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Sensitivity and Specificity of the Bioassay of Estrogenicity in Mammary Gland and Seminal Vesicles of Male Mice

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Summary

Young intact (18 days old) and adult castrated males of CBA and C3H/Di mice were used for measuring the estrogenicity on the basis of growth response of mammary epithelial structures and the weight of seminal vesicles. It was demonstrated that heavier young males had disproportionally heavier seminal vesicles (sex steroid-responsive organs) than small animals at day 33 of age (that is on the day when experimental animals were killed and organs dissected). However, the weight of the spleen (sex steroid-nonresponsive organ) was proportionally related to body weight. To minimize variability in hormone responsiveness, all animals were weighed at the age of 18 days and only males weighing 8±1 g were used for hormone treatment. The percentage area of mammary fat pad occupied by mammary epithelial structures was progressively increased by 17β estradiol from dose 0.01 $\mu g.d^{-1}$. The maximum effective dose of estradiol was 0.1 $\mu g.d^{-1}$ and dose 10 $\mu g.d^{-1}$ of estradiol decreased mammary size to control level (inverted-U-shaped dose-response curve). Progesterone alone stimulated mammary growth only in high doses (500 $\mu g.d^{-1}$ and higher) in young intact males, but had no effect on mammary growth in adult castrated animals. In young intact males, estradiol alone, or progesterone alone decreased the weight of seminal vesicles. No such inhibitory effect of these hormones was noted in adult castrated males. Progesterone acted synergistically with estradiol to produce higher mammary growth compared to that in males treated with estradiol alone. In the presence of progesterone seminal vesicles weight was decreased by estradiol given in such low doses as 0.001 $\mu g.d^{-1}$ of estradiol, which is 10 times lower than that effective in animals treated with estradiol alone. On the other hand, in the adult castrated males a combination of estradiol plus progesterone stimulated seminal vesicles weight. The effects of a combination of estradiol plus progesterone in the mammary gland were mimicked by norethindrone acetate (a synthetic steroid exhibiting progestantial and estrogenic activities) and inhibited by both testosterone and cortisol. Estradiol, progesterone, norethindrone acetate, or testosterone did not affect spleen weight and size of mammary lymph nodes. However, cortisol significantly decreased not only spleen weights but also size of mammary lymph nodes. These results show that simultaneous evaluation of mammary gland growth, seminal vesicles, and the spleen weight in the same animal is suitable for bioassay of estrogenicity as well as for detection of androgenic and antiandrogenic activities.

Key words
Bioassay • Estrogenicity • Male • Mammary gland • Seminal vesicles

Introduction

Man reproductive health has deteriorated in many countries during several last decades. Declining semen quality and increasing incidence of testicular and prostate cancer have been reported. Incidences of hypospadias and cryptorchidism also appear to be increasing. The effects of medicinal products (estrogen-like effect of phtalates on the testes) were also described (Sullivan *et al.* 1993). Similar reproductive problems occur in many wildlife species and livestock. Clinical and laboratory research suggests that these adverse changes may have a common origin in fetal and early postnatal life. Exposure of the male fetus to supranormal levels of estrogens, such as diethylstilbestrol or other hormonally active (e.g. antiandrogenic) chemicals, can result in the above-mentioned reproductive defects (Toppari *et al.* 1996). In a view of many reports of contaminant-associated reproductive and developmental impacts in human and animals, often considered to be caused by endocrine disruption, there is now a major global effort to develop ecotoxicity test guidelines for *in vivo* hazard assessment of endocrine disrupters. The critical objective is to detect chemicals that may be active *in vivo*, thereby taking into account bioavailability and metabolism of a substance in animals. The basic challenge is to demonstrate that a given assay is both biologically meaningful and is reproducible in the hands of the international scientific community (Hutchinson 2000). A screening design should not consist of a single endpoint but rather several endpoints reflective to the mechanism involved (i.e. estrogenicity).

In the following series of experiments, we have examined the sensitivity and specificity of bioassay of estrogenicity on the mammary gland and seminal vesicles of young intact and adult castrated male mice. The dose-response relationships of 17β estradiol and progesterone were determined. In addition, the abilities of norethindrone acetate to mimic and testosterone and cortisol to modulate the organ responses to estradiol and progesterone were also studied.

Methods

Materials, animals, and route of hormone administration are given elsewhere (Škarda 2001). Methods used for surgical procedures, mammary whole-mount preparations and quantitative mammary histology were described previously (Škarda 2001).

Parametric analysis could be used as all measured variables under investigation were sufficiently normally distributed. All data represent means ± S.E.M. Statistically significant difference was determined by ANOVA, followed by Bonferroni test for individual comparisons of the means. The correlation between body weight and seminal vesicles and spleen weights adjusted on the basis of body weight (mg.100 g^{-1} of body weight) in males on day 33 of age was determined using Pearson's correlation coefficient.

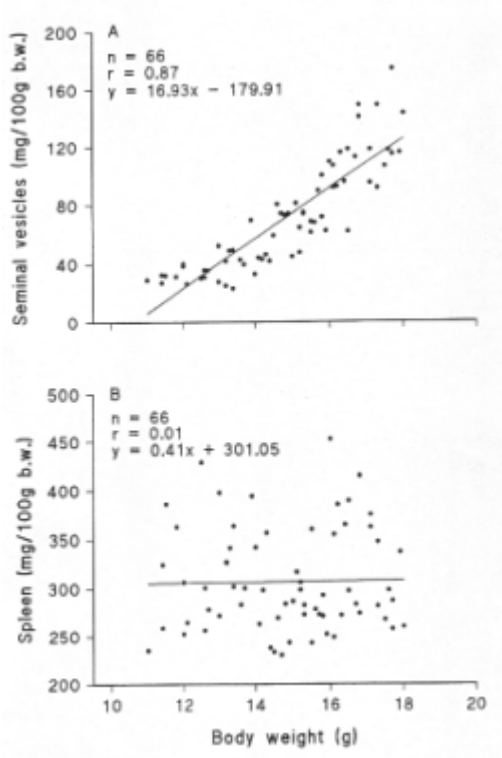

Fig. 1. *Effect of body weight on normalized seminal vesicle and spleen weights in young intact CBA male mice. All animals (n = 66) were weighed and killed on day 33 of age. Seminal vesicles and spleen were removed, weighed and weights were expressed in terms of body weight in mg.100 g^{-1}. The correlation between body weight and normalized organ weights was determined using Pearson's correlation coefficient (r).*

Results

Both age and live weight may affect the sensitivity of sex steroid-responsive organs of prepubertal animals to injected hormones. To reduce the variability of organ responses to hormones, males of the same age were used in our experiments. To study the effect of body

Table 1. Effect of 17β-estradiol (E), progesterone (Prog) and their combinations on the growth of the first inguinal mammary gland, seminal vesicles and spleen weight in young intact and adult castrated male mice

Hormonal treatment ($\mu g.d^{-1}$)	The % area of mammary fat pad occupied by mammary epithelial structures		Seminal vesicles weight (mg.100 g^{-1} of body weight)		Spleen weight (mg.100 g^{-1} of body weight)	
	Intact	Castrated	Intact	Castrated	Intact	Castrated
E 0	1.0±0.1 (32)	1.2±0.1 (8)	145.9±9.2 (14)	51.5±1.6 (4)	547.3±17.9 (14)	504.3±18.3 (4)
0.001	1.2±0.1 (31)	1.2±0.2 (10)	144.1±7.4 (13)	58.5±5.1 (5)	491.2±13.2 (13)	444.0±48.7 (5)
0.01	6.2±0.6*** (29)	3.7±0.7 (8)	66.8±6.0*** (12)	56.6±2.8 (5)	621.1±38.8 (13)	475.2±31.9 (5)
0.1	14.6±0.7*** (25)	10.6±2.1* (10)	27.8±4.1*** (13)	81.0±3.0 (4)	499.4±18.5 (13)	413.4±17.3 (5)
1.0	6.8±0.5*** (24)	8.6±1.8* (8)	29.8±4.2*** (14)	85.1±9.6 (4)	556.4±24.9 (14)	450.3±31.6 (3)
10.0	2.2±0.2 (20)	2.4±0.4 (10)	39.6±3.4*** (10)	54.5±5.4 (5)	469.0±14.3 (10)	488.2±63.3 (5)
Prog 0	1.1±0.1 (38)	1.3±0.1 (43)	152.8+6.3 (18)	44.6±4.1 (31)	552.9±13.7 (18)	464.2±14.5 (31)
125	1.2±0.1 (8)	1.6±0.2 (10)	123.8±14.3 (4)	-	556.8±48.3 (4)	-
250	1.4±0.1 (36)	1.5±0.1 (29)	92.7±6.8*** (15)	58.4±3.7 (10)	595.1±21.2 (15)	408.7±12.8 (10)
500	1.8±0.2** (38)	1.5±0.1 (40)	102.0±5.1***(30)	52.4±3.5 (25)	559.2±14.2 (30)	449.4±14.4 (25)
1000	1.7±0.1* (40)	1.5±0.1 (50)	62.3±3.2*** (18)	49.8±2.4 (26)	594.3±24.5 (19)	508.2±18.2 (26)
0	1.1±0.1 (38)	1.3±0.1 (43)	152.8±6.3 (18)	60.5±3.1 (9)	552.9±13.7 (18)	457.5±25.4 (9)
Prog 500+E 0.001	5.1±0.6 (25)	2.5±0.6 (10)	79.7±7.5*** (13)	64.0±4.4 (5)	550.8±17.0 (13)	388.5±16.7 (5)
0.01	35.6±1.7*** (55)	26.1±3.2*** (18)	34.8±2.8*** (29)	72.6±2.8 (10)	567.2±12.2 (29)	463.4±20.4 (10)
0.1	43.7±1.7*** (29)	39.7±1.9*** (6)	26.2±3.1*** (14)	74.0±5.2 (5)	606.2±25.3 (14)	496.6±28.2 (5)
1.0	37.7±2.3*** (24)	33.9±2.5*** (7)	27.1±1.4*** (13)	93.3±6.2*** (4)	585.0±22.2 (13)	448.3± 23.8 (4)
10.0	16.0±1.5*** (22)	30.7±2.3*** (8)	41.1±8.4*** (12)	96.2±2.0*** (4)	572.6±44.1 (12)	429.2±46.8 (4)

*Young intact (18 days of age) and adult castrated males of C3H/Di strain were subcutaneously injected 50 µl of vehicle (control) or a steroid hormone (50 µl) daily for 15 days. Values are means ± SEM. Differences between values for different doses of hormones were determined by one-way ANOVA followed by the Bonferroni test. An asterisk (*) indicates that the value was significantly different (*P < 0.05, **P < 0.01, ***P < 0.001) from the respective vehicle-treated control group. Numbers in parentheses indicate the number of the first inguinal mammary glands or number of animals supplying seminal vesicles or spleens.*

weight on normalized seminal vesicles (a sex steroid-responsive organ) and spleen (sex steroid-unresponsive organ) weight of males on day 33 of age (i.e. the day when organs of experimental animal were dissected) were used. Figure 1 shows that heavier males have disproportionally heavier (r = 0.87) seminal vesicles than smaller animals, however, spleen weight is more closely related to body weight (r = 0.10). On the basis of these results, all animals were weighed immediately before the experiment on day 18 of age and only those weighing 8±1 g were used.

Table 1 shows the dose-response relationship of 17β estradiol, progesterone and a combination of estradiol plus progesterone on the mammary gland, seminal vesicles and spleen. Figure 2 illustrates typical whole-mounts of the first inguinal mammary glands of steroid hormone treated males. Glands of placebo-treated animals, both intact and castrated, showed no indication of proliferation of ducts beyond rudimentary duct system typical of untreated C3H/Di males. The percentage area of mammary fat pad occupied by mammary epithelial structures was 1.0 to 1.2. Estradiol-treated animals were given 17β estradiol (0.001-10 $\mu g.d^{-1}$) subcutaneously in 50 µl of vehiculum. Glands showed progressive growth of ducts from dose 0.01 $\mu g.d^{-1}$ of estradiol and the maximum effective dose of estradiol was 0.1 $\mu g.d^{-1}$ both in young intact (14.6 %) and adult castrated animals (10.6 %). High dose of estradiol (10 $\mu g.d^{-1}$) decreased percentage of mammary fat pad occupied by mammary epithelial structures: in young intact animals to 2.2 %, in adult castrated animals to 2.4 % (inverted-U-shaped dose-response curve).

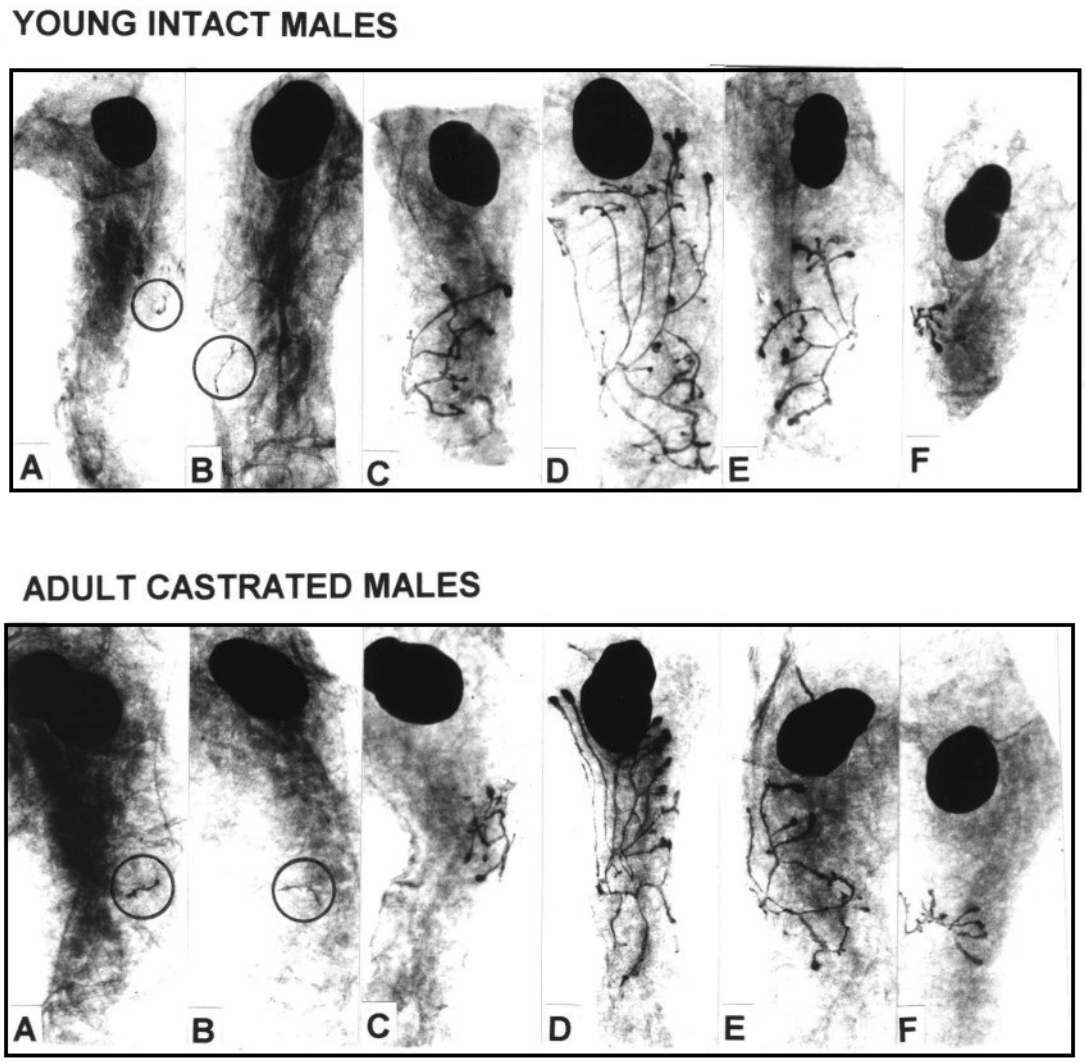

Fig. 2. *Effect of different 17β estradiol dose on the growth of the mammary gland in male mice. Symbols: A = 0; B = 0.001; C = 0.01; D = 0.1; E = 1.0; F = 10.0 $\mu g.d^{-1}$ of estradiol. Other details are in the legend to Table 1.*

Progesterone alone in high doses (500 or 1000 μg.d^{-1}) only slightly stimulated growth of rudimentary mammary duct system (by 0.7 %) in young intact males but had no effect on mammary growth in adult castrated animals.

In immature intact males treated with either estradiol alone or progesterone alone, a distinct decrease ($P<0.001$) in weight of seminal vesicles occurred from dose 0.01 μg.d^{-1} of estradiol (from 145.9 mg in controls to 66.8 mg.100 g^{-1} of body weight in animals injected with estradiol) or from dose 250 μg.d^{-1} of progesterone (from 152.8 mg in controls to 92.7 mg.100 g^{-1} of body weight in animals injected with progesterone). Maximal inhibitory effect of estradiol was achieved at dose 0.1 μg.d^{-1} and progesterone at the highest dose of progesterone used - at 1000 μg.d^{-1}. No such inhibitory effect of estradiol alone and progesterone alone was noted in adult castrated males.

Males treated with estradiol plus progesterone received 500 μg.d^{-1} of progesterone and different doses of 17β estradiol (0.001-10 μg.d^{-1}). Both in young intact and adult castrated males estradiol synergized the effect of progesterone in stimulation of mammary duct growth. The maximum effective dose of estradiol (0.1 μg.d^{-1}) in the presence of progesterone increased percentage of mammary fat pad occupied by mammary epithelial structures to 43.7 % in young intact males and to 39.7 % in adult castrated males. High dose (10 μg.d^{-1}) of estradiol significantly decreased mammary growth even in the presence of progesterone, however, the size of the mammary gland was still significantly larger than in controls (Fig. 3).

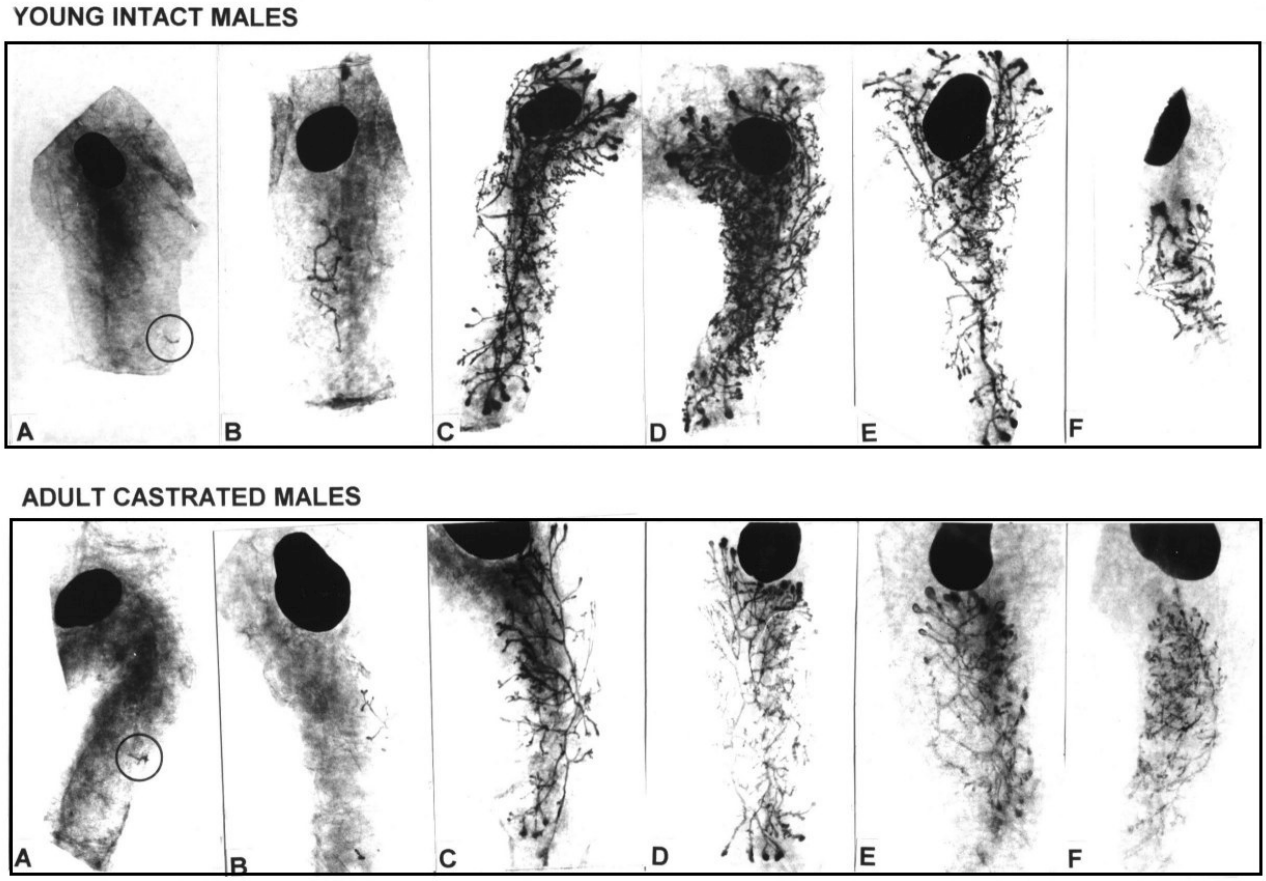

Fig. 3. *Effect of a combination of estradiol plus progesterone on mammary gland growth in male mice. Symbols: A = 0; B-F = progesterone 500 μg.d^{-1} plus 0.001 (B), 0.01 (C), 0.1 (D), 1.0 (E), 10.0 μg.d^{-1} of estradiol (F).*

Seminal vesicles weight was decreased by estradiol in the presence of progesterone (500 μg.d^{-1}) at 0.001 μg.d^{-1} dose of estradiol, which is 10times lower than that effective in animals treated with estradiol alone. On the other hand in the adult castrated males a combination of estradiol plus progesterone at high dose

Table 2. Interactions of testosterone (T) and estradiol (E) plus progesterone (Prog) or norethindrone (NA) on the mammary gland and seminal vesicle growth in young intact and adult castrated males of C3H/Di mice.

	Young intact			**Adult castrated**		
Mammary gland	The % area of mammary fat pad occupied by mammary epithelial structures					
	0	E + Prog	NA	0	E + Prog	NA
T (μg.d^{-1}) 0	1.1±0.2 (20)	26.8±2.4 (106)	33.7±2.1 (30)	1.3±0.2 (18)	24.4±2.7 (34)	23.6±1.7 (51)
50	0.6±0.2 (8)	1.8±0.5*** (10)	28.8±2.7 (20)	1.3±0.4 (5)	0.9±0.1*** (10)	4.8±1.1*** (16)
100	1.0±0.3 (8)	2.2±0.5*** (30)	19.3±2.6*** (25)	1.2±0.3 (8)	1.0±0.2*** (29)	3.1±0.4*** (22)
200	-	2.5±0.8*** (28)	10.0±3.2*** (18)	-	1.4±0.3*** (37)	3.3±1.7*** (7)
300	0.7±0.3 (10)	-	10.0±1.7*** (9)	-	-	-
Seminal vesicles	mg.100 g^{-1} of body weight					
T (μg.d^{-1}) 0	118.2±7.9 (8)	70.6±9.5 (29)	81.3±12.6 (15)	61.3±2.9 (9)	67.3±3.1 (19)	51.0±4.2 (38)
50	--	312.3±37.8*** (5)	318.9±24.1*** (5)	452.1±13.2*** (4)	334.9±24.9*** (5)	455.6±33.0*** (8)
100	397.8±14.0*** (6)	467.7±31.1*** (21)	475.6±16.2*** (13)	528.1±42.6*** (4)	565.3±34.4*** (14)	587.6±47.7*** (12)
200	-	596.7±38.4*** (14)	577.2±23.3*** (10)	978.4±121.7*** (4)	750.4±53.7*** (13)	546.2±40.1*** (4)
300	665.5±54.2*** (5)	-	594.3±58.3*** (5)	-	-	-

Animals were injected placebo or hormones dissolved in placebo for 15 days: NA (25 μg.d^{-1}), E (0.005 μg.d^{-1}), Prog (500 μg.d^{-1}) and T in suggested doses. Other details are in the legend to Table 1.

of estradiol stimulated seminal vesicles weight from 60 mg in controls to 93.3 and 96.2 mg.100 g^{-1} at dose 1.0 and 10 $\mu g.d^{-1}$ of estradiol respectively.

Spleen weights were not affected by estradiol alone and progesterone alone, or by a combination of both.

To test the selectivity of the estradiol and progesterone effect on the mammary duct growth and seminal vesicles weights, we investigated whether it could be mimicked/inhibited by other steroid hormones: norethindrone acetate (a synthetic derivative of 19-nortestosterone exhibiting progestantional and estrogenic activities), and androgen-testosterone and glucocorticoid-cortisol. Norethindrone acetate mimicked effects of a combination of estradiol plus progesterone. Administration of testosterone alone had no effect on mammary growth in both young intact and adult castrated males. However, testosterone significantly increased the weight of seminal vesicles. Estradiol plus progesterone or norethindrone acetate stimulated mammary growth was significantly ($P<0.001$) decreased by testosterone both in young intact and adult castrated males (Table 2).

Cortisol alone had no effect on the percentage area of mammary fat pad occupied by mammary epithelial structures, however, index length x width (l x w) of mammary lymph nodes and spleen weights were significantly decreased ($P<0.05$) in both young intact and adult castrated males. Cortisol alone increased seminal vesicle weights in young intact animals but had no effect on seminal vesicles in adult castrated males. Estradiol or estradiol plus progesterone stimulated growth of the mammary gland was decreased by cortisol in both young intact and castrated animals (Table 3).

Table 3. Interaction of cortisol and 17β estradiol (E), progesterone (Prog) or E plus Prog on sizes of mammary glands and mammary lymph nodes and on weight of spleen and seminal vesicles in C3H/Di mice.

Hormonal treatment ($\mu g.d^{-1}$)	The % area of mammary fat pad occupied by mammary epithelial structures	Mammary lymph node (index l x w)	Spleen (mg.100 g^{-1} of body weight)	Seminal vesicles (mg.100 g^{-1} of body weight)
Young intact				
0	1.0 ± 0.1^{A} (8)	16.0 ± 0.8^{A} (8)	573.9 ± 65.7^{A} (4)	102.9 ± 6.5^{A} (4)
Cortisol (F) 1000	0.9 ± 0.1^{A} (10)	7.1 ± 0.2^{B} (10)	260.1 ± 9.2^{B} (5)	221.4 ± 14.3^{B} (5)
E 0.005	7.4 ± 0.8^{B} (9)	19.5 ± 0.6^{BC} (10)	539.5 ± 10.6^{A} (5)	78.0 ± 7.4^{AC} (5)
E + F	1.3 ± 0.1^{A} (10)	8.5 ± 0.5^{BD} (10)	298.0 ± 13.0^{B} (5)	128.4 ± 7.1^{AD} (5)
Prog 500	1.9 ± 0.8^{A} (9)	10.6 ± 1.1^{BE} (10)	595.0 ± 14.5^{A} (5)	72.5 ± 4.9^{A} (5)
Prog + F	1.5 ± 0.2^{A} (10)	5.4 ± 0.2^{BF} (10)	336.5 ± 8.5^{B} (4)	105.5 ± 13.3^{A} (5)
E + Prog	14.7 ± 1.6^{B} (9)	8.5 ± 0.5^{BG} (8)	585.8 ± 35.7^{A} (5)	66.3 ± 11.0^{AE} (5)
E + Prog + F	4.2 ± 1.0^{A} (9)	5.1 ± 0.3^{BH} (10)	402.0 ± 21.6^{B} (5)	130.0 ± 10.5^{AF} (5)
Adult castrated				
0	1.4 ± 0.1^{A} (10)	21.2 ± 1.1^{A} (10)	564.2 ± 28.0^{A} (5)	62.1 ± 3.9^{A} (5)
F 1000	1.4 ± 0.2^{A} (8)	8.5 ± 0.5^{B} (8)	208.6 ± 12.6^{B} (4)	76.7 ± 16.0^{A} (4)
E 0.005	5.6 ± 1.0^{B} (9)	20.1 ± 0.9^{A} (10)	628.5 ± 35.6^{A} (5)	54.4 ± 1.6^{A} (5)
E + F	1.3 ± 0.2^{A} (10)	8.2 ± 0.6^{B} (10)	242.0 ± 32.1^{B} (5)	54.0 ± 2.6^{A} (5)
Prog 500	1.4 ± 0.1^{A} (8)	19.7 ± 1.3^{A} (8)	599.4 ± 20.1^{A} (4)	52.6 ± 1.5^{A} (4)
Prog + F	1.0 ± 0.1^{A} (10)	7.9 ± 0.5^{B} (10)	183.5 ± 3.9^{B} (5)	57.7 ± 6.2^{A} (5)
E + Prog	11.6 ± 2.1^{B} (9)	18.1 ± 0.7^{A} (10)	581.6 ± 27.9^{A} (5)	42.6 ± 3.3^{A} (5)
E + Prog + F	1.1 ± 0.1^{A} (8)	6.3 ± 0.2^{B} (8)	182.9 ± 11.7^{B} (4)	65.0 ± 5.1^{A} (4)

Index length x width (l x w) was measured on 12 x enlarged photograph of the first inguinal mammary fat pad. Other details are in the legend to Table 1 and 2.

Discussion

In litter-bearing animals such as mice, the variability in growth rate of the offspring is quite usual. Small animals in the litter are unable to compete with larger siblings for colostrum and milk and thus experience dietary restriction, grow slowly and are more likely to have health problems (Hoggard *et al.* 1998). Moreover, these animals could have impaired or delayed growth and functional activities of reproductive systems. Our experiments have shown that heavier prepubertal males have disproportionally heavier normalized weight of seminal vesicles. It could be suggested that the onset of the allometric phase of growth (seminal vesicles are growing faster than the body as a whole) started earlier in these animals probably due to earlier increase in androgen secretion than in small animals. It can thus be expected that response to injected hormones is also different. To ensure the uniformity of response to injected compounds only animals weighing 8±1 g at day 18 of age were used.

Estrogen and progesterone are key regulators of mammary development and their actions are generally considered to be mutually dependent. Earlier studies involving hormone administration pointed to the general conclusion that estrogen is more responsible for the growth of mammary duct system while progesterone, acting in concert with estrogen, is necessary for full alveolar growth (Škarda *et al.* 1989, Imagawa *et al.* 1990). These findings were largely supported by the use of gene-targeting technology to disrupt the genes encoding estradiol (αERKO) and progesterone (PRKO) receptors. The αERKO female mice have lost their capacity to commence the pre- and postpubertal growth of mammary ductal epithelium and PRKO mice were refractory to the progesterone action in lobuloalveolar development after progesterone treatment (Couse and Korach 1999).

In our C3H/Di males, 17β estradiol evoked limited duct growth. The epithelial structures (ducts and terminal end buds, with absent alveoli) occupied only 14.6 % area of mammary fat pad in young intact and 10.6 % in adult castrated animals. Progesterone alone stimulated growth of mammary rudiment slightly but significantly in young intact males but had no effect on mammary growth in adult castrated animals. In contrast progesterone alone evoked large increase of mammary growth in C3H/Di females both in young intact and in adult OV-X animals (Škarda 2002). Thus, mammary gland of males is more suitable for estrogenicity testing than that of females. The difference in mammary growth responses to progesterone in males and females is not understood. It is possible that induction of the progesterone receptor by progesterone in males requires much longer exposure and higher dose of progesterone than in females. In both PRKO and αERKO female mice, the effect of progesterone was not observed unless the gland was exposed to progesterone for a prolonged period of time (Lydon *et al.* 1995, Humphreys *et al.* 1997). The possibility that the effect of progesterone is not direct but rather due to metabolism of progesterone into estrogen cannot be ruled out. However, Plaut *et al.* (1999) demonstrated that *in vivo* progesterone priming did not increase serum estrogen concentration in OV-X mice.

It is recognized in endocrinology that low doses of a hormone can stimulate a tissue, while high doses can have the opposite effect. We report here that estradiol in doses from 0.01 to 1.0 $\mu g.d^{-1}$ stimulated mammary growth in both young intact and adult castrated males. However, when the dose of estradiol was increased to 10 $\mu g.d^{-1}$, mammary growth was not stimulated. We also have demonstrated a similar inverted-U relationship between dose of diethylstilbestrol and mammary growth in mice (Škarda, Mrazíková, Burianová, unpublished results). Potential mechanisms mediating a decrease in mammary gland growth in response to supraphysiological doses of estrogens or estrogenic chemicals may include receptor down-regulation and the capacity for estrogens to bind to receptors for other steroid hormones, as androgen or glucocorticoid receptors, resulting in antagonistic effects mediated via other receptor systems (vom Saal *et al.* 1997). This interpretation is further supported by inhibitory action of both testosterone and cortisol on estradiol or estradiol plus progesterone stimulated mammary growth. However, the possibility that the effects of high doses of estrogens are toxic cannot be ruled out. The inhibition of mammary growth by high doses of estradiol was reduced by simultaneous application of progesterone. This fact allows us to speculate that progesterone activates a series of complex enzyme reactions that decrease estradiol levels within a target cell.

An inverted-U relationship between dose of estrogen chemicals (estrogenic endocrine disruptors) and response should be taken into account when testing for estrogenicity. The use of different doses of tested compounds with and without progesterone is highly recommended.

Diverse animal models and assays have been used to measure estrogenicity. However, all changes in endocrine-sensitive tissues are not necessarily specific e.g. both rodent uterotrophic and human breast cancer cell line MCF-7 assays lack specificity as estrogens and androgens both enhanced uterine growth and MCF-7 cell proliferation (Neumann and Steinbeck 1973, Lippmann *et al.* 1976). These stimulatory, estrogen-like effects of androgens are correlated with the binding of these steroids to the estrogen receptor and are considered as an estrogen receptor mediated mechanism (Hackenberg *et al.* 1993). Therefore, it is important that testing for estrogenicity potential includes the ability to detect androgenic and antiandrogenic activities. This can be done by measuring the increase in seminal vesicles weight and the decrease in estradiol plus progesterone or norethindrone acetate stimulated mammary growth and abolition of testosterone effect in seminal vesicles, uterus and mammary gland by antiandrogens.

Both estradiol and progesterone treatment caused a reduction in seminal vesicles weight in young intact males. In adult castrated males, however, no such inhibitory effect on seminal vesicles weight was noted. Lack of the effect of estradiol and progesterone partly reflects the refractoriness of seminal vesicles to these hormones and partly reflects the very low weight of seminal vesicles in castrated animals, so that not much is left for a further decrease. According to Korach and McLachlan (1995), screening for estrogens with use of males as a model should not be done because estrogens are primarily defined by their ability to increase the mitotic activity of female secondary sex organs. We cannot agree with this statement for two reasons. Firstly, mammary gland of male mouse is equipotential with that of the female when exposed to estradiol plus progesterone. Secondly, rodent uterine assay measures the increase of uterine wet weight that is not only hyperplasia but also water inhibition and hypertrophy, which are also produced by estrogen antagonists (Soto *et al.* 1995), while on the other hand quantitative evaluation of growth of mammary epithelial structures measures cell proliferation only.

In conclusion, estrogens stimulate/inhibit a variety of organ systems and express themselves in many biochemical and physiological endpoints. Our mouse model for detection of estrogenicity consists of the following endpoints: an increase in size of mammary epithelial structures and decrease in seminal vesicles weight. The sensitivity of estrogen assay on the mammary gland and seminal vesicles of prepubertal males is the same as that on female uterus but lower than that on female mammary gland (Škarda 2002). The specificity of the assay is, however, highest in male mammary gland as mammary growth in females is also stimulated by progesterone alone and uterine growth is stimulated not only by estradiol alone or by a combination of estradiol plus progesterone but also by testosterone. On the other hand, testosterone in males inhibited estradiol plus progesterone or nortehindrone stimulated mammary growth and simultaneously seminal vesicle weight was increased. Mammary growth may also be inhibited by glucocorticoids. To determine whether androgens or glucocorticoids cause inhibition of mammary growth, we have to know that glucocorticoids in contrast to androgens have no ability to increase seminal vesicle weight in adult castrated males and that androgens in contrast to glucocorticoids have no ability to decrease size of mammary lymph nodes and spleen weight in both intact prepubertal and adult castrated males. Our model may be also used for evaluation antiestrogenic, antiprogestin and antiandrogenic activities.

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References

COUSE JF, KORACH KS: Estrogen receptor null mice: what have we learned and where will they lead us? *Endocr Rev* **20**: 358-417, 1999.

HACKENBERG R, TURGETT I, FILMER A, SCHULZ K-D: Estrogen and androgen receptor mediated stimulation and inhibition of proliferation by androst-5-ene-3β,17β-diol in human mammary cancer cells. *J Steroid Biochem Mol Biol* **46**: 597-603, 1993.

HOGGARD N, HUNTER L, TRAYHURN P, WILLIAMS L, MERCER JG: Leptin and reproduction. *Proc Nutr Soc* **57**: 421-427, 1998.

HUMPHREYS RC, LYDON J, O'MALLEY BW, ROSEN JM: Mammary gland development is mediated by both stromal and epithelial progesterone receptors. *Mol Endocrinol* **11**: 801-811, 1997.

HUTCHINSON TH, BOWN R, BRUGGER KE, CAMPBELL PM, HOLT M, LANGE R, MCCAHON P, TATTERSFIELD LJ, VAN EGMOND R: Ecological risk assessment of endocrine disruptors. *Environ Health Perspect* **108**: 1007-1014, 2000.

KORACH KS. MCLACHLAN JA: Techniques for detection of estrogenicity, *Environ Health Perspect* **103** (Suppl 7), 5-8, 1995.

LYDON JP, DEMAYO FJ, FUNK CR, MANI SK, HUGHES AR, MONTGOMERY CA Jr, SHYAMALA G, CONNELLY OM, O'MALLEY BW: Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes Development* **9**: 2266-2278, 1995.

PLAUT K, MAPLE R, GINSBURG E, VONDERHAAR B: Progesterone stimulates DNA synthesis and lobulo-alveolar development in mammary glands in ovariectomized mice. *J Cell Physiol* **180**: 298-304, 1999.

ŠKARDA J: Detection of estrogenicity by bioassay on the mouse mammary gland in vivo. *Physiol Res* **50**: 275-282, 2001.

ŠKARDA J: Sensitivity and specificity of bioassay of estrogenicity on mammary gland of female mice. *Physiol Res* **51**: 2002 (in preparation).

ŠKARDA J, FREMROVÁ V, BEZECNÝ I: Progesterone alone is responsible for stimulation of the growth of ducts and of mammary alveolar structures in mice. *Endocrinol Exper* **23**: 17-28, 1989.

SOTO AM, SONNENSCHEIN C, CHUNG K/L, FERNANDEZ M F, OLEA N, OLEA SERANO F: The E-screen assay as a tool to identify estrogens: an update on estrogenic environmental pollutants. *Environ Health Perspect* **103** (Suppl 7): 113-122, 1995.

VOM SAAL FS, TIMMS BG, MONTANO MM, PALANZA P, THAYER KA, NAGEL SC, DHAR MD, GANJAM VK, PARMIGIANI S, WELSHONS WV: Prostate enlargement in mice due to fetal exposure to low doses of estradiol or diethylstilbestrol and opposite effects at high doses. *Proc Natl Acad Sci USA* **94**: 2056-2061, 1997.

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