Uterine histology of the dasyurid marsupial, *Antechinus stuartii*: relationship with differentiation of the embryo

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Uterine samples from *Antechinus stuartii* on days 1, 4, 6, 8, 11, 13, 15, 18, 21 and 23 after ovulation were examined histologically. Animals were pregnant, nonpregnant and unmated, or nonpregnant and mated but found to have only unfertilized eggs on autopsy. The histological parameters used were thickness of the myometrium, endometrial stroma, and endometrial epithelium, and density of uterine stromal glands and of lymphocytes at the endometrial basal lamina. Overall, the fluctuation patterns of these parameters were superficially similar between pregnant and nonpregnant animals (mated or unmated). However, statistically significant differences were detected between pregnant and unmated nonpregnant animals in every parameter examined at nearly every time point except day 13. Comparison of these results with known data on embryonic stages, corpus luteum development and plasma progesterone concentrations revealed that the gravid uterus underwent histological changes co-incident with changes in both progesterone concentration and developmental delay or embryonic arrest. It was concluded that the uterus mediates progesterone-driven changes in embryonic developmental rate. Although determination of number of lymphocytes provided inconclusive evidence of cellular immunity against embryos, the possibility that embryonic signalling to the uterus occurs is discussed.

Introduction

The marsupial reproductive pattern is characterized by a short gestation and a prolonged lactation period. In many known marsupials except macropodids (kangaroos and wallabies), gestation and parturition occur within the luteal phase of the oestrous cycle, which commences with a variably long follicular and ends with a brief post-luteal phase. Neither gestation nor parturition interrupts the oestrous cycle; lactation prevents renewal of the follicular phase. Thus, removal of pouch young from lactating females, and not parturition, results in resumption of the oestrous cycle in polyoestrous species (reviewed in Sharman, 1970; Lee *et al.*, 1982; Tyndale-Biscoe and Renfree, 1987). In monoestrous marsupials, however, removal of pouch young does not provoke resumption of oestrus. In these animals, the follicular phase is not renewed until the next breeding season, as has been shown for *Antechinus stuartii* (Woolley, 1966).

The oestrous cycle in marsupials is largely indifferent to perturbation by pregnancy. In *Didelphis virginiana*, a polyoestrous, polyovular species in which ovulation is spontaneous, the uterine anatomical and histological profile of pregnant animals is similar to that of nonpregnant animals until the terminal stages of pregnancy, when implantation occurs (Fleming and Harder, 1981). There appears to be no endocrine recognition of pregnancy in this species; oestradiol:progesterone ratios are about the same on any given day of the oestrous cycle in both

pregnant and nonpregnant animals (Harder and Fleming, 1981). In polyoestrous dasyurid marsupials, the pattern of changes in body weight, plasma progesterone and uterine histology during the luteal phase which follows the spontaneous ovulation are similar in pregnant and nonpregnant animals (reviewed in Tyndale-Biscoe and Renfree, 1987).

Physiological and anatomical differences have been observed, however, between the gravid and non-gravid uteri of pregnant, monovular, polyoestrous marsupials. In *Trichosurus vulpecula*, for example, the mitotic rate is higher in the endometrium of the gravid uterus (von der Borch, 1963), suggesting that the Graafian follicle may have a local hormonal effect on the ipsilateral uterus (McDonald and Waring, 1982; Young and McDonald, 1982; Curlew and Stone, 1986). The gravid uterus weighs more in this polyoestrous species, a condition attributed to increased progesterone concentrations in the uterine circulation (Curlew *et al.*, 1985). High progesterone may in turn result from increased synthesis of progesterone receptors which, in *Setonix brachyurus* (Owen *et al.*, 1982) and *Macropus eugenii* (Renfree, 1973), appears to be induced by oestrogen (Flint and Renfree, 1982; Harder *et al.*, 1984; Shaw and Renfree, 1984).

The dasyurid marsupial, *Antechinus stuartii*, is monoestrous (Marlow, 1961; Woolley, 1966), polyovular and polytocous (Selwood, 1980). Ovulation is spontaneous (Woolley, 1966); the 27-day gestation period is relatively long (Selwood, 1980). The rate of embryonic development appears to parallel that of corpus luteum formation and is positively correlated with concentrations of progesterone in plasma (Hinds and Selwood, 1990). Woolley (1966) suggested that the rate of corpus luteum

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Table 1. Number of female *Antechinus stuartii* used as source of uterine samples in this study

Status of animal	Days after ovulation										Total
	1	4	6	8	11	13	15	18	21	23	
Pregnant (P)	2	1	1	2	1	1	2	5	4	2	21
Not pregnant/ mated (NPM)	1	0	0	0	1	1	1	1	1	1	7
Not pregnant/ unmated (NPU)	0	1	1	1	0	0	0	0	0	0	3
Total	3	2	2	3	2	2	3	6	5	3	31

development controls the rate of embryonic development, which in *A. stuartii* is arrested *in vivo* and *in vitro* at the four-cell stage and the stage of the early expanding unilaminar blastocyst (Selwood 1981; Selwood and Young, 1983). Because progesterone-supplemented culture medium does not terminate this developmental arrest (Selwood, 1987), Hinds and Selwood (1990) suggested that, if progesterone plays a role in regulating embryonic development, its action must be mediated via the uterus. In this report, we evaluate the differences between the uterine myometrium and endometrium of pregnant and nonpregnant *A. stuartii* during the luteal phase at known stages of gestation. We then correlate these differences with the day after ovulation, the developmental stages of embryos, and with previously determined concentrations of progesterone in plasma during pregnancy.

Materials and Methods

Uteri used in this study were from animals maintained in laboratory colonies at La Trobe University during 1975, 1976, 1978, 1981–1984, 1987 and 1988 under Ministry of Conservation and Natural Resources permit numbers 75/237, 76/91, 78/428, 81/110, 82/32, 82/180, 84/31, 85/107 and 87/56 used for other studies. As such colonies are no longer maintained, the material used was restricted to that already collected.

Uterine samples were obtained from 31 animals determined by urine sampling to be at specific stages after ovulation, following the method described by Selwood (1980). Of these animals, 21 were pregnant (P), seven nonpregnant (unmated) (NPU), and three nonpregnant (mated but found upon dissection to have unfertilized eggs) (NPM) (Table 1). Animals on days 1, 4, 6, 8, 11, 13, 15, 18, 21 and 23 after ovulation were individually anaesthetized with sodium amytal (Lilly, Indianapolis), at 0.08–0.10 mg g⁻¹ body weight (Woolley, 1966). The uteri were excised, rinsed in phosphate-buffered saline and later dissected in serum-supplemented culture medium, into which any embryos present were collected, examined (Selwood, 1980) and used in other studies. The number of corpora lutea in each ovary was determined. This procedure provided additional confirmation that ovulation had occurred and, in the case of pregnant animals, that each uterus was gravid.

Uteri were fixed in Bouin's fixative (Humason, 1967) for 24 h, dehydrated in alcohol and embedded in paraffin. Transverse serial sections at 8 µm were taken of the uterus from the oviduct (anterior) end to a point midway between the anterior and posterior ends of the uterus, where it was widest. Sections were stained in haematoxylin and eosin (BDH Australia, Kilsyth, Victoria) and mounted in Depex (BDH Australia, Kilsyth, Victoria). From each set of slides representing the widest part of a given uterus, a section was chosen randomly. This and two other sections approximately 100 and 200 µm away from the first were examined under brightfield microscopy (Wild M40 inverted microscope, Wild Australia, Prahran, Victoria). Representative sections were photographed using Kodak TMX film, 100 ASA.

The tissues in each uterine section were examined following a uniform procedure for each. Myometrium thickness was measured along a transect drawn perpendicularly between the roughly parallel distal and proximal surfaces of the myometrium. Endometrial stroma thickness was measured in a similar manner, as was the thickness of the endometrial epithelium, which lines the uterine lumen. The density of endometrial glands was estimated by counting the number of sectioned glands encountered along a transect drawn perpendicularly relative to the surfaces of the endometrial stroma. Three sets of these measurements were obtained from each animal. Lymphocytes were counted in a grid superimposed on the sectioned tissue, with one edge of the grid positioned in perfect register with the basal lamina of the endometrial epithelium. A total of ten randomly selected grids, distributed among the three sections evaluated, were counted for every animal.

The numbers of animals examined on specified days after ovulation are shown (Table 1). Effects of reproductive status on uterine parameters were compared by a *t* test. Because the material was examined retrospectively after colonies were disbanded, it was not possible to increase the number of animals examined.

Results

The histological differences detected among uteri from P, NPU, and NPM animals are summarized (Fig. 1). The reproductive

Fig. 1. Uterine histological profile and plasma progesterone levels of (■) pregnant, (△) non-pregnant and mated, and (□) nonpregnant and unmated *Antechinus stuartii* on various days after ovulation. (a) Plasma progesterone concentrations (data from Hinds and Selwood, 1990); (b) thickness of myometrium; (c) thickness of endometrial stroma; (d) thickness of endometrial epithelium; (e) number of stromal glands per transect; (f) density of lymphocytes at basal lamina of endometrial epithelium. Means are presented $\pm$ SEM. Relevant stages of embryonic development are indicated.

states of the animals (days after ovulation) are shown alongside the developmental stages of embryos retrieved from them. Plasma progesterone concentrations from animals of identical reproductive state (days after ovulation) are from Hinds and Selwood (1990).

Myometrium

Myometrial thickness fluctuated in P and NPU animals, that in the former rising by day 8 to a peak on day 11. The myometrium of NPU animals was thickest on day 18 and was

Fig. 2. Transverse section of uterine wall from (a) pregnant and (b) nonpregnant *Antechinus stuartii* 18 days after ovulation showing (a) e, thin endometrial epithelium; g, stromal glands with highly polarized secretory cells; and s, endometrial stroma densely packed with stromal glands and (b) e, thick endometrial epithelium; g, stromal glands with moderately polarized secretory epithelium; and s, oedematous endometrial stroma loosely packed with stromal glands. Scale bars = 50 µm.

significantly thicker ($P < 0.05$) than that of P animals. No obvious histological differences were observed among the myometrial vascular systems of uteri from P, NPU and NPM animals.

Endometrial stroma

Great fluctuations in thickness characterized the endometrial stroma in the three groups of animals examined. The stroma of P animals exhibited more than a sevenfold increase in thickness, and was thinnest on day 1 and thickest on days 6, 18 and 23. The peaks of thickness were mirrored by the stroma of both NPU and NPM animals, although the stroma of P animals was consistently and significantly ($P < 0.05$, $P < 0.01$, or $P < 0.001$) thicker at every peak.

Endometrial epithelium

The endometrial epithelium was low columnar and pseudo-stratified in all sections examined. In NPU animals, it was as thick as, or thicker than, that in P animals at any time after ovulation for which comparisons were available. Epithelial thinning in gravid uteri was significant ($P < 0.05$) on day 15 and very highly so ($P < 0.001$) on days 1 and 18, and appeared to be due to loss of cytoplasmic material on the lumen side of the epithelial surface, which exhibited a ragged outline (Fig. 2a).

This contrasted sharply with the finely villous surface of the endometrial epithelium characteristic of the non-gravid uterus (Fig. 2b).

Endometrial glands

Gland density was significantly greater ($P < 0.05$ on day 11, $P < 0.01$ on days 18 and 23, $P < 0.001$ on days 4, 8, 15 and 21) in P than in NPU or NPM animals on every day of gestation examined except on days 1, 6 and 13. Mitotic figures were observed in the gland epithelium from both gravid and non-gravid uteri at the rate of about one per transverse section (containing from 50 to 300 cells) until day 6, and at frequencies less than that thereafter. On days 16 and 18, haematoxylin-stained, translucent patches were evident in the lumen of endometrial glands from P animals, suggesting the presence of proteinaceous fluid.

Lymphocyte density at endometrial basal lamina

Lymphocytes were clustered in a dense mat in the uterine stroma adjacent to the basal lamina of the endometrial epithelium (Fig. 2). Lymphocyte counts were roughly equal in P and NPU animals, although significantly greater in the former on days 1 ($P < 0.05$) and 15 ($P < 0.01$). After day 15, lymphocyte counts declined in both P and NPU animals, more abruptly

in the latter. Lymphocyte density in NPM animals decreased sharply from days 4 to 8, exhibiting a trend completely the reverse of that in P animals (Fig. 1).

Discussion

The gravid uterus of *A. stuartii* was shown to be histologically distinguishable from its non-gravid counterpart. Although uterine histological profiles from P, NPU and NPM animals were superficially similar, careful inspection revealed that statistically significant differences were detectable at all time points examined except day 13. This instance of minimal (or no) uterine histological differences immediately precedes the period of sustained increase in plasma progesterone in pregnant animals which commences on day 13. This period coincides with the termination of slow or arrested development typical of *A. stuartii* embryos at the expanding, unilaminar blastocyst stage (Hinds and Selwood, 1990). Another time point during which histological differences between gravid and non-gravid uteri are minimal is day 4. Again, this time point immediately precedes, in pregnant animals, a modest but distinctive rise in concentrations of progesterone in plasma (Hinds and Selwood, 1990) and coincides with the termination of arrested development in four-cell *A. stuartii* embryos (Selwood, 1980). It is clear, therefore, that increased concentrations of plasma progesterone correlate strongly not only with resumption of embryonic development, but also with changes in the histological profile of the gravid uterus.

These results are inconsistent with results for *Didelphis virginiana*, the only other marsupial species in which the uterine histology concentrations of progesterone in plasma and corpus luteum development of pregnant and nonpregnant animals have been compared at matched time-points. In *D. virginiana*, gravid uteri differ from non-gravid uteri in one aspect and in one instance only. On day 11 (2 days before parturition), the uterine stroma is significantly thinner in pregnant animals (Fleming and Harder, 1981). An equivalent stage was not sampled in *A. stuartii* but the uterine stroma in *A. stuartii* is considerably thicker ($P < 0.01$) in pregnant animals on day 23, the day of implantation, 3–4 days before parturition.

There is an important point of similarity between *D. virginiana* and *A. stuartii*, however. In both species, concentrations of progesterone in plasma clearly reflect the developmental state of the corpus luteum, in which secretory activity reaches a peak value towards the end of pregnancy (Hartman, 1928; Harder and Fleming, 1981; Hinds and Selwood, 1990). This three-point correlation among corpus luteum, progesterone and embryonic development also occurs in *Dasyurus viverrinus* (Sandes, 1903; Hinds, 1989). In addition, concentrations of progesterone in plasma have been shown to correlate positively with corpus luteum development in *Trichosurus vulpecula* (Thorburn *et al.*, 1971) and endometrial weight in *D. virginiana* (Renfree, 1975).

The uterus is the link between progesterone production and embryonic development. The gravid uterus of *A. stuartii*, however, presents a histological profile quite different from that of non-gravid uteri, suggesting two possibilities: (i) the presence of embryos alters the uterine milieu and, ultimately, its histological profile or (ii) the progesterone rise between days 13 and 15 in

pregnant animals provokes greater changes in the histological profile of the uterus. An embryonic signal would be necessary in either case. In the latter, it would have to be directed at the corpus luteum. The nature of the signal and precisely how it would be transmitted are unclear.

It is clear, however, that embryonic signalling occurs during pregnancy. In eutherian animals, in which implantation typically occurs relatively early in gestation, chorionic gonadotropin production by embryonic trophoblast cells signals the corpus luteum to remain actively secreting progesterone (Dorrington, 1979). This does not occur in marsupials (Tyndale-Biscoe and Renfree, 1987) perhaps because implantation is late or of short duration. The observations that corpus luteum development is the same in pregnant and nonpregnant *D. virginiana* (Fleming and Harder, 1983) and *T. vulpecula* (Pilton and Sharman, 1962) suggest strongly that embryonic signalling, if it occurs, bypasses the corpus luteum.

The presence of acellular investments around the marsupial embryo possibly presents a barrier to signal transmission. Although these investments (zona pellucida, mucoid layer, shell) are permeable to diverse substances, progressively become attenuated, and eventually disappear during pregnancy, they may restrict communication between embryo and uterus to the extent that an immunoprotective role is ascribed to them (reviewed in Tyndale-Biscoe and Renfree, 1987). The comparison of lymphocyte density reported in this study appears to confirm this view, albeit inconsistently, suggesting that a cellular immune response to pregnancy is not manifested in the endometrial basal lamina. We note, however, that lymphocyte counts from pregnant animals are high on day 15, when the primitive endoderm forms in *A. stuartii* embryos (Selwood, 1980) and novel proteins would be synthesized. Perhaps the earliest immune response to pregnancy in marsupials is humoral, or the cellular immune response is manifested in the peripheral circulation (O'Neill, 1985; O'Neill *et al.*, 1985) or in adjacent lymphatics (Clarke, 1984).

In conclusion, gravid and non-gravid uteri exhibit significant histological differences that correlate with embryonic developmental rate, concentrations of progesterone in plasma and corpus luteum formation in *A. stuartii*.

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