A changed responsiveness to oestrogen in ewes with clover disease

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Summary. When clover-infertile ewes are subsequently exposed to non-oestrogenic pasture, they have a reduced fertilization rate, due to an inability to store spermatozoa in the cervix, and the cervical mucus has a reduced spinnbarkeit, caused by a slower response to oestrogenic stimulation. Vaginal cell keratinization and oestrous behaviour occurred more slowly after treatment of affected ewes with oestrogen. Other changes in affected ewes suggest that phyto-oestrogens have permanent mild differentiating effects on adults. Sexual behaviour is masculinized, the cervix takes on a uterine-like appearance and the genital tract becomes permanently oestrogenized. The manner in which these changes relate to the altered responsiveness to oestrogen remains to be clarified.

Introduction

Severe abnormalities of the reproductive system may occur in sheep exposed to subterranean (*Trifolium subterraneum* L.) or red (*Trifolium pratense* L.) clover pastures containing high concentrations of phyto-oestrogens (Bennetts, Underwood & Shier, 1946). These problems constitute the syndrome known as clover disease (Shier & Rossiter, 1949) and include infertility, dystokia and uterine prolapse. Wethers may show enlargement of the bulbo-urethral glands, but rams appear to be unaffected (Bennetts *et al.*, 1946). In recent years, prolapse and dystokia have become uncommon in Western Australia and the term clover disease is now used to describe the permanent infertility in ewes after grazing on pastures of this type.

There are several phyto-oestrogenic compounds. Coumestans are the main phyto-oestrogens in the medicks like lucerne (*Medicago sativa*). In clovers the isoflavones are more important, the main compounds being genistein, biochanin A and formononetin (Beck, 1964). The oestrogenic activity of clover in ruminants depends mainly on the content of formononetin (Millington, Francis & McKeown, 1964), a substance which itself has little oestrogenicity (Shutt & Cox, 1972). However, several lines of work, reviewed by Cox & Braden (1974), have shown that formononetin is metabolized in the rumen to the more active isoflavan, equol, while genistein and biochanin A are metabolized to inactive derivatives.

The sheep are not only infertile while grazing oestrogenic pasture but remain so even after the ewes have been removed from the pasture for several years (Schinckel, 1948). Oestrus and mating are close to normal in clover-affected ewes (Smith, 1971; Lightfoot *et al.*, 1974), but sperm transport is impaired and fewer ova are fertilized (Bennetts *et al.*, 1946; Turnbull, Braden & George, 1966; Lightfoot, Croker & Neil, 1967). There may also be a greater mortality of embryos in ewes with clover disease (Turnbull *et al.*, 1966), though not all authors agree (Kaltenbach & Davies, 1970).

Cervical failure

Most studies on permanent infertility have been carried out on ewes which have grazed oestrogenic pasture for at least three seasons, and then non-oestrogenic pastures for a minimum of 6 months.

Lightfoot *et al.* (1967) showed that poor sperm transport in clover-affected ewes is due to a failure to form a normal reservoir of spermatozoa in the cervix. Spermatozoa are stored in the cervix after mating (Mattner, 1963), and ascend to the oviducts over a period of time. The cervix of clover-affected ewes does not store spermatozoa efficiently, and 24 h after insemination such ewes have less than 5% of the expected number of spermatozoa (Lightfoot *et al.*, 1967). This impaired sperm storage is associated with a change in the cervical mucus. Smith (1971) and Lightfoot *et al.* (1974) found that cotton swabs placed in the vagina took up a greater amount of cervical mucus in clover-affected ewes, than in controls at oestrus, although Smith (1971) found that the amount of dry matter taken up in the swabs was not altered. A more direct study on mucus aspirated by pipette from the cervical os (Adams, 1976a) showed that mucus from affected ewes did not have a higher proportion of water content, but had a lower spinnbarkeit. The total amount of mucus was not increased, but because it had a lower viscosity it could penetrate the cotton swabs more readily. Tampion & Gibbons (1962) showed that the structure of mucus orientates the spermatozoa for migration, and it is suggested that the network structure of cervical mucus (Chretien, Cohen & Psychoyos, 1974) is necessary for formation of the sperm reservoir. The reduced spinnbarkeit of mucus from ewes with clover disease reflects an absence of the normal molecular structure and this prevents the effective orientation for sperm migration (Text-fig. 1).
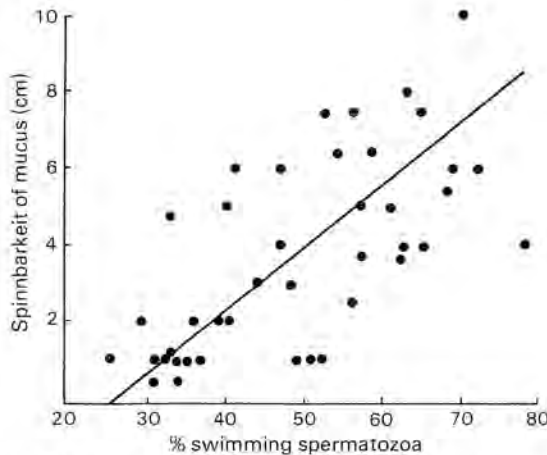
Text-fig. 1. Relationship between the spinnbarkeit of cervical mucus (y) and the proportion of spermatozoa guided to swim along a strand of the mucus *in vitro* (x); $y = 0 \cdot 106x - 1 \cdot 77$; $r = 0 \cdot 60$, $P < 0 \cdot 001$. The mucus was obtained from clover-affected and control ewes at oestrus, as described by Adams (1975).

The cervical folds are reduced in size and cervical glands increase in clover-affected ewes (Adams, 1976b). This change in architecture may also affect maintenance of the sperm reservoir. In addition, Lightfoot *et al.* (1967) found that fewer of the spermatozoa recovered from the cervix of affected ewes were motile. Adams (1976a) could not find any clear-cut toxic effect of mucus from affected ewes on spermatozoa *in vitro*, and it is possible that the loss of viability seen *in vivo* was due to a failure of the mucus to separate the spermatozoa from the flow of leukocytes and fluid from the uterus (Mattner, 1968).

Hormonal changes

Both oestrogen and progesterone influence cervical mucus production (Lindsay & Francis, 1969), and both hormones have been studied in clover-affected ewes.

Oestrogen

The ratio of RNA to DNA in the uterus is dependent on oestrogen (Little & Lambourne, 1976), but no differences have been found between affected and control ewes in the ratio of RNA to DNA in uterine biopsies taken at oestrus or dioestrus. The duration of oestrous behaviour was not altered in clover-affected ewes (Smith, 1971; Lightfoot *et al.*, 1974). The ovulation rate is slightly higher in clover-affected ewes (Adams, Oldham & Heydon, 1979), and because an increased ovulation rate is generally accompanied by a greater production of oestrogen (Wheeler, Baird, Land & Scaramuzzi, 1977) this may be reflected in a slight increase in the amount of oestrogen circulating in such ewes, although there are no differences in ovarian vein concentrations of oestrogens in affected and control ewes (Rodgers *et al.*, 1980).

Taken altogether, the evidence suggests there may be a slight increase in the amount of endogenous oestrogen in affected ewes, but it appears insufficient to cause the large changes seen in cervical mucus (Lindsay & Francis, 1969).

Progesterone

Smith (1975) found no difference between clover-affected and control ewes in peripheral plasma concentrations of progesterone throughout the oestrous cycle. The duration of the cycle was a little more variable in affected ewes (Adams, Lightfoot & Charlick, 1975). The variability probably results from an altered control of the corpus luteum by the uterus, as a result of *hydrops uteri* or endometritis (Adams, Hearnshaw & Oldham, 1981) which are seen only in severely affected animals and are probably incidental to the major cause of infertility. Obst & Seamark (1975) found reduced plasma progesterone at the end of the oestrous cycle in ewes grazing oestrogenic pasture, but it is more likely that this reflected the concurrent intake of phyto-oestrogens than the development of permanent infertility.

Luteinizing hormone (LH)

Findlay *et al.* (1973) found that ovariectomized ewes with clover disease did not give a positive feedback surge of LH in response to oestradiol-17β, although the pituitary response to synthetic gonadotrophin-releasing hormone was unimpaired. Attempts to repeat this work in intact ewes have been unsuccessful (Rodgers *et al.*, 1980; W. A. Chamley, N. R. Adams, R. P. Hooley & R. Carson, unpublished). Furthermore, the duration of the oestrous cycle is only slightly altered (Adams *et al.*, 1975), indicating that a surge of LH sufficient for ovulation does occur normally. Ewes with clover disease have more frequent episodic releases of LH, and higher baseline values of LH (Rodgers *et al.*, 1980). These findings may explain the higher ovulation rate in affected ewes (Adams *et al.*, 1979), but the sensitivity of the ovaries of these ewes to gonadotrophins has not been studied.

Abnormal responses to oestrogen

Considerable effort has been made to determine why clover-affected ewes produce abnormal mucus. Smith (1971) found that after ovariectomy and treatment with progesterone and a single dose of oestradiol benzoate, ewes produced mucus similar to that of controls. This result implied

that the cervical response to hormonal stimulation was normal, but other studies indicated that hormonal levels in affected ewes were also normal. The manner in which cervical mucus responds to treatment with oestrogen was therefore studied. Normal ovariectomized ewes were treated daily with 12·5, 40 or 100 µg oestradiol benzoate and the cervical mucus collected. The volume of mucus and the proportion of water it contained depended mainly on the dose of oestrogen used, but the spinnbarkeit depended primarily on the length of time the ewe had been exposed to oestrogen (Adams & Tang, 1979). These results suggested that the abnormal mucus seen in clover-affected ewes was not caused by a different level of circulating oestrogen, because in ewes with clover disease the volume of mucus and the water content were normal (Adams, 1976a).

Ovariectomized ewes with clover disease were therefore treated with oestrogen for a longer period of time. The spinnbarkeit of mucus was lower in the affected ewes at oestrus, but after ovariectomy followed by 11 days treatment with 10 mg progesterone daily and then a single dose of 25 µg oestradiol benzoate, this difference was less pronounced (Adams, 1979a). Repeated daily treatment with oestradiol benzoate increased the spinnbarkeit in the control ewes, but the increase was much less in those with clover disease, and a significant difference between groups was apparent after treatment for 3 days (Text-fig. 2). Thus the functional lesion appears to be not a different degree of hormonal stimulation, but a decrease in the perceived duration of stimulation. This conclusion is supported by other studies. The degree of keratinization of the vaginal epithelium did not increase as rapidly in affected ewes treated with oestradiol benzoate (Adams, 1979a). Furthermore, when 14 clover-affected and 14 control ovariectomized ewes were treated daily with 40 µg oestradiol benzoate, there was little difference between the groups in the total amount of oestrous behaviour, but the affected ewes were slower to exhibit oestrous behaviour ($P < 0.05$) and to become refractory to oestrogen (Text-fig. 3). This result differs from that of Scaramuzzi, Lindsay & Shelton (1972) who treated normal ewes repeatedly with oestradiol benzoate; the doses of oestrogen which hastened the onset of oestrus also delayed the onset of refractoriness, except at a very high dose. The behavioural change in clover-affected ewes therefore again suggests a slower rate of response to oestrogen or a failure to be 'primed' by a previous oestrogen treatment.
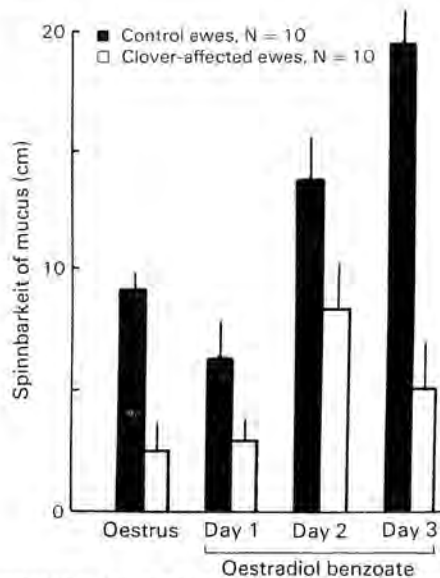
Text-fig. 2. Mean spinnbarkeit (±s.e.m.) of cervical mucus from 10 control and 10 clover-affected ewes at oestrus, and after ovariectomy and progesterone priming, during daily treatments with 25 µg oestradiol benzoate. Data from Adams (1979a).
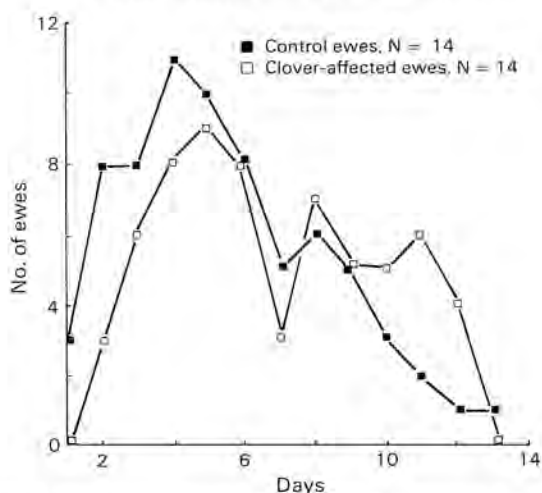
Text-fig. 3. Number of control and clover-affected ovariectomized ewes marked by rams during daily treatment with 40 µg oestradiol benzoate.

Preliminary results suggest that cervical refractoriness to oestrogen is not enhanced in affected ewes. The ovariectomized ewes studied in the experiment on oestrous behaviour were treated for 2 days with 40 µg oestradiol benzoate commencing 13 days after the end of the preceding experiment. Cervical mucus was collected on cotton swabs. The amount of mucus from control ewes declined significantly over the 2-day test period ($0 \cdot 61 \pm 0 \cdot 08$ compared with $0 \cdot 30 \pm 0 \cdot 05$ g; $P < 0 \cdot 05$) but was unchanged in clover-affected ewes ($1 \cdot 05 \pm 0 \cdot 19$ compared with $0 \cdot 87 \pm 0 \cdot 19$ g).

It seems unlikely that the altered responsiveness depends on a changed ability to metabolize oestrogen. Tang & Adams (1978a) found that clearance rates and hepatic conjugation of oestradiol-17β were normal in affected ewes. Furthermore, a changed metabolism of oestrogen might be expected to affect responses to the dose level of oestrogen as well as responses to the duration of exposure.

Developmental changes

The reason why ewes with clover disease respond more slowly to oestrogen is not yet clear. Other changes also occur, which seem best understood as reflecting a permanent differentiation caused by prolonged exposure to phyto-oestrogens even though they are brought about in ewes exposed after puberty.

Cervical histology

Cystic hyperplasia of the endometrial glands is observed in ewes with clover disease (Bennetts *et al.*, 1946), and the cervix may become more like the uterus (Adams, 1976b). The cervical folds fuse so that the crypts are lost, and the lamina propria is filled with cells similar to those seen in uterine stroma. Glands, normally absent from the cervix, are abundant and their histochemical reactions are similar to those of uterine glands (Heydon & Adams, 1977). The surface epithelium has more columnar but fewer goblet cells, and less stratified squamous epithelium (Lightfoot & Adams, 1979). This appearance of the cervix is the opposite of that seen when ewes are first treated with phyto-oestrogen (Adams, 1977). Similar changes have recently been produced in the cranial third of the cervix of 3 ewes treated for 12 weeks with three 3 cm

implants containing oestradiol-17β of the kind described by Karsch *et al.* (1973). The histological change is therefore not due to some aberrant effect of phyto-oestrogen but is a response to oestrogen itself.

The occurrence of uterine-like cells in the cervical fornix and anterior vagina has been described for mice treated with oestradiol-17β during the first 5 days of life (Forsberg, 1969). A similar change is reported for women whose mothers were treated with diethylstilboestrol during pregnancy (Herbst, Kurman & Scully, 1972). These findings show that oestrogen can cause the type of change seen in ewes with clover disease, although such changes occur only during organogenesis in other species.

Masculinization and defeminization of behavioural responses

The most specific type of differentiation caused by oestrogens or aromatizable androgens is a defeminization and masculinization of the sexual behavioural response to hormonal treatment later in life (Gorski, 1973). To see if this occurred in ewes with clover disease, 20 ovariectomized clover-affected ewes and 19 controls were injected daily for 30 days with 5 mg testosterone propionate in oil. The ewes were observed daily with 3 rams and their behaviour recorded. The clover-affected ewes showed a delay to first mount by the ram ($3 \cdot 14 \pm 0 \cdot 51$ compared with $1 \cdot 73 \pm 0 \cdot 15$ (s.e.m.) days, $P < 0 \cdot 05$). The number of days on which ewes were mounted was not significantly different (affected $7 \cdot 6 \pm 1 \cdot 8$ days; control $10 \cdot 3 \pm 2 \cdot 0$ days). Clover-affected ewes showed aggressive behaviour sooner ($15 \cdot 7 \pm 1 \cdot 6$ days) than did controls ($21 \cdot 8 \pm 2 \cdot 1$ days, $P < 0 \cdot 05$).

Male-like behaviour was measured by testing each ewe with 3 oestrous ewes in a small pen after 21 and 28 days of testosterone treatment. Clover-affected ewes showed more courting behaviour on Day 28 ($P < 0 \cdot 05$), while differences in Flehmen and in less hormone-dependent behaviour, such as ano-genital sniffing, were not significant.

Permanent oestrogenization of the genital tract

In mice treated neonatally with a large dose of oestrogen or an aromatizable androgen, the vaginal epithelium becomes permanently keratinized and this persists after ovariectomy (Terenius, Meyerson & Palis, 1969). There is a higher rate of cell turnover (Mori, 1975) and greater RNA, DNA and protein synthesis in the reproductive tract, even after ovariectomy (Kohrman & Greenberg, 1968). Vaginal flushings taken during dioestrus from ewes with clover disease indicated that the ewes appeared to be under oestrogenic stimulation (Tang & Adams, 1978b). Flushings from affected ewes had greater amounts of glycoprotein, and lower levels of enzymes. Further studies on ovariectomized ewes showed that clover-affected ewes have a thicker, more keratinized vaginal epithelium and increased protein and glycoprotein synthesis in the cervix (Tang & Adams, 1981).

Other changes

Affected ewes start to show oestrus at the normal time of year, but oestrous cycles are more erratic in the first half of the breeding season (Adams, 1979c). The amount of cervical mucus produced in response to oestrogen varies with the time of year but is similar in affected and control ovariectomized ewes (Adams, 1980).

Infertile ewes have permanent masculinization of the vulva, including growth of the clitoris and fusion of the ventral commissure (Adams, 1979b). These changes persist after removal from oestrogenic pasture, which again is consistent with a developmental type of change. Therefore, except for the failure to observe clearly defined differences of LH secretion in affected ewes, all of the changes brought about by oestrogen in the fetal or neonatal rodent have their counterpart in

ewes with clover disease, suggesting that the permanent infertility is a result of mild differentiation occurring in the adult.

The manner in which differentiation brings about a changed responsiveness to oestrogen is not yet clear. Both sexes have similar numbers of cytoplasmic oestrogen binding sites in the hypothalamus and pituitary gland, (Barley, Ginsburg, MacLusky, Morris & Thomas, 1977). The principal difference is that, after binding, translocated receptors remain in the nucleus for a shorter time in the male (Whalen & Massicci, 1975; Marrone & Feder, 1977). Because the duration of response is related to the duration of nuclear occupancy by the oestrogen–receptor complex (Bouton & Raynaud, 1979), the altered rate of responsiveness in affected ewes may result from changes in the ability of the nucleus to bind the oestrogen–receptor complex.

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