Progesterone Alone is Responsible for Stimulation of the Growth of Ducts and of Mammary Alveolar Structures in Mice

J. ŠKARDA, V. FREMROVÁ, I. BEZECNÝ

Institute of Animal Physiology and Genetics, Czechoslovak Academy of Sciences, 104 00 Praha 10 — Uhříněves, Czechoslovakia

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The effect of estradiol valerate and progesterone on mammary growth of prepubertal (20, 25 and 30 days of age) female and prepubertal (15, 20 and 30 days of age) and adult male C3H/Di mice were studied. Estradiol treatment had no effect on mammary growth in 30 day old females, but mammary growth of 20 and 25 day old females showed clear inhibition. In contrast, treatment with progesterone alone stimulated both the growth of ducts and formation of alveolar buds. In males, the injection of estradiol valerate resulted in a stimulation of the growth of mammary rudiment. The treatment with progesterone alone stimulated the growth of mammary ducts and the formation of alveolar buds in males treated from day 15 and 20 of age, while mammary growth of those treated from day 30 of age was low and the growth of mammary rudiment of adult males was not stimulated at all.

The mammogenic effect of progesterone was not affected by time of weaning of males but it was inhibited by simultaneous application of testosterone. An age dependent decrease of the mammogenic effect of progesterone in males is related to an increase in plasma concentration of testosterone.

The morphogenesis of mammary gland and the differentiation of mammary epithelial cells are induced or modified by hormones. The ability of mammary tissue to respond to hormones is acquired or lost during

various periods of ontogenesis. Both acquisition and loss of hormone responsiveness of fetal male mammary rudiment to the destructive effect of testosterone was demonstrated by Kratochwil [1977] and Wasner et al. [1983]. In 14-day male fetuses, testicular androgens caused the partial or complete destruction of the mammary buds and consequently, male mice of certain strains (e. g. C3H but not SHN or SLN) postnatally showed either absent or rudimentary mammary glands [Nagasawa et al. 1987a]. In young prepubertal female mice (between 4 and 7 weeks of age) the mammary gland undergoes a surge of growth due to increased ovarian secretory activity [Nandi 1959]. The estrogens and progestins are clasically considered to be direct mediators of mammary cell proliferation *in vivo* [Lyons et al. 1958; Nandi 1959]. Yet, the mechanism of action of these steroid hormones is not understood as neither of these hormones alone showed stimulatory effect on mammary cell proliferation *in vitro* [Nandi et al. 1982].

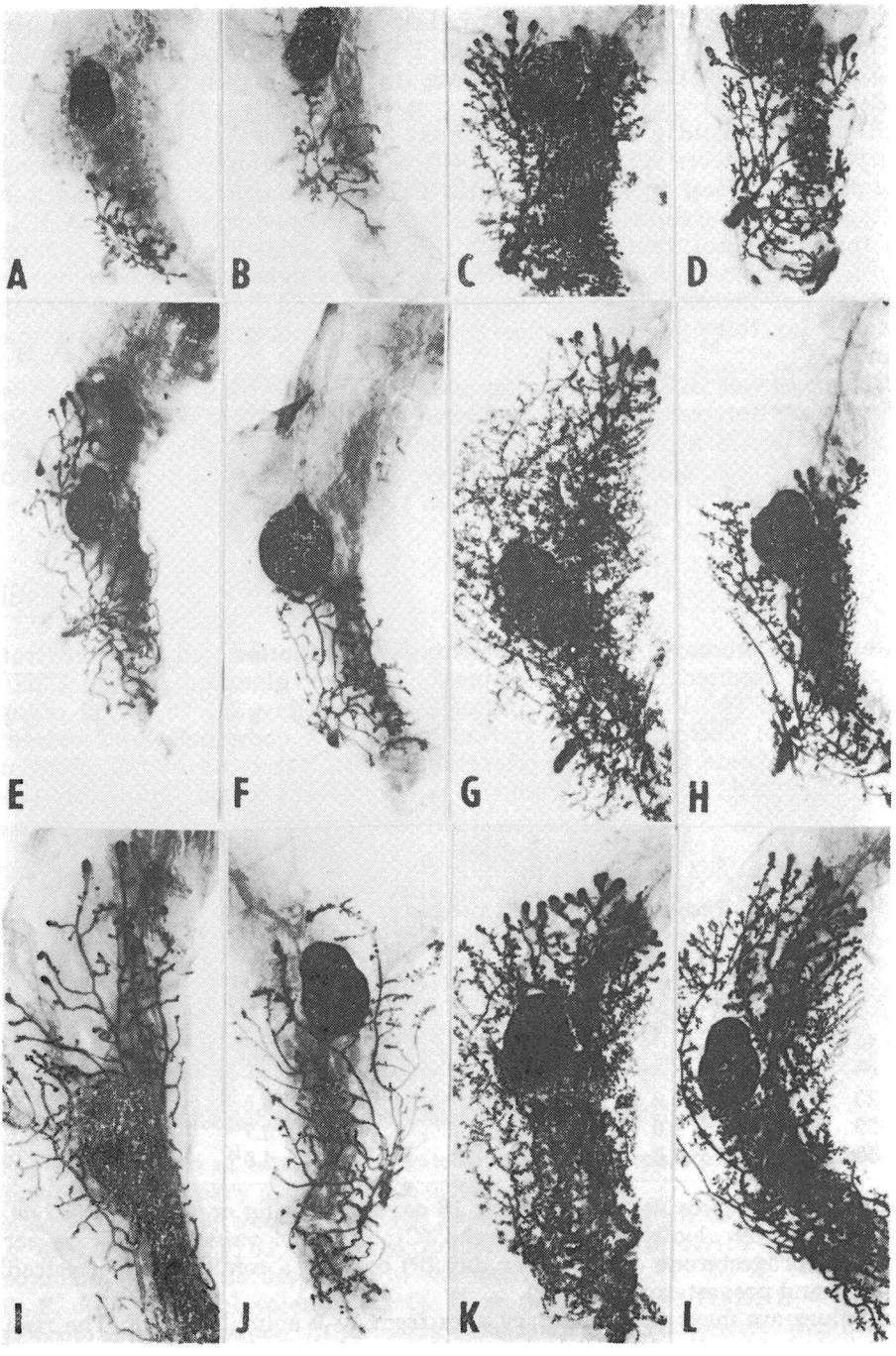
When studying the mammogenic effects of estrogens and progestins alone *in vivo* it should be remembered that, whilst during ontogenesis the mammary tissues of both females and males are continuously exposed to significant levels of estrogens, while progesterone levels are low in females before puberty and very low in both pre- and post-pubertal males [Wuttke 1976; Freeman and Topper 1978]. This gives an opportunity to study the effect of estradiol and progesterone alone on mammary growth in prepubertal females and prepubertal and postpubertal males. The results of this study indicate that progesterone alone stimulates both the growth of ducts and the formation of alveoli in young mice. The mammogenic effect of progesterone alone is however lost in males after 30 days of age.

Materials and Methods

Animals: Mice used in this study were females and males from the first litters of inbred strain C3H/Di weaned at 15, 20 or at 25 days and used for experiments at different ages. Animals (4—9 per group) were injected subcutaneously (in the dorsum) with hormones dissolved in 30—50 μl of olive oil, the females being thus treated for 12 days and males for 19 days. Control animals were injected daily with 50 μl of olive oil vehicle.

Estradiol 17β-valerate - Neofollin (BIOTIKA, Slovenská Lupča, Czechoslovakia) was injected in doses of 3 μg every 3rd day. Progesterone

Fig. 1. The effect of estradiol valerate and progesterone treatment on morphology of the first inguinal mammary gland of young female mice.
Female C3H/Di mice were treated for 12 days, beginning on day 20 (A, B, C, D), 25 (E, F, G, H) or 30 (I, J, K, L) of age, with placebo (Control - A, E, I), estradiol valerate (3 μg per 3 days^{-1} - B, F, J), progesterone (1 mg per day - C, G, K) or a combination of estradiol valerate and progesterone (D, H, L). Representative whole-mounts of mammary glands are shown; magnification 4.65 $\times$.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- Agolutin (BIOTIKA, Slovenská Lupča, Czechoslovakia) was given daily in a dose of 1 mg (3.18 μmol). Testosterone propionate - Agovirin (LÉČIVA, Praha, Czechoslovakia) was injected daily in a dose of 100 μg (0.29 μmol).

Mammary gland growth: At autopsy the bilateral 1st inguinal mammary glands were prepared for wholemount preparations. Mammary glands were fixed in Carnoy's fluid for 2 h, defatted, stained with hematoxylin [Silberstein and Daniel 1982], destained with 10 g l^{-1} HCl in 700 ml l^{-1} ethanol, neutralized with 1 g l^{-1} NH_4OH [Freeman and Topper 1978], dehydrated through graded alcohols to xylene and photographed. The size of mammary tissue area was measured by means of counting squares on foil lying over the enlarged photograph of the mammary gland.

Plasma levels of steroid hormones: Mice were killed at different ages by decapitation and trunk blood was collected. The heparinized blood was centrifuged at 2000 × g for 20 min at 4 °C and plasma was stored at —20 °C until assayed for hormone concentrations by radioimmunoassay [Matoušek et al. 1980; Pícha et al. 1984].

Results

Female mammary gland whole-mount evaluation: Fig. 1 illustrates ductal development in first inquinal mammary gland of female C3H/Di mice treated for 12 days, beginning either at day 20, 25 or 30 of age, with placebo, estradiol, progesterone or with a combination of estradiol and progesterone. In control (placebo treated) mice the mammary gland area increased with age from 5.6 to 26.4 mm^2 (Tab. 1). The glands

Table 1

The effect of estradiol valerate and progesterone treatment on the size of first inguinal mammary gland of young female mice

Age (days)	Treatment			
	C	E	P	E + P
	Mammary gland area (mm^2)			
20	5.6 ± 0.6	2.8 ± 0.3[2)]	38.3 ± 3.5[1)]	24.8 ± 1.6[1)3)]
25	21.6 ± 4.0	8.7 ± 1.7[2)]	62.4 ± 3.7[1)]	33.9 ± 1.7[2)3)]
30	26.4 ± 8.8	25.3 ± 3.9	64.6 ± 4.9[1)]	43.5 ± 4.4[2)3)]

Female C3H/Di mice were treated for 12 days, beginning on day 20 or 25 or 30 of age, with 50 μl olive oil — placebo (C); estradiol valerate — 3 μg per 3 days (E); progesterone — 1 mg per day (P) or with a combination of estradiol valerate and progesterone (E + P).
The values are means of mammary area from 3—9 animals ± S.E. The results were tested using Student's t-test against control ([1)] = $P < 0.01$, [2)] = $P < 0.05$) and P against E + P ([3)] = $P < 0.01$).

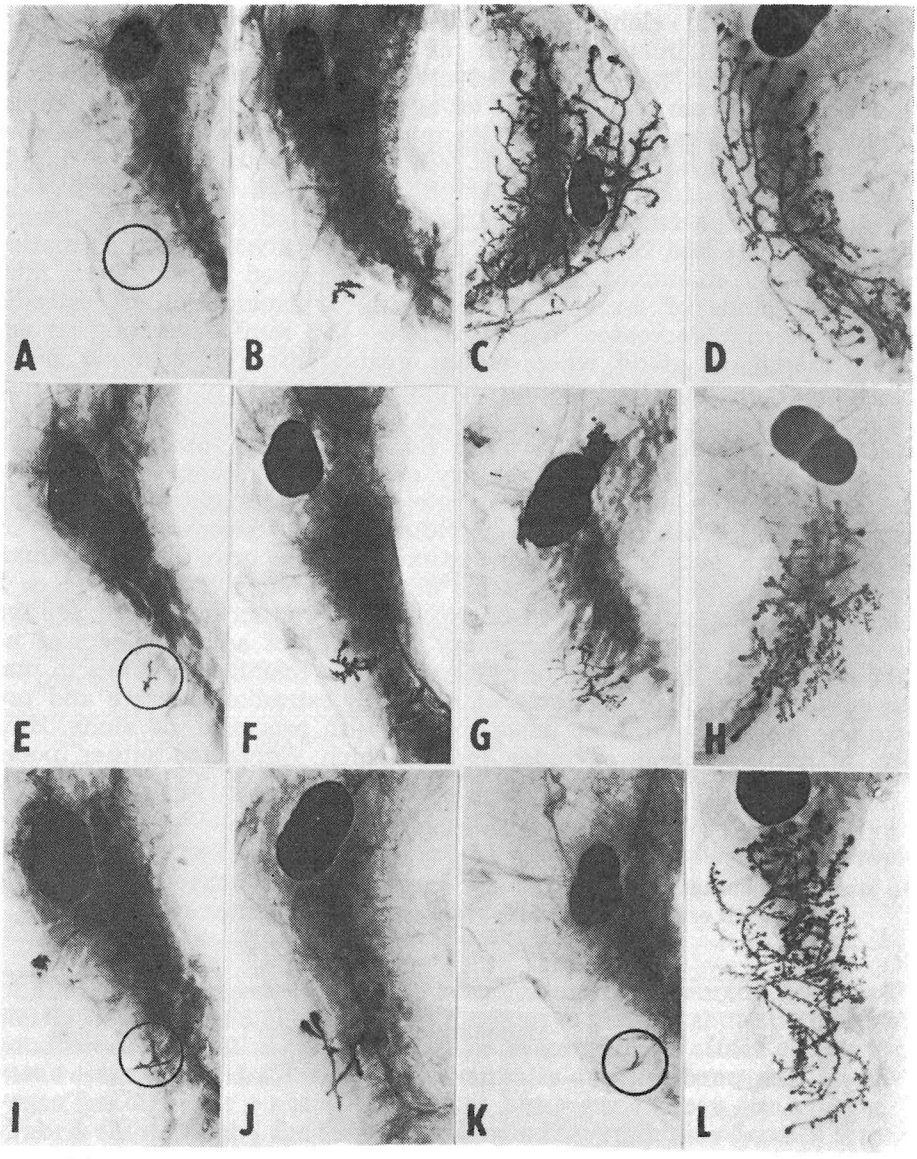

Fig. 2. The effect of estradiol valerate and progesterone treatment on morphology of the mammary gland of male mice.
Male C3H/Di mice were treated for 19 days beginning on day 20 (A, B, C, D) or 30 of age (E, F, G, H) or in the adulthood (I, J, K, L) with the same hormone regimes as described in the legend to Fig. 1: A, E, I = control; B, F, J = estradiol valerate; C, G, K = progesterone; D, H, L = estradiol valerate + progesterone. In the circles are mammary rudiments. Magnification 4.65 ×.

increased through elongation of the ducts beyond the inguinal lymph node and in the amount of branching. All glands showed some end buds but none contained alveolar buds. Mammary glands of animals treated from day 20 or 25 of age with estradiol alone resembled those of placebo treated females with inhibition of growth (Tab. 1). However, mammary glands of females treated with estradiol from day 30 of age showed no inhibition of growth. In contrast, the treatment with progesterone alone from day 20, 25 or 30 resulted in increased length, number of ducts and branches and also induced alveolar bud formation. The size of the mammary gland area was increased to 38.3—64.6 mm^2. Mammary glands of females treated with a combination of estradiol and progesterone decreased in size to 24.8—43.5 mm^2 depending on age.

Male mammary gland whole-mount evaluation: Fig. 2 shows ductal development in the first inguinal mammary gland of male C3H/Di mice treated for 19 days beginning on day 20 or 30 of age or in the adulthood with the same hormones as for the females (above). Mammary glands of all control males were rudimentary or sometimes even absent on one side. The injections of estradiol valerate resulted in slight but significant stimulation of growth of mammary rudiment in all animals (Tab. 2). The treatment with progesterone alone stimulated the growth of mammary ducts and formation of alveolar buds in males treated from day 15 or 20 of age, while mammary growth of those treated from day 30 of age was low and the growth of the mammary rudiment of adult males was not stimulated at all. The degree of development of mammary glands in male mice treated from day 15 or 20 of age with estradiol valerate and progesterone resembled that of those treated with progesterone alone. However, mammary gland of older males which responded either poorly

Table 2

The size of mammary glands of male mice of different ages after treatment with estradiol valerate, progesterone and testosterone

Age (days)	Treatment				
	C	E	P	E + P	P + T
	Mammary gland area (mm^2)				
15	0.03 ± 0.004	—	15.09 ± 1.77[1]	—	0.03 + 0.004[4]
20	0.03 ± 0.004	0.26 ± 0.03[1]	26.46 ± 4.64[1]	20.3 ± 7.7[1]	0.04 ± 0.007[4]
30	0.05 ± 0.008	0.74 ± 0.14[1]	0.81 ± 0.10[1]	22.8 ± 8.9[1][3]	—
A	0.04 ± 0.020	1.89 ± 0.71[2]	0.05 ± 0.01	19.5 ± 3.6[1][3]	—

Male C3H/Di mice were treated for 19 days beginning on day 15 or 20 or 30 of age or in the adulthood (A) with the same hormones as for the females described in the legend to Table 1 and with testosterone propionate — 100 μg per day (T).

The values are means of mammary area from 4—8 animals ± S.E. The results were tested using Student's t-test against control ([1] = $P < 0.01$, [2] = $P < 0.05$), P against E + P ([3] = $P < 0.01$) and P against P + T ([4] = $P < 0.01$).

Table 3

The effects of time of weaning on mammogenic effect of progesterone in prepubertal male mice

Age at weaning (days)	Age at start of progesterone treatment days	
	20	30
	Mammary gland area (mm^2)	
15	28.7 ± 1.7	0.5 ± 0.2
20	30.6 ± 2.2	0.6 ± 0.3
25	31.8 ± 5.3	0.8 ± 0.4

Male C3H/Di mice were weaned at 15 days or 20 days or 25 days of age and progesterone treatment (1 mg per day for 19 days) was started at 20 days or 30 days of age. The values are means of mammary area from 4—6 animals ± ± S.E.

Table 4

Plasma levels of steroid hormones in C3H/Di mice of different ages

Age (days)	Total estrogens		Testosterone	
	Female	Male	Female	Male
	ng ml^{-1}			
15	0.855	0.309	1.06	3.10
30	0.300	0.243	1.14	5.30
adult	—	0.268	—	6.40

The values are the results of radioimmunological assay of hormones in pooled plasma samples from 8—12 animals.

or not at all to progesterone alone, responded well when treated simultaneously with estradiol valerate. Mammary glands showed numerous ductal branches and alveolar buds. The mammogenic effect of progesterone was inhibited by testosterone in males treated from day 15 or 20 of age (Tab. 2).

Tab. 3 shows ductal development of mammary glands of males weaned at 15, 20 or 25 days of age and treated with progesterone alone from day 20 or 30. The mammogenic effect of progesterone decreased with age and was not affected by the time of weaning.

Tab. 4 shows the plasma concentrations of total estrogens and testosterone in mice of different ages. The plasma level of estrogens did not change substantially during development in males but decreased in females. On the other hand, the plasma level of testosterone did not increase in females but increased in males from 3.1 ng ml^{-1} at 15 days of age to 5.3 ng ml^{-1} at 30 days of age, while in adult males it reached 6.4 ng ml^{-1}.

This study demonstrates for the first time that progesterone alone is able to stimulate both the growth of mammary ducts and the formation of alveoli in young mice of both sexes. Estradiol valerate treatment failed to promote the growth of mammary gland in 30 day old females and in 20 and 25 day females was inhibitory. In young males estradiol treatment showed only a low stimulatory effect on mammary growth. This is contrasting with the results obtained in older intact and ovariectomized or castrated mice, when the influence of progesterone was restricted to the stimulation of the formation of alveolar structures [Lyons et al. 1958; Nandi 1959; Freeman and Topper 1978]. However, in ovariectomized animals, Bresciani [1971] demonstrated that progesterone stimulated ^{3}H-thymidine incorporation into DNA in ducts as well as in terminal bud cells, while provoking the appearance of alveoli. The combination of prolactin and progesterone but not prolactin and estrogen increased ^{3}H-thymidine incorporation in rat mammary tissue *in vitro* [Koyama et al. 1972]. Nagasawa et al. [1985] have demonstrated a stimulatory effect of progesterone on mammary gland growth in ovariectomized mice by using pituitary grafts under the kidney capsule. Nandi et al. [1984] found that the combination of prolactin with progesterone but not with estradiol stimulated the proliferation of mouse mammary cells grown within a collagen gel matrix in completely synthetic medium. Recently, Braunsberg et al. [1986] demonstrated that medroxyprogesterone acetate stimulated cell proliferation of human breast cancer cell line.

All these results are in agreement with our conclusion that progesterone is responsible not only for the stimulation of growth of mammary alveoli but also for the stimulation of growth of ducts in intact mice. The effects of progesterone and other steroid hormones are known to depend on the presence of specific receptors. An increase of progesterone receptors in the mammary gland is induced by estrogens [McGuire et al. 1976; Koenders et al. 1977; Horwitz and McGuire 1978; Muldoon 1987]. As plasma concentrations of estrogens are high during the early postnatal development in both sexes [Wuttke 1976; present results - Tab. 4], it may be hypothesized that the concentration of progesterone receptors in mammary tissue will be also high in both sexes. The concentration of estrogen receptors in mammary tissue of young C3H virgin mice is high [Hunt and Muldoon 1977] and it has been shown that prolactin is responsible for their induction [Muldoon 1981, 1987]. By the approach of puberty in female mice the secretion of progesterone increased which may explain the surge of mammary growth between 4—7 weeks of age. In males the secretion of progesterone is not substantially increased during ontogenesis [Wuttke 1976] and therefore mammary growth is not stimulated. However, when progesterone alone is injected until 25 days of age, mammary growth is stimulated. The effect of progesterone is not affected by early weaning of males suggesting that the mammogenic effect of progesterone depends on the age and not on the ingestion of hormonally

active substances in milk. From 30 days of age the mammogenic effect of progesterone alone is no longer seen because an increasing secretion of testosterone inhibits the growth of the mammary gland. This interpretation is supported by the inhibition of the mammogenic effect of progesterone by simultaneous application of testosterone to males at 15 or 20 days of age. Inhibitory effects of testosterone on mammary growth could be mediated via a suppressive effect of testosterone on prolactin secretion and prolactin receptor [Sherman et al. 1977; Kelly et al. 1977]. The effect of progesterone on mammary growth is synergistic with prolactin which is the essential stimulator of mammary growth. It has been shown that prolactin by itself either administered in sufficient doses [Talwalker and Meites 1961] or in animals with three pituitary grafts under the kidney capsule [Nagasawa et al. 1985] is capable of inducing mammogenesis. It was demonstrated that progestins increased the binding of ligand to lactogenic receptors [Murphy et al. 1986].

Our results do not imply that growth factors of hypophyseal, liver or kidney origin [Sirbasku 1978] are of no importance for normal mammary growth. Growth factors of uterine origin may be excluded, however, in view of the progesterone stimulation of mammogenesis in males. The role of estrogen induced growth factor from other tissues in the growth promoting effect of estrogens on mammary tissue is questionable, since progesterone alone can stimulate mammogenesis when progesterone receptors (induced by estrogens) are present. However, the mechanism of progesterone action on growth of mammary gland may involve the progesterone enhancement of epidermal growth factor binding to the surface of mammary epithelial cells [Murphy et al. 1986]. The receptor for epidermal growth factor have been shown to possess tyrosyl kinase activity [Cohen 1983] which is increased during progesterone induced mammary growth in ovariectomized mice [Sheffield et al. 1987]. Moreover, progesterone increases both cAMP content and the activity of cAMP dependent protein kinase (which mediates the physiological effects of cAMP) which, in turn, increase mammary cell proliferation [Sapag-Hagar and Greenbaum 1973; Sheffield et al. 1987].

From the above data it is clear that progesterone does not have to be directly mitogenic on the mammary epithelial cells to be involved in their growth. Nevertheless it could influence mammary epithelial cells directly, i.e. via their progesterone receptors, and initiate a series of events essential to the ultimate growth of the organ [Nandi et al. 1982].

The induction of growth of young male mammary rudiment by progesterone provides another model for studying the mechanism of the effect of steroid hormones on mammary growth and cancerogenesis.

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