PROGESTERONE-INDUCED DEVELOPMENT OF DORMANT BLASTOCYSTS IN THE TAMMAR WALLABY, MACROPSUS EUGENII DESMAREST; MARSUPIALIA

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Summary. Macropus eugenii exhibits embryonic diapause after fertilization at post partum oestrus. The embryo becomes dormant at the blastocyst stage in response to the suckling stimulus of a young in the pouch during the breeding season and embryonic diapause continues during the non-breeding season in the absence of the suckling stimulus. Dormant blastocysts resumed development when progesterone was injected daily for 3 days. Normal dormant blastocysts were recovered from animals up to 30 days after complete ovariectomy, and progesterone-induced resumption of development occurred in blastocysts up to 21 days after ovariectomy. The termination of embryonic diapause in progesterone-injected marsupials is discussed in relation to the hormonal control of delayed implantation in eutherian mammals.

INTRODUCTION

Embryonic diapause was shown to occur in the macropodid marsupials Setonix brachyurus and Macropus eugenii (Protemnodon eugenii) by Sharman (1955a, b). It has since been found to occur, in one form or another, in all macropodid marsupials which have been investigated (Sharman, 1963; Sharman, Calaby & Poole, 1966). In Macropus eugenii, parturition is followed by post partum oestrus and ovulation. Should mating and fertilization occur at this time, the fertilized egg develops to the unilaminar blastocyst stage. The presence of a suckling young in the pouch inhibits further development of the embryo and it enters the diapause state.

In M. eugenii (the tammar), the mean length of the gestation period was 28:4±0:2 days and the mean length of the oestrous cycle in non-fertilized females was 28:4±0:1 days. For both wild and domestic populations, the breeding season extended from January to July and the young occupied the pouch for an average period of 250 days (Berger, unpublished). The period during which a young is suckled in the pouch is called the quiescent phase of lactation and

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during this phase, a dormant blastocyst, 0.24 to 0.33 mm in diameter consisting of about ninety cells, was carried in one of the two uteri.

Loss or removal of a pouch young during the breeding season caused resumption of development in the dormant blastocyst. In this respect, the tammar resembled other macropodid marsupials exhibiting embryonic diapause (Sharman et al., 1966). However, no tammar young vacated the pouch before the close of the breeding season and the dormant blastocyst did not degenerate at the onset of the non-breeding season but was retained whether suckling occurred or not, and resumed development at the onset of the new breeding season (Berger, 1966).

At post partum ovulation, the ruptured follicle was converted into a corpus luteum called the corpus luteum of lactation (Sharman, 1955a). This corpus luteum underwent initial development but remained of small size (approximate diameter 2.5 mm, weight 0.008 mg) during the quiescent phase of lactation. If the pouch young was removed during the breeding season, the corpus luteum of lactation grew by hypertrophy and hyperplasia of its luteal cells, as described for the quokka by Tyndale-Biscoe (1963a), the blastocyst resumed development and a luteal phase was initiated in the uterus. The corpus luteum of lactation persisted during the non-breeding season. The luteal phase, characteristically present during post-segmentation stages of embryonic development in marsupials, was absent during embryonic diapause (Pl. 1, Figs. 1 and 2). It was induced in the uterus of the spayed female marsupial by injection of progesterone (Sharman, 1959) but did not occur if the corpus luteum was removed by surgery soon after it was formed (Pilton & Sharman, 1962). It thus appears that progesterone is necessary for the initiation of the uterine luteal phase in marsupials, that the corpus luteum is the main source of this hormone and that development after the diapause is accompanied by the appearance of a luteal phase in the uterus. It seemed likely that exogenous progesterone would induce development in the quiescent blastocyst of the tammar and the experiments reported here were undertaken to test this hypothesis. As the dormant blastocyst is carried through the non-breeding season, the effects of ovariectomy were also studied.

MATERIALS AND METHODS

Animals

The tammar (Macropus eugeni Desmarest), used in this study, is a small wallaby of the family Macropodidae (kangaroos and their relatives). The average weight of forty-three adult females was 5.8 kg (range 4.5 to 7.2 kg) and the average total length 1002 mm (range 891 to 1090 mm); the average weight of twenty-three adult males was 7.2 kg (range 5.3 to 9.0 kg) and the average total length 1073 mm (range 1014 to 1155 mm). The animals were obtained from the wild population on Kangaroo Island, South Australia, and maintained in open enclosures in which the natural vegetation was supplemented with pellet food, oat seeds and lucerne hay.

Surgery

Anaesthesia was achieved by intravenous injection of pentobarbital sodium
Development of marsupial dormant blastocysts

(Veterinary Nembutal, Abbot, 60 mg/ml) 3 ml/5 kg body weight augmented with open ether when necessary. The ovaries were removed through an incision in the midline of the pouch anterior to the mammary glands. Sterile procedures were used and a prophylactic dose of penicillin (600,000 i.u.) was given post-operatively. To ensure uniformity in reproductive condition, young, if present, were removed from the pouches of animals ovariectomized during the non-breeding season.

**Progesterone injection**

Progesterone in oil (Fawns and McAllen, 10 mg/ml) was given by intramuscular injection. Experimental animals received 1, 2 or 4 mg of progesterone once daily for 3 consecutive days. Controls were injected with peanut oil or distilled water, or were not injected. Three experimental régimes were undertaken:

(i) Intact animals were killed less than 12 days after the first injection. Experimental animals were injected with 1, 2 or 4 mg of progesterone per day for 3 days. Breeding season animals were carrying pouch young (i.e. they were in the quiescent phase) and non-breeding season animals were not lactating (Table 1).

<table>
<thead>
<tr>
<th>Description of animals</th>
<th>No. injected</th>
<th>Dose/day (mg)</th>
<th>Embryo or eggs found at autopsy</th>
<th>Condition of uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Developing Dormant Unfertilized or not found</td>
<td>Luteal Non-luteal</td>
</tr>
<tr>
<td>Quiescent phase, experimental</td>
<td>3</td>
<td>1</td>
<td>3-</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>2</td>
<td>3-</td>
<td>4</td>
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<tr>
<td></td>
<td>3</td>
<td>4</td>
<td>3-</td>
<td>3</td>
</tr>
<tr>
<td>Quiescent phase, sham-injected</td>
<td>2</td>
<td>-</td>
<td>2-</td>
<td>-</td>
</tr>
<tr>
<td>Non-breeding season, experimental</td>
<td>14</td>
<td>1</td>
<td>10-</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2</td>
<td>5-</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4</td>
<td>4-</td>
<td>4</td>
</tr>
<tr>
<td>Non-breeding season, sham-injected</td>
<td>9</td>
<td>-</td>
<td>8-</td>
<td>9</td>
</tr>
</tbody>
</table>

(ii) Intact animals in the quiescent phase receiving 4 mg of progesterone per day for 3 days were subjected to laparotomy 15 or 20 days after the first injection to determine the effect of injected progesterone (Table 2).

(iii) Bilaterally ovariectomized animals in which the viability of dormant blastocysts was tested by injecting 1 or 2 mg of progesterone per day for 3 days. Young were retained in the pouches of animals ovariectomized while in the quiescent phase and non-breeding season animals were not lactating (Table 3).
Table 2
EFFECTS OF THREE CONSECUTIVE DAILY INJECTIONS OF 4 MG PROGESTERONE ON MACROPUS EUGENII IN THE QUIESCENT PHASE (WITH POUCH YOUNG)

<table>
<thead>
<tr>
<th>Experimental procedure</th>
<th>No. injected</th>
<th>Condition of laparotomy</th>
<th>No. ultimately giving birth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pouch young removed 3 days, laparotomy performed 15 days after first injection</td>
<td>6*</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Pouch young removed 10 days, laparotomy performed 15 or 20 days after first injection</td>
<td>4</td>
<td>—</td>
<td>4</td>
</tr>
<tr>
<td>Pouch young not removed, laparotomy performed 15 or 20 days after first injection</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>As above, peanut oil injected controls; pouch young not removed</td>
<td>2</td>
<td>—</td>
<td>2</td>
</tr>
</tbody>
</table>

* Includes three animals given 2 mg progesterone/day.

Table 3
EFFECTS OF THREE CONSECUTIVE DAILY INJECTIONS OF 1 OR 2 MG PROGESTERONE ON MACROPUS EUGENII OVARIECTOMIZED WHILE CARRYING DORMANT BLASTOCYST

<table>
<thead>
<tr>
<th>Experimental procedure</th>
<th>No. injected</th>
<th>Embryos or eggs found at autopsy</th>
<th>Condition of uterus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection</td>
<td>Autopsy (days)</td>
<td>Quiescent phase</td>
<td>Non-breeding</td>
</tr>
<tr>
<td>Progesterone (Days 2, 3, 4)</td>
<td>9 and 11</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>—</td>
<td>14</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Progesterone (Days 14, 15, 16)</td>
<td>23</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Peanut oil (Days 14, 15, 16)</td>
<td>21</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>—</td>
<td>21 and 23</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Progesterone (Days 21, 22, 23)</td>
<td>28 and 30</td>
<td>4</td>
<td>4*</td>
</tr>
<tr>
<td>Peanut oil (Days 21, 22, 23)</td>
<td>28</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>—</td>
<td>30</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

* One animal died during experiment.

Evaluation of results
Animals were killed by injection of excess Nembutal into the heart. The body cavity was opened in the midline and the reproductive system removed. Blastocysts were flushed from the uterus with 0.9% NaCl (Sharman, 1955a) and measured, before fixation, using a dissecting microscope with a calibrated micrometer eyepiece. They were stained by the Feulgen method (Darlington & La Cour, 1960), flattened, and mounted under a coverslip for microscopic examination. The reproductive organs and blastocysts were fixed in alcohol–formalin–acetic acid and transferred to 80% alcohol. The uteri were transferred to alcohol and subsequently weighed. Paraffin sections were cut at 8 µ and
stained in Ehrlich’s haematoxylin and eosin. Eosin–methylene blue staining for basophilia was used to determine if a uterine luteal phase was present (Pilton & Sharman, 1962).

RESULTS

Progesterone-induced development of blastocysts in intact animals

No mitotic figures were found in blastocysts from control animals but were found in all blastocysts except one from the progesterone-injected animals. In blastocysts from control animals, numerous sperm heads were embedded in the egg envelopes surrounding the protoderm (Pl. 2, Fig. 1), but were absent in developing blastocysts from animals in normal pregnancy and progesterone-treated animals (Pl. 2, Figs. 2 and 4). There were no obvious differences in the response of blastocysts of quiescent phase and non-breeding season animals and no obvious differences between animals given 1, 2 or 4 mg of progesterone.

Corpora lutea of experimental and control animals were compared and were not different in size, and neither hyperplasia nor hypertrophy of the luteal cells was observed in corpora lutea from animals with developing blastocysts. Hypertrophy and hyperplasia occurred in the luteal cells of growing corpora lutea associated with blastocysts which resumed development after removal of the pouch young in the quokka (Tyndale-Biscoe, 1963a), red kangaroo (Sharman, 1964) and tammar (Berger, unpublished observations).

The mean weight of uteri from animals in the quiescent phase was 90.8±4.5 mg and from animals in the non-breeding season 66.7±4.3 mg/kg body weight. This difference, while not significant, was reflected in the uteri of experimental animals after progesterone injection. The uteri weighed more after progesterone injection, and uteri from quiescent phase animals were heavier than those from non-breeding season animals receiving the same amount of progesterone (Text-fig. 1). Three daily injections of 1 mg of progesterone were just as effective in terminating diapause as were three injections of 2 or 4 mg. Similarly, 1 mg given for 3 days caused an increase of almost 50% in uterine weight whereas two or four times as much caused little further increase in weight (Text-fig. 1).

The uterine glands of progesterone-treated animals gave a negative reaction for basophilia whereas the glands of control animals gave a positive reaction. A negative reaction for basophilia occurred during the luteal phase in the marsupial Trichosurus (Pilton & Sharman, 1962) and in the tammar (Berger, unpublished results).

During this series of experiments, a total of thirty-nine dormant blastocysts or derived developing embryos was recovered from forty-five animals (i.e. at least 87% of animals had blastocysts) (Table 1). This indicates a high percentage of successful fertilizations of the single egg at post partum oestrus and a high survival rate of blastocysts.

Effect of progesterone on intact animals suckling a pouch young

Only four of the thirteen animals given progesterone were pregnant at laparotomy (Table 2) and none of these gave birth. Neither control animal was pregnant at laparotomy but removal of their pouch young, at the time of operation, resulted in normal gestation and subsequent birth.
Progesterone-induced development of blastocysts in ovariectomized animals

Dormant blastocysts recovered from the uteri of non-injected and peanut-oil-injected, ovariectomized animals did not differ from the dormant blastocysts of intact animals. The viability of similar blastocysts was tested by injecting progesterone into the animals 2 to 21 days after ovariectomy (Table 3). All blastocysts recovered at autopsy were developing.

The nuclei in normal, developing blastocysts of about 2.0 mm diameter were all circular when seen in the flattened, mounted blastocyst (Pl. 2, Fig. 2). Nuclei of two size groups were present, presumably because endoderm had appeared. There were abundant normal mitoses and no evidence of cells with other than the normal diploid number of sixteen chromosomes. This was in contrast to the condition of many of the developing blastocysts recovered after progesterone injection. About a quarter of these were essentially similar to the normal blastocyst described above (Pl. 2, Fig. 4). The remainder had degenerative changes as evidenced by irregularly shaped or pycnotic nuclei and abnormally large nuclei (Pl. 2, Fig. 3). Division was occurring in some nuclei of

EXPLANATION OF PLATE 1

Transverse sections of uteri of control and experimental Macropus eugenii, × 200. In each figure, uterine lumen epithelium and adjacent glands are shown above (a), peripheral glands below (b).

Fig. 1. Non-breeding season, viable dormant blastocyst carried in uterus.

Fig. 2. Luteal phase during active pregnancy.

Fig. 3. Animal ovariectomized during embryonic diapause in non-breeding season and injected with peanut oil.

Fig. 4. Animal ovariectomized during embryonic diapause, given 2 mg progesterone daily for 3 days and killed 7 days after first injection.
most degenerating blastocysts but chromosome counts showed that some cells were at least tetraploid. Other dividing cells, however, showed the normal number of chromosomes and there were apparently normal resting and prophase stages.

The histology of the uteri of quiescent phase and non-breeding season animals carrying dormant blastocysts was similar (Pl. 2, Fig. 1). Little change occurred after ovariectomy except that the peripheral uterine glands of ovariectomized animals (Pl. 1, Fig. 3) tended to be smaller than those of intact animals. Progesterone injection induced a uterine luteal phase in ovariectomized animals (Pl. 1, Fig. 4) like that found in pregnant animals (Pl. 1, Fig. 2).

DISCUSSION

The diapause blastocyst of the tammar, like the delayed blastocyst of rodents, is derived from post partum fertilization, and in both tammar and rodent embryonic development is delayed during the suckling period. Injected progesterone caused development of dormant blastocysts carried by intact or ovariectomized tammars but systemic injection of progesterone was without effect on the rodent blastocysts. Weichert (1940) and Krehbiel (1941) terminated the delay period in lactating rats by injecting oestradiol into intact animals and Whitten (1958) showed that implantation occurred 26 hr after lactating mice were injected with oestradiol. Progesterone maintained delayed implantation in spayed rats until oestradiol was given in addition (Mayer, 1963; Nutting & Meyer, 1963). Yoshinaga (1961) obtained localized implantation in the rat of delayed blastocysts (4 to 5 days after post partum mating) by injecting minute amounts of progesterone into the adipose tissue surrounding the uterine arteries.

Progesterone was also effective in terminating embryonic diapause in other marsupials. Tyndale-Biscoe (1963b), using very much larger daily doses than ourselves, induced development in the diapause blastocysts of five out of twenty ovariectomized quokkas (Setonix) with injected progesterone used alone or in combination with oestradiol. Autopsies were performed 14 to 26 days after the initial injection. Clark (1968) injected intact red kangaroos with progesterone for 3 days and removed their pouch young on Day 4. Premature onset of development occurred and 64% of injected animals gave birth to young derived from dormant blastocysts. Systemic injection of oestradiol also terminated embryonic diapause in intact and ovariectomized tammars (Smith & Sharman, 1969), but fewer developing blastocysts were recovered than after progesterone injection.

EXPLANATION OF PLATE 2

Fig. 1. Dormant blastocyst during embryonic diapause. There are numerous sperm heads in the egg envelopes and none of the nuclei is dividing. × 350.

Fig. 2. Developing blastocyst from untreated animal (normal gestation). Sperm heads are absent, there are dividing nuclei and two size classes of nuclei. × 525.

Fig. 3. Developing blastocyst, showing degenerative changes, recovered 9 days after initial injection of 2 mg progesterone per day. Many nuclei are degenerate and some of them are very large. × 350.

Fig. 4. Blastocyst recovered 9 days after initial injection of 4 mg progesterone per day. Compare with Fig. 2, above. × 525.
In the study reported here of the thirteen intact animals receiving progesterone and maintained alive, only four appeared to be pregnant at laparotomy 15 or 20 days later. These four animals subsequently failed to give birth. Tyndale-Biscoe (1963b) felt that laparotomy carried out during the latter part of pregnancy might result in abortion. All except one of the animals receiving progesterone under the other régimes and killed less than 12 days after the initial injection had developing embryos. It is most probable that the doses of progesterone administered to all the experimental animals in all régimes were sufficient to initiate development but inadequate in most instances to maintain normal pregnancy. The two control animals, not pregnant at laparotomy, gave birth at the expected time after removal of the pouch young. In these two animals, development was not initiated by peanut oil injection and the quiescent blastocysts were retained in the uterus. Removal of the pouch young resulted in normal development and birth.

Complete absence of the ovaries for periods of up to 30 days had no effect on the retention of tammar blastocysts, and the blastocysts were capable of resuming development after periods of up to 21 days in the uteri of completely ovariectomized animals. Tyndale-Biscoe (1963a) reported that neither resorption nor further development of quiescent blastocysts occurred after ovarioectomy in the quokka and that blastocysts of normal appearance were recovered at autopsy from ovariectomized animals. According to Mayer (1963), the blastocysts of rats did not die after spaying on the 4th day, and even after 5 or 6 days' privation of ovarian hormones resumed development when progesterone and oestrogen were administered to the animals. However, Nutting & Meyer (1963) reported very low survival of blastocysts in rats which were ovariectomized on Day 3 of pregnancy and from which hormonal treatment was withheld until Day 9.

Yoshinaga & Adams (1966) ovariectomized mice on Day 1, 2, 3, 4 or 5 of pregnancy but began progesterone injection on the day of operation so did not report on the survival of blastocysts in ovariectomized animals. Implantation was delayed in two-thirds of the Day-1 and Day-2 mice and in all of the Day-3 mice, but implantation occurred in four of seven Day-4, and in all of the Day-5 mice. Progesterone-induced implantation thus occurred after ovarian secretion, presumably of oestrogen, on about Day 4 and a synergistic action of oestrogen and progesterone causing implantation is indicated. Injected oestrogen probably induced development of the rodent blastocyst in synergism with endogenous progesterone since the corpus luteum is apparently functional during delayed implantation in mice (Whitten, 1958).

A luteal phase occurred in the uteri of all progesterone-injected tammars and this was usually accompanied by a developing blastocyst. Resumption of development by the blastocyst was, however, probably not dependent on the initiation of the uterine luteal phase. In the quokka, mitotic activity began in the corpus luteum 2 days after removal of the pouch young and significant growth occurred during the first 4 days after removal of pouch young. Significant enlargement of the outer diameter of the blastocyst occurred between Days 6 and 8 (Tyndale-Biscoe, 1963a). A similar sequence of events occurred during the delayed cycle of reproduction in the tammar (Berger, unpublished).
Development of marsupial dormant blastocysts

Since both progesterone and oestrogen will initiate onset of development in intact or ovariectomized animals, it is possible that they act directly on the blastocyst. It is equally probable that both hormones act by increasing the metabolic activity of the uterus, causing the release of a substance which initiates blastocyst development. Sperm heads, remarkably well preserved, were embedded in the egg envelopes (probably the ‘albumin’ layer) of the quiescent blastocyst (Pl. 2, Fig. 1) and of earlier embryonic stages. These were present in blastocysts which have been dormant for periods of up to 11 months, but they disappeared rapidly as soon as development was resumed (Pl. 2, Fig. 2). This indicates active lysis by some agent present when development begins, which is probably released by the uterus in response to the injected hormone. The connexion between the onset of development and the alteration of the albumin layer has not been established, but it is noteworthy that Krishnan & Daniel (1967) claimed to have isolated a protein fraction, ‘blastokinin’, from the rabbit uterus which stimulated blastocyst development.

The term ‘embryonic diapause’ was adopted to describe the marsupial type of delayed implantation by Tyndale-Biscoe (1963a) who pointed out that implantation did not take place immediately after the resumption of embryonic development as happens in eutherians exhibiting delayed implantation. In the roe deer, a eutherian, anatomical attachment of the foetal placenta to the maternal caruncles takes place some time after resumption of rapid growth by the hitherto delayed blastocyst (Short & Hay, 1966). However, the corpus luteum of the roe deer is functional during the delay period whereas the marsupial corpus luteum is small and does not induce a uterine luteal phase during diapause (Sharman & Berger, 1969). Systemic injection of progesterone was without effect on the delayed blastocysts of the badger and Canivenc & Bonnin-Laffargue (1963) reported that, so far as they were aware, progesterone had proved ineffective in all species in which it was tried. We have shown, however, that injected progesterone will initiate development in dormant blastocysts carried by ovariectomized or intact marsupials. Injected oestrogen had similar effects (Smith & Sharman, 1969). The hormone control of embryonic diapause in marsupials differs from the various types of hormone control of delayed implantation found among eutherian mammals. It also appears that marsupials have different, and probably less specific, hormone requirements for initiation of active embryonic development than have eutherian mammals exhibiting delayed implantation.

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