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Enzyme Activities and Protein and Carbohydrate Concentrations in Cervical Secretions at Dioestrus in Normal Ewes and Ewes with Permanent Phytooestrogenic Infertility

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Abstract

Cervical secretions of clover-affected and control ewes in the luteal phase of the ovarian cycle were obtained by flushing the anterior vagina. The flushings were analysed for proteins, carbohydrates and enzyme activities, and were found to be similar to the secretions of the normal ovine uterus. There was significantly more protein, carbohydrate and acid-soluble glycoprotein but less alkaline phosphatase, *N*-acetylglycosidases (EC 3.2.1.30 and 3.2.1.53) and ribonuclease I in the vaginal flushings of clover-affected ewes.

The observed changes were not due to more inflammation in the cervix of clover-affected ewes as there were fewer bacteria, leukocytes and epithelial cells and no elevation of lysozomal enzyme activities in their flushings. It is suggested that the cervix of the clover-affected ewe behaves as though under a stronger than normal oestrogenic stimulation during dioestrus.

Introduction

Ewes rendered permanently infertile by prolonged ingestion of oestrogenic clover produce abnormal cervical mucus which prevents normal sperm transport and fertilization (Lightfoot *et al.* 1967; Smith 1971). Histological changes of the cervices of these ewes have also been observed by Heydon and Adams (1977). A test on cervical secretions could be expected to be a useful diagnostic method for detecting permanent clover infertility.

Adams (1967*a*) found that mucus from clover-affected ewes had a spinnbarkeit value significantly lower than that from control ewes. Although the mucus spinnbarkeit test was subsequently found to be useful for assessing lowered reproductive performance in ewes exposed to oestrogenic pasture (Adams 1977), it has limitations. Spinnbarkeit varies with the time of the breeding season so that adjustment is needed to compare results obtained at different times. Cervical mucus is abundant only when the ewe is in oestrus and even then some ewes produce so little that collection of an adequate sample is impossible. Because of these limitations it is worthwhile to seek other diagnostic methods and one possible area is the biochemical analysis of cervical secretions during the luteal phase of the reproductive cycle.

In the present experiment proteins, carbohydrates and some enzymes, including lysozomal enzymes, in the luteal-phase cervical secretions obtained by flushing the anterior vagina of clover-affected and control ewes were assayed and compared.

Materials and Methods

Sheep

Seventy control and 70 clover-affected ewes were used. All the ewes originated from the same flock although affected ewes were 5 years old and control ewes were 6 years old. The clover-affected

ewes had grazed the highly oestrogenic Yarloop cultivar of subterranean clover for 3 years and had a lambing rate of 10% to the last mating. None of the ewes had grazed oestrogenic pasture for 4 months prior to or during the study. Control ewes had never been exposed to oestrogenic pasture and were of normal fertility.

Ewes were run with two vasectomized rams fitted with harness and marking crayons. The ewes were examined every 5 days for 2 months for marking by the ram and vaginal flushings were collected at the next examination, i.e. between 5 and 10 days after oestrus. Each ewe was flushed once per oestrous cycle.

Sample Collection

Cervical secretions were obtained by flushing the anterior vagina (Adams 1976*a*) with the following additional steps. After the vagina was opened with a speculum the anterior vagina was flushed with 1 ml 0·15 M saline which was then aspirated into a plastic pipette and stored frozen until analysed. As there was no consistent difference observed in the average amount of cervical secretions during metoestrous from clover-affected and control ewes using the cotton swab technique (Adams 1976*a*), it was assumed that the cervical secretions from clover-affected and control ewes were diluted to the same degree by 1 ml saline. Samples containing blood were discarded. Samples from three to five sheep were pooled before analysis.

Analytical Procedures

Protein was determined by the method of Lowry *et al.* (1951). To remove cells and cell debris, flushings were centrifuged at 700 *g* at 4°C for 15 min and the deposit discarded. Acid-soluble and acid-insoluble proteins were separated by mixing 0·5 ml flushings with 0·1 ml ice-cold 20% (v/v) perchloric acid. The solution was left in ice for 15 min, and the precipitated acid-insoluble proteins were removed by centrifugation. Protein determination on acid-soluble proteins was carried out after removing the perchloric acid either by precipitation with potassium hydroxide or by dialysis against 10 mM phosphate buffer (pH 7·4) at 4°C for 24 h.

Carbohydrate was determined by the orcinol method of Hewitt (1937) using standards made up of an equimolar solution of glucose and fucose. Glucose was determined by the enzyme method of Latzko and Gibbs (1972). Lactate was determined by the method of Barker and Britton (1957).

Enzyme Assays

Alkaline phosphatase (EC 3.1.3.1), lysozome (EC 3.2.1.17), α-amylase (EC 3.2.1.1), ribonuclease I (EC 3.1.4.22) and acid phosphatase (EC 3.1.3.2) were assayed by microradial diffusion as described by Schill and Schumacher (1972). Each well contained 20–60 μg protein in 50 μl of 700-*g* supernatant of cervical flushings. Enzyme activities were expressed as equivalent amounts of purified enzymes obtained from Sigma Chemical Co., St. Louis, U.S.A.

α-L-Fucosidase (EC 3.2.1.51), β-D-galactosidase (EC 3.2.1.23), β-*N*-acetylgalactosaminidase (EC 3.2.1.53) and β-*N*-acetylglucosaminidase (EC 3.2.1.30) were assayed according to the method of Roberts and Parker (1974). The amount of vaginal flushings (700-*g* supernatant) used varied from 50 to 200 μl, containing approximately 60–100 μg protein.

Cell and Bacteria Counts

The relative numbers of bacteria and cells in flushings were determined on smears of 0·01 ml spread uniformly on a circular area of 4 cm^2 on a glass slide, as described by Moir (1951). The number of cells in 20 fields at 100 magnifications and number of bacteria in 20 fields at 1000 magnifications were recorded. The fields were selected at approximately even spacings across a diameter of the circle.

Statistical Analysis

All data in the study were analysed by Student's *t*-test except those on cell and bacteria counts, where the results were not normally distributed and so were analysed by Wilcoxon's Rank test.

Results

Estimation of Enzyme Activities, Protein and Carbohydrate Contents

Enzyme activities

When expressed on a protein basis (in 700-*g* supernatant), the activities or amounts of alkaline phosphatase, ribonuclease I, β-*N*-acetylgalactosaminidase and β-*N*-acetyl-

glucosaminidase were significantly lower in the flushings of clover-affected ewes than in those of control ewes (Table 1). There was no significant difference in the activities of α-L-fucosidase and β-D-galactosidase nor in the amount of amylase between the two groups. There was little or no acid phosphatase or lysozyme detectable in the cell-free fraction of flushings of either group.

Table 1. Quantities and activities of enzymes in 700-g supernatant of flushings of control and clover-affected ewes

Values given are means ± s.e.m. of 12 and 10 samples pooled from 70 control and 70 affected ewes respectively. n.d., Not detectable

Ewes	Enzymes				
	(*a*) Quantity (weight of enzyme/weight of protein)				
	Alkaline phosphatase $\times 10^3$	Lysozyme	α-Amylase $\times 10^6$	Ribonuclease I $\times 10^3$	Acid phosphatase
Control	41·5 ± 11·2	n.d.	10·2 ± 0·9	3·7 ± 1·1	Trace
Affected	4·1 ± 2·4***	Trace	6·5 ± 1·7	0·7 ± 0·3**	Trace
	(*b*) Glycosidase activity (μg p-nitrophenol released per mg protein per 3 h)				
	α-L-Fucosidase	β-D-Galacto-sidase	β-*N*-Acetyl galactosaminidase	β-*N*-Acetyl-glucosaminidase	
Control	15·6 ± 0·9	26·6 ± 1·9	910 ± 130	1940 ± 80	
Affected	13·6 ± 0·9	25·0 ± 2·1	560 ± 90*	1130 ± 110***	

* $P < 0{\cdot}05$. ** $P < 0{\cdot}025$. *** $P < 0{\cdot}001$.

Table 2. Concentration of protein and carbohydrate in flushings from control and clover-affected ewes

Values given are means ± s.e.m. of 12 and 10 samples pooled from 70 control and 70 affected ewes respectively

Ewes	Whole flushing	700-*g* supernatant (700-*g* sup.)	Acid-soluble of 700-*g* sup.	Dialysed acid-soluble of 700-*g* sup.
	(*a*) Protein (μg/ml)			
Control	1339 ± 197	439 ± 52	130 ± 13	118 ± 17
Affected	2655 ± 297***	1292 ± 156***	324 ± 58**	336 ± 73**
	(*b*) Carbohydrate (μg/ml)			
Control	283·6 ± 46·5	38·9 ± 7·1	23·4 ± 2·2	13·8 ± 1·5
Affected	594·4 ± 101·0***	133·1 ± 23·6***	44·2 ± 5·2**	42·4 ± 8·4**

** $P < 0{\cdot}025$. *** $P < 0{\cdot}001$.

Estimation of total protein and carbohydrate, glucose and lactate

Table 2 shows that there was significantly more protein and carbohydrate in the flushings from clover-affected ewes irrespective of the treatment of the flushings. Centrifugation at 700 *g* removed much of the protein and carbohydrate, which was probably contained in cell debris and bacteria. Acid treatment precipitated from one-half to two-thirds of the materials present in the 700-*g* supernatant. The amount of acid-soluble proteins in the vaginal flushings was significantly higher in the clover-

affected than in the control ewes. Dialysis of the acid-treated supernatant did not result in substantial loss of protein or carbohydrate, indicating that the amount of low-molecular-weight peptides and monosaccharides present was small.

There was more glucose in the flushings from clover-affected ewes than in the flushings from the control ewes ($4 \cdot 14 \pm 1 \cdot 85$ *v.* $1 \cdot 75 \pm 0 \cdot 57$ μg/ml) although the difference is not statistically significant. Lactate was present in only trace amounts in both groups.

Bacteria and Cell Type Counts

The mean scores (12 determinations) for bacterial, epithelial and polynuclear cell counts in flushings from control ewes were 743, 167 and 103 respectively, whereas those from clover-affected ewes (9 determinations) were 117, 120 and 43 respectively. Differences in bacterial and epithelial cell counts were statistically significant (Wilcoxon's test, $P < 0 \cdot 01$).

Discussion

The observation by Heydon and Adams (1977) that the histology of the cervix of clover-affected ewes changed to resemble that of the uterus prompted us to analyse the cervical secretions retrieved from vaginal flushings from clover-affected and control ewes for the major substances which have been found in uterine secretions (Wales 1973; Roberts *et al.* 1976). The results showed that most of the substances found in uterine secretions were present in the vaginal flushings of both groups in relative amounts that were very similar to those in the uterine secretions; for example the percentage of total protein which was acid-soluble in the vaginal flushings of clover-affected and control ewes was 12·2 and 9·7 respectively, whereas that in the uterine flushings from normal sheep was 13·8% (Tang and Adams, unpublished data). The amounts of orcinol-reactive carbohydrate and glucose in the vaginal flushings were in the same range as those recorded in the uterine secretion on a protein weight basis by Wales (1973). Also the relative amount of various glycosidases present in the vaginal flushings was similar to that found in the uterine flushings by Roberts *et al.* (1976). However, lactate, which is normally present in relatively large amounts in uterine secretions (Wales 1973), was almost absent from the vaginal flushings of clover-affected and control ewes. It is possible that the substances present in the vaginal flushings were not cervical secretions but were contributions from secretion or blood transudates from the uterus 'leaking' into the vagina through the cervix. However, the facts that in the flushings there was less glucose and more glycosidase compared to serum (Singh and Dutt 1974; Roberts *et al.* 1976), and an absence of lactate compared to uterine secretions (Wales 1973) indicate that those contributions which were probably present were minimal. Therefore, the results of the present experiment show that the cervix of the ewe was mainly responsible for the altered secretory pattern observed in clover-affected ewes.

The present results show that the cervical secretion of clover-affected ewes contains more carbohydrate and protein than that of control ewes. Apart from a permanent physiological change within the cervix of the clover-affected ewe, such an increase in substances secreted could be due to the more common occurrence of cervical inflammation in the clover-affected ewe (Adams 1976*b*). When the vaginal flushings were studied for the presence of bacterial, epithelial and polynuclear leukocytic

cells, we found no indication of greater inflammation in the cervix of clover-affected ewes. Furthermore there was either no difference or a decrease in lysomal enzyme activities such as glycosidases and ribonuclease in the flushings from clover-affected ewes. Also D. L. Watson (personal communication) could not detect any elevation of IgA and IgG_2 in the flushings of clover-affected ewes from the present experiments. It seems therefore that the increase in amount of protein and carbohydrate secreted by the cervix of clover-affected ewes is a direct physiological change caused by prolonged grazing of oestrogenic clover. Thus the estimation of protein and carbohydrate in (cell-free) vaginal flushings of clover-affected ewes during the ovarian luteal phase could be a useful aid in the diagnosis of permanent clover-infertility in sheep.

The fact that there was more protein and carbohydrate left after acid and dialysis treatment in the vaginal flushings from clover-affected ewes than from controls suggests there is an increase in the relative amount of acid-soluble glycoproteins in the flushings. As the cervix of the ewe secretes mucus when stimulated by oestrogen, and cervical mucus consists largely of glycoproteins and proteins (Roberts *et al.* 1976), it seems that the cervix of the clover-affected ewe behaves as though under stronger oestrogenic stimulation than that of the control ewe during the ovarian luteal phase. This is further supported by the decrease in the activities of lysosomal enzymes and alkaline phosphatase in vaginal flushings from clover-affected ewes, as such enzyme activities are consistently low in human cervical mucus under the influence of oestrogens (Schumacher 1973).

Whether the changes in the cervix of clover-affected ewes are due to an imbalance of endogenous sex steroids or to changes in sensitivity of the cervix in responding to steroids awaits further studies. Although Smith (1975) found no difference in peripheral progesterone levels between clover-affected and control ewes, there is no report on the estimation of other hormonal differences between the two groups on which to base a definite conclusion. The minute amount of peripheral oestrodial-17β in the ewe (Scarramuzzi and Land, unpublished data) makes it difficult to detect any changes between clover-affected and control ewes. The alternative that the cervix of the clover-affected ewe changes its sensitivity in responding to steroid hormones is currently under investigation.

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