final administration, the animals were euthanized and the designated organs were weighed. Data are as means±SDs; n=6. Significantly different from control (TP 1 mg/kg/day): *p<0.05, **p<0.01.
formed twice independently, and identical results were noted. These results demonstrate that fenitrothion does not interfere with AR-mediated mechanisms in vivo. Furthermore, to confirm the lack of antiandrogenic effects of fenitrothion, this chemical (3 mg/kg/day) was also assessed in castrated rats treated with 0.25 mg/kg/day of TP, which may have relatively high sensitivity to a weak antiandrogenic agent. As expected, 100 mg/kg/day of p,p'-DDE resulted in significant decreases to 55, 62, and 80% of control values for weights of the ventral prostate, seminal vesicles, and muscles, respectively. In contrast, again, weights of none of the organs were affected by fenitrothion (data not shown).

DISCUSSION

Validation of a 5-day Hershberger assay using young mature male rats

To identify potential endocrine-active chemicals (EACs), screening batteries are designed to determine whether or not the agent being evaluated has the potential to interact not only with the estrogen-hormone system, but the androgen- and thyroid-hormone systems (EDSTAC, 1998). Most effects of EACs appear to be receptor-mediated. The purpose of the Hershberger assay is to screen agents potentially agonist or antagonistic to AR-mediated mechanisms in vivo. The accessory sex glands/tissues are dependent upon androgen

Table 1. For anti-androgenicity: Effect of fenitrothion on body weights, organ weights, cholinesterase activities, and serum androgen levels in castrated male rats daily treated with testosterone propionate.

<table>
<thead>
<tr>
<th>Test chemicals</th>
<th>Doses (mg/kg/day)</th>
<th>TP</th>
<th>Final body weight (g)</th>
<th>Liver (g)</th>
<th>Kidneys (g)</th>
<th>Brain (U/g)</th>
<th>Erythrocyte (U/l)</th>
<th>Serum androgen levels (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>-</td>
<td>391 ± 13</td>
<td>13.9 ± 0.5</td>
<td>2.48 ± 0.2**</td>
<td>NE</td>
<td>NE</td>
<td>≤0.2</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>+</td>
<td>402 ± 15</td>
<td>13.9 ± 1.0</td>
<td>2.94 ± 0.19</td>
<td>11.5 ± 0.67</td>
<td>2259 ± 81</td>
<td>2.1 ± 0.24</td>
</tr>
<tr>
<td>Fenitrothion</td>
<td>0.75</td>
<td>+</td>
<td>406 ± 21</td>
<td>13.8 ± 1.2</td>
<td>2.66 ± 0.19</td>
<td>NE</td>
<td>NE</td>
<td>2.1 ± 0.54</td>
</tr>
<tr>
<td>Fenitrothion</td>
<td>1.5</td>
<td>+</td>
<td>404 ± 17</td>
<td>14.2 ± 1.0</td>
<td>2.71 ± 0.13</td>
<td>NE</td>
<td>NE</td>
<td>2.0 ± 0.41</td>
</tr>
<tr>
<td>Fenitrothion</td>
<td>3</td>
<td>+</td>
<td>406 ± 18</td>
<td>15.1 ± 1.1</td>
<td>2.77 ± 0.21</td>
<td>8.8 ± 0.83**</td>
<td>1518 ± 100**</td>
<td>1.9 ± 0.61</td>
</tr>
<tr>
<td>p,p'-DDE</td>
<td>100</td>
<td>+</td>
<td>401 ± 12</td>
<td>19.6 ± 1.9**</td>
<td>2.6 ± 0.1**</td>
<td>NE</td>
<td>NE</td>
<td>2.3 ± 0.76</td>
</tr>
</tbody>
</table>

Castrated rats administered testosterone propionate (TP, 1 mg/kg/day) daily by subcutaneous injection were administered fenitrothion or p,p'-DDE by oral gavage for 5 days.

Mean±SDs, N=6. NE: Not examined.

**: Significantly different from TP-treated control (p<0.01).

Table 2. For androgenicity: Effect of fenitrothion on body weights, organ weights, and cholinesterase activities in castrated male rats.

| Test chemicals | Doses (mg/kg/day) | TP | Final body weight (g) | Liver (g) | Kidneys (g) | Brain (U/g) | Erythrocyte (U/l) |
|----------------|-------------------|----|-----------------------|-----------|-------------|-------------|-----------------|-----------------|
| Control        | 0                 | -  | 359 ± 10              | 12.5 ± 1.1 | 2.4 ± 0.23  | 11.8 ± 0.38 | 2285 ± 94       |
| Fenitrothion   | 0.75              | -  | 370 ± 16              | 12.9 ± 1.3 | 2.4 ± 0.33  | NE          | NE              |
| Fenitrothion   | 1.5               | -  | 367 ± 9               | 13.9 ± 0.7 | 2.3 ± 0.1   | NE          | NE              |
| Fenitrothion   | 3                 | -  | 366 ± 7               | 12.8 ± 0.9 | 2.4 ± 0.27  | 9.6 ± 0.63**| 1512 ± 139**    |
| MT             | 100               | -  | 364 ± 14              | 13.7 ± 1.0 | 2.8 ± 0.2*  | NE          | NE              |

Castrated rats were administered fenitrothion or methyltestosterone (MT) daily by oral gavage for 5 days.

Testosterone propionate (TP) was not administered.

Mean±SDs, N=6. NE: Not examined.

Significantly different from control: *p<0.05, **p<0.01.
stimulation to gain and maintain weight during and after puberty. In intact animals, it is difficult to assess the effects of weakened androgen signaling on levels of circulating androgens and weights of the accessory sex glands/tissues, due to the existence of negative feedback mechanisms. In fact, a potent antiandrogen flutamide (10 mg/kg/day, 5-day treatment) decreased weights of the accessory sex glands/tissues (Yamada et al., 2000), whereas a weak antiandrogen p,p'-DDE (100 mg/kg/day, 5-day treatment) had no effects on these glands/tissues (Fig. 4). Furthermore, Ashby and Lefèvre (2000a) and O’Connor et al. (1999a) demonstrated that intact male rats (irrespective of age) failed to detect antiandrogenic effects of 100 mg/kg/day of p,p'-DDE with longer terms of exposure on organ weights. On the other hand, the Hershberger assay is designed to detect androgenic and antiandrogenic effects of the test chemicals in critical conditions, with a consistent level of the serum androgens (Hershberger et al., 1953). Therefore, the net effects of test chemicals can be detected. Although the Hershberger assay has been predominantly used by the pharmaceutical industry, studies focused on validation and standardization for screening of EACs are very few in number to date (Ashby and Lefèvre, 2000b; O’Connor et al., 1999a; Yamada et al., 2000). Screening should be designed to avoid false-negatives and false-positives, i.e., screening should be required to consistently predict the adverse findings caused by EAC exposure at critical phases of prenatal development. Many factors affect sensitivity of the Hershberger assay. In the present study, we focused on whether the Hershberger assay with our study protocol can detect endocrine activity of possible EACs at dose levels at which such agents have adverse effects on prenatal development.

When assay systems are validated, selections of reference chemicals and its dose levels are important. In this study, p,p'-DDE was selected as a positive control for antiandrogenicity, since it is a known environmental contaminant and is thought to have weak antiandrogenic activity in vitro and in vivo (Ashby and Lefèvre, 2000b; Gaido et al., 1997; Kelce et al., 1995; O’Connor et al., 1999a; You et al., 1998). p,p'-DDE is the major and persistent metabolite of the widely used pesticide p,p'-DDT. Kelce et al. (1995) found that p,p'-DDE specifically binds to AR and inhibits androgen-induced AR transcriptional activity. The affinity of p,p'-DDE for the AR was about 1/10 that of hydroxyflutamide, the active metabolite of flutamide (a clinically used AR antagonist). p,p'-DDE was approximately 10-fold less potent than T in a yeast-based steroid hormone receptor gene transcription assay (Gaido et al., 1997). These findings indicate that p,p'-DDE is suitable as a weak reference antiandrogen. Furthermore, the effects of p,p'-DDE on male sexual development were examined in rats followed by in utero and lactational exposures; 100 mg/kg/day of p,p'-DDE induced antiandrogenic responses such as reduction in male anogenital distance, increase in retention of male thoracic nipples, and alteration in expression of AR (Kelce et al., 1995; You et al., 1998). Based on these findings, in the present study, 100 mg/kg/day of p,p'-DDE was selected as a positive control for assessing weak antiandrogenic activity as used in a previous study (O’Connor et al., 1999a).

Regarding androgenicity, although several environmental chemicals possibly exhibit interaction with AR in vitro (Kelce et al., 1995; Sohoni and Sumpter, 1998), none of them have to our knowledge been confirmed to have androgenic activity in vivo. Therefore,
pharmaceutical agents were used in the present study. Oral administration was selected for test chemicals, in compliance with the protocol recommended by EDSTAC (1998). First, TP was orally administered at dose levels up to 16 mg/kg/day, but no responses were observed. Finally, we found that oral administration of MT at 100 mg/kg/day significantly increased the weights of ventral prostate, seminal vesicle, and levator ani plus bulbocavernous muscles as strongly as subcutaneous injection with 1 mg/kg/day of TP. Therefore, 100 mg/kg/day of MT was selected as a positive control for androgenicity in subsequent experiments.

The protocol of the present study is similar to that recommended by EDSTAC in the appendix of Chapter 5 of their final report (1998); the major differences between them were (1) animal age, immature vs. mature; (2) treatment period, 7-10 days vs. 5 days; and (3) dose of TP as reference androgen, and 50 μg /rat/day vs. 1 mg/kg/day (in each case the former value was recommended by EDSTAC). As described by O'Connor et al. (1999a), organ weight changes induced by AR antagonists appear to be generally greater in sexually immature rats than in sexually mature rats (Cook et al., 1993; Kelce et al., 1995; Viguier-Martinez et al., 1983a and 1983b). Ashby and Lefèvre (2000b) recommended castration of rats at -6 weeks of age with a 1-week recovery period and a minimum 7-day treatment period, based on results of many experiments. Furthermore, the use of 18-week-old rats and a 10-day dose regime failed to detect the antiandrogenic effect of 200 mg/kg/day of p,p'-DDE (Ashby and Lefèvre, 2000b). On the other hand, Kelce et al. (1997) reported large changes in tissue weights induced by p,p'-DDE (200 mg/kg/day) in 18-week-old rats. However, young mature rats (approximately 10 weeks old; just matured) have not been evaluated. The present study used young mature rats because they have many merits in reliability and feasibility of the Hershberger assay: this protocol may provide a more convenient assay of similar sensitivity. The weight of mature rats is higher than that of immature rats, which increases the accuracy of adjustment of dosing volume by body weight. Body weight gain during the dosing period is smaller in mature than in immature rats, making daily adjustment for body weight unnecessary. The organ sizes of mature rats are larger than those of immature rats, which results in less artifact at trimming of tissue for organ gravimetry. A 5-day treatment period was selected for the present study, since a shorter period of treatment would be preferred in view of animal welfare and suffice for routine screening. Sensitivity of this assay is affected by serum androgen levels in TP-treated rats: levels below or above the suitable levels of serum androgen might either decrease sensitivity due to reducing a range of weight differences or may make the response resistant to androgen antagonists. The dose of TP, which produces sub-maximal response, was selected based on the dose-finding study, as shown in Figs. 1 and 2. Under the conditions of the present study, even mature rats can clearly detect the androgenic activity of MT and the antiandrogenic activity of p,p'-DDE at 100-mg/kg/day level. Although we have not directly compared activities of immature and mature rats, our findings demonstrate that young mature as well as immature rats can be used in the Hershberger assay.

**In vivo assessment of interaction of fenitrothion with AR-mediated mechanisms in the validated Hershberger assay**

Fenitrothion was demonstrated as the first environmental androgen based on findings in a yeast-based *in vitro* assay (Sumptor, 1998). A summary and evaluation of toxicological data for fenitrothion were reviewed by the World Health Organization (WHO) (1992). In that review, no carcinogenic, mutagenic, teratogenic or reproductive effects of the androgenicity of fenitrothion were noted. However, no studies have specifically evaluated the effect of fenitrothion on AR-mediated mechanisms *in vivo*. This is the first study to examine whether fenitrothion interferes with AR-mediated mechanisms in a specific *in vivo* assay system, the Hershberger assay.

The goal of the studies such as the Hershberger and peripubertal male rat assays *etc.* is to assess endocrine modulation in absence of gross toxicity (Ashby and Lefèvre, 2000a). Severe systemic toxicity interferes with endocrine function; e.g., body weight suppression of more than 15-20% confounded endocrine endpoints such as hormone levels and organ weights (O'Connor et al., 1999a, 1999b and 2000). Because it is an organophosphatase, fenitrothion suppresses ChE activity in plasma, erythrocytes, and brain and liver tissues (WHO, 1992). Rats receiving fenitrothion at a dose of 30 mg/kg/day for 14 days exhibited typical signs of cholinergic stimulation such as miosis, muscle fibrillation, salivation, soft stool, and diarrhea during the course of exposure, as well as adrenal hyperfunction, and 4 of 47 rats (in total) died after 6 to 10 consecutive days of exposure (Kunimitsu et al., 1996). At lower doses such as 3 mg/kg/day, rats had no strong cholinergic signs, adrenal hyperfunction, or evi-
Hershberger assay using young mature rats.

dence of immunosuppression despite significant suppression of systemic ChE activities (Kunimatsu et al., 1996). The FAO/WHO (1999) considered that statistically significant inhibition of brain and erythrocyte ChE by 20% or more represents a clear toxicological effect. The US. EPA (1987) demonstrated that criteria for the maximum tolerated dose (MTD) includes significant depression of at least two of the assayed ChE enzyme measurements, i.e., plasma, erythrocytes, or brain ChE levels. In the present study, 3 mg/kg/day of fenitrothion revealed 19-34% suppression of brain and erythrocyte ChE activities with statistical significance, indicating that fenitrothion was systemically absorbed and had biological effects. Although the degree of ChE suppression in the present study meets the criteria of FAO/WHO and US.EPA, this change is unlikely to affect the sensitivity and specificity of the assay, since the doses tested had no severe adverse effects on clinical signs, body weight change, or liver or kidney weights. Therefore, the doses of fenitrothion used in this study were appropriate for determining whether fenitrothion interferes with AR-mediated mechanisms in vivo. Under our conditions, fenitrothion had neither agonistic nor antagonistic effects on AR-mediated mechanisms in vivo, inconsistent with in vitro findings. In vitro assays can provide valuable insights into mechanisms of action of test chemicals, but have limited ability to mimic whole animal chemical metabolism and distribution. The US. EPA (1998) generally gives greater weight to in vivo studies with relevant endpoints than to in vitro studies.

Uptake, metabolism and excretion of fenitrothion were reviewed by WHO (1992). Fenitrothion is rapidly absorbed from the intestinal tract of experimental animals and distributed to various body tissues. The half-life for the dermal absorption of fenitrothion in the monkey was 15-17 hr. The major route of elimination is via the urine, and most of the metabolites are eliminated within 2-4 days in the rat, guineapig, mouse and dog. Fenitrothion has been shown to be metabolized through the major pathways of O-demethylation and by cleavage of the P-O-aryl bond. The major metabolites reported are demethyl fenitrothion, demethyl fenitrooxon, dimethylphosphorothioic acid and dimethylphosphoric acid, and 3-methyl-4-nitrophenol and its conjugates. Fenitrooxon, an active product with ChE suppression effects, is more rapidly excreted. Therefore, fenitrothion and its metabolites are unlikely to remain in the body for a prolonged period (WHO, 1992). In fact, fenitrothion at dietary concentration up to 120 ppm did not cause any impairment in reproductive performance of rats in the 2-generation study (unpublished data; Hoberman, 1990). Although fenitrothion is probably present in the environment, WHO concluded that field observations have revealed no effects of fenitrothion on populations of small wild mammals. These considerations suggest that the effect of this agent detected in the in vitro assay is neither a hazard to mammals nor evidence of risk of endocrine disruption. This is strongly supported by the recent finding that fenitrothion did not alter the age at puberty in male rats when administered for 20 days at 5 or 15 mg/kg/day in peripubertal male rat assay (Ashby and Lefevre, 2000a).

Under the OECD framework, the Hershberger assay is in the initial stages of the validation and standardization process. The findings of the present study may provide information useful to this process. As shown in this study, the 5-day Hershberger assay using young mature (as well as immature) male rats is sensitive enough to detect chemicals with weak androgenic or antiandrogenic activity. Although further studies are required to determine the inter-laboratory reproducibility, our study protocol appears to be technically feasible for routine use as a screening study.

ACKNOWLEDGMENT

The authors gratefully acknowledge the helpful advice of Dr. Earl L. Gray Jr. (US. EPA) for selecting positive control and Dr. William R. Kelce (Searle) for research strategy. The authors would also like to thank Ms. Rie Funabiki for her technical assistance. Finally, we thank all members of the Environmental Health Science Laboratory of Sumitomo Chemical Co., Ltd. for their invaluable work.

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