Development of the Tiered Chemical Screening and Testing Program for Evaluating the Endocrine-disrupting Effects of Chemicals

CONTENTS

GENERAL INTRODUCTION

Certain chemicals, such as industrial chemicals, pesticides, pharmaceuticals, phytochemicals, food supplements, personal care products, and nutraceuticals, among others may have the potential to interfere with normal sexual differentiation and development in humans and wild animals (McLachlan, 1993; McLachlan and Korach, 1995). Consequently, several predictive test methods for the detection of endocrine disrupters are being apprised by international organizations, like the Organization for Economic Cooperation and Development (OECD) and U.S. Endocrine Disruptor Screening and Testing Committee (EDSTAC) (EDSTAC, 1998; OECD, 1997, 1999, 2001, 2003).

The Ministry of Economy, Trade and Industry, Japan (METI) has developed a tiered chemical screening and testing program for evaluating the endocrine-disrupting effects of chemicals (Fig. 1), focusing on effects mediated by estrogen receptors (ER) and androgen receptors (AR). In this program, chemicals are prioritized on the basis of their ER and AR binding affinities in an *in silico* system, and high-priority chemicals are then evaluated for hormonal activity in the program's early screening stage.

In the prioritization stage of this program, prioritization would involve an estimation of the chemical's ability to interact with ER and AR using a three-demension quantitative structure activity model in an *in silico* system, and high-throughput prescreening using AR or ER binding assay in a cell-free *in vitro* system, and receptor-dependent reporter gene expression assays in a whole-cell *in vitro* system. The principle of these *in vitro* predictive tests is based on their ability to detect hormone receptor-mediated interference with hormonal action.

Hormone receptor-binding assays and receptor-mediated reporter gene assays have also been proposed for use in the pre-screening stage and the screening stage to detect ER-mediated and AR-mediated endocrine disruption.

METI has also proposed three short-term *in vivo* rodent assays for use in the

screening stage to detect AR-mediated and ER-mediated endocrine disruption: the Hershberger assay in surgically castrated male rats, the uterotrophic assay in immature female rats, and the "enhanced OECD test guideline no. 407 (enhanced TG407)". The Hershberger assay can be used to screen for (anti-) androgenic compounds using castrated male rats; the assay is based on a gravimetric analysis of accessory sexual glands, like the ventral prostate, seminal vesicles, bulbocavernosus/levator ani muscles, glans penis, and Cowper's gland. The uterotrophic assay can be used to screen for (anti-) estrogenic compounds using immature female rats or surgically ovariectomized mature female rats; this assay is based on a gravimetric analysis of the uterus. The enhanced TG-407 is regarded as an important assay because the test chemicals are orally administered for 28 days and various parameters related to endocrine-mediated effects are examined.

Chemicals that test positive during the screening stage are then definitively tested using an in *vivo* study. A multi-generation reproductive test is candidate for the definitive test. However, the large number of animals and the long test duration that are required for a multi-generation study, as well as the assay's cost performance, are important concerns. Thus, METI has proposed the use of a test protocol in which the test chemical is administered during the embryonic or fetal period and /or the neonatal period an alternative candidate for a definitive test.

The OECD, EDSTAC and METI are now coordinating their efforts on the development of several assays. We are now developing the screening and testing methods to be used in each stage of this program and are also verifying the propriety of this tiered system scheme as an entrusted business from METI.

The efficacy of the uterotrophic assay, Hershberger assay and enhanced TG-407 protocols is now being investigated in an international effort by many laboratories using various chemicals at the OECD's initiation. We are participating in these international validation studies and, in addition, have collected various basic biological data for the development of screening and

testing methods (Takeyoshi *et al.*, 2001; Noda *et al.*, 2002; Sawaki *et al.*, 2003; Yamasaki *et al.*, 2000, 2001a, 2001b, 2001c, 2002a, 2002b, 2002c; 2002d). In an international research effort, special attention to protocol details is imperative to ensure that the results of different laboratories are comparative. In particular, differences in sensitivity of different rodent strains to the test chemicals are one of the major concerns (O'Connor *et al.*, 1999; Xinghua *et al.*, 2000). Charles River, Inc., developed a new rat breeding system, namely the Gold standard system, to obtain a uniform quality in experimental animals. Crj: CD® (SD) IGS rat (SD IGS rat) bred by this system can be used to develop of international protocols. However, while some general biological parameters of this rat have been reported, the development of the female genital system in SD IGS rats during the peripubertal period has not been well characterized. An understanding of the basic biological profile of changes in the female genital system during sexual maturation in the rat is of importance not only for analyzing the results of analysis, but also for standardizing of the assay protocol. In this study, background data on the sexual maturation of female SD IGS rats was collected as part of the international cooperation to develop a standardized assay protocol.

In *vivo* screening methods to detect thyroid function modulators are now being developed in many research laboratories. The "Thyroid Function in Immature Male or Female Rats (pubertal assay)" was, proposed by the EDSTAC as a screening assay to detect hormonal effects not only on male and female sexual maturation, but also on thyroid function (Goldman *et al.*, 2000; Stoker *et al.*, 2000). METI has prepared an enhanced TG-407 for the detection of not only (anti-)estrogenic and (anti-)androgenic chemical activities but of thyroid hormone modulators. However, this test method has some disadvantages, like a low specificity and a low cost-performance, and these disadvantages have yet to be overcome. In a second study, therefore, we examined the applicability of the Hershberger assay protocol for the screening of thyroid function modulators. Alternatively, thyroid hormone is known to regulate neuronal proliferation,

migration, and differentiation in the brain during the fetal and neonatal periods (Bernal and Nunez, 1995). Moreover, pre- or peri-natal hypothyroidism in rats can be induced by various thyroid effectors, and producing characteristic effects like a low body weight, auditory dysfunction, behavioral deficits, and learning impairment (Goldey *et al.*, 1995, 1998; Roegge *et al.*, 2000; Seo *et al.*, 1999). This information suggests that an *in utero*-lactation or neonatal exposure protocol may be particularly suitable as a definitive assay for the detection of thyroid inhibition. Thus, we investigated in *utero*-lactation exposure and neonatal exposure methods as part of preliminary trials to determine whether the endocrine effects of thyroid dysfunction could be detected using these methods and whether the detection of thyroid function modulators could be included in the proposed tiered system.

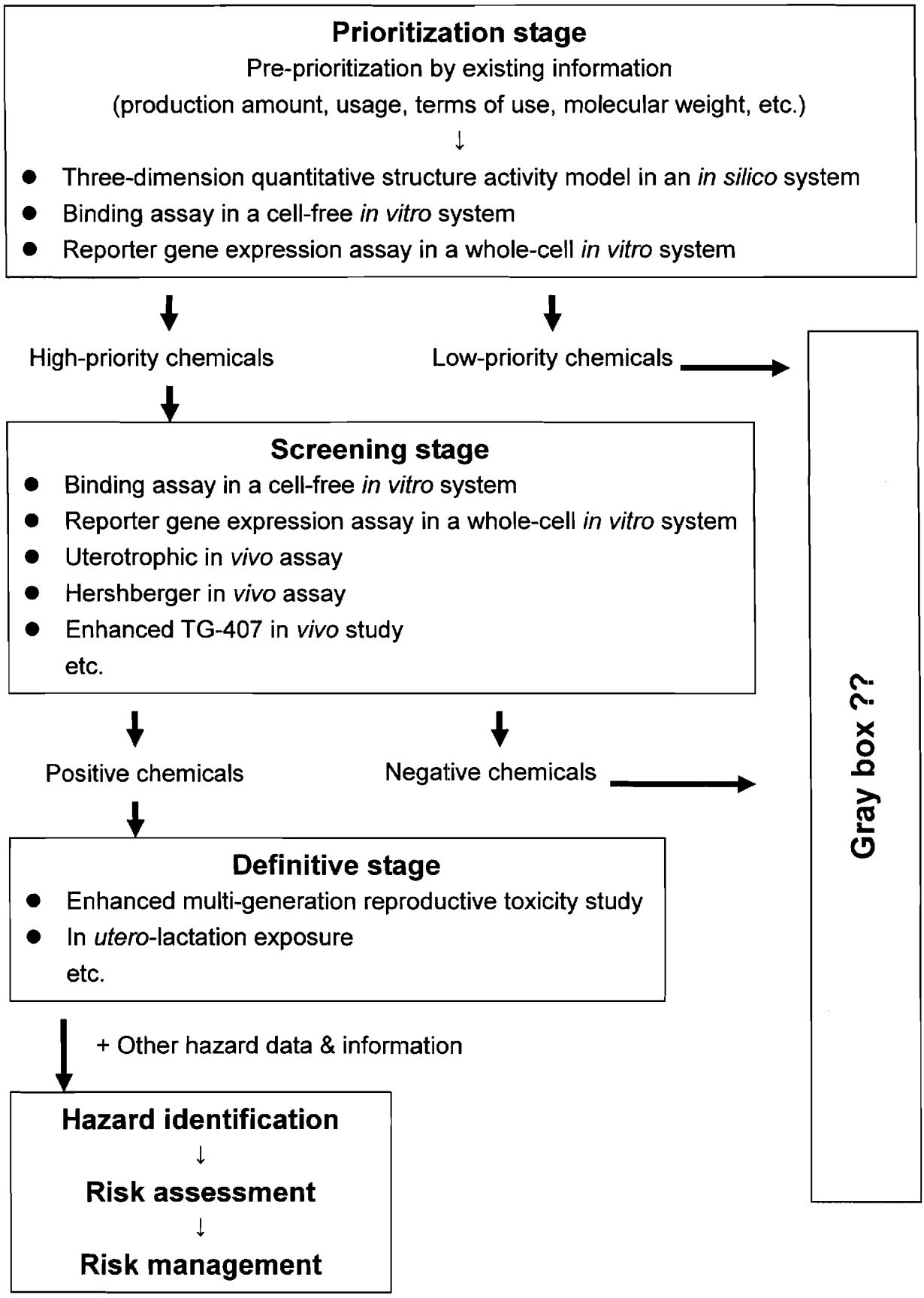

Figure. Outline of the tiered chemical screening and testing program for evaluating the endocrine-disrupting effects of chemicals (draft).

I . Age-Related Changes of Genital Systems in the Female Crj: CD® (SD) IGS Rats during Sexual Maturation

INTRODUCTION

Currently, there is much concern that certain environmental chemicals may have the potential to disturb normal sexual differentiation and development in wild life and humans (Colborn and Clement, 1992; Kelce and Wilson, 1997; McLachlan and Korach, 1995). A uterotrophic assay using immature female rats was proposed by the Organization for Economic Cooperation and Development and Endocrine Disrupter Screening and testing Committee (EDSTAC) of the U.S. Environmental Protection Agency as an *in vivo* screening method to detect the estrogenic or anti-estrogenic activity of the chemicals acting mainly by receptor-mediated mechanisms (Grey, 1998 and Holmes *et al.*, 1998). In the uterotrophic assay, endogenous estrogen-free immature female rat must be employed, because endpoint of the uterotrophic assay is only the uterotrophic action by injection of the test chemicals. Therefore, uterotrophic assay is finished in the endogenous estrogen-free period, i.e., sensitive window of uterus. On the other hand, EDSTAC has recommended another assay, termed the "female pubertal assay" (Goldman *et al.*, 2000). The purpose of this assay is to quantify the effects of environmental chemicals on pubertal development and thyroid function in the immature female rat. Focusing on disruption of the sex hormone system, the proposed endpoints of this assay are the age of vaginal opening, reproductive organ weights and sex-related hormone levels.

The efficacy of several assay protocols is now being investigated in a worldwide effort by many laboratories using various chemicals in the OECD initiation. We participate in these international validation studies, and, in addition, have collected various basic biological data for the development of the screening and testing methods. Which strain is best for these assays, is currently uncertain, because there are differences in sensitivity among rat strains (O'Connor *et al.*,

1999 and Xinghua *et al.*, 2000). Crj: CD® (SD) IGS rat (SD IGS rat) was developed recently under a new breeding system for the purpose of supplying experimental animals with minimal genetic variations. Many researchers have begun to use for regulatory toxicology studies and for various types of biochemical research. However some general biological parameters of this rat have been reported, development of the female genital system in SD IGS rats during peripubertal period has not been well characterized. An understanding of the basic biological profile of changes in the female genital system during sexual maturation in the rat is of importance for not only analyzing the results of assays using female rats but also development of the assay protocols. In the present study, we determined age-related changes in vaginal opening, body weight, the weights of the uterus and ovary together with gross pathological examination, serum 17β-estradiol (E2) and progesterone levels of intact non-treated SD IGS female rats from 21 to 36 days of age, i.e., during the pubertal period. In addition, the genital systems of three rats immediately after vaginal opening were examined microscopically to obtain the preliminary data on the relationship between the first ovulation and vaginal opening.

MATERIALS AND METHODS

- *Animals*

Fourteen timed-pregnant specific-pathogen-free female Crj: CD ® (SD) IGS (SD IGS) rats were purchased from Charles River Japan, Inc. (Hino Breeding Center and Atsugi Breeding Center). Insemination was confirmed by the presence of a sperm-plug in the vagina. The day following overnight mating was designated as pregnant day 0 and the rats were nulliparity at 12 weeks of age. They arrived on pregnant day 14. The animals were housed in an animal room with the temperature set at 23±2℃, the relative humidity at 55±10%, the ventilation rate at 10-15 times/h, and lighting for 12 h daily from 7:00 am to 7:00 pm. Dams were housed individually in hanging stainless steel cages with a wire-mesh floor (260 W x 380 D x 180 H mm) from pregnant day 14 to 17. Neonatal rats were delivered in our laboratory (date of birth designated 0 days of age). Dams and their litters were housed in polycarbonate cages (280 W x 440 D x 150 H mm) with nesting materials (Sun Flake®, Chiba Animal Material Co., Ltd., Japan) from pregnant day 17 until weaning. At 4 days of age each litter was culled to eight female rats. The rats were weaned at 20 days of age. Then, weanlings were ranked by weight and 20 rats each were randomly assigned into 12 experimental groups using a body-weight stratified randomization method to minimize variation in body weights among the groups. After grouping, the weanlings were housed in stainless steel cages with a wire-mesh floor (165 W × 300 D × 150 H mm) hung in a 6-vertical by 6-horizontal cage allocable stainless steel cage rack (1 animal/cage). The animals were allowed free access to autoclaved solid food (before weaning of offspring, CRF-1, after weaning, MF, Oriental Yeast Co., Ltd., Tokyo, Japan), and to chlorinated tap water from an automatic dispenser or supply bottles. All animals were cared for according to the principles outlined in the guide for animal experimentation prepared by *the Japanese Association for Laboratory Animal Science*.

- *Experimental designs*

The twenty intact rats in each group were sacrificed at 21, 23, 24, 28, 29, 30, 31, 32, 33, 34, 35 or 36 days of age. At necropsy, the rats were weighed and blood samples were collected from the abdominal aorta and then euthanized by exanguination under ether deep anesthesia. To reduce the fluctuation of hormonal levels due to stress, the transportation and handling of rats were carried out with great care on the day of necropsy. Macroscopic examinations of the female rat genital system were performed. As 3 rats showed vaginal opening immediately before dissection and corpora lutea-like structures were observed macroscopically, the genital systems of these rats were examined microscopically; the samples were prepared by fixation in neutral-buffered formalin after embedding in paraffin, followed by sectioning and hematoxylin and eosin staining to confirm that they had corpora lutea. The ovaries and uterus of each rat were removed and weighed. The blotted uterine weights, i.e., weight without excess fluid, were also determined by excising the uterine horns and blotting the excess fluid onto filter paper. The sera were stored at -80°C until measurement of 17β-estradiol (E2) and progesterone levels. Until sacrifice, general condition and evidence of vaginal opening were recorded daily and body weight was determined once a week from 21 days of age. The serum E2 level was measured using a commercial radioimmunoassay kit (DPC estradiol double antibody kit, lot No. 300, Diagnostic Products Co., Los Angels, CA, USA), and serum progesterone was measured using a commercial immunoassay kit (Progesterone Enzyme Immunoassay Kit, lot No. 13153A, © Cayman Chemical Company, Ann Arbor, MI, USA).

RESULTS

General conditions and body weights

During daily clinical observations, no abnormalities were noted and mean body weight gradually increased (Fig. 1). The mean value ± standard deviation (SD) of the body weight at weanling 20 days of age was 48.5 grams ± 2.9.

Vaginal opening: The earliest incidence of vaginal opening occurred at 30 days of age, the latest at 35 and the highest at 34 (Table 1).

Organ weights and findings

In general, weight of uterus increased slightly until 29 days of age and then increased rapidly, first peaking at 31 days of age, and was associated with the weight fluctuation (Fig. 2A). The profiles of the absolute uterine weights were similar to relative weights (Data not shown). On macroscopic examinations, watery contents in the uterine lumen, i.e., uterine imbibition, was observed in rats older than 30 day. These findings were considered to be identical to those of the proestrus stage. Most of the rats had high uterine weights. Many rats showed uterine imbibition but not vaginal opening (Fig. 2B, closed triangles). On the other hand, some rats with uterine imbibition already showed vaginal opening (Fig. 2B, closed squares) occurring at later ages during our experimental period, i.e., 35 or 36 days of age, except in one animal.

Absolute ovarian weights gradually increased until 28 days of age and thereafter more increased (Fig. 3A). On the other hand, relative weight increased dramatically from 21 to 24 days of age and then decreased up to 29 days of age followed by an increase from 30 to 34 days of age (Fig. 3B).

Three rats were dissected immediately after vaginal opening was achieved. Figure 4 presents an example of the microscopic findings of genital system when immediately vaginal opening was observed, but it could not found at the routine morning observation. The interval between the time of morning observation and the discovery of vaginal opening was approximately 3 hours. Both ovaries had

multiple corpora lutea consisting of cells with scant cytoplasm containing a small number of fine vacuoles, indicating the corpora lutea to not yet be mature (Fig. 4A). A large proportion of endometrial cell was degenerative, as in the metestrus phase of the endmetrium of normal mature rats (Fig. 4B). In the vagina, cornification, matching the late estrus phase was evident (Fig. 4C).

Serum E2 and progesterone levels

The high initial E2 level at 21 days of age decreased at 28 days of age. Generally, the first peak was seen at 31 days of age, followed by a gradual decrease until 34 days of age (Fig. 5A). The serum E2 level variation after 29 days of age was very large, as was that of uterine weight. There was a significant positive relationship between the serum E2 level and uterine weight after 29 days of age ($r = 0.724$, $p<0.001$). Serum E2 levels tended to be higher in rats with uterine imbibition than in those without it (Fig. 5B). After 31 days of age, serum progesterone levels increased rapidly (Fig. 6).

DISCUSSION

In this study, we measured changes in the frequency of vaginal opening, uterine weight, ovarian weight, and both serum 17β-estradiol (E2) and progesterone levels, seen during the pubertal period in female SD IGS rats.

With the beginning of the elevation of serum E2 level from 28 days of age, uterine and ovarian weights, serum progesterone level started to show drastic change until 31 days of age. Vaginal opening and uterus imbibition was also observed in rats older than 30 days. Especially, uterine weight change correlated with the serum E2 level after 29 days of age. E2 is known to increase uterine weight and promote hypertrophy of the endometrial epithelium (Branham *et al.*, 1993). These changes after 28 days of age were considered to originate in pubertal onset. In general, pubertal onset in rats encompasses the period of vaginal opening and first ovulation (Daston and Kimmel 1998). In female SD IGS rats of this study, pubertal onset was considered to start form from 28 days of age. It is noteworthy that many rats showed uterine imbibition without vaginal opening. This means that uterine imbibition, i.e., proestrus-like change, precedes vaginal opening.

The high initial E2 level at 21 days of age did not have an effect on the uterine weight. This is attributable to the high concentration of α-fetoprotein, which binds up the estrogens available around this age (Andrews and Ojeda 1977 and Meijs-Roelofs and Kramer 1979). It is generally accepted that E2 is inactive when bound to α-fetoprotein. In addition, relative ovarian weight increased dramatically from 21 to 24 days of age and then decreased up to 29 days of age. Meijs-Roelofs reported that the number of follicles with a volume $\geq 100 \times 10^5$ μm^3 increased rapidly until 23 days of age and then more slowly until 27 days of age in contrast to the gradual body weight increase (Meijs-Roelofs *et al.*, 1972). Thus, the decrease in relative ovarian weight after 24 days of age may be attributable to altered numbers of follicles at this stage.

In addition to the experiment described above, three rats were dissected

immediately after vaginal opening was achieved. The histological findings of the genital systems of these rats, especially in terms of the corpora lutea formation and degenerative endometrium are indicative of the metestrus stage. These findings lead us to speculate that the first ovulation in the rat could occur before or at least at the same time as vaginal opening because it is unlikely that multiple corpora lutea formation and endometrial degeneration could occur in such a short interval. This hypothesis is supported by our observation that proestrus-like change precedes vaginal opening. IIt is noteworthy that our hypothesis contrasts with the literature, in which, vaginal opening is generally described as occurring with or shortly before the first ovulation (Daston and Kimmel 1998). However, we have not pinpointed the histology of the genital systems of these rats at the instant of vaginal opening nor have we clarified whether the histological findings of the rat genital system at the instant of vaginal opening are identical to those of mature and normally cycling rats. The further studies will be needed.

In conclusion, we obtained essential data on genital tract development of female Crj: CD® (SD) IGS rats for in *vivo* screening assays that will contribute to detect potential endocrine active chemicals. Our results will contribute to develop the assay protocol using employing the peripubertal female rats, as well as analyses of assays and studies. In addition, it is assumed that the first ovulation precedes or occurs simultaneously with vaginal opening, which opposes the established theory in terms of the timing of the ovulation.

ABSTRACT

The age-related changes of vaginal opening, body weight, the weights of the uterus and ovary, together with histological examination, serum 17β-estradiol (E2) and progesterone levels were examined in intact female Crj: CD® (SD) IGS rats between 21 and 36 days of age to understand the basic biological profile of changes of the female genital system during sexual maturation in the rat for female pubertal assays. With the beginning of the elevation of serum E2 level from 28 days of age, all parameters except body weight started to show drastic change until 31 days of age. The highest incidence of vaginal opening was recorded at 34 days of age. On macroscopic examinations, Most of the rats had high uterine weights. a number of rats showed uterine imbibition but vaginal opening. Immediately after the confirmation of the vaginal opening, the genital systems of three rats were observed microscopically. Both ovaries already had multiple corpora lutea, and degeneration of endometrial epithelial cells was observed. In conclusion, we obtained essential data on genital tract development of female Crj: CD® (SD) IGS rats for in *vivo* screening assays that will contribute to detect potential endocrine active chemicals. In addition, it is assumed that the first ovulation precedes or occurs simulutaneously with vaginal opening.

Table 1. Vaginal Opening in SD IGS Rats during Pubertal Period

	Days of age					
	30	31	32	33	34	35
Number of vaginal opening / examined	1/140	8/120	12/100	3/80	23/60	10/40
Percentage (%)	0.7	6.7	12.0	3.8	38.3	25.0

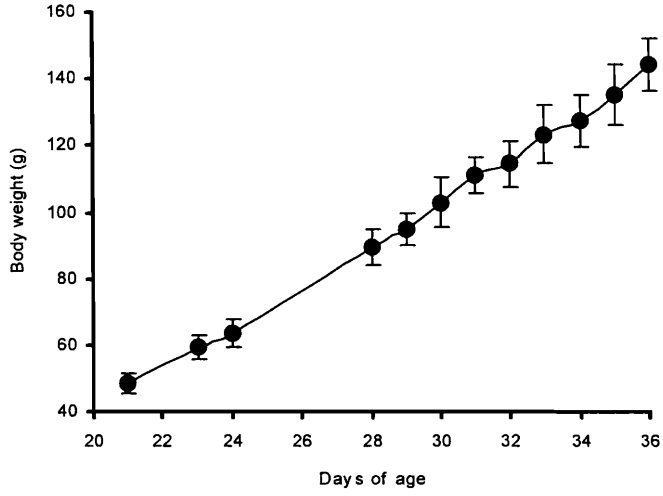

Fig. 1. Body weights of female SD IGS rats during the pubertal period. Points are means and vertical lines represent SD.

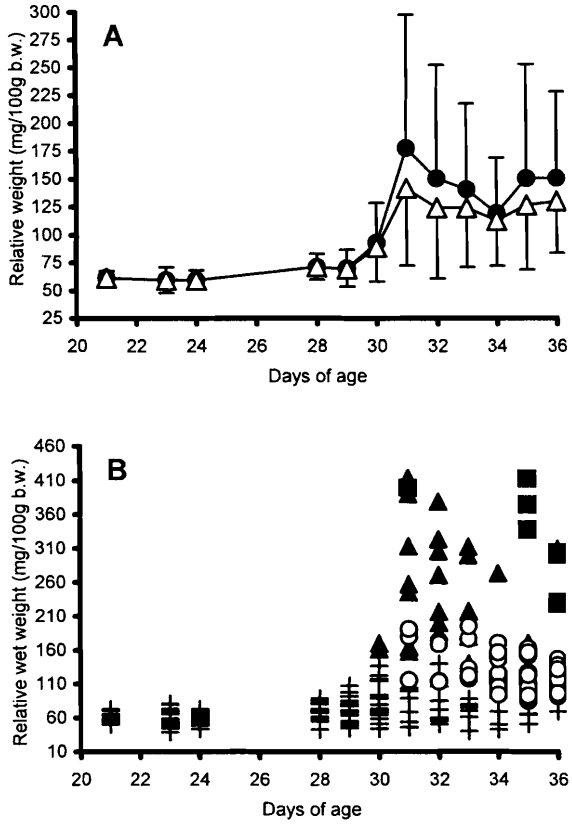

Fig. 2. Relative uterine weight in female SD IGS rats during pubertal period. (A), Uterine weight. Points are means and vertical lines represent SD (n=20). ●, wet weight; △, blotted weight. (B), Individual uterine weights with genital system findings. +, rat with neither vaginal opening nor uterine imbibition; ▲, without vaginal opening but with uterine imbibition; ○, with vaginal opening but without uterine imbibition; ■, with both vaginal opening and uterine imbibition.

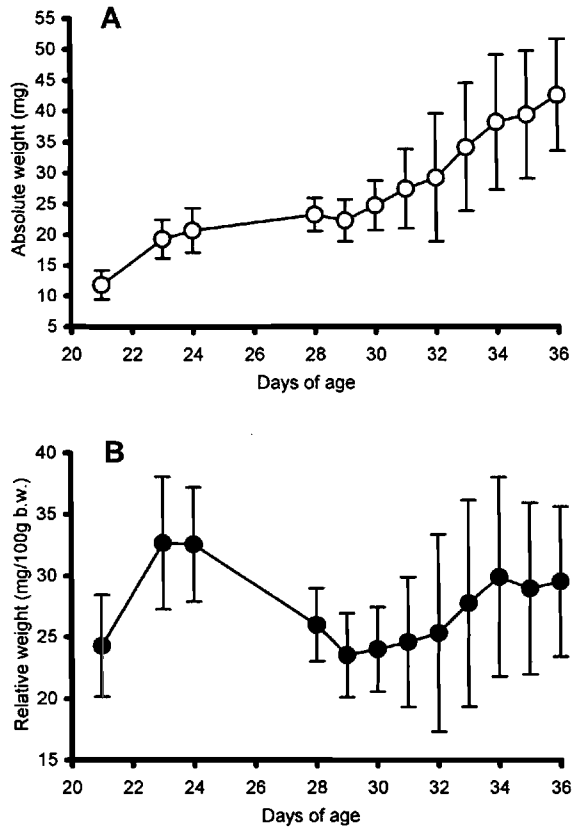

Fig. 3. Ovarian weight in female SD IGS rats during pubertal period. (A), Absolute ovarian weight. (B), Relative ovarian weight. Points are means and vertical lines represent SD (n=20).

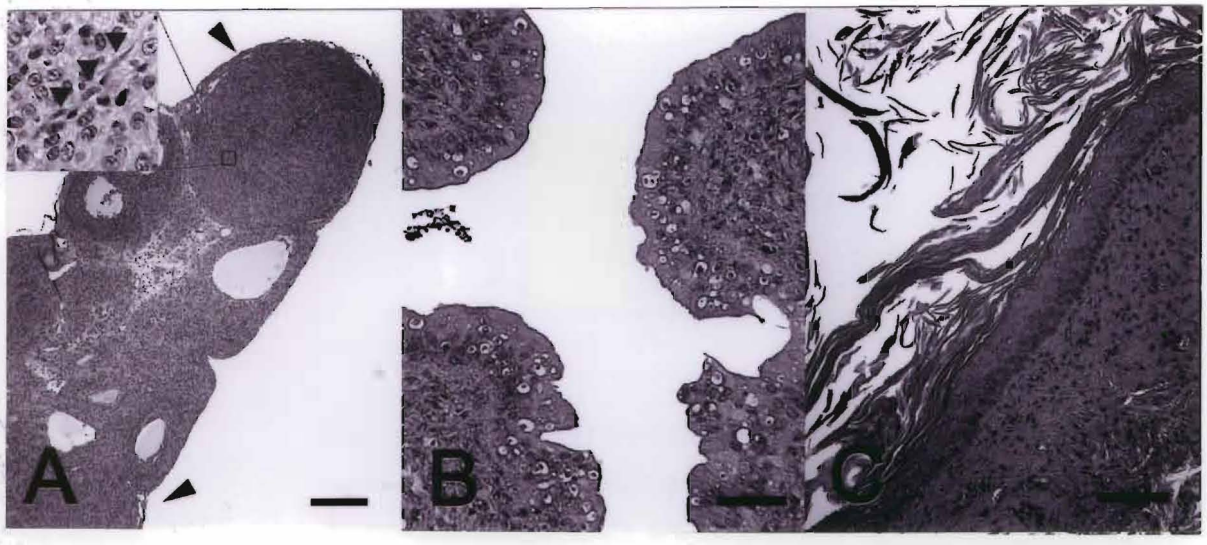

Fig. 4. Microscopic findings of the genital systems of the rat immediately after vaginal opening was noted. (A), Ovary showing a corpus luteum (upper area) and a Graafian follicle (lower area). Arrowheads point a blood vessel, which is a feature of corpora lutea. Cells with relatively rich cytoplasms and prominent nucleoli tightly contact with each other. In contrast, a Graafian follicle consists of granulosa cells with dense nuclei and occasional loose intercellular gaps. H&E staining. Bar = 50 μm. (B), degeneration of endometrial epithelial cells. H&E staining. Bar = 50 μm. (C), Prominent cornification in vagina. H&E staining. Bar = 100 μm.

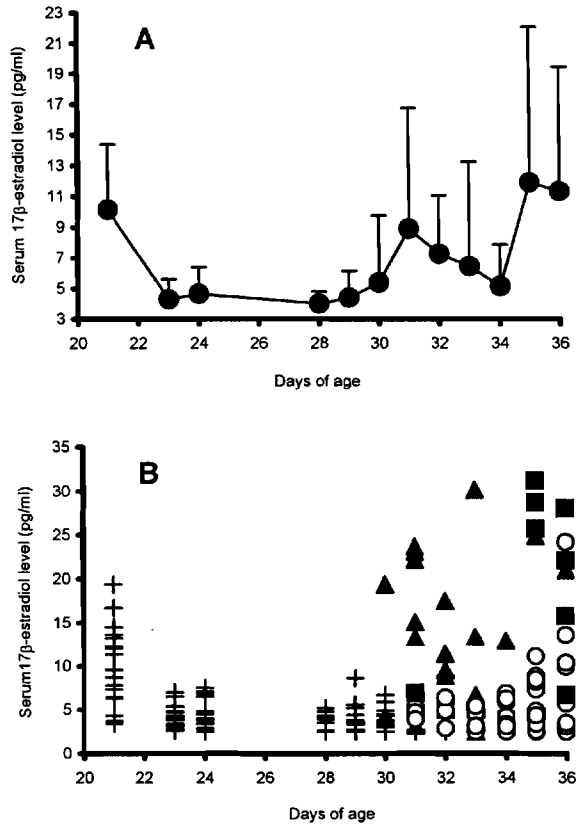

Fig. 5. Serum 17β-estradiol (E2) levels in female SD IGS rats during pubertal period. (A), Serum E2 level. Points are means and vertical lines represent SD (n=20). Detection limit, <2.5 pg/ml, was excluded from the calculation. (B), Relationship between individual serum E2 levels and gross findings. +, rat with neither vaginal opening nor uterine imbibition; ▲, without vaginal opening but with uterine imbibition; ○, with vaginal opening but without uterine imbibition; ■, with both vaginal opening and uterine imbibition.

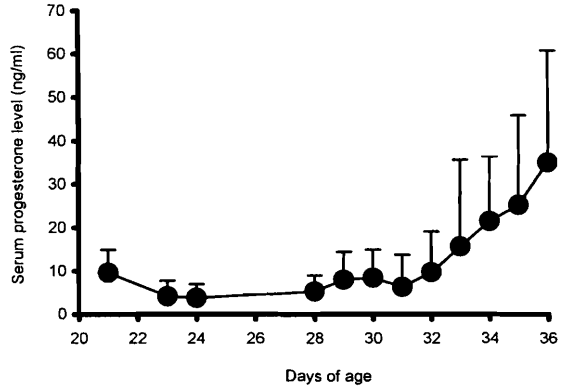

Fig. 6. Serum progesterone level in female SD IGS rats during pubertal period. Points are means and vertical lines represent SD (n=20).

Ⅱ. Ability of the Hershberger Assay Protocol to Detect Thyroid Function Modulators using 3-amino-1,2,4-triazole

INTRODUCTION

Currently, there is a great deal of concern that certain environmental chemicals may have ability to impair normal sexual differentiation and development in humans and wildlife (McLachlan, 1993; McLachlan and Korach, 1995). Endocrine Disruptor Screening and Testing Committee (EDSTAC) is developing a tiered chemical screening and testing program to evaluate for endocrine disrupting effects (EDSTAC, 1998). In this program, high priority chemicals will be evaluated for hormonal activity in the early screening stage, and chemicals positive in the screening stage will be tested for hazard in a definitive test (EDSTAC, 1998; Gray *et al.,* 2002).

A Hershberger assay in surgically castrated male rats was proposed by the Organization for Economic Cooperation and Development (OECD) and EDSTAC of the US Environmental Protection Agency as an in vivo screening method to detect the (anti-) androgenic activity of the chemicals acting mainly via androgen receptor (AR)-mediated mechanisms (Dorfman, 1969; EDSTAC, 1998; Hershberger *et al.,* 1953; OECD, 1997, 2001, 2003). Focusing on disruption of the androgenic system, the proposed endpoints of this assay are the weight of the ventral prostate, seminal vesicles, bulbocavernosus/levator ani muscles, glans penis, Cowper's glands, and liver. The gravimetric endpoint has the advantage of enabling detection of (anti-) androgenic action of a chemical at low cost in a short time. The Hershberger assay will be used in the early stage of a chemical screening and testing program after the *in vitro* screening assays, such as the AR binding assay and the AR reporter gene assay (Gray *et al.,* 2002).

In vitro and *in vivo* screening methods for detection of thyroid function modulators are now under development in many research laboratories, and a

variety of *in vivo* screening methods have been proposed, e.g., enhanced TG407 and male or female pubertal assay. But they have disadvantage such as long experimental periods or low cost-performance, and there have been no breakthroughs thus far. Yamada and colleagues first reported that the enhanced Hershberger assay evaluation of thyroid histopathology and weights, and determination of serum hormone levels, appears to be reliable for screening thyroid modulators with a single dose of propylthiouracil (PTU), 2,2-bis(4-chlorophenyl)-1,1-dichloroethylene (*p,p'*-DDE) and phenobarbital (PB) (Yamada *et al.,* 2004). The original objective of the Hershberger assay was to the screen for (anti-) androgenic activity of the chemicals in male rats subjected to castration, which is essential for detection of androgenic activities. If the Hershberger assay is capable of detecting the (anti-) androgenic activity and thyroid hormone modulating activity of the chemicals at the same time, it will be possible to screen for chemicals hormonal activity rapidly and with better cost performance.

Because of the well-known presence of androgen receptors in the thyroid gland of mammalians (Banu *et al.*, 2002; Pellietier, 2000), the thyroid gland is speculated to be one of the target organs of androgenic compounds. Moreover, testosterone has a stimulatory effect of on the expression of the TSH mRNA (Ross, 1990), and testosterone administration results in a significant decrease in serum T3 and T4 levels in 15-day intact male assays (O'Connor *et al.*, 2000). Thus, if we intend to screen chemicals for (anti-) androgenic activity based on their thyroid hormone modulating activity, effect of castration of male rats on the thyroid should be evaluated. It appears rather unlikely that thyroid specific endpoints can be used to specifically detected (anti-) androgenicity. However, it is necessary to evaluate whether thyroid-active compounds affect the classical parameters of the Hershberger assay and thus confound the detection of (anti-) androgenicity.

We performed 2 experiments. Experiment 1 was designed to assess whether thyroid hormone modulating activity of 3-amino-1,2,4-triazole (AT), which is

known to inhibit the synthesis of T3 and T4 by peroxidase inhibiting in the thyroid gland (Ealey *et al.*, 1984; Krauss and Eling, 1987; Masuda and Goto, 1994; Reader et al., 1987; Santini *et al.*, 2003), could be detected by the thyroid gravimetric method, and whether the dose-dependent AT-induced effect on thyroid gland would be confirmed by the Hershberger assay in castrated male rats. Experiment 2 was designed to assess the influence of castration on the AT-induced effect on thyroid gland, and thus, both castrated and intact male rats were subjected to the same protocol to elucidate the effect of castration on the thyroid.

MATERIALS AND METHODS

Chemicals

3-Amino-1,2,4-triazole (AT, CAS No. 61-82-5, 99% pure) was obtained from Tokyo Kasei Kougyou (Tokyo, Japan), and olive oil was obtained from Fujimi Pharmaceutical Company (Osaka, Japan). The physical stability of AT was assessed with a Fourier transform infrared spectrophotometer (FTS-135, Nippon Bio-Rad Laboratories K.K., Tokyo, Japan), and the stability, homogeneity, and concentration of each AT suspension prepared for administration were confirmed by HPLC.

Animals

Seven-week-old BrlHan WIST@Jcl (GALAS) rats castrated at 6 weeks of age, and 7-week-old intact BrlHan WIST@Jcl (GALAS) rats were purchased from Clear Japan Inc., (Fuji Japan). The animals were housed, three per cage, in stainless steel, wire-mesh cages throughout the study. The rats were weighed, weight-ranked, and randomly assigned to one of the treatment or control groups. Body weight and clinical signs were recorded daily throughout the study. Rats were given ad libitum access to tap water and a commercial diet (MF, Oriental Yeast Co., Tokyo, Japan) and ad libitum access to water from an automatic dispenser. The animal room was maintained at a temperature of 23 ± 2°C and a relative humidity of 55 ± 5%, and it was artificially illuminated with fluorescent light on a 12-h light / dark cycle (0600 - 1800 h). All animals were cared for according to the principles outlined in the guide for animal experimentation prepared by the *Japanese Association for Laboratory Animal Science*.

Study design

Hershberger assay

The experimental design of our study followed the OECD validation of the rodent Hershberger bioassay: phase-2 protocol (OECD, 2002). Six castrated rats

/group were given AT orally at doses of 40, 200, or 1,000 mg/kg daily via a stomach tube for 10 consecutive days beginning at nine weeks of age with or without s.c. injection of 0.2 mg/kg testosterone propionate (TP). The volume of the olive oil solutions of AT was 5 ml/kg for oral administration and 5 ml/kg for s.c. injection. A vehicle control group given olive oil alone was also established. The dosage was adjusted daily for body weight change. Approximately 24 h after the final dose, the animals were killed by bleeding from the abdominal vein under deep ether anesthesia. At necropsy, the ventral prostate, seminal vesicles, bulbocavernosus/levator ani muscles (BC/LA), glans penis, Cowper's gland, and liver were removed and weighed. The thyroid glands and hypophysis were collected and fixed with 10% neutral buffered formalin, and to avoid crushing during weighing because of their fragility, they were weighed approximately 24 hours after fixation.

Quantification of the extent of hypertrophy of the thyroid epithelium in the Hershberger assay

The same experimental procedures as described below, were performed on intact rats and castrated rats.

The rats were given AT at doses of 40 or 200 mg/kg daily. Administration and necropsy were executed according to the same procedure as experiment 1. At necropsy, the thyroid glands and hypophysis were removed and fixed with 10% neutral buffered formalin. To avoid crushing during weighing because of its fragility, the thyroid glands and hypophysis were weighed approximately 24 hours after fixation, stained with hematoxylin and eosin, and examined under a light microscope. Morphometry of the thyroid epithelium was performed to quantify the extent of hypertrophy. The height of the epithelial cells of all rats was measured under a microscope with Scion Image image analysis software (Scion Corporation, MA, USA). 50 points/rat (25 left, 25 right) of the thyroid epithelium were measured.

Statistical analyses

Body weight, organ weights, and height of thyroid follicular epithelium of the rats in the experimental groups and corresponding vehicle control groups were compared i.e., data obtained from the castrated group were compared with data form the castrated control group. Data form the castrated control group and intact control group were also compared. Each endpoint was analyzed by Bartlett's test for homogeneity of variance. If the variances were homogeneous at the 5% level of significance, one-way analysis of variance was performed, and if it revealed a significant difference, the difference between the control group and each of the experimental groups was analyzed by Dunnett's test. If the variances were not homogeneous, the Kruskal-Wallis test was used, and if it showed a significant difference, the difference between the control group and each of the experimental groups was analyzed by the nonparametric Dunnett's test.

RESULTS

Experiment-1: Hershberger assay

General observation revealed no abnormal findings in the AT-treated castrated rats. Irrespective of TP-treatment, the body weight of the AT-treated castrated rats remained normal throughout the study (Table 2).

Organ weight changes are shown in Tables 2 and 3. Absolute and relative thyroid weights in all of the AT groups were statistically increased in a dose dependent manner, regardless of the TP-injection, and increase were statistically significant.

In the classical parameters of the Hershberger assay, absolute seminal vesicle weights were significantly higher in the 200 mg/kg + TP group, and absolute and relative seminal vesicle weights were significantly higher in the 1,000 mg/kg + TP group when compared to the each corresponding vehicle control group. In the 1,000 mg/kg + TP group, relative liver weights were significantly higher. Besides, absolute seminal vesicle weights were significantly lower in the 1,000 mg/kg group, absolute and relative weights were significantly higher in the 40 mg/kg group, absolute glans penis weights and relative BC/LA weights were significantly lower, and absolute and relative liver weights were significantly higher in the 40 mg/kg + TP group, and absolute and relative BC/LA weights were significantly higher in the 200 mg/kg + TP group when compared to the each corresponding vehicle control group.

Gross examination revealed enlargement of the thyroid glands in 4/6 rats in the 40 mg/kg group, in all rats in the 200 and 1000 mg/kg group, in 5/6 rats in the 40 mg/kg +TP group, and in all rats in the 200 and 1000 mg/kg +TP group (Table 4).

Quantification of the extent of hypertrophy of the thyroid epithelium in the Hershberger assay

***Castrated rats**:* General observation revealed no abnormal findings in the AT-treated castrated rats. The body weight of the AT-treated castrated rats

remained normal throughout the study (Fig. 7).

Organ weight changes are shown in Fig 8. Absolute and relative thyroid weights were significantly higher in the 200 mg/kg group. No statistically significant differences were detected in hypophysis weight.

Gross examination revealed enlargement of the thyroid glands in 4/6 rats in the 40 mg/kg group and in all rats in the 200 mg/kg group (Table 3).

The histopathological findings in the thyroid glands of the AT-treated castrated rats are shown illustrated in Figure 9 (left panel), the thyroid glands of all AT-treated rats exhibited hypertrophy and hyperplasia of the thyroid follicular epithelial cells.

The follicular epithelial cell heights are shown in Figure 4. The height of the follicular epithelial cell in all AT-treated groups was increased in a dose-dependent manner by AT (40 mg/kg group, 200.8% of the control; 200 mg/kg group, 225.7% of the control), and the increase were statistically significant.

Intact rats: General observation revealed no abnormal findings in the AT-treated intact rats. The body weight of the AT-treated rats remained normal throughout the study (Fig.7).

Organ weight changes are shown in Figure 8. Absolute and relative thyroid weights were significantly higher in the 200 mg/kg group, and absolute thyroid weight in the 40 mg/kg group. No statistically significant differences hypophyseal weights were detected. Comparison between the VC groups, however, revealed significantly decreased absolute and relative hypophyseal weights in the intact VC group.

Gross examination revealed enlargement of the thyroid glands in all rats in all the AT-treated groups (Table 3). The histopathological findings in the thyroids of the AT-treated intact rats are shown in Figure 9 (right panel). The thyroid glands of all AT-treated animals exhibited hypertrophy and hyperplasia of the thyroid follicular epithelial cells.

The height of the follicular epithelial cell is shown in Fig 10. Follicular epithelial

cell height in all AT-treated groups was significantly increased in a dose-dependent manner by AT (40 mg/kg group, 149.6% of the control; 200 mg/kg group, 195.0% of the control).

DISCUSSION

We examined the applicability of the Hershberger assay 10-day administration protocol to screening of chemicals for a thyroid hormone modulator. The thyroid peroxidase inhibitor, AT, a widely used herbicide found to produce thyroid and liver tumors in rodents and classified as possibly carcinogenic to humans, was investigated to acquire further information about its mechanism of action (Mattioli *et al.,* 1994). In this study, AT was used as the test substance to determine whether that of the Hershberger assay can detect thyroid hormone modulation caused by a mechanism different from that of PTU, *p,p'*-DDE, and PB, such as by the mechanisms of modulation of iodination of thyroglobulin and of the coupling reaction and metabolism enhancers. Both castrated and intact male rats were subjected to the same protocol to elucidate the effect of castration on the thyroid.

The organ weight changes showed that thyroid weight increased in all AT-treated groups in a dose-dependent manner, and thyroid gland enlargement in the AT-treated rats. Histologically, the thyroid glands of all AT-treated rats exhibited hypertrophy and hyperplasia of the follicular epithelial cells, and their height increased in a dose-dependent manner in both the castrated and intact rats. AT is known to inhibit the synthesis of T3 and T4 by peroxidase inhibiting in the thyroid gland (Ealey *et al.*, 1984; Krauss and Eling, 1987; Masuda and Goto, 1994; Reader et al., 1987; Santini *et al.*, 2003). It has been reported that after the administration of AT to rats is followed by enlargement of the thyroid gland, a decrease in colloid content, and proliferation of the follicular epithelium (Masuda and Goto, 1994). The results of the present study showed that the Hershberger assay 10-day administration protocol clearly detected thyroid hormone modulating effect of AT on the thyroid glands. Moreover, the reproducibility of the results was demonstrated by experiments 1 and 2. Yamada and colleagues first reported that the Herberger assay enhanced by evaluation of thyroid histopathology and weights and serum hormone levels appears to be a reliable

method of screening for thyroid modulators (Yamada *et al.*, 2004). PTU is not only an inhibitor of type I deiodinase inhibitor, but also an inhibitor of iodination of thyroglobulin and of the coupling reaction, whereas peroxidase inhibition seems not to occur under in vivo inhibition (Moura *et al.,* 1990; Shiroozu *et al.*, 1983; Taurog *et al.,* 1995). *p,p'*-DDE and PB are the thyroid hormone metabolism enhancers (Barter and Klaassen, 1994; Capen, 1997; McClain *et al.*, 1989). Thus, the thyroid inhibiting effects of several thyroid modulators have already been detected by the Hershberger assay. It therefore seems possible to use the Hershberger assay to assess the potency of anti-thyroid chemicals and perhaps to bioassay them.

The workshop on Screening Methods for Chemicals that Alter Thyroid Hormone Action, Function and Homeostasis (DeVito *et al.*, 1997, 1999) concluded that at least a 2- to 6-week dosing period is necessary to be able to observe consistent thyroid responses. Other assays, such as the male and female pubertal assays (Goldman *et al.*, 2000; Stoker *et al.*, 2000) and enhanced TG 407(OECD, 1999), require a 20- to 30-day administration period and are not convenient screening methods for evaluating large numbers of chemicals for the thyroid-hormone-modulating-activity. If the Hershberger assay is used to screen for thyroid function modulating activity and (anti-) androgenic compounds at the same time, more chemicals can be screened for its (anti-) androgenic activity and/or thyroid hormone modulating activity.

It was reported that castration induced a dramatic increase in the size and proportion of LH-immunoreactive cells present within the adenohypophysis of control rats (Kirby, J.D. *et al.*, 1997) and the castration induces an immediate increase in the serum levels and pituitary content of the gonadotropins, luteinizing hormone and follicle-stimulating hormone as well as a concomitant rise in the steady state levels of the messenger RNAs directing their synthesis (Emanuele *et al.*, 1996; Valenti *et al.*, 1997). It is speculated that an increased hypophysis weight in castrated rats may be a result of the removal of testicular negative feedback. Histologically, however, the thyroid glands of all AT-treated

rats exhibited follicular epithelial cell hypertrophy and hyperplasia, and the height of the follicular epithelium of the thyroid glands increased in a dose-dependent manner in both the castrated and intact rats. Moreover, results of morphometry of the thyroid epithelium showed almost same sensitivity in both the castrated rats and intact rats. These results suggest that the effect of AT can be detected by the Hershberger assay 10-day administration protocol regardless of castration. The other assays to detect the thyroid modulating effect, such as male or female pubertal assay (Goldman *et al.*, 2000; Stoker *et al.*, 2000), and enhanced TG 407(OECD, 1999) are used in intact rats. Our results emphasize the possibility of applying the Hershberger assay protocol to the detection of thyroid modulators. At the same time, the 10-day oral administration protocol in intact male rats may provide a new approach to screening for thyroid hormone modulators. In terms of the preventing of surgical intervention of animals, it is valuable to develop screening methods that do not involve surgical invasion, such as castration, ovariectomy, etc. Thus, further study is needed, including evaluation of other compounds with mechanisms of action different from those of AT, such as thyroid hormone metabolism enhancers and thyroid hormone antagonist etc.

In addition, assessment of the (anti-) androgenic action of AT in seminal vesicle weight revealed a significant increase in the 200 and 1000 mg/kg +TP groups in a dose-dependent manner. The mechanisms and biological or toxicological significance of the increase of seminal vesicle weight in AT + TP groups were unclear and there were no AT related changes in other androgen sensitive organs, but this seminal vesicle weight change was not considered to be disregarded because dose dependency was confirmed, although the study to determine the reproducibility of the data will be needed to confirm this findings in the present study. About other organs weight changes, excluded the seminal vesicle weights change in the AT + TP groups, were judged to be of no toxicologically significant, since there were no dose relationships. Based on this finding, it might be appropriate to concluded that the androgen action of TP is

reinforced or synergized. The original Hershberger assay is a screening method that evaluates the hormonal activity of chemicals based on changes in accessory sex organs alone (Dorfman, 1969; Gray *et al.*, 2002; Hershberger *et al.*, 1953; OECD, 1997), and it is impossible to speculate on the mechanism of the changes in the accessory sex organs. We performed the AR binding assay and AR reporter gene assay of AT, and AT showed lack of binding affinity to AR and did not exhibit any agonist or antagonist activity in the reporter gene assay in these *in vitro* assays (data not shown). These results point to the existence of an endocrine disrupting mechanism other than the AR-mediated mechanism in the Hershberger assay and suggest the importance of the *in vitro* assay to evaluate the androgen action by the Hershberger assay.

In conclusion, we wish to emphasize the that Hershberger assay has the ability to be used to the assess the potency, and possibly to bioassay not only of the (anti-)androgenic activity of chemicals but of thyroid hormone modulators although the further studies will be needed to confirm the reproducibility of the findings in the present study, and evaluated another endpoints, such as hormonal evaluation, and another chemicals with thyroid hormone modulating effects to evaluate the value of the Hershberger assay protocol as a method to screen thyroid function modulators. Moreover, 10-day oral administration protocol in intact male rats may enable a new approach to screening for thyroid hormone modulators, although other compounds with mechanisms different of action those of from AT, such as thyroid hormone metabolism enhancers, and thyroid hormone antagonists should be tested.

ABSTRACT

In vivo screening methods for detection of thyroid function modulators are now under development in many research laboratories. We assessed the applicability of the Hershberger assay protocol to screen for thyroid function modulators. In the experiment 1, castrated male BrlHan WIST@Jcl (GALAS) rats were administered a potent thyroid peroxidase inhibitor, 3-amino-1,2,4-triazole (AT), in doses of 0, 40, 200 and 1,000 mg/kg/day with gravimetric endpoint, and in the experiment 2, castrated and intact male rats were administered in doses of 0, 40 and 200 mg/kg/day with quantification of the extent of hypertrophy of the thyroid epithelium to assess the effects of castration, by gavage to 8 week old for 10 consecutive days. At necropsy of both experiments, the thyroid glands and hypophysis were collected and fixed with 10% neutral buffered formalin. To avoid crushing during weighing because of their fragility, the thyroid glands and hypophysis were weighed approximately 24 hours after fixation with 10% neutral-buffered formalin. All animals were sacrificed approximately 24 h after the final dose. In the experiment 2, the thyroid glands of all animals were stained with hematoxylin and eosin for histological examination and morphometry of follicular epithelial height. In the experiment 1, absolute and relative thyroid weights in all of the AT groups were statistically increased in a dose dependent manner, regardless of the TP-injection. In the experiment 2, the results showed a significant increase in thyroid weight in the 200 mg/kg groups of both castrated and intact rats. Hypophyseal weight was unaltered by AT, but comparison of greater the vehicle-treated groups showed that the hypophyseal weight of the castrated rats was greater than that of the intact rats. Enlarged thyroid glands were observed in the AT-treated rats at necropsy. Histological examination of the thyroid glands of all AT-treated animals showed hypertrophy and hyperplasia of the follicular epithelial cells, and the height of follicular epithelium of the thyroid glands increased in a dose-dependent manner in both of the castrated and intact rats. In the experiment 1, assessment of the (anti-) androgenic action of AT in

seminal vesicle weight revealed a significant increase in the 200 and 1000 mg/kg +TP groups in a dose-dependent manner. These results suggest that the effect of AT can be detected by the Hershberger assay 10-day administration protocol and may be useful for screening for thyroid function modulators regardless of whether the animals have been castrated.

Table 2. Absolute organ weight in AT-treated castrated male rats.

Dose (mg/kg/day)	Thyroid mg	Ventral prostate mg	Seminal vesicle mg	bulbocavernosus /levator ani muscles mg	Glans penis mg	Cowper's galnd mg	Liver g	Final body weight g
VC	16.4 ± 5.9	15.1 ± 1.7	29.3 ± 4.2	146.0 ± 17.1	39.0 ± 3.0	3.9 ± 1.5	11.0 ± 0.6	281.2 ± 13.3
40	27.8 ± 8.8*	14.1 ± 6.1	27.1 ± 3.0	148.4 ± 19.2	41.3 ± 4.2	5.3 ± 0.9	12.0 ± 0.8*	285.3 ± 15.7
200	50.2 ± 11.0**	17.1 ± 1.9	25.3 ± 2.8	144.2 ± 23.1	38.2 ± 5.3	4.5 ± 0.8	11.6 ± 0.9	282.4 ± 13.5
1,000	56.5 ± 19.1**	15.9 ± 2.5	24.0 ± 4.1*	139.8 ± 24.6	39.6 ± 4.4	4.9 ± 1.0	11.2 ± 1.1	277.6 ± 19.4
VC +TP	14.8 ± 2.3	108.2 ± 9.4	232.2 ± 30.2	368.7 ± 23.8	78.5 ± 6.4	23.7 ± 3.8	11.0 ± 0.9	288.7 ± 14.1
40 +TP	27.1 ± 6.2##	118.9 ± 14.2	275.5 ± 66.1	344.7 ± 28.0	70.5 ± 5.9#	24.3 ± 3.2	12.6 ± 1.2#	298.8 ± 12.6
200 +TP	45.8 ± 16.8##	109.0 ± 13.8	285.4 ± 45.0#	317.5 ± 14.8##	71.4 ± 5.1	23.3 ± 3.8	11.7 ± 1.1	286.9 ± 11.9
1,000 +TP	45.4 ± 9.3##	113.3 ± 25.6	307.5 ± 39.5##	348.9 ± 32.2	73.3 ± 3.4	26.3 ± 2.3	12.2 ± 1.5	289.2 ± 14.2

AT, 3-amino-1,2,4-triazole; VC, vehicle control; TP, testosterone propionate.

*, **Significantly different from the castrated vehicle control at $p<0.05$ and at $p<0.01$, respectively.

#, ##Significantly different from the intact vehicle control at $p<0.05$ and at $p<0.01$, respectively.

Table 3. Relative organ weight organ weight in AT-treated castrated male rats.

Dose (mg/kg/day)	Thyroid mg/100g b.w.	Ventral prostate mg/100g b.w.	Seminal vesicle mg/100g b.w.	bulbocavernosus /levator ani muscles mg/100g b.w.	Glans penis mg/100g b.w.	Cowper's galnd mg/100g b.w.	Liver g/100g b.w.
VC	5.8 ± 1.7	5.4 ± 0.8	10.5 ± 1.8	52.0 ± 6.7	13.9 ± 1.3	1.4 ± 0.5	3.9 ± 0.2
40	9.9 ± 3.3*	4.9 ± 2.0	9.5 ± 0.9	51.9 ± 4.6	14.5 ± 1.2	1.8 ± 0.3	4.2 ± 0.2*
200	17.7 ± 3.2**	6.1 ± 0.8	9.0 ± 1.4	50.9 ± 6.0	13.6 ± 2.3	1.6 ± 0.3	4.1 ± 0.2
1,000	20.1 ± 5.5**	5.7 ± 0.8	8.6 ± 1.2	50.1 ± 6.0	14.4 ± 2.3	1.8 ± 0.4	4.0 ± 0.2
VC +TP	5.2 ± 0.9	37.7 ± 4.9	81.0 ± 14.6	127.9 ± 9.2	27.3 ± 3.3	8.2 ± 1.4	3.8 ± 0.2
40 +TP	9.1 ± 1.9##	39.9 ± 5.7	92.2 ± 21.5	115.5 ± 9.3#	23.7 ± 2.4	8.2 ± 1.4	4.2 ± 0.3#
200 +TP	15.9 ± 5.2##	38.0 ± 4.9	99.6 ± 16.3	110.8 ± 6.6##	24.9 ± 1.8	8.1 ± 1.1	4.1 ± 0.2
1,000 +TP	15.8 ± 3.6##	39.3 ± 9.4	106.5 ± 13.9#	120.7 ± 10.6	25.4 ± 1.7	9.1 ± 0.9	4.2 ± 0.3#

AT, 3-amino-1,2,4-triazole; VC, vehicle control; TP, testosterone propionate.

*, **Significantly different from the castrated vehicle control at $p<0.05$ and at $p<0.01$, respectively.

#, ##Significantly different from the intact vehicle control at $p<0.05$ and at $p<0.01$, respectively.

Table 4. Gross pathological findings in AT-treated rats.

	Experiment-1								Experiment-2					
	AT				AT+TP				Castrated			Intact		
Doses (mg/kg/day)	VC	40	200	1,000	VC	40	200	1,000	VC	40	200	VC	40	200
No. of animals	6	6	6	6	6	6	6	6	6	6	6	6	6	6
No abnormalities detected	6	2	0	0	6	1	0	0	6	2	0	6	0	0
Thyroid Enlargement	0	4	6	6	0	5	6	6	0	4	6	0	6	6

AT, 3-amino-1,2,4-triazole; VC, vehicle control; TP, testosterone propionate.

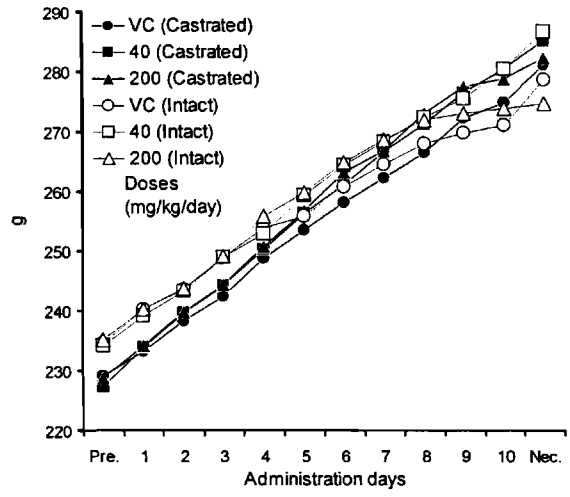

Fig. 7. Body weight changes in AT-treated castrated and intact male rats.

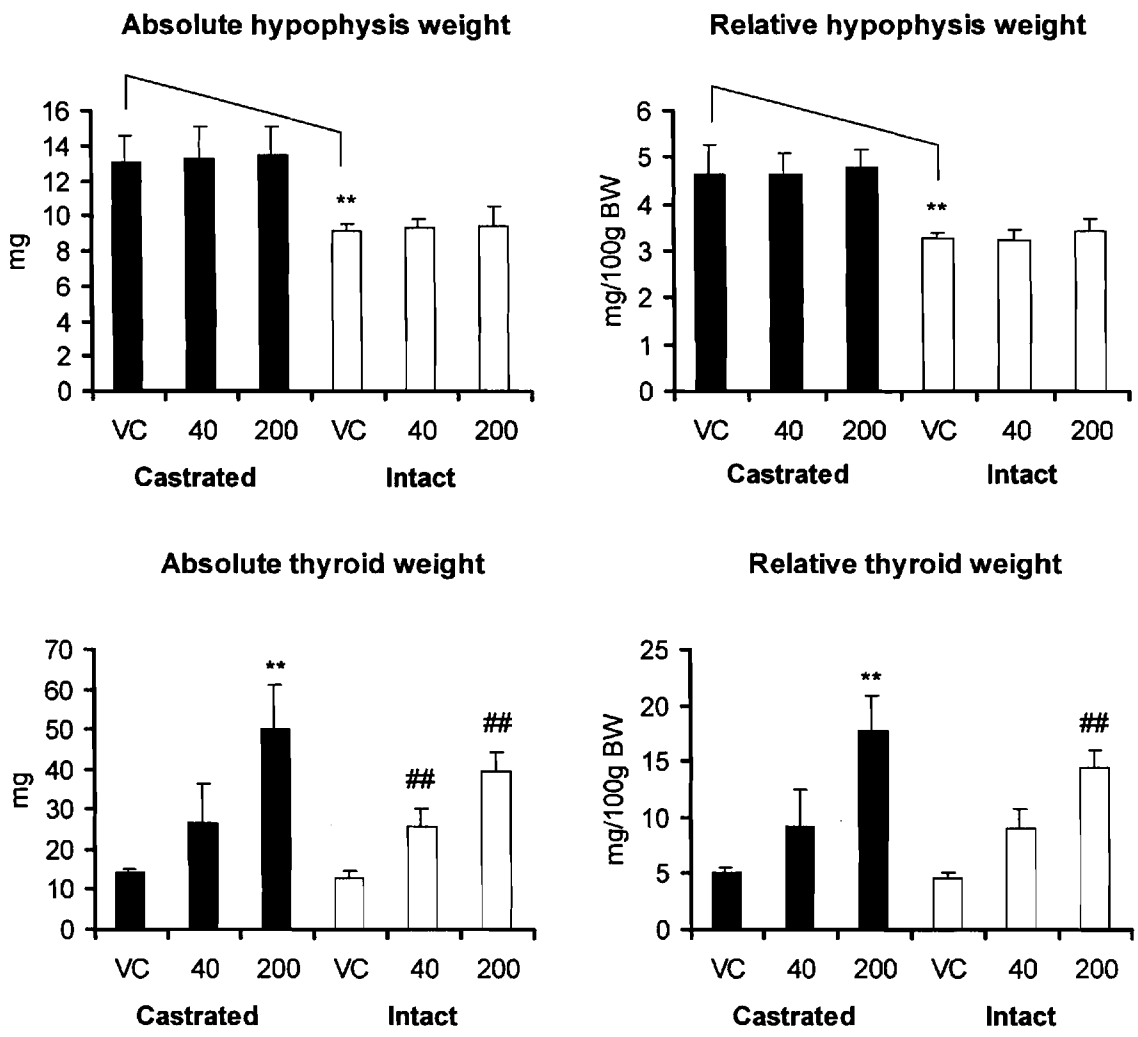

Fig. 8. Hypophyseal and thyroid weights of AT-treated castrated and intact male rats.

**Significantly different from the castrated vehicle control at $p<0.01$.

##Significantly different from the intact vehicle control at $p<0.01$.

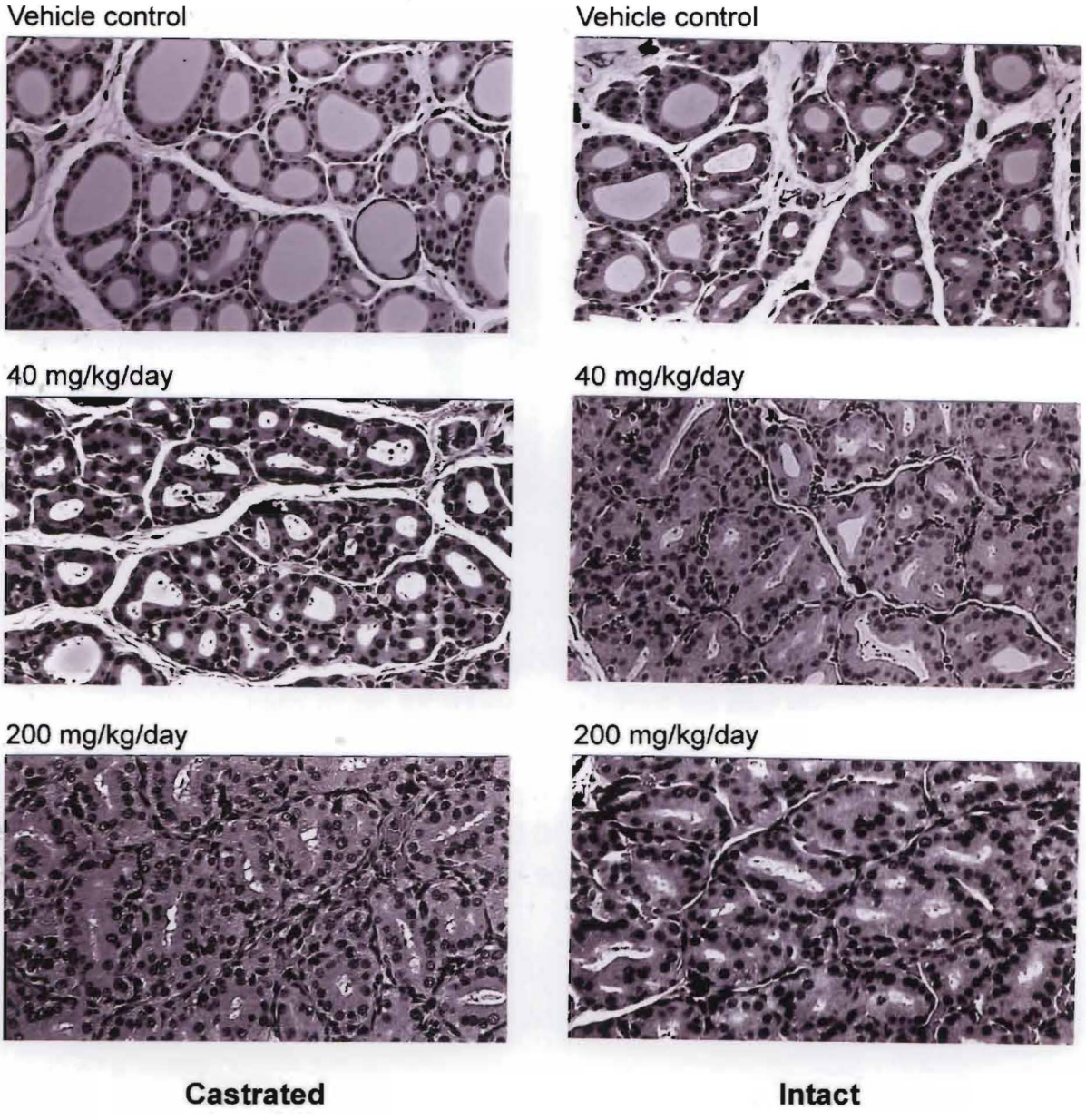

Fig. 9. Micrographs of the thyroid gland of an AT-treated castrated rat and an AT-treated intact male rat. Magnification (125 x)

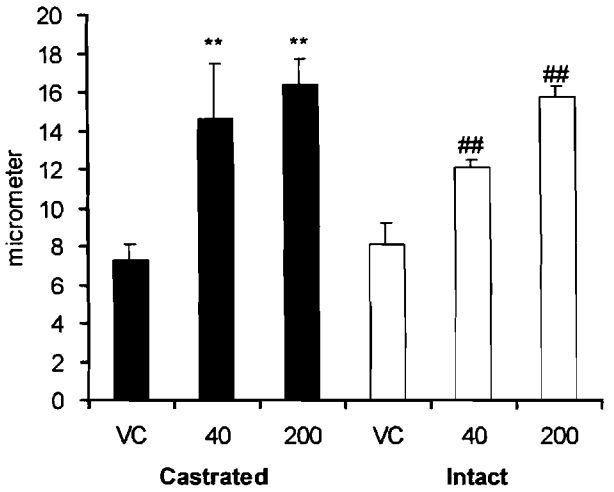

Fig. 10. Height of thyroid follicular epithelial cells of an AT treated castrated rat and an AT-treated intact male rat.

**Significantly different from the castrated vehicle control at $p<0.01$.

##Significantly different from the intact vehicle control at $p<0.01$.

Ⅲ. Preliminary Evaluation of *In utero*-lactation Exposure using Thyroid Inhibiter, 6-n-propyl-2-thiouracil.

INTRODUCTION

Several *in vivo* screening methods, like the uterotrophic assay, the Hershberger assay, and the enhanced TG-407, are being developed by the Organization for Economic Cooperation and Development (OECD, 1999, 2001) to detect chemicals with endocrine-disrupting activities. In addition, the Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) has developed other screening assays to detect hormonal effects on thyroid function (Goldman *et al.*, 2000; Stoker *et al.*, 2000). Moreover, an in *utero*-lactation screening protocol is being considered by the U.S. Environmental Protection Agency as an alternative assay because of the unique sensitivity of fetuses and neonates (Gray *et al.*, 2002) or neonates has been recognized for nearly 100 years (Gardner, 1975). Recently, several epidemiological studies have linked attention deficit hyperactivity disorder or lower IQ levels in children with higher dietary intakes of contaminated fish and background exposure to polychlorinated biphenyls, dioxins, and/or co-contaminants (Jacobson and Jacobson, 1996; Kooistra *et al.*, 2001; Rovet and Hepworth, 2001). In rats, pre- or peri - natal hypothyroidism caused by PTU produces delays in physical development, hearing disturbances, behavioral deficits, and learning impairments (Akaike et al., 1991; Goldey *et al.*, 1995; Tamasy *et al.*, 1986). Most reports studying PTU have involved administration in water or in food, and reports on the oral gavage of 6-n-propyl-2-thiouracil (PTU) during the fetal organogenesis and lactation period are very rare, although the amount and duration of treatment can be precisely controlled compared to treatment as drinking water or in food to their mother. In this study, dams were administered PTU by oral gavage during the fetal organogenesis and lactation period, and the ability of the *in utero*-lactation assay to detect the anti-thyroid effects was evaluated.

MATERIALS AND METHODS

Chemicals

6-n-propyl-2-thiouracil （PTU, CAS No. 51-52-5, Lot No. 99H2509） was purchased from Sigma Chemical Company, (US). The PTU was mixed with olive oil (Lot No. 014OOY; Fujimi Pharmaceutical, Company. Osaka, Japan).

Animals

Pregnant SPF female Crj: CD ® (SD) IGS (SD IGS) rats, purchased from Charles River Japan, Inc. (Hino Breeding Center, Japan), were used in this study. The rats were housed in polycarbonate cages (280 mm wide x 440 mm deep x 150 mm high) containing nesting materials (Sun Flake®; Chiba Animal Material Co., Ltd., Japan) and allowed to acclimate for one week. On gestational day 6 (GD 6), the dams were ranked by weight and 3 dams were randomly assigned to each of 5 experimental groups using a body-weight stratified randomization protocol. The body weight of the dams was recorded on GD 6, 7, 14, and 20 and on postnatal day (PND) 0, 7, 14, and 21. The day that dams completed her delivery by 15:00 was designated as PND 0. At the completion of delivery, the newborns were counted (both dead and alive neonates), and the gestational age was determined. On PND 4, the litter size was culled to 4 of each sex. In litters with 8 or fewer pups, all pups were reared. If the number of either sex was less than 4, a total number of pups were reared. The offspring were weaned on PND 21. The weanlings were housed in hanging stainless steel cages with a wire-mesh floor (260 mm wide x 380 mm deep x 180 mm high). The body weight of offspring was measured on PND 0, 4, 7, 14, 21, 28, 35 and 40. Rats were provided with a commercial diet (MF; Oriental Yeast Co., Tokyo, Japan) ad libitum and an automatic water supply or bottled water ad libitum. The animal room was maintained at a temperature of 23 ± 20℃, a relative humidity of 55 ± 5%, and was artificially illuminated with fluorescent light on a 12- h light / dark cycle (7:00 – 19:00 h). All animals were cared for according to the principles

outlined in the guide for animal experimentation prepared by the *Japanese Association for Laboratory Animal Science*.

Dosage and dosing period

PTU was administered orally at a defined time to inseminated rats (n=3, each dosage groups) at dosages of 0, 0.0032, 0.016, 0.08 and 0.4 mg/kg body weight on GD 7 through to lactation day 18. The dosages were based on the latest body weight of the rat. Controls were given the same volume of olive oil alone.

Physical development

To assess the development of physical landmarks, the ano-genital distance of all surviving pups was measured and pinna unfolding was evaluated on PND 4 before culling; thereafter, the growth of hair, eruption of the lower incisors, and eye opening were noted from PND 8, 11, and 14 onwards, respectively.

Reflex ontogeny

All surviving pups were examined for pain response on PND 5, negative geotaxis on PND 8, startle response (60 dB, 1,000 and 20,000 Hz) using an audiometer (PA-1, Nagashima Medical Equipment Co., Ltd.) on PND 13, and air righting reflex on PND 16.

Open-field test

Each weanling was subjected to an open-field test for 3 consecutive days, beginning on PND 21, using gray circular field (80 cm ϕ x 40 cm high) consisting of 19 sectors. The intensity of the illumination at the center of the apparatus was set at 500 lux. The latency time (time required to escape from the center sector), the frequencies of ambulation (from one sector to another), and the number of rearing, defecation and urination episodes during a 2-min period were counted.

Biel - type water T-maze test

A Biel - type water T-maze test was performed for each weanling beginning on PND 30 at a water temperature of 23±2℃. On the first day, the weanlings were given triplicate swimming trials in a straight course, and the time required to reach the goal was recorded. On the following 3 days, the weanlings were subjected to 4 maze trials per day using the Biel's original pathways and the number of errors and the time required to reach the goal, up to 180 seconds, were recorded. Experiments where the whole body of the test animal reentered the starting grid were counted as errors.

Autopsy

All offspring in each group were weighed and then euthanized by exanguination under ether deep anesthesia and carefully autopsied on PND 40. The brain, hypophysis, thyroid glands with parathyroid glands, testes, epididymidis, ovaries and uterus were removed and weighed.

Histopathological examinations

The brain, hypophysis, thyroid glands with parathyroid glands, testes, epididymidis, ovaries and uterus of the offspring were fixed in 10% neutral buffered formalin and were examined histopathologically.

Statistics

Body weight and organ weight data were analyzed using a Bartlett test for homogeneity of variance. When the variances were homogeneous at a significance level of 5%, a one-way analysis of variance was performed. If a significant difference was obtained, the differences between the control group and each of the treatment groups were analyzed using a Dunnett test. When the variances were not homogeneous, a Kruskal-Wallis test was used. If a significant difference was obtained, the differences between the control group and each of the treatment groups were analyzed using a nonparametric Dunnett

test. Fisher's exact test was applied to the sex ratios of live newborns or fetuses and all other parameters that were represented as ratios. Analyses were performed using the mean litter values prior to weaning and those of individual pups after weaning. A P value of 0.05 was adopted as the level of significance.

RESULTS

General condition and body weight change

No abnormalities were observed in the dams with regard to clinical signs or body weight changes during pregnancy or nursing. Nine males and 13 females in the vehicle control group, 12 males and 12 females in the 0.0032 mg/kg group, 10 males and 14 females in the 0.016 mg/kg group, 12 males and 12 females in the 0.08 mg/kg group and 14 males and 10 females in 0.4 mg/kg group were weaned on PND 21. In the offspring, no abnormalities were noted, based on clinical observations. The body weights of males in the 0.4 mg/kg/day group and in females of the 0.016 and 0.4 mg/kg/day groups were significantly lower, compared to the control groups for each sex, throughout the study (Table 5).

Physical development

The results of the physical development tests are shown in Table 6. Incisor eruption was significantly delayed in male and female offspring belonging to the 0.4 mg/kg/day group.

Reflex ontogeny

The results of the reflex ontogeny tests are shown in Table 7. The lack of a startle response on PND 13 was observed in male and female offspring belonging to the 0.4 mg/kg/day group.

Open-field test

PTU administration did not appear to have a notable effect on the open-field test results, though significant differences were sporadically seen for some items (data not shown).

Biel-type water T-maze test

The results of of Biel-type water T-maze test are shown in Table 8. On the first

day, a prolonged swimming time for the straight course was observed in females belonging to the 0.4 mg/kg/day group. On the following test days, an increase the number of errors and prolonged swimming times for the maze courses were observed in females belonging to the 0.4 mg/kg group.

Gross changes

No abnormalities in the dams that received PTU, or their offspring were observed.

Organ weight

The organ weight changes in the offspring are shown in Tables 9, 10. In males, increase in the relative thyroid weight and a decrease of absolute testes weight were observed in the 0.4 mg/kg/day group, while increase, in the absolute and relative seminal vesicle weight were observed in the 0.08 and 0.0032 mg/kg/day groups. In females, increases in the relative brain and thyroid weights were observed in the 0.4 mg/kg/day group, while an increase in the relative brain weight was also observed in the 0.016 mg/kg/day group. There are no abnormalities in the thyroid weights of the dams were observed.

Histolopathological examinations

Hypertrophy and hyperplasia of the follicular epithelium was observed in all dams in the 0.4 mg/kg/day group (Table 11). In the offspring, flattening of follicular epithelium (8/14), decrease of resorptive colloid droplets (10/14), degeneration of follicular epithelium (8/14), hyperplasia of follicular epithelium (1/14), in the males, belonging to the 0.4 mg/kg/day group, flattening of follicular epithelium (1/12), decrease of resorptive colloid droplets (1/12), degeneration of follicular epithelium (2/14) in the males, belonging to the 0.08 mg/kg/day group were observed (Fig. 11, Table 12). No other organ abnormalities were seen in the males or females.

DISCUSSION

The *in utero*-lactation assay is an alternative assay for the detection of endocrine-disrupting chemicals that has been recommended for consideration by the EDSTAC. However, some concerns exist regarding the large number of animals and the long test duration that are required as well as the assay's sensitivity and specificity as a screening test method (Gray *et al.*, 2002). On the other hand, thyroid hormone is known to regulate neuronal proliferation, migration, and differentiation in the brain during the fetal and neonatal periods (Bernal and Nunez, 1995). This information implies that the in *utero*-lactation assay may be particularly suitable as a screening method for the detection of thyroid inhibitors. This study is designed to obtain preliminary data on the potential functional and pathological hazards to the next generation that may arise in offspring from exposure of the dam during pregnancy and lactation period by oral gavage. The observation items, those are considered to have comparatively high specificity for the thyroid hormone inhibition, were selected. In this preliminary study, hormonal evaluations were not performed because of the small sample size (3 dams/group). The hormonal evaluation of dams and their offspring will be performed in the future studies.

PTU does not produce major malformations (Dlav-citrin and Ornoy, 2002) and its inhibitory mechanisms are comparatively well-known: namely, PTU directly interferes with the first step in thyroid hormone biosynthesis in the thyroid gland and inhibits the conversion of peripheral thyroxin (T4) to triiodothyronine (T3). Although PTU is commonly used in experimental models of congenital hypothyroidism, the administration of PTU by oral gavage in dams during the entire period of fetal organogenesis and lactation has not been previously reported. The oral gavage has the advantage that the amount and duration of PTU treatment can be precisely controlled compared to PTU treatment as drinking water to their mother. Thus, we performed an in *utero*-lactation assay using PTU to evaluate the suitability of this method as a screening test for the

detection of the thyroid hormone inhibitor.

Pre- or peri-natal hypothyroidism in rats can be induced by various thyroid effectors and produces characteristic effects, such as a low body weight, auditory dysfunction, behavioral deficits, and learning impairment (Goldey *et al.*, 1995, 1998; Roegge *et al.*, 2000; Seo *et al.*, 1999), similar to the hypothyroidism in rats evoked by PTU (Akaike *et al.*, 1991; Goldey *et al.*, 1995; Tamasy *et al.*, 1986). Some developmental effects, like eye opening for example, are thought to have a different mechanism of action (Goldey and Crofton, 1998). In this study, hypertrophy and hyperplasia of the follicular epithelial cells in the thyroid of dams in the 0.4 mg/kg/day group, increase in the relative thyroid weights of the offspring belonging to the 0.4 mg/kg/day group, flattening of follicular epithelium, decrease of resorptive colloid droplets, degeneration of follicular epithelium, hyperplasia of follicular epithelium in the male offspring belonging to the 0.4 mg/kg/day group, and an increase in errors and prolonged swimming times in the Biel type water T-maze test in female offspring belonging to the 0.4 mg/kg/day group were determined to be authentic endocrine-mediated effects of PTU. Histpathologically, abnormalities were also observed in the 0.08 mg/kg/day group. Contrary to our expectations, no significant differences were observed in the outcomes of the open-field test. Akaike reported that rats with neonatal hypothyroidism induced by PTU showed an increase in ambulation at 6 weeks of age in the open-field test (Akaike *et al.*, 1991). The reproducibility of these results and the experimental conditions should be further investigated. Furthermore, comparative studies using other methods of testing emotional and learning ability may be needed. Identifying appropriate endpoints to detect the influence of thyroid effects on emotion is an important issue, since emotion and perception are two of the main functions of the brain.

The present histpathological results did not show any major adverse effects on the reproductive systems of either sex. Although some organs weight showed significant differences, when compared to the controls, whether these results are caused solely by hormonal imbalances evoked by PTU remains uncertain.

Neonatal hypothyroidism in a rat model showed initial disturbance in the onset of mesenchymal cell differentiation into adult Leydig cells (Siril Ariyaratne *et al.*, 2000), the inhibition of testosterone production (Chiao *et al.*, 2002), and disturbed folliculogenesis (Dijkstra *et al.*, 1996). Thus, neonatal hypothyroidism has an evident effect on the reproductive systems of both sexes. These findings imply that the effects of thyroid effectors on reproductive systems may only become evident upon sexual maturation.

In this study, the utility of the in *utero*-lactation assay was demonstrated, and a dose of 0.4 mg/kg/day of PTU was determined to be the minimal required dosage to produce clear anti-thyroid effects on auditory function, learning ability, and thyroid weight and thyroid histopathology, without causing any major malformations. Moreover, histpathological abnormalities were observed in some offspring belonging to the 0.08 mg/kg/day group.

The histopathological effects of PTU on the thyroids of rats are poorly understood, even though the thyroid is one of the target organs of PTU. In this study, the histopathological findings in the thyroids of the offspring were not the same as those in the dams. Although we could not explain the mechanism for the difference in the effects seen in the offspring and the dams, the effect of PTU during *in utero* through lactation exposure is apparently different from that resulting from exposure in homeostatically mature rats. Thus, the ability of this assay to detect effects that cannot be seen in the mature rats, including developmental neurotoxicity, emphasizes the importance of this assay.

A two-generation reproductive study of PTU in which groups of 20 male and female adult SD rats were given PTU in drinking water at concentrations of 0, 0.0001, 0.0004, or 0.0015 w/v% has been reported (Nehrebeckyj *et al.*, 2001). In this study, the most sensitive endpoint was a decrease in ano-genital distance in male offspring in the 0.0001 w/v% group (0.1 mg/kg/day, according to the authors). In the study evaluated the effects of developmental hypothyroidism on auditory and motor function in the rats received PTU in drinking water at concentrations of 0, 1, 5, or 25 ppm from GD 18 until PND 21, only slight and

transient effects on eye opening and ontogeny of motor activity and decreased serum thyroxin levels in the 5 ppm treatment group (0.17 mg/kg/day, according to the authors) has been reported (Goldey *et al.*, 1995). In the present study, hearing disturbances, an elevated relative thyroid weight, an increase in the number of errors in the Biel-type water T-maze test were observed in the 0.4 mg/kg/day group. Histopathologically, flattening of follicular epithelium, decrease of resorptive colloid droplets, degeneration of follicular epithelium, hyperplasia of follicular epithelium was observed in the 0.4 mg/kg/day group. Moreover, histpathological abnormalities were also observed in some offspring belonging to the 0.08 mg/kg/day group. Thus, it was considered that there were no sensitivity differences between in *utero*-lactation assay and other study conducted by administration in drinking water.

In conclusion, the present results indicate that the in *utero*-lactation assay is a useful method for detecting the thyroid hormone antagonist activity of chemicals, with the Biel-type water T-maze test, thyroid weight and histopathological examinations of dams and offspring as sensitive endpoints. Based on the present results, a study to determine the reproducibility of the data in a much larger number of dams will be performed to confirm the findings in the present study, and evaluated another endpoints, such as hormonal evaluation of dams and their offspring, sexual developmental landmarks and fertility of the offspring to fully evaluate the effects of in *utero* - lactational oral administration.

ABSTRACT

In this preliminary study, the ability of the *in utero*-lactation exposure for the detection of thyroid effectors was evaluated by treating three dams/group with 6-n-propyl-2-thiouracil (PTU), a known thyroid antagonist by oral gavage at doses of 0, 0.0032, 0.016, 0.08 and 0.4 mg/kg/day during fetal organogenesis and lactation. Hearing disturbances and an elevated relative thyroid weight were observed in offspring of the both sexes in the 0.4 mg/kg/day group. The Biel-type water T-maze test showed an increase in the number of errors made by females in the 0.4 mg/kg/day group. Histopathologically, flattening of follicular epithelium, decrease of resorptive colloid droplets, degeneration of follicular epithelium, hyperplasia of follicular epithelium was observed in males belonging to the 0.4 mg/kg/day group. In the dams, hypertrophy of the follicular epithelium of the thyroid was observed in the 0.4 mg/kg/day group. Although we could not explain the mechanism for the difference in the effects seen in the offspring and the dams, the effect of PTU during *in utero* through lactational administration is apparently different from that resulting from exposure in homeostatically mature rats. Most reports studying PTU have involved in water or in food, and reports on the oral gavage of PTU during the fetal organogenesis and lactation period are very rare. This assumes that dosages > 0.4 mg/kg/day would also produce clear anti-thyroid effects by oral gavage and, possibly, emphasizes that dosages < 0.4 mg/kg/day did not have a noticeable effect. The present results are likely to become a benchmark for the development of *in utero*-lactation exposure for the detection of thyroid effectors.

Table 5. Body weights of offspring

Sex	Dose of PTU (mg/kg/day)	Days of age							
		0	4	7	14	21	28	35	40
Male	VC	7.0 ± 0.9 (12)	12.6 ± 1.4 (11)	19.8 ± 1.9 (9)	38.5 ± 3.4 (9)	61.9 ± 4.1 (9)	105.6 ± 16.3 (9)	169.0 ± 25.1 (9)	221.0 ± 30.0 (9)
	0.0032	7.0 ± 0.1 (18)	11.9 ± 0.6 (18)	18.9 ± 0.7 (12)	38.7 ± 1.8 (12)	64.1 ± 2.6 (12)	111.2 ± 6.4 (12)	181.5 ± 10.8 (12)	236.0 ± 13.2 (12)
	0.016	6.8 ± 1.0 (14)	11.1 ± 2.6 (14)	16.5 ± 3.4 (10)	34.3 ± 4.2 (10)	54.9 ± 7.7 (10)	96.8 ± 12.0 (10)	160.4 ± 17.5 (10)	212.4 ± 18.8 (10)
	0.08	6.7 ± 0.4 (18)	10.9 ± 0.8 (18)	18.1 ± 1.3 (12)	37.9 ± 0.9 (12)	60.5 ± 1.5 (12)	106.2 ± 4.9 (12)	174.3 ± 8.3 (12)	228.1 ± 12.3 (12)
	0.4	6.6 ± 0.3 (25)	9.4 ± 0.9 (25)	15.2 ± 1.8 (14)	33.2 ± 2.5 (14)	49.5 ± 2.0 (14)*	80.0 ± 16.0 (14) **	134.4 ± 26.9 (14) **	184.5 ± 33.6 (14)**
Female	VC	6.8 ± 0.5 (19)	12.0 ± 1.5 (17)	19.2 ± 1.9 (13)	38.3 ± 3.5 (13)	62.0 ± 4.2 (13)	100.9 ± 5.7 (13)	150.1 ± 7.0 (13)	179.1 ± 10.6 (13)
	0.0032	6.7 ± 0.6 (18)	11.5 ± 1.7 (18)	18.3 ± 1.9 (12)	38.5 ± 2.7 (12)	63.1 ± 4.5 (12)	106.1 ± 7.8 (12)	160.8 ± 10.0 (12)	194.9 ± 10.7 (12)
	0.016	6.3 ± 0.6 (21)	10.2 ± 2.0 (21)	15.4 ± 2.8 (14)	32.0 ± 3.4 (14)*	51.3 ± 6.7 (14)*	86.8 ± 8.5 (14) **	132.0 ± 9.9 (14) **	162.4 ± 9.5 (14) **
	0.08	6.4 ± 0.3 (22)	10.5 ± 0.6 (22)	17.7 ± 0.7 (12)	36.8 ± 0.4 (12)	58.0 ± 3.8 (12)	97.2 ± 7.8 (12)	146.0 ± 12.5 (12)	176.1 ± 14.7 (12)
	0.4	6.3 ± 0.2 (15)	9.1 ± 0.9 (15)	14.8 ± 1.3 (10)	31.8 ± 1.8 (10)*	47.8 ± 1.8 (10)**	75.6 ± 13.5 (10) **	117.6 ± 20.2 (10) **	150.2 ± 22.4 (10)**

Value in parentheses represents the number of rats examined.

Day of birth = N 0, Day of weaning = N 21.

*, **Significantly different from vehicle control at $p<0.05$ and at $p<0.01$, respectively.

Table 6. Physical development of offspring

Sex	Items	Dose of PTU (mg/kg/day)				
		VC	0.0032	0.016	0.08	0.4
Male	Pinna unfolding	11/11 (100.0)	18/18 (100.0)	14/14 (100.0)	18/18 (100.0)	25/25 (100.0)
	Growth of hair	9/9 (100.0)	12/12 (100.0)	10/10 (100.0)	12/12 (100.0)	14/14 (100.0)
	Incisor eruption	9/9 (100.0)	12/12 (100.0)	9/10 (90.0)	11/12 (91.7)	3/14 (21.4)*
	Eye opening	8/9 (88.9)	12/12 (100.0)	8/10 (80.0)	12/12 (100.0)	14/14 (100.0)
Female	Pinna unfolding	17/17 (100.0)	18/18 (100.0)	21/21 (100.0)	22/22 (100.0)	15/15 (100.0)
	Growth of hair	13/13 (100.0)	12/12 (100.0)	14/14 (100.0)	12/12 (100.0)	10/10 (100.0)
	Incisor eruption	13/13 (100.0)	12/12 (100.0)	14/14 (100.0)	11/12 (91.7)	4/10 (40.0)*
	Eye opening	13/13 (100.0)	12/12 (100.0)	12/14 (85.7)	12/12 (100.0)	9/10 (90.0)

Value in parentheses represents percentage of the number of rats examined.

* Significantly different from vehicle control at $p < 0.05$

Table 7. Reflex ontogeny of offspring

Sex	Items	Dose of PTU (mg/kg/day)				
		VC	0.0032	0.016	0.08	0.4
Male	Pain response	9/9 (100.0)	12/12 (100.0)	10/10 (100.0)	12/12 (100.0)	14/14 (100.0)
	Negative geotaxia	9/9 (100.0)	11/12 (91.7)	10/10 (100.0)	12/12 (100.0)	14/14 (100.0)
	Startle response					
	1,000 Hz	9/9 (100.0)	11/12 (91.7)	10/10 (100.0)	12/12 (100.0)	1/14 (7.1)*
	20,000 Hz	9/9 (100.0)	12/12 (100.0)	6/10 (60.0)	12/12 (100.0)	0/14 (0.0)*
	Air righting reflex	9/9 (100.0)	12/12 (100.0)	10/10 (100.0)	12/12 (100.0)	14/14 (100.0)
Female	Pain response	13/13 (100.0)	12/12 (100.0)	14/14 (100.0)	12/12 (100.0)	10/10 (100.0)
	Negative geotaxia	13/13 (100.0)	12/12 (100.0)	14/14 (100.0)	12/12 (100.0)	10/10 (100.0)
	Startle response					
	1,000 Hz	13/13 (100.0)	12/12 (100.0)	14/14 (100.0)	12/12 (100.0)	1/10 (10.0)*
	20,000 Hz	13/13 (100.0)	12/12 (100.0)	10/14 (71.4)	11/12 (100.0)	0/10* (0.0)
	Air righting reflex	13/13 (100.0)	12/12 (100.0)	14/14 (100.0)	12/12 (100.0)	10/10 (100.0)

Value in parentheses represents percentage of the number of rats examined.

* Significantly different from vehicle control at $p < 0.05$.

Table 8. Water multiple T-maze test findings of offspring

Sex		Trial day	Dose of PTU (mg/kg/day) VC	0.0032	0.016	0.08	0.4
	Number of rats examined		9	12	10	12	14
	Straight course						
	Swimming time (sec.)	1st	10.1[a]	10.6	11.5	10.3	12.9
	Maze course						
Male	Swimming time (sec.)	2nd	32.0[a]	28.7	29.1	29.4	41.1
		3rd	16.5	17.0	19.8	18.6	22.4
		4th	12.9	13.3	15.1	13.1	17.5
	Number of selecting errors	2nd	2.5[b]	2.5	2.5	3.0	3.8
		3rd	0.5	0.5	1.3	0.8	1.8
		4th	0.0	0.0	0.0	0.0	1.0
	Number of rats examined		13	12	14	12	10
	Straight course						
	Swimming time (sec.)	1st	8.2[a]	8.7	10.1	9.6	11.7 **
	Maze course						
Female	Swimming time (sec.)	2nd	27.4[a]	25.5	33.3	30.2	47.3 **
		3rd	16.2	18.1	16.6	19.4	30.4 **
		4th	13.5	14.1	14.4	15.5	24.8 **
	Number of selecting errors	2nd	2.0[b]	2.0	3.0	3.0	4.0 **
		3rd	0.5	1.0	0.8	1.0	3.5 **
		4th	0.0	0.0	0.0	0.0	1.5 **

a, Harmonic mean; b, median.

** Significantly different from vehicle control at $p < 0.01$.

Table 9. Organ weight of male offspring

Dose (mg/kg/day)	Number of offspring	Brain	Hypophysis	Thyroid	Ventral prostate	Seminal vesicle	Testis	Epididymis	Final body weight (g)
VC	(mg)	1835.3 ± 90.9	9.6 ± 1.4	16.7 ± 5.0	106.9 ± 23.8	92.4 ± 39.9	1801.2 ± 249.9	229.3 ± 28.0	221.0 ± 30.0
	(mg/100gb.w.)	844.7 ± 121.6	4.4 ± 0.5	7.6 ± 2.0	48.5 ± 8.4	41.1 ± 15.2	816.6 ± 45.1	105.2 ± 16.4	
0.0032	(mg)	1866.3 ± 59.0	10.8 ± 1.1	17.0 ± 2.8	117.9 ± 21.4	132.4 ± 20.4 **	1886.3 ± 135.1	246.5 ± 29.9	236.0 ± 13.2
	(mg/100gb.w.)	793.1 ± 51.6	4.6 ± 0.4	7.2 ± 1.0	50.0 ± 8.9	56.5 ± 10.6 *	800.5 ± 57.6	104.8 ± 14.8	
0.016	(mg)	1835.9 ± 135.3	8.6 ± 0.8	16.4 ± 3.8	112.1 ± 22.8	110.3 ± 29.0	1609.9 ± 226.8	217.8 ± 29.1	212.4 ± 18.8
	(mg/100gb.w.)	866.4 ± 37.1	4.1 ± 0.3	7.8 ± 2.2	52.7 ± 8.9	51.7 ± 11.0	756.1 ± 61.8	103.2 ± 15.4	
0.08	(mg)	1894.5 ± 51.5	9.9 ± 1.1	19.0 ± 3.4	123.2 ± 17.7	130.9 ± 29.5 **	1804.3 ± 100.2	228.5 ± 26.3	228.1 ± 12.3
	(mg/100gb.w.)	832.4 ± 43.2	4.4 ± 0.5	8.3 ± 1.6	54.0 ± 7.3	57.3 ± 12.1 *	792.8 ± 57.5	100.4 ± 12.5	
0.4	(mg)	1879.0 ± 73.2	8.9 ± 1.9	17.7 ± 4.1	103.6 ± 26.6	92.5 ± 20.7	1536.9 ± 160.9**	228.7 ± 26.5	184.5 ± 33.6**
	(mg/100gb.w.)	1051.3 ± 203.4 **	4.8 ± 0.6	9.6 ± 1.6 *	58.1 ± 19.0	51.2 ± 12.1	848.2 ± 101.2	126.5 ± 18.7 **	

*, **Significantly different from vehicle control at $p<0.05$ and at $p<0.01$, respectively.

Table 10. Organ weights of female offspring

Dose (mg/kg/day)	Number of offspring	Brain	Hypophysis	Thyroid	Uterus	Ovary	Final body weight
VC	(mg)	1739.1 ± 62.3	10.1 ± 1.3	14.6 ± 3.0	245.1 ± 62.8	52.6 ± 8.8	179.1 ± 10.6
	(mg/100gb.w.)	973.2 ± 49.3	5.6 ± 0.7	8.2 ± 1.6	137.2 ± 35.1	29.5 ± 5.4	
0.0032	(mg)	1797.5 ± 65.1	11.2 ± 1.1	14.6 ± 1.2	295.0 ± 85.3	63.2 ± 10.2	194.9 ± 10.7
	(mg/100gb.w.)	925.3 ± 67.5	5.8 ± 0.6	7.5 ± 0.6	152.3 ±47.0	32.5 ± 5.4	
0.016	(mg)	1748.2 ± 99.8	10.0 ± 1.3	15.8 ± 3.1	252.2 ± 64.0	49.3 ± 9.3	162.4 ± 9.5 **
	(mg/100gb.w.)	1076.7 ± 32.5 **	6.2 ± 0.6	9.8 ± 2.0	155.1 ± 37.6	30.3 ± 5.1	
0.08	(mg)	1773.8 ± 65.1	10.1 ± 1.6	15.7 ± 2.7	260.2 ± 49.0	53.4 ± 10.8	176.1 ± 14.7
	(mg/100gb.w.)	1011.4 ± 60.9	5.7 ± 0.8	8.9 ± 1.4	149.0 ± 31.7	30.4 ± 5.6	
0.4	(mg)	1787.0 ± 72.8	9.3 ± 2.0	15.4 ± 3.2	269.8 ± 55.8	52.0 ± 15.3	150.2 ± 22.4 **
	(mg/100gb.w.)	1210.4 ± 157.5 **	6.2 ± 0.7	10.3 ± 1.7 *	182.2 ± 44.7	34.5 ± 8.2	

*, **Significantly different from vehicle control at $p<0.05$ and at $p<0.01$, respectively.

Table 11. Summary of histopathological changes of dams

Findings	Doses of PTU (mg/kg/day)				
	VC	0.0032	0.016	0.08	0.4
Number of animals examined	3	NE	NE	3	3
Thyroid					
No abnormalities detected	3			3	0
Hypertrophy and hyperplasia of follicular epithelium	0			0	3

NE, not examined.

Table 12. Summary of histopathological changes of male offspring

Findings	Doses of PTU (mg/kg/day)				
	VC	0.0032	0.016	0.08	0.4
Number of animals examined	9	NE	NE	12	14
Thyroid					
No abnormalities detected	9			10	3
Flattening of follicular epithelium	0			1	8
Decrease of resorptive colloid droplets	0			1	10
Degeneration of follicular epithelium	0			2	8
Hyperplasia of follicular epithelium	0			0	1
Hypophysis					
No abnormalities detected	9			NE	14
Brain					
No abnormalities detected	9			NE	14

NE, not examined.

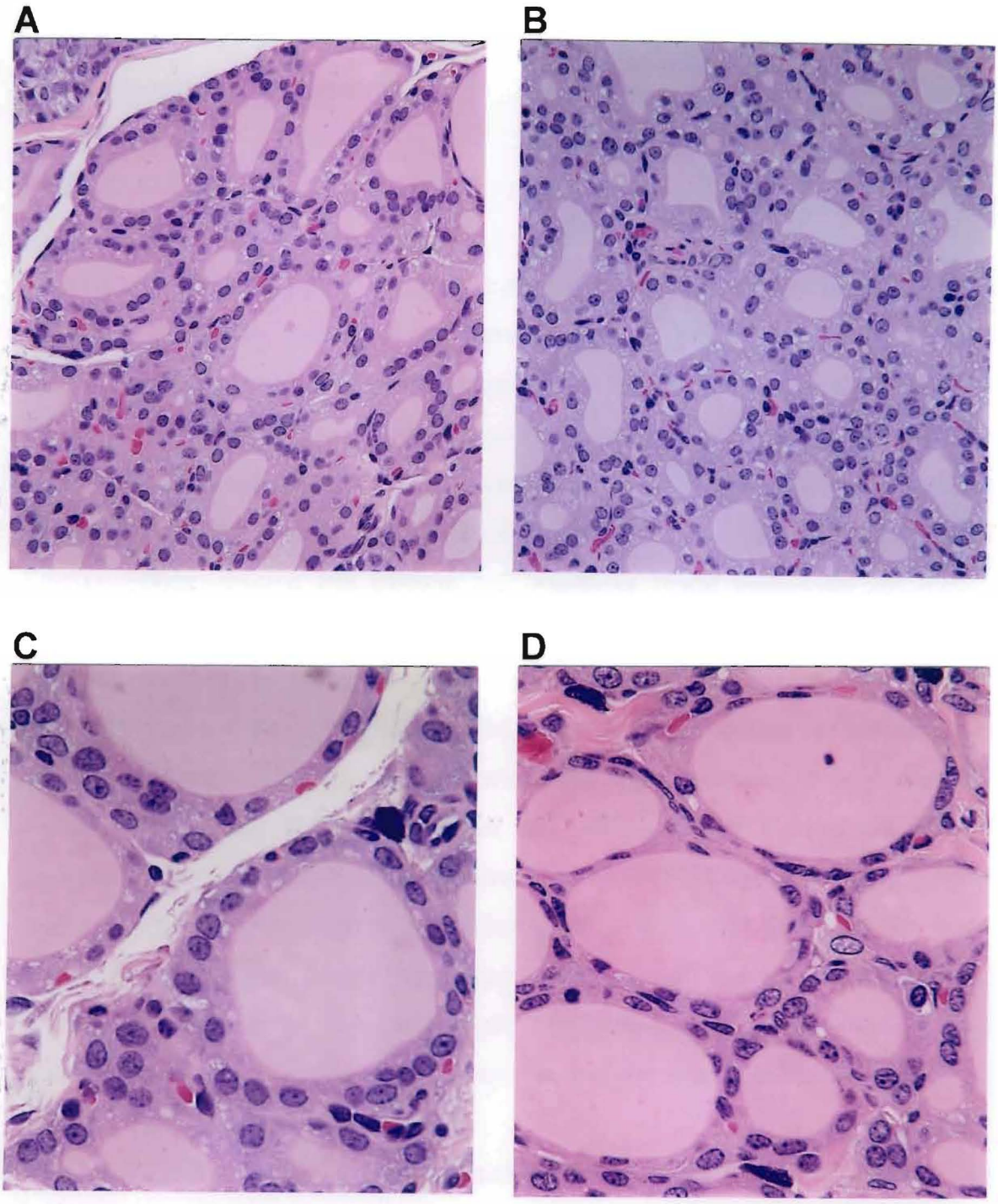

Fig. 11. Micrographs of the thyroid gland of dams (A, vehicle control; B, 0.4 mg/kg/day group) and their male offspring (C, vehicle control; D, 0.4 mg/kg/day group). The thyroid glands of all PTU-treated dams exhibited hypertrophy and hyperplasia of the thyroid follicular epithelial cells. In contrast, an increased number of follicles filled with colloid were observed in the thyroid gland of offspring in the 0.4 mg/kg/day group. The thyroid follicles of offspring in the 0.4 mg/kg/day group are containing abundant colloid, and lined by flattened epithelium. Magnification, A and B, 80 x; C and D, 160 x.

IV. Effect of Neonatal Oral Exposure of Thyroid Inhibiter, 6-n-propyl-2-thiouracil

INTRODUCTION

Since a considerable number of chemicals have been reported to have possible endocrine-disrupting activities in humans and animals (McLachlan 1993; McLachlan and Korach, 1995), the Organisation for Economic Co-operation and Development (OECD) and the Endocrine Disrupter Screening and Testing Advisory Committee (EDSTAC) have proposed various *in vivo* test methods to detect endocrine-mediated effects (EPA, 1998; OECD, 1999; OECD, 2001; OECD, 2003). Among the various test methods being examined, an assay involving the administration (subcutaneous injection) of estrogenic compounds, diethylbestrol, ethinyl estradiol, clomiphene or tamoxifen for 5 days beginning on postnatal day 1 (PND 1, birthday = PND 1) was performed by several groups, and the endocrine effects of these compounds were detected (Branham *et al.,* 1988; Iguchi *et al.,* 1989; Medlock *et al.,* 1988). This method is particularly noteworthy because it can detect the endocrine effects of chemicals during early neonatal life. Numerous endocrine disrupters are thought to enter the body through an oral route, like the ingestion of contaminated maternal milk, food and drinks. However, most reports studying endocrine disrupters have involved subcutaneous injections, and reports on the oral gavage of endocrine disrupters are very rare, to our knowledge.

The compound 6-n-propyl-2-thiouracil (PTU) induces hypothyroidism by inhibiting thyroid iodination and has been used as a typical thyroid antagonist (Dijkstra *et al.,* 1996; Hardy *et al.,* 1996; Kirby *et al.*, 1997; Marty *et al.,* 1999; O'Connor *et al.,* 1999). In adult rats, changes related endocrine disruption, like delayed preputial separation, altered hormone levels, increased thyroid weight, and altered thyroid histology, have been previously reported in repeated-dose toxicity studies involving oral gavege (Marty *et al.,* 2001; Yamasaki *et al.,* 2002).

However, the use of oral gavage during the neonatal period, has not been reported.

We performed the present assay as a preliminary trial using PTU for 5 days, starting on PND 1, to examine whether the endocrine effects of thyroid dysfunction could be detected after early neonatal oral administration.

MATERIALS AND METHODS

Chemicals

6-n-propyl-2-thiouracil (PTU, CAS No. 51-52-5, 99% pure) was obtained from the Sigma Chemical Company (Tokyo, Japan), and the olive oil was obtained from Fujimi Pharmaceutical Company (Osaka, Japan).

Animals

Fifty 11-week-old Crj:CD (SD) IGS female rats at 11 weeks of age were purchased from Charles River Japan, Inc. (Shiga, Japan) and mated at 14 weeks of age with Crj:CD (SD) IGS male rats. After detecting a vaginal plug or sperm in a vaginal smear, the dams were divided into four experimental groups consisting of 10 dams per group. The pups that were subsequently born were used in this study. The litters were randomly culled to eight males per dam on PND 7. When the number of pups of either sex was insufficient, the litter size was adjusted to eight, regardless of sex. No adjustment was made when the number of pups per dam was less than eight. The pups were kept in polycarbonate pens with the dams until weaning. All pups were weaned after 21 days and then housed, three per cage, in stainless steel, wire-mesh cages throughout the study; water was provided automatically, and a commercial diet (MF, Oriental Yeast Co., Tokyo, Japan) was provided ad libitum. The animal room was maintained at a temperature of 23 ± 20℃ and a relative humidity of 55 ± 5%; the room was artificially illuminated with fluorescent light according to a 12-hour light/dark cycle (06:00 – 18:00 h). All animals were cared for according to the principles outlined in the guide for animal experimentation prepared by the Japanese Association for Laboratory Animal Science.

Experimental design

Rats were treated by oral gavage with 0, 0.01, 0.1 or 1 mg/kg/day of PTU for 5 days, starting on PND 1. Oral gavage in the newborn was easily accomplished

using an Atom Indwelling Feeding Tube for infant (3Fr; ATOM medical Company, Ltd. Tokyo, Japan) and a 1-mL disposable tuberculin syringe (Terumo Corporation, Tokyo, Japan). The newborn was restrained so that its throat and abdomen formed a straight line, and the PTU was administered. The dosages were selected based on the results of our previous studies examining enhanced TG 407 and pubertal assays using PTU (Yamasaki *et al.*, 2002e). The volume of the olive oil solutions containing the PTU was 5 ml/kg for the oral administration. The concentration and stability of PTU were confirmed. Animals were killed by exsanguination under ether anesthesia on PND 61, and blood samples were obtained from the abdominal aorta and used for the hormone analysis. The following tests were performed: clinical signs and body weight changes, incisor eruption and eye opening, negative geotaxis and auditory startle response, open-field test, water maze test, preputial separation, serum hormonal analysis, organ weight changes, and histopathology. The pup numbers used for each observation are show in Table 13. Four male pups out of eight animals per dam were used in this study; the remainder were used in other studies. These four pups per dam were subjected to observations of body weight changes, incisor eruption and eye opening, preputial separation, organ weight changes and histopathology; two of the four rats were used in the negative geotaxis, auditory startle response and water maze tests, and the remaining two rats were used in the open-field tests.

Clinical signs were recorded daily in all animals. Body weight was measured on PND 0, 1, 5, 7, 14 and 21 before weaning. After weaning, body weight was measured once a week throughout the study.

Incisor eruption and eye opening were examined as indicators of physical development on PND 12 and 14, respectively.

Negative geotaxis and auditory startle response tests were performed to examine the pups, reflexes and as sensory function tests. Negative geotaxis was examined 3 times on PND 8. In this test, each pup was placed head downward on a piece of plywood (30 cm x 30 cm) at a 20° incline, and the time required for

the animal to reverse its position was recorded, up to 3 seconds. The auditory startle response test was performed on PND 13. Each pup was placed in a test cage and, after a 10 - minute acclimation period, a noise level of 60 dB, 1 kHz, and 20 kHz was produced using an audio meter; a total of 5 trials (intertrial interval = 5 seconds) were performed.

An open-field test was performed to examine motor activity. Each pup performed the open-field test on 3 consecutive days, beginning on PND 23. An 80-cm ϕ x 40-cm high gray circle with 19 areas was used. The intensity of the illumination was set at 500 lux. The number of ambulations, rearing and grooming episodes, and the presence or absence of defecation or urination were observed for 2 minutes.

The Biel-type water T-maze test was performed to examine learning and memory. The Biel-type water T-maze test was performed using each pup, beginning on PND 29, using a water temperature of 22 ± 2°C. On the first day, the pups were trained three times using a straight course, and their swimming behavior was observed. On the following 3 days, the pups were given 4 maze trials per day, and the number of errors made during a period of 180 seconds was counted. Experiments where the whole body of the test animal reentered the starting grid were counted as errors.

Preputial separation was evaluated by examining the penis and attempting to retract the prepuce to determine if it had separated from the glans penis; the animal weight on the day of preputial separation was recorded. Observations were performed daily, beginning on PND 23, until preputial separation was first noted.

The animals were killed on PND 61 by bleeding from the abdominal vein under deep ether anesthesia. After necropsy, the following organs were weighed: testes, epididymes, ventral prostate, seminal vesicle plus coagulating gland with fluid, bulbo cavernosus/levator ani muscle, glans penis, liver, kidneys, pituitary, thyroids, adrenals and brain. The thyroid and pituitary were weighed after organ fixation. The thyroid, pituitary and brain were fixed in 10% neutral buffered

formalin at the time of necropsy and examined histopathologically.

A hormonal analysis was performed on the animals used in the open-field test. Thyroid-stimulating hormone (TSH), thyroxin (T4) levels, and triiodothyronine (T3) were measured at the end of each test period. The T3 and T4 levels were determined using an automatic immunoassay system (IMX, Abott Laboratories), while the TSH level was measured using a microplate reader (UV Max, Molecular Devices).

Statistics

Body weights, hormone levels, swimming time in the water maze test and organ weights were analyzed using Bartlett's test for homogeneity of variance. When the variances were homogeneous at a significance level of 5%, a one-way analysis of variance was performed. If this test yielded a significant difference, the differences between the control group and each of the treatment groups were analyzed using Dunnett's test. When the variances were not homogeneous, the Kruskal-Wallis test was used. If this test yielded a significant difference, the differences between the control group and each of the treatment groups were analyzed using a nonparametric Dunnett's test. Countable data such as open-field, water maze except for swimming time, and preputial separation results were analyzed using the Kruskal-Wallis test. If this test yielded a significant difference, the differences between the control group and each of the treatment groups were analyzed using a nonparametric Dunnett's test. Date of incisor eruption and eye opening, negative geotaxis and auditory startle response were analyzed by the Chi-square test.

RESULTS

No abnormal general observations were seen in the rats given PTU.

The body weight of the rats receiving PTU remained normal throughout the study (Tables 14, 15).

No differences between the control group and any of the PTU groups were seen with regard to incisor eruption and eye opening, negative geotaxis, auditory startle response and preputial separation (data not shown).

In the open-field test, no effects of PTU were identified for any of the items (data not shown).

The results of the Biel-type water T-maze test are shown in Table 16. On the first day, the swimming ability of the animals was normal, and no significant differences in the swimming time for the straight course were observed between the control group and any of the PTU. On the following days, no significant differences between the control group and any of the PTU groups were seen with regard to the number of errors and the swimming time for the maze courses.

Regarding the gross findings, the thyroid was slightly enlarged in the 1 mg/kg/day dosage group.

The changes in organ weight are shown in Table 17. Absolute and relative thyroid weights increased significantly in the 1 mg/kg/day dosage group. Furthermore, the absolute brain weight increased significantly in the 0.1 and 1 mg/kg/day dosage groups, while the relative brain weight increased in the 1 mg/kg/day dosage group.

In the hormone analysis, the T4 level increased significantly in the 0.01 and 1 mg/kg/day dosage groups, and a tendency to increase was also detected in the 0.1 mg/kg/day dosage group (Table 18). The TSH level decreased significantly in the 0.1 mg/kg/day group, and a tendency to decrease was also observed in the 1 mg/kg/day dosage group.

The histopathological changes are shown in Table 19 and illustrated in Fig. 12. In the 1 mg/kg/day group, an increased number of follicles filled with colloid was

observed in the thyroid gland. These follicles, containing abundant colloid, were lined by flattened epithelium.

DISCUSSION

Estrogenic compounds like diethylbestrol, ethinyl estradiol, clomiphen, tamoxifen and 17β-estradiol have been administered to neonatal rats for short periods of time, enabling the endocrine-mediated effects of these compounds to be detected (Branham *et al.,* 1988; Iguchi *et al.,* 1989; Medlock *et al.,* 1988). Hardy et al. administered PTU to rats via their diet beginning after birth and continuing until weaning and observed a testicular disturbance (Hardy *et al.,* 1996). Furthermore, Dijkstra et al. administered PTU to rats via drinking water for 40 days, starting after birth, and observed ovarian changes and elevated in TSH levels (Dijkstra *et al.*, 1996). However, weight and histological changes in the thyroids were not described in these studies, and no previous reports have investigated pups exposed to PTU by gavage a short period of time. To our knowledge, the neonatal administration of a thyroid hormone modulator by gavage is very rare. In this study, we studied the effect of neonatal oral gavage of PTU.

Endocrine-mediated effects related to PTU were detected in the histopathological examination and hormone analysis in the present neonatal exposure assay. Hypothyroidism in rats (induced by various thyroid hormone modulators, such as barbital, PTU, and methimazole) is characterized by an increase in TSH and a decrease in T3 and T4 levels (Ching 1981; Valle *et al.,* 1985). The "Enhanced OECD Test Guideline No. 407 (enhanced TG407)" by the OECD and the "Pubertal Development and Thyroid Function in Immature Male or Female Rats (pubertal assay)" by the EDSTAC have been developed as screening assays for detecting hormonal effects on thyroid function (OECD, 2001; Goldman *et al.,* 2000; Stoker *et al.,* 2000). In our previous study using the enhanced TG 407 and pubertal assays with PTU, an increase in the TSH level, a decrease in the T3 and T4 levels, an increase in thyroid weight, hypertrophy of the follicular epithelial cells of the thyroid, an increase in pituitary weight, and an increase in the number of basophilic cells in the pituitary were detected

(Yamasaki *et al.,* 2002). The pituitary changes can be attributed to the decrease in T3 and T4 levels produced by the PTU treatment followed by an increase in the TSH level, resulting in the hypertrophy of the follicular epithelial cells of the thyroid. However, the hormonal and histological changes seen in the thyroid in the present study were opposite to the results of the enhanced TG 407 and pubertal assays described above. Furthermore, no hyperactivity changes in the pituitary, such as an increase in weight or an increase in the number of basophilic cells, were detected in the present study. Strangely, the hormonal and histological changes in the thyroid observed in this study were similar to findings in adult rats given a hyperthyroidism chemical, thyroxin, for 28 days (OECD, 2003). Although we could not clarify the reason for of the changes observed in this study, the changes produced by PTU in the pups and adult animals appear to be different.

In rats, a decrease in body weight, hearing loss and learning disabilities were observed in neonates born by dams who had been exposed to PTU during their pregnancy and lactation periods (Akaike *et al.,* 1991; Casba, 1991). In the present study, no abnormal results for physical growth and development, reflexes and sensory function, motor activity, or learning and memory were detected. The reasons for these negative results in this study are unclear. However, the short term of PTU treatment (only 5 days) in the present study may be partially responsible.

The importance of thyroid function for normal brain development in fetuses or neonates has been recognized for nearly 100 years (Gardner, 1975). Many endocrine disrupters are thought to enter the body through an oral route, like the ingestion of contaminated maternal milk, food and drinks. Thus, the present data on the neonatal oral administration of a thyroid antagonist may be meaningful, because the amount and duration of treatment can be precisely controlled compared to treatment as drinking water or in food to their mother. However, to fully evaluate the effects of neonatal oral administration, further experiments, such as the measurement of T3, T4 and TSH levels immediately after the

termination of PTU administration or longer periods of PTU treatment, are necessary. Moreover, other compounds with different mechanisms from that of PTU, such as thyroid antagonist and thyroid hormone metabolism enhancers, should be also tested.

ABSTRACT

To detect the endocrine-mediated effects of 6-n-propyl-2-thiouracil (PTU), dosages of 0, 0.01, 0.1, and 1 mg/kg/day of PTU were administered b y oral gavage to male rats for 5 days starting on the first postnatal day. Serum thyroxin (T4) levels increased in all groups given PTU, while thyroid-simulating hormone (TSH) levels decreased in the 0.1 and 1 mg/kg/day groups. An increase in the thyroid weight and an increased number of follicles filled with colloid in the thyroid gland were also detected in the 1 mg/kg/day group. On the other hand, no abnormalities in physical growth and development, reflexes and sensory function, motor activity, or learning and memory were observed.

Table 13. Pup numbers used in each observation

Observations	Doses (mg/kg/day)			
	0	0.01	0.1	1
Clinical signs and body weight	40	40	38	36
Incisor eruption and eye opening	40	40	38	36
Negative geotaxis and auditory startle response	20	20	20	18
Open-field test	20	20	20	18
Biel-type water T-maze test	20	20	18	18
Preputial separation	40	40	38	36
Hormonal analysis	19	19	20	17
Organ weights	38	35	38	36
Histopathology	40	40	38	36

Table 14. Mean body weight before weaning

Doses	Postnatal days					
(mg/kg/day)	0	1	5	7	14	21
VC	6.3 ± 0.7	6.8 ± 0.9	11.0 ± 1.0	14.2 ± 1.3	33.9 ± 1.7	55.4 ± 3.3
0.01	6.4 ± 0.4	6.9 ± 0.6	11.4 ± 1.2	14.9 ± 1.3	35.1 ± 2.8	56.4 ± 4.0
0.1	6.7 ± 0.6	7.4 ± 0.9	12.4 ± 1.8	16.0 ± 2.6	35.8 ± 2.4	57.9 ± 3.4
1	6.3 ± 0.6	6.9 ± 0.8	11.5 ± 1.4	14.9 ± 1.7	34.2 ± 2.4	55.5 ± 4.2

Mean±SD (g).

Table 15. Mean body weight after weaning

Doses	Postnatal days						
(mg/kg/day)	21	28	35	42	49	56	61
VC	55.4 ± 3.3	96.7 ± 8.0	159.9 ± 12.9	229.7 ± 16.7	293.2 ± 20.0	352.9 ± 25.1	390.7 ± 28.7
0.01	56.4 ± 4.0	98.3 ± 8.7	160.3 ± 14.9	226.2 ± 20.3	289.0 ± 24.5	349.3 ± 28.0	386.9 ± 32.6
0.1	57.9 ± 3.4	100.0 ± 6.8	162.3 ± 12.0	229.6 ± 16.6	294.4 ± 20.0	354.2 ± 24.6	392.3 ± 29.3
1	55.5 ± 4.2	97.4 ± 8.2	158.5 ± 14.1	224.7 ± 18.8	288.8 ± 24.9	347.9 ± 28.7	384.9 ± 32.7

Mean±SD (g).

Table 16. Summary results of Biel-type water T-maze test

		Doses (mg/kg/day)			
	Trial day	0	0.01	0.1	1
Straight course					
Swimming ability	1st	NAD	NAD	NAD	NAD
Swimming time (sec.)	1st	10.1	9.9	9.4	11.9
Maze course					
Swimming time (sec.)	2nd	33.8	32.9	36.2	41.1
	3rd	21.1	19.2	21.1	22.3
	4th	17.3	15.7	17.2	17.8
Number of selecting errors	2nd	3.3	2.8	3.4	3.5
	3rd	1.4	0.8	0.8	1.1
	4th	0.8	0.4	0.4	0.4

NAD, no abnormalities detected.

Swimming time indicate the harmonic mean.

Number of selecting errors indicate the median.

Table 17. Thyroid and brain weights

	Doses (mg/kg/day)			
	0	0.01	0.1	1
Organ weights				
Thyroids				
Absolute (mg)	18.5±2.92	18.8±3.27	19.5±2.80	22.7±4.57**
Relative (mg/100g)	4.74±0.77	4.87±0.85	4.99±0.70	5.91±1.22**
Brain				
Absolute (mg)	2014.0±83.2	2018.1±70.9	2052.9±63.4*	2060.9±64.6*
Relative (mg/100g)	517.5±34.3	523.3±35.6	525.8±37.0	538.5±39.7*
Final body weights (g)	390.7±28.7	387.7±32.7	392.3±29.3	384.9±32.7

Mean±SD.

*, ** Significantly different from control at $p<0.05$ and $p<0.01$, respectively.

Table 18. Serum thyroid-stimulating hormone (TSH), thyroxin (T4) or triiodothyronine (T3)concentration

	Doses (mg/kg/day)			
Items	0	0.01	0.1	1
TSH (ng/ml)	11.2±3.3	11.3±2.1	8.96±2.5*	9.48±2.26
T4 (μg/dl)	5.75±0.68	6.65±0.99**	6.31±0.81	6.47±0.86**
T3 (ng/dl)	50±10	57±30	50±10	50±10

mean±SD

*, ** Significantly different from control at $p<0.05$ and $p<0.01$, respectively.

Table 19. Histopathological findings

	Doses (mg/kg/day)			
Organ findings	0	0.01	0.1	1
No abnormalities detected	40	40	38	20
Thyroid				
Increased follicles filled with colloid	3	5	4	16

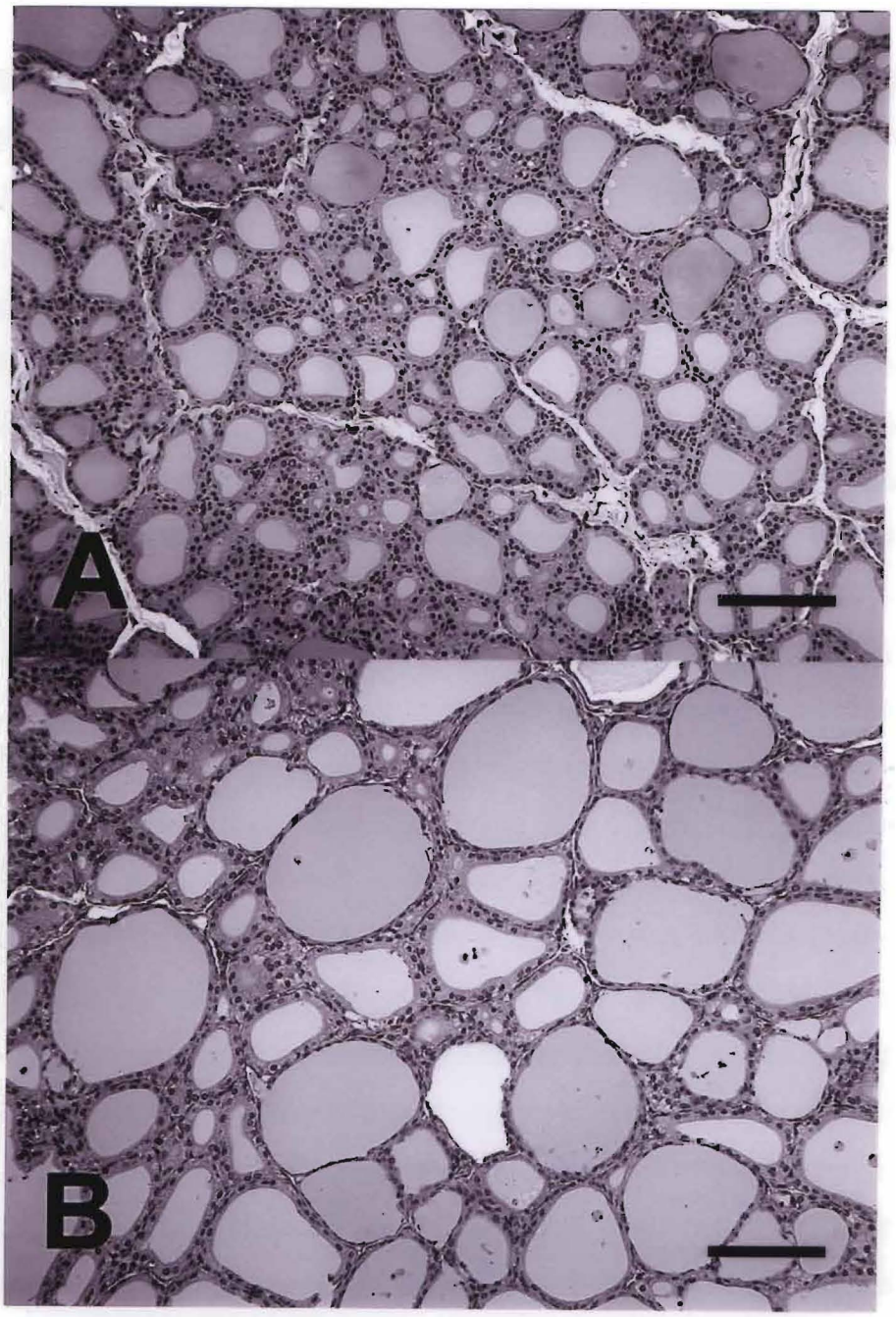

Fig. 12. Histopathological changes in the thyroid glands of vehicle control group (A) and 1 mg/kg group (B). In the 1 mg/kg/day group, an increased number of follicles filled with colloid was observed in the thyroid gland. These follicles, containing abundant colloid, were lined by flattened epithelium. H&E staining. Bar = 100 μm.

GENERAL CONCLUSIONS

Currently, an OECD Task Force on Endocrine Disruptor Testing and Assessment (EDTA) has been established to provide a focal point within the OECD to identify and recommend priorities for the development and validation of screening methods for endocrine active chemicals. As a result, the development of several assay protocols is being advanced with international cooperation, focusing on detection of (anti-) estrogenic and (anti-) androgenic chemical activities. The EDTA has recommended that the Hershberger assay, uterotrophic assay and enhanced TG-407 be used to start work on international validation (Gray *et al.*, 2002). We are participating in these international validation studies and, furthermore, have collected various basic biological data for development of the screening and testing methods (Takeyoshi *et al.*, 2001; Noda *et al.*, 2002; Sawaki *et al.*, 2003; Yamasaki *et al.*, 2000, 2001a, 2001b, 2001c, 2002a, 2002b, 2002c; 2002d; 2002e). As part of this international effort, we first determined age-related changes in vaginal opening, body weight, the weights of the uterus and ovary with gross pathological findings, and serum 17β-estradiol (E2) and progesterone levels in intact non-treated SD IGS female rats, a newly established strain bred by Charles River, Inc., during the pre-pubertal period. Beginning with an elevation in the serum E2 level from about 28 days of age, all the parameters, except body weight, started to undergo drastic changes until 31 days of age. The highest incidence of vaginal opening was recorded at 34 days of age (Noda *et al.*, 2002). Endogenous estrogen-free immature female rat must be used in the uterotrophic assay, which is recommended by the OECD, because the endpoint of the uterotrophic assay is the uterotrophic action of the injected test chemical. Therefore, an accurate evaluation of the test results is difficult in the presence of endogenous estrogen. The present results clarified that the endogenous estrogen-free period, i.e., sensitive window of uterus, is up to the 28 days of age, although some variation is to be expected. Thus, when a uterotrophic assay is utilized to screen for the (anti-) estrogenic activity of

chemicals, the test substance should be injected before pubertal onset, which begins around the 28 days of age. Moreover, the present results are also variable to the evaluation of assays employing peripubertal female rats, including the enhanced TG-407, female pubertal assays, in *utero* - lactation exposure, multi-generation studies, because the influence of the test chemicals on sexual differentiation is one of the endpoints used in these assays (Noda *et al.*, 2002).

Thyroid hormone is known to regulate neuronal proliferation, migration, and differentiation in the brain during the fetal and neonatal periods (Bernal and Nunez, 1995). Thus, the detection of chemicals that may disrupt thyroid hormone, as well as estrogen and androgen, is a high - priority. Although several predictive assay protocol have been proposed, a standardized assay protocol for the detection of thyroid hormone modulators has not been established. We evaluated the ability of the Hershberger assay protocol to detect thyroid function modulators. The original objective of the Hershberger assay was to the screen for (anti-) androgenic chemical activities. Because AR is known to be present in the thyroid glands of mammals (Banu *et al.*, 2002; Pellietier, 2000), the thyroid gland is thought to be one a target of androgenic compounds. If the Hershberger assay is capable of simultaneously detecting the (anti-) androgenic activities and the thyroid hormone - modulating activities of test chemicals, it could serve as a rapid and cost - effective screening method for detecting the hormonal activities of chemicals. Our results showed a significant increase in thyroid weights of both castrated and intact rats receiving a dose of 200 mg/kg/day of AT. The hypophyseal weight was unaltered by the administration of AT, but a comparison of vehicle-treated groups showed that the hypophyseal weight of the castrated rats was greater than that of the intact rats. Enlarged thyroid glands were observed in the AT-treated rats by necropsy. Histological examinations of the thyroid glands in all the AT-treated animals showed hypertrophy and hyperplasia of the follicular epithelial cells, and the height of the follicular epithelium of the thyroid glands increased in a dose-dependent manner in both of the castrated

and intact rats. These results suggest that the effect of AT can be detected by the Hershberger assay using a 10-day administration protocol and that this assay may be useful for screening for thyroid function modulators, regardless of whether the animals have been castrated. At the same time, the 10-day oral administration protocol in intact male rats may provide a new approach to screening for thyroid hormone modulators. In terms of limiting cruelty to animals, the development of screening methods that do not involve surgical invasion, such as castration, is very important. Thus, further study is needed, including the evaluation of other compounds with mechanisms of action that are different from those of AT, such as thyroid hormone metabolism enhancers and thyroid hormone antagonists (Noda *et al.*, 2005b).

The U.S. Environmental Protection Agency (EPA) has funded a two-generation reproductive and developmental study using PTU at doses of approximately 0.1, 0.5 and 1.2 mg/kg/day (Gio Batta Gori, personal communication). However, some concerns exist regarding the large number of animals and the long test duration that are required, in addition to concern regarding the test's cost performance. To develop an enforceable and high-performance, low-cost definitive test method, we performed in *utero*-lactation exposure and neonatal exposure were performed as preliminary trials to determine whether the endocrine effects of thyroid dysfunction could be detected by these assays, and to determine whether thyroid function modulators could be screened for using METI's tiered system.

Several epidemiological studies have linked attention deficit hyperactivity disorder or lower IQ levels in children with higher dietary intakes of contaminated fish and background exposure to polychlorinated biphenyls, dioxins, and/or co-contaminants, which are assumed to be thyroid modulators (Jacobson and Jacobson, 1996; Kooistra *et al.*, 2001; Rovet and Hepworth, 2001). Thus, we evaluated the use of in *utero*-lactation exposure and neonatal exposure protocol for the detection of adverse effects on learning ability and emotional function. An in *utero*-lactation exposure revealed hearing disturbances in offspring of both

sexes that received 0.4 mg/kg/day of PTU. In addition, a Biel-type water T-maze test showed an increase in the number of errors made by females in the 0.4 mg/kg/day group (Noda *et al.*, 2005a). These results indicated that the *in utero*-lactation exposure may be a useful method for detecting chemicals with thyroid hormone antagonist activities. However, contrary to our expectations, no significant differences in the learning ability and emotional function test outcomes were observed in the neonatal exposure (Noda *et al.*, 2005c, under review). The reasons for the absence of a significant change in the neonatal exposure are unclear. However, the short term of PTU treatment (only 5 days) may be partially responsible. On the other hand, many endocrine disrupters are thought to enter the body through an oral route, like the ingestion of contaminated maternal milk, food and drinks. Thus, the present data regarding the neonatal oral administration of a thyroid antagonist might have been meaningful, since the amount and duration of treatment were precisely controlled, compared to situations in which PTU was added to the drinking water or food given to the mothers. However, to fully evaluate the effects of neonatal oral administration, further experiments, such as the measurement of T3, T4 and TSH levels immediately after the termination of PTU administration or longer periods of PTU treatment, are necessary.

The histopathological transgeneration effects of PTU on the thyroids of rats are poorly understood, even though the thyroid is a target organ of PTU. In an in *utero*-lactation exposure, a flattening of the follicular epithelium, a decrease in resorptive colloid droplets, the degeneration of the follicular epithelium, and hyperplasia of the follicular epithelium were observed in males belonging to the 0.4 mg/kg/day group (Noda *et al.*, 2005a). In the dams, hypertrophy of the follicular epithelium of the thyroid was observed in the 0.4 mg/kg/day group (under review). Although the mechanism responsible for the differences in the effects seen in the offspring and the dams could not be explained, the effect of PTU administered *in utero* - lactation exposure was apparently different from that exposed in mature female rats. Interestingly, similar thyroid findings can be

observed in a neonatal exposure (Noda *et al.*, 2005c, under review). Possible explanations for the thyroid findings seen in *in utero*- lactation exposure and neonatal exposure are mentioned as follows. Kawaoi and Tsuneda reported that PTU administration in pregnant rats beginning from GD 8 resulted in hyperplasia with an unclear follicular structure and degenerative changes in the epithelium of the fetal thyroid glands (Kawaoi and Tsuneda, 1986). Although only the fetal thyroid was examined in their experiments, their thyroid findings are similar to the present thyroid findings. Kawaoi and Tsuneda concluded that the thyroid findings in fetuses were caused by the maternal administration of PTU and an increased secretion of TSH from the fetal hypophysis (Kawaoi and Tsuneda, 1986). Newborn rats treated with TSH showed similar findings, i.e. the development of more exocytotic vesicles and an enlarged endoplasmic reticulum filled with products in the thyroid gland up until the first postnatal week (Gruszczynska *et al.,* 1993). The presence of follicles filled with colloid in the thyroid glands of the offspring in the present study was thought to originate from an increase in TSH secretion from the hypophysis during the neonatal period; this effect of PTU is irreversible. Moreover, neonatal hyperthyroidism has been reported to result in a permanent decrease in pituitary reserves of TSH (Varma and Crawford, 1979). The lowered TSH status in our examination was assumed to originate from a permanent decrease in the pituitary reserve of TSH. On the other hand, the hormonal imprinting is known to take place during the perinatal period, when the appropriate hormones and developing hormone receptors come in contact for the first time (Casba, 1980, 1994). The encounter of hormones and their receptors results in the complete maturation of the receptor–hormone complex, and, consequently, the formation of receptor binding capacity and cell response characteristics during adulthood (Casba, 1985, 1991). The contradiction between the T3 and T4 statuses and that of TSH in the present results may indicate the injury of the receptor hormone connection as a result of the absence of imprinting during the neonatal period. The above results suggest that histopatholgical examination of the thyroid may be a reliable

endpoint for detecting thyroid hormone modulating activity *in vivo*. The present results are likely to become a benchmark for the development of definitive study methods for the detection of thyroid modulators, although numerous problems remain to be overcome.

In conclusion, we obtained essential data on genital tract development in female IGS rats that will be useful not only for *in vivo* screening assays, but also for definitive studies. These findings will contribute to the detection of potential endocrine-active chemicals. Moreover, the present results suggest that the Hershberger assay, using a 10-day administration protocol, may be useful for screening for thyroid function modulators, and in *utero*-lactation exposure may be a reliable protocol for use as a definitive study.

The tiered chemical screening and testing program proposed by METI for the detection of the endocrine-disrupting chemical activities appears to be reliable for the detection of not only the ER- mediated and AR-mediated effects, but for thyroid modulating chemical activities.

SUMMARY

The Ministry of Economy, Trade and Industry (METI) in Japan has developed a tiered chemical screening and testing program for evaluating the endocrine-disrupting effects of chemicals, focusing on effects mediated by estrogen receptors (ER) and androgen receptors (AR). In this program, chemicals are prioritized on the basis of their ER and AR binding affinities in an *in silico* system, and high-priority chemicals are then evaluated for hormonal activity in the program's early screening stage. Chemicals that test positive during the screening stage are then definitively tested using an in *vivo* study. METI has also proposed three short-term *in vivo* rodent assays for use in the screening stage to detect AR-mediated and ER-mediated endocrine disruption: the Hershberger assay in surgically castrated male rats, the uterotrophic assay in immature female rats, and the "enhanced OECD test guideline no. 407 (enhanced TG407)". The efficacy of the uterotrophic assay, Hershberger assay and enhanced TG-407 protocols is now being investigated in an international effort by many laboratories using various chemicals at the OECD's initiation. An understanding of the basic biological profile of changes in the female genital system during sexual maturation in the rat is of importance not only for analyzing the results of analysis, but also for standardizing of the assay protocol. In this study, background data on the sexual maturation of female SD IGS rats was collected as part of the international cooperation to develop a standardized assay protocol.

In vivo screening methods for detection of thyroid function modulators are now under development in many research laboratories. We assessed the applicability of the Hershberger assay protocol to screen for thyroid function modulators. Castrated male BrlHan WIST@Jcl (GALAS) rats were administered a potent thyroid peroxidase inhibitor, 3-amino-1,2,4-triazole (AT), in doses of 0, 40, and 200 mg/kg/day by gavage to 8 week old for 10 consecutive days. The results showed a significant increase in thyroid weight in the 200 mg/kg groups of both

castrated and intact rats. Hypophyseal weight was unaltered by AT, but comparison of greater the vehicle-treated groups showed that the hypophyseal weight of the castrated rats was greater than that of the intact rats. Enlarged thyroid glands were observed in the AT-treated rats at necropsy. Histological examination of the thyroid glands of all AT-treated animals showed hypertrophy and hyperplasia of the follicular epithelial cells, and the height of follicular epithelium of the thyroid glands increased in a dose-dependent manner in both of the castrated and intact rats. These results suggest that the effect of AT can be detected by the Hershberger assay 10-day administration protocol and may be useful for screening for thyroid function modulators regardless of whether the animals have been castrated. Moreover, we investigated in *utero*-lactation exposure and neonatal exposure methods as part of preliminary trials to determine whether the endocrine effects of thyroid dysfunction could be detected using these methods and whether the detection of thyroid function modulators could be included in the proposed tiered system. An in *utero*-lactation exposure revealed hearing disturbances in offspring of both sexes that received 0.4 mg/kg/day of PTU. In addition, a Biel-type water T-maze test showed an increase in the number of errors made by females in the 0.4 mg/kg/day group. These results indicated that the *in utero*-lactation exposure may be a useful method for detecting chemicals with thyroid hormone antagonist activities. However, contrary to our expectations, no significant differences in the learning ability and emotional function test outcomes were observed in the neonatal exposure. In an in *utero*-lactation exposure, a flattening of the follicular epithelium, a decrease in resorptive colloid droplets, the degeneration of the follicular epithelium, and hyperplasia of the follicular epithelium were observed in males belonging to the 0.4 mg/kg/day group. In the dams, hypertrophy of the follicular epithelium of the thyroid was observed in the 0.4 mg/kg/day group. Although the mechanism responsible for the differences in the effects seen in the offspring and the dams could not be explained, the effect of PTU administered *in utero*- lactation exposure was apparently different from that exposed in mature

female rats. Interestingly, similar thyroid findings can be observed in a neonatal exposure. The above results suggest that histopatholgical examination of the thyroid may be a reliable endpoint for detecting thyroid hormone modulating activity *in vivo*. The present results are likely to become a benchmark for the development of definitive study methods for the detection of thyroid modulators, although numerous problems remain to be overcome.

In conclusion, we obtained essential data on genital tract development in female IGS rats that will be useful not only for *in vivo* screening assays, but also for definitive studies. These findings will contribute to the detection of potential endocrine-active chemicals. Moreover, the present results suggest that the Hershberger assay, using a 10-day administration protocol, may be useful for screening for thyroid function modulators, and in *utero*-lactation exposure may be a reliable protocol for use as a definitive study. The tiered chemical screening and testing program proposed by METI for the detection of the endocrine-disrupting chemical activities appears to be reliable for the detection of not only the ER- mediated and AR-mediated effects, but for thyroid modulating chemical activities.

ACKNOWLEGEMENTS

The author's grateful thanks are due to: Professor Susumu Tateyama, and Dr. Ryoji Yamaguchi, Department of Veterinary Pathology, Facluty of Agriculture, Miyazaki University, for helpful discussions and suggestions, and critical review of this manuscript, as well as valuable support in the course of the investigation; Professor Akinori Shimada, Facluty of Agriculture, Tottori University, for helpful discussions and suggestions, and critical review of this manuscript.

The author would like to thank Takako Muroi, Saori Takakura, Hideo Mitoma, Satoko Sakamoto, Yuko Kuwano, Keiji Shiraishi, and Masakuni Sawaki, for their technical assistance; Kanji Yamasaki, Masahiro Takeyoshi, Takaaki Umano, Atsushi Minobe and Mineo Takatsuki, for their management of this project; and also the members of Chemicals Evaluation and Research Institute, Japan., for their valuable contributions.

This study was supported by a Grant-in-Aid from the Ministry of Economy, Trade and Industry, Japan.

REFERENCES

Akaike, M., Kato, N., Ohno, H., and Kobayashi, T. (1991). Hyperactivity and spatial maze learning impairment of adult rats with temporary neonatal hypothyroidism. Neurotoxicol Teratol. 13, 317-322.

Andrews, W.W., and Ojeda, S.R. (1977). On the Feedback Actions of Estrogen on Gonadotropin and Prolactin Release in Infantile Female Rats. Endocrinology 101, 1517-1523.

Banu, S.K., Govindarajulu, P., and Aruldhas, M.M. (2002). Testosterone and estradiol up-regulate androgen and estrogen receptors in immature and adult rat thyroid glands *in vivo*. Steroids. 67, 1007-1014.

Barter, R.A., and Klaassen, C.D. (1994). Reduction of thyroid hormone levels and alteration of thyroid function by four representative UDP-glucuronosyltransferase inducers in rats. Toxicol. Appl. Pharmacol. 128, 9-17.

Bernal, J., and Nunez, J. Thyroid hormones and brain development. (1995). Eur J Endocrinol. 133, 390-398.

Branham, W.S., Zehn, D.R., Chen, J.J., and Sheehan, D.M. (1988). Uterine abnormalities in rats exposed neonatally to diethylbestrol, ethynylestradiol, or clomiphene citrate. Toxicolog.y 51, 201-212.

Branham, W.S., Zehr, D.R. and Sheehan, D.M. (1993). Differential Sensitivity of Rat Uterine Growth and Epithelium Hypertrophy to Estrogens and Antiestrogens. P. S. E. B. M. 203, 297-303.

Capen, C.C. (1997). Mechanistic data and risk assessment of selected toxic endpoints of the thyroid gland. Toxicol. Pathol. 25, 39-48.

Casba, G. (1980). Phylogeny and ontogeny of hormone receptors: the selection theory of receptor formation and hormonal imprinting. Biological Reviews. 55, 47-63.

Casba, G. (1985). Receptor ontogeny and hormonal imprinting. Experientia. 42, 750-759.

Casba, G. (1991). Interaction between the genetic programme and environmental influencesin the perinatal critical period. Zoological Sciences. 8, 813-825.

Casba, G. (1994). Phylogeny and ontogeny of chemical signaling: Origin and development of hormone receptors. International Review of Cytology. 155, 1-48.

Chiao, YC., Cho, WL., and Wang, PS. (2002). Inhibition of testosterone production by propylthiouracil in rat Leydig cells. Biol Reprod. 67, 416-422.

Ching, M. (1981). Effect of barbital on the pituitary-thyroid axis. J. Endocrinol. Invest. 4, 389-392.

Colborn, T. and Clement, C (Eds). (1992). Chemically-Induced Alterations in Sexual and Functional Development. Princeton Scientific Publishing.

Daston,G. and Kimmel, C. (1998). An Evaluation and Interpretation of Reproductive Endpoints for Human Health Risk Assessment. International life sciences institute health and environmental sciences institute developmental

and reproductive toxicology committee.

DeVito, M., Crofton, K., and McMaster, S. (1997). Workshop report: Screening methods for chemicals that alter thyroid hormone action, function and homeostasis. Durham, NC, June 23-25, 1997. United States Environmental Protection Agency. EPA/600/R-98/057.

DeVito, M., Biegel, L., Brouwer, A., Brown, S., Brucker-Davis, F., Cheek, A.O., Christensen, R., Colborn, T., Cooke, P., Crissman, J., Crofton, K., Doerge, D., Gray, E., Hauser, P., Hurley, P., Kohn, M., Lazar, J., McmMaster, S., McClain, M., McConnell, E., Meier, C., Miller, R., Tietge, J., and Tyl, R. (1999). Screening methods for thyroid hormone disruptors. *Environ. Health Perspect.* 107, 407-415.

Ealey, P.A., Henderson, B., Loveridge, N. (1984). A quantitative study of peroxidase activity in unfixed tissue sections of the guinea-pig thyroid gland. Histochem. J. 16 (2), 111-122

EDSTAC. (1998). Endocrine Disruptor Screening and Testing Committee (EDSTAC). Final report.

Emanuele, N.V., Jurgens. J., La Paglia, N., Williams. D.W., Kelley, M.R. (1996). The effect of castration on steady state levels of luteinizing hormone-releasing hormone (LHRH) mRNA and proLHRH processing: time course study utilizing semi-quantitative reverse transcription/polymerase chain reaction. J Endocrinol. 148, (3), 509-15.

Gardner, LI. (1975). Historical notes on cretinism. In: Gardner LI, ed. Endocrine and genetic diseases of childhood and adolescence. 2nd ed. Philadelphia: W.B. Saunders. 234-238.

Goldey, ES., and Crofton, KM. (1998). Thyroxine replacement attenuates hypothyroxinemia, hearing loss, and motor deficits following developmental exposure to Aroclor 1254 in rats. Toxicol Sci. 45, 94-105.

Goldey, ES., Kehn, LS., Lau, C., Rehnberg, GL., and Crofton, KM. (1995). Developmental exposure to polychlorinated biphenyls (Aroclor 1254) reduces circulating thyroid hormone concentrations and causes hearing deficits in rats. Toxicol Appl Pharmacol 135, 77-88.

Goldey, ES., Kehn, LS., Rehnberg,, GL., and Crofton, KM. (1995). Effects of developmental hypothyroidism on auditory and motor function in the rat. Toxicol Appl Pharmacol. 135, 67-76.

Goldman, J.M., Laws, S.C., Balchak, S.K., Cooper, R.L. and Kavlock, R.J. (2000). Endocrine-Disrupting Chemicals: Pubertal Exposures and Effects on Sexual Maturation and Thyroid Activity in the Female Rat. A Focus on the EDSTAC Recommendations. Critical Reviews in Toxicology, 30, 135-196.

Grey, L.E., Jr. (1998). Tiered Screening and Testing Strategy for Xenoestrogens and Antiandrogens. Toxicol. Lett. 102-103, 677-680.

Gray, L.E., Ostby, J., Wilson, V., Lambright, C., Bobseine, K., Hartig, P., Hotchkiss, A., Wolf, C., Furr, J., Price, M., Parks, L., Cooper, R.L., Stoker, T.E., Laws, S.C., Degitz, S.J., Jensen, K.M., Kahl, M.D., Korte, J.J., Makynen, E.A., Tietge, J.E., Ankley, G.T. (2002). Xenoendocrine disrupters-tiered screening and testing Filling key data gaps. Toxicology. 181-182, 371-82.

Gruszczynska, M., Thang, T.C., and Csaba, G. (1993). Response to thyroid stimulating hormone in 1-week-old rat thyroid gland after pretreatment with

the same hormone in newborn conditions (hormonal imprinting). Cytobios. 74, 298-299.

Hardy, M.P., Sharma, R.S., Arambepola, N.K., Sottas, C.M., Russell, L.D., Bunick, D., Hess, R.A., Cooke, P.S., 1996. Increased proliferation of Leydig cells induced by neonatal hypothyroidism in the rat. J. Androl. 17, 231-238.

Hershberger, L.G., Shipley, E.G., eyer, R.K. (1953). Mytrophic activity of 19-nortestosterone and other steroids determined by modified levator ani muscle method. Proc. Soc. Exp. Biol. Med. 83, 175-180.

Holmes, P., Humfrey, C. and Scullion, M. (1998). Non-Regulatory Test Models Proposed for Further Development/adoption. In Appraisal of Test Methods for Sex-Hormone Disrupting Chemicals Capable of Affecting the Reproductive Process. MRC Institute for Enviroment and Health.

Iguchi, T., Todoroki, R., Yamaguchi, S., and Takasugi, N., (1989). Changes in the uterus and vagina of mice treated neonatally with antiandrogen. Acta Anat. 136, 146-154.

Jacobson, JL., and Jacobson, SW. (1996). Intellectual impairment in children exposed to polychlorinated biphenyls in utero. N Engl J Med. 335, 783-9.

Kawaoi, A., and Tsuneda, M. (1986). Effects of thyroidectomy and administration of propylthiouracil (PTU) or thyrotropin (TSH) to pregnant rats on the functional development of the fetal thyroid gland. An immunihistochemical study. Endocrinol Japon. 33(6), 835-841.

Kirby, J.D., Arambepola, N., Porkka-Heiskanen, T., Kirby, Y.K., Rhoads, M.L., Nitta, H., Jetton, A.E., Iwamoto, G., Jackson, G.L., Turek, F.W., and Cooke,

P.S. (1997). Neonatal hypothyroidism permanently alters follicle-stimulating hormone and luteinizing hormone production in the male rat. Endocrinology 138, 2713-2721.

Kelce, W.R. and Wilson, E.M. (1997). Enmironmental Antiandrogens: Developmental Effects, Molecular Mechanisms, and Clinical Implications. J. Mol. Med. 75, 198-207.

Kirby, J.D., Arambepola, N., Porkka-Heiskanen, T., Kirby, Y.K., Rhoads, M.L., Nitta, H., Jetton, A.E., Iwamoto, G., Jacson, G.L., Turek, F.W., and Cooke, P.S. (1997). Neonatal hypothyroidism permanently alters follicle-stimulating hormone and luteinizing hormone production in the male rat. Endocrinology. 138, 2713-2712.

Kohrle, J. (1990). Thyrotropin (TSH) action on thyroid hormone deiodination and secretion: One aspect of thyrotropin regulation of thyroid cell biology. Horm. Metab. Res. Suppl. 23, 18-28.

Kooistra, L., Stemerdink, N., van der Meere, J., Vulsma, T., and Kalverboer, AF. (2001). Behavioural correlates of early-treated congenital hypothyroidism. Acta Paediatr. 90, 1141-1146.

Krauss, R.S., and Eling, T.E. (1987). Macromolecular binding of the thyroid carcinogen 3-amino-1,2,4-triazole (amitrole) catalyzed by prostaglandin H synthase, lactoperoxidase and thyroid peroxidase. Carcinogenesis 8 (5), 659-664

Marty, M.S., Crissman, J.W., and Carney, E.W. (1999). Evaluation of the EDSTAC female pubertal assay in CD rats using 17β-estradiol, steroid biosynthesis inhibitors, and a thyroid inhibitor. Toxicol. Sci. 52, 269-277.

Marty, M.S., Crissman, J.W., and Carney, E.W. (2001). Evaluation of the male pubertal assay's ability to detect thyroid inhibitors and dopaminergic agents. Toxicol. Sci. 60, 63-76.

Masuda, H., and Goto, N. (1994). Comparative study of goitrogenic actions of 3-substituted 1, 2, 4-triazoles in rats. J. Vet. Med. Sci. 56(2), 341-346.

Mattioli, F., Robbiano, L., Fazzuoli, L. and Baracchini, P. (1994). Studies on the mechanism of the carcinogenic activity of amitrole. Fundam Appl Toxicol. Jul;23(1):101-6.

McClain, R.M., Levin, A.A., Posch, R., and Downing, J.C. (1989). The effect of Phenobarbital on the metabolism and excretion of thyroxine in rats. Toxicol. Appl. Pharmacol. 99, 216-228.

McLachlan, J.A. (1993). Functional toxicology: a new approach to detect functionally active xenobiotics. Environ. Health Prespect. 101, 386-387.

McLachlan, J. A. and Korach K. S. (1995). Estrogens in the Environment III: Global Health Implications. Environ. Health Prespect. 103 (Suppl. 7), 3-178.

Medlock, K.L., Sheehan, D.M., Nelson, C.J., and Branham, W.S. (1988). Effects of postnatal DES treatment on uterine growth, development, and estrogen receptor levels. J. Steroid Biochem. 29, 527-532.

Meijs-Roelofs, H.M. and Kramer, P. (1979). Maturation of the Inhibitory Feedback Action of Oestrogen on Follicle-Stimulating Hormone Secretion in the Immature Female Rat: a Role for Alpha-Fetoprotein. J. Endocrinol. 81, 199-208.

Meijs-Roelofs, H.M., Uilenbroek, J.Th.J, Osman, P and Welschen, R. (1972). Serum Levels of Gonadotropins and Follicular Growth in Prepuberal Rats. Amsterdam, Excerpta Medica. 267, 3-11.

Moura, E, G., Pazos-Moura, C, C., Dorris, M, L. and Taurog, A. (1990). Lack of effect of propylthiouracil and methylmercaptoimidazole on thyroglobulin biosynthesis. Proc Soc Exp Biol Med.194(1):48-53.

Noda, S., Sawaki, M., Shiraishi, K., Yamasaki, K., and Yamaguchi, R. (2002). Age-related changes of genital systems in the female Crj:SD®(SD)IGS rats during sexual maturation. J Vet Med Sci. 64, 315-319.

Noda, S., Muroi, T., Takakura, S., Sakamoto, S., Takatsuki, M., Yamasaki, K., Tateyama, S., and Yamaguchi, R. (2005a). Preliminary evaluation of *In utero*-lactational Assay using 6-n-propyl-2-thiouracil. Arch Toxicol. (in press; published online, 31, March).

Noda, S., Muroi, T., Mitoma, H., Takakura, S., Sakamoto, S., Takatsuki, M., Yamasaki, K., Tateyama, S., and Yamaguchi, R. (2005b). Ability of the Hershberger assay protocol to detect thyroid function modulators. Arch Toxicol. (in press).

Noda, S., Muroi, T., Tateyama, S., Yamaguchi, R and Yamasaki, K. (2005c). Effect of neonatal oral gavage of 6-n-propyl-2-thiouracil. Arch Toxicol. (under review).

O'Connor, J.C., Frame, S.R., Davis, L.G. and Cook, J.C. (1999). Detection of the Environmental Antiandrogen p,p'-DDE in CD and Long-Evans Rats Using a Tier 1 Screening Battery and a Hershberger Assay. Toxicol. Sci. 51, 44-53.

OECD. (1997). Draft detailed review paper: Appraisal of test methods for sex-hormone disrupting chemicals. Environmental Health and Safety Publications, Paris.

OECD, (1999). EMSG OECD draft proposal for testing of adequacy of an enhanced OECD protocol, Repeated dose (28 days) toxicity (oral) study, based on OECD 407, April 19-20.

OECD, 2001. Third meeting of the validation management group for the screening and testing of endocrine disrupters (mammalian effects), March 26-27.

OECD, 2002. Final report of the work towards the validation of the rat Hershberger assay: phase-1, androgenic response to testosterone propionate, and anti-androgenic effects of flutamide. ENV/JM/TG/EDTA(2002)1/REV2. Paris.

OECD, 2003. Fourth meeting of the validation management group for the screening and testing of endocrine disupters (mammalian effects), April 14-15.

Pelletier, G. (2000). Localization of androgen and estrogen receptors in rat and primate tissues. Histol. Histopathol. 15, 1261-1270.

Reader, S.C., Carroll, B., Robertson, W.R., and Lambert, A. (1987). Assessment of the biopotency of anti-thyroid drugs using porcine thyroid cells. 36 (11), 1825-1828.

Roegge, CS., Seo, BW., Crofton, KM., and Schantz, SL. (2000).

Gestational-lactational exposure to Aroclor 1254 impairs radial-arm maze performance in male rats. Toxicol Sci. 57, 121-130.

Ross, D.S. (1990). Testosterone increases TSH-beta mRNA, and modulates alpha-subunit mRNA differentially in mouse throtrophic tumor and castrate rat pituitary. Horm. Metab. Res. 22, 163-169.

Rovet, JF., and Hepworth, SL. (2001). Dissociating attention deficits in children with ADHD and congenital hypothyroidism using multiple CPTs. J Child Physiol Psychiatry. 42, 1049-1056.

Santini, F., Vitti, P., Ceccarini, G., Mammoli, C., Rosellini, V., Pelosini, C., Marsili, A., Tonacchera, M., Agretti, P., Santoni, T., Chiovato, L., Pinchera, A. (2003). In vitro assay of thyroid disruptors affecting TSH-stimulated adenylate cyclase activity. J. Endocrinol. Invest. 26 (10), 950-955

Sawaki, M., Noda, N., Muroi, T., Mitoma, H., Takakura, S., Sakamoto, S., and Yamasaki, K. (2003). Evaluation of an in utero through lactational exposure protocol for detection of estrogenic effects of ethinyl estradiol on the offspring of rats: preliminary trial. Reprod Toxicol 17, 335-343.

Seo, BW., Sparks, AJ., Medora, K., Amin, S., and Schantz, SL. (1999). Learning and memory in rats gestationally and lactationally exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Neurotoxicol Teratol. 21, 231-239.

Shiroozu, A., Taurog, A., Engler, H. and Dorris, M, L. (1983). Mechanism of action of thioureylene antithyroid drugs in the rat: possible inactivation of thyroid peroxidase by propylthiouracil. Endocrinology. Jul;113(1):362-70.

Siril Ariyaratne, HB., and Ian Mason, J, (2000). Mendis-Handagama SM. Effects of thyroid and luteinizing hormones on the onset of precursor cell differentiation into leydig progenitor cells in the prepubertal rat testis. Biol Reprod. 63, 898-904.

Stoker, T.E., Parks, L.G., Gray, L.E., and Cooper, P.L. (2000). Endocrine-disrupting chemicals: prepubertal exposures and effects on sexual maturation and thyroid function in the male rat. a focus on the EDSTAC recommendations. Crit. Rev. Toxicol. 30, 197-252.

Taurog, A., Dorris, ML., Hu, W, X., and Guziec, F, S, Jr. (1995). The selenium analog of 6-propylthiouracil. Measurement of its inhibitory effect on type I iodothyronine deiodinase and of its antithyroid activity. Biochem Pharmacol. Mar 1;49(5):701-9.

Takeyoshi, M., Yamasaki, K., Sawaki, M., Nakai, M., Noda, S., and Takatsuki, M. (2001). The efficacy of endocrine disruptor screening tests in detecting anti-estrogenic effects downstream of receptor-ligand interactions. 126 (2), 91-98.

Tamasy, V., Meisami, E., Vallerga, A., and Timiras, PS. (1986). Rehabilitation from neonatal hypothyroidism: spontaneous motor activity, exploratory behavior, avoidance learning and responses of pituitary--thyroid axis to stress in male rats. Psychoneuroendocrinology. 11, 91-103.

Valle, L.B., Oliveria-Filho, R.M., Romaldini, J.H., and Lara, P.F., (1985). Pituitary-testicular axis abnormalities in immature male hypothyroid rats. J. Steroid Biochem. 23, 253-257.

Valenti, S., Sarkissian, A., Giusti, M., Giordano, G., Dahl, K.D. (1997).

Immunoreactive and bioactive LH release from pituitaries of intact or castrated male rats: effect of in vitro GnRH and KCl administration. J Endocrinol Invest. 20 (7), 381-386.

Varma, S.K., and Crawford, J.D. (1979). Long-term perspectives of thyroxine administration in neonatal rats. Horm Res. 10(6), 327-335.

Yamada, T., Kunimatsu, T., Miyata, K., Yabushita, S., Sukata, T., Kawamura, S., Seki, T., Okuno, Y., Mikami, N. (2003). Enhanced rat Hershberger assay appears reliable for detection of not only (anti-)androgenic chemicals but also thyroid hormone modulators. Toxicol Sci. 79, 64-74.

Yamasaki, K., Sawaki, M., Noda, S., Muroi, M., and Maekawa, A. (2000). Immature rat uterotrophic assay of diethylstilbestrol, ethynyl estradiol and atrazine. J Toxicol Pathol 13, 145-149.

Yamasaki, K., Sawaki, M., Noda, S., and Takatsuki, M. (2001a). Effects of age and weaning on the immature rat uterotrophic assay using ethylestradiol. Exp Anim 50, 87-89.

Yamasaki, K., Sawaki, M., Noda, S ,and Takatsuki, M.(2001b). Effects of olive, Corn, sesame or peanut oil on the body weights and reproductive organ weights of immature male and female rats. Exp Anim 50, 173-177.

Yamasaki, K., Sawaki, M., Noda, S., and Takatsuki, M. (2001c). Preputial separation and glans penis changes in normal growing Crj: CD (SD) IGS rats. Reprod Toxicol 15, 533-536.

Yamasaki, K., Sawaki, M., Noda, S., and Takatsuki, M. (2002a). Uterotrophic and Hershberge assays for n-butylbenzene in rats. Arch Toxicol. 75, 703-706.

Yamasaki, K., Sawaki, M., Noda, S., Wada, T., Hara, T., and Takatsuki, M. (2002b). Immature uterotrophic assay of estrogenic compounds in rats given different phytoestrogen content diets and the ovarian changes in the immature rat uterotrophic of estrogenic compounds with ICI 182,780 or antide. Arch Toxicol. 76, 613-620.

Yamasaki, K., Sawaki, M., Noda, S., Imatanaka, M., and Takatsuki, M. (2002c). Subacute oral toxicity study of ethynyl estradiol and bisphenol A based on the draft protocol for the "Enhanced OECD Test Guideline no. 407". Arch Toxicol. 76, 65-74.

Yamasaki, K., Takeyoshi, M,. Noda, S., and Takatsuki, M. (2002d). Changes of serum alpha2u-globulin in the subacute oral toxicity study of ethynyl estradiol and bisphenol A based on the draft protocol for the "Enhanced OECD Test Guideline No. 407". Toxicology. 176, 101-112.

Yamasaki, K., Tago, Y., Nagai, K., Sawaki, M., Noda, S. and Takatsuki, M. (2002e) Comparison of toxicity studies based on the draft protocol for the "Enhanced OECD Test Guideline no. 407" and the research protocol of "Pubertal Development and Thyroid Function in Immature Male Rats" with 6-n-propyl-2-thiouracil. Arch. Toxicol., 76, 495-501.

Xinghua, L., Rosemary,S., Nira B.J., Andrea C.G,, Peter C. M. Y., Kenneth, P. N. and Robert, M.B. (2000). Strain Differences in Vaginal Responses to the Xenoestrogen Bisphenol A. Environ. Health Prespect. 108, 243-247.