Table 2a Absolute and relative organ weights in male rats treated with S-421 for 28 days and 14 days recovery period

	28 days dosing test					14 days recovery test	
Group(mg/kg)	0	10	40	160	640	0	640
Effective No.	5	5	5	5	5	5	5
Body weight g	215±16	222±13	228±7.0	220±7.0	191±15**	258±13	232±18*
Absolute organ weight							
Brain g	1.79±0.07	1.81±0.05	1.77±0.06	1.74±0.04	1.71±0.03*	1.83±0.04	1.77±0.03*
Heart g	0.64±0.06	0.66±0.05	0.66±0.02	0.64±0.02	0.57±0.07	0.73±0.04	0.68±0.05
Lung g	0.80±0.11	0.85±0.11	0.78±0.04	0.78±0.06	0.70±0.06	0.97±0.20	0.82±0.12
Liver g	6.40±0.50	6.92±0.47	7.79±0.42	8.85±0.11**	13.73±0.78**	7.61±0.88	7.25±0.59
Kidney g	1.48±0.14	1.53±0.13	1.65±0.12	1.75±0.09**	1.86±0.14**	1.55±0.09	1.65±0.13
Spleen g	0.43±0.03	0.45±0.05	0.45±0.02	0.42±0.05	0.35±0.04**	0.51±0.02	0.45±0.03**
Testis g	2.54±0.13	2.51±0.19	2.53±0.11	2.48±0.15	2.42±0.08	2.64±0.23	2.55±0.11
Pituitary mg	5.9±0.5	6.9±0.8	6.3±1.0	7.2±1.2	5.3±1.2	7.4±1.2	7.0±0.8
Adrenal gl. mg	32.4±3.7	30.9±4.5	31.2±2.4	29.5±3.6	26.0±1.9	33.6±3.8	27.3±1.8**
Salivary gl. g	0.38±0.04	0.40±0.02	0.38±0.03	0.37±0.02	0.29±0.03**	0.41±0.02	0.37±0.04*
Thymus g	0.33±0.05	0.37±0.05	0.38±0.05	0.33±0.05	0.32±0.04	0.32±0.03	0.29±0.07
Relative organ weight							
Brain (g/100g B.W.)	0.83±0.04	0.81±0.04	0.78±0.02	0.79±0.02	0.90±0.07*	0.71±0.03	0.77±0.05*
Heart (g/100g B.W.)	0.30±0.01	0.30±0.01	0.29±0.01	0.29±0.01	0.30±0.02	0.28±0.01	0.29±0.02
Lung (g/100g B.W.)	0.38±0.03	0.38±0.04	0.34±0.02	0.36±0.02	0.37±0.04	0.37±0.08	0.35±0.04
Liver (g/100g B.W.)	2.98±0.04	3.12±0.13	3.41±0.10	4.02±0.12**	7.23±0.51**	2.94±0.20	3.12±0.11
Kidney (g/100g B.W.)	0.69±0.03	0.69±0.02	0.72±0.04	0.79±0.02**	0.98±0.07**	0.60±0.02	0.71±0.02**
Spleen (g/100g B.W.)	0.20±0.02	0.20±0.02	0.20±0.01	0.19±0.02	0.19±0.02	0.19±0.01	0.19±0.01
Testis (g/100g B.W.)	1.19±0.08	1.13±0.08	1.11±0.06	1.12±0.06	1.27±0.08	1.02±0.09	1.10±0.04
Pituitary (mg/100g B.W.)	2.8±0.3	3.1±0.4	2.8±0.4	3.3±0.6	2.8±0.6	2.9±0.5	3.0±0.5
Adrenal gl. (mg/100g B.W.)	15.1±1.3	13.9±2.1	13.7±1.0	13.4±1.5	13.7±1.3	13.0±1.3	11.8±0.6*
Salivary gl. (g/100g B.W.)	0.17±0.01	0.18±0.00	0.17±0.01	0.17±0.01	0.15±0.01*	0.16±0.01	0.16±0.02
Thymus (g/100g B.W.)	0.15±0.02	0.16±0.02	0.17±0.02	0.15±0.02	0.17±0.02	0.13±0.01	0.13±0.02

Values represent mean ±S.D.
* and ** show significant difference from the control at $p<0.05$ and $p<0.01$, respectively.

Table 2b Absolute and relative organ weights in female rats treated with S-421 for 28 days and 14 days recovery period

Group(mg/kg)	28 days dosing test					14 days recovery test	
	0	10	40	160	640	0	640
Effective No.	5	5	5	5	5	5	5
Body weight g	148±9	154±8	148±7	149±6	143±8	167±9	149±8**
Absolute organ weight							
Brain g	1.64±0.06	1.68±0.02	1.65±0.06	1.62±0.04	1.65±0.06	1.70±0.07	1.63±0.08
Heart g	0.46±0.03	0.48±0.03	0.51±0.05	0.47±0.02	0.47±0.03	0.49±0.03	0.47±0.04
Lung g	0.60±0.04	0.64±0.09	0.62±0.05	0.64±0.04	0.61±0.03	0.74±0.12	0.59±0.06*
Liver g	4.16±0.49	4.22±0.28	4.55±0.32	6.61±0.39**	11.37±0.53**	4.62±0.88	5.49±0.37*
Kidney g	0.99±0.02	1.08±0.07*	1.07±0.04	1.20±0.09**	1.41±0.03**	1.07±0.10	1.12±0.07
Spleen g	0.32±0.03	0.34±0.03	0.34±0.05	0.35±0.04	0.29±0.03	0.35±0.04	0.35±0.03
Ovary mg	41.6±6.2	48.2±5.7	45.3±5.0	50.2±3.4	49.2±5.5	49.2±6.4	44.7±2.7
Pituitary mg	8.1±2.3	9.6±1.5	8.9±1.6	8.4±0.6	6.7±1.8	10.1±3.0	9.6±1.5
Adrenal gl. mg	38.5±2.8	38.8±2.2	40.6±2.1	43.1±1.6*	36.1±4.5	41.8±2.3	32.8±4.2**
Salivary gl. g	0.26±0.03	0.28±0.02	0.27±0.02	0.29±0.02	0.26±0.02	0.29±0.03	0.28±0.04
Thymus g	0.30±0.03	0.27±0.06	0.28±0.04	0.32±0.02	0.29±0.01	0.28±0.05	0.26±0.04
Relative organ weight							
Brain (g/100g B.W.)	1.11±0.05	1.10±0.05	1.12±0.03	1.09±0.04	1.16±0.08	1.02±0.02	1.10±0.06*
Heart (g/100g B.W.)	0.31±0.02	0.31±0.00	0.35±0.05	0.32±0.02	0.33±0.01	0.30±0.01	0.32±0.01**
Lung (g/100g B.W.)	0.41±0.03	0.42±0.04	0.42±0.04	0.43±0.03	0.43±0.02	0.44±0.07	0.40±0.04
Liver (g/100g B.W.)	2.80±0.18	2.74±0.07	3.08±0.15	4.43±0.26*	7.99±0.51**	2.74±0.40	3.70±0.37**
Kidney (g/100g B.W.)	0.67±0.03	0.70±0.02	0.72±0.01	0.81±0.08*	0.99±0.07**	0.64±0.03	0.75±0.03**
Spleen (g/100g B.W.)	0.22±0.02	0.22±0.01	0.23±0.03	0.23±0.03	0.21±0.02	0.21±0.01	0.23±0.02*
Ovary (mg/100g B.W.)	28.0±3.0	31.3±2.1	30.7±3.4	33.7±2.2*	34.5±3.8**	29.6±5.0	30.0±1.4
Pituitary (mg/100g B.W.)	5.5±1.6	6.2±1.0	6.0±0.8	5.6±0.4	4.7±1.2	6.0±1.6	6.5±1.0
Adrenal gl. (mg/100g B.W.)	26.1±3.3	25.3±1.9	27.5±0.8	29.0±2.0	25.3±2.3	25.0±1.0	22.2±3.6
Salivary gl. (g/100g B.W.)	0.18±0.02	0.18±0.02	0.18±0.01	0.19±0.02	0.18±0.01	0.17±0.01	0.18±0.02
Thymus (g/100g B.W.)	0.20±0.02	0.17±0.03	0.19±0.02	0.22±0.02	0.21±0.01	0.16±0.03	0.18±0.02

Values represent mean ±S.D.

* and ** show significant difference from the control at $p<0.05$ and $p<0.01$, respectively.

Table 3 Summary for Histopathological Findings in Male and Female Rats Treated with S-421 for 28 days and 14 days Recovery Period

		Male							Female						
		28 days dosing test					recovery		28 days dosing test					recovery	
Group (mg/kg)		0	10	40	160	640	0	640	0	10	40	160	640	0	640
Effective No.		5	5	5	5	5	5	5	5	5	5	5	5	5	5
Liver															
degeneration		0	0	0	3	5	0	0	0	0	0	1	5	0	0
swelling	±	0	0	0	3	3	0	0	0	0	0	1	3	0	0
	+	0	0	0	0	2	0	0	0	0	0	0	2	0	0
vacuolization	±	0	0	0	0	3	0	0	0	0	0	0	2	0	0
	+	0	0	0	0	2	0	0	0	0	0	0	0	0	0
microgranuloma		0	0	0	1	2	1	1	2	1	2	1	2	2	1
focal necrosis	±	0	0	0	0	3	0	0	0	0	0	0	1	0	0
	+	0	0	0	2	2	0	0	0	0	0	0	1	0	0
Anisonucleosis		0	0	0	0	5	0	0	0	0	0	0	5	0	0

±: slight, +: moderate.

しかし，近年S-421は，調査母乳の半数以上[2,3]，屋内汚染指標の電気掃除機中の全集塵から検出され[4]，広範囲な屋内汚染が進行していることが判明し，更に，土壌中の半減期は，約200～800日と長く，分解しにくく蓄積性である[1]ことが確認された．更に，今回の試験の結果，比較的多量のS-421を継続的に摂取させた場合，肝臓の顕著な重量増加および軽度の局限性壊死および空胞変性がみられた．一方，ピレスロイド系殺虫剤は他の殺虫剤であるカーバメイト剤や有機リン剤と混合することにより相乗効果がみられ，この効果は，抵抗性解除ともいうべき薬剤感受性の回復機構によるものである．しかし，この効果は，他方では動物に対しても種々の化学物質に対して毒性強化に転化する可能性が高い[10]ことから，その意味では安全性に対して慎重に検討する必要がある．

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Original

Hypospadias and Incomplete Preputial Separation in Male Rats Induced by Prenatal Exposure to an Anti-androgen, Flutamide

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Abstract: Hypospadias was induced in male Sprague-Dawley rats by prenatal exposure to 30 mg/kg/day of flutamide from gestational days 14 to 17, or from 18 to 21. Their external genitalia were examined histopathologically and compared to untreated controls. On postnatal day 6, the glans penis of untreated controls was bordered with epithelium. On postnatal day 22, papillae were recognized on the glans penis side, and cornification started close to the tip of the papilla on postnatal day 35. On postnatal day 42 cornification spread to the surface of the glans penis. The cornification progressed from tip to base of the glans penis, and from dorsal to ventral. When cornification reached the base of the glans penis, separation of the double layered epithelia was complete and the animal was considered sexually mature. Flutamide treatment on gestational days 14–17 induced a defect in the ventral half of the glans penis (cleft phallus) and cleft in the ventral prepuce (cleft prepuce) in the male pups, while treatment on gestational days 18–21 induced cleft phallus without apparent abnormalities in the prepuce. The external urethral orifice opened at the ventral end of the glans penis (hypospadias) in both treatment groups. In male pups with cleft phallus, cornification of the dorsal epithelium followed by separation of the prepuce occurred, while separation of the ventral part of glans penis did not occur because epithelium was not formed at the ventral part of the glans penis. Consequently, the onset of puberty was not decided in these animals. These findings indicate that the defect of the ventral half of the phallus is the reason why the time of sexual maturation was not decided, and that there is a difference between the phallus and prepuce in the sensitive period concerning the development of flutamide-induced malformations.
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Key words: flutamide, anti-androgen, rat, prenatal exposure, hypospadias

Introduction

Preputial separation, which is observed as separation of the prepuce from the glans penis, has been used as a sign of puberty in the male rat. Histological observation on the progress of preputial separation after cornification at the lining of prepuce and surface of glans penis was well described using Long-Evans rats in 1942[1]. Preputial separation is thought to be dependent on androgens, because castration blocked preputial separation, and the addition of testosterone (TS) or dihydrotestosterone (DHT) recovered the effect of castration[1,2]. In recent years, preputial separation has been used as an endpoint to evaluate endocrine disrupting chemicals. Although the observation of preputial separation is a useful tool for detecting sexual maturation, anti-androgenic chemicals induce hypospadias in male rats by intrauterine exposure. The time of sexual maturation is determined by complete separation of the prepuce from the ventral surface of the glans penis, but in males with hypospadias, puberty is undetermined because this complete separation in the glans penis is not evident[3].

The purpose of this study was to reveal the histological process of normal and abnormal preputial separation, as well to reveal the reason why the time of sexual maturation cannot be decided in males with hypospadias induced by prenatal exposure to flutamide (FLU), an anti-androgenic chemical, in Sprague-Dawley rats. FLU was administered on gestational days (GD) 14–17 (expected to be the most sensitive period for hypospadias) or on GD 18–21 (thought to be a less sensitive period for hypospadias).

Materials and Methods

Sprague-Dawley rats (Crj:CD (SD) IGS), 30 males and 33 females, 11 weeks of age, were obtained from Charles

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River Japan, Inc. (Atsugi, Japan). All animals were acclimatized to laboratory conditions and quarantined for one week before mating. Rats used for this study were selected based upon general condition, appearance and behavior during the acclimatization period. Animals were housed individually in wire-bottom metal cages (220 × 270 × 190 mm) and kept in a barrier sustained animal room that was maintained at 21.0 – 25.0°C and 40.0 – 75.0% relative humidity with a 12-hour artificial light cycle (lighting from 7:00 to 19:00). Fifteen changes of room air per hour were provided. Commercial diet CE-2 (CLEA Japan, Inc., Tokyo) and water (Hadano City) were available ad libitum throughout the study. The protocol of the present study was approved by the Animal Use Committee of the Hatano Research Institute.

The untreated control group consisted of 18 females. While the second group, consisting of 9 females, was treated orally with 30 mg/kg/day of FLU (Sigma Chemical Co., St. Louis, USA) dissolved in corn oil (Nacalai Tesque, Inc., Kyoto, Japan) from GD 14 to 17, the third group, consisting of 6 females, was treated with the same dose from GD 18 to 21. From the result of a preliminary study the dosage was decided as 30 mg/kg/day, because pregnant rats died after administration of 100 mg/kg/day of FLU. To obtain pregnant animals, 12-week-old females were cohabited overnight on a 1:1 basis with males 12 weeks of age or older. Females were considered to be at GD 0 when daily examination revealed a vaginal plug. All pregnant animals were housed in cages with animal bedding (PAPER CLEAN®, Japan SLC, Inc., Shizuoka) from GD 18 until postpartum day 10, and allowed to give birth. On postnatal day (PND) 6 (PND 0 is the day of delivery) all female pups were discarded. Body weights of male pups were measured on PND 0, 6, 22, 35 and 56. Progress of preputial separation of male pups was observed macroscopically from PND 35.

Control male pups were sacrificed by exsanguination under anesthesia on PND 6, 22, 35, 42 and 56 (number of pups in group 1 were 6, 16, 15, 4 and 24, respectively). Male pups from FLU-treated females were sacrificed under anesthesia on PND 6 and 56 (number of pups in group 2 were 18 and 41, respectively, and those in group 3 were 8 and 21, respectively). After macroscopic examination, the prepuce and penis were dissected and fixed with 0.1 mol/L phosphate buffered 10% formalin solution. Sagittal slices of the prepuce and penis were embedded in paraffin, and sections were stained with hematoxylin-eosin (H & E) for histopathological examination.

Results

On macroscopic examination, the glans penis of control males was covered with prepuce, and the prepuce could be completely retracted to expose the glans penis until PND 46 (Figs. 1A, 1B). Prepuce of males prenatally exposed to FLU on GD 14–17 had a cleft at the ventral part (cleft prepuce), and the glans penis was observed from the cleft (Fig. 1C). The ventral part of the glans penis of these males was incompletely formed (cleft phallus) and the os penis was often exposed (Fig. 1D). The incidence of cleft prepuce was 80% (33/41), and the incidence of cleft phallus was 90% (37/41). Cleft prepuce is usually observed with cleft phallus. Another 4 males showed no cleft on their prepuce or phallus, while preputial separation was delayed or incomplete on PND 56. Although there was no cleft at the prepuce of males exposed to FLU on GD 18–21 (Fig. 1E), the ventral part of the glans penis was incompletely formed (cleft phallus, Fig. 1F), and incidence of the cleft phallus in this group was 100% (21/21). Body weight gains of males were not affected by FLU exposure.

Upon histological examination of untreated controls on PND 6, the glans penis was bordered with specific epithelium (Fig. 2A). The epithelium consisted of outer and inner basal layers (Fig. 2B). The outer layer lined the inside of the prepuce and the inner layer covered the glans penis. The urethra was located in the center of the glans penis and the os penis was observed between the dorsal surface of the glans penis and the urethra (Fig. 2A). On PND 22, there were many papillary processes from the glans penis (arrows in Fig. 2C), and the surface of these processes was covered with squamous epithelial cells. At this point the two basal layers lost their parallel arrangement. On PND 35, the epithelial layer consisted of stratified squamous epithelium, and the surface of the papillary processes (arrows in Fig. 2D) was covered with cornified cells. The cornified layer was limited to the surface of these processes. On PND 42, epithelial cells between the papillae also cornified, and both surfaces of the penis and prepuce consisted of keratinized stratified squamous epithelium. Cornification and separation were incomplete at the basal part of the glans penis. Separation at the ventral surface of the glans penis was more delayed than that at the dorsal surface. On PND 56 cornification was complete from the tip to the base of the glans penis and the ventral surface also showed cornified layers (Figs. 2E, 2F). The preputial separation was complete across the entire surface of the glans penis.

Histological examination of males prenatally exposed to FLU on GD 14–17 revealed a cleft at the ventral surface of genital tubercle on PND 6 (arrow in Fig. 3A). The urethra was not located in the center of the glans penis, but instead was observed at the ventral surface of the glans penis. The dorsal part of the glans penis was bordered by epithelium as observed in controls, while the ventral part was covered with urethral epithelium. This finding indicates that the ventral half of glans penis was not formed (comparison with controls as shown by an asterisk (*) in Fig. 2A). The cavernous body of the penis was tortuous and also observed in PND 56 males exposed to FLU on GD 14–17 (Fig. 3B). The dorsal surface of the glans penis and prepuce of PND 56 males were covered with keratinized stratified squamous epithelium, and the prepuce was separated from the glans penis (Fig. 3B). The ventral part of the glans penis and ventral epithelium were not formed between the urethra and subcutis, the ventral surface of the glans penis was not covered with squamous epithelium and the preputial

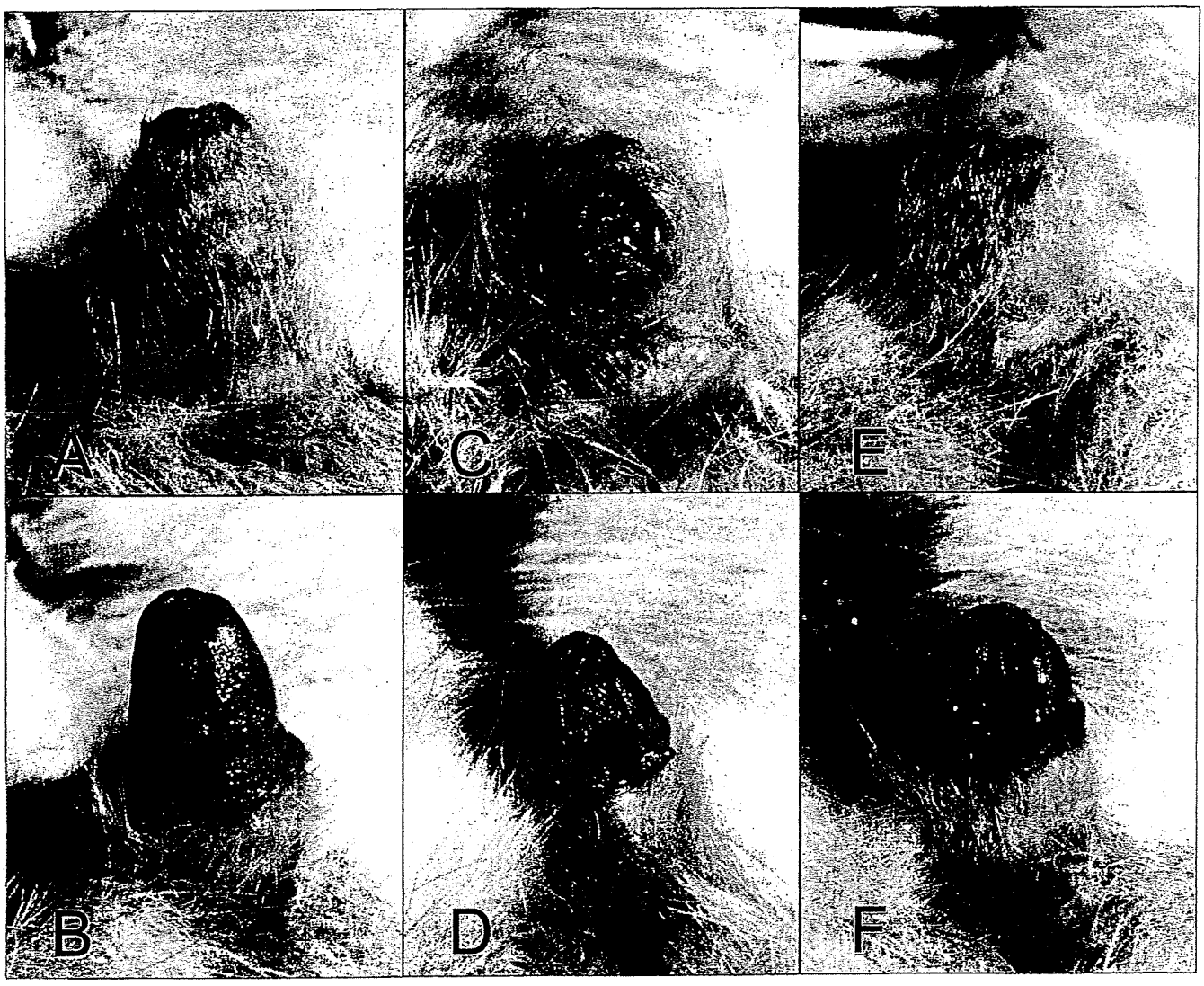

Fig. 1. Ventral surface of genital tubercle (A, C and E) and glans penis (B, D and F) of males at PND 56. A and B: Control rat. Prepuce is completely retracted. C and D: Male rat prenatally exposed to FLU on GD 14–17. Ventral side of the prepuce has a cleft, and the glans penis is observed from the cleft. Ventral part of the glans penis is incompletely formed (cleft phallus) and os penis is observed. E and F: Male rat prenatally exposed to FLU on GD 18–21. Prepuce does not have a cleft at the ventral side. Glans penis shows cleft phallus and os penis is observed.

separation did not progress at the ventral part. The external urethral orifice opened at the ventral surface of the glans penis (hypospadias). The preputial tissue was hypoplastic and the tip of the penis was not overlain with prepuce.

The glans penis of PND 6 males exposed to FLU on GD 18–21 was covered with skin, and a cleft was not observed at the ventral part of genital tubercle (Fig. 3C). The dorsal part of the glans penis was bordered by epithelium, but the ventral part of the glans penis and ventral epithelium were not formed between the urethra and subcutis (comparison with controls as shown by an asterisk (*) in Fig. 2A). The tortuous structure of the cavernous body was indistinct. The prepuce overlaid the glans penis of PND 56 males exposed on GD 18–21 (Fig. 3D). The dorsal surface of the glans penis of these males was covered with keratinized stratified squamous epithelium, and the prepuce was separated from the glans penis. The ventral part of the glans penis and ventral epithelium were not formed between the urethra and subcutis, and preputial separation did not progress at the ventral part. In these rats the external urethral orifice opened at the ventral surface of the glans penis.

Discussion

As described above, preputial separation in untreated rats initiated from cornification of the epithelium on the penile side lying in the dual phasic epithelium between the glans penis and prepuce. Cornification began at the surface very close to the apex of the papillary process from the glans penis, and when the cornification reached the next papilla the prepuce separated from the glans penis. Preputial separation progressed from the tip of the glans penis towards its base,

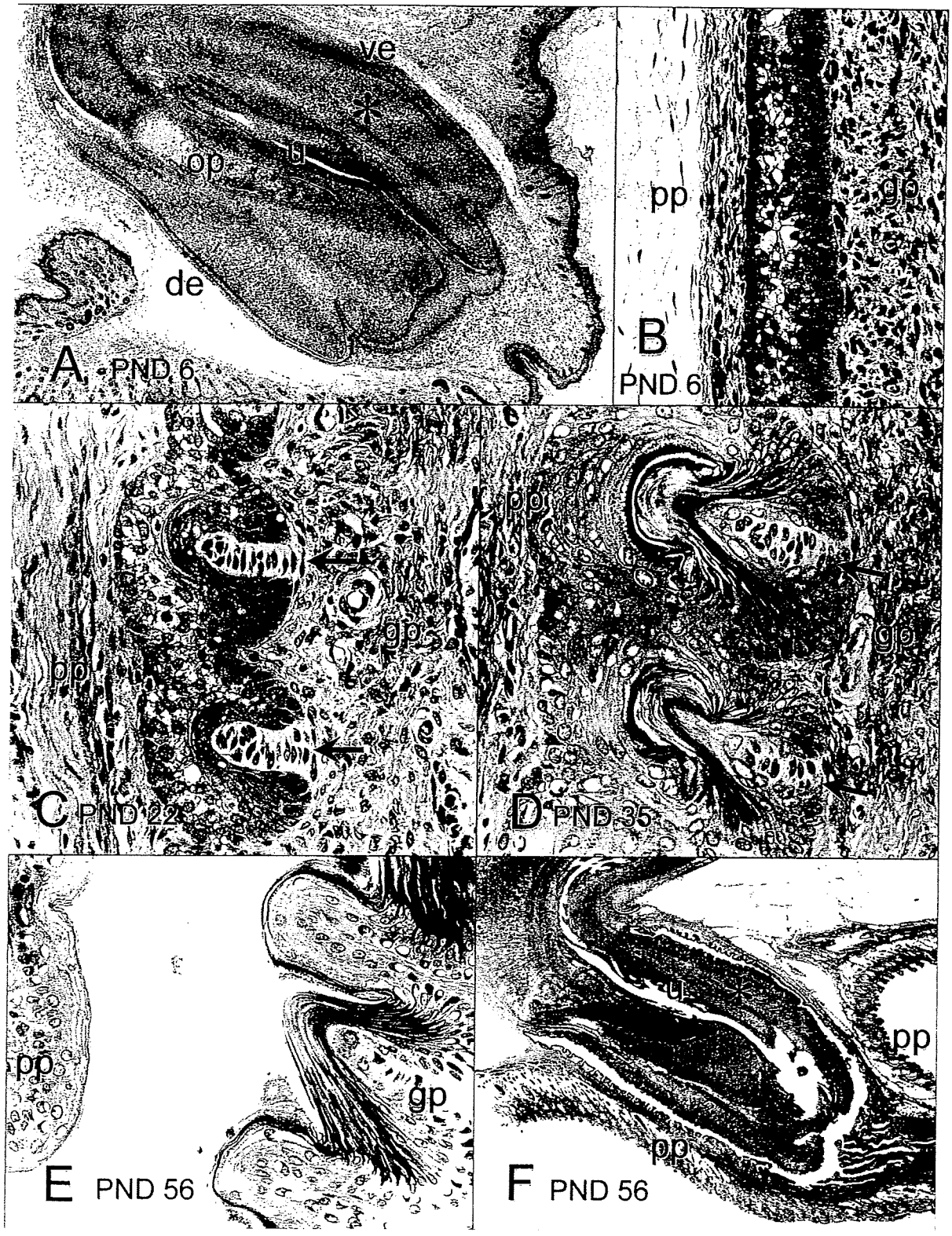

Fig. 2. Sagittal sections of the genital tubercle from control males.
A: Genital tubercle of a male on PND 6. Glans penis is bordered with dorsal epithelium (de) and ventral epithelium (ve). Urethra (u) is located in the center of the glans penis. op: os penis. *: ventral half of the glans penis.
B, C, D and E: Epithelium between the dorsal part of the glans penis (gp) and prepuce (pp) on PND 6, 22, 35 and 56, respectively. Epithelium of PND 6 consisted of outer and inner basal layers (B). Squamous epithelial cell of PND 22 is covering the papillary processes (arrows) from the glans penis (C). Epithelial layer of PND 35 consists of stratified squamous epithelium, and cornified layer is covering the papillary processes (arrows in D). Whole surface of both the glans penis and prepuce is covered with cornified layer on PND 56 (E).
F: Glans penis and prepuce on PND 56. Preputial separation is completed. Urethra (u) is located in the center of the glans penis.
*: ventral half of the glans penis. H & E. Magnification, A: × 35, B, C and D: × 400, E: × 330, F: × 9.

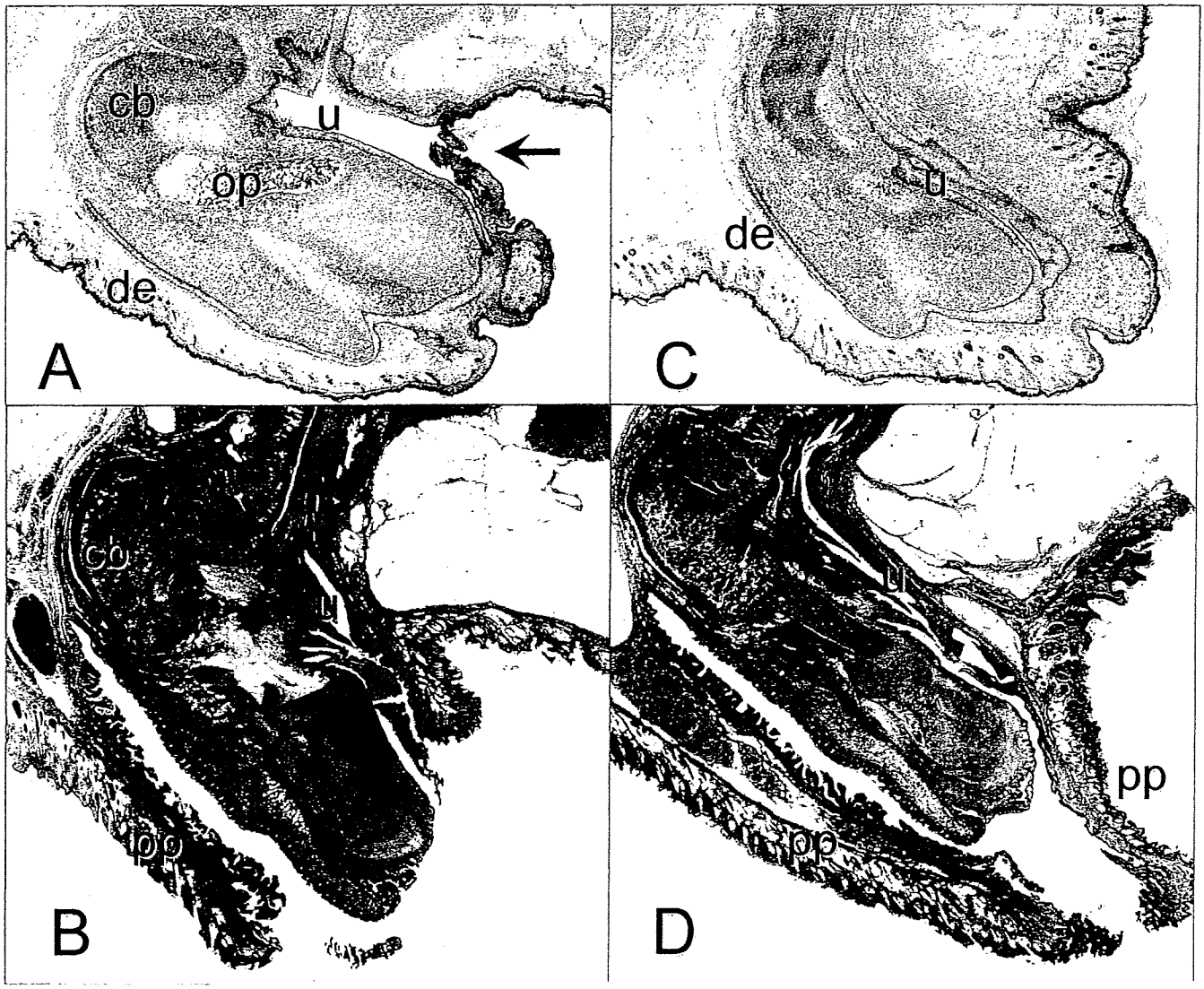

Fig. 3. Sagittal sections of genital tubercle from males prenatally exposed to FLU on GD 14–17 (A, B) or GD 18–21 (C, D), sacrificed on PND 6 (A, C) and PND 56 (B, D).
A: There is a cleft (arrow in the figure) at the ventral side of genital tubercle. Urethra (u) is observed at the ventral surface of the glans penis. Dorsal part of the glans penis is bordered with epithelium (de). Cavernous body (cb) shows tortuous structure.
B: Prepuce (pp) is separated from the glans penis at the dorsal part. Prepuce is hypoplastic, and the glans penis is not completely overlain with the prepuce. Urethra is located between the glans penis and subcutis.
C: Cleft is not formed at the ventral side of genital tubercle. Dorsal part of the glans penis is bordered with epithelium (de). Ventral part of the glans penis and ventral epithelium is not formed. Urethra is located between the glans penis and subcutis.
D: Prepuce is separated from the glans penis at the dorsal part. The ventral part of the glans penis and ventral epithelial layer is not formed. The glans penis is completely overlain with the prepuce.
H&E. Magnification, A and C: × 27; B and D: × 10.

and also from the dorsal to ventral surface of the glans penis. Histological features observed in controls of this study were almost the same as shown in Long-Evans rats[1]. Complete separation was not observed in animals exposed to FLU in their fetal period, since they had a cleft phallus at their ventral surface of the glans penis. Histopathological examination revealed defects in the ventral part of the glans penis and lack of an epithelial layer at the ventral part in newborn rats.

Induction of hypospadias has been reportedly caused by various chemicals, which include anti-androgens such as FLU, vinclozolin and finasteride. FLU is a well-known potent androgen receptor antagonist and is used as a nonsteroidal anti-androgen drug for the treatment of prostate cancer. FLU inhibits TS and DHT binding to the intracellular androgen receptor and prenatal/perinatal exposure to FLU induces abnormalities in the genital tract such as hypospadias, agenesis of the prostate, epididymis and vas deferens[2,4,5]. Vinclozolin, a fungicide, is also an androgen receptor antagonist, and induces hypospadias in

rats by perinatal (GD 14 to day 3 postpartum)[6] or prenatal (for 2 days in GD 12–21)[7] administration. Finasteride, which inhibits 5α-reductase conversion of TS to DHT, also induces hypospadias in male rats exposed from GD 15 to day 21 postpartum, and based on this finding DHT is thought to be involved in the development of external genitalia[8]. While the Wolffian ducts are dependent on TS, their derivatives such as the epididymis, vas deferens or seminal vesicles were not affected by intrauterine exposure to finasteride[8]. Hypoplastic change in the genital tubercle was reported in fetuses exposed to finasteride from GD 6 to 20[9]. Wedge shaped mesenchymal tissue between rectum and urogenital sinus failed to develop in these fetuses, and the urethra opened near the base of the tubercle. This finding indicates that the mesenchymal wedge may be the most sensitive area to loss of the effect of DHT in male fetuses. Our study also revealed a defect in the ventral part of the glans penis, in which preputial separation could not progress, and this is the reason why the time of sexual maturation could not be decided in males with hypospadias.

Androgen receptors are detectable in the mesenchymal cells of the rat urogenital tubercle from fetal day 14 onwards[10]. In many studies of sexual differentiation and male reproductive organ malformation, dosing starts from GD 12 or 14. The most sensitive period to induce hypospadias is reportedly GD 15–16 with 400 mg/kg of vinclozolin exposure, and the incidence was 42% (10/24), while only weak sensitivity (11%, 1/9) was found with treatment on GD 17–18[7]. Similar results were obtained in our study of vinclozolin (unpublished data)[11]. Exposure to 100 mg/kg of vinclozolin on GD 14–17 induced cleft phallus with cleft prepuce, and the incidence was 85%, but exposure on GD 18–21 induced no abnormalities in external genitalia. Pregnant females or newborn pups died after exposure to 200 mg/kg of vinclozolin. Finasteride exposed rats also showed similar results[8]. Male pups exposed to 20 mg/kg of finasteride on GD 16–17 showed hypospadias, and the incidence was 39% (14/36), while incidence in the GD 18–19 group was 0% (0/36).

Many reports describe the malformation of the phallus as hypospadias, but details and the incidence of the prepuce malformations are not clear. In our study, pregnant rats were administered with 30 mg/kg of FLU on GD 14–17, which was considered to be the sensitive period, and the incidence of cleft phallus and cleft prepuce was compared to the exposure on GD 18–21. Male pups exposed to FLU on GD 14–17 showed cleft phallus with hypospadias (90%) and cleft prepuce (80%), while males exposed on GD 18–21 had cleft phallus with hypospadias (100%) without apparent abnormality in the prepuce. A lower dose of FLU also showed similar results with lower incidence (unpublished data)[11]. Exposure of 10 mg/kg of FLU on GD 14–17 induced cleft phallus (58%) and cleft prepuce (25%), while exposure on GD 18–21 induced cleft phallus (25%) without apparent abnormality in the prepuce. These findings show that the period of sensitivity to FLU in terms of phallus malformation is different from vinclozolin and finasteride, and also that there are differences among the sensitive periods between the phallus and prepuce concerning FLU-induced malformations.

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Workshop 6.2

Hormonally active agents and plausible relationships to adverse effects on human health*

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Abstract: A hormonally active compound was first identified in the book *Silent Spring* by Rachel Carson in 1962, implicating the effect of pesticides such as DDT and the derivatives. Nearly four decades later, the book *Our Stolen Future* by Theo Colborn et al., and other pertinent publications have revisited and broadened the issue regarding a variety of possible chemicals and the area exposed. Translation and publication became available in Japan within the last four years. Since then, Japan joined the member countries involved in the global issue of endocrine disruptors, the "environmental hormone".

Although a significant number of chemicals possessing a hormone-like action have been recognized for many years, and the action of their biological plausibility related to the receptor-mediated effects strongly suggests possible human effects comparable to hormonal changes in wildlife, little is known about evidences or adversities in experimental animals and humans. The most essential key to resolving these dilemmas may be to understand the mechanism of actions (i.e., a possible low-dose issue). In other words, the mechanism at the low-dose effect may be resolved simultaneously by the mechanism of three major questions linked to the low-dose issue; namely, threshold, possible oscillation, and additive and/or synergistic action.

INTRODUCTION

The objective of this paper is to summarize all currently available information on hormonally active agents and plausible relationships to adverse effects on human health from the standpoints of the mechanisms of action of these chemicals.

It is not uncommon to come across agrochemicals and industrial chemicals that have hormone-mimic effects. These chemicals, the so-called "environmental hormones", often accumulate at detectable levels in the environment, and it has been feared that they may have adverse effects not only on wildlife but also on human beings. Following reports of feminization and decreased colony size of wild creatures, and reports suggesting a possible association of these chemicals with abnormalities of reproductive organs and oncogenesis in humans, attention has focused on the possibility that these occurrences may be associated with exposure to endocrine-disrupting chemicals (EDCs). In this connection, we would like to draw the attention of the reader to a Japanese translation of the book *Our Stolen Future*, written by Theo Colborn et al.

*Report from a SCOPE/IUPAC project: Implication of Endocrine Active Substances for Human and Wildlife (J. Miyamoto and J. Burger, editors). Other reports are published in this issue, *Pure Appl. Chem.* 75, 1617–2615 (2003).

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This paper will review the subjects related to EDCs, the courses of arguments regarding the possible hazards of these chemicals, and current medical subjects pertaining to them.

CHEMICALS WITH HORMONE-MIMIC ACTIONS

Substances with hormone-mimic effects can be divided into four groups:

- hormones found in vivo;
- medicines with hormone-mimic actions manufactured for use in hormonal therapy, etc.;
- plant hormones known to exert phytoestrogen-like actions; and
- chemicals found in environments that can interact with hormone receptors.

In addition, substances that do not interact with hormone receptors but exert effects on gonads by their modifying effects on steroid metabolism may be deemed as hormone-mimics in the broader sense of the term. In this paper, however, emphasis shall be placed on the hormone-mimic actions mediated by receptors that play essential roles in the mechanism of actions of hormone-mimics.

CHARACTERISTICS OF THE RECEPTOR-MEDIATED ACTIONS OF HORMONE-MIMICS

The receptor-mediated actions of hormone-mimics are fundamentally characterized by the similarity in structures of the receptors involved, crossing the barrier of animal species. These characteristics allow us to speculate the possibility that the actions of these chemicals exerted in nature may also occur in humans.

Second, since similarities in the structure of various sex steroids and hormones are also known, it is possible that each individual hormone-mimic exerts diverse effects by acting on male hormone receptors, female hormone receptors, and nuclear receptors (including many orphan receptors), etc.

Third, many of these chemicals are excreted from the living body in the form of conjugated inactive substances instead of as degraded metabolites. They may also be eliminated in the unchanged form. Therefore, if feces and urine containing these substances are eliminated into river water, it is plausible to imagine that even inactivated hormones can sometimes become active and exert hormone-mimic actions in the environment. This is one of the characteristics unique to this class of chemicals.

Receptor-mediated responses involve many unresolved questions. Various undefined elements may be involved, including the relationship between receptor binding and signals, the relationship between receptor-ligand binding (ligand: substances that can bind to receptors) and the dissociation of ligands from receptors, signal cross-talks, involvement of unknown nuclear receptors, etc.

The actions of these chemicals add to the effects of intrinsic hormones. For this reason, these chemicals may exert their actions in a way different from that known for other chemicals that do not have structural or functional counterparts in vivo. For example, stimulation of hormone receptors by these extrinsic chemicals may modify homeostasis in vivo, leading to down-modulation of the physiological stimulation of these receptors by the intrinsic ligands. Therefore, the influence of the continued effects of environmental hormones needs special study.

PITFALL IN THE EFFECTS OF HORMONE-MIMICS

We must distinguish the interactions of endocrine hormone-mimics with hormone receptors from the hazards caused to endocrine tissue. Bearing this in mind, let us now summarize the problems related to the effects of hormone-mimics.

Antagonistic effects maintaining homeostasis

The endocrine system is regulated by homeostatic mechanisms. It is not uncommon for the effects of small amounts of hormone-mimics to interfere slightly with these mechanisms, often with no adverse influence; this is well known. However, this is not always the case. There seems to be a group of genes that act antagonistically to each other in the maintenance of homeostasis.

With the uterotrophic assay, which is used to check for estrogenic activity, the ovary is removed in advance and the blood level of the intrinsic female hormone is reduced to the minimum. Under the thus-created extremely shrinking state of the uterus, the test substance (a chemical or hormone) is administered to evaluate for its effects on the inflation of the uterus. This test (checking for growth of the uterus in ovariectomized animals) is designed to evaluate the hormone activity and effects of hormone-mimics under conditions of blockade of homeostasis.

This test method itself is valid. However, there is no sufficient rational evidence that indicates that the responses observed under such indirect control conditions of the living body can serve as an indicator of the health hazards of hormone-mimics. Although the ovo-testes seen in lower vertebrates may be used if the effects observed were to be valid as such an indicator, there is no consensus on what is valid as an indicator of the health hazards of EDCs when mammals are used as experimental animals.

Down-regulation of the expression of receptors

It is known that the expression of gene-encoding receptors is down-regulated by continuous stimuli, leading to reduced receptor activity. This can lead to a paradoxical outcome wherein the effects observed in the presence of low levels of a substance are not seen at high levels of the same substance. If this phenomenon occurred in individual organisms, the dose–response relationship will be nonlinear.

This means that extrapolation of results obtained at high levels of the chemicals, to conditions where low levels of the same substance are present, would be difficult. It is needed to test the validity of this hypothesis; analysis of the mechanisms underlying this phenomenon if the hypothesis were indeed valid, are thus important. Studies to resolve these questions are now under way.

Data gap concerning the effects of female hormones

In mature women, there are high levels of physiological hormones in vivo, and these are subject to cyclic control. It has been proposed that girls with inadequate physical growth begin menstruation at lower ages and undergo sexual maturation earlier than usual, and that hormone-mimics in these subjects can precipitate breast cancer.

The weak links in this hypothesis have been pointed out, and it has been shown experimentally that estrogen by itself may be teratogenic, although this tendency has been shown to be weak. It is known that organisms are programmed such that excessive exposure to estrogens during the intrauterine period or other developmental stages is avoided.

There are many open questions as to the mechanism by which mature females remain physiologically stable, even when exposed daily to high levels of estrogen (400 pM/l). Some additional dramatic effects may be needed to disturb this homeostatic physiology.

Multigeneration tests and effects on fetuses

It has been shown that exposure to hormones or hormone-mimics during intrauterine or early neonatal periods can lead to irreversible changes in the pattern of development. This susceptibility period is short, extending from the 13^{th} gestational day to about one week after birth. These effects are the so-called "intrauterine window effects."

In animal studies involving observation of experimental animals for two or more generations, no effects of EDCs have been demonstrated. The question therefore arises as to why window effects are observed during the short period mentioned above. It is unknown whether or not these effects really do occur, and if they do, how they are produced.

Delayed growth of the thalamic nucleus specific to males (called sexual dimorphic nucleus) is seen in male rats treated with female hormones. We may say that under conditions of homeostasis of the physiological hormones in mature individuals, exposure to dose levels that usually cause only reversible changes can lead to irreversible changes, if the exposure occurs during genesis, morphogenesis, or functional development. However, there are no ample data endorsing this view in humans.

Considering the biological plausibility inferred from the experimental data accumulated to date*, we may say that there are no sufficient data that clearly rule out this view. Close attention has therefore been paid to these effects in children.

New theories of methodology, focusing on effects in fetuses and children, are now being developed, primarily in the United States, or the World Health Organization, within the framework of children's program, etc.

HEALTH HAZARDS AT LOW LEVELS OF EXPOSURE

Chemicals used for agriculture or industrial purposes are marketed, in general, only after their effects on living beings have been investigated. We may therefore understand that they are used on the premise that the possibility of these chemicals exerting hazardous effects on health at relatively high-dose levels has been almost ruled out. Nevertheless, problems with EDCs have begun to be highlighted. These problems may not be confined to those related to the accumulation of these substances through food chains in the ecosystem, but also to the additional possibility that these chemicals may exert effects at low-dose levels even if they have been declared safe at high-dose levels. The latter possibility may apply, however, only to some cases and not to others.

We may say that a major issue pertaining to EDCs that must be resolved urgently is whether or not they pose health hazards at low-dose levels. This issue can be summarized into the following three questions:

- presence/absence of threshold level;
- presence/absence of synergistic or additive effects; and
- possibility of extrapolation of high-dose effects to low-dose levels (i.e., presence/absence of a linear dose–response relationship).

No clear-cut answers have as yet emerged to these questions. Considering the above-mentioned characteristics of the effects of hormones, it is plausible to imagine how difficult it may be to resolve these questions.

To determine if these chemicals exerted hazardous effects on health at low-dose levels, the following basic questions may need to be considered; their biological plausibility is hardly denied.

- Regarding the presence or absence of threshold levels, it seems likely that many chemicals suspected of being EDCs can easily permeate across the cell membrane, which is composed of phospholipids. Therefore, assuming that one receptor molecule reacts with one chemical molecule, the lower limit of the dose level exerting the chemical's effects would be extremely low.

  Of course, since the probability of the binding of a ligand to the receptor will be low if the dose level is low, we cannot say that there is no threshold level for the effects seen in the low-

*Biological plausibility: Likelihood of a phenomenon as judged by considering the difference or similarity of elements of reactions in individual organisms, on the basis of the results of a series of related biological experiments. (Cf. probability)

dose-level range. In fact, for bisphenol A (which has been attracting close attention because of its hazardous effects on health at low-dose levels), the presence/absence of a threshold level has not yet been reported. It seems rational, therefore, to assume that these health hazards occur in a very low-dose-level range.

- If we consider not only the affinity of each substance for the receptor, but also the nonlinearity of responses (e.g., waveform responses as a result of reduced receptor expression following an increase in dose level), it is possible to assume that there are U-shaped or reverse U-shaped reactions, or oscillational dose–response curves. Interim data endorsing such a view are being accumulated.
- Regarding the possibility of synergistic or additive effects, the observation of additive effects among different nuclear receptors has been reported. Data yielded by analysis of interactions between receptor signals also suggest such a possibility. In fact, the dose–response curves for some composite materials were reported to be additive, but not synergistic.

Thus, the questions on health hazards at low-dose levels have several aspects:

- type of receptor-mediated actions of the hormone mimics;
- diverse reactive characteristics on the part of the receptors;
- diverse modification during expression of intracellular signals; and
- factors involved in irreversible changes related to morphogenesis and functional development.

Resolution of all these aspects of the question will lead to clarification of the mechanism of actions of the substances from each of the aforementioned standpoints. While these questions are among the hottest research themes at present, they are certainly unlikely to be resolved easily.

At a workshop held in North Carolina, USA, in October 2000, health hazards of chemicals at low-dose levels were discussed. Investigators for and against the possibility of these substances posing health hazards at low-dose levels gave detailed accounts of their studies, and no definitive conclusions could be reached, as the arguments of both sides appeared to be tenable.

This means that reports affirming the plausibility of these substances posing health hazards at low-dose levels in animal experiments cannot be immediately rejected. The workshop concluded by pointing out the necessity of paying attention to the possible hazards on fetuses and neonates.

HEALTH HAZARDS OF HORMONE-MIMICS TO HUMANS

The possibility of health hazards of hormone-mimics to human beings have not been supported by adequate epidemiological data, and the number of cases for which the data clearly endorse such effects is quite small. The U.S. National Research Council (NRC) emphasizes the necessity of conducting further epidemiological studies on this topic (NRC, 1999).

In conclusion, this paper summarizes the current knowledge concerning the health hazards of hormone-mimics to humans. Reports dealing with the effects of these substances on humans are confined to those pertaining to the effects of dioxins and polychlorinated biphenyls (PCBs); the validity and usefulness of these results have not yet been established.

The following information is based on case studies conducted to date.

Health hazards of dioxins

Regarding health hazards of dioxins, two-year dosing studies revealed weight loss and liver damage, and three-generation reproductive studies in rats disclosed intrauterine death and a decrease in litter size. Onset of endometriosis in rhesus monkeys has also been reported.

A causal relationship of EDCs to the following episodes in humans has been suggested: biased male-to-female ratio in children born in the dioxin-exposed Seveso area of Italy, and increased inci-

dence of cleft palate in the Diemerzeedijk district of the Netherlands, probably due to steroids. In both of these cases, the U.S. Environmental Protection Agency (USEPA) did not affirm a causal relationship, and classified them as cases requiring special attention.

No consensus has been reached concerning the relationship of hypothyroidism observed in the inhabitants along Lake Michigan to the ingestion of PBB- (polybrominated biphenyls-) contaminated fish.

Effects on mature females (e.g., increased incidence of breast cancer)

No reports affirm the effects of dioxins on mature human females (e.g., effects on breast cancer or endometriosis as discussed below). There are many unresolved questions on this topic. However, none of the studies conducted in mature experimental animals revealed data endorsing the plausibility of occurrence of such effects. On the other hand, it is known that the age at menarche is lower and the incidence of breast cancer higher in females exposed to dioxins. Some investigators cite these data when discussing the health hazards of dioxins. It is also known that females exposed to dioxins are often taller.

In European countries, a height increase of about 3.5 mm per year and an approximately one-year decrease in the age at menarche have been reported during the past 30 years. It is difficult to identify the influence of extrinsic endocrine factors on these changes, and no studies addressing this issue have been reported to date. Although several studies have been published concerning the effects of female hormone preparations, including pills used for contraception and hormone replacement therapy in postmenopausal women, no studies have provided data that establish the effects of EDCs.

Endometriosis

Endometriosis is a disease of unexplained origin that is seen in primates with sexual cycles. It has been pointed out that this disease tends to be more severe in individuals exposed to dioxins (2,3,7,8-tetrachlorodibenzo-*p*-dioxin [TCDD] and to PCBs). Data yielded from experiments in rhesus monkeys are used as evidence to corroborate the causal relationship between dioxins and endometriosis. Thus, we cannot rule out the biological plausibility of these effects. However, no reports affirming the causal relationship in humans have been published.

Possibility of other effects on humans

Biological plausibility has also been considered for the following effects of hormone-mimics on humans: qualitative dysfunction of human sperm, effects on neurobehavior of neonates, and immune functions. The effects on immune functions have been suggested by reports of cases with Yu-sho (PCB intoxication).

CONCLUSION

The International Program of Chemical Safety (IPCS), a section of the World Health Organization, has released a Web site publication "Global Assessment of the State-of-the Science of Endocrine Disruptors" (GAED), June 2002 (URL: <http://ehp.niehs.hih.gov/who/>). WHO/IPCS started the GAED program in March 1998 after the publication of *Our Stolen Future* (Theo Colbone et al., 1996). The publication took three years to edit; covering a policy to document all the published pertinent literatures, to summarize them as descriptive manner solely based on those published literatures. Twenty-seven expert scientists and 20 independent peer-reviewers participated in editing the GAED.

Other reports on nonylphenol and octylphenol, released by the Japanese Ministry of Environment (MoE), revealed an "ovotestes" formation that was observed in the assay of the laboratory experimen-

tal fish (*Medaka*) exposed to doses close to those recorded in the monitoring fields in the MoE surveillance. Further, phthalates, such as di-(2-ethylhexyl)phthalate, di-cyclohexylphthalate, and butylbenzylphthalate, as selected and prioritized chemicals by the MoE, showed some unique data in different endpoints, including mRNA expression, in dose ranges lower than those no observed effect levels (NOELs) and/or no observed adverse effect levels (NOAELs) reported previously.

The effects of EDCs on human health are unknown at this moment. However, due to the biologically plausible data currently accumulated, the existence of endocrine disruptions under certain circumstances seems to be a reality. Thus, by the time of the SCOPE/IUPAC symposium, the EDC research for the next stage may shift from plausibility to possibility, and put forward further mechanistic research.

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Implications of Endocrine Active Substances for Humans and Wildlife—a SCOPE/IUPAC Project

- Molecular Mode of Action of Nuclear Receptors: Fundamentals for Understanding the Action of Endocrine Active Substances
- Environmental Fate and Metabolism of Endocrine Active Substances
- Effects of Endocrine Active Chemicals in Rodents and Humans, and Risk Assessments for Humans
- Effects of Endocrine Active Substances in Wildlife Species
- Effectiveness of QSAR for Prescreening of Endocrine Disruptor Hazard
- Toxbogenomics as a Rational Approach to Endocrine Disruptor Research
- The Need for Establishing Integrated Monitoring Programs
- Simple, Rapid Assay for Conventional Definitive Testings of Endocrine Disruptor Hazard
- Precautionary Principle/Approach and Weight of Evidence in Endocrine Disruptor Issues
- Risk Management Options for Endocrine Disruptors in National and International Programs

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Special Topic Issue

Health Hazards of Endocrine-Disrupting Chemicals on Humans as Examined from the Standpoints of Their Mechanisms of Action

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Abstract: Hormonally active compounds were first recognized in "*Silent Spring*" by Rachel Carson in 1962, which implicated pesticides, such as DDT and derivatives. Nearly four decades later, the book "*Our Stolen Figure*," by Theo Colborn *et al.*, and other pertinent publications have revisited and broadened the issue to a variety of chemicals and areas exposed. Translations of these books have just become available in Japan in the past three or four years, and since then Japan has started to join the debate and/or discussion of the global issue of endocrine disruptors—"Environmental Hormones." Although significant numbers of chemicals possessing a hormone-mimicking action have been recognized for many years and based on biological plausibility their receptor-mediating effects strongly suggest effects in humans similar to those seen in wildlife, little is known about the experimental evidence related to human adverse effects. The key issue in resolving the dilemmas posed by the biological plausibility and poor experimental evidence may be to clarify their mechanism of actions at low levels. In other words, the mechanisms of the possible low-dose effects may be resolved simultaneously by defining three major properties threshold, oscillation, and additive-synergism.

Key words: Receptor; Hormone mimics; Homeostasis; Effects at low dosage; Human hazards

Introduction

The objective of this paper is to summarize all the currently available information on the possible hazards of endocrine-disrupting chemicals (EDs) on human health from the stand-

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The Japanese text is a transcript of a lecture originally aired on October 12, 2001, by the Nihon Shortwave Broadcasting Co., Ltd., in its regular program "Special Course in Medicine".

points of the mechanisms of actions of these chemicals.

It is not uncommon to come across agrochemicals and industrial chemicals that have hormone-mimicking effects. These chemicals, the so-called "environmental hormones," often accumulate at detectable levels in the environment, and it has been feared that they may have adverse effects on living beings. Following reports of feminization and decreased colony size of wild creatures, and reports suggesting a possible association of these chemicals with abnormalities of reproductive organs and oncogenesis in human, attention has been focused on the possibility that these occurrences may be associated with exposure to EDs. In this connection, a Japanese translation of the book entitled "*Our Stolen Future*," written by Theo Colborn *et al.*, was published some time ago.

This paper will review the problems related to EDs, the courses of arguments regarding the harmful effects of these chemicals, and current medical topics pertaining to them.

Chemicals with Hormone-Mimicking Actions

Substances with hormone-mimicking effects can be divided into four groups: (1) hormones found *in vivo*, (2) medicines with hormone-mimic actions manufactured for use in hormonal therapy, etc., (3) plant hormones known to exert phytoestrogen-like actions, and (4) chemicals found in environments that can interact with hormone receptors.

In addition, substances which do not interact with hormone receptors but exert effects on gonads by their modifying effects on steroid metabolism may be deemed as hormone-mimics in the broader sense of the term. In this paper, however, emphasis shall be placed on the hormone-mimicking actions mediated by receptors which play essential roles in the mechanism of actions of hormone-mimics.

Characteristics of the Receptor-Mediated Actions of Hormone-Mimics

The receptor-mediated actions of hormone-mimics are fundamentally characterized by the similarity in the structures of the receptors involved, crossing the barrier of species. This characteristic allows us to estimate the possibility of the actions of these chemicals exerted in nature also occurring in humans.

Secondly, since similarities in the structure to various sex steroids and hormones are also known, it is possible that each individual hormone-mimic exerts diverse effects by acting on male hormone receptors, female hormone receptors, receptors in the nuclei (including some unknown receptors), etc.

Thirdly, many of these chemicals are eliminated from the living body in the form of conjugated inactive substances instead of as degraded metabolites. They may also be eliminated in the unchanged form. Therefore, if feces and urine containing these substances are eliminated into river water, it is plausible to imagine that even inactivated hormones can sometimes become active and exert hormone-mimic actions in the environment. This is one of the characteristics unique to this class of chemicals.

Receptor-mediated responses involve many unresolved questions. Various undefined elements may be involved, including the relationship between receptor binding and signals, the relationship between receptor-ligand binding (ligand: substances that can bind to receptors) and the dissociation of ligands from receptors, signal cross-talks, involvement of unknown nuclear receptors, etc.

The actions of these chemicals add to the effects of intrinsic hormones. For this reason, these chemicals may exert their actions in a way different from that known for other chemicals which do not have structural or functional counterparts *in vivo*. For example, stimulation of hormone receptors by these extrinsic chemicals may modify homeostasis *in vivo*, leading

to weakening of the physiological stimulation of these receptors by the intrinsic substances. Therefore, the influence of the continued effects of environmental hormones needs special study.

Pitfall in the Effects of Hormone-Mimics

We must distinguish the interactions of endocrine hormone-mimics with hormone receptors from the hazards caused to endocrine tissue. Bearing this in mind, let us now summarize the problems related to the effects of hormone-mimics.

1. Antagonistic effects on the maintenance of homeostasis

The endocrine system is regulated by homeostatic mechanisms. It is not uncommon for the effects of small amounts of hormone-mimics to interfere slightly with these mechanisms, often with no adverse influence; this is well-known. However, this is not always the case. There seems to be a group of genes that act antagonistically to each other in the maintenance of homeostasis.

With the uterus growth test, which is used to check for estrogenic activity, the ovary is removed in advance and the blood level of the intrinsic female hormone is reduced to the minimum. Under the thus-created extremely undeveloped state of the uterus, the test substance (a chemical or hormone) is administered to check for its effects on the growth of the uterus. This test (checking for growth of the uterus in ovariectomized animals) is designed to evaluate the hormone activity and effects of hormone-mimics under conditions of blockade of homeostasis.

This test method itself is valid. However, there is no sufficient rational evidence that indicates that the responses observed under such indirect control conditions of the living body can serve as an indicator of the health hazards of hormone mimics. Although the ovotestes seen in lower vertebrates may be used if the effects observed were to be valid as such an indicator, there is no consensus on what is valid as an indicator of the health hazards of ED's when mammals are used as experimental animals.

2. Down-regulation of the expression of receptors

It is known that the expression of genes encoding receptors is down-regulated by stimuli, leading to reduced receptor sensitivity. This can lead to a paradoxical outcome wherein the effects observed in the presence of low levels of a substance are not seen at high levels of the same substance. If this phenomenon occurred in individual organisms, the dose-response relationship will be non-linear.

This means that extrapolation of results obtained at high levels of the chemicals to conditions where low levels of the same substance are present would be difficult. It is needed to test the validity of this hypothesis, and analysis of the mechanisms underlying this phenomenon if the hypothesis were indeed valid, are thus important. Studies to resolve these questions are now under way.

3. Data gap concerning the effects of female hormones

In mature women, there are high levels of physiological hormones *in vivo*, and these are subject to cyclic control. It has been proposed that girls with inadequate physical growth begin menstruation at lower ages and undergo sexual maturation earlier than usual, and that hormone-mimics in these subjects can precipitate breast cancer.

The weak links in this hypothesis have been pointed out, and it has been shown experimentally that estrogen by itself may be teratogenic, although this tendency has been shown to be weak. It is known that organisms are programmed such that excessive exposure to estrogens during the intrauterine period or other developmental stages is avoided.

There are many open questions as to the