Organizational and Activational Effects of Phytoestrogens on the Reproductive Tract of the Ewe (43837)

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Abstract. **Ewes exposed to phytoestrogens may display two forms of infertility, categorized as temporary or permanent. Temporary infertility results from actions of estrogen that are similar to the activational effects of estrogen in most species of mammals. The permanent infertility results from changes to the cervix which are analogous to the organizational effects of estrogen reported in other species treated during organogenesis. However, in the ewe these effects may be produced after organogenesis by prolonged treatment during adult life. It has recently become apparent that the level of nutrition and metabolic hormones influence the degree of uterus-like histological change in the cervix produced by prolonged treatment with estrogen. It is hypothesized that, under some nutritional conditions, the hormonal milieu in adult ewes may simulate hormonal patterns that are normally experienced by fetal lambs *in utero*, thereby allowing the cervix of the adult ewe to give an organizational response to estrogen.** [P.S.E.B.M. 1995, Vol 208]

Infertility in female sheep grazed on estrogenic subterranean clover (*Trifolium subterraneum L.*) was described almost 50 years ago (1), as part of the condition known as "clover disease." The fertility of male sheep was rarely affected. It soon became apparent that there were two forms of infertility: a temporary form that resolved when sheep were returned to nonestrogenic pasture and a permanent form that became progressively worse with continued exposure. The biological basis for these two different forms of infertility has been determined over the past 15 years. Adult female sheep can show either activational or organizational responses to estrogen, and these have different effects on fertility. This review summarizes knowledge of these reponses, and describes briefly the epidemiology of infertility.

Both coumestan and isoflavone compounds can be estrogenic in sheep. Coumestans such as coumestrol occur in alfalfa or annual medics, particularly in plants suffering from a foliar disease. Isoflavone compounds, including genistein, biochanin A and formononetin, occur in subterranean clover or red clover (*Trifolium pratense*). Genistein and biochanin A are usually broken down by microbial activity in the rumen of sheep, while formononetin is converted to the isoflavan equol, which is rapidly absorbed and is responsible for most of the estrogenic activity in ruminants. Subterranean clover is an annual plant that contains estrogenic isoflavones only while it is green and growing. The concentrations of isoflavones depend primarily on the genotype, and are relatively independent of disease status of the plant.

Activational Effects of Phytoestrogens

Functional Change. Fertility is reduced in sheep that are mated on estrogenic pasture, although these effects normally resolve within 3 to 5 weeks after return to nonestrogenic pasture. Most of the decrease in fertility arises from decreased twinning rate (2), probably caused by direct effects of phytoestrogen on the ovarian follicles. Follicular function may be compromized to the extent that some ewes fail to ovulate at all. Embryo mortality may be slightly increased due to abnormalities of ovum transport and uterine function (3). Effects on the central nervous system are relatively slight. There are no substantiated reports of estrus behaviour produced by phytoestrogens, and neither isoflavones nor coumestans have much effect on

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plasma concentrations of luteinizing hormone (LH) (4).

Morphological Change. Coumestans and isoflavones produce morphological changes typical of stimulation with steroidal estrogens such as 17β-estradiol in all the estrogen target organs (5, 6). Mammary gland enlargement and hypertrophy of the teats is very common. Milk or clear yellow fluid is frequently observed in the mammary gland. In breeds of sheep other than the Merino, reddening of the vaginal mucous membrane and swelling of the vulva may be observed (6), associated with an increase in thickness and keratinization of the vaginal epithelium. The cervix also enlarges, and mucus cells accumulate in the surface epithelium. Uterine weight increases linearly with increasing dose of phytoestrogen, and the cellular hypertrophy of the mucosa and muscular layer may be accompanied by subacute endometritis. There is histological atresia of the ovarian follicles, accompanied by hypertrophy of the granulosa cells.

Cellular hypertrophy has also been observed in a number of other tissues, including the pituitary, adrenal, and thyroid gland. Microgliosis and neuronal hyperchromicity was observed in the hypothalamus of ewes after several weeks exposure to estrogenic pasture (5). Similar changes can be produced in rats after treatment with a large dose of 17β-estradiol (7).

Organizational Effects

Prolonged exposure to estrogenic pasture may cause a permanent decrease in fertility in the ewe. This has been studied experimentally using sheep exposed to estrogenic subterranean clover pastures for three or more years, the pasture being green and estrogenic for periods of 5 to 7 months in each year. After exposure, animals were removed to nonestrogenic pasture for 6 to 18 months before study to ensure that all the activational change wrought up by estrogen had regressed. Several groups of animals prepared in this way were used in the studies described below.

Functional Changes. The infertility results primarily from a failure of ewes to conceive, due to impaired transport of spermatozoa through the cervix (8). Other stages of the reproductive process such as twinning rate, embryo mortality, the proportion of ewes that come on heat, or the duration of the breeding season are only slightly affected.

The cervix normally acts as a reservoir for spermatozoa during the period between insemination and ovulation in the ewe. Cervical mucus that is penetrable to sperm and has appropriate viscoelasticity is central to this process, allowing spermatozoa to penetrate the cervix and guiding their migration towards the mucosa. The spinnbarkeit, which is the length to which a strand of mucus can be drawn out before it breaks, is a measure of the viscoelasticity of the mucus. In infertile ewes, cervical mucus is watery and easily penetrable, but the spinnbarkeit of cervical mucus is reduced (9). The low viscoelasticity results in the loss of spermatozoa from the cervix, presumably through the vagina, so fewer sperm reach the oviduct and the chance of conception is reduced.

The low spinnbarkeit results from an abnormal response of the cervix to estrogen. In control ovariectomized ewes, treatment with estrogen causes an increase in water content of cervical mucus while continued treatment over a 3-day period is required to increase the spinnbarkeit (10). Infertile ovariectomized ewes do not show an increase in spinnbarkeit with continued exposure to estrogen (9), but they do produce more mucus than controls in the absence of estrogen. Thus, mucus secretion from the cervix of infertile ewes has a reduced ability to respond to estrogen but also becomes less dependent on the presence of estrogen.

Careful studies can detect abnormal responses to estrogen in other target tissues. Sexual behaviour in ovariectomized infertile ewes treated with androgen or estrogen is slightly masculinized and defeminized compared with ovariectomized controls (11). The LH surge response to estrogen treatment is reduced, and minor abnormalities are observed in the tonic secretion of LH. In the ewe, though estrogen normally suppresses the secretion of LH during seasonal anestrus, it does not do so during the breeding season. Tonic concentrations of LH are greater in infertile ewes during anestrus, but are somewhat lower than controls during the breeding season (12). The infertile ewes appear to have an impaired ability to distinguish the breeding season; however, the abnormality may simply reflect a reduced responsiveness to estrogen.

Morphological Changes. The loss of function in the cervix is accompanied by gross morphological change. Exposure to estrogen after puberty causes the cervix to become shorter and broader, which is accompanied by extensive histological change resulting in a complete alteration in cervical architecture. The cervical folds, which are present well before puberty, become blunter and fuse together (Fig. 1). Coiled tubular glands which stain histochemically like uterine glands, particularly in their distal portions, develop underneath the folds (13). Some glands may become cystic. The area of lamina propria tissue in the endocervix increases, and stromal cells accumulate under the epithelium. The surface epithelium also changes, becoming predominantly plain columnar cells, with fewer mucus cells, ciliated cells, or stratified squamous cells (14). All of these changes lead to an appearance that is very similar to that of the uterus (14). Once the change occurs, it does not resolve.

The appearance of the uterus is less affected. The uterine horns may become more coiled and sharply

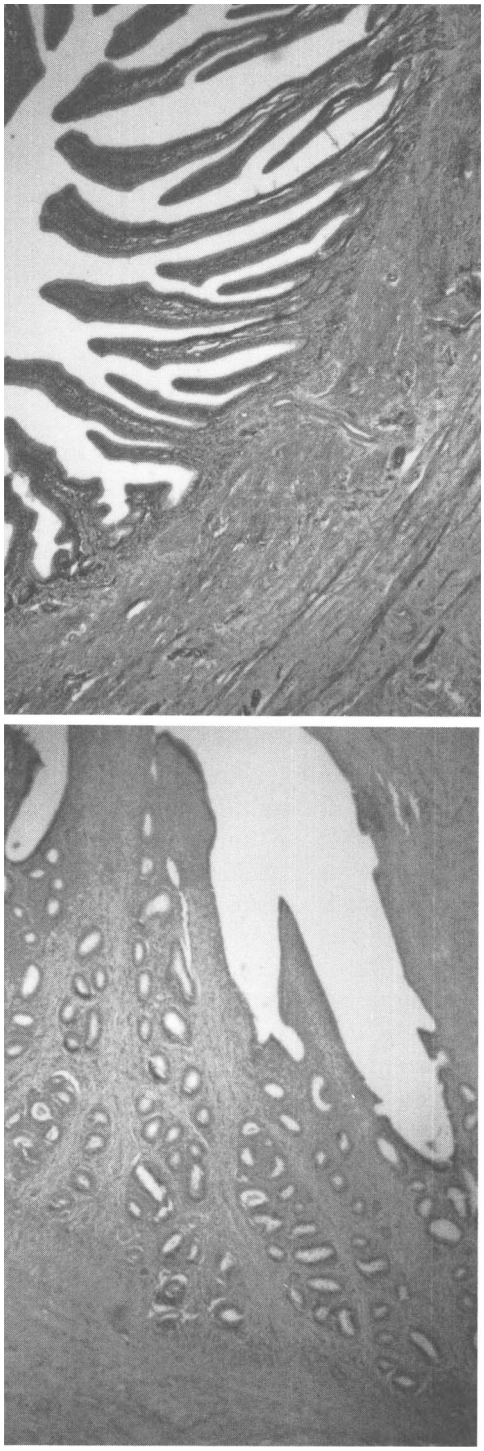

Figure 1. Cross-section through the mid-cervix of a control ewe (a) and a ewe after prolonged exposure to estrogen (b). The cervical folds become thicker and fuse together leaving tubular glands.

delineated (15), but histological changes in the uterus are relatively slight. Uterine glands may become cystic, and occasionally groups of glands grow outwards and penetrate the myometrium, causing adenomyosis. Mild endometritis is very common, probably because a loss of cervical integrity permits bacterial invasion of the uterus from the vagina. The stratified squamous epithelium of the vagina has fewer layers and is less keratinized. The external genitalia may undergo masculinization, with fusion of the lips of the vulva at the lower commissure and hypertrophy of the clitoris in about 2% of ewes (16).

Identical morphological changes can be produced in ewes after exposure to 17β-estradiol for periods of 80 to 180 days, followed by a period of 60 or more days after estrogen withdrawal before tissues were collected (15). Interestingly, the number of cervical glands increased while the animals were exposed to estrogen, but most of the fusion of the cervical folds and accompanying change of the architecture occurred after estrogen was withdrawn (17).

Organizational Effects of Estrogen in Other Species. The permanent changes described above are analogous in many ways to the changes in other species resulting from exposure to estrogen during organogenesis. Estrogen treatment of mice during neonatal life can cause a loss of cervical structure and the appearance of uterine-like epithelial cells and glandular structures in the cervical fornix and upper vagina (18). A similar phenomenon has been observed in women exposed to diethylstilbestrol during fetal life (19). Detailed studies in rats and mice treated with estrogen neonatally have shown other abnormalities including hypoplasia in the adult uterus (20) and oviduct. It appears that high concentrations of estrogen can prevent normal differentiation of the female reproductive tract, with morphological effects on the cervix very similar to changes seen in permanently infertile ewes.

Less work has been carried out on the functional abnormalities in the genital tract that occur after treatment with estrogen during organogenesis. Mori (21) examined the function of vaginal and uterine epithelium in adult ovariectomized mice that had been treated neonatally with estrogen. He found that the effect of estrogen in increasing mitotic rate of epithelial cells was impaired when the mice were retreated as adults, but in the absence of estrogen the mitotic rate in ovariectomized adults was greater in neonatally treated animals than in controls. This reduced estrogen responsiveness, accompanied by an increased constitutive function, is similar to the change in cervical mucus secretion in permanently infertile ewes described above.

Control of the Organizational Change. In other species, organizational effects of estrogen have been reported only during the period of organogenesis. The mechanism that permits the ewe to mount an organizational response to estrogen during adult life is unknown. The response of sheep to estrogen is relatively complex compared with many species, and clues to the mechanism may lie in some of these modulations.

For example, the cervical mucus response to estrogen rapidly becomes refractory to continued treatment with estrogen in sheep (10), but the significance of this is unknown. Furthermore, the amount of sexual behaviour and the amount of mucus produced by the cervix in response to estrogen are both greater during the breeding season than in anestrus. However, we have found that the degree of histological change in the cervix, as measured by number of glands, is independent of the time of the breeding season.

Recently, we observed that the extent of uterine-like histological change in the cervix in ewes treated with 17β-estradiol for 80 days was affected by the level of nutrition the ewes were receiving. Furthermore, daily treatment with 3 international units of insulin ultralente interacted with nutrition, reducing the effects of estradiol in ewes that were fed below maintenance but exacerbating the effects of estrogen in ewes fed above maintenance (Fig. 2). The complexity of these effects indicates that another hormonal system such as insulin-like growth factor (IGF) may be involved.

Such effects of nutrition on the organizational response to estrogen have not been recorded before, but they are in accord with field evidence. Permanent infertility is particularly severe in areas with a mediterranean climate, where sheep undergo large annual changes in liveweight. Within these areas, the condition appears worst in sheep that are exposed to phytoestrogens during the autumn, when their liveweight is lowest, even though the greatest exposure to estrogen occurs during spring when clover growth is at its maximum. It could be speculated that under certain nutritional conditions the IGF system in the cervix of the adult ewe may achieve conditions similar to those that occur normally during fetal life, permitting estrogen to cause organizational rather than activational change.

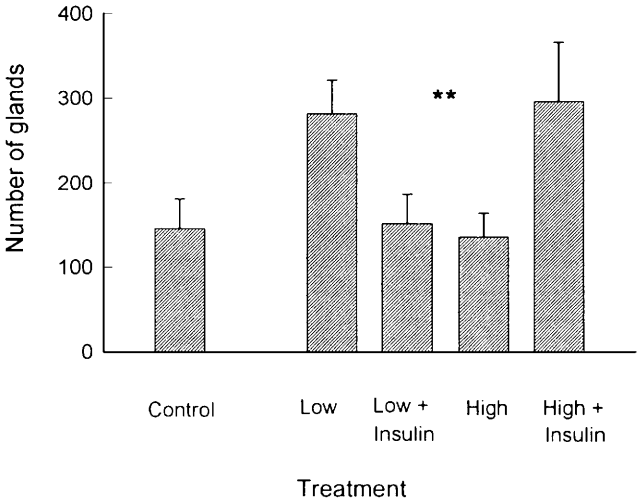

Figure 2. Number of glandular cross-sections in histological sections of cervix of ewes either not treated with estrogen (control) or treated with 17β-estradiol and fed below (low) or above (high) maintenance and injected daily with saline or insulin (Insulin). **The interaction between feeding level and insulin was statistically significant (ANOVA; $P < 0.01$).

Significance and Epidemiology

In southern Australia, particularly the south western corner, rainfall is restricted to winter and spring. The dry summers make it impossible to grow perennial pastures, so that animal production relies on annual pasture, of which subterranean clover is the dominant legume. Some cultivars of subterranean clover containing high concentrations of formononetin are very well adapted and are still widespread, so in Australia several million ewes are still exposed to estrogenic pasture.

In this environment, sheep are mated in summer on dry nonestrogenic pasture so that lambs are born 5 months later onto green pasture. As the clover is not estrogenic when it is dry, temporary infertility is less important economically than the permanent infertility. The economic importance of permanent infertility to sheep farming in this area has been examined in surveys which related the decrease in fertilization rate (22), the reduction in the spinnbarkeit of cervical mucus (23), or the severity of uterine-like changes in cervical histology (24) to the reproductive performance of the flocks. All of these studies indicate that the condition is widespread, causing at least 500,000 ewes to fail to lamb each year in Australia.

The economic importance of temporary infertility has proven more difficult to assess. The infertility is manifested largely as a reduced twinning rate, but in sheep the twinning rate is very dependent on the nutritional regime, and so is not easy to predict in individual flocks. It is probable that the condition is underreported in many areas of the world where there are transient increases in pasture estrogenicity.

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