Effects of corpus luteum removal on progesterone, oestradiol-17 β and LH in early pregnancy of the tammar wallaby, *Macropus eugenii*

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Summary. The quiescent corpus luteum of female tammars was reactivated by removal of the pouch young (RPY). The reactivated corpus luteum was ablated 3 days after RPY. Plasma progesterone and oestradiol concentrations were measured by radio-immunoassay in these and in sham-operated controls. Excision of the CL abolished the rise in progesterone seen at Day 5–6 in the sham-operated animals (130.7 ± 56.6 vs 452.4 ± 176.0 pg/ml, mean $\pm$ s.d.). By contrast, oestradiol-17 β values increased within 6–16 h of CL excision to 16.3 ± 6.9 pg/ml and remained high for 1–3 days while in the sham-operated animals there were less sustained and more variable peaks of 10–20 pg/ml between Days 3 and 5 (mean 12.0 ± 3.6 pg/ml at Day 4–5). We conclude that the early transient increase in peripheral plasma of progesterone is of luteal origin but the source of the oestradiol remains unknown.

Keywords: corpus luteum; progesterone; oestradiol-17 β ; early pregnancy; marsupial

Introduction

It is generally recognized that the corpus luteum (CL) is the main source of circulating progesterone in the tammar wallaby: luteal progesterone content and concentration rises during pregnancy concomitant with increase in size of the CL (Renfree *et al.*, 1979). Peripheral plasma progesterone concentrations mirror the luteal concentrations and for the first 5 days of the cycle are <200 pg/ml (Hinds & Tyndale-Biscoe, 1982). There is a transient rise of progesterone of about 450 pg/ml lasting 1–2 days on Days 5–8 after removal of the pouch young followed by a return to basal levels. From Day 10 concentrations rise to around 500 pg/ml until parturition or oestrus.

This peak *in vivo* is reflected in in-vitro incubations of luteal tissue by an increased production rate at Day 5 after removal of pouch young (Hinds *et al.*, 1983), and luteal tissue can synthesize progesterone from suitably labelled precursors at all stages of pregnancy (Renfree *et al.*, 1984). Removal of the CL at Day 18 after removal of pouch young reduces plasma progesterone from 630 to <200 pg/ml within 24 h (Findlay *et al.*, 1983), and at Day 23 from about 600 to <200 pg/ml (Harder *et al.*, 1985).

The origin of circulating oestradiol-17 β during pregnancy is less clear. Whilst the post-partum rise in oestradiol from <10 to 20 pg/ml at oestrus is clearly of follicular origin (Shaw & Renfree, 1984; Harder *et al.*, 1984, 1985), there is also a transient rise to 16 pg/ml at around Day 5 after removal of pouch young when there are no developing follicles present (Shaw & Renfree, 1984). A similar transient rise in oestradiol has been observed in early pregnancy in wild-shot animals with blastocysts reactivating after seasonal quiescence (Flint & Renfree, 1982).

The increase in plasma oestradiol coinciding with the rise in progesterone at Day 5 suggests a luteal origin for the oestrogens also. This has been proposed by Evans *et al.* (1980) and Renfree *et al.* (1982), who observed that exogenous oestradiol but not progesterone or androstenedione could mimic the inhibition of follicular growth by the quiescent CL in tammar from which the CL had

been removed. However, Renfree *et al.* (1984) could not detect aromatase in luteal tissue at Day 0 or between Days 11 and 25 after removal of pouch young, and CL contained only minute quantities of oestradiol. Likewise, the content of oestradiol in CL from mid- to late pregnant tammar was very low (Renfree *et al.*, 1984; Shaw & Renfree, 1984). The highest ovarian oestradiol content is found in ovaries with Graafian follicles (Harder *et al.*, 1984; Shaw & Renfree, 1984).

This study was designed to determine whether peripheral plasma progesterone and oestradiol during early pregnancy and the oestrous cycle in the tammar wallaby are of a luteal or non-luteal origin by removing the CL before the observed transient rises in these two hormones.

Materials and Methods

Animals. The tammar used in this study were part of a breeding colony established at Monash University with animals originally from Kangaroo Island, South Australia. Husbandry and handling were as previously described (Renfree & Tyndale-Biscoe, 1978). The females were housed with males throughout this study.

Pouch young were removed (Day 0) from 11 lactating females to terminate quiescence at 09:00–10:00 h in March (i.e. during the breeding season). At 3 days after removal of young, the reactivated CL was removed from 6 tammar and another 5 were sham-operated as previously described (Young & Renfree, 1979). Animals were checked at regular intervals from Day 25 to Day 80 (i.e. the period equivalent to 3 pregnant or oestrous cycles of 26–30 days each) for births and/or mating plugs.

Collection of blood samples. Blood samples of 4–5 ml were taken from one of the lateral tail veins into heparinized syringes. The animals were bled daily from 2 days before removal of pouch young to Day 3 when blood was taken before and 4–6 h after operation, at 8-h intervals from Day 3 to 6, twice on Day 7, and then daily to Day 13. Blood haematocrit showed no significant changes over the duration of the experiment so it was not necessary to replace the red cells. Plasma was separated by centrifugation and stored at -20°C until assayed for progesterone, oestradiol and LH concentration by radioimmunoassays (RIA).

Hormone assays. The progesterone assay was that of Shaw & Renfree (1984) re-validated as described below for antiserum S334 provided by Dr R. I. Cox (CSIRO Division of Animal Production, Prospect, NSW, Australia). The antiserum was raised against progesterone-11-hemi-succinate-bovine serum albumin and had the following percentage cross-reactivities: progesterone, 100%; 11β -hydroxyprogesterone, 27%; 17α -hydroxyprogesterone, 0.3%; 20α -hydroxy-4-pregnene-3-one, 0.3%; 20β -hydroxy-4-pregnene-3-one, 0.3%; pregnenolone, 0.6%; $3\beta,17$ -dihydroxy-5-pregnene-20-one, <0.02%; 5β -pregnane-3 $\alpha,20\beta$ -diol, 0.03%; 5β -pregnane-3 $\alpha,2\alpha$ -diol, 0.03%; 5β -pregnane-3,20-dione, 1.4%; cortisol, 0.01%; corticosterone, 5.8% and 11 -deoxycorticosterone, 6.9%. Progesterone was measured by transferring 1 ml plasma to 20-ml screw-capped glass tubes with 8 ml hexane (Analar, B.D.H. Australia, Port Fairy, Victoria) and shaking for 10 min on a horizontal tube shaker at about two 3 cm excursions/sec. The phases were allowed to separate and the aqueous layer was frozen in a bath of solid CO_2 /ethanol. The hexane was decanted into 13×100 mm test tubes and dried under a stream of dried, filtered air. The extracts were dissolved in 250 μl of assay buffer (0.1 M-phosphate, pH 7.0; with 9.0 g NaCl/l, 1 g gelatin/l, 1 g NaN_3 /l) and allowed to equilibrate for at least 4 h. After equilibration, duplicate 0.1 ml samples of the extract were assayed as described by Shaw & Renfree (1984).

Efficiency of the extraction procedure was 75% as determined by recovery of radioactive progesterone equilibrated with plasma. Parallelism between standard and endogenous progesterone was determined by comparison of the buffer standard curve with a series of samples made by diluting a pool of plasma with high progesterone content with plasma previously treated with charcoal to remove endogenous steroids. Sensitivity of the assay, calculated as the lowest standard which differed from the zero standard by more than twice the standard deviation of the zero standard was 20 pg/ml for 4 assays. Accuracy was determined by addition of 0.5, 1, 3 or 5 ng progesterone to aliquants from a plasma pool which were extracted and assayed as described above. The average measured concentrations were within 10% of the expected values. Precision of the assay was evaluated by multiple determination of aliquants from 2 plasma pools included in every assay. The inter-assay coefficients of variation were 2.4% and 12.6% from 2 plasma pools in 4 assays, and intra-assay coefficient of variation was 12.4%. All samples from each animal were measured in the same assay.

Oestradiol was measured by the method of Shaw & Renfree (1984), validated for tammar plasma, using antiserum S6181 provided by Dr R. I. Cox (CSIRO Division of Animal Production, Prospect, NSW, Australia). Efficiency of the extraction procedure was 92%. Sensitivity of the assay was 4 pg/ml. The inter-assay coefficients of variation were 8.76% and 9.4% for 2 plasma pools in 4 assays, and intra-assay coefficient of variation was 5.33%. All samples from each animal were measured in the same assay.

Luteinizing hormone was measured by the heterologous double-antibody radioimmunoassay described by Sutherland *et al.* (1980) and validated for use with tammar plasma. The radio ligand was NIADDK rat LH-I-6 and the antibody was rabbit anti-ovine LH (GDN-15) purchased from Professor G. D. Niswender (Colorado State University, Fort Collins, CO, U.S.A.). The standard was NIH-oLH-S23. The assay sensitivity was 0.3 ng/ml and inter- and intra-assay coefficients of variation were 6.5 ($n = 3$) and 4.5% respectively.

Fig. 1. Plasma concentrations of progesterone (●) and oestradiol-17β (○) in 6 tammar before and after ablation of the corpus luteum (arrow and vertical line on Day 3) reactivated by removal of the pouch young (RPY) on Day 0.

Results

Progesterone concentrations

The mean ($\pm$ s.d.) concentration of progesterone measured before removal of pouch young was 217.1 ± 107.2 pg/ml ($n = 33$) with transient (single sample) high values seen in 2 of the 11 animals. After excision of CL on the morning of Day 3 after removal of pouch young progesterone concentration fell by different amounts in 5 of 6 tammar and the mean concentration at the afternoon sample was 127 pg/ml (Fig. 1, Table 1). In the other animal plasma progesterone concentrations remained unchanged at the afternoon sample. Subsequently, progesterone concentrations remained low for the duration of the experiment and no animal without CL had an early peak in progesterone. In the sham-operated group there was no marked effect on progesterone on the day of operation. The early progesterone peak was observed in each animal of the sham-operated group with the peak occurring on Day 4 in one animal, on Day 7 in one animal, and on Days 5–6 in 3 animals (Fig. 2).

Oestradiol concentrations

The mean ($\pm$ s.d.) concentration of oestradiol measured before removal of pouch young was 10.1 ± 6.5 pg/ml ($n = 33$) but with considerable between-animal variation. Oestradiol concentration rose to oestrous values (16.3 ± 6.9 pg/ml) in 5 animals within 16 h of operation (Fig. 1). In the remaining animal (No. 1696) oestradiol rose by 16 h but not to the same extent (Fig. 1). Oestradiol values remained high for up to 48 h then declined but continued to fluctuate for several days. The mean oestradiol concentration in the sham-operated animals at Days 4–5 was 12.0 ± 3.6 pg/ml, but there were considerable fluctuations within and between animals, with a number of small peaks occurring during the period of thrice daily sampling (Fig. 2), so that only 2 (Nos 0770 and 1297) had a single marked peak.

Fig. 2. Plasma concentrations of progesterone (●) and oestradiol-17β (○) in 5 tammars before and after sham excision (arrow and vertical line on Day 3) of the corpus luteum reactivated by removal of the pouch young on Day 0.

Table 1. Mean ($\pm$ s.d.) progesterone, oestradiol-17β and LH concentrations measured immediately before CL excision (CL-X) or sham operation on Day 3 after removal of the pouch young, on the afternoon of Day 3 and early morning of Day 4

		Progesterone		Oestradiol-17β		LH	
Sample		Sham	CL-X	Sham	CL-X	Sham	CL-X
Day 3	10:00 h	277.1 $\pm$ 63.2	207.2 $\pm$ 79.3	8.4 $\pm$ 5.0	9.4 $\pm$ 4.3	0.48 $\pm$ 0.42	2.11 $\pm$ 2.46
	16:00 h	258.5 $\pm$ 36.6	127.3 $\pm$ 45.2	8.4 $\pm$ 3.1	16.3 $\pm$ 6.9	0.37 $\pm$ 0.25	0.65 $\pm$ 0.68
Day 4	06:00 h	295.1 $\pm$ 101.3	137.4 $\pm$ 22.3	8.6 $\pm$ 4.1	18.9 $\pm$ 5.9*	0.21 $\pm$ 0.06	0.70 $\pm$ 0.80

Each post-treatment value within groups was tested for significant differences from the pre-operation value by Student's *t* test for paired samples (Scheffler, 1979).
*Significantly different from the pre-operation value, *P* < 0.01.

LH concentrations

The mean ($\pm$ s.d.) concentration of LH measured before operation was 1.1 $\pm$ 1.1 ng/ml (*n* = 13) (Fig. 3); one animal (No. 1654) had a very high LH value (4.8 ng/ml) before removal of pouch young and was the source of most of the variation around the mean. With this animal eliminated from the data the basal pre-operative LH concentration was 0.9 $\pm$ 0.4 ng/ml (*n* = 12) which was comparable with 0.52 $\pm$ 0.34 ng/ml in the sham-operated animals. Tammar 1654 also had the lowest concentrations of oestradiol-17β of any animal in the experiment. Sham-operated animals showed no change in LH concentration after operation but in all animals from which

Fig. 3. Plasma concentrations of LH (NIH-oLH-23) in tammars before and after excision of the CL ($N = 6$) and sham excision ($N = 5$) of the corpus luteum reactivated by removal of the pouch young (RPY) on Day 0. The time of operation is indicated by the vertical line. Values are mean $\pm$ s.e.m.; error bars are not shown when less than the size of the symbol.

the CL had been excised LH concentration declined within 6 h and remained low with some fluctuations for the duration of the experiment (Fig. 3).

Resumption of reproductive activity

In the sham-operated group 4/5 animals had pouch young on Day 36. By back dating from pouch young estimated ages, the day of birth was between Day 27 and Day 30, which is within the normal range. The remaining animal did not give birth but had a mating plug at the same time, also within the expected time. In the experimental group 2 tammars had new pouch young on Day 45 (Nos 1696 and 1709) and another two on Day 52 (Nos 1654 and 1666). By subtracting the period of 29 days from oestrus to birth (Merchant, 1979), it appears that the new ovulations occurred 16–23 days after removal of CL. New pouch young or a mating plug were not detected in the remaining 2 animals by Day 80 after removal of pouch young.

Discussion

The transient increases in peripheral plasma progesterone and oestradiol at Days 5–7 previously reported (Hinds & Tyndale-Biscoe, 1982; Shaw & Renfree, 1984) were also observed in this study. However, oestradiol concentrations were more variable in individual animals, and the mean of 12.0 ± 3.6 pg/ml does not accurately reflect the fluctuations which appear with the increased frequency of sampling. The mean rise is less than that of 16 pg/ml reported by Shaw & Renfree (1984) and a clear-cut peak at around Days 4 or 5 was only observed in 2 animals. Excision of CL at Day 3 after removal of pouch young abolished the rise in progesterone seen at Day 5–6 in the sham-operated animals (130.7 ± 56.5 pg/ml vs 452.4 ± 176.0 pg/ml), but did not depress the circulating oestradiol values. After removal of CL, there was a rapid (6–16 h) increase in plasma oestradiol from <10 pg/ml to 16.3 ± 6.9 pg/ml (Table 1), and the rise was sustained for 2–3 days.

These results confirm that the corpus luteum is responsible for the increases in peripheral plasma progesterone during early as well as late pregnancy, and also show that the increase in production rate of progesterone by luteal tissue *in vitro* (Hinds *et al.*, 1983) is reflected *in vivo*. However, the oestradiol in peripheral plasma cannot be of luteal origin.

These results support the findings from in-vitro incubations of luteal tissue with suitable radioactive precursors, in which progesterone but not oestradiol can be detected (Renfree *et al.*, 1984). Furthermore, although luteal tissue contains oestradiol and oestrone, albeit in small quantities (< 100 pg) (Renfree *et al.*, 1984; Shaw & Renfree, 1984), from the present results it is clear that this luteal oestrogen is not the source of the observed transient rise.

Paradoxically, the quiescent corpus luteum of diapause is able to prevent early follicular development while it itself is inhibited by the sucking stimulus. Removal of the CL leads to a new ovulation 15–18 days later (Tyndale-Biscoe & Hawkins, 1977). The time taken to ovulate after CL excision in the present study was slightly longer (16–23 days). Since inhibition of follicular development may be mimicked by administration of physiological doses of oestradiol-17 β (but not progesterone) after CL removal (Evans *et al.*, 1980; Renfree *et al.*, 1982) by a negative feedback of oestradiol on the hypothalamic–pituitary axis (Horn *et al.*, 1985), it is possible that the rise in oestrogen after surgery temporarily inhibited immediate resumption of the events leading to follicular maturation in some animals. However, sham operation on Day 3 did not affect oestrogen concentrations (Table 1), nor did it influence the time of birth, confirming previous detailed results (Young & Renfree, 1979).

The tammar adrenal gland has aromatase activity (Callard *et al.*, 1982), as does the ovarian cortex (Renfree *et al.*, 1984), with highest oestradiol concentrations being found in ovaries with Graafian follicles (Harder *et al.*, 1984). Although ovaries at Day 0 contain oestradiol (Shaw & Renfree, 1984), follicles on Day 3 after removal of pouch young are small, and since oestradiol values of 12.7 pg/ml are found after ovariectomy (Flint & Renfree, 1982), it seems possible that the immediate increase in circulating oestradiol after excision of the CL is of adrenal origin.

The exact role of the transient rises in both progesterone and oestradiol at Days 5–7 remains unclear. Uterine metabolic reactivation is measurable at Day 4 after removal of pouch young (Shaw & Renfree, 1986), and blastocyst metabolism increases by Day 5 (Thorner *et al.*, 1981; Shaw & Renfree, 1986). The initiation of uterine and embryonic metabolism after diapause has therefore already occurred by the time the transient rises are observed. Furthermore, while exogenous progesterone alone can reactivate and support embryonic development (Renfree & Tyndale-Biscoe, 1973), oestradiol in pharmacological (Smith & Sharman, 1969) or physiological doses (Fletcher *et al.*, 1988) does not. It is possible that initiation of development after diapause may be due to the earlier, although very variable, increases in plasma progesterone and oestradiol observed in this study. Once diapause has ended, either or both steroids may be the essential signals for the maintenance of early development because removal of the corpus luteum before Day 8 after removal of pouch young results in embryonic loss and failure of the pregnancy (Tyndale-Biscoe, 1963, 1970).

We conclude that the early transient rise of progesterone is of luteal origin, consistent with the in-vitro data. A consistent rise in oestradiol-17 β was not demonstrated in this study, despite 8-h sampling intervals on Days 3–6. Perhaps only low, fluctuating levels of oestrogen are required in concert with the progesterone peak for reactivation. The relative importance of these two steroids in early pregnancy of the tammar remains unclear.

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