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Effects of Long-Term Implantation of Vaginal Concretions on the Cervicovaginal Epithelium of Mice[1] (41947)

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Abstract. In two experiments, neonatal female BALB/cCrgl or BALB/cfC3HCrgl mice were given subcutaneous injections of 5 μg 17β-estradiol or sesame oil for the first 3 days of life and were ovariectomized at 60 days of age, at which time *vaginal concretions* (Experiments I and II) or *silica* (Experiment II) were implanted intravaginally. Mice were examined at 12 months of age. Three abnormal cervicovaginal epithelial responses were noted: persistent vaginal stratification/cornification (PVS); prominent vaginal squamocolumnar junction (SCJ); epithelial pegs, downgrowths, or lesions (dysplasias). PVS, not present in unimplanted controls, occurs in at least half of the members of the neonatally estrogen-treated groups; implants of concretions or silica did not increase its incidence significantly. Although SCJ was observed in implanted but not in unimplanted controls, its incidence was significantly higher in neonatally estrogen-treated mice than in either control group. The elevated incidence in neonatally estrogen-treated mice was not increased further by implantation of concretions or silica. In neonatally estrogenized mice, the subsequent implantation of a concretion significantly increased the incidence of cervicovaginal abnormalities. Increased PVS and SCJ are teratological consequences of neonatal exposure to a small amount of estrogen; on the other hand, increased dysplasias may, in part, be responses of the estrogenized vaginal epithelium to the concretions.

In recent years, there has been a renewed interest (1–3) in the development of concretions (calculi) in the reproductive tract of female mice. First described by this laboratory in 1962 (4), vaginal concretions occur in adult mice of numerous strains: A, BALB/c, BALB/cfC3H, C3H, C57BL, and CD-1, after neonatal sex hormone treatment. The presence of vaginal concretions is correlated with persistent cornification (4), metaplastic, eroding, or inflammatory cervicovaginal lesions (1, 4), increased occurrence of vaginal lesions in neonatally sex hormone-treated ovariectomized mice (5), female clitoridal hypospadias and patent connections between the urethra and vaginal lumen (1, 4), and immunosuppression characterized by reduced mitogen responsiveness (3).

The association between long-term exposure to concretions and vaginal epithelial hyperplasia raises the question of a role for the former in the development of the latter; i.e., is the concretion an "irritating" stimulus resulting in increased epithelial proliferation? The present study was undertaken to ascertain whether the presence of a vaginal calculus or an inert equivalent would result in vaginal downgrowths and lesions in mice treated neonatally with small amounts (threshold or subthreshold) of estradiol, known to induce persistent vaginal cornification.

Materials and Methods. *Experiment I.* Commencing within the first 24 hr after birth, three daily subcutaneous injections of 5 μg 17β-estradiol (E_2) (Calbiochem, La Jolla, Calif.) in 0.02 ml sesame oil (Hain's cold-pressed, Los Angeles, Calif.) were administered to female BALB/cfC3HCrgl mice. All mice were weaned at 1 month of age and ovariectomized at 60 days.

Immediately after ovariectomy, dried vaginal concretions collected from previous experiments ranging in dry weight from 8.2 to 21.2 mg (mean = 14 mg) were implanted into the vaginae of approximately half of the mice. The presence of the concretion within the vaginal lumen was monitored monthly by examination with blunt curved forceps. If the concretion was absent, a replacement concretion was inserted. The control mice,

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those without implanted concretions, were also probed for the presence of concretions.

Animals were maintained in a temperature-monitored animal facility with an artificially illuminated 12-hr day, fed water and standard laboratory chow (Berkeley Diet, Feed Stuff Inc., San Francisco, Calif.) *ad libitum,* and housed approximately five to a plastic cage.

All animals were killed at 12 months of age and their reproductive tracts were fixed in Bouin's fluid. Serial parasagittal paraplast (Sherwood Medical, St. Louis, Mo.) sections, 7-μm thick, were stained with Harris' hematoxylin and eosin. At the time of killing, half of the concretion-implanted mice were found to have lost their concretions.

Experiment II. Newborn female BALB/cCrgl mice received three daily subcutaneous injections of 5 μg E_2 in 0.02 ml sesame oil or the oil vehicle alone, beginning within the first 18 hr after birth. Litter size was adjusted to around five or six pups per mother and litters were randomly assigned to treatment groups. All mice were weaned at 30 days and ovariectomized at 60 days of age.

Directly after castration, each treatment group was divided into three subgroups: an unimplanted control group, a vaginal concretion-implanted group, and a silica-implanted group. Vaginal concretions were collected from neonatally treated females in previous experiments and stored in 70% ethanol. They ranged in dry weight from 10 to 15 mg (mean = 12 mg). Silica granules of approximately equivalent weight (10–15 mg) were washed for 1 hr with 1 *N* HCl followed by 1 hr in 1 *N* NaOH, dried, and stored in 70% ethanol before implantation. All implanted animals were examined every 2 weeks for 2 months and then weekly for 8 months with a blunt forceps to monitor for the presence of implants. If no implant was found, a replacement implant was inserted. Animals were housed four to six to a plastic cage covered with a filter cap (Isocap, Lab Products, Rochelle Park, N.J.) and fed water and standard laboratory chow (Purina Rat Chow) *ad libitum* in a temperature-controlled (72°F) animal facility with an artificially illuminated 12-hr day.

At 12 months of age all animals were killed, the presence of an implant was confirmed, and the reproductive tract was fixed in Bouin's fluid. As in Experiment I, 7-μm-thick serial parasagittal paraplast sections of the reproductive tract were stained with hematoxylin and eosin. The data were statistically analyzed using the Wilcoxon rank sum test of independent proportions or the χ^2 test.

Results. Three abnormal cervicovaginal responses were recognized in this study: persistent vaginal stratification/cornification (PVS), prominent squamocolumnar junction (SCJ), and epithelial pegs, downgrowths, or lesions (dysplasias) (Table I). PVS was characterized by stratified and often cornified squamous epithelium found lining the distal two-thirds of the vagina, frequently extending cranially to the endocervix, in the neonatally treated ovariectomized females (Fig. 2). Neonatal estrogen treatment alone resulted in PVS in about half of all mice in both series (Table I). The implantation of either vaginal concretion or silica tended to increase its incidence slightly but not significantly (Table I). In Experiment II, control oil-treated females without subsequent implantation had typical castrate vaginal linings with two exceptions: one female with a prolapsed reproductive tract and one female with an atypical response (not included in table). One female control in each of the implant groups (silica and concretion) displayed PVS (Table I). Implantation with either concretion or silica did not significantly increase PVS in control groups. However, neonatal estrogen regardless of implantation significantly increased the incidence of PVS over control groups (χ^2, $P < 0.005$). All animals not exhibiting PVS had mucified vaginae with one to three epithelial layers (Fig. 1).

The squamocolumnar junction was defined as a distinct boundary between the stratified squamous epithelium of the more distal vagina and the columnar cells underlain by basal cells of the proximal vagina (Fig. 3, Table I). The endocervical epithelium was sometimes reduced to one to two layers of cuboidal cells. Neonatal E_2 treatment without subsequent implant resulted in different SCJ incidences in Experiment I and Experiment II (Table I). Experiment II showed an overall increased incidence compared with Experiment I, but in neither series were the neonatally E_2-treated implant-bearing mice dif-

TABLE I. EFFECT OF VAGINAL IMPLANT ON CERVICOVAGINAL EPITHELIUM OF 12-MONTH-OLD OVARIECTOMIZED MICE

Neonatal treatment[a]	Post-ovariectomy implant[b]	No. of mice developing: Persistent vaginal stratification (%)	Abnormal squamocolumnar junction (%)	Abnormal cervicovaginal epithelium[c] (%)
Experiment I				
5 μg E_2	None	10/20 (50)	7/20 (35)	0/20 (0)
5 μg E_2	Concretion	15/19 (79)	8/19 (42)	7/19 (37)[d]
Experiment II				
5 μg E_2	None	10/15 (67)	13/15 (87)	1/15 (7)
5 μg E_2	Concretion	18/21 (86)	15/21 (70)	7/21 (33)[e]
5 μg E_2	Silica	9/13 (69)	10/13 (77)	2/13 (15)
5 μg E_2	Combined	27/34 (79)[f]	25/34 (74)[f]	9/34 (27)[f]
Control	None	0/15 (0)	0/15 (0)	0/15 (0)
Control	Concretion	1/16 (6)	4/16 (25)[g]	0/16 (0)
Control	Silica	1/17 (6)	3/17 (18)	0/17 (0)
Control	Combined	2/33 (6)	7/33 (21)	0/33 (0)

[a] Newborn female BALB/cCrgl or BALB/cfC3HCrgl mice were given three subcutaneous injections of 5 μg E_2 or sesame oil (control).

[b] All animals ovariectomized at 60 days unimplanted (none) or implanted with concretions or silica. Data were combined where no significant difference was found between concretion or silica implantation groups combined.

[c] Cervicovaginal pegs, downgrowths, and lesions.

[d] E_2 + concretion > E_2, Wilcoxon rank sum test: $P < 0.005$.

[e] E_2 + concretion > E_2, Wilcoxon rank sum test: $P < 0.03$.

[f] E_2 combined > control combined, χ^2: $P < 0.001$.

[g] control + concretion > control none, χ^2: $P < 0.025$.

ferent from the unimplanted E_2-treated mice. Control females without implant did not show SCJ; however, the controls receiving concretion implants (Table I) showed a low incidence, significantly different from the unimplanted controls (χ^2, $P < 0.025$).

Epithelial abnormalities (pegs, downgrowths, and lesions consisting of irregular projections of epithelium extending into the stroma with occasional loss of basement membrane) were limited to the neonatally E_2-treated groups (Fig. 4). The presence of an implanted concretion in both series increased the occurrence of abnormal lesions significantly: in Experiment I, $P < 0.005$; in Experiment II, $P < 0.03$. The incidence after silica implantation was not significantly increased.

Discussion. The neonatal estrogen treatment used herein, although low, was sufficient to elicit two significant ovary-independent responses in some adult mice: persistent vaginal stratification/cornification and an altered squamocolumnar junction. This dose may be regarded as near-threshold inasmuch as many estrogen-treated animals showed only a castrate-appearing vaginal lining. The abnormal responses were limited to some neonatally estrogen-treated or implanted animals; neither of these abnormalities was seen in unimplanted ovariectomized control mice. Implantation of either concretions or silica increased the incidence of PVS and SCJ; however, the increase was significant only in the control oil-treated group. The squamocolumnar junction probably represents the margin between the mullerian duct-derived proximal vagina and the urogenital sinus-derived distal vagina. It becomes abnormally prominent as a result of neonatal exposure to low estrogen dosage. Thus, these responses, although promoted by concretion implantation, are the consequences of either a direct effect of neonatal estrogen on the selection/proliferation of an abnormal vaginal epithelial cell population or an indirect effect mediated by an altered hormonal milieu in the adult.

The association between vaginal concretions and the occurrence of PVS and cervicovaginal lesions has been discussed previ-

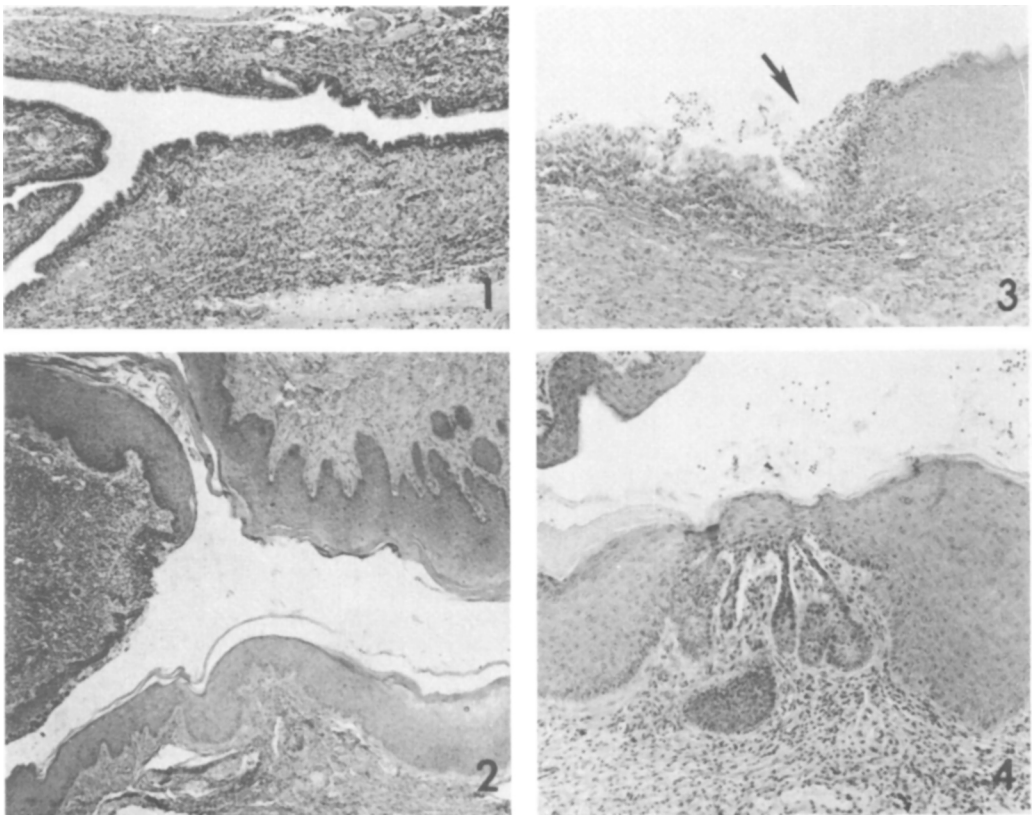

FIG. 1. Typical castrate vagina of a 12-month-old ovariectomized mouse given neonatal estrogen; cervix on left. H & E, ×40

FIG. 2. Stratification and cornification in the vagina of a 12-month-old ovariectomized mouse given neonatal estrogen and subsequently implanted with a concretion; cervix on left. H & E, ×40

FIG. 3. Distinct squamocolumnar junction (arrow) located in the vagina of a 12-month-old ovariectomized mouse given neonatal estrogen. H & E, ×80

FIG. 4. Epithelial hyperplasia and downgrowths in the vagina of a 12-month-old ovariectomized mouse given neonatal estrogen and subsequently implanted with a concretion. H & E, ×80

ously (5–7), since the description of vaginal crystals and concretions following neonatal estrogen treatment more than 20 years ago in this journal (4). Antenatal or neonatal sex hormone exposure is necessary for the formation of both vaginal concretions and epithelial abnormalities (1, 2, 5–10). The incidence of concretions diminishes after ovariectomy of neonatally hormone-treated mice (5, 6, 8), suggesting a relationship between the hormonal status of the animal and the genesis or continued deposition of concretions.

In the present study, low neonatal estrogen alone was able to induce abnormal epithelial pegs and downgrowths in only one animal (Experiments I and II combined: 1/35 or 3%). Implantation with concretion significantly increased this incidence to 30%. A nonsignificant rise in epithelial abnormalities was noted after implantation with silica. Thus a partial contribution of nonspecific physical irritation to the formation of cervicovaginal downgrowths and lesions remains possible.

The chemical composition of collected vaginal concretions reveals that their major components are magnesium, phosphorus, calcium, potassium, and organic matter (1, 2, 4). There is still no definitive explanation for the formation of concretions. Organic and inorganic contributions from the uterus and/or urethra or an alteration in calcium

metabolism combined with the appropriate vaginal environment (alkaline pH, bacteria, a matrix of accumulated desquamated cells) may be involved (1, 2, 4). A clearcut association between hypospadias, which might allow urine to enter the vaginal lumen, and the occurrence of concretions is lacking (1, 2).

A role of concretions in the promotion of abnormal epithelial development has been suggested previously (11–13); the data presented herein substantiate this possibility and raise the issue of the increased sensitivity of the cervicovaginal area exposed perinatally to estrogen to subsequent physicochemical stimuli.

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1. Warner MR, Warner RL, Clinton CW. Reproductive tract calculi, their induction, age incidence, composition, and biological effects in BALB/cCrgl mice injected as newborns with estradiol-17β. Biol Reprod **20**:310–322, 1979.
2. Lamb JC IV, Newbold RR, McLachlan JA. Visualization by light and scanning electron microscopy of reproductive tract lesions in female mice treated transplacentally with diethylstilbestrol. Cancer Res **41**:4057–4062, 1981.
3. Ways SC. Immunological Aspects of Development of Genital Tract Lesions and Tumors in Neonatally Diethylstilbestrol-treated Female Mice. Ph.D. thesis, 1982.
4. Takasugi N, Bern HA. Crystals and concretions in the vaginae of persistent-estrous mice. Proc Soc Exp Biol Med **109**:622–624, 1962.
5. Jones LA, Bern HA. Long-term effects of neonatal treatment with progesterone, alone and in combination with estrogen, on the mammary gland and reproductive tract of female BALB/cfC3H mice. Cancer Res **37**:67–75, 1977.
6. Takasugi N, Bern HA. Tissue changes in mice with persistent vaginal cornification induced by early postnatal treatment with estrogen. J Natl Cancer Inst **33**:855–865, 1964.
7. Wong LM, Bern HA, Jones LA, Mills KT. Effect of later treatment with estrogen on reproductive tract lesions in neonatally estrogenized female mice. Cancer Lett **17**:115–123, 1982.
8. Jones LA, Bern HA. Cervicovaginal and mammary gland abnormalities in BALB/cCrgl mice treated neonatally with progesterone and estrogen, alone or in combination. Cancer Res **39**:2560–2567, 1979.
9. Kimura T, Nandi S. Nature of induced persistent vaginal cornification in mice. IV. Changes in the vaginal epithelium of old mice treated neonatally with estradiol or testosterone. J Natl Cancer Inst **39**:75–93, 1967.
10. McLachlan JA, Newbold RR, Bullock BC. Long-term effects on the female mouse genital tract associated with prenatal exposure to diethylstilbestrol. Cancer Res **40**:3988–3999, 1980.
11. Bern HA, Jones LA, Mills KT, Kohrman A, Mori T. The use of the neonatal mouse in studies of the longterm effects of early exposure to hormones and other agents. J Toxicol Environ Health **1**(Suppl.):103–116, 1976.
12. Takasugi N. Effect of estrogen on vaginal epithelium of ovariectomized adult mice receiving early postnatal injections of estrogen. J Fac Sci Univ Tokyo Sect 4, **10**:403–408, 1964.
13. Bern HA, Talamantes FJ Jr. Neonatal mouse models and their relation to disease in the human female. In: Herbst AL, Bern HA, eds. Developmental Effects of Diethylstilbestrol (DES) in Pregnancy. New York, Thieme-Stratton, Chap 10:p129, 1981.

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