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Proportions of Nucleic Acids in the Uteri of Ewes with Clover Disease and the Effect of Oestrogen after Ovariectomy

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Abstract

In entire ewes, the ratio of RNA to DNA in the uterus was higher at oestrus than at dioestrus but did not differ between control and clover-affected ewes at either stage of the oestrous cycle.

After ovariectomy the ratio of RNA to DNA in the uteri decreased in both control and clover-affected ewes, but the decrease was greater in the control ewes ($P < 0{\cdot}01$). Subsequently when ewes received a single injection of oestradiol-17β, a similar rise in uterine RNA/DNA ratio occurred in the two groups. However, following injection of the ewes with oestradiol-17β for 3 days, the ratio of uterine RNA to DNA was higher in control than in clover-affected ewes ($P < 0{\cdot}05$). Thus, the uteri from clover-affected ewes undergo less regression after ovariectomy and yet show less response to repeated oestrogenic stimulation than do those of control ewes. This pattern of response is consistent with the hypothesis that the permanent changes caused by phyto-oestrogens in ewes are analogous to the differentiation which normally occurs during organogenesis.

Introduction

After prolonged grazing on oestrogenic clover pasture, ewes may exhibit permanent infertility (Schinkel 1948) as part of the syndrome called clover disease. The infertility appears mainly due to an impaired function of the cervix (Lightfoot *et al.* 1967) which produces abnormal mucus (Adams 1976).

In affected ewes on non-oestrogenic pasture, the cervix continues to act as though it is under oestrogenic stimulation during dioestrus (Tang and Adams 1978). Ovariectomized-affected ewes have an impaired responsiveness to repeated treatment with oestradiol benzoate which prevents the production of cervical mucus with a normal spinnbarkeit (Adams 1979) and the responsiveness of the hypothalamus to oestrogen is also slightly decreased (Adams 1978). Recently we reported that rates of protein and DNA synthesis were greater in the genital tracts of ovariectomized clover-affected ewes than in those of ovariectomized controls (Tang and Adams 1981). However, it is not known whether the uterus in clover-affected ewes responds normally to oestrogenic stimulation. Since the exact nature of the altered responsiveness to oestrogen in ewes with permanent clover infertility is not clear, the following studies were carried out to try to elucidate this problem. The ratio of RNA to DNA in the uterus is a sensitive indication of oestrogenic stimulation (Heald *et al.* 1970; Little and Lambourne 1976; Miller 1976) and was used to determine whether there was any abnormality in responsiveness to oestrogen in the uterus of clover-affected ewes.

Materials and Methods

Entire Ewes

Eight-year-old South Australian Merino ewes were drawn from the flock described by Rossiter and Marshall (1974). Nine had grazed the highly oestrogenic Dinninup cultivar of subterranean clover for 4 years, and only one had produced a lamb at their last lambing. They had not been exposed to oestrogenic pasture for 2 years before the present study. The 12 control ewes of the same age had grazed the very slightly oestrogenic Northam A cultivar of subterranean clover and had produced 12 lambs from their last mating.

The ewes were run on non-oestrogenic pasture with vasectomized rams fitted with a harness and crayon and were inspected daily at 1000 h. Ewes which had been marked by the ram were removed from the group and laparotomy was performed with the animals under sodium thiamylal anaesthesia. Large Graafian follicles were observed in the ovaries of seven clover-affected and eight control ewes and a recently ruptured follicle was present in at least one ovary in each of the remaining ewes. A complete segment of uterine horn about 0·5 cm long was excised from the anterior uterine horn contralateral to the ovary with the recently ruptured follicle or that with the largest follicle and this was frozen immediately in liquid nitrogen. The incision in the uterus was sutured.

Two months later, the ewes were again run with harnessed vasectomized rams and inspected daily. Six clover-affected ewes and eight controls were slaughtered 7 days after being marked by the ram and the dioestral sample of uterine horn was taken from the unoperated side. Part of the uterine horn was fixed in formol–saline and then embedded in paraffin. Histological sections were cut at 6 μm and stained with haematoxylin and eosin.

Ovariectomized Ewes

The 6-year-old Merino ewes originated from a single flock. For 3 years, the 17 clover-affected ewes had grazed the oestrogenic Yarloop cultivar of subterranean clover and only two had lambed to the last mating while the 18 control ewes of the same age had grazed non-oestrogenic pasture during this period and 14 had lambed to the last mating. For the last 2 years all ewes had been grazed together on non-oestrogenic pasture.

The ewes were ovariectomized during the normal breeding season 6 weeks before the study began. Five clover-affected and five control ewes were injected s.c. with 25 μg oestradiol-17β in 1 ml ethanol–saline (1 : 9 v/v) 24 h before slaughter. Another five clover-affected and five control ewes were injected with the same amount of oestradiol-17β daily for 3 days before slaughter. The remaining ewes were slaughtered without any injection. Portions of uterine horn were prepared for histological examination as described above.

Estimation of Nucleic Acids

The whole uterus from ovariectomized ewe or a segment of the uterine tissue taken from the entire ewe was blotted dry and minced finely. About 1 g of tissue was weighed and homogenized in 5 ml ice-cold water. An equal volume of 10% (w/v) perchloric acid was added, mixed and left in ice for 15 min. The suspension was centrifuged at 800 *g* for 15 min at 4°C. The resultant pellet was washed with 10 ml ethanol followed by 10 ml ether to remove lipids, washed twice in 10 ml 5% (w/v) ice-cold perchloric acid and then digested with 10% perchloric acid at 70°C for 20 min with frequent stirring. The resultant digest was used for the estimation of DNA and RNA according to Ceriotti (1955).

Results were analysed statistically by Student's *t*-test.

Results

Two of the affected ewes had hydrops uteri, but after histological processing there was no detectable difference between control and affected ewes in the relative proportions of endometrium, myometrium or epithelium. Affected ewes could be distinguished histologically only by the occasional appearance of dilated or cystic glands.

In the entire ewes, the DNA content of the uterus was significantly lower ($P < 0{\cdot}01$) but the amount of RNA stayed reasonably constant, so the uterine RNA/DNA ratio was greater ($P < 0{\cdot}001$) at oestrus than at dioestrus (Table 1). There were no differences between clover-affected and control ewes in respect to either the content of DNA or RNA/DNA ratio in the uterus at either oestrus or dioestrus.

Table 1. Mean values (± s.e.m.) for nucleic acid concentration in uterine samples from clover-affected and control ewes at oestrus and dioestrus

** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$, significantly different from dioestrus samples (Student's *t*-test)

Group	Dioestrus		Oestrus	
	DNA (mg/g wet wt)	RNA/DNA (wt/wt)	DNA (mg/g wet wt)	RNA/DNA (wt/wt)
Control	8·07±0·44	0·49±0·04	5·66±0·35	0·67±0·02
Clover-affected	7·64±0·70	0·49±0·05	6·35±0·26	0·62±0·02
Both groups combined	7·86±0·37	0·49±0·03	6·00±0·24**	0·65±0·02***

There was no difference in the amount of DNA per gram of uterine tissue between control and clover-affected ewes after ovariectomy (Table 2). The ratio of RNA to DNA in the uterus was significantly higher in clover-affected than in control ewes (Table 2). As seen from Table 2, the ratio of RNA to DNA increased by a similar

Table 2. Mean values (± s.e.m.) for nucleic acid concentration in uterine samples from ovariectomized clover-affected and control ewes before and after oestradiol-17β injection

* $P < 0{\cdot}025$, ** $P < 0{\cdot}01$, significantly different from control ewes (Student's *t*-test)

Treatment	Group	DNA (mg/g wet wt)	RNA/DNA (wt/wt)
Untreated	Control	14·1±0·9	0·204±0·004
	Clover-affected	12·2±1·0	0·231±0·009**
Single treatment	Control	7·5±0·3	0·75 ±0·03
	Clover-affected	6·9±0·3	0·74 ±0·02
Three treatments	Control	5·7±0·3	1·073±0·024
	Clover-affected	6·6±0·4	0·838±0·008*

amount in ovariectomized control and ovariectomized clover-affected ewes given a single injection of oestradiol. However, when similar ewes were injected daily with oestradiol-17β for 3 days, the uteri of the two classes of ewes responded differently. The uterine RNA/DNA ratio was greater in control than in clover-affected ewes.

Discussion

The nucleic acid composition of the uterus depends closely on hormonal control (Heald *et al.* 1970; Miller 1976). Further, Little and Lambourne (1976) and Cox *et al.* (1978) have demonstrated that the uterine RNA/DNA ratio is a sensitive indicator of the degree of oestrogenic stimulation in ovariectomized ewes. As shown in the results, the change in RNA/DNA ratio occurs because the amount of RNA increases

at a rate similar to the increase in total weight of tissue, whilst the DNA does not. There is no indication that the absolute amount of DNA decreases, but the other components of the tissue (water, protein and RNA) increase (Miller 1976) in response to oestrogen. The marked difference between the uterine nucleic acid content at oestrus and dioestrus in entire ewes in the present study suggests that the ratio is also a sensitive indicator of oestrogenic stimulation in entire animals. Since the uterine RNA/DNA ratio varies in accord with the circulating level of oestrogen (Little and Lambourne 1976), the similarity between entire clover-affected and control ewes in the uterine nucleic acid content at either stage of the oestrous cycle indicates that there was no great difference between the groups in the degree of oestrogenic response in the tissues. Thus, the changes in ewes with permanent clover infertility cannot be attributed to a continuation of the previous oestrogenic stimulation within the animal.

The uteri of ewes atrophy after ovariectomy (Little and Lambourne 1976) resulting in a lower ratio of uterine RNA/DNA. In the present study the uterine ratio of RNA to DNA was significantly greater in ovariectomized clover-affected ewes than controls. This and the fact that the uterus from ovariectomized clover-affected ewes has a higher rate of protein and glycoprotein synthesis (Tang and Adams 1981) indicate that the uteri of ovariectomized clover-affected ewes did not regress as much as did those of the control ewes and thus functioned as though still under slight oestrogenic stimulation. However, mild bacterial endometritis is common in ewes with clover infertility (Adams 1975) and this may have had some influence on the results.

In ovariectomized ewes, the increase in uterine RNA/DNA ratio was slightly less in clover-affected than in control ewes given one injection of oestradiol-17β. This difference was accentuated with time, so that a significant difference occurred after three daily doses of oestradiol-17β. A similar impairment of responses to multiple oestrogenic stimulation occurs in the cervix and vagina (Adams 1979). The cervix may be more severely affected than the uterus in clover disease because an alteration in the response of the cervix can be readily demonstrated in entire ewes at oestrus (Adams 1976), while there was no difference between affected and control ewes in respect to uterine RNA/DNA at oestrus.

Lobl and Maenza (1975) reported that when neonatal female rats were treated with testosterone propionate they underwent abnormal sexual differentiation and became permanently infertile. At maturity the uterus of these rats was of normal size, but it did not regress completely after ovariectomy, and it showed an impaired growth response to multiple, but not to single, injections of oestradiol. It is not clear why the difference should take 3 days to appear, but the present study shows that a very similar phenomenon occurs in sheep with clover disease. Thus the results are consistent with the hypothesis (Tang and Adams 1981) that the changes which occur in clover disease are a result of the phyto-oestrogen having the type of differentiating action on the ewe which is normally seen with sex steroids on their target tissues during organogenesis. Since a similar change in responsiveness to oestrogen also occurs in the cervix, it is possible that this differentiating action of the phyto-oestrogen is responsible for the functional abnormality in the cervix which results in infertility.

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