Long-Term Reproductive Tract Alterations in Female Mice Treated Neonatally with Coumestrol (43835)

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Abstract. **The neonatal mouse has proven to be an excellent model to ascertain the deleterious effects of estrogenic compounds on the developing reproductive tract. This system has been used to determine the morphological consequences after neonatal exposure to coumestrol (a plant estrogen).**

The role of estrogens in carcinogenesis (as a cocarcinogen or a tumor promotor) is unresolved at this time. Many studies suggest that estrogens are capable of functioning in this manner and recent evidence in cellular and molecular oncology revealed that estrogens act by genetic and epigenetic mechanisms in cancer cells. This review discusses the long-term morphological alterations in the reproductive tract of mice exposed neonatally to coumestrol. [P.S.E.B.M. 1995, Vol 208]

The presence in food of naturally occurring chemical agents possessing carcinogenic and mutagenic properties has attracted the attention of the environmentalist, the nutritionist, the physician, and the consumer. Agents such as nitrosamine, aflatoxin, polycyclic aromatic hydrocarbons, and estrogens can all be shown to be cancer-promoting under certain conditions (1). The existence of estrogenic activity in plant extracts was first recognized in the late 1920s (2). Dietary estrogen consumption in humans has become a matter of considerable concern.

During the early 1970s, diethylstilbestrol (DES) was commonly used in the United States as a growth stimulant in meat production (3). The finding of abnormal reproductive changes both in human females resulting from possible exposure neonatally to DES (4, 5) and in mice exposed neonatally to DES or 17β-estradiol (6, 7) prompted a ban on the use of DES in meat production by the Food and Drug Administration (FDA) (38 Fed. Reg. 10485, April 27, 1973) (8). As a result of these findings, the National Cancer Institute issued a press release warning pregnant women against the hazards of eating beef liver because of possible estrogen contamination.

It was later established that the concentration of phytoestrogen in food is high in comparison with the amounts of DES used in meat production. Currently, the dietary estrogen to which humans are exposed is more likely to be naturally occurring plant estrogens or their metabolites in food rather than synthetic estrogens such as DES (3).

The intrauterine exposure of human females to synthetic estrogen (principally DES) from the 1940s through the 1960s has led to the recognition of an abnormal reproductive tract syndrome which appears in adolescence and later in life (9). DES-exposed daughters may show uterine changes (e.g., T-shaped uterus, hooded cervix, etc.), interference with normal conception and pregnancy, vaginal adenosis and cervico ectropion, and a low but significant incidence of vaginal clear cell adenocarcinoma (9).

The neonatal mouse has proven to be a valuable model system for the study of estrogen- and DES-induced long-term changes (10, 11, 12, 13, 14, 15). The long-term reproductive tract alterations after neonatal exposure to estrogenic hormones in female mice include ovary-dependent and -independent persistent

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vaginal cornification, hyperplastic lesions, and cervicovaginal cancer (9, 16, 17, 18, 19). Additional abnormalities such as adenosis (heterotopic columnar epithelium) in the cervicovaginal region have also been described (9, 19). DES administered to mice transplacentally has comparable effects (15, 20, 21). Analysis of the effects of perinatal exposure of mice to hormones may elucidate the mechanisms by which intrauterine exposure to sex hormones induce pathological effects in humans (9, 12, 13, 14, 15, 19).

Phytoestrogens occur in a variety of plants. These estrogens may be ingested directly or as constituents of the tissues of animals that ingest the plant sources (22). Coumestrol was first isolated from landino clover (23), and has been detected in a variety of different plants, including alfalfa (24). Coumestrol causes uterine enlargement in a mouse biological assay (25), results in decreased ovulatory rate (26) when injected into castrate mice, and increases embryo degeneration (27) when fed to pregnant mice. Coumestrol binds with less affinity to estrogen receptors than 17β-estradiol does, but more avidly than other phytoestrogens (27). Medlock et al. (28) have shown that exposure of rats to 100 μg coumestrol on Day 1–5 resulted in an increase in luminal epithelial height on postnatal Day (PND) 5 and premature gland appearance on PND 8.

This review summarizes the reproductive tract alterations in female mice after neonatal treatment with coumestrol.

Precocious Vaginal Opening

The initial study demonstrated that complete vaginal opening (29) was advanced to PND 1–5 and PND 5–12 in the DES- and coumestrol-treated groups, respectively, as compared with controls (ranging from PND 23 to PND 42). Subsequent studies with various doses of coumestrol administered for the first 5 days of life revealed a dose-dependent effect on initial vaginal opening (30). All doses of coumestrol advanced vaginal opening before that of the controls (30).

In mice, vaginal opening is a normal maturational event which is accompanied by a short estrous cycle. Vaginal opening is the result of rising titers of estrogen produced by the ovary under the influence of a functioning hypothalamo-hypophysial axis (31). Precocious vaginal opening was demonstrated in mice exposed to the lowest daily neonatal treatment of coumestrol (10^{-3} μg) (31).

Persistent Vaginal Cornification

Neonatal estrogenization has manifested itself in a number of reproductive tract alterations in rodents. The disruption of the estrous cycle by possible chemical neural lesion(s) in the brain that impair the hypothalamo-hypophysial-ovarian axis (9) rendering these animals anovulatory and exhibiting prolonged periods of cornification is one example of these changes. This syndrome appears to be permanent.

Persistent vaginal cornification (PVC) is a characteristic genital tract response to neonatal estrogenization. Animals with the condition are described as exhibiting either ovary-dependent or -independent PVC. In ovary-dependent PVC, low neonatal estrogen treatment appears to exert its effect on the developing hypothalamo-hypophysial-ovarian axis, resulting in the ovaries secreting continuous low levels of estrogen (9). In ovary-independent PVC, high neonatal estrogen dose appears to act directly on the vaginal epithelium promoting this epithelium to exhibit prolonged periods of cornification, and ovariectomy of these animals does not abolish the PVC of the epithelium (9).

Neonatally coumestrol-treated female mice elicited similar alterations in the estrous cycle. Female mice given 100 μg of coumestrol (PND 1–5) exhibited long periods of cornification at 60 and at 150 days of age (29). PVC occurred in the presence or absence of ovaries. It was demonstrated that this dose (100 μg of coumestrol) elicited ovary-independent PVC. Subsequent studies, established that doses as low as 10^{-2} μg of coumestrol resulted in ovary-independent PVC (30). However, the ovary-dependent dose was not as obvious. Doses $\geq$5 μg of coumestrol were shown to be statistically different from the controls, while dose $\leq$1 μg of coumestrol (excluding the 10^{-2}-μg group) were not (30).

In order to establish if PVC seen at 60 days of age would persist later in life, subsequent vaginal smears were examined at 20 to 22 months of age. These observations established that neonatal doses of 5, 50, and 100 μg coumestrol resulted in extended periods of cornification (30). Castrate-type smears were observed in ovariectomized untreated controls and in the majority of DMSO-treated controls. Mice receiving the higher doses ($\geq$25 μg) consistently had ovary-independent PVC at 20 and 22 months of age.

Morphological Alterations

Neonatal coumestrol treatment is effective in causing a number of morphological alterations in the female reproductive tract. These changes were seen as early as 40 days of age and were maintained throughout the animal's life. These structural changes become more enhanced in aged animals. The following synopses will describe the reproductive tract alterations that occurred after neonatal exposure to coumestrol.

Ovary. Ovarian changes observed in female mice exposed to various doses of coumestrol exhibited hemorrhagic and poly ovular follicles (30). Animals receiving higher doses ($\geq$25 μg), lacked corpora lutea (30). In aged mice (20 to 22 months of age) similar

morphological changes were observed, but many of the prominent corpora lutea exhibited by neonatally coumestrol-treated animals at PND 40 were greatly reduced as compared with combined controls (30). The notion that female mice are anovulatory after early estrogenization (9) may not hold true in the case of coumestrol exposure.

Uterus. Initially, it was established that neonatal coumestrol exposure of female C57BL/Crgl mice (terminated at 13 months of age) resulted in uterine squamous metaplasia and the replacement of the fibromuscular wall with collagen deposition (32). These alterations (after neonatal treatment with various doses of coumestrol) were more pronounced at 20–22 months of age. These structural changes were prevalent in the intact animals (doses $\geq$50 μg of coumestrol neonatally). Thus, neonatal doses of $\geq$50 μg coumestrol resulted in ovary-dependent uterine squamous metaplasia (30). Neonatal DES resulted in either ovary-dependent and -independent uterine squamous metaplasia, depending on dose (33). Ostrander et al. noted in 1985 (24) that in the absence of the ovary, uterine squamous metaplasia induced by high neonatal DES was abolished. The uterine squamous metaplasia seen at 20–22 months did not exhibit keratinization, in contrast to that seen in neonatally coumestrol-treated animals at 13 months of age (30, 32).

Cervicovaginal. Neonatal exposure to coumestrol resulted in cervicovaginal downgrowths and pegs, as well as cervicovaginal adenosis (30, 32). In humans, adenosis may be replaced by a rapidly proliferating stratified squamous epithelium (9). The fate of adenosis-like lesions in mice is uncertain. Adenosis was not seen at 13 months of age in mice treated neonatally with coumestrol (32). These alterations have been described in 10-month-old and younger mice exposed neonatally to estrogen (10). The occurrence of adenosis in animals at 20–22 months of age does not support the hypothesis that adenosis in mice is an age-related lesion that is replaced by squamous metaplasia.

Summary

Neonatal estrogenization in rodents is effective in causing numerous reproductive tract changes. These phenomena are well characterized for synthetic (i.e., DES) and biological estrogens (i.e., E_2) in the neonatal mouse model. The outcome of phytoestrogen treatment and the possible mechanism of action in the neonatal mouse model parallels the morphological consequences described in the classical estrogen experiments. However, there are a number of differences that can be extracted from various studies that may suggest some variation in the course of coumestrol action. The literature presents that the effects of coumestrol on the uterus are greatly enhanced by endogenous estrogen (via the ovary), as described in this review. The appearance of vaginal adenosis and uterine squamous metaplasia in intact, aged, neonatally coumestrol-treated animals was more prevalent than in the ovariectomized, aged, neonatally coumestrol-treated animals. To date, there is no study which shows that chronic exposure to coumestrol following neonatal coumestrol-treatment will ultimately give rise to an enhancement of these morphological alterations or frank tumors. The mechanism of action, as well as the metabolic processing, of coumestrol is not clear at this time. Yet, it is evident at the level of the ovaries that these changes parallel what is expected after neonatal exposure to estrogen, but the severity of these abnormalities (such as ovarian hemorrhagic follicles) is more frequent in neonatally coumestrol-treated mice. The neonatal mouse model has been an instrumental tool in assessing the risk of the reproductive tract alterations after early exposure to coumestrol. However, future research may address the following issues: (i) whether perinatal exposure to coumestrol affects embryonic development; (ii) whether coumestrol's interactions with estrogen receptor or the estrogen response element affect gene expression; and (iii) whether coumestrol stimulates or inhibits regulation of specific proteins and growth factors. These may be the underlying factors which will provide additional information on the mechanism of action of this phytoestrogen and how it relates to the classical estrogen (E_2 and DES) story.

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