Physiol. Res. 50: 275-282, 2001

Detection of Estrogenicity by Bioassay on the Mouse Mammary Gland in Vivo

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Received April 27, 2000
Accepted October 13, 2000

Summary

The wide chemical diversity of estrogenic compounds precludes an accurate prediction of estrogenic activity on the basis of chemical structure or radioimmunological assay and thus requires that the potency of these compounds is defined by bioassay. The mammary duct growth response in intact prepubertal and adult gonadectomized female and male mice of the C3H/Di strain was used to assess the estrogenicity of synthetic compounds or their derivatives. The vehicle for tested compounds should be free of estrogenic and other hormonal effects. Olive oil or sunflower oil exerted estrogenic activities and were thus unsuitable as vehicles for the tested compounds. The absence of estrogenic activity, high solubility of different steroid hormones, and the low incidence of the inflammatory reactions at the injection site were achieved by using a vehicle containing benzyl alcohol, benzyl benzoate, butylhydroxyanisole, butylhydroxytoluene, ethyl oleate and ethanol. The bioassay was primarily designed to examine the effect of tested compounds on mammogenesis. The duration of hormone treatment was chosen to be long enough for induction of duct growth but too short to induce lobuloalveolar differentiation. Females were treated for 10 days, males for 15 days. The proportional volume occupied by mammary epithelial structures was estimated by the modified Chalkley's technique. The mean coefficient of variation of quantitative evaluation of 10 different mammary glands obtained by two operators varied between 3.2 and 17.4 %. The mean coefficient of variation of quintuplicate determinations of each mammary gland by one operator was 10.1 %, and 11.1 % by the other. The correlation coefficient between results of two operators was 0.994. Estrogens are primarily defined by their ability to increase the mitotic activity of female secondary sex organs. However, our results have shown that progesterone alone, if administered in a high dose, stimulates mammary growth in both intact prepubertal and OV-X female mice similarly as the synthetic progestatial steroid norethindrone with inherent estrogenic properties. In contrast, progesterone alone had no effect, in young intact or adult castrated males, but norethindrone did stimulate mammary growth. These results demonstrated that the mammary gland of males is a suitable model for estrogen screening.

Key words
Mammary gland • Mouse • Estrogenocity • Bioassay • Progesterone

Introduction

Many biological indicators have been developed for the *in vivo* characterization of substances with hormonal action. Routine models used for the evaluation of hormonal and antihormonal activities of synthesized compounds or animal and plant products used in food, pharmaceutical and cosmetic industry include their effects on growth, structure and function of the pituitary glands, uterus, prostate, seminal vesicles, kidneys, the liver and thymus (Neumann and Steinbeck 1974, Philibert *et al.* 1991). However, the mammary gland has not been routinely used in spite of the fact that it contains all 5 classes of steroid hormone receptors, receptors for peptide hormones and receptors for growth factors.

Because of the multiple hormonal interactions required for its growth and differentiation, the mammary gland may be considered a complex hormone target organ in the mammalian body. Each hormone possesses the ability to alter relatively specific biological events or changes in structure of the mammary gland that may be indicative of the presence of specific hormone activity (Imagawa *et al.* 1990). This makes the mammary gland a suitable system for the routine evaluation of hormonal and antihormonal activities of newly synthesized compounds and their metabolites or endocrine disrupting chemicals in the environment and in animal and human diets.

The developmental stages of the mammary gland can easily be studied in species where glands develop in two dimensions such as mouse, rat, or rabbit by means of whole-mounts. Recently we have developed a simple and reliable quantitative histology evaluation of mammary gland growth responses to substances with hormonal or antihormonal activities (Škarda and Lavriv 1998). Our objective herein was to assess rapidly the estrogenicity using the mammary gland of mice *in vivo*.

Methods

Material

2,2,2-tribromoethanol (Fluka Chemie AG, Buchs, Switzerland) and t-amylalcohol (Sigma-Aldrich, Prague, Czech Republic) were used for preparation of Avertin. Progesterone, norethindrone and methyl salicylate were purchased from Sigma-Aldrich (Prague, Czech Republic). Hematoxylin was bought from Merck (Darmstadt, Germany). Heavy duty Kapak/Scotchpak heat sealable pouches (Stock No. 502) from KAPAK Corporation (Minneapolis, MN, U.S.A.) were gifts from Professor Evelyn M. Rivera, Michigan State University, East Lansing, U.S.A. Corn and sesame oils were bought from Fluka Chemie AG (Buchs, Switzerland). Olive oil was bought from two sources - one from Fluka Chemie AG, the second from Prague Pharmacy Store. The commercial vehicle for progesterone (benzyl alcohol 20 g, benzyl benzoate 70 g, sunflower seed oil 910 g) was a gift from Biotika (Slovenská Lupča, Slovakia), and the commercial vehicle for testosterone had the following composition: benzyl alcohol 24 g, benzyl benzoate 242 g, butylhydroxyanisole 2 g, butylhydroxytoluene 2 g, ethyl oleate 806 g, ethanol 28 g. The commercial vehicle for 17α-hydroxy-progesterone caproate (benzyl alcohol 26 g, benzyl benzoate 320 g, butylhydroxyanisole 1 g, butylhydroxy-toluene 1 g, ethyl oleate 375 g, castor oil 512 g, ethanol 30 g) was purchased from Spofa a.s. (Prague, Czech Republic).

Animals

C3H/Di mice were from our own colony. Females were placed with littermate males at 2 months of age and the sequential pregnancy-lactation system was used. Animals were maintained on a 12 h light/12 h dark lighting schedule (lights at 6:00-18.00h) in our vivarium. They were fed a nutritionally complete pelleted diet purchased from TOP Velaz (Lysá nad Labem, Czech Republic). The diet and water were allowed *ad libitum* and the daily routine in the vivarium was constant. To minimize variation of the dose-response relationship of injected hormones, all young animals were weighed at the age of 18 days and only animals weighing 8±1 g were either randomly distributed into the treated groups or used for further breeding.

Development of the female mammary gland begins in the fetus with mammary anlagen (promordium) giving rise to primary and secondary sprouts. From birth till 3 to 4 weeks of age, the growth of ducts is slow but thereafter the size and arborization of the ducts increases rapidly (Imagawa *et al.* 1990). Thus, when intact females were used for the experiments, hormone treatment was initiated on day 18 or 19 of age. To attain a better slope of the dose-response curve, adult virgin females were ovariectomized (OV-X) between 21 to 24 days of age, i.e. before the allometric growth phase of the mammary glands.

Male mice lack nipples due to mesenchymal condensation around the fetal mammary buds, resulting

in the partial or complete destruction of the mammary buds (Wasner *et al.* 1983). During ontogenesis, residual mammary rudiments experience a different degree of branching in males of some strains (SLN, GR/A) while in others (Swiss, C3H/He, C3H/Di), the mammary gland remains undeveloped throughout life. However, under appropriate hormonal stimulation, mammary rudiments of males of the Swiss and C3H/Di strains display a considerable developmental potential (Freeman and Topper 1978, Nagasawa *et al.* 1987, Škarda *et al.* 1989). In our intact males, hormone treatment was initiated on day 18 of age, i.e. before a substantial increase of testicular androgen secretion in males (Škarda *et al.* 1989). When adult males were used, they were castrated 10 to 20 days before the administration of tested compounds.

Vehicle and route of hormone administration

In the present experiments, we tested estrogenic properties and solubility of steroid hormones in sesame oil, corn oil, olive oil, and three commercial oil mixtures as a vehicle for steroid hormones. The vehicle (placebo, 50 µl) or a steroid hormone (50 µl) were injected subcutaneously using a 1 ml glass tuberculin syringe fitted with a 24-gauge needle. The oily vehicle tends to leak from the site of injection and hence needle should be inserted under the skin from the sacral region to cranial thoracic region where the dose is deposited. Injections were given daily in the morning between 8:00 to 9:00 to avoid stress of handling as recommended by Sillence and Etherton (1989). The estrogenic activity of the vehicles was determined in the presence and absence of progesterone. The duration of the period of hormone treatment was chosen to be long enough for inducing duct growth (the quantitative changes) but too short to induce lobuloalveolar differentiation (qualitative changes). Females were treated for 10 days, males for 15 days.

Surgical procedures

Anesthesia was induced by intraperitoneal injection of 15-30 µl per g of body weight of 2.5 % Avertin (250 µl of stock solution of 1 g of 2,2,2-tribromoethanol in 1 ml of t-amyl alcohol was added to 9.75 ml of saline and solubilized by gentle warming and mixing). When the animals ceased to react, they were placed on an operating board. The females were bilaterally ovariectomized by the paralumbal route, the males were castrated *via* the scrotal route.

Mammary whole-mount preparations

One day after the last injection, the mice were weighted and killed by cervical dislocation and exsanguinated. The first inguinal mammary fat-pads were removed and transferred into warm (35 °C) tap water.

Mammary glands were spread as flat as possible on a piece of copy machine paper, blood was pressed out off the vessels and gland was covered by another piece of paper and put into Carnoy's fixative (2 h) in Petri dishes. Then, the pieces of paper were removed and glands were defatted by shaking for 3 h in triple exchanged acetone. Then, 100 % ethanol was added and glands hydrated through graded alcohols to 70 % ethanol. For hematoxylin staining, to demonstrate mammary epithelial structures, a dye solution contained 0.65 g $FeCl_3$ in 67.5 ml H_2O; 8.7 ml stock hematoxylin (10 % hematoxylin in 95 % ethanol); 1000 ml 95 % ethanol (Silberstein and Daniel 1982); 10 ml of concentrated HCl. The glands were stained for 2-3 h and transferred into tap water (1 h in 2 changes), then washed in 50 % ethanol and destained in 1 % HCl in 50 % ethanol for 30-60 min. After destaining glands were blue by washing in tap water for 5- to 10 min and dehydrated through graded alcohols to mixture of 100 % ethanol plus methylsalicylate (1:1, v/v) and stored in methylsalicylate. Whole-mounts were individually sealed (with small amount of methylsalicylate) into a heavy duty heat sealable pouches (KAPAK Corporation) and photographed.

Quantitative mammary histology

A modification of Chalkley's morphological analysis (Chalkley 1943) was used to determine the percentage area of the mammary fat pad occupied by mammary epithelial structures on enlarged (x 12) photographs of the mammary whole-mount preparations. A foil divided by 10 parallel vertical lines (2.35 cm distance between lines) was laid over the print (Fig. 1). The percentage of the mammary epithelial area was determined by random presentation of "fields" from the photographs to 25 Chalkley's points placed on the Petri dish (4.7 cm in diameter). The center of the Petri dish was shifted from one vertical line to another one along the middle of the mammary gland. The contacts of points with mammary epithelial structures were counted for 20 fields (two replicate counts were made per vertical line). In this way, an area of 4.70 x 25.85 cm over each whole-mount was counted and the percent area of

mammary epithelial structure (AMES) was calculated from

$$\%\text{AMES} = \frac{\text{total number of contacts}}{\text{total number of points}} x100$$

where the total number of points is 500 (25 points per field x 2 fields per vertical line x 10 vertical lines).

Specificity and sensitivity of bioassay

The estrogenicity bioassay using mammary duct growth measures mammary epithelial cell proliferation. In young intact or adult castrated males, the stimulation of duct growth is a specific estrogen response, while on the other hand, in OV-X females the stimulation of duct growth may also be achieved by progesterone alone, if high dose (1000 μg/day) is given. Testosterone alone has no effect on mammary growth (Škarda unpublished data). The percentage mammary fat pad occupied by mammary epithelial structures is increased in young intact females by 17β estradiol at dose 0.001 μg/day and in OV-X females at dose 0.01 μg/day, while uterine growth was significantly stimulated by 10 times higher dose. In young intact males, the size of mammary epithelial structures was increased at dose 0.01 μg/day, in adult castrated males at dose 0.1 μg/day (Škarda unpublished data).

Statistical analysis

All variables under investigation were sufficiently normally distributed so that parametric analysis could be used. All data represent either means ± S.E.M. or means ± S.D. Statistically significant difference was determined by ANOVA, followed by the Bonferroni test for individual comparisons of the means. Differences between animals treated with placebo and those treated with placebo plus a hormone were analyzed by the t-test. The agreement between results of quantitative histology obtained by two operators was assessed by calculation of the coefficient of variation and correlation coefficient.

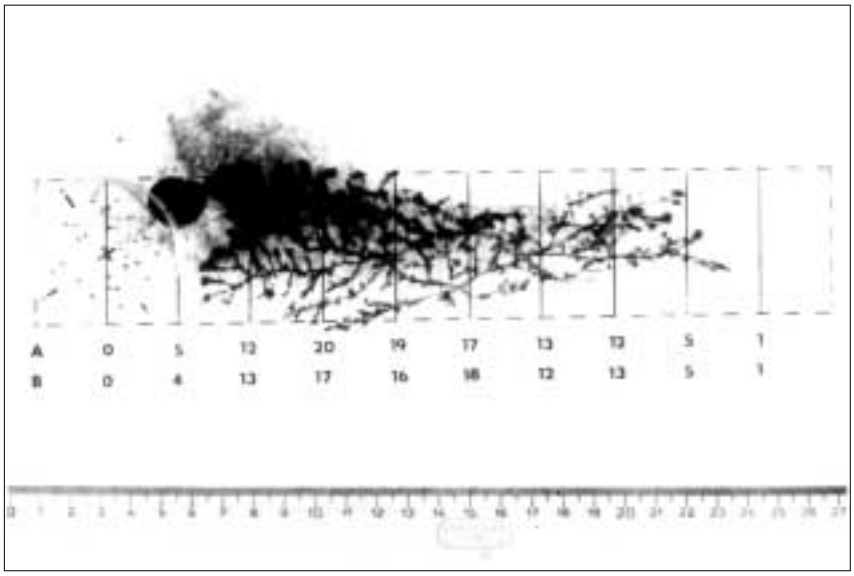

Fig. 1. *Position of foil with vertical lines and Petri dish with Chalkley's points on enlarged photograph of the mammary whole-mount during quantitative histology.*

Results

The proportionate volume occupied by mammary epithelial structures was estimated by a random presentation of points throughout the inguinal mammary fat pad of whole-mounts and summing up the number of points lying in the epithelial structure (Fig. 1). Table 1 compares the results of the percentage area of the mammary fat pad occupied by mammary epithelial structures on enlarged photographs obtained by two operators. There was no significant difference between means of the percentage areas of the mammary tissues obtained by two operators over a wide range of sizes of the mammary glands. The mean coefficient of variation

of the quantitative evaluation of 10 different mammary glands performed by two operators varied between 3.2 and 17.4 %. The mean coefficient of variation of 5 determinations of each mammary gland by one operator was 10.1 %, by the other 11.1 %. The correlation coefficient between results of two operators was 0.994. Using our modified Chalkley's method, the percentage area of one mammary fat pad occupied by mammary epithelial structures may be determined by a skilled technician in 1 to 2 min.

Table 1. Percentage area of mammary fat pad occupied by mammary epithelial structures in whole-mount preparation obtained by two operators for 10 glands and their coefficient of variation

Mammary gland No.	Norethindrone acetate (µg/d)	Operator I		Operator II		Mean coefficient of variation
		Mean ± S.D.	Coefficient of variation	Mean ± S.D.	Coefficient of variation	
1	6.25	6.3±0.7	11.4	9.8±0.7	7.4	9.4
2	3.13	2.4±0.4	15.0	3.2±0.5	15.4	15.2
3	6.25	5.2±0.6	11.2	7.3±0.8	11.3	11.3
4	3.13	3.2±0.6	17.8	4.4±0.7	16.9	17.4
5	25.00	31.9±1.4	4.3	29.2±0.6	2.1	3.2
6	12.50	18.2±1.4	7.6	19.9±0.5	2.5	5.1
7	6.25	13.2±0.7	5.1	14.4±1.3	8.8	7.0
8	3.13	2.4±0.3	11.1	3.3±0.7	22.3	16.7
9	3.13	2.4±0.2	7.4	3.6±0.5	14.8	11.1
10	12.50	19.1±1.8	9.7	21.9±2.2	9.9	9.8
Mean coefficient of variation			10.1		11.1	

Prepubertal intact C3H/Di male mice were treated with different doses of norethindrone acetate for 15 days. Percent area of mammary fat pad occupied by mammary epithelial structure was determined by two operators as described in Materials and Methods. The values in the Table represent the average of 5 determinations ± SD. Correlation coefficient between the results of two operators was 0.994.

Table 2. Effects of different vehicles on mammary growth responses to progesterone in male mice

Vehicle	Placebo	Progesterone (1000 µg/day)	Significance
Corn	1.3±0.2 (5)	1.4±0.2 (5)	NS
Sesame oil	1.2±0.1 (5)	1.7±0.2 (5)	NS
Olive oil (Fluka)	1.6±0.1 (5)	3.3.±0.5 (6)	NS
Olive oil (Prague)	10.8±1.8 (6)	42.0±1.8 (4)	P<0.001
Commercial vehicle A)	1.4±0.1 (4)	23.6±2.0 (5)	P<0.001
Commercial vehicle B)	1.5±0.1 (4) NS	1.2±0.3 (5)	NS
Commercial vehicle C)	1.2±0.2 (5) NS	1.3±0.3 (4)	NS

Intact C3H/Di male mice at age of 18 days were daily treated (s.c.) with either placebo (vehicle) or placebo plus progesterone for 15 days. Values are means ± S.E.M. of the percentage area of mammary fat pad occupied by mammary epithelial structures. Numbers in parentheses indicate the number of animals supplying the whole-mounts of first inguinal mammary glands. Commercial vehicle for progesterone (A), for 17α hydroxyprogesterone caproate (B), for testosterone (C). The composition of commercial vehicles is described in Materials and Methods.

Table 3. Effect of norethindrone acetate (NA) on the mammary gland growth in intact and gonadectomized male and female C3H/Di mice

	Mammary epithelial structure (%)	
	Control	**NA**
Young intact male	1.0±0.1 (38)	40.8±3.1 (43)
Adult castrated male	1.4±0.1 (15)	25.5±2.4 (17)
Young intact female	13.4±1.5 (34)	35.7±2.4 (28)
Adult ovariectomized female	11.5±1.1 (5)	29.1±1.4 (5)

At the age of 18 to 19 days intact animals received daily 50 μl vehicle (control) or norethindrone acetate s.c. (25 μg/day) for 10 days (females) or 15 days (males). Adult males were castrated 15 days before initiation of treatment. When adult females were used, they were ovariectomized between 21 to 24 days of age. Values are means ± SEM of the percentage area of mammary fat pad occupied by mammary epithelial structures. Numbers in parentheses indicate the number of animals supplying whole-mounts of the first inguinal mammary glands.

The solubility of progesterone or different analogues of steroid hormones (up to 1000 μg per 50 μl) in vegetable oils was low and it was necessary to dissolve them by heating at 100-120 °C for more than 30 min. Steroids dissolved at ambient temperature crystallized during several hours. When the substance of a steroid was first dissolved in a small amount of acetone and then vegetable oil was added, the incidence of the inflammatory reactions at the injection site was high. The best solubility and the low incidence of inflammatory reactions at the injection site were achieved by using commercial vehicles. Further experiments were designed to test vehicles for estrogenic activities in the mammary gland. As the mammogenic potency of estrogens depended upon whether synergizing progestagens were present, the vehicles were administered alone or with progesterone. In males, one preparation of olive oil alone (bought in a Prague pharmacy) stimulated growth of the mammary gland and thus suggested the presence of estrogenic activity in the oil. When progesterone was added to the vehicles, mammary growth was stimulated not only in animals injected with above mentioned olive oil but also when added to the commercial vehicle containing sunflower oil (commercial vehicle for progesterone) (Table 2). When progesterone, dissolved in estrogen-free vehicles (corn and sesame oils and in commercial vehicles for 17α-hydroxyprogesterone caproate or for testosterone) was injected in males, no stimulation of mammary gland growth was detected. In females, however, progesterone (1000 μg/day) stimulated growth of the mammary gland even when commercial vehicle free of estrogenic activity (vehicle for testosterone) was used. In intact prepubertal females, progesterone increased the percentage area of mammary fat pad occupied by mammary ducts from 13.0±1.0 % in placebo-treated to 30.5±1.5 % in progesterone-treated animals. In OV-X animals, progesterone increased area of mammary ducts from 10.7±1.4 % in placebo-treated to 26.3±2.6 % in progesterone-treated animals. The ability of the male mammary gland to respond to norethindrone acetate – the synthetic progestatial steroid with inherent estrogenic properties (Table 3) – have demonstrated the suitability of the male mammary gland as a model for evaluating the estrogenicity of widely diverse chemicals.

Discussion

In general, in litter-bearing animals such as mice, the variability in growth rate of the offspring is affected by the birthweight, health and by the lactational performance of the dam. Small animals are unable to compete with larger siblings for the colostrum and milk. They thus experience dietary restriction, grow slowly and are more likely to have health problems. Moreover, animals experiencing dietary restrictions during neonatal and prepubertal life have severely impaired reproductive systems, e.g. low weight of uteri, ovaries, testes, seminal vesicles, delayed puberty (Hoggard *et al.* 1998, Škarda, unpublished results) and one can expect that their response to injected hormones would be very variable. To ensure the uniformity of response to injected compounds, the animals were weighted at day 18 of age and only animals weighing 8±1 g were used for experiment or for further breeding.

A modified Chalkley's technique described in this paper is both sufficiently rapid and accurate for evaluation of mammary gland growth. Agreement in results between operators is good over a wide range of percent areas of mammary fat pad occupied by mammary epithelial structures. For ten mammary whole-mounts with percentage area ranging from 2.4 % to 31.9 % the average coefficient of variation of quintuplicate determination for two operators was 10.6 %. The

coefficient of variation decreased as the percent area occupied by mammary epithelial structures increased. There was a high level of correlation (r = 0.994) between results of two operators. Our modification Chalkley's technique may be used for evaluation of mammary gland growth on a monitor connected with a microscope without having to prepare photographs of the mammary whole-mounts.

The potency of steroid hormones and their analogues depends on the vehicle employed, the route of administration, factors that affect the rate of absorption, metabolism and excretion, and the sensitivity of the final indicator used for measuring the efficacy (Zarrow *et al.* 1964). As a rule, steroid hormones are administered subcutaneously in oily solutions, however, many of the vegetable oils used in the diet or as vehicles for application of the hormone exhibit some estrogenic properties (Booth *et al.* 1960) that can interfere with assays for hormone activity. In estimations of estrogenic and/or other interfering endocrine activity, proper controls should be carefully maintained. In our experiments, a high level of estrogenic activity was detected in the olive oil bought in a Prague pharmacy by stimulation of growth of mammary rudiments in males injected with olive oil alone. The low level of estrogenic activity in a commercial vehicle containing sunflower oil was detected by stimulation of male mammary gland growth in the presence of the vehicle plus progesterone.

Estrogens are primarily defined by their ability to increase the mitotic activity of female secondary sex organs. The gold standard for the detection of functional estrogenicity is still mouse uterine bioassay (Korach and McLachlan 1995), in spite of the fact that uterine growth is also stimulated by androgens (Neumann and Steinbeck 1974). The bioassay of estrogenicity on the male mammary gland is more specific as testosterone alone has no effect on mammary gland growth and estradiol- or estradiol plus progesterone-stimulated growth is inhibited by testosterone. The sensitivity of the estrogen assay on mammary gland is at least ten times higher than that on the uterus in both young intact and adult OV-X females. The sensitivity of estrogen assay on the mammary gland of males is the same as that on the female uterus but lower than that on the female mammary gland. The specificity of the assay is, however, higher in the 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 a combination of estradiol plus progesterone but also by testosterone alone. On the other hand, testosterone inhibited estradiol plus progesterone-stimulated growth of the mammary gland in males (Škarda, unpublished results). Our results have shown that progesterone alone, if given in high doses, stimulates mammary growth in both intact prepubertal and OV-X female mice unprimed with estrogens, similarly as synthetic progestatial steroid norethindrone with inherent estrogenic properties. In contrast, progesterone alone has no effect in young intact and adult castrated males but norethindrone stimulates mammary growth. These results demonstrate that females (both intact and OV-X) either still have enough endogenous estrogens or they metabolize injected progesterone to estrogens. On the basis of our results, it is clear that screening for estrogens using mammary gland of C3H/Di males as a model should be performed. In this organ, estrogens act directly and not *via* a negative feedback to inhibit gonadotropins that results in lowering androgen secretion.

In vivo testing is on the decline with improvements *in vitro* testing (Welshons *et al.* 1992, Soto *et al.* 1995). The use of cell cultures avoids the complexity of the whole animal. However, there is little evidence in general terms to suggest that it is, from all aspects, an alternative to testing on living animals. Some newly synthesized hormone analogues may display a clear effect *in vitro*, but are totally devoid of activity *in vivo* (Philibert *et al.* 1991). Weak structure-activity relationship of environmental estrogens (plant and fungal products, breakdown products of detergents, pesticides, plasticizers and variety of chlorinated compounds) requires defining the identity and potency of these chemicals by bioactivity (Heppel *et al.* 1995). An effective bioassay could be developed by combining modifications of existing techniques to assess the hormonal and/or antihormonal activities of different substances simultaneously on two to three organs (one of them is mammary gland) in both male and female secondary sex organs.

Acknowledgements

I express my thanks to Ing. Jitka Janečková, Eva Urbanová and Dr. Pavlo Lavriv for their excellent technical assistance. This work was funded by the Grant Agency of the Czech Republic (No. 505/94/0009 and No. 523/99/9843).

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