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Sensitivity and Specificity of Bioassay of Estrogenicity on Mammary Gland and Uterus of Female Mice

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Summary

Young intact (18 days of age) and adult ovariectomized (OV-X, ovariectomized between 21 to 24 days of age) C3H/Di mice were used to measure the estrogenicity on the basis of the growth response of mammary epithelial structures and weight of the uterus. The percentage area of the mammary fat pad occupied by mammary epithelial structures was progressively increased by 17β estradiol from dose 0.001 $\mu g.d^{-1}$. The maximum effective dose of estradiol was 0.01 $\mu g.d^{-1}$ and the dose 10 $\mu g.d^{-1}$ of estradiol decreased mammary size to control levels (inverted-U-shaped dose-response curve). Progesterone alone progressively stimulated mammary growth in young intact females from dose 125 $\mu g.d^{-1}$, in adult OV-X animals from dose 1000 $\mu g.d^{-1}$. Both in young intact and adult OV-X animals, uterine weight progressively increased during estradiol treatment. Progesterone alone had no effect on uterine weight in young intact animals; in adult OV-X animals, uterine weight was increased starting from dose 250 $\mu g.d^{-1}$. Progesterone acted synergistically with estradiol to produce higher mammary growth than that in females treated with estradiol alone. The effects of a combination of estradiol plus progesterone in the mammary gland were mimicked by norethindrone acetate and inhibited by cortisol in both young intact and adult OV-X animals. Testosterone inhibited estradiol plus progesterone stimulated growth of mammary gland only in OV-X animals, but stimulated uterine weights in both young intact and adult OV-X animals. Spleen weight and size of mammary lymph nodes were not affected by estradiol, progesterone, norethindrone acetate or testosterone, but were decreased by cortisol. Cortisol also decreased the percent area of the mammary fat pad occupied by mammary epithelial structures, but had no effect on weight of the uterus. These results show that bioassay of estrogenicity in females is not specific. Mammary and uterine growth is stimulated not only by estrogens but also by progesterone and testosterone, respectively.

Key words

Bioassay • Estrogenicity • Mammary gland • Uterus • Female mice

Introduction

Animal reproduction is a complex and vulnerable process at all stages of development. Recently, a number of endocrinologists, ecologists and toxicologists have called attention to the potential hazardous effects that estrogen-like and antiandrogenic drugs and certain other environmental chemicals may have on human and animal health. These chemicals have been called endocrine disrupters because they are thought

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to mimic natural hormones, inhibit the action of hormones, or alter the normal regulatory function of the immune, nervous and endocrine systems (Crisp *et al.* 1998). Due to the complexity of the mechanisms involved in tissue responsiveness to compounds with steroid hormone agonist and antagonist actions, a powerful *in vivo* test has to be used for evaluating the entire complexity of a possible response. *In vitro* assays are useful for supplementing the routinely used *in vivo* approaches for studying expected or defined effects. However, they are not suitable for revealing hitherto unknown actions of a given drug on the complex reproductive process (Riecke and Stahlmann 2000).

In the present studies, we examined the sensitivity and specificity of bioassay of estrogenicity on the mammary gland and uterus of young intact and adult ovariectomized female mice. The dose-response relationship 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.

Materials and Methods

Materials, animals and route of hormone administration have previously been published (Škarda 2001). Methods used for surgical procedures, mammary whole-mount preparations and quantitative mammary histology have been described (Škarda 2001).

Statistical analyses for all variables under investigation were performed as described by Škarda (2002).

Results

Table 1 shows the dose-response relationships of 17β estradiol, progesterone and a combination of estradiol plus progesterone on the mammary gland, uterus and spleen. Glands of placebo treated young intact females contained ducts branched four to five times to form secondary, tertiary and quarternary ducts. The duct system of adult OV-X females was similar to that in young intact prepubertal females. Glands of estradiol-treated animals showed a progressive lengthening and branching of ducts from the dose 0.001 μg. The maximum effective dose of estradiol was 0.01 $\mu g.d^{-1}$ in young intact and 0.1 $\mu g.d^{-1}$ in adult OV-X animals. Expansions of duct ends ranged in size from large end buds (club ends), generally at the periphery of the parenchymal area, to small end buds throughout the parenchymal area. The percentage area of the mammary fat pad occupied by mammary epithelial structures increased from about 11 % to 24.9 % in young intact and to 23.1 % in adult OV-X females ($P < 0.001$). However, high doses of 17β estradiol (10 $\mu g.d^{-1}$) had the opposite effect (inverted-U-shaped dose-response curve): in young intact females the percentage area decreased to 8.6 %; in adult OV-X females to 11.4 %.

In young intact and adult OV-X animals, uterine weight was significantly increased starting from dose 0.01 $\mu g.d^{-1}$ of estradiol. In contrast to the effect on the mammary gland, a significant inhibitory effect of high doses of estradiol on uterine growth was not observed.

Progesterone alone showed a significantly increasing expansion and branching of mammary ducts from dose 125 $\mu g.d^{-1}$ in young intact females and from dose 1000 $\mu g.d^{-1}$ in OV-X animals. Uterine weight was not affected by progesterone alone in young intact females, however, in adult OV-X mice progesterone increased uterine weight from dose 250 $\mu g.d^{-1}$.

In both young intact and adult OV-X mice 17β estradiol acted synergistically with progesterone to produce a higher mammary growth rate (side branching of the ducts and formation of lobulo-alveolar structures) than that observed in animals which had received injections of either progesterone alone or estradiol alone. The % area of mammary fat pad occupied by mammary epithelial structures was increased to 38.8 % in young intact and to 44.9 % in adult OV-X mice. The fact that a decline of duct growth in young intact females at a dose 10 $\mu g.d^{-1}$ of estradiol plus 500 μg of progesterone contrasted with animals treated with 10 $\mu g.d^{-1}$ of estradiol alone was nonsignificant suggests that progesterone counteracted the inhibitory effect of high doses of estradiol in these animals.

When 17β estradiol was combined with progesterone, uterine weight did not differ from that observed in young intact mice injected with estradiol alone. However, uterine growth was significantly stimulated in adult OV-X mice, by a combination of estradiol plus progesterone at a dose of estradiol 0.001 $\mu g.d^{-1}$, i.e. at a 10 times lower dose than was effective in animals treated with estradiol alone.

Table 1. Effect of 17β estradiol (E), progesterone (Prog) and their combinations on the growth of the first inguinal mammary gland and uterine and spleen weight in young intact and adult ovariectomized (OV-X) female C3H/Di mice

Hormonal treatment ($\mu g.d^{-1}$)		The % area of mammary fat pad occupied by mammary epithelial structures		Uterine weight (mg.100 g^{-1} of body weight)		Spleen weight (mg.100 g^{-1} of body weight)	
		Intact	OV-X	Intact	OV-X	Intact	OV-X
Estradiol	0	11.3±0.9 (20)	10.3±0.5 (30)	102.9±5.8 (10)	32.0±3.5 (14)	615.0±21.1 (10)	452.9±21.0 (14)
	0.001	16.0±1.2* (18)	16.4±1.9** (10)	124.5±14.0 (8)	55.0±8.8 (5)	621.6±26.9 (9)	553.4±42.7 (5)
	0.01	24.9±1.2*** (16)	23.1±1.5*** (34)	288.3±28.9*** (8)	120.8±11.5*** (18)	573.9±25.2 (8)	478.6±12.6 (13)
	0.1	21.5±0.9*** (16)	19.4±1.6*** (10)	335.3±12.8*** (8)	149.5±8.0*** (5)	643.7±56.8 (8)	449.6±19.0 (5)
	1.0	19.1±0.8*** (18)	18.2±1.8*** (10)	354.2±13.7*** (9)	216.8±23.1*** (5)	651.7±22.9 (9)	388.2±17.8 (5)
	10.0	8.6±0.5 (21)	11.4±1.1 (8)	293.0±8.2*** (10)	274.0±20.9*** (4)	582.8±36.6(10)	532.4±24.7 (4)
Prog	0	13.1±0.5 (42)	11.1±0.6 (30)	121.2±5.8 (20)	33.2±2.3 (19)	614.7±15.7 (20)	544.9±30.4 (19)
	125	19.8±1.1** (21)	8.4±1.2 (8)	156.7±6.6 (5)	34.6±4.4 (4)	622.6±10.3 (5)	492.5±28.3 (4)
	250	26.7±1.0*** (39)	11.3±1.0 (17)	127.8±6.9 (10)	51.4±4.5* (9)	620.7±21.0 (9)	485.8±24.1 (9)
	500	28.8±1.1*** (38)	13.2±1.2 (25)	144.5±6.8 (23)	57.5±3.7*** (24)	665.1±18.6 (23)	556.5±31.2 (24)
	1000	30.5±1.9*** (25)	19.1±1.0*** (27)	134.8±6.9 (24)	61.0±3.1*** (20)	687.0±17.6 (24)	504.5±14.9 (20)
Prog 500+E	0	13.1±0.5 (42)	11.1±0.6 (30)	121.2±5.8 (20)	33.2±2.3 (19)	614.7±15.7 (20)	544.9±30.4 (19)
	+E 0.001	23.9±1.9*** (22)	24.9±1.9*** (10)	124.1±3.1 (11)	76.5±5.6*** (5)	609.9±33.3 (11)	661.1±31.5 (5)
	+E 0.01	34.7±1.2*** (30)	44.9±3.1*** (28)	230.1±11.5*** (3)	141.7±13.6*** (15)	695.4±22.5 (18)	476.7±15.4 (10)
	+E 0.1	38.8±2.1*** (20)	32.8±1.3*** (22)	219.0±14.5*** (10)	162.2±10.8*** (11)	597.4±25.9 (10)	579.5±13.7 (6)
	+E 1.0	33.0±1.3*** (20)	34.3±1.3*** (12)	281.9±107.5***(10)	180.9±9.5*** (6)	572.1±29.3 (10)	598.1±28.6 (6)
	+E 10.0	27.5±1.4*** (16)	23.8±1.3*** (12)	336.8±11.7*** (8)	220.6±11.7*** (5)	591.9±16.8 (8)	457.8±30.6 (5)

*Young intact (18 days of age) and adult OV-X (ovariectomy at 22-24 days of age) females received subcutaneous injections of 50 µl vehicle (control) or hormones (50 µl) for 10 days. Values are means ±S.EM. Differences between values for different doses of hormones were determined by one-way ANOVA followed by the Bonferroni test. Asterisks indicate 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 uterus or spleen.*

Table 2. Interaction of testosterone (T) and 17β estradiol (E) plus progesterone (Prog 500) or norethindrone acetate (NA) on mammary and uterine growth in young intact and adult OV-X females of C3H/Di mice

	Young intact mice			Adult OV-X mice		
	0	E + Prog	NA	0	E + Prog	NA
Mammary gland	**The % area of mammary fat pad occupied by mammary epithelial structures**					
T (μg.d^{-1}) 0	12.4±0.9 (12)	27.0±2.4 (42)	29.0±2.3 (9)	10.7±1.4 (10)	28.3±1.3 (10)	26.8±1.3 (10)
50	-	-	-	6.6±0.4 (8)	24.5±2.1 (20)	18.7±1.3** (10)
100	10.2±0.9 (8)	26.4±1.6 (40)	-	7.6±0.9 (10)	20.7±2.2. (19)	19.6±1.1*** (10)
200	-	-	-	9.5±1.2 (10)	15.0±1.8** (20)	18.7±1.3** (7)
300	9.2±1.1* (10)	-	20.9±1.5 (8)	-	-	-
Uterus	**mg.100 g^{-1} of body weight**					
T (μg.d^{-1}) 0	102.9±5.8 (10)	120.8±5.1 (5)	249.8±15.2 (5)	46.6±2.4 (5)	80.9±7.2 (9)	134.4±13.0 (5)
50	-	-	-	146.4±2.3*** (5)	147.0±9.2*** (10)	145.7±21.2 (5)
100	246.5±12.1*** (5)	238.0±15.3*** (5)	-	164.9±10.9*** (5)	170.9±13.4*** (10)	182.6±9.9 (5)
200	-	-	-	207.7±15.9*** (5)	184.4±10.8*** (10)	227.1±12.6** (4)
300	315.8±9.7*** (5)	-	425.2±45.6*** (4)	-	-	-

Animals were injected placebo or hormones dissolved in placebo for 10 days: NA (12.5 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.

To test the selectivity of the estradiol and progesterone effect on mammary duct growth and uterine weight, we investigated whether it could be mimicked/ inhibited by other steroid hormones.

Testosterone alone in doses 50 to 200 μg.d^{-1} did not affect mammary growth in either young intact or adult OV-X animals. However, uterine weight was greater ($P<0.001$) in females given 50 μg or more testosterone daily than in the controls. The estradiol plus progesterone stimulated mammary growth was not significantly decreased by testosterone in young intact animals, however, mammary growth was significantly decreased in adult OV-X animals given testosterone in a dose 200 μg.d^{-1}. Testosterone did not affect norethindrone acetate stimulated mammary growth in young intact females. In adult OV-X animals, however, norethindrone acetate stimulated mammary growth which was significantly inhibited by testosterone in a dose 50 μg or higher (Table 2).

Cortisol alone or in combination with estradiol or progesterone or estradiol plus progesterone significantly ($P<0.05$) decreased the % area of mammary fat pad occupied by mammary epithelial structures, and the index of length x width (l x w) of mammary lymph nodes and spleen weight but had no effect on weight of the uterus (Table 3).

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

Hormonal treatment (μg.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)	Uterus (mg.100 g^{-1} of body weight)
Young Intact Mice				
0	13.4±1.5^{A} (10)	12.9±0.8^{A} (10)	634.3±54.7^{A} (5)	89.9±7.0^{A} (5)
Cortisol (F)1000	6.2±0.6^{B} (10)	8.7±0.5^{B} (10)	261.8±15.7^{B} (5)	85.0±12.3^{A} (5)
E 0.005	15.5±1.2^{A} (8)	12.0±0.7^{A} (8)	620.5±29.5^{A} (5)	123.9±2.8^{A} (5)
E + F	6.7±0.2^{B} (10)	7.6±0.5^{B} (10)	250.2±11.2^{B} (5)	118.1±10.4^{A} (5)
Prog 500	23.5±2.0^{B} (10)	9.1±0.4^{B} (6)	607.6±28.5^{A} (5)	126.0±2.3^{B} (8)
Prog + F	10.7±1.2^{A} (10)	6.9±0.5^{B} (10)	338.6±13.0^{B} (5)	131.9±5.5^{B} (5)
E + Prog	30.3±1.6^{BC} (10)	14.5±1.0^{A} (10)	778.7±67.6^{A} (5)	194.5±11.5^{B} (5)
E + Prog + F	21.2±1.6^{BD} (10)	7.6±0.4^{B} (10)	328.4±13.3^{B} (5)	194.2±9.1^{B} (5)
Adult OV-X Mice				
0	10.7±1.4^{A} (10)	18.0±2.0^{A} (10)	646.5±104.3^{A} (5)	26.7±2.9^{A} (5)
F 1000	6.5±0.4^{A} (10)	6.6±0.4^{B} (10)	218.3±12.2^{B} (5)	32.6±8.1^{A} (5)
E 0.005	23.4±1.7^{B} (6)	16.7±1.2^{A} (10)	546.0±18.6^{A} (5)	87.1±27.0^{B} (5)
E + F	8.9±1.0^{A} (8)	3.7±0.4^{B} (8)	202.9±14.7^{B} (5)	109.2±9.3^{B} (5)
Prog 500	8.8±1.2^{A} (10)	17.1±1.9^{A} (9)	766.4±70.5^{A} (5)	53.0±9.9^{A} (5)
Prog + F	6.0±0.6^{A} (8)	4.7±0.4^{B} (8)	260.7±4.5^{B} (4)	46.7±2.4^{A} (4)
E + Prog	29.4±1.1^{B} (10)	16.6±1.0^{A} (10)	604.3±24.5^{A} (5)	112.8±3.8^{B} (5)
E + Prog + F	7.1±0.4^{A} (10)	6.5±0.7^{B} (10)	172.5±6.5^{B} (5)	86.3±5.5^{B} (5)

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

A range of various species such as chicken, rat, mouse, trout and reptiles have been used in estrogenicity testing. The most widely used rodent bioassay measures the increase in uterine weight in the rat (Korach and McLachlan 1995) in spite of that lacks specificity as both estrogens and androgens enhanced uterine growth (Neumann and Steinbeck 1973, present results). Estrogen-like effects of androgens were correlated with

the binding of these steroids to the receptor and are considered as an estrogen receptor mediated mechanism (Hackenberg *et al.* 1993). Therefore, it is important that testing for the estrogenicity potential includes the ability to detect androgenic and antiandrogenic activities. This can be done by measuring changes in the weight of seminal vesicles (a decrease by estrogens and antiandrogens, an increase by androgens) and in mammary growth (an increase by compounds exhibiting the activity of estrogens or estrogens plus progesterone and a decrease by androgens and cortisol) in young intact and adult castrated males of C3H/Di strains (Škarda 2002), C3H/HeN or CBA strain (Škarda, unpublished). The sensitivity of estrogen assays in the female mammary gland was at least 10 times higher than that in the uterus of both young intact and adult OV-X females. The percentage area of the mammary fat pad occupied by mammary epithelial structures showed a distinct biphasic response to the application of estradiol. Estradiol concentrations of 0.001 to 1.0 $\mu g.d^{-1}$ stimulated mammary growth, but concentrations above 1 $\mu g.d^{-1}$ were inhibitory in both young intact and adult OV-X animals. Potential mechanisms mediating the reduction in mammary gland growth may include receptor down-regulation and the capacity for estrogens to bind to receptors for other steroid hormones, androgen or glucocorticoid receptors, resulting in antagonistic effects mediated *via* other receptor systems in response to supraphysiological doses of estrogens (vom Saal *et al.* 1997). This interpretation is further supported by the inhibitory action of both testosterone and cortisol on estradiol or estradiol plus progesterone or norethindrone (a synthetic derivative of 19-nortestosterone exhibiting progestational and estrogenic activities) stimulated mammary growth. The disadvantage of estrogenicity testing on the female mammary gland is the stimulation of duct growth and lobuloalveolar development in animals primed not only by estrogen alone but also by progesterone alone. In contrast, progesterone in young intact and adult castrated males alone has a low or no stimulatory effect on mammary growth (Škarda 2002) and may be used for estrogenicity assays with a high degree of specificity.

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