Aust. J. Biol. Sci., 1979, **32**, 409–14

Enzyme Histochemistry of the Sheep Uterus during the Oestrous Cycle

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Abstract

Enzyme histochemical techniques were applied to frozen sheep uteri from different stages of the oestrous cycle. The localization and activities of succinate, lactate, glucose-6-phosphate, and isocitrate ($NADP^+$) dehyrogenases and acid and alkaline phosphatases were studied in the luminal and glandular epithelia, caruncle and myometrium. Enzyme activity in the sections was scored on a scale of 0–5.

In general the enzyme activity in the uterine caruncles and epithelia was higher than in the myometrium. The myometrium did not show any alkaline phosphatase activity and isocitrate dehydrogenase ($NADP^+$) activity was negligible. The low activities of acid phosphatase and lactate dehydrogenase and the moderate levels of glucose-6-phosphate and succinate dehydrogenases in the myometrium were constant.

The caruncular tissue showed high levels of phosphatases and glucose-6-phosphate dehydrogenase, moderate levels of lactate and succinate dehydrogenases, and low levels of isocitrate dehydrogenase ($NADP^+$) throughout the oestrous cycle. Much lower phosphatase and isocitrate dehydrogenase ($NADP^+$) levels were found in the epithelium of deep glands compared with superficial glands.

The high activity of acid and alkaline phosphatases in the luminal epithelium and the superficial glands was constant from mid-cycle to ovulation, but a significant decrease was observed immediately after ovulation. The level of dehydrogenases in epithelia was generally high and did not change during the oestrous cycle.

Introduction

Many histochemical studies of the human uterus, particularly of the endometrium under normal and abnormal conditions have been reported (Filipe and Dawson 1968; Connell 1972; Boutselis 1973). In other species some hydrolytic and oxidative enzymes have been localized in the uteri of the cow, pig, rat, guinea-pig and rabbit (Velardo and Rosa 1963; Marinov and Lovell 1968; Veznik *et al.* 1972). Limited information is available on the enzyme histochemistry of the sheep uterus during the oestrous cycle and only acid and alkaline phosphatases (Hadek 1958) and carbonic anhydrase (Lutwak-Mann and Averill 1954) appear to have been investigated.

Quantitative data obtained by biochemical studies (R. N. Murdoch and White 1968) lack specificity of localization which histochemistry can provide and this is of special interest in a heterogeneous tissue such as the uterus containing luminal and glandular epithelia, stroma, muscular layers and blood vessels.

This paper describes the histochemical localization and semi-quantification of succinate (succinate: (acceptor) oxidoreductase; E.C. 1.3.99.1), lactate (L-lactate: NAD^+ oxidoreductase; E.C. 1.1.1.27), isocitrate ($NADP^+$) (*threo*-D_s-isocitrate: $NADP^+$ oxidoreductase (decarboxylating); E.C. 1.1.1.42) and glucose-6-phosphate

(D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase, E.C. 1.1.1.49) dehydrogenases and acid (orthophosphoric-monoester phosphohydrolase, acid optimum, E.C. 3.1.3.2) and alkaline (orthophosphoric-monoester phosphohydrolase, alkaline optimum, E.C. 3.1.3.1) phosphatases in the uterus of the sheep during the oestrous cycle. A preliminary report has been presented elsewhere (Zamiri and Blackshaw 1977).

Materials and Methods

Sheep uteri were obtained from a local abattoir within 30 min of slaughter, frozen in liquid nitrogen and transferred to the laboratory on dry ice. Ovaries, matched to the frozen tissue, were used for identification of the stage of the cycle (Restall 1964).

Frozen sections (10 μm) from the mid-segment of the right horn of the uterus were cut in a Lipshaw cryostat at $-25°C$, mounted on microscope slides, air-dried and used for the histochemical localization of enzymes. All chemicals used for histochemistry were obtained from Sigma (St. Louis, Missouri, U.S.A.).

Acid and alkaline phosphatases were localized according to Pearse (1968) using sodium-α-napthyl phosphate (1 mg/ml) and Fast Blue BB (1 mg/ml) in 100 mM acetate buffer, pH 5·0 (acid phosphatase) or in 100 mM Tris–HCl, pH 10 (alkaline phosphatase), both containing 75 mg/ml polyvinylpyrrolidone. Fresh incubation medium (0·5 ml) was pipetted onto the sections which were incubated for 45 min at 37°C (acid phosphatase) or at room temperature (alkaline phosphatase).

The optimal incubation medium for dehydrogenases was prepared according to Pearse (1972) and contained nitro-blue-tetrazolium, substrate, sodium azide or cyanide, polyvinylpyrrolidone, phenazine methosulfate, and NAD^+(P) in 100 mM Tris–HCl buffer in amounts as listed in the following tabulation:

	Dehydrogenase			
	Succinate	Lactate	Isocitrate ($NADP^+$)	Glucose-6-phosphate
pH	7·4	7·2–7·4	7·2–7·4	7·4
Nitro-blue-tetrazolium (mg/ml)	0·5–1	1	1	0·5–1
Sodium azide (mM)	10	—	—	10
Sodium cyanide (mM)	—	10	10	—
NAD (mg/ml)	—	1	—	—
NADP (mg/ml)	—	—	1	1
Substrate (mM)	200	200	100	100
Phenazine methosulfate (mg/ml)	0·5	0·5	0·5	0·5
Polyvinylpyrrolidone (mg/ml)	—	150	150	150
Incubation time at 37°C (min)	25–30	10–15	30–40	25–30

Control sections were incubated in a medium containing all ingredients except the substrate. Following incubation the sections were rinsed in distilled water, fixed in formol saline (3 min), rinsed with running tap water (1 min), mounted in Farrant's medium and sealed with a cover-slip.

To semiquantify enzymatic activity, two sections from different stages of the oestrous cycle were randomly selected and scored (125×) on a scale of 0–5, from no activity (0), very weak (1) to very strong (5) activity. The scores were then analysed by the analysis of variance (Snedecor and Cochran 1967).

Enzyme activity was determined in the stroma, luminal epithelium, glandular epithelium, caruncle and circular and longitudinal layers of the myometrium. In sections stained for isocitrate dehydrogenase ($NADP^+$) and acid and alkaline phosphatases, the epithelia of superficial and deep glands were scored separately, as they showed marked differences in staining.

Results

Control sections, incubated for non-specific staining, showed no phosphatase activity and only a small amount of non-specific tetrazolium reduction was present in the case of dehydrogenases. Some activity was found in the stroma for all enzymes studied, but strong staining was seen in that part of the stroma nearer to the uterine lumen.

Alkaline Phosphatase

The caruncle showed strong alkaline phosphatase activity (mean ±s.e. = 3·9±0·1) at all stages of the oestrous cycle with no change in activity pattern (Table 1). The activity in the luminal epithelium fell from a very high activity near mid-cycle (day −7) to a moderate level on day −2, increased to very high levels on day 0 and then fell to very low levels by day +4 ($P<0·01$).

Table 1. Alkaline and acid phosphatase activity (mean score ±s.e.) in different uterine structures of the ewe during the oestrous cycle

* Significantly lower than day 0 ($P<0·05$). ** Significantly lower than other values ($P<0·01$)

				Alkaline phosphatase			
Days from ovulation (0)	−7	−3	−2	−1	0	+1	+4
No. of animals	6	5	6	4	2	4	3
Luminal epithelium	4·8±0·4	4·6±0·5	3·0±0·4	5·0±0·5	4·5±0·8	1·7±0·5**	1·0±0·6**
Superficial glands	4·2±0·4	3·0±0·5	2·8±0·5	4·5±0·5	4·5±0·8	1·5±0·5**	0·7±0·6**
Caruncle	3·5±0·3	4·2±0·3	3·7±0·3	4·0±0·4	4·5±0·5	3·5±0·4	3·7±0·4
				Acid phosphatase			
Days from ovulation (0)	−7	−3	−2	−1	0	+1	+4
No. of animals	6	5	5	4	2	4	3
Luminal epithelium	4·8±0·4	5·0±0·4	4·4±0·4	4·7±0·4	5·0±0·6	2·7±0·4**	2·0±0·5**
Superficial glands	4·7±0·4	4·4±0·4	4·6±0·4	4·7±0·4	5·0±0·6	3·0±0·4**	2·0±0·5**
Deep glands	0·8±0·4*	0·6±0·4*	1·8±0·4	0·5±0·5*	3·0±0·5	2·0±0·6	1·2±0·5*
Caruncle	4·7±0·2	5·0±0·2	4·8±0·2	4·0±0·2	5·0±0·3	4·8±0·2	5·0±0·3
Myometrium	1·2±0·4	1·6±0·4	2·2±0·4	1·7±0·5	3·0±0·6	2·5±0·5	3·0±0·5

The enzyme activity in the superficial glands was constant from day −7 to day 0, but it decreased significantly ($P<0·01$) after ovulation. The level of activity in the deep glands was much lower than in the superficial glands and showed very weak activity throughout the oestrous cycle. No activity was present in the myometrium.

Acid Phosphatase

There was little difference in the high acid phosphatase activity of the caruncle and the luminal and superficial glandular epithelia from day −7 to day 0; after this time caruncular activity remained high (mean ±s.e. = 4·8±0·1), but the epithelial activity decreased ($P<0·01$) to moderate levels by day +1 and then fell to weak levels by day +4 (Table 1).

Weak to moderate activity occurred in the myometrium without any definite changes in the pattern of activity (mean ±s.e. = 2·2±0·2). The lowest activity was in the epithelium of deep glands; on day 0 these glands showed moderate staining, but by day +4 the activity had dropped to the level seen before ovulation ($P<0·05$).

Dehydrogenases

There was no significant effect of the stage of the cycle on the activity of dehydrogenases. The results are, therefore, expressed as mean ±s.e. for each uterine structure.

The activity of glucose-6-phosphate dehydrogenase was high in the luminal epithelium (4·7±0·1), glandular epithelium (4·1±0·1) and caruncles (4·3±0·1), and moderate in the circular (3·0±0·1) and longitudinal (2·6±0·1) layers of the myometrium. Lactate dehydrogenase activity was high in the luminal (4·1±0·1) and glandular (3·9±0·1) epithelia and weak in the caruncles (2·1±0·1) and the muscle (2·2±0·1).

Succinate dehydrogenase activity was high in the luminal (4·7±0·1) and glandular (4·2±0·1) epithelia, and moderate in the caruncles (3·6±0·1) and circular layer of the myometrium (3·3±0·1). In the longitudinal layer of the myometrium the activity of succinate dehydrogenase was higher ($P<0·01$) on day 0 (4·0±0·5) and day +1 (3·0±0·5) compared with other stages of the cycle (1·9±0·3).

Isocitrate dehydrogenase ($NADP^+$) activity in the glandular epithelium was confined mainly to superficial glands, and only negligible activity was found in the epithelium of deep glands. The highest activity was seen in the luminal epithelium (3·6±0·2) followed by the superficial glandular epithelium (2·7±0·2) and the caruncle (1·7±0·2). No activity was detected in the myometrium.

Discussion

The histochemical findings for alkaline phosphatase do not agree with those of Hadek (1958) who found increased activity (Gomori method) during pro-oestrus and oestrus with maximum activities at early dioestrus but no activity at mid and late dioestrus.

Marinov and Lovell (1968) reported the presence of alkaline phosphatase in the luminal epithelium of the cow uterus with higher concentrations during the mid-cycle. Higher alkaline phosphatase activity was found in the epithelium of superficial glands than in the epithelium of deep glands, a finding similar to that for alkaline phosphatase and acid phosphatase in the present work.

In a recent histochemical study on the cow endometrium Veznik *et al.* (1972) found low levels of alkaline phosphatase and acid phosphatase in the endometrium during the follicular phase and increased activity during the luteal phase, but did not specify the relative activity of different structures.

Alkaline phosphatases catalyse the hydrolysis of a variety of phosphate esters (Stadtman 1961) and have been associated with carbohydrate metabolism (Boshier 1969) and with the transfer of solutes across the membranes of secretory cells (Moog 1946; Bradfield 1950). Acid phosphatase is also involved in the hydrolysis of phosphate esters. The high alkaline phosphatase and acid phosphatase activities observed around day 0 in our studies may be related to the hormonal profiles necessary for oestrual behaviour and successful fertilization (Able *et al.* 1976). Also, the subsequent rise in activity towards the end of the cycle may have some function in preparing the uterus for implantation of the embryo in the case of fertile matings or for the next oestrual period in the case of non-fertile matings.

R. N. Murdoch and White (1968) suggested that alkaline phosphatase and acid phosphatase were under the control of progesterone as their activities followed the

growth and regression of the corpus luteum; however, in early pregnancy when progesterone production is high, no association between alkaline phosphatase and progesterone levels was found, although acid phosphatase increased (R. N. Murdoch 1970).

In our studies alkaline phosphatase and acid phosphatase activities in epithelia dropped sharply from day 0 to day +4, the period of minimal oestrogen and progesterone production, but the activities were high at other stages of the cycle. In the ewe, several surges of oestrogen have been reported during the oestrous cycle (Emmens and Gidley-Baird 1977) and it is likely that oestrogen (perhaps in association with progesterone) may control enzyme induction in several uterine structures. Nevertheless, alkaline phosphatase and acid phosphatase activity in the caruncle was high at all times during the cycle and showed no cyclical variations.

The activity of dehydrogenases in the uterus was constant during the cycle; however, in the longitudinal layer of the myometrium the activity of succinate dehyrogenase was highest around the time of ovulation. Biochemically, no changes were seen in the total activities of glucose-6-phosphate dehydrogenase and lactate dehydrogenase of the sheep uterus during the oestrous cycle, but succinate dehydrogenase activity was maximal during the luteal phase (R. N. Murdoch and White 1968). In another study (B. E. Murdoch and O'Shea 1978) the activity of succinate dehydrogenase in the caruncle and endometrium did not change during the oestrous cycle.

Our histochemical study shows higher dehydrogenase activity in the epithelial tissue compared to the caruncular tissue. The level of succinate dehydrogenase determined by biochemical methods (R. N. Murdoch and White 1968) was greater in the caruncles than in the intercaruncular endometrium, but the reverse was true for lactate dehydrogenase; however, glucose-6-phosphate dehydrogenase activity was similar in both structures. The differences between histochemical and biochemical findings may reflect the enzymatic activity of the stromal tissue which was homogenized with the epithelial tissue for biochemical determinations.

While succinate dehydrogenase activity in the human endometrium has been associated with progesterone (Ishihara *et al.* 1964) or with oestrogen (Fawzy and Baradi 1967), there are also reports of a lack of cyclical activity of this enzyme and also of lactate dehydrogenase, glucose-6-phosphate dehydrogenase and isocitrate dehydrogenase ($NADP^+$) (Filipe and Dawson 1968).

From these histochemical observations, it is not possible to relate the activity of dehydrogenases to the hormonal profile during the sheep oestrous cycle. The lack of cyclical activity may be due to the low sensitivity of histochemical techniques in detecting the changes in the activity of a particular enzyme.

The high levels of dehydrogenases in the uterine epithelial tissue may represent a potentially high metabolic rate of the uterus during the oestrous cycle. This high enzymatic activity may have a role in supplying metabolites such as Krebs' cycle intermediates in the uterine fluid (histotrophe) which can be used by the developing conceptus once it enters the uterine environment (Cook and Hunter 1978).

Studies using microfluorometric methods are now in progress to determine the activity of these enzymes in histologically defined areas of the freeze-dried sections from uteri of sheep with known stages of the oestrous cycle.

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Manuscript received 29 September 1978, accepted 24 May 1979