Table 4. Relative Organ Weights and Body Weights of PND 56 Male Rats Prenatally Exposed to Chemicals

Chemical Dosing period	Group	Males	Litters	Body weight (g)	Testes (mg/g)	Epididymides (mg/g)	Ventral prostate (mg/g)	Seminal Vesicles (mg/g)
FLU								
GD 14-17	Control	12	3	350.2 ± 10.6	8.674 ± 0.105	1.439 ± 0.054	0.791 ± 0.007	1.464 ± 0.188
	1 mg/kg	12	3	331.1 ± 2.2	8.433 ± 0.313	1.481 ± 0.026	0.829 ± 0.060	1.605 ± 0.065
	10 mg/kg	12	3	341.4 ± 27.5	7.982 ± 0.819	1.410 ± 0.091	0.818 ± 0.105	1.657 ± 0.284
GD 18-21	Control	12	3	333.0 ± 15.5	8.364 ± 0.084	1.499 ± 0.066	0.855 ± 0.023	1.651 ± 0.227
	1 mg/kg	12	3	350.0 ± 21.9	8.164 ± 0.115	1.456 ± 0.083	0.798 ± 0.143	1.694 ± 0.164
	10 mg/kg	7	3	346.6 ± 29.3	8.447 ± 0.438	1.460 ± 0.080	0.641 ± 0.115	1.642 ± 0.278
DDE								
GD 14-17	Control	12	3	350.2 ± 10.6	8.674 ± 0.105	1.439 ± 0.054	0.791 ± 0.007	1.464 ± 0.188
	10 mg/kg	12	3	359.1 ± 38.6	8.330 ± 0.125	1.447 ± 0.113	0.862 ± 0.092	1.703 ± 0.206
	30 mg/kg	16	4	368.9 ± 38.3	7.763 ± 0.492**	1.431 ± 0.062	0.946 ± 0.082	1.672 ± 0.201
	100 mg/kg	16	4	340.4 ± 23.4	8.785 ± 0.229	1.465 ± 0.097	0.743 ± 0.102	1.601 ± 0.280
GD 18-21	Control	14	4	338.5 ± 15.6	8.291 ± 0.374	1.444 ± 0.095	0.882 ± 0.062	1.817 ± 0.032
	10 mg/kg	16	4	342.9 ± 15.6	8.308 ± 0.186	1.490 ± 0.058	0.758 ± 0.052	1.794 ± 0.270
	30 mg/kg	14	4	352.7 ± 21.6	7.927 ± 0.285	1.443 ± 0.075	0.903 ± 0.120	1.785 ± 0.142
	100 mg/kg	4	1	339.9	9.027	1.582	1.003	1.816
VZ								
GD 14-17	Control	20	5	355.2 ± 19.5	7.177 ± 0.212	1.463 ± 0.073	0.982 ± 0.089	1.729 ± 0.262
	10 mg/kg	19	5	352.9 ± 6.1	7.486 ± 0.358	1.514 ± 0.080	0.931 ± 0.047	1.752 ± 0.068
	30 mg/kg	19	5	344.6 ± 18.9	7.809 ± 0.699	1.489 ± 0.139	0.992 ± 0.124	1.840 ± 0.308
	100 mg/kg	20	1	369.2 ± 6.4	7.404 ± 0.608	1.489 ± 0.062	0.755 ± 0.093**	1.675 ± 0.213
GD 18-21	Control	20	5	363.8 ± 49.2	7.766 ± 0.955	1.523 ± 0.169	0.970 ± 0.131	1.869 ± 0.210
	10 mg/kg	16	4	358.5 ± 32.8	7.768 ± 0.493	1.596 ± 0.166	1.007 ± 0.178	1.996 ± 0.327
	30 mg/kg	16	4	357.4 ± 16.1	7.525 ± 0.405	1.597 ± 0.084	0.934 ± 0.095	1.830 ± 0.339
	100 mg/kg	15	4	357.2 ± 19.7	7.609 ± 0.309	1.517 ± 0.066	0.963 ± 0.118	1.771 ± 0.119
DES								
GD 14-17	Control	19	5	347.0 ± 23.9	7.980 ± 1.152	1.454 ± 0.106	0.983 ± 0.132	1.922 ± 0.086
	0.1 μg/kg	12	3	353.6 ± 11.7	7.802 ± 0.485	1.581 ± 0.043	1.091 ± 0.147	1.902 ± 0.239
	1 μg/kg	20	5	358.3 ± 22.7	7.639 ± 0.619	1.527 ± 0.105	1.004 ± 0.081	1.850 ± 0.092
	10 μg/kg	16	4	363.0 ± 23.6	7.966 ± 0.554	1.626 ± 0.088	1.037 ± 0.102	2.120 ± 0.187
	100 μg/kg	16	4	373.5 ± 13.7	7.337 ± 0.523	1.532 ± 0.084	0.913 ± 0.029	1.888 ± 0.243
	300 μg/kg	12	4	350.4 ± 20.1	7.888 ± 0.320	1.592 ± 0.062	0.718 ± 0.058**	1.885 ± 0.251
GD 18-21	Control	16	4	343.8 ± 11.2	8.006 ± 0.610	1.609 ± 0.102	1.115 ± 0.040	1.872 ± 0.144
	0.1 μg/kg	20	5	353.3 ± 20.7	8.369 ± 1.418	1.491 ± 0.072	1.100 ± 0.199	1.853 ± 0.263
	1 μg/kg	16	4	370.1 ± 25.7	7.110 ± 0.642	1.517 ± 0.161	1.033 ± 0.094	1.866 ± 0.238
	10 μg/kg	20	5	354.8 ± 15.0	7.959 ± 0.389	1.633 ± 0.116	1.050 ± 0.113	1.974 ± 0.290
	100 μg/kg	20	5	354.9 ± 10.2	7.457 ± 0.337	1.577 ± 0.079	0.753 ± 0.081**	1.743 ± 0.136
	300 μg/kg	7	2	316.5 ± 2.8	8.228 ± 0.183	1.664 ± 0.025	0.668 ± 0.134**	1.991 ± 0.364
EE								
GD 14-17	Control	16	4	364.1 ± 19.7	7.912 ± 0.032	1.583 ± 0.122	0.954 ± 0.095	1.918 ± 0.116
	10 μg/kg	16	4	333.8 ± 21.5	7.693 ± 0.280	1.477 ± 0.076	0.967 ± 0.145	1.877 ± 0.185
	100 μg/kg	20	5	354.9 ± 17.0	7.745 ± 0.189	1.538 ± 0.086	0.980 ± 0.165	1.993 ± 0.197
GD 18-21	Control	19	5	358.5 ± 14.1	7.677 ± 0.424	1.477 ± 0.112	0.949 ± 0.092	1.967 ± 0.185
	10 μg/kg	15	4	372.0 ± 16.2	7.752 ± 0.454	1.539 ± 0.073	1.036 ± 0.124	2.106 ± 0.062
	100 μg/kg	16	4	364.8 ± 6.2	7.791 ± 0.475	1.618 ± 0.062	0.923 ± 0.089	2.070 ± 0.173
TAM								
GD 14-17	Control	16	4	364.1 ± 19.7	7.912 ± 0.032	1.583 ± 0.122	0.954 ± 0.095	1.918 ± 0.116
	0.03 mg/kg	15	4	356.8 ± 26.7	7.808 ± 0.311	1.491 ± 0.116	0.996 ± 0.159	1.948 ± 0.285
	0.1 mg/kg	7	2	360.7 ± 13.4	7.320 ± 0.038	1.458 ± 0.117	0.903 ± 0.014	1.659 ± 0.076
GD 18-21	Control	19	5	358.5 ± 14.1	7.677 ± 0.424	1.477 ± 0.112	0.949 ± 0.092	1.967 ± 0.185
	0.03 mg/kg	12	3	359.8 ± 44.0	7.142 ± 0.660	1.469 ± 0.030	0.932 ± 0.174	1.869 ± 0.277
	0.1 mg/kg	11	3	376.4 ± 49.8	7.475 ± 0.202	1.513 ± 0.020	0.949 ± 0.036	1.731 ± 0.093

Value: Mean ± S.D. calculated using the litter as the unit.

FLU: flutamide; DDE: *p,p'*-dichlorodiphenyldichloroethylene; VZ: vinclozolin; DES: diethylstilbestrol; EE: ethynylestradiol; TAM: tamoxifen; GD: gestational day; PND: postnatal day.

**: significantly different from control, $p<0.01$.

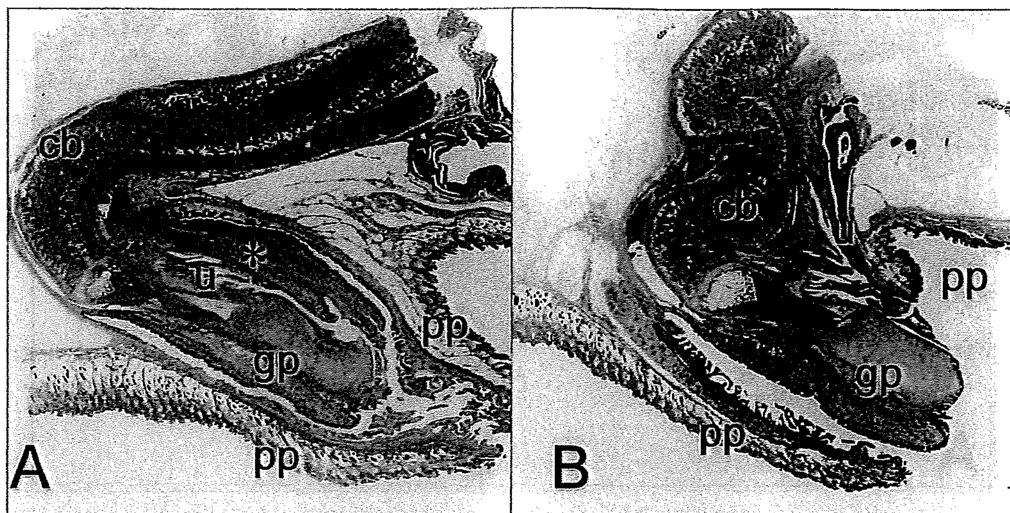

Fig. 3. Sagittal sections of the prepuce and penis of male rats sacrificed on PND 56.
A: Control male rat. The prepuce (pp) is separated from the glans penis (gp). The urethra (u) is located in the center of the glans penis. *: ventral half of the glans penis. cb: cavernous body. H&E staining, magnification: × 6. B: Male rat from a dam exposed to 100 mg/kg vinclozolin on GD 14–17. The prepuce (pp) is separated from the glans penis (gp) at the dorsal part, and the urethra (u) is located between the glans penis and subcutis. The prepuce is hypoplastic and the glans penis is not completely covered by the prepuce. The cavernous body (cb) of the penis is tortuous and bent. H&E staining, magnification: × 6.

difference in AGD. Body weights at the time of AGD measurement did not show any significant difference.

5) Organ weight

Relative organ weights of males prenatally exposed to chemicals are shown in Table 4. Changes in absolute organ weights were similar to changes in relative organ weights. Relative weights of the ventral prostate were decreased significantly in males from dams exposed to 100 mg/kg of VZ on GD 14–17, 300 μg/kg of DES on GD 14–17, and 100 and 300 μg/kg of DES on GD 18–21. Although there was a significant decrease in relative organ weight of the testes in the DDE group, a dose dependent relationship was not detected.

6) Pathological examination

Histopathological examination of representative males from dams exposed to 10 mg/kg of FLU or 100 mg/kg of VZ on GD 14–17 revealed a defect in the ventral half of the glans penis (cleft phallus). The urethra was not located in the center of the glans penis, but instead was observed at the ventral surface of the penis (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. 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 preputial separation did not progress at the ventral aspect. The external urethral orifice opened at the ventral surface of the penis (hypospadias). These males with cleft phallus showed a tortuous cavernous body of the penis (Fig. 3B). On PND 6, hypoplasia of the ventral half of the glans penis and tortuous cavernous body were observed in male pups from dams exposed to 100 mg/kg of VZ on GD 14–17 (Fig. 4B).

Ectopic testes were induced by FLU and VZ, and showed severe atrophy of the seminiferous tubule in PND 56 males. Severe prostatitis and seminal vesiculitis were observed in males from dams exposed to 10 mg/kg of FLU on GD 18–21. Neutrophils, lymphocytes and macrophages infiltrated the prostate, seminal vesicles, and surrounding tissues of five animals which died (PND 44, 45, 52, 55 and 56) and two sacrificed (PND 56) males. In some cases, hemorrhage was observed in the muscular layer of the urinary bladder. The males with severe prostatitis and vesiculitis did not show hypoplasia in the ventral half of the glans penis.

Postnatal exposure

1) Preputial separation

The day of preputial separation and the body weights on the day of preputial separation were statistically analyzed using both the litter as the unit and the individual data. There were no significant changes on the day of preputial separation of males exposed to FLU, DDE or VZ on PND 1–5 (Table 5). On the other hand, the day of preputial separation was significantly delayed in groups exposed to 10 and 30 mg/kg of FLU and 30 mg/kg of VZ on PND 35–39. Statistical analyses using the individual data revealed additional significances in the delay of preputial separation in the groups exposed 300 mg/kg of DDE on PND 17–21 and 100 mg/kg on PND 35–39, and 100 mg/kg of VZ on PND 35–39. In the DDE group, 10 of 12 males exposed to 300 mg/kg of DDE on PND 35–39 died before maturation.

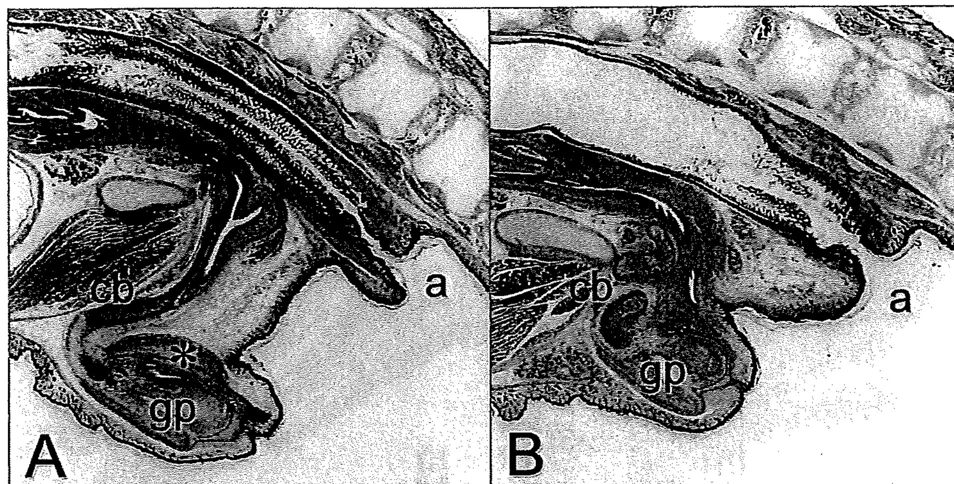

Fig. 4. Sagittal sections of the genital tubercle of males sacrificed on PND 6.
A: Control male rat. Glans penis (gp) is bordered with dorsal and ventral epithelium. The urethra is located in the center of the glans penis. a: anus. *: ventral half of the glans penis. H&E staining, magnification: × 8. B: A male rat from a dam exposed to 100 mg/kg vinclozolin on GD 14–17. The dorsal part of the glans penis is bordered with epithelium. The urethra is observed at the ventral surface of the glans penis (gp) and the cavernous body (cb) shows tortuous structure. a: anus. H&E staining, magnification: × 8.

Preputial separation in two surviving males (PND 48 and 50) was delayed compared to controls. A significant delay was not detected in males exposed to FLU or VZ on PND 17–21.

DES induced a significant delay in the 300 μg/kg group exposed on PND 1–5 or PND 35–39, while 2 of 16 males in the 100 μg/kg group and 3 of 16 males in the 300 μg/kg group exposed on PND 1–5 showed incomplete preputial separation on PND 56. In statistical analyses using the individual data, significant delays of preputial separation were also observed in the group exposed to 100 μg/kg of DES on PND 1–5 or 35–39. There were no significant delays in males exposed on PND 17–21.

In EE treatment, preputial separation was significantly delayed in males of the 100 μg/kg group exposed on PND 1–5, and 1 of 12 males in the 10 μg/kg group and 10 of 12 males in the 100 μg/kg group had incomplete separation on PND 56. Administration of 10 μg/kg of EE on PND 6–10 or PND 11–15 was added to the experimental protocol to define the sensitive period, because PND 1–5 exposure induced a marked effect on preputial separation. Although a delay in preputial separation was observed after PND 6–10 exposure, there was no significant difference between the PND 11–15 exposed group and controls. The 3 mg/kg TAM treated groups exposed on PND 1–5 also showed a significant delay in preputial separation, and 5 of 16 males in the 1 mg/kg group and 14 of 15 males in the 3 mg/kg group had incomplete separation on PND 56. Slight delay in preputial separation was also observed in the 0.3 mg/kg TAM group, and 2 of 16 males had incomplete separation on PND 56. In the statistical analyses using the individual data, significant delays of preputial separation were also observed in the groups exposed to 10 μg/kg of EE and 1 mg/kg of TAM on PND 1–5. No influence of EE and TAM treatments on PND 17–21 or PND 35–39 was detected.

Body weights on the day of preputial separation showed a higher value in the groups with a delay of separation.

2) Organ weight

Relative organ weights of males postnatally exposed to the chemicals are presented in Table 6 using the litter as the unit and in Table 7 using the individual data. Absolute organ weights showed similar changes to those of relative organ weights. Relative weights of the ventral prostate were decreased significantly in males exposed to 30 mg/kg of FLU on PND 35–39. No significant changes were observed in males exposed to FLU on PND 1–5 or 17–21, and DDE and VZ in any period.

DES caused a significant reduction in the relative weight of the ventral prostate in the 100 and 300 μg/kg groups exposed on PND 1–5. No significant changes were observed in males exposed to DES on PND 17–21 or PND 35–39. EE caused reductions in the testes, ventral prostate and seminal vesicles of males in the 100 μg/kg group and seminal vesicles of males in the 10 μg/kg group exposed to EE on PND 1–5. No significant changes in relative weight were observed in males exposed on PND 17–21 or PND 35–39. TAM treatment on PND 1–5 led to reductions in body and reproductive organ weights in the 3 mg/kg group and the ventral prostate weight in the 1 mg/kg group. No weight reductions were observed in the reproductive organs of males exposed to TAM on PND 17–21 or PND 35–39. Although the statistical analyses using the individual data showed significant results in the lower dose group and the other organs, there were no significances in the groups exposed to VZ.

Table 5. Preputial Separation and Body Weights of Male Rats Postnatally Exposed to Chemicals

Chemical	Dosing period	Group	Litters	PND of preputial separation	BW (g) on the day of PPS	Males	PND of preputial separation	BW (g) on the day of PPS	Incomplete separation
FLU	PND 1-5	Control	4	44.4 ± 2.3	268.4 ± 8.9	16	44.4 ± 2.4	268.4 ± 21.2	0
		1 mg/kg	4	42.7 ± 2.4	246.5 ± 21.6	16	42.7 ± 2.3	246.5 ± 22.4	0
		10 mg/kg	4	42.8 ± 2.1	257.6 ± 15.2	16	42.8 ± 2.1	257.6 ± 21.4	0
		30 mg/kg	4	43.9 ± 1.1	252.7 ± 19.6	15	44.0 ± 2.0	252.7 ± 27.9	0
	PND 17-21	Control	3	44.3 ± 0.4	258.3 ± 7.9	12	44.3 ± 1.4	258.3 ± 19.0	0
		1 mg/kg	3	45.0 ± 2.9	256.7 ± 20.1	12	45.0 ± 3.2	256.7 ± 29.7	0
		10 mg/kg	3	43.2 ± 1.9	250.6 ± 17.5	12	43.2 ± 2.0	250.6 ± 23.5	0
		30 mg/kg	3	43.6 ± 2.5	256.5 ± 28.2	12	43.6 ± 2.5	256.5 ± 26.9	0
	PND 35-39	Control	4	43.5 ± 1.4	253.1 ± 16.9	16	43.5 ± 1.7	253.1 ± 18.6	0
		1 mg/kg	4	44.6 ± 0.8	272.2 ± 9.6	16	44.6 ± 1.4	272.2 ± 20.4	0
		10 mg/kg	4	46.8 ± 1.2**	287.8 ± 9.2*	15	46.8 ± 2.0**	287.9 ± 19.6**	0
		30 mg/kg	4	47.1 ± 1.4**	294.5 ± 24.7*	16	47.1 ± 2.2**	294.5 ± 30.0**	0
DDE	PND 1-5	Control	3	42.3 ± 1.1	239.8 ± 8.6	12	42.3 ± 1.7	239.8 ± 13.5	0
		10 mg/kg	3	42.0 ± 0.7	243.3 ± 8.4	12	42.0 ± 1.3	243.3 ± 17.5	0
		30 mg/kg	3	40.9 ± 1.2	227.3 ± 7.5	12	40.9 ± 1.7	227.3 ± 15.4	0
		100 mg/kg	3	42.1 ± 0.2	242.2 ± 14.3	11	42.1 ± 1.2	240.8 ± 19.9	0
		300 mg/kg	3	42.2 ± 0.6	248.5 ± 13.5	12	42.2 ± 0.9	248.5 ± 17.8	0
	PND 17-21	Control	3	43.6 ± 0.1	243.4 ± 2.6	12	43.6 ± 0.8	243.4 ± 16.2	0
		10 mg/kg	3	43.8 ± 0.9	256.4 ± 10.5	12	43.8 ± 1.4	256.4 ± 15.5	0
		30 mg/kg	3	43.9 ± 1.1	246.1 ± 16.9	11	43.9 ± 1.6	245.1 ± 21.2	0
		100 mg/kg	3	44.5 ± 0.3	246.7 ± 3.8	12	44.5 ± 1.3	246.7 ± 19.5	0
		300 mg/kg	4	45.6 ± 1.4	261.0 ± 20.3	13	45.5 ± 1.4**	259.1 ± 21.5	0
	PND 35-39	Control	3	42.1 ± 1.2	210.0 ± 21.7	12	42.1 ± 1.3	210.0 ± 22.2	0
		10 mg/kg	3	43.1 ± 1.4	238.4 ± 19.7	12	43.1 ± 1.3	238.4 ± 20.0**	0
		30 mg/kg	3	43.4 ± 2.7	232.3 ± 11.1	12	43.4 ± 2.5	232.3 ± 18.1*	0
		100 mg/kg	3	46.4 ± 3.1	264.7 ± 5.1**	12	46.4 ± 3.3**	264.7 ± 16.4**	0
VZ	PND 1-5	Control	4	41.9 ± 0.9	244.3 ± 9.5	16	41.9 ± 1.5	244.3 ± 16.3	0
		10 mg/kg	3	42.8 ± 0.9	253.7 ± 19.0	12	42.8 ± 1.4	253.7 ± 24.9	0
		30 mg/kg	4	42.5 ± 1.6	252.7 ± 17.9	15	42.3 ± 1.7	251.2 ± 19.2	0
		100 mg/kg	4	41.6 ± 0.1	242.4 ± 13.1	16	41.6 ± 1.0	242.4 ± 15.9	0
	PND 17-21	Control	4	44.6 ± 1.4	249.3 ± 14.9	16	44.6 ± 2.2	249.3 ± 19.6	0
		10 mg/kg	4	44.1 ± 0.9	253.6 ± 6.1	16	44.1 ± 1.8	253.6 ± 13.4	0
		30 mg/kg	4	43.8 ± 0.9	257.7 ± 10.5	16	43.8 ± 1.8	257.7 ± 19.2	0
		100 mg/kg	4	42.6 ± 0.8	242.2 ± 14.6	16	42.6 ± 1.2**	242.2 ± 16.1	0
	PND 35-39	Control	4	43.6 ± 1.9	248.0 ± 4.7	16	43.6 ± 2.0	248.0 ± 14.2	0
		10 mg/kg	3	43.9 ± 0.8	256.4 ± 17.1	12	43.9 ± 1.4	256.4 ± 18.7	0
		30 mg/kg	3	46.8 ± 0.7*	267.4 ± 22.2	12	46.8 ± 1.4**	267.4 ± 29.0	0
		100 mg/kg	3	45.5 ± 1.1	261.5 ± 12.5	12	45.5 ± 1.7*	261.5 ± 17.9	0
DES	PND 1-5	Control	4	44.4 ± 1.9	259.5 ± 27.5	16	44.4 ± 2.5	259.5 ± 30.4	0
		10 μg/kg	4	44.9 ± 0.8	261.4 ± 25.1	16	44.9 ± 1.5	261.4 ± 27.5	0
		100 μg/kg	4	48.9 ± 3.4	311.8 ± 42.0	16	48.9 ± 4.0**	311.8 ± 46.0**	2 (2)
		300 μg/kg	4	49.9 ± 3.0*	288.0 ± 33.5	16	49.9 ± 3.9**	288.0 ± 39.8	3 (2)
	PND 17-21	Control	4	44.5 ± 1.3	242.7 ± 16.0	15	44.5 ± 2.1	241.2 ± 21.7	0
		10 μg/kg	3	46.3 ± 1.6	250.4 ± 7.2	12	46.3 ± 2.3	250.4 ± 19.9	0
		100 μg/kg	4	44.8 ± 0.9	243.3 ± 11.3	16	44.8 ± 1.1	243.3 ± 15.3	0
		300 μg/kg	4	45.4 ± 1.0	253.1 ± 12.7	16	45.4 ± 2.0	253.1 ± 22.2	0
	PND 35-39	Control	4	44.4 ± 1.9	251.6 ± 19.3	16	44.4 ± 2.2	251.6 ± 24.6	0
		10 μg/kg	3	43.9 ± 0.8	240.8 ± 32.7	12	43.9 ± 1.6	240.8 ± 32.5	0
		100 μg/kg	4	46.3 ± 1.4	243.3 ± 14.8	16	46.3 ± 2.4*	243.3 ± 19.8	0
		300 μg/kg	4	48.7 ± 2.2*	267.5 ± 34.1	16	48.7 ± 2.6**	267.5 ± 34.0	0
EE	PND 1-5	Control	4	43.3 ± 0.6	267.5 ± 4.8	16	43.3 ± 1.6	267.5 ± 17.4	0
		10 μg/kg	3	47.9 ± 4.3	282.7 ± 38.6	12	47.9 ± 4.6*	282.7 ± 39.3	1 (1)
		100 μg/kg	3	55.4 ± 1.0*	365.8 ± 9.1	12	55.4 ± 1.5**	365.8 ± 16.3**	10 (3)
	PND 6-10	Control	3	43.8 ± 1.1	245.7 ± 15.3	12	43.8 ± 1.8	245.7 ± 18.3	0
		10 μg/kg	4	48.4 ± 1.2**	287.3 ± 10.3**	16	48.4 ± 3.5**	287.3 ± 35.2**	1 (1)
	PND 11-15	Control	3	43.2 ± 0.9	257.0 ± 10.9	12	43.2 ± 1.2	257.0 ± 12.3	0
		10 μg/kg	4	44.1 ± 1.0	264.5 ± 14.1	16	44.1 ± 1.2	264.5 ± 19.3	0
	PND 17-21	Control	3	44.0 ± 0.8	243.7 ± 22.0	12	44.0 ± 1.1	243.7 ± 23.7	0
		10 μg/kg	2	43.8 ± 0.7	239.4 ± 20.9	8	43.8 ± 1.9	239.4 ± 18.8	0
		100 μg/kg	3	43.8 ± 0.8	252.7 ± 6.6	12	43.8 ± 1.7	252.7 ± 13.0	0
	PND 35-39	Control	4	44.3 ± 0.4	251.7 ± 16.9	16	44.3 ± 1.1	251.7 ± 22.5	0
		10 μg/kg	4	43.4 ± 1.5	240.0 ± 18.1	16	43.4 ± 1.9	240.0 ± 22.6	0
		100 μg/kg	4	45.9 ± 1.9	254.0 ± 24.2	16	45.9 ± 2.2	254.0 ± 28.3	0
TAM	PND 1-5	Control	4	43.3 ± 0.6	267.5 ± 4.8	16	43.3 ± 1.6	267.5 ± 17.4	0
		0.3 mg/kg	4	46.1 ± 4.5	276.8 ± 51.9	16	46.1 ± 4.6	276.8 ± 54.0	2 (1)
		1 mg/kg	4	49.8 ± 5.1	291.3 ± 47.9	16	49.8 ± 5.0**	291.3 ± 50.2	5 (2)
		3 mg/kg	4	55.8 ± 0.5**	323.0 ± 23.5	15	55.7 ± 1.0**	325.2 ± 29.6**	14 (4)
	PND 17-21	Control	4	44.6 ± 1.4	249.3 ± 14.9	16	44.6 ± 2.2	249.3 ± 19.6	0
		0.3 mg/kg	3	43.3 ± 1.3	244.4 ± 15.1	11	43.2 ± 2.0	243.3 ± 25.6	0
		1 mg/kg	4	43.9 ± 1.8	243.1 ± 3.8	15	43.7 ± 3.2	243.2 ± 27.3	0
		3 mg/kg	3	43.7 ± 2.1	246.5 ± 14.4	12	43.7 ± 2.3	246.5 ± 17.8	0
	PND 35-39	Control	4	43.6 ± 1.9	248.0 ± 4.7	16	43.6 ± 2.0	248.0 ± 14.2	0
		0.3 mg/kg	3	45.2 ± 0.8	246.0 ± 27.7	12	45.2 ± 2.1	246.0 ± 29.7	0
		1 mg/kg	3	44.5 ± 1.3	235.4 ± 13.9	12	44.5 ± 3.2	235.4 ± 26.3	0
		3 mg/kg	3	44.6 ± 1.3	231.6 ± 10.2	12	44.6 ± 1.6	231.6 ± 14.2	0

Value: Mean ± S.D. using the litter as the unit or using individual data.

PND: postnatal day; PPS: preputial separation; FLU: flutamide; DDE: *p,p'*-dichlorodiphenyldichloroethylene; VZ: vinclozolin; DES: diethylstilbestrol; EE: ethynylestradiol; TAM: tamoxifen; BW: body weight.

Incomplete separation: number of animals (litters) with incomplete separation on PND 56.

*: significantly different from control, p<0.05; **: significantly different from control, p<0.01.

Table 6. Relative Organ Weights and Body Weights of PND 56 Male Rats Postnatally Exposed to Chemicals (Using the Litter as the Unit)

Chemical	Dosing period	Group	Litters	Body weight (g)	Testes (mg/g)	Epididymides (mg/g)	Ventral prostate (mg/g)	Seminal Vesicles (mg/g)
FLU	PND 1-5	Control	4	384.1 ± 19.6	7.281 ± 0.714	1.400 ± 0.079	1.025 ± 0.137	2.008 ± 0.289
		1 mg/kg	4	378.1 ± 15.6	7.536 ± 0.457	1.511 ± 0.121	0.986 ± 0.175	1.895 ± 0.239
		10 mg/kg	4	389.8 ± 28.3	7.441 ± 0.713	1.443 ± 0.074	0.998 ± 0.112	1.774 ± 0.216
		30 mg/kg	4	371.4 ± 17.0	7.594 ± 0.583	1.438 ± 0.087	1.050 ± 0.099	1.806 ± 0.244
	PND 17-21	Control	3	376.2 ± 5.9	7.446 ± 0.481	1.438 ± 0.082	0.989 ± 0.051	1.758 ± 0.220
		1 mg/kg	3	358.8 ± 11.3	7.389 ± 0.075	1.489 ± 0.214	1.007 ± 0.093	1.835 ± 0.266
		10 mg/kg	3	372.1 ± 14.1	7.508 ± 0.469	1.451 ± 0.062	0.951 ± 0.188	1.918 ± 0.064
		30 mg/kg	3	376.4 ± 16.4	7.707 ± 0.172	1.484 ± 0.040	1.043 ± 0.041	1.923 ± 0.349
	PND 35-39	Control	4	377.3 ± 30.6	7.654 ± 0.302	1.520 ± 0.039	1.068 ± 0.076	2.065 ± 0.132
		1 mg/kg	4	382.8 ± 15.8	8.108 ± 0.773	1.603 ± 0.153	1.097 ± 0.112	2.108 ± 0.099
		10 mg/kg	4	378.5 ± 19.9	7.742 ± 0.725	1.407 ± 0.118	1.051 ± 0.121	1.774 ± 0.235
		30 mg/kg	4	381.1 ± 15.5	7.951 ± 0.378	1.398 ± 0.093	0.866 ± 0.106*	1.788 ± 0.131
DDE	PND 1-5	Control	3	362.5 ± 6.2	7.760 ± 0.192	1.447 ± 0.068	0.809 ± 0.084	1.942 ± 0.217
		10 mg/kg	3	382.2 ± 10.3	7.122 ± 0.178	1.395 ± 0.067	0.852 ± 0.113	1.874 ± 0.138
		30 mg/kg	3	367.2 ± 27.2	7.670 ± 0.261	1.443 ± 0.091	0.928 ± 0.074	1.893 ± 0.259
		100 mg/kg	3	369.8 ± 19.6	7.458 ± 0.358	1.421 ± 0.052	0.826 ± 0.020	1.792 ± 0.049
		300 mg/kg	3	374.0 ± 13.3	8.024 ± 0.356	1.398 ± 0.086	0.749 ± 0.011	1.783 ± 0.185
	PND 17-21	Control	3	358.6 ± 11.8	8.522 ± 0.979	1.539 ± 0.052	0.815 ± 0.136	1.724 ± 0.319
		10 mg/kg	3	377.0 ± 7.0	7.804 ± 0.504	1.465 ± 0.128	0.893 ± 0.006	1.820 ± 0.284
		30 mg/kg	3	359.7 ± 12.9	7.864 ± 0.469	1.497 ± 0.098	0.864 ± 0.058	1.674 ± 0.171
		100 mg/kg	3	362.8 ± 2.4	8.263 ± 0.261	1.502 ± 0.012	0.840 ± 0.059	1.751 ± 0.095
		300 mg/kg	4	361.9 ± 33.6	8.344 ± 0.668	1.423 ± 0.088	0.848 ± 0.127	1.550 ± 0.114
	PND 35-39	Control	3	333.5 ± 26.1	7.844 ± 0.454	1.520 ± 0.116	0.925 ± 0.205	1.834 ± 0.318
		10 mg/kg	3	356.0 ± 22.3	7.362 ± 0.622	1.323 ± 0.115	0.875 ± 0.017	1.831 ± 0.110
		30 mg/kg	3	347.2 ± 40.7	7.500 ± 0.215	1.376 ± 0.028	0.908 ± 0.067	1.807 ± 0.172
		100 mg/kg	3	353.5 ± 23.0	7.823 ± 0.247	1.409 ± 0.108	0.847 ± 0.065	1.523 ± 0.249
VZ	PND 1-5	Control	4	367.5 ± 20.6	7.990 ± 0.894	1.601 ± 0.142	1.049 ± 0.151	2.151 ± 0.180
		10 mg/kg	3	382.0 ± 32.7	7.392 ± 0.531	1.477 ± 0.047	1.006 ± 0.055	2.059 ± 0.083
		30 mg/kg	4	380.1 ± 9.5	7.986 ± 0.644	1.500 ± 0.133	0.910 ± 0.118	2.049 ± 0.093
		100 mg/kg	4	377.9 ± 17.6	7.373 ± 0.152	1.582 ± 0.109	1.030 ± 0.094	2.116 ± 0.082
	PND 17-21	Control	4	357.5 ± 26.3	7.791 ± 0.654	1.432 ± 0.058	0.967 ± 0.094	1.903 ± 0.300
		10 mg/kg	4	366.8 ± 9.9	7.981 ± 0.225	1.562 ± 0.080	0.987 ± 0.018	2.041 ± 0.239
		30 mg/kg	4	378.3 ± 17.4	7.629 ± 0.610	1.550 ± 0.147	0.891 ± 0.105	1.931 ± 0.237
		100 mg/kg	4	373.2 ± 25.5	8.066 ± 0.507	1.532 ± 0.137	1.018 ± 0.103	2.105 ± 0.135
	PND 35-39	Control	4	369.8 ± 20.8	7.711 ± 0.696	1.502 ± 0.053	0.895 ± 0.041	1.752 ± 0.115
		10 mg/kg	3	373.3 ± 39.0	7.420 ± 0.420	1.572 ± 0.114	0.910 ± 0.048	1.911 ± 0.081
		30 mg/kg	3	361.7 ± 32.0	8.125 ± 0.253	1.546 ± 0.018	0.982 ± 0.042	1.885 ± 0.053
		100 mg/kg	3	363.1 ± 5.5	7.719 ± 0.280	1.508 ± 0.088	0.941 ± 0.058	1.907 ± 0.083
DES	PND 1-5	Control	4	367.5 ± 25.0	8.089 ± 0.473	1.560 ± 0.151	1.064 ± 0.097	1.948 ± 0.141
		10 μg/kg	4	365.7 ± 24.8	7.296 ± 0.588	1.391 ± 0.100	0.917 ± 0.139	1.870 ± 0.364
		100 μg/kg	4	383.6 ± 13.7	8.277 ± 1.331	1.431 ± 0.121	0.769 ± 0.088**	1.668 ± 0.537
		300 μg/kg	4	343.6 ± 19.7	7.840 ± 1.413	1.585 ± 0.206	0.829 ± 0.076*	1.323 ± 0.086
	PND 17-21	Control	4	343.3 ± 32.2	7.753 ± 0.315	1.554 ± 0.155	0.925 ± 0.046	1.952 ± 0.302
		10 μg/kg	3	337.2 ± 20.4	7.906 ± 0.530	1.432 ± 0.075	0.922 ± 0.083	1.781 ± 0.050
		100 μg/kg	4	348.9 ± 19.6	6.940 ± 0.696	1.455 ± 0.105	0.900 ± 0.095	1.939 ± 0.281
		300 μg/kg	4	352.8 ± 13.8	7.309 ± 0.751	1.417 ± 0.093	0.933 ± 0.099	1.935 ± 0.317
	PND 35-39	Control	4	363.7 ± 38.2	7.750 ± 0.452	1.542 ± 0.103	1.052 ± 0.045	2.303 ± 0.295
		10 μg/kg	3	344.9 ± 40.5	7.472 ± 0.696	1.593 ± 0.146	1.035 ± 0.113	1.963 ± 0.221
		100 μg/kg	4	330.4 ± 23.8	7.920 ± 0.561	1.556 ± 0.024	0.987 ± 0.160	1.910 ± 0.240
		300 μg/kg	4	336.5 ± 36.0	7.986 ± 0.322	1.611 ± 0.100	0.970 ± 0.075	2.017 ± 0.201
EE	PND 1-5	Control	4	391.7 ± 14.7	7.908 ± 0.276	1.533 ± 0.097	1.024 ± 0.084	1.947 ± 0.140
		10 μg/kg	3	367.0 ± 22.8	7.681 ± 0.568	1.477 ± 0.094	0.850 ± 0.087	1.548 ± 0.136*
		100 μg/kg	3	374.0 ± 6.1	6.920 ± 0.110*	1.394 ± 0.112	0.700 ± 0.096**	1.220 ± 0.123**
	PND 17-21	Control	3	353.9 ± 32.7	7.154 ± 0.245	1.456 ± 0.062	0.908 ± 0.127	1.978 ± 0.041
		10 μg/kg	2	357.0 ± 27.3	7.201 ± 0.472	1.366 ± 0.099	1.013 ± 0.143	1.875 ± 0.179
		100 μg/kg	3	373.1 ± 21.3	6.980 ± 0.316	1.521 ± 0.049	1.053 ± 0.160	2.118 ± 0.156
	PND 35-39	Control	4	357.4 ± 26.0	7.766 ± 0.479	1.567 ± 0.098	1.078 ± 0.174	1.954 ± 0.102
		10 μg/kg	4	353.6 ± 9.4	7.586 ± 0.739	1.569 ± 0.131	0.960 ± 0.084	1.910 ± 0.236
		100 μg/kg	4	347.1 ± 27.6	7.637 ± 0.700	1.517 ± 0.119	0.936 ± 0.104	1.783 ± 0.262
TAM	PND 1-5	Control	4	391.7 ± 14.7	7.908 ± 0.276	1.533 ± 0.097	1.024 ± 0.084	1.947 ± 0.140
		0.3 mg/kg	4	368.7 ± 20.3	7.153 ± 0.691	1.498 ± 0.084	0.813 ± 0.120	1.625 ± 0.271
		1 mg/kg	4	356.4 ± 22.4	6.756 ± 1.014	1.454 ± 0.198	0.790 ± 0.128*	1.601 ± 0.242
		3 mg/kg	4	325.6 ± 25.8**	5.011 ± 0.351**	1.159 ± 0.042**	0.596 ± 0.112**	1.197 ± 0.257**
	PND 17-21	Control	4	357.5 ± 26.3	7.791 ± 0.654	1.432 ± 0.058	0.967 ± 0.094	1.903 ± 0.300
		0.3 mg/kg	3	365.3 ± 22.5	7.788 ± 0.213	1.524 ± 0.155	0.935 ± 0.178	1.937 ± 0.160
		1 mg/kg	4	351.9 ± 31.7	7.990 ± 0.766	1.541 ± 0.119	0.972 ± 0.264	1.871 ± 0.139
		3 mg/kg	3	363.5 ± 15.9	7.457 ± 0.510	1.516 ± 0.095	0.927 ± 0.131	2.060 ± 0.090
	PND 35-39	Control	4	369.8 ± 20.8	7.711 ± 0.696	1.502 ± 0.053	0.895 ± 0.041	1.752 ± 0.115
		0.3 mg/kg	3	348.8 ± 37.5	7.357 ± 0.352	1.548 ± 0.074	0.900 ± 0.075	1.733 ± 0.126
		1 mg/kg	3	348.8 ± 37.4	8.189 ± 0.358	1.581 ± 0.136	0.959 ± 0.079	1.909 ± 0.235
		3 mg/kg	3	339.8 ± 10.0	8.159 ± 0.212	1.626 ± 0.133	0.934 ± 0.119	1.866 ± 0.138

Value: Mean ± S.D. calculated using the litter as the unit.

FLU: flutamide; DDE: *p,p'*-dichlorodiphenyldichloroethylene; VZ: vinclozolin; DES: diethylstilbestrol; EE: ethynylestradiol; TAM: tamoxifen; PND: postnatal day.

*: significantly different from control, $p<0.05$; **: significantly different from control, $p<0.01$.

Table 7. Relative Organ Weights and Body Weights of PND 56 Male Rats Postnatally Exposed to Chemicals (Using the Individual Data)

Chemical	Dosing period	Group	Males	Body weight (g)	Testes (mg/g)	Epididymides (mg/g)	Ventral prostate (mg/g)	Seminal Vesicles (mg/g)
FLU	PND 1-5	Control	15	383.1 ± 24.2	7.294 ± 0.799	1.397 ± 0.105	1.014 ± 0.170	1.986 ± 0.304
		1 mg/kg	16	378.1 ± 21.8	7.536 ± 0.528	1.511 ± 0.130	0.986 ± 0.194	1.895 ± 0.255
		10 mg/kg	16	389.8 ± 34.8	7.441 ± 0.751	1.443 ± 0.111	0.998 ± 0.146	1.774 ± 0.249
		30 mg/kg	15	371.0 ± 27.8	7.574 ± 0.684	1.431 ± 0.107	1.049 ± 0.112	1.801 ± 0.276
	PND 17-21	Control	12	376.2 ± 15.8	7.446 ± 0.542	1.438 ± 0.124	0.989 ± 0.124	1.758 ± 0.292
		1 mg/kg	11	359.1 ± 21.3	7.388 ± 0.609	1.511 ± 0.205	1.016 ± 0.132	1.862 ± 0.307
		10 mg/kg	12	372.1 ± 27.8	7.508 ± 0.590	1.451 ± 0.084	0.951 ± 0.185	1.918 ± 0.170
		30 mg/kg	12	376.4 ± 20.4	7.707 ± 0.428	1.484 ± 0.100	1.043 ± 0.127	1.923 ± 0.388
	PND 35-39	Control	16	377.3 ± 33.2	7.654 ± 0.377	1.520 ± 0.067	1.068 ± 0.094	2.065 ± 0.238
		1 mg/kg	16	382.8 ± 26.3	8.108 ± 0.811	1.603 ± 0.165	1.097 ± 0.172	2.108 ± 0.301
		10 mg/kg	15	378.4 ± 26.7	7.778 ± 0.859	1.411 ± 0.139	1.053 ± 0.192	1.755 ± 0.324*
		30 mg/kg	16	381.1 ± 22.1	7.951 ± 0.487	1.398 ± 0.114*	0.866 ± 0.154**	1.788 ± 0.266*
DDE	PND 1-5	Control	12	362.5 ± 14.6	7.760 ± 0.369	1.447 ± 0.090	0.809 ± 0.130	1.942 ± 0.267
		10 mg/kg	12	382.2 ± 32.2	7.122 ± 0.498*	1.395 ± 0.108	0.852 ± 0.169	1.874 ± 0.226
		30 mg/kg	12	367.2 ± 26.8	7.670 ± 0.442	1.443 ± 0.102	0.928 ± 0.112	1.893 ± 0.292
		100 mg/kg	11	367.9 ± 28.3	7.460 ± 0.544	1.422 ± 0.074	0.826 ± 0.098	1.789 ± 0.214
		300 mg/kg	12	374.0 ± 26.9	8.024 ± 0.658	1.398 ± 0.096	0.749 ± 0.084	1.783 ± 0.288
	PND 17-21	Control	12	358.6 ± 24.0	8.522 ± 1.002	1.539 ± 0.142	0.815 ± 0.171	1.724 ± 0.334
		10 mg/kg	12	377.0 ± 25.9	7.804 ± 0.660	1.465 ± 0.131	0.893 ± 0.077	1.830 ± 0.308
		30 mg/kg	11	358.8 ± 24.7	7.830 ± 0.568	1.489 ± 0.109	0.865 ± 0.095	1.676 ± 0.174
		100 mg/kg	12	362.8 ± 18.3	8.263 ± 0.382	1.502 ± 0.064	0.840 ± 0.103	1.751 ± 0.211
		300 mg/kg	13	359.1 ± 38.0	8.731 ± 0.864	1.430 ± 0.124	0.841 ± 0.159	1.558 ± 0.178
	PND 35-39	Control	12	333.5 ± 26.1	7.844 ± 0.731	1.520 ± 0.124	0.925 ± 0.209	1.834 ± 0.331
		10 mg/kg	12	356.0 ± 24.1	7.362 ± 0.634	1.323 ± 0.112**	0.875 ± 0.084	1.831 ± 0.153
		30 mg/kg	12	347.2 ± 41.8	7.500 ± 0.372	1.376 ± 0.069*	0.908 ± 0.097	1.807 ± 0.238
		100 mg/kg	12	353.5 ± 26.9	7.823 ± 0.492	1.409 ± 0.156	0.847 ± 0.119	1.523 ± 0.247**
VZ	PND 1-5	Control	15	368.9 ± 30.7	7.917 ± 1.053	1.590 ± 0.158	1.039 ± 0.153	2.168 ± 0.277
		10 mg/kg	12	382.0 ± 35.0	7.392 ± 0.688	1.477 ± 0.100	1.006 ± 0.137	2.059 ± 0.160
		30 mg/kg	15	379.6 ± 16.8	8.020 ± 0.761	1.505 ± 0.136	0.917 ± 0.130	2.048 ± 0.302
		100 mg/kg	15	376.3 ± 24.8	7.361 ± 1.030	1.589 ± 0.134	1.025 ± 0.187	2.115 ± 0.195
	PND 17-21	Control	16	357.5 ± 28.1	7.791 ± 0.655	1.432 ± 0.086	0.967 ± 0.133	1.903 ± 0.317
		10 mg/kg	16	366.8 ± 24.6	7.981 ± 0.658	1.562 ± 0.126	0.987 ± 0.101	2.041 ± 0.302
		30 mg/kg	16	378.3 ± 26.0	7.629 ± 0.831	1.550 ± 0.182	0.891 ± 0.117	1.931 ± 0.287
		100 mg/kg	15	373.2 ± 30.3	8.056 ± 0.710	1.527 ± 0.146	1.022 ± 0.156	2.117 ± 0.277
	PND 35-39	Control	16	369.8 ± 25.8	7.711 ± 0.746	1.502 ± 0.098	0.895 ± 0.153	1.752 ± 0.190
		10 mg/kg	12	373.3 ± 35.7	7.420 ± 0.647	1.572 ± 0.122	0.910 ± 0.100	1.911 ± 0.231
		30 mg/kg	12	361.7 ± 31.7	8.125 ± 0.562	1.546 ± 0.076	0.982 ± 0.114	1.885 ± 0.154
		100 mg/kg	12	363.1 ± 14.2	7.719 ± 0.393	1.508 ± 0.134	0.941 ± 0.132	1.907 ± 0.203
DES	PND 1-5	Control	16	367.5 ± 31.8	8.089 ± 0.914	1.560 ± 0.174	1.064 ± 0.155	1.948 ± 0.262
		10 µg/kg	16	365.7 ± 26.6	7.296 ± 0.829	1.391 ± 0.152*	0.917 ± 0.167*	1.870 ± 0.390
		100 µg/kg	16	383.6 ± 23.0	8.277 ± 2.095	1.431 ± 0.127	0.769 ± 0.129**	1.668 ± 0.527
		300 µg/kg	15	343.0 ± 38.6	7.893 ± 2.723	1.569 ± 0.271	0.835 ± 0.120**	1.321 ± 0.232**
	PND 17-21	Control	15	340.4 ± 35.0	7.783 ± 0.497	1.566 ± 0.156	0.927 ± 0.097	1.962 ± 0.353
		10 µg/kg	12	337.2 ± 26.7	7.906 ± 0.674	1.432 ± 0.105*	0.922 ± 0.132	1.781 ± 0.209
		100 µg/kg	16	348.9 ± 24.8	6.940 ± 0.743**	1.455 ± 0.118	0.900 ± 0.109	1.939 ± 0.283
		300 µg/kg	16	352.8 ± 20.5	7.309 ± 0.846	1.417 ± 0.121**	0.933 ± 0.117	1.935 ± 0.346
	PND 35-39	Control	15	360.3 ± 36.2	7.793 ± 0.609	1.539 ± 0.168	1.052 ± 0.194	2.299 ± 0.488
		10 µg/kg	12	344.9 ± 39.7	7.472 ± 0.686	1.593 ± 0.155	1.035 ± 0.140	1.963 ± 0.240
		100 µg/kg	16	330.4 ± 26.3	7.920 ± 0.736	1.556 ± 0.089	0.987 ± 0.193	1.910 ± 0.351
		300 µg/kg	16	336.5 ± 36.5	7.986 ± 0.777	1.611 ± 0.153	0.970 ± 0.134	2.017 ± 0.260
EE	PND 1-5	Control	16	391.7 ± 17.3	7.908 ± 0.437	1.533 ± 0.131	1.024 ± 0.128	1.947 ± 0.269
		10 µg/kg	12	367.0 ± 22.9**	7.681 ± 0.690	1.477 ± 0.103	0.850 ± 0.110**	1.548 ± 0.270**
		100 µg/kg	10	372.7 ± 15.4*	6.919 ± 0.402**	1.387 ± 0.149*	0.692 ± 0.114**	1.223 ± 0.166**
	PND 17-21	Control	12	353.9 ± 32.3	7.154 ± 0.561	1.456 ± 0.126	0.908 ± 0.147	1.978 ± 0.207
		10 µg/kg	8	357.0 ± 27.4	7.201 ± 0.538	1.366 ± 0.101	1.013 ± 0.149	1.875 ± 0.277
		100 µg/kg	12	373.1 ± 23.1	6.980 ± 0.530	1.521 ± 0.101	1.053 ± 0.187	2.118 ± 0.245
	PND 35-39	Control	16	357.4 ± 29.4	7.766 ± 0.783	1.567 ± 0.118	1.078 ± 0.196	1.954 ± 0.286
		10 µg/kg	16	353.6 ± 17.8	7.586 ± 0.738	1.569 ± 0.149	0.960 ± 0.147	1.910 ± 0.347
		100 µg/kg	16	347.1 ± 31.1	7.637 ± 0.870	1.517 ± 0.144	0.936 ± 0.123 *	1.783 ± 0.267
TAM	PND 1-5	Control	16	391.7 ± 17.3	7.908 ± 0.437	1.533 ± 0.131	1.024 ± 0.128	1.947 ± 0.269
		0.3 mg/kg	16	368.7 ± 27.9*	7.153 ± 0.772	1.498 ± 0.115	0.813 ± 0.161**	1.625 ± 0.352*
		1 mg/kg	16	356.4 ± 28.8**	6.756 ± 1.135*	1.454 ± 0.219	0.790 ± 0.181**	1.601 ± 0.403*
		3 mg/kg	15	327.9 ± 29.0**	5.043 ± 0.550**	1.162 ± 0.114**	0.606 ± 0.124**	1.218 ± 0.371**
	PND 17-21	Control	16	357.5 ± 28.1	7.791 ± 0.655	1.432 ± 0.086	0.967 ± 0.133	1.903 ± 0.317
		0.3 mg/kg	11	364.5 ± 29.6	7.768 ± 0.452	1.530 ± 0.189	0.950 ± 0.196	1.936 ± 0.244
		1 mg/kg	15	354.0 ± 31.8	7.913 ± 0.812	1.530 ± 0.126	0.945 ± 0.243	1.873 ± 0.236
		3 mg/kg	12	363.5 ± 21.1	7.457 ± 0.901	1.516 ± 0.121	0.927 ± 0.212	2.060 ± 0.374
	PND 35-39	Control	16	369.8 ± 25.8	7.711 ± 0.746	1.502 ± 0.098	0.895 ± 0.153	1.752 ± 0.190
		0.3 mg/kg	12	348.8 ± 37.5	7.357 ± 0.457	1.548 ± 0.095	0.900 ± 0.102	1.733 ± 0.173
		1 mg/kg	12	348.8 ± 42.4	8.189 ± 0.787	1.581 ± 0.134	0.959 ± 0.141	1.909 ± 0.291
		3 mg/kg	12	339.8 ± 16.1*	8.159 ± 0.596	1.626 ± 0.134*	0.934 ± 0.139	1.866 ± 0.194

Value: Mean ± S.D. calculated using the individual data.

FLU: flutamide; DDE: *p,p'*-dichlorodiphenyldichloroethylene; VZ: vinclozolin; DES: diethylstilbestrol; EE: ethynylestradiol; TAM: tamoxifen; PND: postnatal day; *: significantly different from control, $p<0.05$; **: significantly different from control, $p<0.01$.

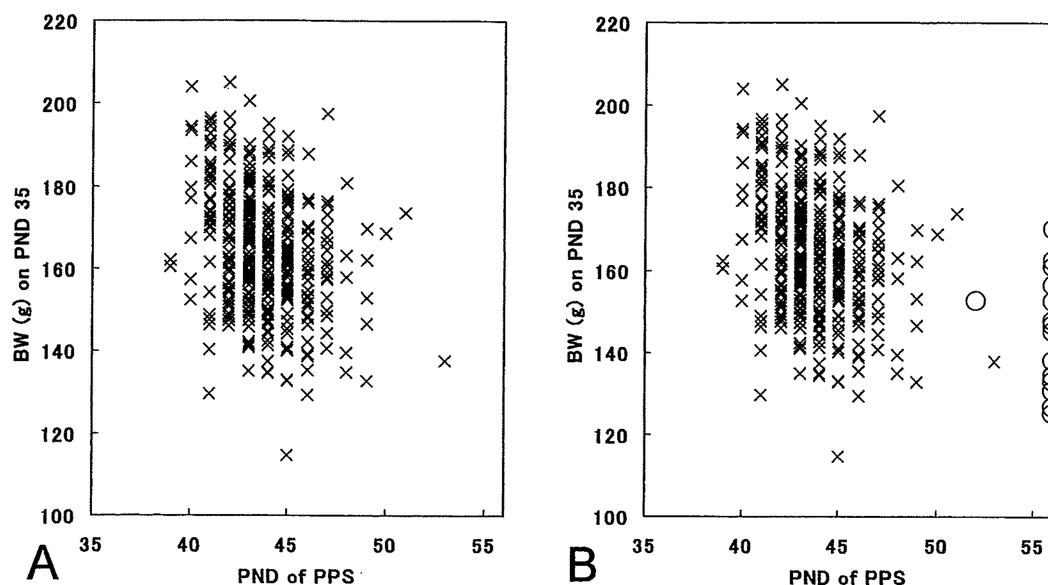

Fig. 5. Correlation diagram between the day of preputial separation and body weight of control and dosed rats.
A: There is a negative correlation between PND of preputial separation (PPS) and body weight on PND 35. Number of cases = 366, Pearson's correlation coefficient: r = –0.30. B: Preputial separation (PPS) of males exposed to 3 mg/kg tamoxifen on PND 1–5 is delayed and the data are plotted as "O" outside the range of control data "X".

3) Pathological examination

Unilateral or bilateral cloudy white discoloration of the efferent ductule as well as testis enlargement was increased in the groups exposed to EE and DES on PND 1–5. Histopathological examination revealed retention of sperm or inflammatory cells in the lumen of the efferent ductule and edema in adipose tissue surrounding the dilated ductule. Organs of the EE or TAM groups showed no remarkable histological changes except for a relative atrophic appearance.

Correlation between preputial separation and body weight

The control animal data in this study (n=366) are symbolized by "X" on the scattergram of Fig. 5A. PND of preputial separation and body weight on PND 35 showed a negative correlation (Pearson's correlation coefficient: r = –0.30). The data from animals treated with 3 mg/kg TAM, symbolized by "O", are compared in Fig. 5B. Body weight on PND 35 in this group showed significant reduction. Preputial separation of TAM treated males was delayed, and the data were outside the range of the control data.

Discussion

Preputial separation in untreated rats initiates from cornification of the epithelium lying between the glans penis and prepuce[1,2]. The cornification progresses from the tip of the glans penis towards its base and from the dorsal surface to ventral aspect of the glans penis. Preputial separation is considered complete when cornification reaches the ventral end of the glans penis. However, complete separation was not observed in animals exposed to FLU or VZ in their fetal period, since they had a cleft phallus and hypospadias. Induction of hypospadias is reportedly caused by FLU, VZ and finasteride[9,14–19]. FLU, a well-known potent androgen receptor antagonist, is used as a non-steroidal, anti-androgen drug for the treatment of prostate cancer. FLU inhibits TS and DHT binding to the intracellular androgen receptor, and prenatal/perinatal FLU exposure induces abnormalities in the genital tract of rats such as hypospadias and agenesis of the prostate, epididymis, and vas deferens[9,14,15]. The fungicide, VZ, is also an androgen receptor antagonist. It induces hypospadias in rats after perinatal or prenatal administration[16,17]. Finasteride, which inhibits 5α-reductase conversion of TS to DHT, also induces hypospadias in male rats exposed to it from GD 15 to day 21 postpartum[18] or GD 6–20[19], and based on this finding, DHT is thought to be involved in the development of the external genitalia.

The most sensitive period to induce hypospadias is reportedly GD 15–16 with 400 mg/kg of VZ[17], while only weak sensitivity was found with treatment on GD 17–18. Finasteride-exposed rats also showed similar results[18]. In our present study, exposure to 100 mg/kg of VZ on GD 14–17 induced hypospadias and a cleft phallus with cleft prepuce, but exposure on GD 18–21 did not induce any abnormalities in the external genitalia. At a higher dose of 200 mg/kg, VZ caused the death of the pregnant females or newborn pups (data not shown). Exposure to 10 mg/kg of FLU on GD 14–17 induced hypospadias with a cleft phallus and cleft prepuce, while exposure on GD 18–21 induced hypospadias and a cleft phallus without a cleft prepuce.

Higher doses of FLU induced the same abnormality as our previous study[2]. Although both FLU and VZ are androgen receptor antagonists, their sensitive periods differed: cleft phallus was caused by FLU administration until later in pregnancy. FLU exposed males without the malformation showed a hypoplastic penis and a delay in preputial separation, but prenatal exposure to other chemicals did not affect the preputial separation.

Postnatal chemical exposure influenced preputial separation in a variety of ways. Anti-androgen, FLU caused a delay when administered on PND 35–39, and statistical analyses using individual data of DDE and VZ also showed significant delays of preputial separation, but neonatal exposure to these chemicals on PND 1–5 did not influence the time of preputial separation. On the other hand, neonatal administration of EE and TAM induced a marked delay or incomplete separation. DES exposure during both PND 1–5 and PND 35–39 caused a delay in separation. Preputial separation is thought to be dependent on the continued presence of androgen after PND 35, since castration on PND 35 blocks preputial separation and the addition of TS or DHT reverses the effects of castration[3]. Male rat serum testosterone levels have been reported to decrease after birth and to increase at the prepubertal stage[11]. Testosterone during fetal life is thought to act to masculinize the genitalia, and testosterone at puberty may act on the maturation of the target organ. Prenatal exposure to the anti-androgens used in the present study resulted in malformation of the external genitalia, and the effect of their prepubertal exposure was a delay in preputial separation. These results may indicate that anti-androgens effect on male rats in a relatively higher level of serum testosterone, and that male rats are not sensitive to neonatal exposure to anti-androgenic chemicals.

Neonatal exposure of estrogenic chemicals is known to induce marked effects on male rats. Delay of preputial separation and decrease of testis and prostate weight with a reduction of plasma testosterone levels have been reported in rats neonatally administered estradiol benzoate[20]. Neonatal treatment of DES or EE has also been reported to cause dose-dependent reductions in plasma testosterone levels and testis weights in adulthood[21]. An estrogen receptor is found in the male reproductive tract[22,23]. These findings suggest that estrogen is relevant to the growth of the male reproductive organs. The delay of preputial separation induced by DES, EE and TAM exposures on PND 1–5 in the present study is thought to be caused by estrogen-related effects, and the delay may have not only been caused by the direct effects on the genital tract but also by effects on the systemic endocrine function. Prepubertal exposure to DES induced a delay of preputial separation in male rats in the present study, the same as observed for anti-androgenic chemicals. Serum testosterone levels in adult or prepubertal male rats have been reported to be increased by FLU[24,25], DDE[5] and VZ[24] treatment, and the increase is thought to be caused by their anti-androgenic effects. It has been reported that serum testosterone levels in adult male rats treated with DES are decreased[26], thus, testosterone reduction may be the reason for the delay of preputial separation seen in the DES group prepubertally treated in our present study. EE and TAM have also been reported to decrease serum testosterone levels[25,27], but these chemicals did not delay preputial separation in our present study. The reason for the different effects of DES and EE/TAM treatments were not revealed in our study.

A negative correlation between the body weight on PND 35 and the day of preputial separation was demonstrated in the control males (Fig. 5A). This diagram shows a tendency for males of higher body weight to complete preputial separation earlier than males of lower body weight. Ashby and Lefevre[8] thought that there was a marked dependence of the day of preputial separation on the initial body weight of the test animals, and that delays in preputial separation can only be interpreted with confidence when they are not accompanied by losses in body weight. In our present study, males exposed to 3 mg/kg TAM on PND 1–5 showed significantly lower body weight on PND 35, and their preputial separation was delayed. The delay may not depend on the reduced weight gain, since 14 males out of 15 did not show complete separation of the prepuce on the day of autopsy, PND 56, and their data were outside the range of control data.

AGD was reduced by FLU and VZ exposures on GD 14–17. Reduction of AGD by anti-androgen has been reported, and AGD is thought to have high sensitivity to anti-andorogens[17]. In the present study, however, there were no apparent changes in AGD after DDE exposure. DES also caused a decrease in AGD with GD 18–21 exposure, and there was a difference between the results of DES and EE/TAM exposure. Histological examination revealed a tortuous and bent cavernous body of the penis seen in the sagittal section of males prenatally exposed to FLU or VZ and sacrificed on PND 6. This morphological change may be a reason for the reduction in AGD.

The relative weights of the ventral prostate in males prenatally exposed to VZ or DES were decreased significantly. In these groups, prostate aplasia or hypoplasia was observed in the group exposed to VZ on GD 14–17 and DES on GD 18–21. Postnatal FLU exposure decreased the relative weight of the ventral prostate in the group dosed on PND 35–39. No apparent effect was detected in males postnatally exposed to DDE or VZ. PND 1–5 exposure of EE or TAM induced a reduction in the weight of the testis and other reproductive organs. Although the statistical analyses using the individual data showed significant results in the lower dose group and the other organs, there were no significant differences in the groups exposed to VZ. These findings indicate that preputial separation is more useful than the measurement of organ weight as an endpoint in detecting endocrine active chemicals under the conditions used in this study.

Prenatal exposure of anti-androgens FLU and VZ induced hypospadias, and the time of preputial separation could not be determined. Although GD 18–21 exposure was expected to produce a very low incidence of hypospadias,

FLU exposure on GD 18–21 induced the abnormality. Other chemicals did not induce hypospadias or delays of the preputial separation. From these results, it is unclear if preputial separation after prenatal exposure is a useful way for detecting endocrine active chemicals. On the other hand, prepubertal exposure to FLU, DDE and VZ caused delays in preputial separation, and neonatal exposure to EE and TAM induced delays in separation with a reduction in organ and body weight gain. Both neonatal and prepubertal exposure to DES caused a delay in preputial separation. These results indicate that neonatal (PND 1–10) and prepubertal exposure may be useful for detecting endocrine active chemicals by observing preputial separation, and that continuous administration of chemicals from PND 35 to the day of preputial separation may be more effective for prepubertal exposure.

In conclusion, the usability of preputial separation to detect endocrine active chemicals after prenatal exposure to them is still unclear. Postnatal exposure, however, may be a useful method for a screening assay to detect endocrine active chemicals by preputial separation, and postnatal exposure is dependent on both neonatal (PND 1–10) and prepubertal continuous exposure.

Acknowledgements: This work was supported by Health and Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare of Japan.

References

1. Lyons WR, Berlin I, and Friedlander S. Cornification of balano-preputial epithelium in normal rats and in castrated rats treated with testosterone propionate. Endocrinology. **31**: 659–663. 1942.
2. Yoshimura S, Yamaguchi H, Konno K, Ohsawa N, Noguchi S, and Chisaka A. Hypospadias and incomplete preputial separation in male rats induced by prenatal exposure to an anti-androgen, flutamide. J Toxicol Pathol. **17**: 113–118. 2004.
3. Korenbrot CC, Huhtaniemi IT, and Weiner RI. Preputial separation as an external sign of pubertal development in the male rat. Biol Reprod. **17**: 298–303. 1977.
4. Yamada T, Sunami O, Kunimatsu T, Kamita Y, Okuno Y, Seki T, Nakatsuka I, and Matsuo M. Dissection and weighing of accessory sex glands after formalin fixation, and a 5-day assay using young mature rats are reliable and feasible in the Hershberger assay. Toxicology. **162**: 103–119. 2001.
5. O'Connor JC, Frame SR, Davis LG, and Cook JC. Detection of the environmental antiandrogen *p,p'*-DDE in CD and Long-Evans rats using a tier I screening battery and a Hershberger assay. Toxicol Sci. **51**: 44–53. 1999.
6. Sunami O, Kunimatsu T, Yamada T, Yabushita S, Sukata T, Miyata K, Kamita Y, Okuno Y, Seki T, Nakatsuka I, and Matsuo M. Evaluation of a 5-day Hershberger assay using young mature male rats: methyltestosterone and *p,p'*-DDE, but not fenitrothion, exhibited androgenic or antiandrogenic activity *in vivo*. J Toxicol Sci. **25**: 403–415. 2000.
7. Yamasaki K, Sawaki M, Ohta R, Okuda H, Katayama S, Yamada T, Ohta T, Kosaka T, and Owens W. OECD validation of the Hershberger assay in Japan: phase 2 dose response of methyltestosterone, vinclozolin, and *p,p'*-DDE. Environ Health Perspect. **111**: 1912–1919. 2003.
8. Ashby J and Lefevre PA. The peripubertal male rat assay as an alternative to the Hershberger castrated male rat assay for the detection of anti-androgens, oestrogens and metabolic modulators. J Appl Toxicol. **20**: 35–47. 2000.
9. McIntyre BS, Barlow NJ, and Foster PMD. Androgen-mediated development in male rat offspring exposed to flutamide *in utero*: permanence and correlation of early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues. Toxicol Sci. **62**: 236–249. 2001.
10. Gaytan F, Pinilla L, Romero JL, and Aguilar E. Differential effects of the administration of human chorionic gonadotropin to postnatal rats. J Endocrinol. **142**: 527–534. 1994.
11. Paz GF, Winter JSD, Reyes FI, and Faiman C. Developmental pattern of testosterone production by the rat testis. Steroids. **36**: 675–688. 1980.
12. Watanabe C, Kuwagata M, Yoshimura S, Azegami J, Kojima K, Ono H, and Nagao T. An improved technique for repeated gavage administration to rat neonates. Congenit Anom (Kyoto). **43**: 177–179. 2003.
13. Wada K. Method and problem in measurement of AGD and preputial separation (in Japanese). Annual Report of Hatano Research Institute. **25**: 109–114. 2002.
14. Mylchreest E, Sar M, Cattley RC, and Foster PMD. Disruption of androgen-regulated male reproductive development by di(*n*-butyl) phthalate during late gestation in rats is different from flutamide. Toxicol Appl Pharmacol. **156**: 81–95. 1999.
15. Miyata K, Yabushita S, Sukata T, Sano M, Yoshino H, Nakanishi T, Okuno Y, and Matsuo M. Effects of perinatal exposure to flutamide on sex hormones and androgen-dependent organs in F1 male rats. J Toxicol Sci. **27**: 19–33. 2002.
16. Gray LE Jr, Ostby JS, and Kelce WR. Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rat. Toxicol Appl Pharmacol. **129**: 46–52. 1994.
17. Wolf CJ, LeBlanc GA, Ostby JS, and Gray LE Jr. Characterization of the period of sensitivity of fetal male sexual development to vinclozolin. Toxicol Sci. **55**: 152–161. 2000.
18. Clark RL, Anderson CA, Prahalada S, Robertson RT, Lochry EA, Leonard YM, Stevens JL, and Hoberman AM. Critical developmental periods for effects on male rat genitalia induced by finasteride, a 5α-reductase inhibitor. Toxicol Appl Pharmacol. **119**: 34–40. 1993.
19. Anderson CA and Clark RL. External genitalia of the rat: normal development and the histogenesis of 5α-reductase inhibitor-induced abnormalities. Teratology. **42**: 483–496. 1990.
20. Bellido C, Gaytan F, Aguilar R, Pinilla L, and Aguilar E. Prepuberal reproductive defects in neonatal estrogenized male rats. Biol Reprod. **33**: 381–387. 1985.
21. Atanassova N, McKinnell C, Walker M, Turner KJ, Fisher JS, Morley M, Millar MR, Groome NP, and Sharpe RM. Permanent effects of neonatal estrogen exposure in rats on reproductive hormone levels, Sertoli cell number, and the

efficiency of spermatogenesis in adulthood. Endocrinology. **140**: 5364–5373. 1999.
22. Fisher JS, Millar MR, Majdic G, Saunders PT, Fraser HM, and Sharpe RM. Immunolocalisation of oestrogen receptor-α within the testis and excurrent ducts of the rat and marmoset monkey from perinatal life to adulthood. J Endocrinol. **153**: 485–495. 1997.
23. Saunders PTK, Fisher JS, Sharpe RM, and Millar MR. Expression of oestrogen receptor beta (ER β) occurs in multiple cell types, including some germ cells, in the rat testis. J Endocrinol. **156**: R13–17. 1998.
24. Yu WJ, Lee BJ, Nam SY, Ahn B, Hong JT, Do JC, Kim YC, Lee YS, and Yun YW. Reproductive disorders in pubertal and adult phase of the male rats exposed to vinclozolin during puberty. J Vet Med Sci. **66**: 847–853. 2004.
25. Okuno Y and Miyata K. Effects of an endocrine disruptor on male reproductive organs. J Toxicol Pathol. **14**: 79–82. 2001.
26. Goyal HO, Braden TD, Mansour M, Williams CS, Kamaleldin A, and Srivastava KK. Diethylstilbestrol-treated adult rats with altered epididymal sperm numbers and sperm motility parameters, but without alterations in sperm production and sperm morphology. Biol Reprod. **64**: 927–934. 2001.
27. Gill-Sharma MK, Balasinor N, and Parte P. Effect of intermittent treatment with tamoxifen on reproduction in male rats. Asian J Androl. **3**: 115–119. 2001.

J Toxicol Pathol 2004; 17: 113–118

Original

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

Shinsuke Yoshimura[1], Hajime Yamaguchi[1], Kazunori Konno[1], Noriko Ohsawa[1], Satoshi Noguchi[1], and Akiko Chisaka[1]

[1]Hatano Research Institute, Food and Drug Safety Center, 729–5, Ochiai, Hadano, Kanagawa 257–8523, Japan

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.
(J Toxicol Pathol 2004; **17**: 113–118)

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

Received: 26 January 2004, Accepted: 15 April 2004
Mailing address: Shinsuke Yoshimura, Laboratory of Toxicology, Hatano Research Institute, Food and Drug Safety Center, 729–5, Ochiai, Hadano, Kanagawa 257–8523, Japan
TEL: 81-463-82-4751 FAX: 81-463-82-9627
E-mail: yoshimura.s@fdsc.or.jp

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

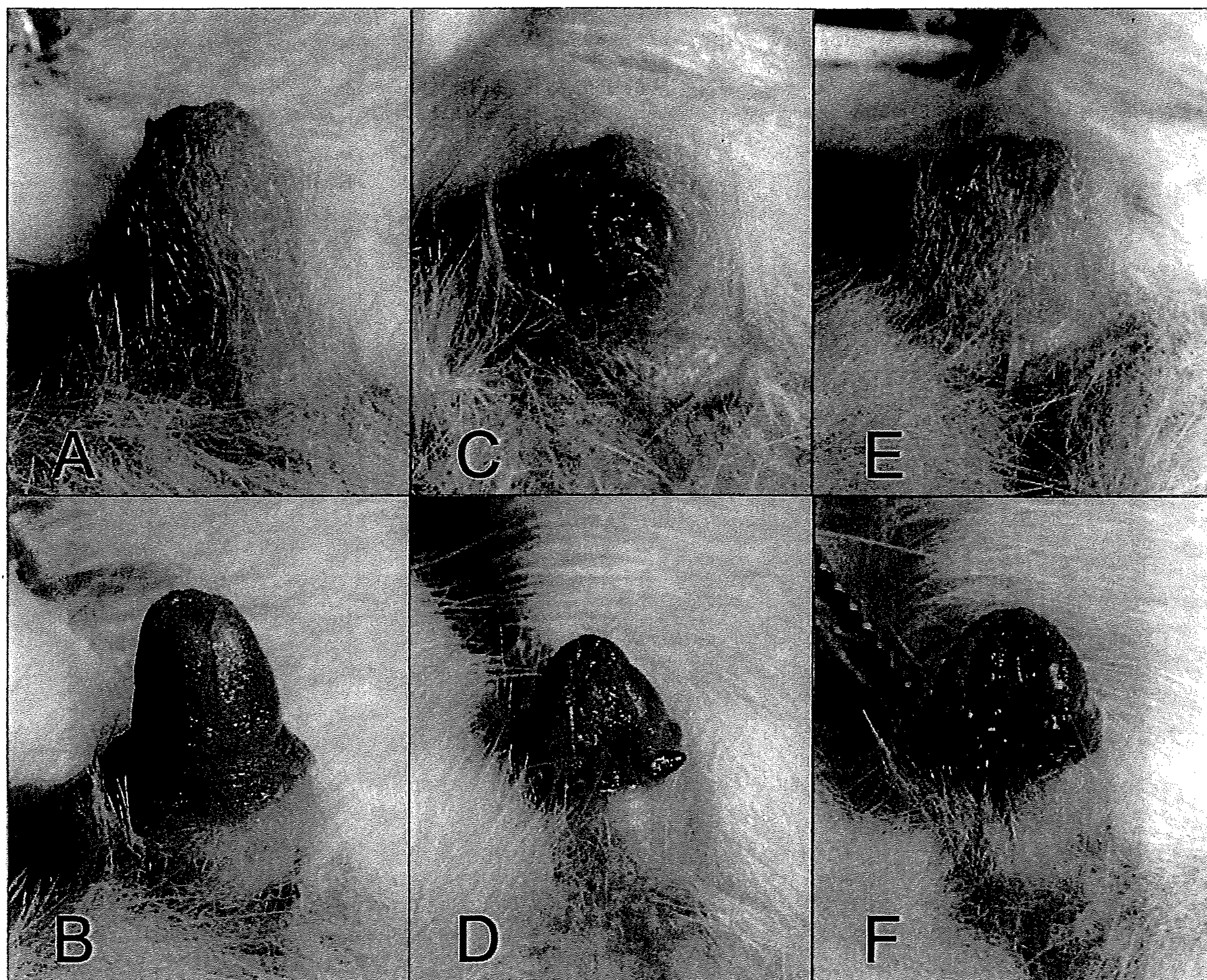

Fig. 1. Ventral surface of genital tubercle (A, C and E) and glans penis (B, D and F) of males at PND 56. A and B: Control rat. Prepuce is completely retracted. C and D: Male rat prenatally exposed to FLU on GD 14–17. Ventral side of the prepuce has a cleft, and the glans penis is observed from the cleft. 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,

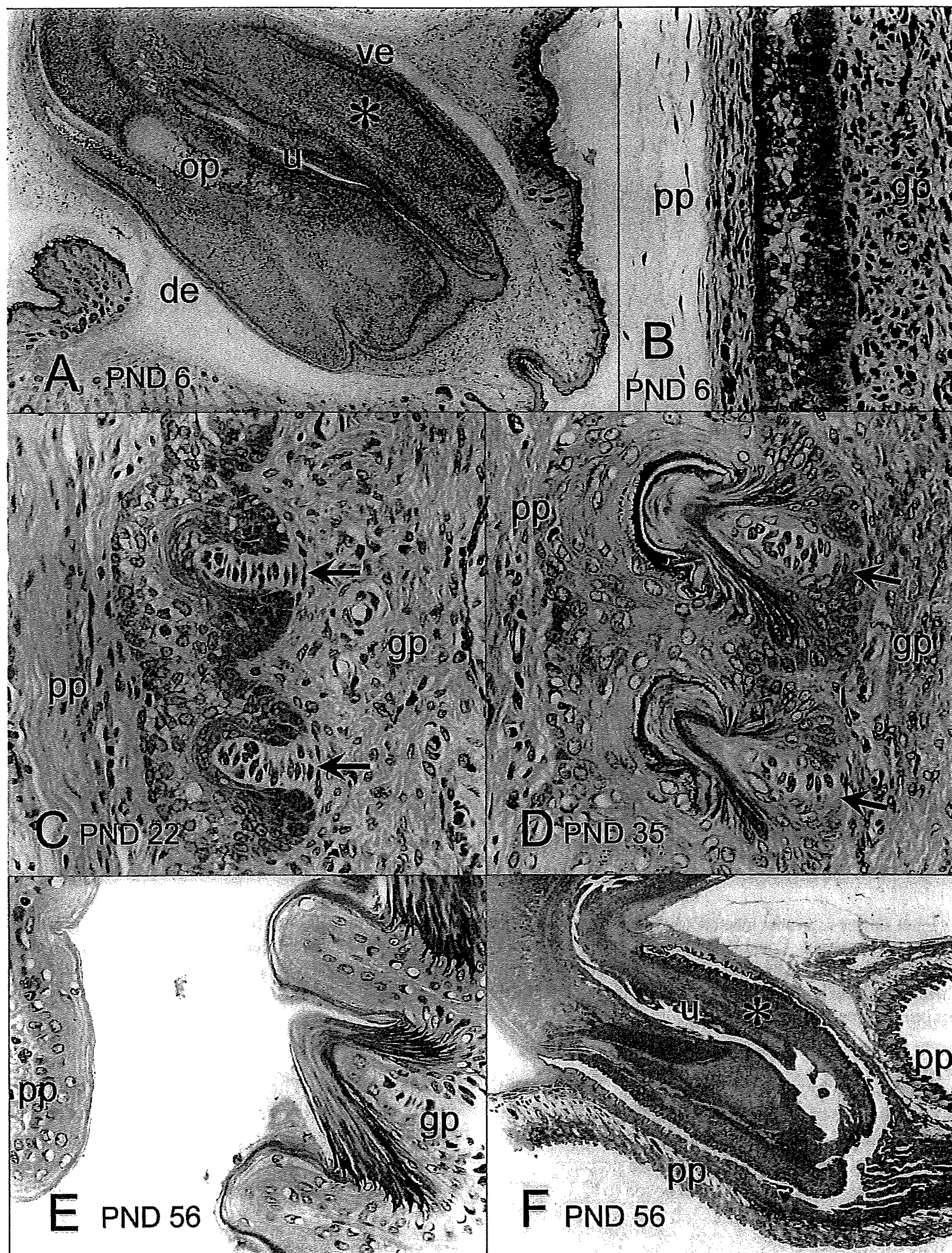

Fig. 2. Sagittal sections of the genital tubercle from control males.
A: Genital tubercle of a male on PND 6. Glans penis is bordered with dorsal epithelium (de) and ventral epithelium (ve). Urethra (u) is located in the center of the glans penis. op: os penis. *: ventral half of the glans penis.
B, 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.

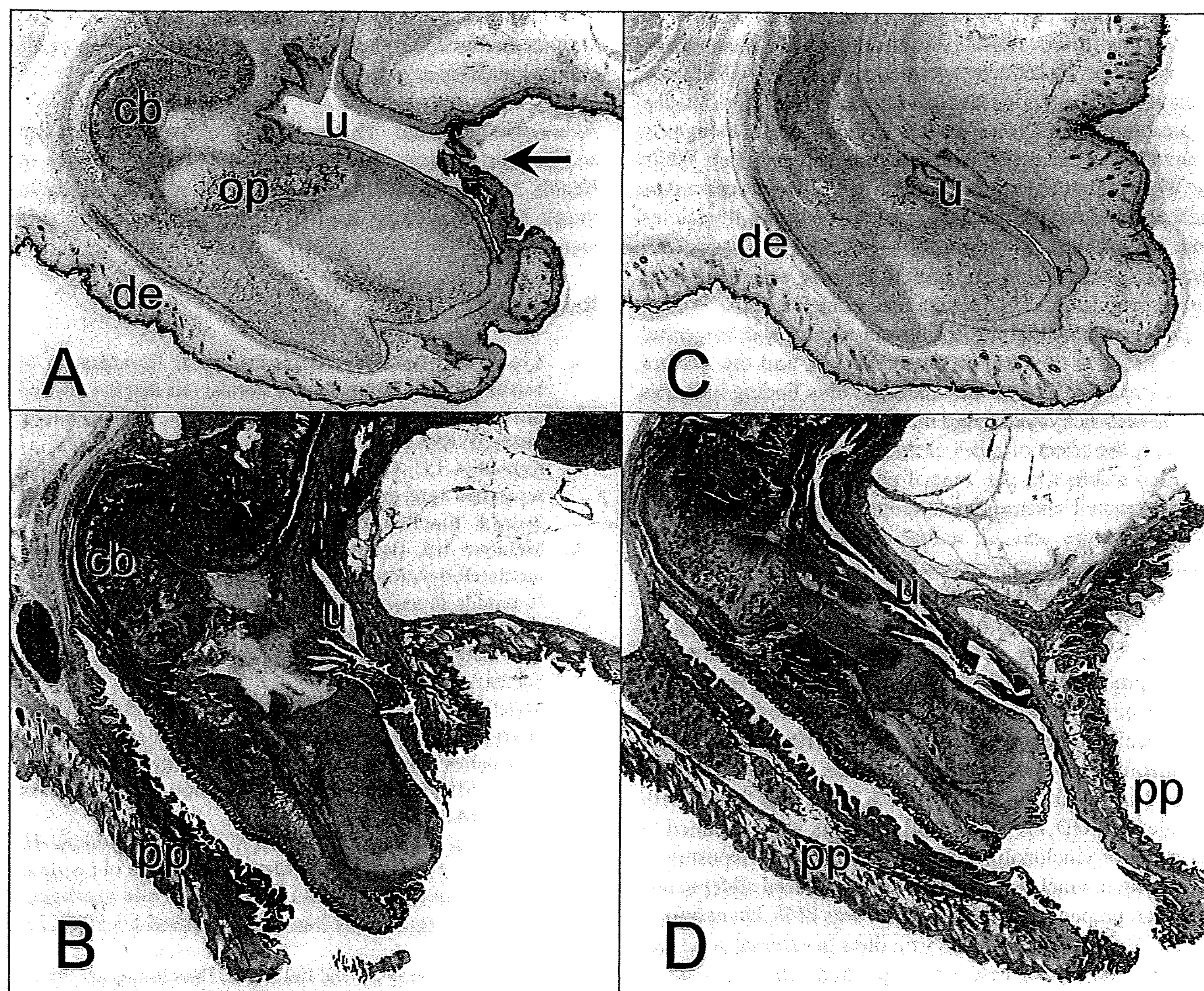

Fig. 3. Sagittal sections of genital tubercle from males prenatally exposed to FLU on GD 14–17 (A, B) or GD 18–21 (C, D), sacrificed on PND 6 (A, C) and PND 56 (B, D).
A: There is a cleft (arrow in the figure) at the ventral side of genital tubercle. Urethra (u) is observed at the ventral surface of 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.

Acknowledgments: This work was supported by Health and Labour Sciences Research Grants from the Ministry of Health, Labour and Welfare of Japan. We would also like to thank Dr. Masayoshi Kanisawa for proofreading this manuscript.

References

1. Lyons WR, Berlin I, and Friedlander S. Cornification of balano-preputial epithelium in normal rats and in castrated rats treated with testosterone propionate. Endocrinology 1942; **31**: 659–663.
2. Korenbrot CC, Huhtaniemi IT, and Weiner RI. Preputial separation as an external sign of pubertal development in the male rat. Biol Reprod 1977; **17**: 298–303.
3. McIntyre BS, Barlow NJ, and Foster PMD. Androgen-mediated development in male rat offspring exposed to flutamide *in utero*: permanence and correlation of early postnatal changes in anogenital distance and nipple retention with malformations in androgen-dependent tissues. Toxicol Sci 2001; **62**: 236–249.
4. Mylchreest E, Sar M, Cattley RC, and Foster PMD. Disruption of androgen-regulated male reproductive development by di(*n*-butyl) phthalate during late gestation in rats is different from flutamide. Toxicol Appl Pharmacol 1999; **156**: 81–95.
5. Miyata K, Yabushita S, Sukata T, Sano M, Yoshino H, Nakanishi T, Okuno Y, and Matsuo M. Effects of perinatal exposure to flutamide on sex hormones and androgen-dependent organs in F1 male rats. J Toxicol Sci 2002; **27**: 19–33.
6. Gray LE Jr, Ostby JS, and Kelce WR. Developmental effects of an environmental antiandrogen: the fungicide vinclozolin alters sex differentiation of the male rat. Toxicol Appl Pharmacol 1994; **129**: 46–52.
7. Wolf CJ, LeBlanc GA, Ostby JS, and Gray LE Jr. Characterization of the period of sensitivity of fetal male sexual development to vinclozolin. Toxicol Sci 2000; **55**: 152–161.
8. Clark RL, Anderson CA, Prahalada S, Robertson RT, Lochry EA, Leonard YM, Stevens JL, and Hoberman AM. Critical developmental periods for effects on male rat genitalia induced by finasteride, a 5α-reductase inhibitor. Toxicol Appl Pharmacol 1993; **119**: 34–40.
9. Anderson CA and Clark RL. External genitalia of the rat: normal development and the histogenesis of 5α-reductase inhibitor-induced abnormalities. Teratology 1990; **42**: 483–496.
10. Bentvelsen FM, Brinkmann AO, van der Schoot P, van der Linden JETM, van der Kwast TH, Boersma WJA, Schröder FH, and Nijman JM. Developmental pattern and regulation by androgens of androgen receptor expression in the urogenital tract of the rat. Mol Cell Endocrinol 1995; **113**: 245–253.
11. Yoshimura S. Effects of prenatal or postnatal exposure to chemicals on preputial separation of male rats (unpublished).

Meeting Report: Validation of Toxicogenomics-Based Test Systems: ECVAM–ICCVAM/NICEATM Considerations for Regulatory Use

Raffaella Corvi,[1] Hans-Jürgen Ahr,[2] Silvio Albertini,[3] David H. Blakey,[4] Libero Clerici,[5] Sandra Coecke,[1] George R. Douglas,[4] Laura Gribaldo,[1] John P. Groten,[6] Bernd Haase,[7] Karen Hamernik,[8] Thomas Hartung,[1] Tohru Inoue,[9] Ian Indans,[10] Daniela Maurici,[1] George Orphanides,[11] Diana Rembges,[5] Susanna-Assunta Sansone,[12] Jason R. Snape,[13] Eisaku Toda,[14] Weida Tong,[15] Joost H. van Delft,[16] Brenda Weis,[17] and Leonard M. Schechtman[18,19]

[1]European Centre for the Validation of Alternative Methods (ECVAM), Institute for Health and Consumer Protection (IHCP), Joint Research Centre of the European Commission (JRC), Ispra, Italy; [2]Bayer HealthCare AG, Wuppertal, Germany; [3]Hoffmann-La Roche, Basel, Switzerland; [4]Environmental Health Centre, Health Canada, Ottawa, Ontario, Canada; [5]Physico-Chemical Exposure, IHCP, JRC, Ispra, Italy; [6]TNO, Utrecht, the Netherlands; [7]QIAGEN, Hilden, Germany; [8]U.S. Environmental Protection Agency, Washington, DC, USA; [9]National Institute of Health Sciences, Tokyo, Japan; [10]Health Safety Executive, London, United Kingdom; [11]Syngenta, Macclesfield, United Kingdom; [12]European Molecular Biology Laboratory, European Bioinformatics Institute, Hinxton, Cambridge, United Kingdom; [13]AstraZeneca, Brixham, United Kingdom; [14]Organisation for Economic Co-operation and Development, Paris, France; [15]Food and Drug Administration, National Center for Toxicological Research, Jefferson, Arkansas, USA; [16]University of Maastricht, Maastricht, the Netherlands; [17]National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services, Research Triangle Park, North Carolina, USA; [18]U.S. Interagency Coordinating Committee on the Validation of Alternative Methods, Research Triangle Park, NC, USA; [19]U.S. Food and Drug Administration, National Center for Toxicological Research, Rockville, Maryland, USA

This is the report of the first workshop "Validation of Toxicogenomics-Based Test Systems" held 11–12 December 2003 in Ispra, Italy. The workshop was hosted by the European Centre for the Validation of Alternative Methods (ECVAM) and organized jointly by ECVAM, the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), and the National Toxicology Program (NTP) Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM). The primary aim of the workshop was for participants to discuss and define principles applicable to the validation of toxicogenomics platforms as well as validation of specific toxicologic test methods that incorporate toxicogenomics technologies. The workshop was viewed as an opportunity for initiating a dialogue between technologic experts, regulators, and the principal validation bodies and for identifying those factors to which the validation process would be applicable. It was felt that to do so now, as the technology is evolving and associated challenges are identified, would be a basis for the future validation of the technology when it reaches the appropriate stage. Because of the complexity of the issue, different aspects of the validation of toxicogenomics-based test methods were covered. The three focus areas include *a*) biologic validation of toxicogenomics-based test methods for regulatory decision making, *b*) technical and bioinformatics aspects related to validation, and *c*) validation issues as they relate to regulatory acceptance and use of toxicogenomics-based test methods. In this report we summarize the discussions and describe in detail the recommendations for future direction and priorities. ***Key words:*** **acceptance, alternatives, biomarker, predictive test, regulatory use, standardization, toxicogenomics, toxicology, validation. *Environ Health Perspect* 114:420–429 (2006). doi:10.1289/ehp.8247 available via *http://dx.doi.org/* [Online 17 August 2005]**

Toxicogenomics, an emerging field in molecular toxicology, offers the promise of new approaches to identify and characterize such factors as the biologic activity of new and existing chemicals and drugs and could play an important role in hazard assessment for human health. This revolutionary field can potentially affect many scientific and medical areas, including the development of a new generation of alternative predictive testing and screening methods that could lend themselves to the reduction, refinement, and replacement of animals used for such purposes.

The European Centre for the Validation of Alternative Methods (ECVAM), the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), and the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) are currently investigating the specific considerations necessary for adequate validation of toxicogenomics-based test methods. The primary objective of ECVAM and ICCVAM/NICEATM is to facilitate development, validation, and regulatory acceptance of new, revised, and alternative test methods that reduce, refine, and replace the use of animals (referred to as the three Rs; Russell and Burch 1959) in testing while maintaining and promoting scientific quality and the protection of human health, animal health, and the environment. The efforts of such organizations as ICCVAM/NICEATM and ECVAM have helped foster the principles of the three R's and have contributed to progress in the use of alternative methods for regulatory, research, and educational purposes.

Experience in the validation of conventional alternative test methods has led to an understanding that new and innovative approaches likely will be necessary to standardize test methods based on toxicogenomics and to evaluate the scientific validity and regulatory applicability of such test methods. It is envisioned that the entire validation process will be more complex and challenging than that typically encountered thus far for other alternative test methods. This is because not only will the technology itself need to be standardized and validated, but the methods that are based upon the technology and their predictive aspects will also need to undergo validation if they are to be employed in regulatory decision-making processes. In addition the validation process must be able to accommodate the anticipated rapid changes in technology that could affect the performance of the test method and its reliability for a specific purpose.

Toxicogenomics-based methods are being widely applied in toxicology and biomedical research. Because data are already being generated using these technologies, it is both timely and important to address the subject of validation now with the aim of establishing a foundation that will facilitate future regulatory acceptance of scientifically validated

Address correspondence to R. Corvi, European Centre for the Validation of Alternative Methods, IHCP, Joint Research Centre of the European Commission, Via E. Fermi 1, 21120 Ispra, Italy. Telephone: 39-0332-785266. Fax: 39-0332-785845. E-mail: raffaella.corvi@jrc.it

Supplemental Material is available online (http://ehp.niehs.nih.gov/members/2005/8247/suppl.pdf).

We gratefully acknowledge W.S. Stokes, Director of the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods, for contributions to the planning and organization of the workshop as well as insightful and constructive comments.

This document represents the consensus of the participants' views expressed as individual scientists and does not necessarily represent the policies and procedures of their respective institutions.

The authors declare they have no competing financial interests.

Received 22 April 2005; accepted 17 August 2005.

toxicogenomics-based test methods. By addressing the critical validation issues early, and in parallel with the evolutionary and maturation phases of the technologic development of toxicogenomics-based methods, it should be possible to preempt many potential pitfalls and data gaps encountered with retrospective method evaluations that could impede validation of this promising research and regulatory tool. Such a strategy will also facilitate early buy-in and confidence in the technologies by the regulatory arena in its quest for new, improved, and relevant methods by which to help ensure human health, protect the environment, and demonstrate responsiveness to animal welfare issues.

In consideration of all these related issues, ECVAM and ICCVAM/NICEATM held the first of a planned series of workshops to address the validation principles that lend themselves to toxicogenomics-based test methods, for example, gene expression technologies and associated bioinformatics. Given the complexity of the rapidly evolving toxicogenomics field, a variety of issues were addressed. These included but were not limited to *a*) differences in and evolution of technology platforms including changes in genome coverage for model species; *b*) quality assurance (QA) and Good Laboratory Practice (GLP) compliance; *c*) technology standardization, transferability, and reproducibility; *d*) relevance to *in vivo* biological responses; *e*) yardsticks against which toxicogenomics responses should be measured; *f*) data evaluation, statistical approaches, and databases; *g*) validation approaches; and *h*) regulatory acceptability.

To begin to examine these complex issues, three breakout groups were formed. Each group concentrated on different aspects of the validation of toxicogenomics-based test methods, and the discussions were shared with the other participants in plenary sessions. The three focus areas were *a*) biological validation of toxicogenomics-based test methods for regulatory decision making, *b*) technical and bioinformatics aspects related to validation, and *c*) validation issues as they relate to regulatory acceptance and use of toxicogenomics-based test methods.

Validation of Toxicogenomics: Focus on the Biological Systems

The biological issues related to the validation of toxicogenomics-based test methods involved two strategies proposed for developing and validating such methods so that they can be employed to support regulatory decision making. One strategy involves phenotypic anchoring of gene expression changes to identify molecular mechanisms and candidate biomarkers of toxicity (i.e., single genes, proteins, or biological pathways). A second strategy involves the identification and validation of predictive gene expression signatures of toxicity. Validation considerations specific to data quality and cross-platform and interlaboratory variability that are common to both strategies were identified. It is acknowledged that any new toxicogenomics-based methods will need to address established validation criteria for determination of reliability and relevance (Balls et al. 1995; ICCVAM 1997, 2003) as well as articulate the advantages and limitations of a given toxicogenomics-based test method. In addition biological validation of such a test method, that is, assessment of the concordance of gene changes with biological events, is essential but is contingent upon validation of the technology itself, which is addressed elsewhere in this article.

Strategy 1: use of toxicogenomics data to define mechanism and identify biomarkers. Toxicogenomics offers the opportunity to enhance existing toxicity prediction strategies through elucidation of biological mechanisms around critical events. This sentiment is captured in the recent U.S. Environment Protection Agency (EPA) and U.S. Food and Drug Administration (FDA) strategies regarding the inclusion of genomics data in submissions of regulated substances (U.S. EPA 2002; U.S. FDA 2005). Although these agencies currently preclude basing regulatory decision making on genomics data alone, they do encourage the voluntary submission of well-documented, quality genomics data. Both agencies are considering the use of submitted data on a case-by-case basis for assessment purposes (e.g., to help elucidate mechanism of action or contribute to a weight-of-evidence approach) or for populating relevant comparative databases by encouraging parallel submissions of genomics data and traditional toxicologic test results. This approach is appropriate given the state of scientific knowledge of toxicogenomics and the requisite need for a clear understanding of the toxicologic relevance of the gene expression signals detected by this technology. There is a small but rapidly increasing number of published reports demonstrating a linkage between gene expression changes and adverse phenotypic changes (Huang et al. 2003; Orphanides 2003). These reports provide qualitative evidence of the power of genomics to link phenotype with gene expression, thereby contributing to an understanding of mechanism of action. Some such reports demonstrate the predictive power of these data to classify compounds. However, they fail to address adequately quantitative dose- and time-dependent (e.g., threshold) responses that are the hallmark of toxicologic evaluation, making their immediate acceptance in regulatory arenas circumspect.

Nonetheless, toxicogenomics data may eventually be useful in hazard and risk assessment if data quality and validity can be adequately substantiated. Some regulators are finding that these data have the potential to add to the body of knowledge about compound mechanism of action. With appropriate dose- and time-dependent measurements, gene and protein changes can be used to mark the molecular events that occur as an organism moves through the continuum from exposure to response. The obvious benefit is the identification of early markers of response, including responses that mark the point of departure from adaptation to toxicity. In addition, it may be possible to detect unforeseen effects at very low doses or in unexpected tissues (Brown et al. 2002). This is important because changes in gene or protein expression alone are not sufficient to differentiate toxicity from biologic adaptation after exposure to an exogenous compound. The challenge for predictive toxicology is to link changes in gene and protein expression to sequential changes in phenotype, both adaptive and adverse, in a manner that is consistent with the underlying biologic mechanisms. For example, gene expression profiling has been used to classify hepatotoxins based on mechanism of action and to differentiate early, presumably adaptive, responses from later responses that are reflective of toxicity (Hamadeh et al. 2002a, 2002b; Waring et al. 2001, 2003). The gene expression changes correlated well with changes in histopathology and clinical chemistry, supporting the liver as target organ for the test compounds.

Although good technical progress has been made in recent years, additional proof-of-principle studies are needed for the regulatory community to become more accepting of the use of toxicogenomics data as part of the regulatory decision-making process. It would be important to demonstrate, for instance, that toxicogenomics not only can confirm what is already known about specific compounds and toxic end points (i.e., phenotypic anchoring) but also can accurately predict toxicity for unknown compounds. The task is to present regulatory scientists with new knowledge gained from toxicogenomics approaches in a familiar context. Ideally, at least in the short term, the focus will be the identification of single, or small sets of, genes or proteins that serve as biomarkers of response, as opposed to signatures of response that are the typical output of microarray experiments. Simple biomarkers of response are favored over complex expression signatures because they are familiar in toxicology assessment, are easy to maintain over time (e.g., are independent of the microarray platform), and can be readily validated. Validation strategies for toxicogenomics-based markers can be modeled after protocols for existing biomarkers. Thus, global gene expression technologies such as microarrays can be used to identify a specific gene marker,

or a suite of markers, that can then be validated by conventional methods such as Northern blot analysis, *in situ* hybridization, and quantitative polymerase chain reaction. This approach has advantages because regulatory agencies such as the U.S. FDA have proposed procedures to address gene and protein biomarkers, and other organizations, such as the Organisation for Economic Co-operation and Development (OECD 2005), are embarking on establishing similar guidance (Supplemental Material, Section 1; http://ehp.niehs.nih.gov/members/2005/8247/suppl.pdf).

Proof-of-principle studies could be conducted concurrently with existing regulatory test methods using similar samples of test compounds. In such situations, it may be appropriate to use *in vivo* systems, which are widely accepted by the regulatory community. Parallel *in vitro* studies could be conducted in situations where an appropriate test system is available. It may be wise to focus initial efforts on defining relationships between gene expression changes and toxicity for individual compounds or compound classes with well-defined end points. The experimental design should address conventional aspects of dose and time (dose response), species and strain susceptibility, group size and sex, and selection of end points for study (e.g., histopathology, clinical chemistry). Numerous commercial microarray platforms offer genomewide coverage for model systems such as rat, mouse, *Caenorhabditis elegans*, and humans. Commercial microarrays are also available for genes that are highly expressed in specific tissues (e.g., liver, breast) and during specific biological processes such as metabolism (e.g., P450 enzymes). Both genomewide and dedicated arrays can be used with RNA samples from *in vivo* and *in vitro* (tissue and cell culture) systems, enabling parallel studies to be conducted with a single microarray platform. This is important because the results of microarray experiments can vary depending on the array design and the selection and performance of gene probes on the array. Encouraging results on cross-platform comparisons and between-laboratory reproducibility are now emerging (Bammler et al. 2005; Chu et al. 2004; Irizarry et al. 2005; Larkin et al. 2005; Yauk et al. 2004). Toxicogenomics studies conducted in parallel and comparative systems can demonstrate the biologic relevance of *in vitro* models as surrogates for *in vivo* models without the need to address cross-platform (technologic) issues (Boess et al. 2003; Huang et al. 2003). Although initial efforts should focus on defining simple gene and protein biomarkers for specific compound classes, end points, and model systems, the end goal is to establish a compendium of compound-specific knowledge that transcends technology platform. Ideally, the markers should be robust enough to withstand technologic advances in toxicology that add to the existing knowledge about the compound. Once sufficient and adequately validated data are available, toxicogenomics can become part of a hierarchical approach to compound assessment.

The use of toxicogenomics to identify (screen) compounds with the potential to cause adverse effects may present opportunities to reduce the need for full animal tests, or perhaps refine animal use, and/or reduce the numbers of animals needed when *in vivo* tests are necessary. Of course, the statistical power of any test will influence the number of animals used in an *in vivo* test as well. Screening-type assessments may be appropriate for priority setting, dose setting, chemical ranking, and so forth. The extent of validation required for screening tests may be different than that required for full replacement tests because negative compounds might still undergo full animal testing. Establishing a compendium of compound-specific information will enable regulators and sponsors to access what is known about a compound across multiple test systems, species, and end points, thereby improving the biological relevance of regulatory decisions to safeguard human health and the environment.

Strategy 2: use of gene expression signatures to predict toxicity. Toxicogenomics holds great promise for improving predictive toxicologic assessments. Gene expression profiling has been used to classify compounds by chemical class and mechanism (Hughes et al. 2000; Scherf et al. 2000; Steiner et al 2004; Thomas et al. 2001), tumors by origin and type (Chung et al. 2002), and breast cancer patients for follow-up chemotherapy (van 't Veer et al. 2002). In all cases, classification was based on a set of discriminatory gene elements, between 10 and several hundred, identified from a larger pool of genes on a microarray. The pattern of gene expression, not the measurement of a single or a small set of genes, was the basis for classification. A variety of gene expression analysis algorithms were used to discriminate samples based on gene expression signature. In all cases, the compound class or tumor status was known *a priori*, and gene expression signatures for known samples were used to predict classification for other known but blinded samples (Blower et al. 2002; Brindle et al. 2002). Such models are currently being developed in the private sector (e.g., Gene Logic, Iconix) and are commercially available but cannot, as yet, be exploited by regulators and the scientific community because the underlying data sets and algorithms have not been made available outside the private sector.

Predictive model development will require an extensive "training" set of gene expression measurements for classes of model compounds in a variety of test systems, both *in vivo* and *in vitro*, at multiple doses and time points. Initial studies can be conducted concurrently with conventional testing systems as a way to confirm model predictions. In the short term, it is unlikely that sufficient data will be available for gene expression signatures to replace conventional approaches. Until then, such data can be used as part of a hierarchical approach to toxicity testing in conjunction with accepted methods routinely used for regulatory purposes. In the long-term, sufficient data should accumulate from well-designed validation studies such that gene expression signatures could be part of a battery of tests that reduce or replace animal procedures.

Model validation will necessitate multiple independent data sets and application of sophisticated statistical approaches. Acceptance of these models will require that research and regulatory communities have access to the data analysis tools used to build the models, and that they become familiar with the limitations and uncertainties of using these complex computational models. Confidence in and acceptance of these models will also require rigorous performance standards and appropriate controls to ensure reproducibility and stability over time (see below) and adequate sensitivity and specificity to discriminate toxic from nontoxic responses. Initial model development could easily be accelerated through coordinated sector-spanning efforts. Coordinated efforts across academia, government, and industry partnerships will accelerate progress in defining gene sets that are robust and discriminatory both within and across technology platforms. This is an ideal scenario given the rapidly advancing pace of technology development.

An important aspect of any toxicogenomics validation strategy is the need to measure the range of biological variability of gene responses for a given test system. Ideally, this should be accomplished by one species, tissue, and end point at a time, in order to adequately assess cross-species differences that often hamper risk assessments. Measurements of biologic variability under baseline and toxicant-challenged conditions will enable regulators to better discriminate biologically relevant responses from baseline homeostatic fluctuation. This is an important issue for toxicogenomics, as studies conducted on cell culture populations demonstrate a wide range of biological variability in gene expression measurements for individual cells under both baseline and challenged conditions (Kuang et al. 2004). Therefore, it is necessary to define criteria to adequately address biological variability in a data submission and to establish whether the burden of maintaining these data is that of the regulator or sponsor.

The recommendations related to the biological validation of toxicogenomics-based test methods are listed in Table 1.

Standardization and Validation of Toxicogenomics-Based Methods: Focus on the Technology

Considerations given to validation of the technology encompassed the technical and bioinformatics issues related to the validation of toxicogenomics-based test methods. The starting premise adopted was that with the availability of bioinformatics expertise, biological data generated from toxicogenomics studies could be interpreted with a high degree of confidence. The ultimate aim was to identify a strategic approach that would enable credible biological observations and consequential judicious regulatory decisions, and that this approach would be independent of the toxicogenomic platform used. Moreover, standardization and validation of toxicogenomic platforms were seen as essential for identifying and reducing technologic artifacts. Standardization would also be required to increase the certainty by which biological observations could be extrapolated across and between different microarray platforms. It is therefore important to build on the learning of previous and ongoing efforts in standardization of toxicogenomics (reviewed by Sansone et al. 2004).

Three distinct levels where validation is necessary were identified (see Figure 1 and discussion below). The first level of validation is the responsibility of the array manufacturer or provider and has to be performed only once. This can be seen as a "one-off validation" and relates to both the microarray quality and the instrumentation. The second level of validation is the responsibility of both the experimental toxicologist and the array manufacturer or provider. This can be seen as "routine validation" or best practice to allow data comparability. It encompasses quality control (QC) aspects of the critical experimental components and is a process that occurs on a regularly scheduled basis. The third level of validation, that is, determination of reliability and relevance, is needed every time a change is introduced into the test procedure. Performance standards developed based upon the original test method would serve as the criteria against which the revised method would be compared. Despite these multilevel validation needs, it was repeatedly emphasized that significant technologic development and progress in microarray platforms are still under way and that efforts to validate and standardize these technologic platforms must not be at the expense of innovation.

One-Off Validation

The one-off validation is the responsibility of the array manufacturer or array provider. This is required to ensure that the array platform being used is robust and that the inherent variability within the platform is transparent to the user and the regulator (Figure 1). The following were identified as being necessary for microarray-based toxicogenomics to be used in regulatory assessments:

- Microarrays should be fabricated in accordance with the principles of Good Manufacturing Practice (GMP).
- Specifications and performance criteria for all instrumentation and method components should be available.
- All quality assurance/quality control (QA/QC) procedures should be transparent, consistent, comparable, and reported.
- The array should have undergone sequence verification, and the sequences should be publicly available.
- All data should be exportable in a MAGE (MicroArray and Gene Expression)-compatible format.

Routine Validation

Routine validation is an ongoing process that is the responsibility of the experimental toxicologist and the array manufacturer or provider (Rockett and Hellmann 2004). Again, for microarray-based toxicogenomic assays to be used in regulatory decision making the following important factors were identified (Figure 1):

- Oligos, cDNAs, or clones that are arrayed should be randomly sequence-verified to ensure that no errors are introduced between batch syntheses. This verification process should be recorded and reported by the manufacturer
- All reagent components should be identified. Reagents should be prepared according to GMP and/or GLP as appropriate. Data regarding batch variability should also be recorded and reported
- Common reference RNA standards (housekeeping genes) should be adopted to facilitate comparison between array platforms. This may be achieved in collaboration with the international Microarray Gene Expression Data (MGED) Society and other related efforts (see below).

Biological standards. Performance standards, test component standards, and QC measures are key components of any validation strategy for a toxicologic test method. Establishing standards is particularly important for gene expression technologies due to the inherent technologic and biological "noise" in these systems. Commonly used biological standards are reference RNAs that are competitively hybridized with the sample of interest in two-channel array formats, and *in vitro* RNA transcripts that are "spiked into" RNA samples of interest in either one-channel or two-channel array formats. Establishing accepted RNA standards will address concerns of regulatory reviewers about data quality and variability within and between laboratories and across different technology platforms. The standards will also provide a common benchmark for regulators to assess platform performance over time. To achieve this goal, we must establish standards that maintain a defined level of accuracy, sensitivity, specificity, and reproducibility across platforms.

Reference RNAs can be derived from tissue extracts, cell lines, or both and serve a variety of purposes. Workshops sponsored by governments and industry have focused on defining the specifications for reference RNAs for clinical and regulatory applications (Joseph 2004). The consensus is a that multiple RNA standards are needed to measure the accuracy, dynamic range, sensitivity, and specificity of varied technology platforms under varied conditions. Important questions are whether regulatory agencies will define preferred sources of RNA standards, and, if so, who will generate and maintain baseline information about these

Table 1. Recommendations: focus on biological systems.

- Encourage increased use of toxicogenomics-based approaches to define the mechanistic context of toxic responses to exogenous compounds
- Promote greater understanding of the relationships between gene expression responses and altered phenotype, considering the biological pathways affected, dose response, and the point of departure from adaptive to toxic response
- Favor the identification of biomarkers that are independent of technology platform but acknowledge the potential strengths of pathway analysis
- Characterize the range and extent of biological variability of responses for the test systems (e.g., diurnal effects, animal care and use, age-related context)
- Encourage the immediate use of toxicogenomics-based approaches in conjunction with conventional toxicity testing approaches
- Explore the extent to which toxicogenomics can address cross-species responses and specific disease states
- Promote the conduct of parallel and comparative *in vivo* and *in vitro* studies to identify *in vitro* systems that can serve as surrogates for *in vivo* systems
- Characterize predictive toxicology models with respect to parameters such as dose, time, study design, relevance; characterize the system to fulfill validation criteria
- Promote the identification of gene and protein biomarkers as early (prognostic) markers as a refinement to existing toxicity testing methods
- Establish a compendium of toxicant information based on gene expression responses for model compounds across multiple species, end points, and test systems
- Foster the development of effective partnerships between academic, government, and industry groups to promote collaborative efforts to validate toxicogenomics-based test methods and generate sufficient high-quality data to support regulatory decision making