J. Toxicol. Pub. Health
Vol. 17, Supplement, pp. 313-317, 2001

Hormone-Mimic Chemicals and Their Possible Endocrine Disruption - Development of Testing Methods -

Kiyoshi Imai*

Food and Drug Safety Center, Hatano Research Institute 729-5 Ochiai, Hadano, Kanagawa, 257-8523, Japan

ABSTRACT *: The Ministry of Health and Welfare of Japan has set up six research groups concerning the endocrine disrupting chemicals. One of these projects was "A study on development of testing methodology for health effects due to exposure of environmental endocrine disruptors". In this paper, three topics are described. In OECD collaboration for pre-validation of uterotrophic assay, the most sensitive response to ethnyl estradiol was noted in the ovarectomized rats treated subcutaneously for 7 days. Secondly, it was suggested that changes of the serum α_{2u}-globulin level may be a sensitive parameter for detecting the estrogenic activities of chemicals. Finally, development of the sexually dimorphic nucleus of preoptic area in the brain of male rats was inhibited by the treatment with estrogenic chemicals, and their masculine behaviors and reproductive abilities were impaired after sexual maturation. In conclusion, these parameters are considered to be sensitive endpoints for testing estrogenic chemicals.*

Key Words *: Endocrine disruptors, OECD pre-validation test, Uterotrophic assay, Serum α_{2u}-globulin, SDN-POA*

I. INTRODUCTION AND BACKGROUND ASPECTS

The Ministry of Health and Welfare of Japan has focused on the chemicals with estrogenic activities since a decade ago and established a research project, "A Study on the Crisis Management of Chemicals", chaired by Dr. M. Setaka in 1996. In 1998, six research project groups concerning endocrine disrupting chemicals were also set up. One of these project was "A Study on Development of Testing Methodology for Health Effects due to Exposure of Environmental Endocrine Disruptors".

At that time, the interministrial liaison meeting among twelve ministries in Japan was also set up to develop and coordinate research activities on the endocrine disruptors. The Ministry of Health and Welfare has mainly focused on the human health effects, such as human exposure, action mechanisms, health effect surveillance, and hazard identification method. The main subjects of the other ministries are listed in the Table 1.

The research project, "A Study on Development of Testing Methodology for Health Effects due to Exposure of Environmental Endocrine Disruptors", consists of five subgroups focusing on *in vitro* screening methods, toxicokinetics and pharmacology of estrogenic substances, reproductive and developmental abnormalities including neuro-behaivioral changes and carcinogenicity, collaboration for OECD validation test for endocrine disruptor, and surveillance of literature for constructing data-base about endocrine disruptors. In this paper, three topics in this field are introduced.

*To whom correspondence should be addressed

II. INTERNATIONAL COLLABORATION FOR A OECD PRE-VALIDATION TEST

Recently, OECD proposed a testing strategy for endocrine disruptors. To see the effects *in vivo*, three test methods were adopted. Those are uterotrophic assay based on trophic reaction of the uterus to estrogenic activities, Hershberger test based on trophic action of para-testicular adnex tissue, and 28-day repeated dose toxicity test enhanced for special parameters to examine the endocrine functions (enhanced TG 407). These test methods have been evaluated by international collaboration. Under the interministrial liaison, six institutions in Japan participated in the

Table 1. Japanese interministry collaboration on endocrine disruptors

Ministry of Health & Welfare	Human effects
	human exposure/action mechanism
	human health effect surveillance
	hazard indentification method
Ministry of Environment	Wildlife & environment
	environmental exposure
	wildlife exposure assessment
	risk assessment of environment chemicals
	Developing reptotox study for pesticides
Ministry of Trade & Industry	Screening & testing
Ministry of Labor	Labor's effect
Ministry of Transportation	Marine protection
Ministry of Agriculture	Effects of agricultural products
	Effects on agro-eco system
	Action mechanism through pesticides
Ministry of Construction	River, sewage, biludings, etc
Ministry of Science & Technol.	Developing national-wide research
Ministry of education & Cult.	Promotion of basic research

pre-validation of the uterotrophic assay, 5 in Hershberger assay, and 5 in TG 407 enhanced.

In the OECD uterotrophic assay, four protocols are proposed. Protocol A and B were regulated to use mature female rats, and ethynyl estradiol was administered subcutaneously as a positive control chemical at dose levels of 0.01, 0.03, 0.1, 0.3, 1, 3, or 10 μg/kg for 3 or 7 days after ovarectomy. Protocol C and C' were conducted using immature female rats, and ethinyl estradiol was administered subcutaneously or orally for 3 days at the same dose levels in Protocol A and B (Table 2).

As a result, the most sensitive response to ethynyl estradiol was obtained in the test according to the Protocol C', and the response to ethynyl estradiol was reduced in order of the Protocol C, B and A (Table 3).

Concerning interlaboratory differences of the uterotrophic assay, there was principally no remarkable difference among the participating laboratories in the results of uterotrophic assay for ethynyl estradiol, except one or two laboratories. However, the actual key variable factors of these laboratories seemed to be the differences in vehicle for test substance administration, rodent diet, and housing conditions.

Table 2. OECD pre-validation test protocol for uterotrophic assay
Experimental protpcol
Animals: Sprague-dawley rats
Treatment groups:

	Animals	Treatment
Protocol A	immature rats	oral dosing x 3 days
Protocol B	immature rats	subcutaneous x 3 days
Protocol C	OVX mature rats	subcutaneous x 3 days
Protocol C'	OVX mature rats	subcutaneous x 7 days

Chemicals and dose levels: ethynyl estradiol, 0.01, 0.03, 0.1, 0.3, 1, 3, or 10 μg/kg.

Table 3. Results of OECD pre-validation test for uterotrophic assay in Food and Drug Safety Center, Japan

	ED_{50} (μg/kg/day)			
	Wet		Blotted	
	Absolute	Relative	Absolue	Relative
Protocol A	3.81	3.24	1.75	1.60
Protocol B	0.70	0.69	0.57	0.58
Protocol C	1.00	1.01	0.48	0.52
Protocol C'	0.57	0.67	0.31	0.35

III. EFFECTS OF ESTROGENIC CHEMICALS ON THE SERUM α_{2u}-GLOBULIN LEVELS

α_{2u}-globulin (AUG) is usually found in mature male rats and the decrease of serum AUG levels of adult rats are known to be marked by the administration of estrogenic chemicals (Biswas and Ghosh *et al.*, 1983) or by castration (Kulkarni and Gubits *et al.*, 1985). To evaluate possibilities for developing a new screening method of endocrine disruptors, Takeyoshi and Anai *et al.* (2000) had examined the changes in serum AUG levels after the administration of a strong estrogenic chemical, diethylstilbestrol (DES).

DES was administered by gavage to the Sprague-Dawley male rats at dose levels of 0.01, 0.1, or 1 μg/kg for 14 days. After treatment with the chemical,

Table 4. Serum AUG levels, hepatic AUG mRNA levels and histological findings of the testis in rats treated with diethlstilbesterol at doses of 1, 0.1, or 0.01 mg/kg for 14 days

Group	Serum AUG level (μg/ml)	Hepatic AUG mRNA level#	Pathological findings of the testis											
			Degeneration of pachytene spermatoctes				Inhibition of Spermiation				Retention of elongated spermatids			
			-	±	+	++	-	±	+	++	-	±	+	++
Control (n=5)	333.2±167.0[1]	5109±450	5	0	0	0	5	0	0	0	5	0	0	0
0.01 mg/kg (n=4)	186.5±65.4	5024±191	4	0	0	0	4	0	0	0	4	0	0	0
0.1 mg/kg (n=5)	34.0±12.5*	2608±1483*	1	2	2	0	4	1	0	0	4	1	0	0
1 mg/kg (n=5)	16.8±1.8*	333±201**	0	0	5	0	0	2	2	1	0	2	2	1

[1]Mean±SD, ±: slight, +: moderate, ++: marked.
#: Hepatic AUG mRNA levels were estimated by RT-PCR technique.
*: Signicicantly different from the control, $P<0.05$.
**: Significantly different from the control, $P<0.01$.

serum AUG levels and AUG messenger RNA contents in the liver were determined by the enzyme-immunoassay and the reverse transcription and polymerase chain reaction method (RT-PCR), respectively. In addition, pathological examination of the testis was also performed.

No significant decrease in testicular weight was detected in any of the DES treated groups, while the serum levels of AUG were decreased in dose-dependent manner. AUG messenger RNA levels in the liver were also decreased, and degeneration of pachytene spermatocytes and inhibition of spermiation were noted in 0.1 and 1 mg/kg treated groups (Table 4).

These results suggested that changes of the serum AUG levels in intact male rats may be a sensitive parameter for detecting the estrogenic activities of the chemicals.

IV. EFFECTS OF ESTROGENIC CHEMICALS ON THE SEXUALLY DIMORPHIC NUCLEUS OF THE PREOPIC AREA

After Takasugi (1970) had shown that neonatal treatment with estrogens caused a permanent impairment of male reproductive organs, functional and cellular changes of reproductive organs have been reported in neonatally estrogenized animals of both sexes (Takasugi, 1976; Ohta, 1977). For males, sexual behavior in rats has been shown to be closely associated with sexually dimorphic nucleus of the preoptic area (SDN-POA) in the hypothalamus of the brain (Dohler, 1991). The volume of SDN-POA of male rats is about 3 times larger than that of female rats (Gorski and Gordon *et al.*, 1978), and a bilateral lesions in this area caused loss of sexual behavior (Heimer and Larsson *et al.*, 1996).

To examine the effects of neonatal exposure to estrogenic chemicals on the development of SDN-POA and reproductive functions such as copulation and fertility, Nagao and Saito *et al.* (1999) had conducted an interesting experiment. In this experiment, male pups were subcutaneously given 2 μg/g of estradiol benzoate from neonatal day 1 to 5. In addition, 50 to 1000 μg/kg of DES, 20 and 40 mg/kg of tamoxifen were subcutaneously injected and 125 to 1000 mg/kg of nonylphenol were administered by gavage to male neonates for 5 days. At the 12 weeks of age, the animals were examined for reproductive functions including reproductive abilities and masculine sexual behaviors. Then, these animals were sacrificed for histological examination of SDN-POA, (Table 5).

As a result, the volume of SDN-POA in rats treated

Table 5. The effects of estrogenic chemicals on the SDN-POA and reproductive function

Experimental Protocol
Animals: Sprague-Dawley rats
Treatment

Age of Rats
0 Neonatal (days) 1 2 3 4 5 — Adult (weeks) 12

↑ ↑ ↑ ↑ ↑ — ↑
Treatment of chemicals — Functional observations and histological studies

Test chemicals		
Estradiol benzoate	2 μg/g/day	subcutaneous injection
Diethylstilbesterol	50 to 1000 μg/kg/day	subcutaneous injection
Tamoxifen	20 and 40 mg/kg/day	subcutaneous injection
Nonylphenol	125 to 1000 mg/kg/day	oral administration

Table 6. Effects of estradiol benzoate on the reproductive ability of male rats

Group	No. of rats examined (A)	No. of rats copulated (B)	Copulation index (B/A x100)	No. of fertile rats	Fertility index
Control	25	25	100	25	100
EB 2 mg/g	22	0*	0*	-	-

In all cases treated males were cohibited with untreated females.
*: Significantly different from the control, P<0.01.

Table 7. Effects of estradiol benzoate on the masculine behavior of male rats

Behavioral items	Control	EB 2 μg/g
No. of rats tested	10	10
No. of rats showing mount behavior	10	10
No. of mounts per rat	33.2±6.3[1]	36.4±8.3
Latency (s) of the first mount	192.2±53.0	326.8±72.2*
No. of rats showing intromissions	10	10
No. of intromissions per rat	12.8±5.7	4.2±2.3*
Latency (s) of the first intromission	489.4±113.5	1755.6±199.3*
No. of rats showing ejaculations	6	0
No. of ejaculations per rat	4.3±2.3	-
Latency (s) of the first ejaculation	2009.3±265.4	-

[1]Mean±SE. *: Significantly different from the control, P<0.01.

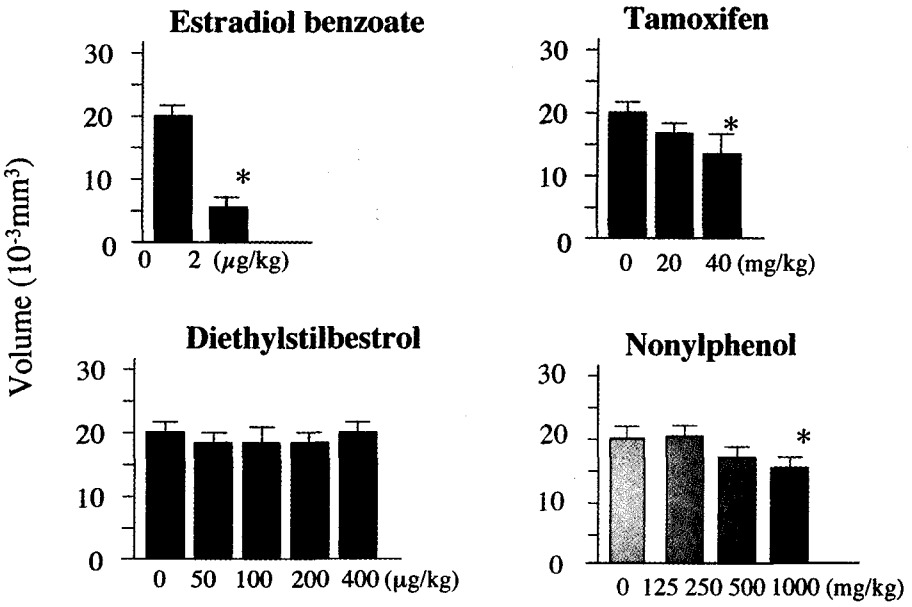

Fig. 1. The volume of the SDN-POA in male rats treated with endocrine disrupting chemicals at postnatal period.

with estradiol benzoate was decreased to 35.7% of that of control males. In addition, estradiol benzoate treated animals failed to copulate with untreated females during the 2-week mating period (Table 6), and masculine sexual behaviors such as the number of intramissions were also significantly decreased (Table 7). In male rats treated with the other estrogenic chemicals, tamoxifen and nonylphenol, showed the same effects on the SDN-POA after the treatment with high dose levels, but DES showed no inhibitory effect on the SDN-POA (Fig. 1).

These results indicate that neonatal exposure to some of estrogenic chemicals affects the SDN-POA of male and that reproductive functions are also impaired after sexual maturation.

V. ASSESSMENT OF ENDOCRINE DISRUPTING CHEMICALS

The endocrine functions are well conducted by the down-regulation through the hypothalamus and the pituitary and upper-regulation on the negative feed back mechanisms. Recently, it was clarified that estrogen had regulatory effects on the prolactin synthesis in the mammary gland and the pituitary, and the TSH production in the pituitary. Therefore, we should adopt a new concept for regulation of endocrine functions to consider the action mechanism of the endocrine disrupting chemicals.

For fetal or neonatal experimental animals, exposure to potent estrogenic chemicals caused a disturbance in sexually related neural tissues and impairment of the reproductive functions, showing so called "Window effects". Therefore, the effects of endocrine disrupting chemicals are considered to be stage specific on the developmental and the differential process.

Some estrogenic substances showed a low-dose response with so called "U- shaped dose response curve" on the experimental animals. However, there is little direct evidence to indicate that exposures to ambient levels of estrogenic chemicals are affecting human reproductive health. Therefore, it is also important to confirm the U-shaped health effects of estrogenic chemicals both in human and wildlife.

According to these evidences, the positive results on *in vitro* and *in vivo* screening methods do not necessarily suggest adverse effects of the chemicals on the human and wildlife. When the chemicals show negative results on these tests, it has probably no effect on the human and wildlife.

In conclusion, chemicals must be examined using all kinds of available screening methods to assess the effects on human and wildlife and evaluated based on all kinds of the available knowledge.

REFERENCES

Biswas, N.M., Ghosh, P.K., Ghosh K.K. and Neutaus, W. (1983): Effect of α_{2u}-globulin on serum concentration of gonadotrophins and testicular activity on estrogen-treated rats. *J. Endocrinol.*, **96**, 321-327.

Dohler, K.D. (1991): The pre- and postnatal influence of hormones and neurotransmitters on sexual differentiation of the mammalian hypothalamus. *Int. Rev. Cytol.*, **131**, 1-57.

Gorski, R.A., Gordon, J.H., Shryne, J.E. and Southman, A.M. (1978): Evidence for a morphological sex difference within the medial preoptic area of the rat brain. *Brain Res.*, **148**, 333-346.

Heimer, L. and Larsson, K. (1966): Impairment of mating behavior in male rats following lesions in the preoptic-anterior hypothalamic continuum. *Brain Res.*, **67**, 248-263.

Kulkarni, A.B., Gubits, R.M. and Feigelson, P. (1985): Developmental and hormonal regulation of α_{2u}-globulin gene transcription. *Proc. Natl. Acad. Sci. USA*, **82**, 2579-2582.

Nagao, T., Saito, Y., Usumi, K., Kuwagata, M. and Imai, K. (1999): Reproductive function in rats exposed neonatally to bisphenol A and estradiol benzoate. *Repro. Toxicol.*, **13**, 303-311.

Ohta, Y. (1977): Response of testis to androgen and gonadotropins in neonatally estrogenized and androgenized mice. *Endocrinol. Jpn.*, **24**, 287-294.

Takasugi, N. (1970): Testicular damages in neonatally estrogenized adult mice. *Endocrinol. Jpn.*, **17**, 277-281.

Takasugi, N. (1976): Cytological basis for permanent vaginal changes on mice treated neonatally with steroid hormones. *Int. Rev. Cytol.*, **44**, 193-224.

Takeyoshi, M., Anai, S. and Shinoda, K. (2000): Changes in serum α_{2u}-globulin levels in male rats given diethylstilbestrol and applicability to a screening test for endocrine-disrupting chemicals. *Arch. Toxicol.*, **74**, 48-53.