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Toxicity of compounds with endocrine activity in the OECD 421 reproductive toxicity screening test

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Samenvatting

De problematiek rond de hormoonontregelaars doet de vraag rijzen of voor de humane risicoschatting gevoeliger testmethoden nodig zijn om de reproductietoxische eigenschappen van xenobiotica met endocriene eigenschappen vast te kunnen stellen. In principe ligt het voor de hand dat stoffen die de balans van de geslachtshormonen verstoren ook effecten op de voortplanting en de embryonale ontwikkeling zullen hebben. Wij hebben zes bekende en verdachte hormoonontregelaars getetst in een bestaande OECD screeningstest voor reproductietoxiciteit, om vast te stellen of deze test deze stoffen als reproductietoxisch zou scoren. Het protocol omvat dagelijkse orale blootstelling van ouderdieren vanaf twee weken voorafgaand aan het paren, tot zes dagen postnataal voor moederdieren, en een totale blootstellingsduur van 28 dagen voor vaderdieren. Geteste stoffen waren het anticonceptivum Ethynyloestradiol, het fytooestrogeen Coumestrol, het surfactant 4-Tert-octylphenol, het plastic monomeer Bisphenol A, het fungicide Vinclozoline, en de weekmaker Butylbenzylftalaat. Doseringen werden gekozen op grond van de relatieve potentie op het niveau van de oestrogeenreceptor. Zodoende werden alle stoffen behalve Ethynyloestradiol getest bij doseringen die veel hoger liggen dan enige te verwachten blootstelling van de mens. Vijf stoffen kwamen uit de test duidelijk als reproductietoxisch naar voren, met effecten op vruchtbaarheid, luteinisatie, spermatogenese en foetale ontwikkeling. 4-Tert-octylphenol bleek lethaal bij de gekozen dosering en had weinig effect bij een tienvoudig lagere dosering. Deze data suggereren dat hormoonontregelaars waarschijnlijk effectief zijn in bestaande testen voor reproductietoxiciteit die gebruikt worden voor humane risicoschatting, bij doseringen die onder de in deze testen vastgestelde maximaal toe te passen dosering liggen. Hormonale eigenschappen van xenobiotica, vastgesteld op het niveau van de hormoonreceptor, dienen beoordeeld te worden in combinatie met dierexperimentele gegevens over algemene en reproductietoxiciteit voor een zinvolle risicoevaluatie en risico management van deze stoffen.

Summary

The issue of endocrine disruption has raised the question whether in view of human risk assessment more sensitive test methods are needed to detect the reproductive toxic properties of xenobiotic compounds with endocrine properties. In principle it would seem obvious that compounds which disturb sex hormone homeostasis will also affect reproduction and prenatal development. We have studied six known and alleged endocrine disruptors in an existing reproductive toxicology screening test to check whether this test would score these compounds as reproductive toxicants. The protocol involves parental dosage from two weeks premating to 6 days postnatally for dams, and a total of 28 days exposure in sires. Compounds tested were the contraceptive Ethynylestradiol, the phytoestrogen Coumestrol, the surfactant 4-Tert-octylphenol, the plastic monomer Bisphenol A, the fungicide Vinclozolin, and the plasticizer Butylbenzylphthalate. Compound dosage was chosen on the basis of relative potency at the estrogen receptor level. Therefore, apart from ethynylestradiol all compounds were tested at dosages much higher than any likely exposure to be expected in man. Five compounds were clearly scored as reproductive toxicants, affecting one or more parameters such as fertility, luteinization, spermatogenesis, and fetal development. 4-Tert-octylphenol appeared lethal at the dosage selected and had little effect at a tenfold lower dosage. These data suggest that endocrine disruptors are likely to be effective in existing OECD reproductive toxicity test systems used for human risk evaluation, at dosages below the maximal dosages to be used according to the test protocols. Endocrine properties of xenobiotic compounds, assessed at the hormone receptor level, should be combined with in vivo data on their reproductive and general toxicity for proper risk evaluation and risk management of these compounds.

1. Introduction

Synthetic compounds with endocrine activity have been suggested to cause reproductive effects in wildlife animal populations. A similar association has been hypothesized for exposure to such endocrine disrupters and human reproductive disorders (Colborn et al., 1993). Many compounds that were mentioned in this respect have been in use for decades and have not been specifically tested for their hormone-disrupting properties and reproductive toxicity. In addition, for new compounds, reproductive toxicity assessment is not required until a certain production level is reached. In order to update the knowledge as regards the endocrine properties of xenobiotics, a quick assay is needed which incorporates the relevant sensitive endpoints for endocrine activity. Specific hormone-related end points such as estrogen receptor affinity and activation capacity have been suggested as sensitive and pertinent end points. The question emerges whether existing regulatory test systems do or do not contain the relevant and sensitive endpoints for detection of endocrine activity and its toxic consequences.

Many assays have been proposed for endocrine disruption screening (OECD 1997). Specific assays which determine single parameters such as hormone receptor binding in vitro or uterine hypertrophy in vivo lack the generality of response parameters necessary for estimating the possible reproductive risk of exposure to these compounds. Complete multigeneration studies and developmental toxicity studies are time-consuming and expensive. The OECD 421 reproductive toxicity screening assay was developed for quick and rough assessment of the reproductive toxic potential of high production volume chemicals. It is also recommended for use at base set production level if general toxicology assessment suggests possible reproductive toxicity of a compound. Compounds should be tested to a maximum dose level of 1000 mg/kg bodyweight per dagy. Using the protocol, males and females are dosed during premating, mating, postmating, pregnancy and postnatal stages and then parents and pups are necropsied and parental sex organs are analyzed histologically. In view of its characteristics this test may also be useful for screening compounds for sex hormone disturbing properties.

We have tested six alleged endocrine disrupters in this assay at dosages selected on the basis of their estrogenicity relative to ethynylestradiol, and for comparison we additionally determined sex hormone levels and histopathology of pup sex organs.

Ethynylestradiol is widely used in contraceptive medicine. It causes a maximal estrogenic response in the rat uterotrophic assay after three daily doses of 100 µg/kg bw (Odum et al., 1997), and this dose was also used in the present study.

Coumestrol is a naturally occurring plant bioflavonoid, which mimics estradiol in the uterotrophic assay with a hundredfold lower potency (Markaverich et al., 1995). Therefore, in the present study coumestrol was dosed daily at 10 mg/kg bw.

4-Tert-octylphenol represents a large group of alkylphenols, which are widely used as surfactants and plastic additives. They have varying estrogenic potency depending on their structural characteristics. The compound selected here was the most potent alkylphenol found in a yeast cell human estrogen receptor activation assay, the activity being at least 3 orders of magnitude lower as compared to estradiol (Routledge

and Sumpter 1997). We therefore planned testing 4-tert-octylphenol at 1000 mg/kg bw.day.

Bisphenol A is used as a monomer in the manufacture of polycarbonate plastics. In developmental toxicity studies it was ineffective in rats at 640 mg/kg bw.day and induced resorptions in mice at 1250 mg/kg bw.day (Morrissey et al., 1987). In an estrogen replacement assay using the rat uterine estrogen receptor, bisphenol A had an affinity approximately 1:2000 that of estradiol (Krishnan et al., 1993). This compound was tested at 1000 mg/kg bw.day.

Vinclozolin is a fungicide used on fruits, vegetables and vines. It inhibits sexual differentiation in male rats in an antiandrogenic manner. An effective dose of 100 mg/kg given daily during the second half of gestation has been reported in rats (Gray et al., 1994). We employed the same daily dose in the present study.

Butylbenzylphthalate is a representative of a large group of phthalic esters, which are used widely as plasticizers in e.g. medical devices and food packaging. Using the OECD 421 protocol we have shown that this compound induces postimplantation loss at 1000 mg/kg bw.day (Piersma et al., 1995). We have used this dose again in the present study.

2. Methods

2.1 Chemical compounds and doses

The contraceptive ethynyl-estradiol (Sigma E8476) was used at 0.1 mg/kg body weight per day. The phytoestrogen coumestrol (Fluka 27885) was used at 10 mg/kg bw.day. The detergent 4-tert-octylphenol (Fluka 75070) was used at 100 mg/kg bw.day. The plastic component bisphenol A (Acros 15824) was used at 1000 mg/kg bw.day. The fungicide vinclozolin (Brunschwig-Ehrenstorfer C179200) was used at 100 mg/kg bw.day. The plasticizer butylbenzylphthalate (BBP, Merck 821030) was used at 1000 mg/kg bw.day. Compounds were administered by gavage using corn oil (Sigma C8267) as the vehicle at 1 ml per 200 g body weight. A control group was dosed with the same volume of corn oil only.

2.2 Animals and husbandry

Wistar (WU) Hsd/Cpb spf rats, age 12-15 weeks at the onset of dosing, were kept individually in macrolon type 3 and 4 cages, with standard RMH-GS feed and tap water available at libitum. Temperature was 20-24 °C, humidity was 50-75%, and a 12-12 hour light-dark cycle was installed, with the light period starting at 7.00 AM. Animals were checked daily for signs of ill health. Dosing was started after 2 weeks acclimatization followed by randomization of rats over treatment groups.

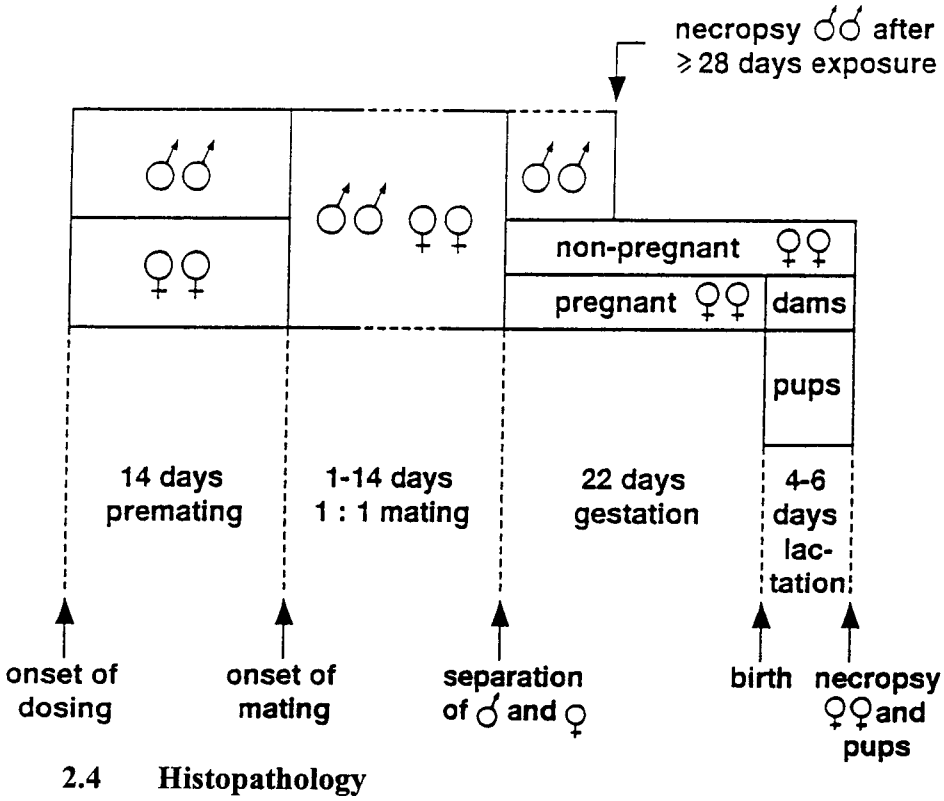
2.3 Experimental protocol

The OECD 421 protocol (figure 1) was used. Some extra parameters were added to be able to study in more detail possible effects on sex hormone levels and sex organ morphology. Briefly, after dosing both sexes by gavage for 14 days, males and females

were paired (1:1), four pairs per dosage group, and allowed to mate for a maximum of 14 days, whilst dosing was continued. If daily vaginal sperm detection showed evidence of mating, animals were separated. Nonmating pairs were separated after 14 days. Males were dosed further and killed and necropsied after a total dosing period of 28 days. Sex organs were removed, weighed, and analyzed histologically. Dosing of females was continued until postpartum day 6, when females were killed and necropsied. Uteri and ovaria were removed and weighed, corpora lutea and implantation sites counted, and uteri were analyzed histologically. Pups were counted, sexed, weighed and examined for external malformations on days 1 and 6 after birth. Parental body weights were recorded weekly with the exception of the mating period. Parental circulating sex hormones were determined at termination, and pup anogenital distance was measured and pup sex organs were analyzed histologically. Dosing was different from the general protocol for two compounds: 4-tert-octylphenol was dosed from 12 days premating onward due to extreme toxicity of a tenfold higher dose, and coumestrol was dosed for the first ten days premating only due to unforseen limited availability of the compound.

figure 1

Schematic representation of the OECD421 protocol



2.4 Histopathology

Tissues sampled for histopathology were trimmed and processed routinely for paraffin embedding. Organs included uteri and testes from dams and sires, respectively, as well as from offspring. Sections (5μm) were cut and stained with haematoxylin and Eosin (H&E).

2.5 Endocrinology

Hormones were measured by radioimmunoassay (RIA). Estradiol was determined with the Estradiol Double Antibody kit from Diagnostic products Corporation (KE2D1; DPC; Los Angeles CA, USA); progesterone was determined with the Coat-A-Count Progesterone RIA kit (TKPG1; DPC); testosterone was determined with the Coat-A-Count Testosterone RIA kit (TKTT1; DPC). Luteinizing hormone (LH) and follicle stimulating hormone (FSH) were analyzed using reagents (LH: 125I-rLH-15, rLH-RP2 and anti-rLH-S6; FSH: 125I-rFSH-15, rFSH-RP1 and anti-rFSH-S11) kindly provided by the NIDDK, National Hormone & Pituitary Program (Dr.Parlow, CA, USA).

3. Results

Body weights and food consumption (Tables 1-3), necropsy data (Table 4-5), histopathology (Table 6 and 7 and Plate 1), and sex hormone levels (Tables 8-9), are discussed below for each compound tested.

3.1 Ethynyl-estradiol

This compound, administered at 0.1 mg/kg bw.day, caused decreased body weight gain and food consumption in both sexes in the premating phase and decreased body weight gain over the complete dosing period. No pregnancy occurred in 4/4 pairs. The endometrium of exposed dams showed increased cellularity and basophilia, with apoptotic bodies, mitotic figures and focal metaplasia. The lamina propria was collagen-rich with polymorphonuclear granulocytes. The testis of exposed sires was reduced to one third of control weight, and showed severe inhibition of spermatogenesis and atrophy of interstitial cells. In sires, circulating estradiol was increased, whereas FSH and LH were variably decreased. Testosterone was decreased by two orders of magnitude which was consistent with histopathological Leydig cell hypoplasia and decreased mature sperm numbers. In females, estradiol was increased over pregnant controls. FSH, LH and progesterone were relatively low. The increases observed in estradiol were not significant, and may have been caused by cross-reactivity of ethynylestradiol in the estradiol assay.

3.2 Coumestrol

As a consequence of unforseen limited availability of this compound it was administered at the first ten dosing days only. No effects on food consumption and body weight were found. Three out of 4 dams became pregnant. Remarkably, at necropsy the number of corpora lutea was about 50% increased over controls, suggesting premature luteinization. Pup testis weight was decreased over controls but histology was normal. Sire testis weight tended to be somewhat reduced but testis histology was unremarkable. Hormone levels were not changed at necropsy.

3.3 4-Tert-octylphenol

One dose of this compound at 1000 mg/kg bw was lethal to the animals. As a consequence, new rats were enrolled into the study which were dosed from 12 days premating onward, at 100 mg/kg bw.day. This dosage affected food consumption and body weight gain of dams and sires. Two out of 4 dams became pregnant. At necropsy pregnant dams showed a small increase of corpora lutea over controls, suggesting possibly premature luteinization. Histological analysis showed no abnormalities in both sexes. LH was somewhat low in dams, and testosterone was decreased in sires.

3.4 Bisphenol A

Food consumption and body weight gain were markedly reduced in the first dosing week for both dams and sires. Over the complete dosing period body weight gain was reduced for both sexes. Pregnancy was achieved in 3/4 pairs. At necropsy one pregnant dam had 30 corpora lutea, suggesting premature luteinization. In dams, FSH and LH tended towards an increase, whereas in sires no sex hormone changes were observed. Histological analyses did not reveal abnormalities.

3.5 Vinclozolin

Food consumption and body weight gain were only marginally reduced over the complete dosing period in both sexes. Pregnancy occurred in 3/4 pairs. Necropsy of dams did not reveal abnormalities. Mean pup weight at day 1 and at day 6 after birth was reduced over controls. Sex ratio at day 1 was shifted dramatically towards a majority of fenotypically female pups, as determined by measurement of anogenital distance. At necropsy, the sex ratio appeared normal as evidenced by internal sex organs. Pup testis weight was decreased over controls but histology was normal. Sire testis histology revealed unaffected spermatogenesis, but a clear increase was observed in number and size of interstitial cells. Progesterone and FSH appeared low in dams, and FSH and LH were clearly increased in sires, accompanied with increased average testosterone levels.

3.6 Butylbenzylphthalate

Food consumption and body weight gain were relatively low in the first dosing week in dams and sires. Body weight gain was reduced in the last week of pregnancy and over the total dosing period in the dams. Sire body weight gain over the total dosing period was unaffected. Pregnancy occurred in 3/4 pairs. Remarkably, at necropsy the number of corpora lutea was about 50% increased over controls, suggesting premature luteinization. The number of live pups per dam was severely reduced. Body weights and testis weights of surviving pups were severely reduced also. Sire testis weights were reduced and histopathology revealed focal degenerative changes in testicular tubules, with slightly increased size and number of interstitial cells. Progesterone levels were decreased in dams, whereas FSH and LH tended towards an increase. In sires LH and FSH were increased, with no change in testosterone levels.

4. Discussion

This study was initiated to determine whether compounds with endocrine disrupting properties can be detected with classical parameters in reproductive toxicology. The experimental results clearly show that five out of six diverse xenobiotic compounds are indeed readily detected as they affected one or several parameters used in routine reproductive toxicity testing. Ethynylestradiol precluded pregnancy and affected testis weight and histology, coumestrol and bisphenol A affected corpora lutea numbers, vinclozolin affected offspring sex organ development and sire testicular histology, and butylbenzylphthalate affected live pup numbers and sire testis histology. These affected end points are all regularly studied in guideline-based routine reproductive toxicity testing protocols. The only reproductive effect observed after exposure to 4-tert-octylphenol was a questionable increase in corpora lutea. However, we had to lower the dose of this compound due to extreme toxicity, which suggests that other toxicity than endocrine-mediated reproductive effects may be more important for this compound. Such a finding underscores that in the process of risk assessment endocrine activity of compounds should not be regarded separately, but as part of their complete toxicity profile. In addition it should be stressed that apart from ethynylestradiol, all compounds were tested at dosages much higher than any likely exposure to be expected in man. These results do not support the need for additional endocrine parameters to increase the sensitivity of existing test systems for alleged endocrine disrupters.

We have modified the OECD 421 protocol in some aspects to accomodate the specific question of the present study. The number of pairs of rats was reduced from 8 in the protocol to 4 in this study, as animals could be saved in view of the fact that at the high doses used clear or no effects were expected. Secondly, only one dose per compound was used instead of the prescribed 3 doses and a control group, as the effectiveness of a range of compounds was studied rather than the toxicity profile of one compound. As another addition to the protocol, the histology of pup sex organs was also studied, as they were available at necropsy anyway. Newborn uterine histology can be changed by estrogen exposure (Branham et al., 1985) and testicular size and sperm production were affected in pups a.o. by octylphenol and butylbenzylphthalate in a similar exposure protocol as in the OECD 421 test although animals were necropsied 3 weeks after birth (Sharpe et al., 1995). In our study, pup sex organs collected at postnatal day 6 appeared all similar to the controls histologically, except for those exposed to vinclozolin. The size and stage of development of the organs at this stage therefore appear not optimal for assessment of endocrine effects. A further addition to the protocol included the assessment of sex hormones in the circulation in sires and dams at necropsy. In general, the hormonal data showed a large variability, which complicates the interpretation of the data. The small group sizes (2 to 4) have contributed to this situation. Nevertheless, FSH, LH and testosterone levels in sires, and FSH, LH and progesterone in dams did show some probably real effects after exposures to some of the compounds tested. Estradiol did not show any significant change in this study. Given the variability in physiological hormone concentrations and the observed sensitivity of the standard OECD 421 parameters, hormone levels did not contribute to the sensitivity of the protocol as a whole.

After the issue of endocrine disruption emerged, a wealth of test systems have been put forward for rapid assessment of endocrine properties of compounds. Most

systems have been directed towards detection of estrogenic activity, with in vitro estrogen receptor binding and activation assays being most prominent. These assays are sensitive enough to detect estrogenic activity many orders of magnitude below that of the physiologic estrogens circulating in the complete organism. The question is what such low activities at the receptor level mean for effectiveness of the compound after in vivo exposure in animals and man. External exposure level, uptake, absorption, distribution, biotransformation, and excretion may limit the availability of the compound at the estrogen receptor, and even if internal exposure occurs, normal sex hormone homeostasis may compensate for the exposure. In vivo test systems such as the uterotrophic assay which only cover the estrogenic pathway, still give an incomplete picture of the compounds' properties, as other mechanistic routes may also lead to the reproductive toxic effects that have been suggested in relation to endocrine disruption. Therefore, although specific in vitro and in vivo tests are certainly useful in hazard identification and prioritization of compounds with relatively high estrogen receptor binding affinity and receptor activation properties for further testing, only in vivo reproductive toxicity testing can be the basis for proper risk evaluation and risk management of these compounds.

The question whether in view of human risk assessment new test systems should be developed or new end points added to existing test systems for adequate detection of endocrine disruptors is a timely one. It is to be expected that compounds that affect sex hormone homeostasis beyond the limits of normal regulation will cause developmental toxicity or fertility effects. The present study was designed to check this expectation. The existing reproductive toxicity screening test OECD 421 was employed, in which merely simple classical parameters of reproduction and development are incorporated. We have shown that five out of six diverse xenobiotic compounds with diverse endocrine activity are clearly scored in this test as reproductive toxicants. The sixth compound was lethal at the dose selected on the basis of its estrogenicity. Although these findings should not readily be generalized for all xenobiotic compounds, they support the notion that existing reproductive toxicity tests are probably adequate for detection of compounds mentioned in relation to endocrine disruption that may pose a reproductive risk for the human population.

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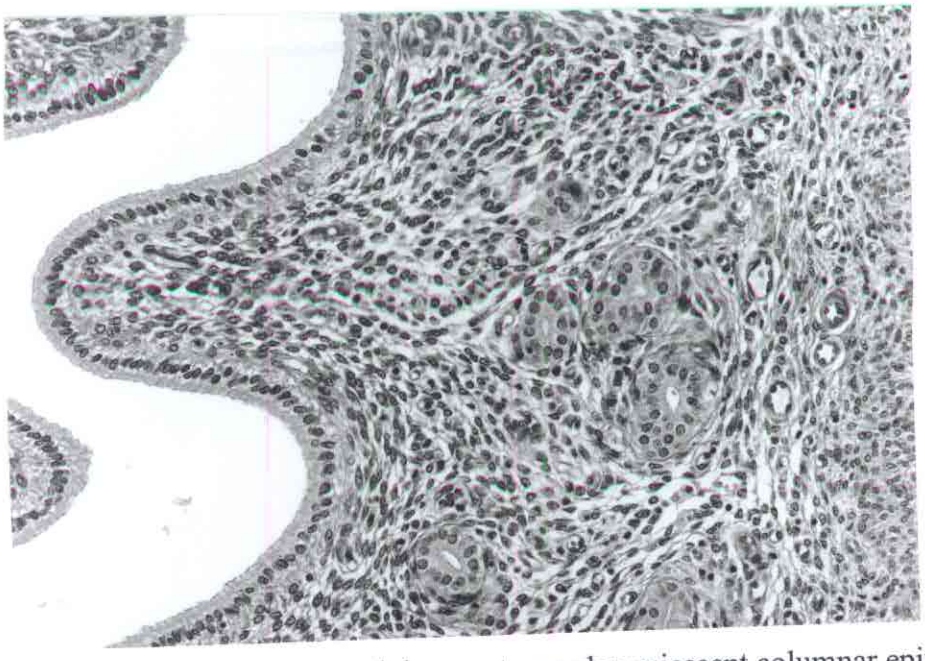
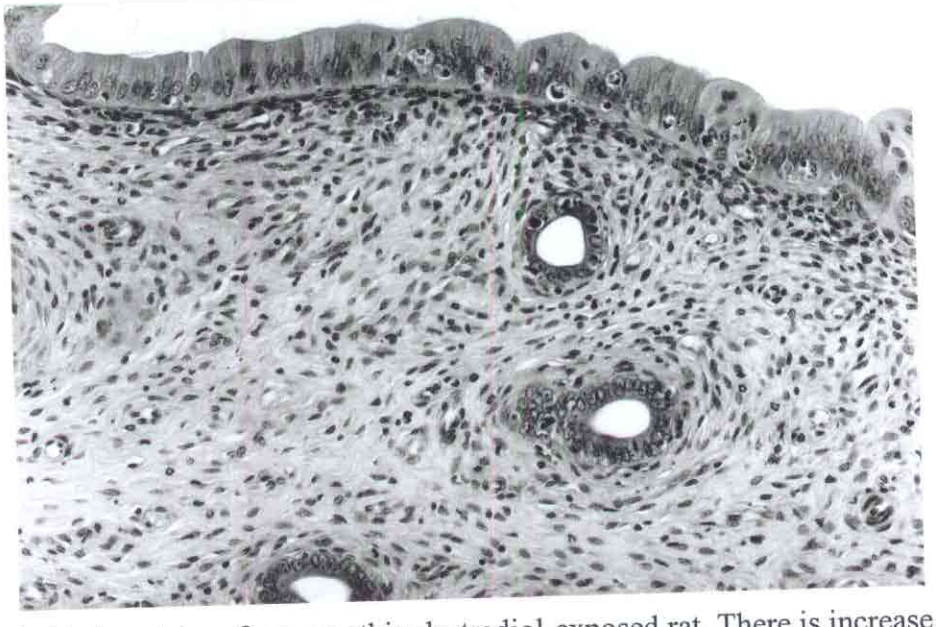
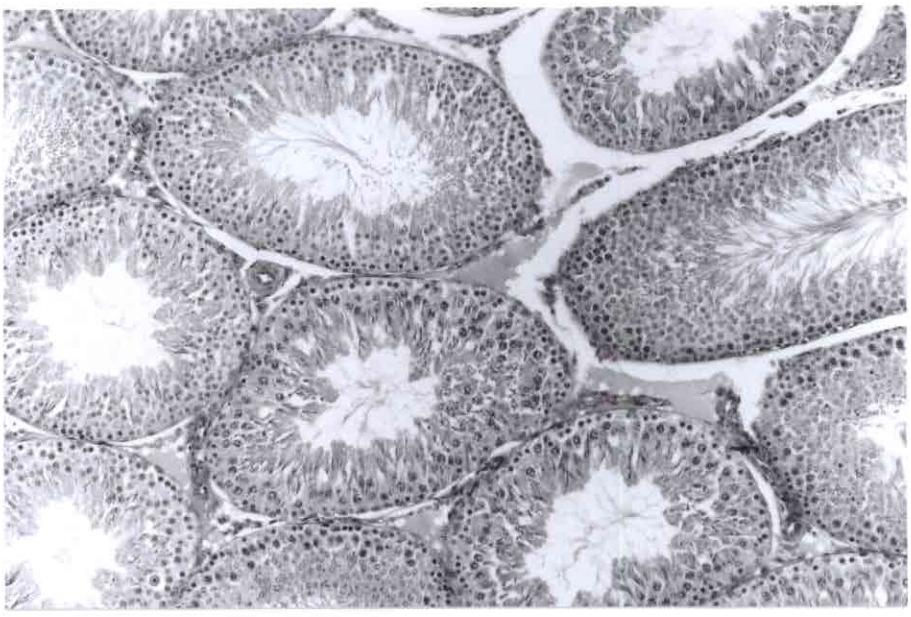
Appendix 1 Histology of uterus and testis



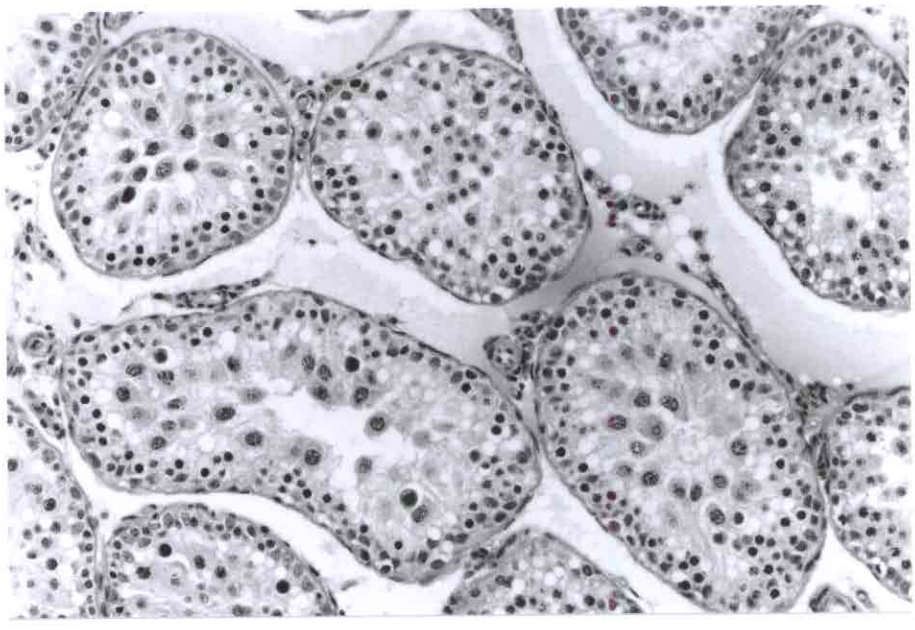
a. Endometrium of a control dam: note regular quiescent columnar epithelium (including uterine glands) and highly cellular propria with minimal collagen.



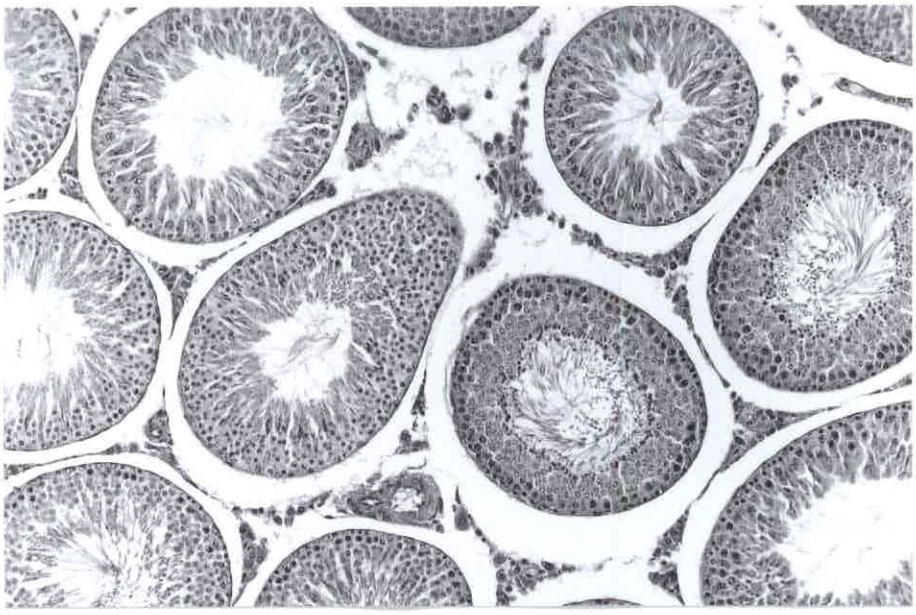
b. Endometrium from an ethinylestradiol-exposed rat. There is increase in cellularity and basophilia of the epithelium, with many apoptotic bodies and mitotic figures, and focal metaplasia. Propria is collagen-rich and contains polymorphnuclear granulocytes.



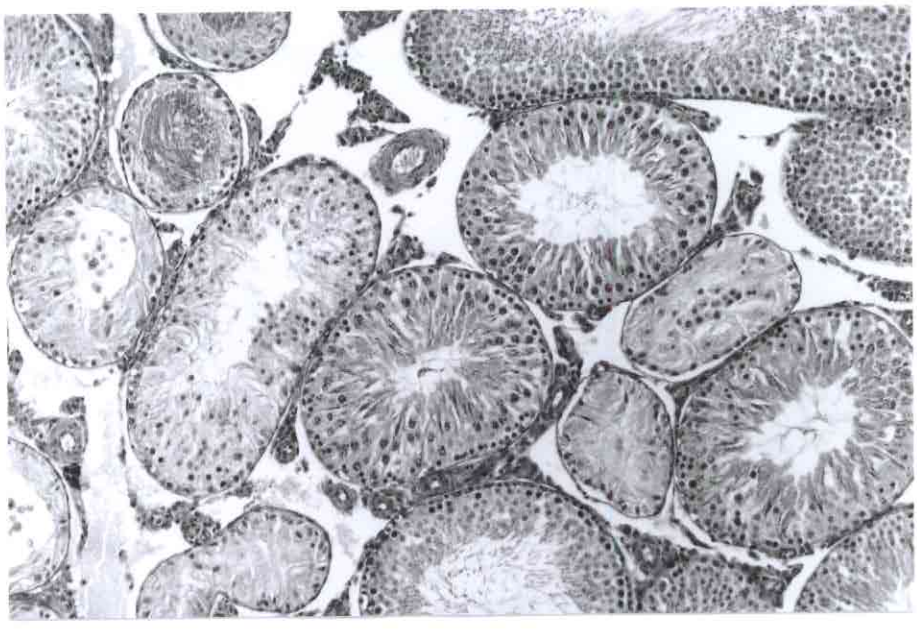
c. Control male. Normal testicular tubules showing various stages of spermatogenesis. Note interstitial cells.



d. Testis from an ethinylestradiol-exposed rat. There is severe inhibition of spermatogenesis and atrophy of interstitial cells.



e. Testis of a vinclozolin-exposed rat. The process of spermatogenesis seems unaffected, but there is clear increase in number and size of interstitial cells.



f. Testis of a BBP-exposed rat. Degenerative features are found in a number tubules, and the number and size of interstitial cells are slightly increased.

Appendix 2 Tables

Table 1. Summarized body weight and food consumption data

compound dose (mg/kg bw.day)	OIL 0		EES 0.1		COU 10		OCT 100		BIS 1000		VIN 100		BBP 1000	
	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean
sires														
body weight gain (g)														
day 0-7 premating	4	18	3	-32	4	21	4	1*	3	-24	4	12	4	7
day 7-14 premating	4	22	3	-3	4	11	4	6	3	19	4	10	4	25
day 0-28	4	62	3	-41			4	3**	3	8	4	45	4	54
food consumption (g)														
day 0-7 premating	4	160	3	83	4	158	4	75*	3	79	4	150	4	136
day 7-14 premating	4	156	3	109	4	162	4	125	3	137	4	142	4	178
dams														
body weight gain (g)														
day 0-7 premating	4	4	4	-6	4	0	4	4*	4	1	4	2	4	7
day 7-14 premating	4	7	4	9	4	4	4	-5	4	18	4	6	4	10
not pregnant														
day 0-7	0	-	4	1	-	-	2	-5	1	17	1	33	1	22
day 7-14	0	-	4	2	-	-	2	5	1	7	1	3	1	10
day 14-21	0	-	4	2	-	-	2	-2	1	-13	1	-1	1	2
pregnant														
day 0-7	4	28	0	-	-	-	2	24	3	17	3	24	3	26
day 7-14	4	34	0	-	-	-	2	11	3	20	3	29	3	30
day 14-21	4	67	0	-	-	-	2	58	2	2	3	73	3	16
food consumption (g)														
day 0-7 premating	4	94	4	70	4	93	4	50*	4	76	4	106	4	94
day 7-14 premating	4	96	4	80	4	102	4	76	4	102	4	105	4	103
not pregnant														
day 0-7	0	-	4	77	-	-	2	84	1	104	1	139	1	105
day 7-14	0	-	4	75	-	-	2	102	1	103	1	135	1	109
day 14-21	0	-	4	68	-	-	2	75	1	72	1	107	1	108
pregnant														
day 0-7	4	112	0	-	-	-	2	100	3	111	3	117	3	125
day 7-14	4	129	0	-	-	-	2	112	3	122	3	123	3	137
day 14-21	4	140	0	-	-	-	2	106	2	125	3	133	3	127

* day 2-7 premating

** day 2-28

Table 2. Individual body weight, food consumption and necropsy data sires

		before mating							necropsy		
		day 0		day 7			day 14		day 28		
compound	rat no.	bw (g)	food given (g)	bw (g)	rest food (g)	food given (g)	bw (g)	rest food (g)	testis wt (g)	bw (g)	testis wt/bw
OIL	29	458	384	473	233	368	492	225	5.60	510	1.10
	30	465	408	486	243	394	508	230	5.52	533	1.04
	31	420	389	436	239	359	465	208	4.81	495	0.97
	32	461	414	481	239	354	499	189	5.77	514	1.12
EES	33	501	436	465	352	378	469	257	1.22	456	0.27
	34	465	437	428	359	394	418	294	1.09	402	0.27
	35	417	450	395	363	408	392	301	3.04	401	0.76
	36	*									
COU	37	391	399	407	244	369	421	208	5.90	nd	nd
	38	397	490	425	337	363	434	203	5.23	nd	nd
	39	415	468	438	300	351	450	184	4.86	nd	nd
	40	391	420	408	263	342	416	182	4.96	nd	nd
	45	*									
	46	378	385	357	306	386	377	256	5.00	388	1.29
	47	405	432	383	345	423	391	289	4.96	403	1.23
	48	416	385	386	313	363	415	216	5.19	432	1.20
VIN	49	361	403	361	277	348	367	231	4.49	384	1.17
	50	434	447	442	287	359	449	210	5.06	478	1.06
	51	416	308	433	147	313	443	166	5.31	470	1.13
	52	391	416	414	265	348	430	194	5.16	450	1.15
BBP	53	432	414	442	273	341	468	164	4.51	485	0.93
	54	401	381	402	244	320	424	149	4.02	453	0.89
	55	424	396	427	275	339	459	145	5.23	490	1.07
	56	415	371	427	228	319	445	148	4.11	460	0.89
		day 2 (!)									
		bw (g)	food given (g)								
OCT	41	446	318	442	241	305	446	177	5.05	452	1.12
	42	440	360	429	304	370	436	261	5.48	432	1.27
	43	465	364	473	282	334	477	214	4.90	454	1.08
	44	411	387	420	302	345	429	203	5.44	434	1.25

* minated after dosing error

Table 3. Individual body weight and food consumption data dams

		before mating							after mating										pregnant
		day 0		day 7			day 14		d0		d7			d14			d21		
compound	rat no.	bw (g)	food given (g)	bw (g)	rest food (g)	food given (g)	bw (g)	rest food (g)	bw (g)	food given (g)	bw (g)	rest food (g)	food given (g)	bw (g)	rest food (g)	food given (g)	bw (g)	rest food (g)v	food given (g)	
OIL	1	230	256	230	158	276	238	176	244	249	271	140	256	299	126	415	357	283	340	yes
	2	215	269	222	176	276	230	182	226	377	251	266	301	279	182	384	353	241	342	yes
	3	185	314	191	226	304	194	214	196	414	227	301	368	266	238	365	335	229	426	yes
	4	214	274	215	176	263	225	163	231	424	258	310	365	300	229	335	367	188	398	yes
EES	5	197	308	191	246	319	198	249	199	277	195	216	394	198	318	318	196	268	268	no
	6	200	303	202	224	293	218	196	216	343	221	247	321	213	250	354	216	275	452	no
	7	195	281	187	218	288	195	212	199	462	194	383	383	200	305	305	203	236	333	no
	8	228	283	216	207	270	219	192	213	227	219	157	425	227	351	351	231	279	336	no
COU	9	215	271	216	179	255	219	151	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	yes
	10	224	236	221	138	250	231	140	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	yes
	11	223	303	225	209	279	230	180	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	yes
	12	193	259	193	173	264	192	168	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	yes
BIS	17	235	236	234	150	246	257	130	261	412	279	289	289	306	160	414	270	278	467	yes
	18	206	271	196	213	294	220	202	230	302	248	204	464	279	338	397	nd	nd	nd	yes
	19	208	250	209	172	275	217	173	241	226	257	115	308	259	198	280	298	166	207	yes
	20	195	250	208	170	267	226	170	215	384	232	280	280	239	177	402	226	330	330	no
VIN	21	224	268	232	160	280	228	166	267	267	300	128	416	303	281	434	302	327	327	no
	22	240	246	237	140	238	245	137	237	340	261	223	265	289	136	378	344	249	417	yes
	23	214	261	216	153	218	232	121	231	328	255	217	256	289	136	408	374	266	423	yes
	24	228	248	230	148	255	235	148	239	349	263	227	257	288	137	430	368	302	454	yes
BBP	25	187	271	181	190	271	193	179	203	356	225	251	343	235	234	450	237	342	383	no
	26	204	266	213	180	251	222	155	216	378	234	275	319	262	199	493	286	369	492	yes
	27	218	293	235	187	261	240	150	239	383	267	253	317	291	186	445	301	320	385	yes
	28	214	298	221	197	258	236	144	232	262	265	119	174	303	13	477	316	344	407	yes
		day 2 (l)																		
		bw (g)	food given (g)																	
OCT	13	230	237	226	195	267	218	208	216	274	239	184	253	237	159	365	235	294	447	no
	14	213	249	218	205	256	219	179	214	268	233	174	260	248	158	389	291	295	361	yes
	15	228	192	232	133	257	230	166	254	369	221	292	409	232	299	299	231	221	253	no
	16	232	220	241	165	256	231	181	229	366	257	260	281	263	159	276	335	158	368	yes

Table 4. Summarized necropsy data dams and pups

Compound	OIL	EES	COU	OCT	BIS	VIN	BBP
Dose (mg/kg bw.day)	0	0.1	10	100	1000	100	1000
dams							
Females mated	4	4	4	4	4	4	4
Females achieving pregnancy	4	0	3	2	3	3	3
Day 0 (day of birth)							
Dead foetuses/dam (mean)	0		0	0	0	0.3	0
Live foetuses/dam (mean)	13.0		12.7	10.0	11.3	12.3	3.0
Day 6 (necropsy day)							
Corpora lutea/dam (mean)	14.8		23.3	18.5	19.0	16.0	21.7
Implants/dam (mean)	13.5		14.0	11.5	9.5	14.0	11.3
Live pups/dam (mean)	13.3		12.7	10.0	10.5	12.0	1.0
pregnant uterus weight (g)	0.48		0.41	0.49	0.44	0.43	0.67
not pregnant uterus weight (g)		0.54	0.43	0.59	0.38	0.64	0.50
pups							
Day 1 (after birth)							
Number examined	52		38	20	21	37	3
Body weight (g) (mean)	7.3		7.5	7.4	7.1	6.3	5.8
sex ratio (f/m) (mean)	0.93		1.24	0.67	1.63	36.00	0.00
Day 6 (necropsy day)							
Number examined	53		38	20	21	36	3
sex ratio (f/m) (mean)	0.96		0.90	0.67	2.00	0.80	2.00
Body weight (g) (mean)	13.7		13.6	14.2	12.5	11.5	11.0
Mean anogenital distance (female) (mm)	3.1		2.9	2.7	2.9	2.9	3.6
Mean anogenitale distance (male) (mm)	6.3		5.8	6.1	5.8	3.8	3.1
Mean uterus weight (mg)(n)	16 (6)		13 (8)	18 (5)	20 (6)	12 (9)	13 (2)
Mean testis weight (mg)(n)	57 (9)		46 (9)	58 (7)	52 (6)	33 (8)	15 (1)

Table 5. Individual necropsy data dams and pups

				dag 0 (day of birth)		dag 6 (necropsy day)			
compound	rat no.	vaginal plug date	birth date	dead pups	live pups	corpora lutea	implantation sites	uterus weight (g)	live pups
OIL	1	29-Dec-97	20-Jan-98	0	12	13	13	0.50	12
	2	24-Dec-97	16-Jan-98	0	14	16	15	0.49	14
	3	23-Dec-97	15-Jan-98	0	12	15	13	0.41	13
	4	23-Dec-97	15-Jan-98	0	14	15	13	0.52	14
EES	5	5-Jan-98	not pregnant					0.64	
	6	23-Dec-97	not pregnant					0.45	
	7	25-Dec-97	not pregnant					0.47	
	8	3-Jan-98	not pregnant					0.61	
COU	9	25-Dec-97	16-Jan-98	0	15	29	17	0.54	15
	10		19-Jan-98	0	11	26	11	0.36	11
	11	23-Dec-97	15-Jan-98	0	12	15	14	0.33	12
	12	23-Dec-97	not pregnant					0.43	
OCT	13	23-Dec-97	not pregnant					0.67	
	14	23-Dec-97	15-Jan-98	0	8	17	9	0.44	8
	15	5-Jan-98	not pregnant			6	0	0.50	
	16	25-Dec-97	17-Jan-98	0	12	20	14	0.55	12
BIS	17	25-Dec-97	16-Jan-98	0	15	30	13	0.52	15
	18	30-Dec-97	died 18-Jan-98*	0	13*	nd	nd	nd	nd
	19	2-Jan-98	25-Jan-98	0	6	8	6	0.36	6
	20	27-Dec-97	not pregnant					0.38	
VIN	21	1-Jan-98	not pregnant					0.64	
	22	25-Dec-97	16-Jan-98	0	10	14	10	0.40	10
	23	25-Dec-97	16-Jan-98	1	13	16	16	0.43	13
	24	25-Dec-97	16-Jan-98	0	14	18	16	0.46	13
BBP	25	29-Dec-97	not pregnant			1	1	0.50	
	26	23-Dec-97	16-Jan-98	0	3	34	8	0.39	3
	27	23-Dec-97	not observed	0	0	15	14	0.66	0
	28	23-Dec-97	not observed	0	0	16	12	0.97	0

* dosing error, fetuses were counted after termination

Table 6. Histopathology of maternal uterine endometrium

compound	rat no.	basophilia	hyperplasia	apoptosis	nucleus *	hypochromatin	pmn**
OIL	1	-	-	-	c		
	2	-	-	-	c		
	3	-	-	-	c		
	4	-	-	-	c		
EES	5	++		+	b	+	++
	6	++	++	++	b	++	++
	7	++	++	++	b	++	++
	8	++	+	+	b	++	++
COU	9	-	-	-	c	-	-
	10	-	-	-	c	-	-
	11	-	-	-	c	-	-
	12	+	+	+	b	+	++
OCT	13	++	+	+	b	+	+
	14	-	-	-	c	-	-
	15	+	+	+	c	+	+
	16	-	-	-	c	-	-
BIS	17	-	-	-	c	-	-
	19	-	-	-	b	+	-
	20	++	+	-	b	++	+
VIN	21	++	++	++	b	++	++
	22	+	+	+	c	+	-
	23	+	+	-	c	+	-
	24	+	+	-	c	+	-
BBP	25	++	++	++	b	++	++
	26	-	-	-	b	+	-
	27	++	++	+	b	++	++
	28	++	+	-	b	++	++

- Normal
+ Increased
++ Highly increased
* Nucleus located centrally (c) or basally (b) in the cell.
** pmn: polymorphonuclear granulocytes

Table 7. Histopathology of sire testis

compound	rat no.	degeneration type *	Leydig cells	remarks
OIL	29	focal 3-4		near suspensory ligament
	30	no anomalies		
	31	focal 3-4		near suspensory ligament
	32	no anomalies		
EES	33	diffuse 3	hypoplasia	maturation block
	34	diffuse 3	hypoplasia	
	35	focal 2		few mature sperm
COU	37	diffuse 3-4 unilateral		maturation block
	38	focal 2-4 subcapsular		
	39	no anomalies		
	40	no anomalies		
OCT	41	no anomalies		
	42	focal 5		near suspensory ligament
	43	no anomalies		
	44	no anomalies		
BIS	46	no anomalies		
	47	no anomalies		
	48	focal 5-7	slight hypoplasia	near suspensory ligament
VIN	49	no anomalies	hyperplasia	
	50	no anomalies	hyperplasia	
	51	no anomalies	hyperplasia	
	52	no anomalies	hyperplasia	some tubules 5
BBP	53	diffuse 2-5, subcapsular	hyperplasia	<10% of tissue affected
	54	diffuse 2-7, subcapsular	hyperplasia	<10% of tissue affected
	55	diffuse 2-5, subcapsular	hyperplasia	<10% of tissue affected
	56	diffuse 2-5, subcapsular	hyperplasia	<10% of tissue affected

* rate of degeneration on scale 1-8, with 1-4 being reversible conditions (Yang-Dar and McEntee, 1987)

Table 8. Summarized endocrinology data

sires

	estradiol (nmol/l)			testosterone (nmol/l)			rFSH (µg/l)			rLH (µg/l)		
	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD
OIL	4	0.04	0.00	4	10.8	3.9	4	167	15	4	1.10	0.14
EES	3	0.08	0.03	3	0.2	0.3	3	103	105	3	0.67	0.59
COU	4	0.06	0.02	4	12.9	9.9	4	152	105	4	1.28	1.02
OCT	4	0.06	0.01	4	4.5	1.4	4	131	42	4	1.03	0.46
BIS	3	0.05	0.01	3	7.3	8.1	3	179	24	3	1.10	0.26
VIN	4	0.06	0.01	4	16.3	11.8	4	284	24	4	2.33	0.61
BBP	4	0.06	0.02	4	12.2	4.2	4	230	13	4	1.85	0.84

pregnant females

	estradiol (nmol/l)			progesterone (nmol/l)			rFSH (µg/l)			rLH (µg/l)		
	n	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD
OIL	3	0.04	0.02	3	168	51	3	85	2	3	0.90	0.17
EES*	4*	0.08	0.08	4*	12	11	4*	43	50	4*	0.68	0.31
COU	3	0.02	0.00	3	203	110	3	28	49	3	0.67	0.15
OCT	2	0.02	0.01	2	216	99	2	46	65	2	0.35	0.49
BIS	2	0.02	0.01	2	119	46	2	106	13	2	1.40	0.85
VIN	3	0.01	0.01	3	16	19	3	27	47	3	0.83	0.21
BBP	3	0.11	0.11	3	76	91	3	125	217	3	1.47	0.47

* nonpregnant females

Table 9. Individual endocrinology data

compound	rat no.	sex	pregnant	nmol/l estradiol	nmol/l progesterone	µg/l rFSH	µg/l rLH	nmol/l testosterone
OIL	1	f	yes	0.05	218	82	1.0	
OIL	3	f	yes	0.04	169	86	0.7	
OIL	4	f	yes	0.02	117	86	1.0	
EES	5	f	no	0.03	5.9	83	0.4	
EES	6	f	no	0.05	15	90	1.1	
EES	7	f	no	0.04	1.2	<75	0.7	
EES	8	f	no	0.20	25	<75	0.5	
COU	9	f	yes	0.02	156	85	0.7	
COU	10	f	yes	0.02	329	<75	0.5	
COU	11	f	yes	0.02	124	<75	0.8	
COU	12	f	no	0.05	58	108	3.8	
OCT	13	f	no	0.09	22	<75	1.1	
OCT	14	f	yes	0.02	286	92	0.7	
OCT	15	f	no	0.04	114	102	2.0	
OCT	16	f	yes	0.03	145	<75	ND	
BIS	17	f	yes	0.02	151	115	2.0	
BIS	19	f	yes	0.03	86	97	0.8	
BIS	20	f	no	0.14	18	<75	3.1	
VIN	21	f	no	0.04	16	447	1.2	
VIN	22	f	yes	<0.02	5.1	<75	0.6	
VIN	23	f	yes	<0.02	5.3	82	0.9	
VIN	24	f	yes	0.02	38	<75	1.0	
BBP	25	f	no	0.08	85	244	3.7	
BBP	26	f	yes	0.05	181	<75	1.3	
BBP	27	f	yes	0.04	23	375	2.0	
BBP	28	f	yes	0.23	24	<75	1.1	
OIL	29	m		0.05	2.3	182	1.2	5.5
OIL	30	m		0.04	3.5	165	1.2	12
OIL	31	m		0.04	3.7	173	1.1	11
OIL	32	m		0.05	2.0	147	0.9	15
EES	33	m		0.10	2.8	<75	<0.2	<0.3
EES	34	m		0.10	3.3	100	0.9	<0.3
EES	35	m		0.05	2.7	210	1.1	0.6
COU	37	m		0.06	1.5	ND	ND	6.1
COU	38	m		0.09	20	211	2.2	27
COU	39	m		0.05	1.2	162	0.9	5.4
COU	40	m		0.05	4.0	233	2.0	13
OCT	41	m		0.06	1.1	183	0.8	3.9
OCT	42	m		0.06	1.2	96	0.9	3.0
OCT	43	m		0.06	1.6	99	0.7	4.9
OCT	44	m		0.07	6.8	147	1.7	6.3
BIS	46	m		0.04	3.0	171	1.0	2.5
BIS	47	m		0.05	3.9	206	1.4	17
BIS	48	m		0.05	1.4	159	0.9	2.7
VIN	49	m		0.07	5.4	297	2.8	33
VIN	50	m		0.06	3.2	312	2.9	5.3
VIN	51	m		0.06	1.6	262	1.8	13
VIN	52	m		0.04	1.5	266	1.8	14
BBP	53	m		0.07	2.7	249	1.6	18
BBP	54	m		0.03	2.2	225	3.1	13
BBP	55	m		0.08	1.3	224	1.3	8.3
BBP	56	m		0.06	1.9	221	1.4	10

ND note determined due to insufficient sureum sample volume

bad duplicates	
LH	values
rat 14	0.5 - 0.9
FSH	values
rat 1	67 - 97
rat 4	71 - 102
rat 6	48 - 131